การผลิตและการทดสอบประสิทธิภาพรีคอมบิแนนท์โปรไบโอติกยีสต์ที่ผลิตเอนไซม์ Delta 6 desaturase ในปลา

นางสาวอารยา แจ้งไพร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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PRODUCTION AND EFFICACY OF RECOMBINANT PROBIOTIC YEAST PRODUCING DELTA 6 DESATURASE IN FISH

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Animal Production Technology

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PRODUCTION AND EFFICACY OF RECOMBINANT PROBIOTIC YEAST PRODUCING DELTA 6 DESATURASE IN FISH

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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อารยา แจ้งไพร : การผลิตและการทดสอบประสิทธิภาพรีคอมบิแนนท์โปรไบโอติกยีสต์ที่ผลิตเอนไซม์ Delta 6 desaturase ในปลา (PRODUCTION AND EFFICACY OF RECOMBINANT PROBIOTIC YEAST PRODUCING DELTA 6 DESATURASE IN FISH) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.สุรินทร บุญอนันธนสาร, 234 หน้า.

การวิจัยในครั้งนี้มีวัตถุประสงค์เพื่อผลิตและทดสอบประสิทธิภาพรีคอมบิแนนท์โปรไบโอติก *Saccharomyces cerevisiae* (RY-Δ6) ผลิตเอนไซม์ delta 6 desaturase (Δ6) ในปลา การทดลองที่ 1 และ 2 เป็นการสร้างชุดยีนที่ผลิตเอนไซม์ delta 6 desaturase (Δ6) จากปลานิล (*Oreochromis niloticus*) พบว่าโปรโมเตอร์ translational elongation factor (p*TEF*) เป็นโปรโมเตอร์ที่เหมาะสมในการผลิตเอนไซม์ Δ6 ในการเปลี่ยนกรดไขมัน C18:2n6 เป็น C18:3n6 และกรดไขมัน C18:3n3 เป็น C18:4n3 ในกระบวนการสังเคราะห์กรดไขมันกลุ่ม n6-PUFA และ n3-PUFA ตามลำดับ จึงได้ทำการผลิตรีคอมบิแนนท์ยีสต์ *S. cerevisiae* (RY-Δ6) ซึ่งมียีน Δ6 ในจีโนมและทำการตรวจสอบโดยการวิเคราะห์ลำดับนิวคลีโอไทด์ ทำการวิเคราะห์การแสดงออกของยีน *Oni-fads2* ที่ระดับการถอดรหัส (transcription) และการแปรรหัส (translation) ของรีคอมบีแนนท์ยีสต์ RY-Δ6 ด้วยเทคนิค RT-PCR และ Western blot ตามลำดับ รีคอมบีแนนท์ยีสต์ RY-Δ6 มีการแสดงกิจกรรมของเอนไซม์ Δ6 ในกระบวนการสังเคราะห์กรดไขมันของ n6-PUFA และ n3-PUFA นอกจากนี้สารสกัดหยาบ (crude extract) จากรีคอมบีแนนท์ยีสต์ RY-Δ6 มีการแสดงกิจกรรมของเอนไซม์ Δ6 และมีการเปลี่ยนแปลงองค์ประกอบของกรดไขมันในน้ำมันถั่วเหลืองและน้ำมันเมล็ดลินซีด โดยเพิ่มองค์ประกอบของกรดไขมัน LC-PUFA ใน n6-PUFA และ n3-PUFA ดังนั้นโดยสรุปรวมรีคอมบีแนนท์ยีสต์ RY-Δ6 น่าจะมีความสามารถในใช้การเป็น cell factory เพื่อการผลิต LC-PUFA

การทดลองที่ 3 เป็นการศึกษาผลของการเสริมรีคอมบีแนนท์ยีสต์ RY-Δ6 เป็นสารเสริมโปรไบโอติกในปลานิล โดยมี 7 กลุ่มทดลอง ประกอบด้วยอาหารพื้นฐาน อาหารพื้นฐานเสริมด้วยยีสต์ที่ไม่ถูกทรานซ์ฟอร์ม (NT) ที่ระดับ 10^6 และ 10^8 เซลล์ต่อกรัมอาหารตามลำดับ อาหารพื้นฐานเสริมด้วยรีคอมบีแนนท์ยีสต์ RY-Δ6 ที่ระดับ 10^6 และ 10^8 เซลล์ต่อกรัมอาหาร ตามลำดับ อาหารพื้นฐานเสริมด้วยน้ำมันปลา และอาหารทางการค้า โดยทำการเลี้ยงปลาด้วยอาหารทดลองดังกล่าวเป็นระยะเวลา 90 วัน ผลการศึกษาพบว่า การเสริมยีสต์ NT และรีคอมบีแนนท์ยีสต์ RY-Δ6 สามารถพัฒนาสมรรถนะการเจริญเติบโตและค่าภูมิคุ้มกันให้ดีขึ้นได้ และสามารถเพิ่มความสูงของ villus ในลำไส้ ($P<0.05$) อาหารเสริมยีสต์ NT และรีคอมบีแนนท์ยีสต์ RY-Δ6 สามารถเปลี่ยนแปลงจุลินทรีย์ในลำไส้ของปลาได้ อย่างไรก็ตามไม่พบความแตกต่างอย่างมีนัยสำคัญทาง

สถิติขององค์ประกอบทางเคมีของตัวปลาและเนื้อ ค่าเคมีในเลือด และค่าทางโลหิตวิทยาระหว่างกลุ่มทดลองทั้งหมด ($P<0.05$) นอกจากพบว่าอาหารเสริมรีคอมบีแนนท์ยีสต์ RY-Δ6 มีผลต่อการเพิ่มการสะสมกรดไขมัน C18:3n6 และ C18:4n3 ในตับและเนื้อของปลา ($P<0.05$)

การทดลองที่ 4 เป็นการศึกษาผลของการใช้รีคอมบีแนนท์ยีสต์ RY-Δ6 ในการเสริมให้กับอาร์ทีเมีย เพื่อใช้เป็นอาหารมีชีวิตสำหรับปลากะพงขาววัยอ่อน (*Lates calcarifer*) จากผลการทดลองพบว่า อาร์ทีเมียที่เสริมด้วยน้ำมันถั่วเหลือง หรือน้ำมันลินซีด ร่วมกับรีคอมบีแนนท์ยีสต์ RY-Δ6 สามารถพัฒนาสมรรถนะการเจริญเติบโต และอัตราการรอดชีวิตของปลากระพงวัยอ่อนให้สูงขึ้นได้ ($P<0.05$) รีคอมบีแนนท์ยีสต์ RY-Δ6 สามารถเปลี่ยนแปลงจุลินทรีย์ในลำไส้ของปลาได้ รีคอมบีแนนท์ยีสต์ RY-Δ6 มีผลทำให้เกิดการเพิ่มกรดไขมัน C18:3n6 C18:4n3 C20:4n6 Σn3-PUFA และ Σn6-PUFA ในตัวปลาได้ นอกจากนี้อาร์ทีเมียที่เสริมน้ำมันลินซีดร่วมกับรีคอมบีแนนท์ยีสต์ RY-Δ6 สามารถเพิ่มความต้านทานต่อความเครียดของปลา เมื่อปลากระพงวัยอ่อนถูกทดสอบความเครียดด้วยแอมโมเนีย

ในการศึกษานี้สรุปได้ว่า รีคอมบีแนนท์ยีสต์ RY-Δ6 มีแสดงออกของกิจกรรมเอนไซม์ Δ6 และสามารถนำไปใช้เพื่อเป็น cell factory ในการผลิต LC-PUFA นอกจากนี้รีคอมบีแนนท์ยีสต์ RY-Δ6 มีศักยภาพในการใช้เป็นรีคอมบิแนนท์โปรไบโอติกในอาหารปลา เพื่อเพิ่มปริมาณ LC-PUFA ได้ นอกจากนี้ควรมีการศึกษาต่อไปเกี่ยวกับการใช้รีคอมบีแนนท์ยีสต์ RY-Δ6 ในอาหารของสัตว์น้ำชนิดอื่น ๆ

สาขาวิชาเทคโนโลยีการผลิตสัตว์ ลายมือชื่อนักศึกษา อารยา

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ARAYA JANGPRAI : PRODUCTION AND EFFICACY OF RECOMBINANT PROBIOTIC YEAST PRODUCING DELTA 6 DESATURASE IN FISH. THESIS ADVISOR : ASSOC. PROF. SURINTORN BOONANUNTANASARN, Ph.D., 234 PP.

RECOMBINANT YEAST/PROMOTER/*FADS2*/*ACT*/*PGK*/*TEF*/TILAPIA

The present study aimed to investigate the production and efficacy of recombinant probiotic *Saccharomyces cerevisiae* (RY-Δ6) producing delta 6 desaturase in fish. In experiments I-II, construction of the expression vector which produced delta 6 desaturase activity (Δ6) from Nile tilapia (*Oreochromis niloticus*) was performed. The translational elongation factor (p*TEF*) promoters was observed to be a suitable promoter to express Δ6 to convert C18:2n6 to C18:3n6 and C18:3n3 to C18:4n3 in biosynthesis pathways of n6- and n3-PUFAs, respectively. Subsequently, recombinant *S. cerevisiae* (RY-Δ6) which stably contained the Δ6 gene in its genome was generated and confirmed by DNA sequencing. The heterogeneous expression of RY-Δ6 at the transcription and translation levels were detectable by RT-PCR and Western blot analysis, respectively. RY-Δ6 exhibited Δ6 in biosynthesis pathways of both n6- and n3-PUFAs. In addition, the crude extract of RY-Δ6 exhibited Δ6 and modified fatty acid composition of soybean and linseed oils by increasing the fatty acids of LC-PUFA in n6- and n3-PUFAs. Combined with these results, RY-Δ6 could have the potential to be used as a cell factory to produce LC-PUFA.

Experiment III investigated the effects of the dietary supplementation of RY-Δ6 as probiotic in Nile tilapia. Seven dietary treatments including basal diet, basal diets

supplemented with non-transformed yeast (NT) at 10^6 and 10^8 CFU/g feed, basal diets supplemented with RY-Δ6 at 10^6 and 10^8 CFU/g feed, basal diet supplemented with fish oil and a commercial diet were fed to fish for 90 days. Supplementation of both NT and RY-Δ6 led to significantly improved growth performance and immune parameters and increased intestinal villus height ($P<0.05$). Dietary NT and RY-Δ6 modulate intestinal microbiota. However, there were no significant differences in the chemical composition in the whole body and meat, blood chemistry and hematological indices among experimental treatments ($P<0.05$). Additionally, dietary RY-Δ6 increased the accumulation of C18:3n6 and C18:4n3 in liver and meat ($P<0.05$).

Experiment IV evaluated the effects of the use of RY-Δ6 to enrich *Artemia* for use as live feed for seabass (*Lates calcarifer*) larvae. *Artemia* co-enriched with soybean oil or linseed oil and RY-Δ6 improved the growth performance and survival rate of seabass larvae ($P<0.05$). The RY-Δ6 modulated intestinal microbiota. RY-Δ6 led to increased C18:3n6, C18:4n3, C20:4n6, ∑n3-PUFA and ∑n6-PUFA in the whole body of fish. In addition, co-enriched *Artemia* with linseed oil and RY-Δ6 could improve stress resistance of fish when they were subjected to challenge with ammonia.

In conclusion, the present study generated RY-Δ6 expressing Δ6 and demonstrated its use as a cell factory to produce LC-PUFA. In addition, RY-Δ6 has the potential to be used as dietary recombinant probiotics in fish feed to increase LC-PUFA contents. Further investigations to examine their uses in other aquafeed are required.

School of Animal Production Technology Student's Signature Arayaa

Academic Year 2017 Advisor's Signature

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CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF TABLES (Continued)

Table **Page**

LIST OF TABLES (Continued)

Table **Page**

LIST OF TABLES (Continued)

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LISTS OF ABBREVIATIONS

ACH50	=	Alternative complement haemolytic 50 activity
ADG	=	Average daily gain
ANOVA	=	Analysis of variance
BUN	=	Blood urea nitrogen
cm	=	Centimeter
° C	=	Degree celsius
CFU	=	Colony-forming units
DO	=	Dissolved oxygen
EU	=	European union
FCR	=	Feed conversion ratio
FE	=	Feed efficiency
FI	=	Feed intake
× g	=	Gravity force
g	=	Gram
g kg-1	=	Gram per kilogram
g L-1	=	Gram per liter
h	=	Hour
Hb	=	Hemoglobin
Ht	=	Hematocrit
IU	=	International unit
L-1	=	Liter

LIST OF ABBREVIATIONS (Continued)

L L-1	=	Liter per liter
L min-1	=	Liter per minute
m	=	Meter
mg	=	Miligram
mg kg-1	=	Miligram per kilogram
mg L-1	=	Miligram per liter
mg mL-1	=	Miligram per milliliter
min	=	Minute
ml	=	Mililiter
mm	=	Millimeter
mM	=	Millimolar
mmol L-1	=	Millimole per liter
mol L-1	=	Mole per liter
M	=	Molar
MRS	=	de Man, Rogosa and Sharpe
µg mL-1	=	Microgram per mililiter
µL	=	Microliter
µm	=	Micrometer
µmol L-1	=	Micromole per liter
nm	=	Nanometer
NaCl	=	Sodium chloride
OD	=	Optical density

LIST OF ABBREVIATIONS (Continued)

ppm	=	Part per million
PBS	=	Phosphate buffer saline
PCA	=	Plate count agar
PER	=	Protein efficiency ratio
rpm	=	Revolutions per minute
RBC	=	Red blood cells
SD	=	Standard deviation
SGR	=	Specific growth rate
CF	=	Condition factor
TCBS	=	Thiosulfate citrate bile salts sucrose
Total Ig	=	Total immunoglobulin
U L-1	=	Unit per liter
U mg-1	=	Unit per miligram
WG	=	Weight gain

CHAPTER I

INTRODUCTION

Long-chain polyunsaturated fatty acids (LC-PUFAs) are precursors of eicosanoids that are used to produce prostaglandins and thromboxanes, as well as major components of membrane phospholipids. LC-PUFAs play pivotal roles in various processes such as the immune system, neurotransmitter biosynthesis and cholesterol metabolism (Simopoulos, 2000; Abedi and Sahari, 2014; Li and Hu, 2009). Sources of LC-PUFAs includes plants, microalgae and fish (Abedi and Sahari, 2014) and fish are an excellent source of very long-chain PUFAs (VLC-PUFAs) (Lenihan-Geels et al., 2013). Consequently, the biosynthesis of LC-PUFAs has been intensively studied in a number of fish species which comprises of multiple rounds of desaturation and elongation (Tocher et al., 2006). Consequently, enzymes involved in LC-PUFA synthesis pathways have been characterized and their applications have been explored (Tocher, 2003; Tanomman et al., 2013; Hastings et al., 2001; Santigosa et al., 2009; Zheng et al., 2005; Tocher et al., 2006; Seiliez et al., 2003; Seiliez et al., 2001). According to the advances in biotechnological techniques, the molecular information and function of enzymes that are involved in VLC-PUFAs in fish would provide application to produce VLC-PUFAs for food and feed industries.

Most freshwater fish are capable of producing LC-PUFAs using C18-PUFA as substrate, and LC-PUFA biosynthesis pathways have been intensively studied in various fish species including Nile tilapia, carp, seabass, Atlantic salmon, Atlantic cod,

seabream and rainbow trout (Sargent et al., 1999; Glencross, 2009; Eyster, 2007; Tocher, 2003; Tanomman et al., 2013; Hastings et al., 2001; Santigosa et al., 2009; Zheng et al., 2005; Tocher et al., 2006; Seiliez et al., 2003; Seiliez et al., 2001). Among the enzymes involved in LC-PUFA biosynthesis, fatty acid delta 6 desaturase (Δ6), which converts C18:2n6 and C18:3n3 to C18:3n6 and C18:4n3, respectively, catalyses the first and rate-limiting step of VLC-PUFA synthesis (Tocher et al., 2006). The *fads2* gene encoding Δ6 has been cloned and characterised from a number of fish including tilapia (*Oni-fads2*). *Oni-fads2* encodes the bifunctional Δ5 and Δ6 enzyme, hence recombinant yeast expressing *Oni-fads2* could provide a single-cell microbial factory as an alternative source of LC-PUFAs (Tanomman et al., 2013; Zheng et al., 2005; Gonzalez-Rovira et al., 2009; Monroig et al., 2010).

Recombinant protein technology using *Saccharomyces cerevisiae* as a host has been employed to investigate the biological activities of desaturase and elongase enzymes (Tanomman et al., 2013; Zheng et al., 2005; Gonzalez-Rovira et al., 2009; Monroig et al., 2010). Additionally, recombinant protein production can overcome the limitations imposed by extraction of natural sources. The yeast *S. cerevisiae* is generally recognized as safe (GRAS) organism and widely used as host for heterologous protein expression (Martinez et al., 2012). Functional protein production by recombinant *S. cerevisiae* has been successfully applied to functional food, animal feed, biopharmaceutical and chemical industries (Kim et al., 2015; Vieira et al., 2018). Metabolic engineering of *S. cerevisiae* to express enzymes involved in LC-PUFA synthesis can generate microbial cell factories for the sustainable production of LC-PUFAs, with implications for global food safety and food security.

Probiotics are live microorganisms that exert beneficial effects on animals. In general, probiotics pass all the way through gastrointestinal tract and colonize in intestine. Some probiotics produce bacteriocin or inhibitory compounds to prevent the adhesion of pathogenic bacteria, therefore modifying the intestinal microbiota. Consequently, probiotics have the potential to be a biotherapeutic approach for the improvement of host health (Fuller, 1989; Verschuere et al., 2000; FAO/WHO, 2016; Salinas et al., 2005; Balcazar et al., 2007; Wongsasak et al., 2015; Boonanuntanasarn et al., 2016). In addition, probiotics improve the efficiency of feed utilization by improving digestive enzyme activity and the growth response of host, resulting in increased feed utilization, and growth performance in animal (Tovar-Ramírez et al., 2002; Wang and Xu, 2006; Askarian et al., 2011). There have been reports describing the use of *S. cerevisiae* as a probiotic dietary supplement in aquatic feeds in Nile tilapia (*Oreochromis mossambicus*), Japanese flounder (*Paralichthys olivaceus*) and striped catfish (*Pangasianodon hypophthalmus*) (Lim et al., 2005; Liu et al., 2012; Boonanuntanasarn et al., 2018; Taoka et al., 2006; Wache et al., 2006; Essa et al., 2011). Since *S. cerevisiae* is capable of using as host for recombinant protein production, *S. cerevisiae* would provide practical applicability for recombinant probiotic.

In this study, to develop recombinant yeast expressing Δ6, the promoter should be optimised to maximise recombinant protein production. A highly constitutive promoter that can drive high-level *Oni-fads2* expression is required to develop a recombinant *S. cerevisiae* cell factory with strong Δ6 activity for large-scale industrial fermentation. Well-defined endogenous and glucose-based promoters such as those driving expression of actin (p*ACT*), phosphoglycerate kinase (p*PGK*) and translational elongation factor (p*TEF*) genes were tested.

To overcome the limitation of extrachromosomal expression plasmid vector in *S. cerevisiae*, stable expression of recombinant yeast (RY-Δ6) was established by making the construct that could integrated into yeast genome.

Further investigations were to examine the use of RY-Δ6 as recombinant probiotic for dietary supplementation in fish. Nile tilapia (*O. niloticus*) is an important economic fish of Thailand. Global tilapia production has increased year by year to provide a sustainable fish food (FAO, 2013). RY-Δ6 producing delta 6 desaturase was therefore tested its uses to increase VLC-PUFA in tilapia. Microencapsulation of RY-Δ6 was performed and examined its used as dietary probiotic effects. Furthermore, the use of microencapsulation of RY-Δ6 as recombinant probiotic for marine fish larvae was conducted. Seabass (*Lates calcarifer*) is an important economic fish in tropical countries. Seabass is brackish water fish in the nursing of seabass larvae very important because seabass larvae sensitive on water quality especially ammonia, its led to stress on fish larvae. Microencapsulation of RY-Δ6 was used to enrichment *Artimia* with substrate oils including soybean oil and linseed oil. Subsequently, the effects of the use *Artimia* co-enriched with RY-Δ6 and oils on growth performance, survival rate, fatty acid profile and stress resistance to ammonia were investigated. This studies would provide the potential use of RY-Δ6 expressing delta 6 desaturase as dietary recombinant probiotics is aquafeed.

1.1 Research objectives

1.1.1 To study the performance of three promoters (p*ACT*, p*PGK* and p*TEF*) for the expression of *Oni-fads2*.

1.1.2 To produce recombinant *S. cerevisiae* RY-Δ6 stably expressing *Oni-fads2* which exerted Δ6 desaturation activity.

1.1.3 To study the effects of the use of dietary microencapsulation recombinant *S. cerevisiae* (RY-Δ6) as probiotic on growth performance, whole body composition, meat composition, health status (such as hematological, blood chemical and immune parameters), microbial populations, histological (intestinal villus height and number of goblet cells) in intestinal tract and accumulation of fatty acid profile in meat and liver of Nile tilapia.

1.1.4 To study the effect of the use of RY-Δ6 to enrich *Artemia* on growth performance, survival rate, fatty acid profile and stress resistance of Seabass larvae.

1.2 Research hypothesis

1.2.1 The recombinant *S. cerevisiae* expressing *Oni-fads2* driven by three promoters (p*ACT*, p*PGK* and p*TEF*) could exhibit Δ6 activity with different performances.

1.2.2 The recombinant *S. cerevisiae* (RY-Δ6) that stably expressed *Oni-fads2* could exert Δ6 desaturation activity.

1.2.3 The microencapsulation recombinant probiotic *S. cerevisiae* (RY-Δ6) could exhibit probiotic effects including improvement of growth performance, whole body composition, meat composition, health status and morphology of intestine as well as modulation intestinal microbiota and accumulation of fatty acid profile in meat and liver of Nile tilapia.

1.2.4 The enrichment *Artemia* with RY-Δ6 could increase growth performance, survival rate, accumulate fatty acid profile and improve stress resistance of seabass larvae when it was used as live feed.

1.3 Scope of the study

To develop recombinant yeast expressing Δ6, the promoter should be optimised to maximise recombinant protein production. The ability of promoters to drive *Oni-fads2* expression for maximal Δ6 activity was evaluate by quantitative determination of fatty acid composition in cultured cells grown in media supplemented with exogenous fatty acid substrate. Subsequently, the production recombinant *S. cerevisiae* RY-Δ6 stably expressing *Oni-fads2* was conducted. The microencapsulation of RY-Δ6 was achieved. The potential use of microencapsulated RY-Δ6 as dietary probiotic was performed in Nile tilapia (freshwater fish) and seabass larvae (brackish water fish).

1.4 Expected results

The recombinant *S. cerevisiae* RY-Δ6 stably expressing *Oni-fads2* driven by the strong promoter could have potential to be a yeast factory for the sustainable production of LC-PUFAs. Additionally, the RY-Δ6 expressing *Oni-fads2* has potential to be uses as dietary recombinant probiotics in aquafeed.

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CHAPTER II

LITERATURE REVIEW

Long-chain polyunsaturated fatty acids (LC-PUFAs) are precursors of eicosanoids that are used to produce prostaglandins and thromboxanes, as well as major components of membrane phospholipids. LC-PUFAs play pivotal roles in various processes such as the immune system, neurotransmitter biosynthesis and cholesterol metabolism (Simopoulos, 2000; Abedi and Sahari, 2014; Li and Hu, 2009). Sources of LC-PUFAs includes plants, microalgae and fish (Abedi and Sahari, 2014), and fish are an excellent source of very long-chain PUFAs (VLC-PUFAs) (Lenihan-Geels et al., 2013). Consequently, the biosynthesis of LC-PUFAs has been intensively studied in a number of fish species, and comprises multiple rounds of desaturation and elongation (Tocher et al., 2006). Enzymes involved in LC-PUFA synthesis pathways have been characterised and their applications have been explored. Recently, researches are focus on the application of the PUFA biosynthesis pathway for animal production in order to increase PUFA content in animal product.

2.1 Polyunsaturated fatty acid (PUFA) biosynthesis pathway

The PUFA biosynthesis is divided into 2 groups: n-6 PUFA and n-3 PUFA. It has mechanism to change the fatty acid precursor linoleic acid or C18: 2n-6 in n-6 PUFA and Alpha-linolenic acid or C18:3n-3 in n-3 PUFA by the delta 6 desaturase to increase the double bond in the chain. Carbon as C18:3n-6 and C18: 4n-3 and then the

elongase will add to the long line of carbon as C20:3n-6 and C20:4n-3 and the delta 5 desaturase will add to the double bond in the carbon as C20: 4n-6 or arachidonic acid (AA) and C20:5n-3 or eicosapentaenoic acid (EPA). The elongase will add to the long line of carbon as C22:4n-6 and C22:5n-3 and the delta 4 desaturase will add to the double bond in the carbon as C22:5n-6 and C22:6n3 or DHA (Pereira, 2003) shown in Fig 2.1. This research will focus delta 6 desaturase enzyme or *fads2* gene because *fads2* gene is first step enzyme in PUFA biosynthesis.

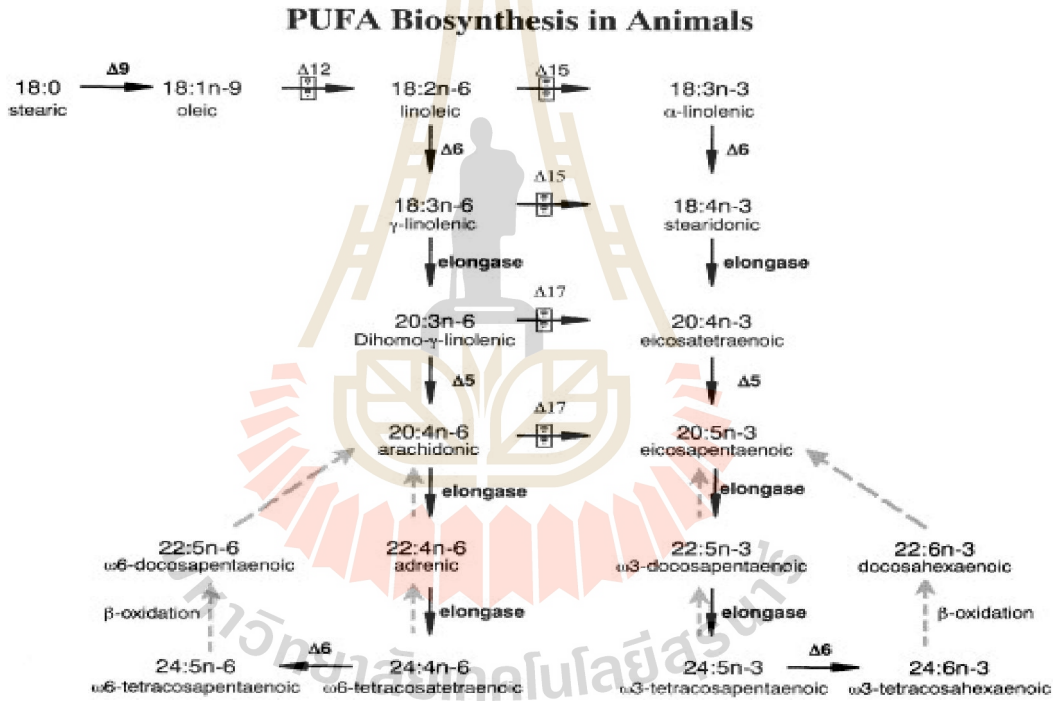

Figure 2.1 Polyunsaturated fatty acid biosynthesis (Pereira, 2003).

Most freshwater fish and marine fish are capable of producing LC-PUFAs from C18-PUFA as substrate, and LC-PUFA biosynthesis pathways have been intensively studied in various fish species including Nile tilapia, carp, seabass, Atlantic salmon, Atlantic cod, sea bream and rainbow trout (Sargent et al., 1999; Glencross, 2009;

Eyster, 2007; Tocher, 2003; Tanomman et al., 2013; Hastings et al., 2001; Santigosa et al., 2009; Zheng et al., 2005; Tocher et al., 2006; Seiliez et al., 2003; Seiliez et al., 2001) (Table 2.1). Among the enzymes involved in LC-PUFA biosynthesis, fatty acid delta 6 desaturase (Δ6), which converts C18:2n6 and C18:3n3 to C18:3n6 and C18:4n3, respectively, catalyses the first and rate-limiting step of VLC-PUFA synthesis (Tocher et al., 2006). The *fads2* gene encoding Δ6 has been cloned and characterised from a number of fish including tilapia (*Oni-fads2*). *Oni-fads2* encodes the bifunctional Δ5 and Δ6 enzyme, hence recombinant yeast expressing *Oni-fads2* could provide a single-cell microbial factory as an alternative source of LC-PUFAs (Tanomman et al., 2013; Zheng et al., 2005; Gonzalez-Rovira et al., 2009; Monroig et al., 2010).

Table 2.1 The conversion efficiency of recombinant delta 6 fatty acid desaturase enzyme in fish.

Species	FA substrate	Product	Conversion ratio	References
Freshwater fish				
Tilapia	18:2n6	18:3n6	30.47	Tanomman et al.
(*Oreochromis niloticus*)	18:3n3	18:4n3	7.07	(2013)
Carp	18:2n6	18:3n6	1.5	Hastings et al.
(*Cyprinus carpio*)	18:3n3	18:4n3	7	(2001)
Marine fish				
Seabass	18:2n6	18:3n6	8.2	Santigosa et al.
(*Dicentrarchus labrax*)	18:3n3	18:4n3	10.4	(2009)
Atlantic salmon	18:2n6	18:3n6	14	Zheng et al.
(*Salmo salar*)	18:3n3	18:4n3	60	(2005)
Atlantic cod	18:2n6	18:3n6	18	Tocher et al.
(*Gadus morhua*)	18:3n3	18:4n3	33	(2006)
Sea bream	18:2n6	18:3n6	12.2	Seiliez et al.
(*Sparus aurata*)	18:3n3	18:4n3	23.1	(2003)
Rainbow trout	18:2n6	18:3n6	3.6	Seiliez et al.
(*Oncorhynchus mykiss*)	18:3n3	18:4n3	31.5	(2001)

2.2 Recombinant protein technology

Recombinant protein technology using *S. cerevisiae* as a host has been employed to investigate the biological activities of desaturase and elongase enzymes (Tanomman et al., 2013; Zheng et al., 2005; Gonzalez-Rovira et al., 2009; Monroig et al., 2010). Additionally, recombinant protein production can overcome the limitations imposed by extraction of natural sources. The yeast *S. cerevisiae* is generally recognized as safe (GRAS) organism and widely used as host for heterologous protein expression (Martinez et al., 2012). Functional protein production by recombinant

S. cerevisiae has been successfully applied to functional food, animal feed, biopharmaceutical and chemical industries (Kim et al., 2015; Vieira et al., 2018). Metabolic engineering of *S. cerevisiae* to express enzymes involved in LC-PUFA synthesis can generate microbial cell factories for the sustainable production of LC-PUFAs, with implications for global food safety and food security.

To develop recombinant yeast expressing Δ6, the promoter should be optimised to maximise recombinant protein production. In general, inducible promoters have low basal activity but expression is increased upon induction. To develop recombinant yeast as a cell factory for expressing *Oni-fads2*, the inducer should be easy to manipulate, stable, inexpensive, and have no negative effect on cell growth. Heterologous expression of *Oni-fads2* has been demonstrated in *S. cerevisiae* using the galactose-inducible GAL1 promoter (p*GAL1*) (Tanomman et al., 2013). However, expression from p*GAL1* requires a medium containing low glucose (Tanomman et al., 2013; Zheng et al., 2005; Gonzalez-Rovira et al., 2009; Monroig et al., 2010), which is a major disadvantage because *S. cerevisiae* prefers glucose and fructose as carbon sources, and galactose is expensive for use for industrial applications. A highly constitutive promoter that can drive high-level *Oni-fads2* expression is required to develop a recombinant *S. cerevisiae* cell factory with strong Δ6 activity for large-scale industrial fermentation. Well-defined endogenous and glucose-based promoters such as those driving expression of actin (p*ACT*), phosphoglycerate kinase (p*PGK*) and translational elongation factor (p*TEF*) genes are candidates (Randez-Gil et al., 1995; Kingman et al., 1990; Peng et al., 2015; Nevoigt et al., 2006; Xie et al., 2015). However, the recombinant *S. cerevisiae* carried extrachromosomal plasmid (non-integrative plasmid) are instability due to plasmid loss (Kilonzo et al., 2009; Aherm et al., 1988), segregational instability

and/or allele segregation (Impoolsup et al., 1989; Patnaik, 2000; Friehs, 2004). Consequently, there would be in gene expression within the population of recombinant yeast (Da Silva and Srikrishnan, 2012; Karim et al., 2013), especially during long term and large-scale industrial cultivations.

To overcome the limitation of extrachromosomal expression plasmid vector in *S. cerevisiae*, stable expression of recombinant yeast was established by making the construct that could integrated into yeast genome at gene for ribosomal RNA loci. The ribosomal RNA gene are multicopy genes which employed and increase the chance of the expression cassetted to integrate in the yeast genome. It was revealed that the location of integration is one of the main factor for integration efficiency. For instance, with delta locus integration, multi- copy integration of heterologous genes were observed at the Ty retrotransposon delta sites in the yeast genome (Da Silva and Srikrishnan, 2012; Sakai et al., 1990; Shi et al., 2014; Yamada et al., 2010; Yuan and Ching, 2013; Yuan and Ching, 2014). High copy number of recombinant gene in chromosome of yeast were observed with high expression protein level (Fuji et al., 1990; Klabunde et al., 2003; Liu et al., 2012).

2.3 Probiotics

Probiotics are live microorganisms that exert beneficial effects on animals. In general, probiotics pass all the way through gastrointestinal tract and colonize in intestine. Some probiotics produce bacteriocin or inhibitory compounds to prevent the adhesion of pathogenic bacteria, therefore modifying the intestinal microbiota. Consequently, probiotics have the potential to be a biotherapeutic approach for the improvement of host health (Fuller, 1989; Verschuere et al., 2000; FAO/ WHO, 2016;

Salinas et al., 2005; Balcazar et al., 2007; Wongsasak et al., 2015; Boonanuntanasarn et al., 2016). In addition, probiotics improve the efficiency of feed utilization by improving digestive enzyme activity and the growth response of host, resulting in increased feed utilization, and growth performance in animal (Tovar-Ramírez et al., 2002; Wang and Xu, 2006; Askarian et al., 2011).

2.4 Use of *S. cerevisiae* as probiotic in fish aquaculture

Yeast used as probiotic in aquaculture which have been used as single-cell protein sources, feed additives and probiotics in aquatic animals (Barnes et al., 2006; Reyes-Becerri et al., 2008; Nayak, 2011 for review, see Gatesoupe, 2007). In fact, a number of live yeasts were found to be a part of microbiota in freshwater and marine fish gut (for review, see Blackburn and Avery, 2003). Since *S. cerevisiae* is capable of using as host for recombinant protein production, *S. cerevisiae* would provide practical applicability for recombinant probiotic. There have been reports describing the use of *S. cerevisiae* as a probiotic dietary supplement in aquatic feeds in Nile tilapia (*O. mossambicus*), Japanese flounder (*P. olivaceus*) and striped catfish (*P. hypophthalmus*) (Lim et al., 2005; Liu et al., 2012; Boonanuntanasarn et al., 2018; Taoka et al., 2006; Wache et al., 2006; Essa et al., 2011), which supplemented yeast as probiotic to fish diet could increase growth performance, modulate hematological parameters and increase immune of fish. Therefore, yeast has been recognized to have important effects on immunostimulant functions of fish (Table 2.2, 2.3, 2.4).

Table 2.2 The supplementation of yeast as probiotics on growth performance of fish.

Species	Level (%)	Initial weight (g)	Final weight (g)	SGR (%/day)	FCR	Survival (%)	References
Nile tilapia	control	0.33±0.088	7.22±0.11^{d}	3.673±0.015^{c}	1.72±0.028^{a}	96.7±1.7	Tawwab et al. (2008)
(*O. niloticus*)	0.25	0.34±0.033	8.00±0.06^{c}	3.760±0.019^{bc}	1.61±0.073^{ab}	95.0±2.9	
	0.5	0.34±0.033	8.50±0.23^{bc}	3.832±0.023^{b}	1.55±0.052^{b}	100.0±0.0	
	1.0	0.33±0.088	9.90±0.29^{a}	4.049±0.011^{a}	1.48±0.034^{c}	95.0±2.9	
	2.0	0.33±0.012	10.05±0.11^{a}	4.067±0.030^{a}	1.39±0.045^{cd}	100.0±0.0	
	5.0	0.32±0.058	10.00±0.29^{a}	4.098±0.054^{a}	1.35±0.032^{d}	96.7±1.7	
Nile tilapia	Control	26.11±0.99	170.89±1.55^{c}	2.40±0.13^{c}	1.60±0.22	-	Abdelsamee Goda et al. (2012)
	2	26.36±1.13	197.64±1.06^{b}	2.54±0.39^{b}	1.54±0.16	-	
	4	26.60±1.65	213.40±1.58^{a}	2.61±0.17^{a}	1.51±0.15	-	
African catfish	Control	34.26±0.41	760.2±5.1^{d}	-	1.39±0.05^{a}	92.96±1.06	Essa et al. (2011)
(*C. gariepinus*)	1	34.26±0.40	796.1±4.5^{c}	-	1.32±0.05^{ab}	93.77±0.71	
	1.5	34.33±0.38	840.7±4.2^{b}	-	1.30±0.06^{ab}	93.93±0.49	
	2	34.27±0.42	913.3±4.8^{a}	-	1.26±0.04^{b}	94.00±0.48	
Beluga	Control	11.2±0.6	29.2±2.6^{b}	2.27±0.21^{b}	2.053±0.10^{a}	87.0±4.2	Hoseinifar et al. (2011)
(*Huso huso*)	1	11.4±0.5	36.9±4.8^{ab}	2.79±0.33^{ab}	1.83±0.11^{ab}	92.6±3.2	
	2	11.4±0.4	38.5±3.4^{a}	2.89±0.22^{a}	1.79±0.07^{b}	93.5±4.2	

Means with different superscripts in each column differ significantly from each other ($P<0.05$).

Table 2.3 The supplementation of yeast as probiotics on hematological parameters of fish.

Species	Level	RBC ($x10^6$ cell/µL)	Hemoglobin content (g/dl)	Hematocrit (%)	Albumin (g/L)	Globulin (g/L)	References
Nile tilapia	Control	1.74 ± 0.138^{b}	5.03 ± 2.93^{b}	12.67 ± 0.917^{b}	16.2 ± 0.9^{c}	19.9 ± 2.3^{d}	Tawwab et al.
(*O. niloticus*)	0.25	1.81 ± 0.087^{b}	5.43 ± 2.89^{b}	13.43 ± 0.921^{b}	16.8 ± 0.8^{c}	30.2 ± 0.7^{c}	(2008)
	0.5	1.59 ± 0.045^{b}	4.80 ± 3.21^{b}	12.23 ± 0.984^{b}	17.3 ± 0.9^{bc}	41.4 ± 2.1^{ab}	
	1.0	2.54 ± 0.142^{a}	7.50 ± 4.07^{a}	16.90 ± 0.322^{a}	19.7 ± 0.8^{a}	46.4 ± 4.5^{a}	
	2.0	2.58 ± 0.170^{a}	7.78 ± 3.05^{a}	16.70 ± 0.153^{a}	18.7 ± 1.5^{ab}	37.5 ± 2.6^{b}	
	5.0	2.41 ± 0.058^{a}	8.43 ± 4.88^{a}	16.87 ± 0.406^{a}	18.6 ± 0.5^{ab}	36.5 ± 3.1^{b}	
Beluga	Control	0.74±16.12	6.56±0.85	19.71±18.53	-	-	Hoseinifar et al.
(*H. huso*)	1	0.72±0.75	6.55±0.63	20.46±1.06	-	-	(2011)
	2	0.70±19.82	6.77±0.84	21.45±1.21	-	-	

Means with different superscripts in each column differ significantly from each other ($P<0.05$).

Table 2.4 The supplementation of yeast as probiotics on immune parameters of fish.

Species	Type of yeast	Level	NBT test (mg/ml)	Lysozyme (10^3 Units/l)	Extracellular superoxide anion (nmol O_2^-)	Intracellular superoxide anion (O.D. at 620 nm)	References
Hybrid striped bass		Control	1.99^{c}	1246.7	0.897	2.679^{c}	Li et al. (2003)
Morone chrysops X	*S. cerevisiae*	1%	2.59^{ab}	1073.3	1.039	3.694^{b}	
M. saxatilis	Brewer's yeast	2%	3.09^{a}	965	1.293	4.511^{ab}	
		4%	2.31^{bc}	1155	1.091	4.86^{a}	
Hybrid striped bass		Control	1.20	473	7.21^{c}	1.28	Li et al. (2004)
M.chrysops X	*S. cerevisiae*	1%	1.40	515	11.22^{a}	1.55	
M. saxatilis	Brewer's yeast	2%	1.58	472	10.55^{a}	1.66	
	Grobiotic^{TM}AE	1%	1.28	448	9.71 ^{a,b}	1.79	
		2%	1.31	640	8.34 ^{b,c}	1.59	

Means with different superscripts in each column differ significantly from each other ($P<0.05$).

Nile tilapia (*O. niloticus*) is an important economic fish of Thailand. Global tilapia production has increased year by year to provide a sustainable fish food (FAO, 2013) (Figure 2.2). In tilapia feed, vegetable oil has been utilized as a fat source for both essential fatty acids and energy producing nutrients (Lim et al., 2011). The global demand for biofuels has led to increased demand for oil-based energy, thereby affecting the use of common plant-based oils in aquaculture feed. Thus, the investigation of LC-PUFA biosynthetic pathways, especially Δ6 desaturation, at molecular level in tilapia has attracted great interest. It would provide biological information of fatty acid bioconversion process in fish.

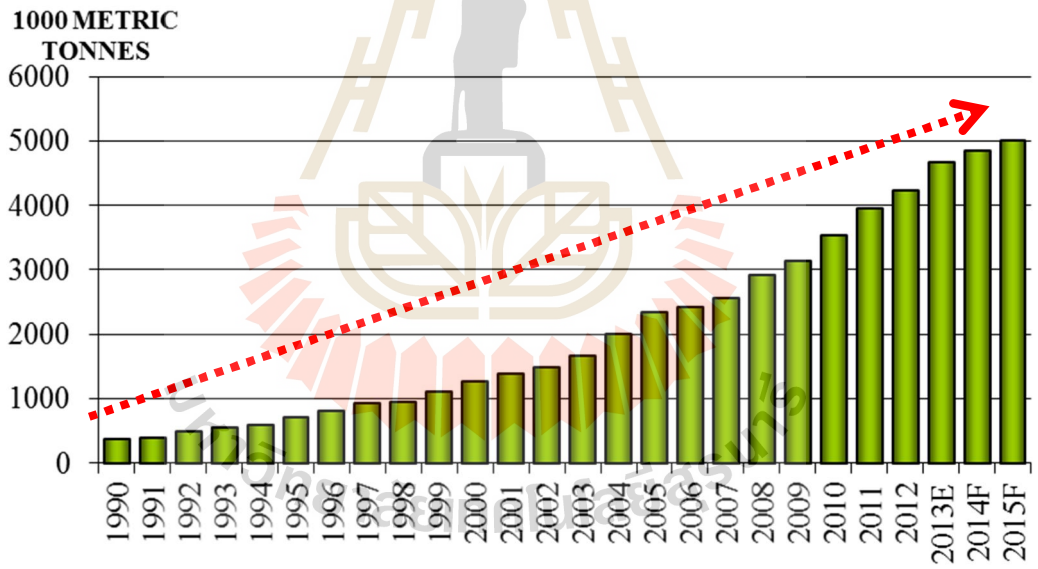

Figure 2.2 Tilapia global aquaculture production (FAO, 2013).

Recombinant protein producing delta 6 desaturase would provide an useful approach to produce sustainable LC-PUFA sources. Microencapsulation of probiotic for the use as dietary supplementation has been one of the powerful techniques to attribute the survival of probiotic passage through the adverse conditions of host gut

(Shori, 2017). Microencapsulation is a process in which cells are incorporated within an encapsulating matrix, which are generally food grade biopolymers such as guar gum, alginate, and pectin. Immobilization of probiotic in an encapsulating matrix would contribute the reduction of cell death when exposure to gastric solution during stomach passage. Additionally, the polymer matrix could control the release of entrapped probiotics in intestine. Currently, a number of microencapsulated innovations have been applied for industrial probiotics (Cook et al., 2012; Ditthab and Boonanuntanasarn, 2016). Microencapsulation of *S. cerevisiae* using guar gum as encapsulation materials following freeze drying was revealed to extend the shelf life of *S. cerevisiae* up to 16 weeks (Brinker and Reiter, 2011). There's possibly a very interesting the microencapsulation of recombinant *S. cerevisiae* for supplement diet of tilapia.

Recently, the production of fish larvae relies heavily on the brine shrimp *Artemia* as the main live food organism used in commercial hatcheries (Sorgeloos et al., 1998; Wouters et al., 2009; Cobo et al., 2015). *Artemia* contains high protein and fatty acid; however, the amount n3-PUFAs were not enough to be used as live feed for marine fish (Figueiredo et al., 2009; Lèger and Sorgeloos, 1992). Therefore, enhancement of n-3 LC-PUFA levels could be achieved through enrichment techniques. Consequently, enriched *Artemia* was improved their nutritional value which therefore were be able to use for feeding fish larvae (Sorgeloos et al., 1998; Wouters et al., 2009). The larvae which were fed enriched *Artemia* had high growth performance and survival rate (Leger and Sorgeloos, 1994; Xu et al., 1993; Rees et al., 1994; Kyuungin et al., 2000; Immanuel et al., 2001) (Table 2.5). Additionally, the probiotics were enriched to *Artemia* and fed to fish larvae found. *Artemia* enriched with probiotics led to improve growth performances and immune systems in silver carp, persian sturgeon, *Catla catla*

and Pejerrey (Adineh et al., 2011; Iranshahi et al., 2012; Divya et al., 2014; Fuhr et al., 2016) (Table 2.6). Additionally, the supplement *Artemia* and rotifer enriched with probiotics had increased number of total bacteria and *Bacillus* sp. in gastrointestinal tract of Pacific white shrimp (Jamali et al., 2015) (Figure 2.3) in the same way with Immanuel et al. (2007) found that *Penaeus monodon* shrimp fed enriched *Artemia* with *Lactobacillus* sp. and *Saccharomyces* sp. had the number of *Bacillus* sp and yeast in hepatopancreas higher than shrimp were fed non-enrich *Artemia*, when shrimp were challenged with *V. harveyi* found that shrimp fed enriched *Artemia* with *Lactobacillus* sp. and *Saccharomyces* sp. could decrease the number of *V. harveyi* in hepatopancreas when compare with shrimp were fed non-enrich *Artemia* (Table 2.7).

Table 2.5 The enrichment *Artemia* with essential oils on growth performance and survival rate of fish larvae.

Species	*Artemia* enriched	Total length (mm)	Total weight (mg)	Survival (%)	References
Senegal sole	DHA-non	10.6±1.3^{ab}	3.0±0.4	77.0±8.4	Villalta et al.
(*Solea senegalensis*)	DHA-low	10.2±1.2^{bc}	2.7±0.9	85.2±15.6	(2005)
	DHA-medium	10.9±0.8^{a}	3.1±0.3	72.2±2.0	
	DHA-high	9.8±1.0^{c}	2.4±0.4	89.8±8.0	
Pacific bluefin tuna	OA:DHA(1:0)	14.9±1.3^{c}	31.7±6.7^{c}	6.5±1.0^{b}	Seoka et al.
(*Thunnus orientalis*)	OA:DHA (3:1)	16.9±1.5^{bc}	44.0±11.7^{bc}	16.5±6.0^{b}	(2007)
	OA:DHA (7:5)	16.9±1.5^{bc}	42.9±10.5^{bc}	13.2±1.3^{b}	
	OA:DHA (0:1)	17.1±1.4^{b}	47.7±8.7^{b}	36.4±1.9^{a}	
	Yolk sac	24.1±4.9^{a}	159.3±85.5^{a}	34.4±0.6^{a}	
Japanese flounder	Oleic acid (OA)	13.5±1.84^{d}	-	36.6	Furuita et al.
(*Paralichthys olivaceus*)	OA:EPA(7:1)	14.8±1.92^{cd}	-	54.7	(1999)
	OA:EPA(3:1)	14.4±1.88^{b}	-	43.1	
	OA:EPA(1:1)	14.9±2.35^{cd}	-	46.2	
	OA:DHA(3:1)	14.8±2.54^{cd}	-	44.3	
	OA:DHA(1:1)	15.5±2.75^{ab}	-	52.4	
	DHA	16.0±2.22^{a}	-	51.5	

Means with different superscripts in each column differ significantly from each other ($P<0.05$), [1]= $P<0.001$.

Table 2.5 The enrichment *Artemia* with essential oils on growth performance and survival rate of fish larvae (Continued).

Species	***Artemia* enriched**	**Total length (mm)**	**Total weight (mg)**	**Survival (%)**	**References**
Striped trumpeter[1]	0% fish oil	9.70±0.09^{c}	1.06±0.05^{c}	43.1±3.8	Bransden et al.
(*Latris lineata*)	9% fish oil	10.04±0.16^{bc}	1.24±0.14^{bc}	33.3±6.0	(2005)
	38% fish oil	10.24±0.12^{ab}	1.33±0.13^{bc}	43.9±10.6	
	57% fish oil	10.40±0.37^{ab}	1.51±0.16^{ab}	42.2±20.2	
	77% fish oil	10.57±0.07^{a}	1.63±0.16^{a}	23.3±10.2	

Means with different superscripts in each column differ significantly from each other ($P<0.05$), [1]= $P<0.001$.

Table 2.6 The enrichment *Artemia* with probiotics on growth performance and survival rate of fish larvae.

Species	*Artemia* enriched	Level	Final weigh (mg)	SGR (%)	Survival (%)	References
Persian Sturgeon	*Saccharomyces* sp.	0	389.780±54.35^{b}	10.05±2.34^{c}	88.33±1.53^{b}	Iranshahi et
(*Acipenser persicus*)		5 x10^{10} CFU/ml	400.89±75.85^{a}	10.28±2.17^{bc}	89.00±1.73^{ab}	al. (2012)
		5.30 x10^{10} CFU/ml	487.22±69.40^{a}	11.64±2.27^{a}	92.00±2.00^{a}	
		5.48 x10^{10} CFU/ml	427.56±75.48^{a}	10.87±1.69^{b}	80.03±1.00^{c}	
Silver carp	Yeast : Bacilli	0	155.88±15.51^{c}	7.84±0.29^{b}	98.45±10.28	Adineh et
(*Hypophthalmichthys*	bacteria	1:1x10^{5} CFU/ml	200.23±26.83^{a}	8.54±0.42^{a}	99.36±6.13	al. (2011)
molitrix)		2:2x10^{5} CFU/ml	194.13±2.61^{ab}	8.47±0.03^{a}	98.64±14.78	
		3:3x10^{5} CFU/ml	160.92±21.25^{bc}	7.90±0.38^{b}	97.12±10.12	
		4:4x10^{5} CFU/ml	212.77±11.5^{a}	8.73±0.15^{a}	99.48±17.24	
Pejerrey	Control	0	84.5±6.24^{b}	12.6±0.42^{b}	98.4±0.45	Fuhr et al.
(*Odontesthes*	*Saccharomyces* sp.	1000 mg/L	102.13±9.5^{a}	13.5±0.23^{a}	98.6±0.95	(2016)
argentinensis)	*Candida utilis*	1000 mg/L	93.6±6.5^{ab}	13.1±0.25^{ab}	98.4±0.98	

Means with different superscripts in each column differ significantly from each other ($P<0.05$).

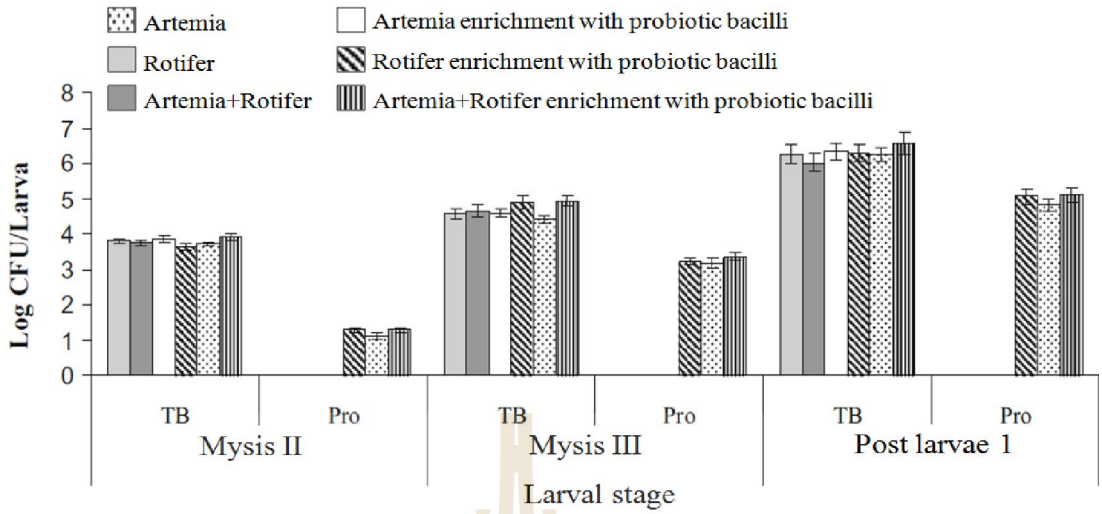

Figure 2.3 Effect of supplement *Artemia* and rotifer enriched with probiotics on microbial population (total bacteria, TB) and *Bacillus* sp. (Pro) in gastrointestinal tract of Pacific white shrimp (*Litopenaeus vannamei*) (Jamali et al., 2015).

Table 2.7 The enrichment *Artemia* with probiotics on microbial population in hepatopancreas of *Penaeus monodon* shrimp after challenging with *V. harveyi*.

Species	*Artemia* enriched	Total bacteria (CFU/g)	*Bacillus* sp. (CFU/g)	Yeast (CFU/g)	*V. harveyi* (CFU/g)	References
Penaeus monodon	Control	$1.2x10^{6}±6x10^{3}$	$1.2x10^{2}±3.05$	$1.7x10^{3}±2x10$	$1.0x10^{5}±5x10^{3}$	Immanuel et al. (2007)
	Lactobacillus sp. (600 mg/L)	$1.6x10^{7}±5.6x10^{5}$	$1.07x10^{5}±3.4x10^{3}$	$6x10^{3}±7x10$	$5.2x10^{2}±9.0x10$	
	Saccharomyces sp. (600 mg/L)	$1.3x10^{6}±8.5x10^{4}$	$6.1x10^{2}±3x10$	$8x10^{6}±5x10^{3}$	$4.6x10^{2}±9x10$	

These findings suggested that, the enrichment *Artemia* with oil (rich HUFA) and probiotic which increase the nutritional contents improve growth performance, survival rate and immune of fish larvae. The researcher attempt to study to using the RY-Δ6 to enrich *Artemia* for cultured seabass larvae which important in commercial of aquaculture.

Seabass (*Lates calcarifer*) is an important economic fish in tropical countries. It grows to comparatively large size with delicate flavoured flesh and commands premium price in the market (FAO, 2012). In the nursing of seabass larvae very important because Seabass larvae sensitive on water quality especially ammonia, its led to stress on fish larvae. The stress condition can be due to environmental and anthropogenic stressors (Dederen et al., 1986; Modesto and Martinez, 2010). Ammonia toxicity is one of the most common environmental stressors during fish culture especially in aquaculture Intensive systems due to high stocking densities. Ammonia stress causes several physiological changes including oxygen consumption, homeostasis and immune suppression which impair normal growth, health status and survival rate (de Lourdes Cobo et al., 2014; Racotta and Hernandez-Herrera, 2000). Therefore, the enrichment *Artemia* with RY-Δ6 would be a practical way to improve health under stressful conditions.

At present, researchers became interested in recombinant probiotics for animal production. Both the enzyme improves digestion and absorption, which can reduce pollution, vaccines to prevent and to increase productivity (Akinalp et al., 2007; Lim et al., 2005; Liu et al., 2011; Piyaviriyakul et al., 2002; Tsai et al., 1994; Hayami et al., 1989; Maiti et al., 2012; Yan et al., 2015). Recombinant probiotic is a probiotic that has been modified from genetic material (DNA) by using genetic engineering technology.

The recombinant yeast vitellogenin (rVtg) with the ability to produce vitellogenin, which is a precursor of yolk protein in fish, which supplement rVtg as probiotic in tilapia diet had increased growth performance and survival rate when compare with control group (Lim et al., 2005). In the same way the investigated effect the recombinant yeast contained fish growth hormone could increase growth performance of giant catfish, Japanese flounder, striped mullet and tuna (Liu et al., 2011; Piyaviriyakul et al., 2002; Tsai et al., 1994; Hayami et al., 1989). Additionally, the recombinant probiotic could improve the health status and immune of common carp and red crucian carp (Maiti et al., 2012; Yan et al., 2015). Demonstrating that the recombinant probiotics could be used as potential feed additives to improve growth performance health status and immune system of fish.

In this study, development recombinant yeast *S. cerevisiae* expressing *Oni-fads2* and tested its used as probiotic in fish. We compare the performance of three promoters (p*ACT*, p*PGK* and p*TEF*) for the expression of *Oni-fads2*. *S. cerevisiae* expressing *Oni-fads2*. Subsequently, production recombinant *S. cerevisiae* RY-Δ6 stably expressing *Oni-fads2*. Using the recombinant *S. cerevisiae* RY-Δ6 as dietary probiotic, investigation the effects of supplement the microencapsulation RY-Δ6 as probiotic on growth performance, whole body composition, meat composition, health status, microbial populations, histology in intestinal tract and fatty acid profile in Nile tilapia. Additionally, evaluate to use of RY-Δ6 to enrich *Artemia* on growth performance, survival rate, fatty acid profile and stress resistance of seabass larvae. This studies would provide the RY-Δ6 expressing *Oni-fads2* has potential to be uses as dietary recombinant probiotics and uses in other aquafeed or animal feed are required.

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CHAPTER III

ENGINEERING OF *SACCHAROMYCES CEREVISIA*E PROMOTERS FOR HETEROLOGOUS EXPRESSION OF FATTY ACID DELTA 6 DESATURASE FROM NILE TILAPIA (*OREOCHROMIS NILOTICUS*)

3.1 Abstract

Metabolic engineering of *Saccharomyces cerevisiae* for expression of fatty acid delta 6 desaturase (*fads*) from Nile tilapia (*Oreochromis niloticus*), an enzyme involved in long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis, has potential for sustainable production. In general, promoters have significant influence on recombinant protein production. Herein, we compared the performance of actin (p*ACT*), phosphor-glycerate kinase (p*PGK*) and translational elongation factor (p*TEF*) promoters for driving *Oni-fads2* expression. Exogenous substrate C18:2n6 was used to determine Δ6 activity by quantitatively determining the C18:3n6 product. The results showed that p*TEF* was the strongest promoter for *Oni-fads2* expression. The uptake mechanism of exogenous fatty acid substrates was dependent on the concentration of fatty acid supplementation, but substrate concentration had little effect on Δ6 desaturation efficiency. Recombinant *S. cerevisiae* cells expressing *Oni-fads2* driven by p*TEF* were tested with the substrate C18:3n3, and Δ6 desaturation efficiently converted C18:3n3 to C18:4n3. Furthermore, crude extracts of recombinant yeast exhibited Δ6

activity. Thus, recombinant *S. cerevisiae* cells expressing *Oni-fads2* driven by the strong p*TEF* promoter have potential as a yeast factory for the sustainable production of LC-PUFAs.

3.2 Introduction

Long chain polyunsaturated fatty acids (LC-PUFAs) are precursors of eicosanoids that are used to produce prostaglandins and thromboxanes, as well as major components of membrane phospholipids. LC-PUFAs plays pivotal roles in various processes such as the immune system, neurotransmitter biosynthesis and cholesterol metabolism (Simopoulos, 2000; Abedi and Sahari, 2014; Li and Hu, 2009). Sources of LC-PUFAs includes plants, microalgae and fish (Abedi and Sahari, 2014), and fish are an excellent source of very long-chain PUFAs (VLC-PUFAs) (Lenihan-Geels et al., 2013). Consequently, the biosynthesis of LC-PUFAs has been intensively studied in a number of fish species, and comprises multiple rounds of desaturation and elongation (Tocher et al., 2006). Enzymes involved in LC-PUFA synthesis pathways have been characterised and their applications have been explored. Recombinant protein technology using *S. cerevisiae* as a host has been employed to investigate the biological activities of desaturase and elongase enzymes (Tanomman et al., 2013; Zheng et al., 2005; Gonzalez-Rovira et al., 2009; Monroig et al., 2010).

Recombinant protein production can overcome the limitations imposed by extraction of natural sources. The yeast *S. cerevisiae* is a generally recognised as safe organism that is widely used as a host for heterologous protein expression (Martinez et al. , 2012). Functional protein production by recombinant *S. cerevisiae* has been successfully applied to functional food, animal feed, biopharmaceutical and chemical

industries (Kim et al., 2015; Vieira et al., 2018). Metabolic engineering of *S. cerevisiae* to express enzymes involved in LC-PUFA synthesis can generate microbial cell factories for the sustainable production of LC-PUFAs, with implications for global food safety and food security. Most freshwater fish are capable of producing LC-PUFAs from C18-PUFA as substrate, and LC-PUFA biosynthesis pathways have been intensively studied in various fish species including Nile tilapia (Sargent et al., 1999; Glencross, 2009; Eyster, 2007; Tocher, 2003; Tanomman et al., 2013). Among the enzymes involved in LC-PUFA biosynthesis, fatty acid delta 6 desaturase (Δ6), which converts C18:2n6 and C18:3n3 to C18:3n6 and C18:4n3, respectively, catalyses the first and rate-limiting step of VLC-PUFA synthesis (Tocher et al., 2006). The *fads2* gene encoding Δ6 has been cloned and characterised from a number of fish including tilapia (*Oni-fads2*). *Oni-fads2* encodes the bifunctional Δ5 and Δ6 enzyme, hence recombinant yeast expressing *Oni-fads2* could provide a single-cell microbial factory as an alternative source of LC-PUFAs (Tanomman et al., 2013; Zheng et al., 2005; Gonzalez-Rovira et al., 2009; Monroig et al., 2010).

To develop recombinant yeast expressing Δ6, the promoter should be optimised to maximise recombinant protein production. In general, inducible promoters have low basal activity but expression is increased upon induction. To develop recombinant yeast as a cell factory for expressing *Oni-fads2*, the inducer should be easy to manipulate, stable, inexpensive, and have no negative effect on cell growth. Heterologous expression of *Oni-fads2* has been demonstrated in *S. cerevisiae* using the galactose-inducible GAL1 promoter (p*GAL1*) (Tanomman et al., 2013). However, expression from p*GAL1* requires a medium containing low glucose (Tanomman et al., 2013; Zheng et al., 2005; Gonzalez-Rovira et al., 2009; Monroig et al., 2010), which is a major disadvantage

because *S. cerevisiae* prefers glucose and fructose as carbon sources, and galactose is expensive for use for industrial applications. A highly constitutive promoter that can drive high-level *Oni-fads2* expression is required to develop a recombinant *S. cerevisiae* cell factory with strong Δ6 activity for large-scale industrial fermentation. Well-defined endogenous and glucose-based promoters such as those driving expression of actin (p*ACT*), phosphor-glycerate kinase (p*PGK*) and translational elongation factor (p*TEF*) genes are candidates (Randez-Gil et al., 1995; Kingman et al., 1990; Peng et al., 2015; Nevoigt et al., 2006; Xie et al., 2015). Therefore, in the present study, we compared the performance of these three promoters for the expression of *Oni-fads2*. Conversion of exogenous C18:2n6 and C18:3n3 substrates to C18:3n6 and C18:4n3, respectively, was monitored to evaluate Δ6 activity, and the influence of the concentration of exogenous substrate on Δ6 activity was investigated. Furthermore, Δ6 activity was also determined using crude recombinant yeast extracts.

3.3 Objective

To study the performance of three promoters (p*ACT*, p*PGK* and p*TEF*) for the expression of *Oni-fads2*. Investigation of Δ6 activity by conversion of exogenous C18:2n6 and C18:3n3 substrates to C18:3n6 and C18:4n3, respectively, and determine Δ6 activity by using crude recombinant yeast extracts.

3.4 Materials and methods

3.4.1 Strain and culture media

S. cerevisiae cells were grown in YPD rich medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose) at 30 °C with shaking at 200 rpm in Erlenmeyer

flasks. After 24 h, *S. cerevisiae* cells were collected by centrifugation at 12,000 rpm for 5 min at 4 °C, washed three times with 0.85% NaCl, frozen and stored at -80 °C for genomic DNA and total RNA extraction, or at -20 °C for fatty acid extraction.

3.4.2 Construction of expression plasmids

S. cerevisiae strain DSY-5 (*MATalpha leu 2 trp1 ura3-52 his3 PGAL1-GAL4pep4 prb1-1122*) was used throughout this study. *S. cerevisiae* cells were grown in YPD rich medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose) at 30 °C with shaking at 200 rpm for 24 h.

Actin (p*ACT*) and phosphoglycerate kinase (p*PGK*) promoters was cloned from *S. cerevisiae* genomic DNA. *S. cerevisiae* cells (50 mg) were suspend in lysis buffer (40 mM TRIS-acetate, 20 mM sodium acetate, 1% w/v sodium dodecyl sulphate [SDS], pH 7.8) containing 0.04 g of glass beads (450-600 µm) and disrupted using two cycles of bead-beating for 5 min. After bead-beating, 0.5 µl of 100 mg/ml proteinase K (Promega, Madison, WI, USA) was added, and the mixture was incubated at 37 °C for 30 min. Cell lysis was performed using phenol-chloroform extraction and isopropanol precipitation. After centrifugation at 12,500 rpm for 30 min at 4 °C, the DNA pellet was resuspended in 300 µl of 1× TE buffer (10 mM TRIS-HCl, pH 8.0, 1 mM EDTA) containing 30 µg of RNase A (Life Technologies Inc., Carlsbad, CA, USA). The DNA solution was treated with RNase at 37 °C for 30 min, and phenol-chloroform purification and ethanol precipitation were performed, followed by centrifugation, and the DNA pellet was resuspended in nuclease-free water and adjusted a concentration of 50 ng/µl for PCR. DNA fragments of p*ACT* and p*PGK* promoters were cloned by PCR using primers ACT-F1 and ACT-R1, and PGK-F1 and PGK-R1, respectively, (Table 3.1). PCR was performed in a 50 µl volume containing 5 µl of genomic DNA template,

400 µM of each dNTP, 5 pmol of each primer, 2.5 mM $MgCl_2$, 1.0× LA Taq buffer, and 2.5 U LA Taq (TaKaRa Shuzo, Shiga, Japan). Reactions were carried out at 95 °C for 3 min, followed by 40 cycles of 45 s at 95 °C, 45 s at 59 °C, and 90 s at 72 °C, and a final elongation step at 72 °C for 5 min. PCR products of the expected size were isolated and purified using UltraClean 15 (MO Bio Laboratories, Solana Beach, CA, USA). PCR-amplified DNA fragments were cleaved with *Sac* I and *Spe* I restriction enzymes to generate expression vectors.

Plasmid *EvTC* (Figure 3.1A), containing the p*TEF* promoter and the cytochrome C1 terminator (*CYC1*), was obtained from the p427TEF yeast expression vector (Dualsystems Biotech, Schlieren, Switzerland). To construct plasmid *EvAC* and *EvPC* (Figure 3.1B, 3.1C), p*TEF* was digested with *Sac* I and *Spe* I, and ligated with digested p*ACT* or p*PGK* fragments. The p*ACT* and p*PGK* regions in plasmids *EvAC* and *EvPC*, respectively, were verified by DNA sequencing, and plasmids *EvTC*, *EvAC* and *EvPC* were digested with *Eco* RI and *Sal* I, and purified.

Total RNA was extracted from 50 mg of Nile tilapia liver using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Approximately 2 µg of total RNA was used to synthesise cDNA using a First-strand cDNA Synthesis Kit (GE Healthcare, Buckinghamshire, UK). The coding region of *Oni-fad2* was amplified with PCR using cDNA as template and two gene-specific primers TiF6RY_F and TiF6RY_R (Table 3.1). PCR was performed using LA Taq (TaKaRa) following the conditions described earlier. The amplicon of the expected size was isolated and purified using UltraClean 15 (MO Bio Laboratories). The PCR amplified *Oni-fads2* fragment was cleaved with *Eco* RI and *Sal* I and purified. Subsequently, to generate *EvT-Δ6-C*, *EvA-Δ6-C* and *EvP-Δ6-C*, the digested *Oni-fads2*

fragment was ligated into the *Eco* RI and *Sal* I sites of digested plasmids *EvTC*, *EvAC* and *EvPC*, respectively. All plasmid vectors were verified by DNA sequencing before transformation.

Table 3.1 Sequences of PCR primers used in this study.

Primer	Sequence	Primer order
ACT-F1	5'-GTTAACGCATGCGAGCTCGCATGCAGATC TTAATACGACTCACTATAGGGGTGTGGGGAA GCGGGTAAGCTGCC-3'	Clone Actin promoter
ACT-R1	5'-CCCGGGGGATCCTGATCAAGCGTGAAAAA TCTAA AAGCTGAT-3'	Clone Actin promoter
PGK-F1	5'-GTTAACGCATGCGAGCTCGCATGCAGATC TTAATACGACTCACTATAGGGGAAGTACCTT CAAAGAATGGGGTC-3'	Clone PGK promoter
PGK-R1	5'-CCCGGGGGATCCTGATCAGTTTTATATTTG TTGTA AAAAGTA-3'	Clone PGK promoter
TiF6RY_F	5'-ACTAGTGAATCCGAATTCATGGGAGGTGG AAGCC AGCAGACGG-3'	Clone *Oni-fads2*
TiF6RY_R	5'-AGATCTAAGCTTGTCGACTCTAGATCATTT ATGGA GATATGCATCCAGCCAG-3'	Clone *Oni-fads2*
Actin-F	5'-GACGACGCTCCTCGTGCTGTCTTCC-3'	RT-PCR
Actin-R	5'-GGGGCAACTCTCAATTCGTTGTAGA-3'	RT-PCR

3.4.3 Yeast transformation and RT-PCR

Plasmids *EvTC*, *EvAC*, *EvPC, EvT-Δ6-C, EvA-Δ6-C* and *EvP-Δ6-C* were separately transformed into *S. cerevisiae* DSY-5 using an S. c. Easy Comp Transformation Kit (Invitrogen). Four transformed clones were selected on YPD containing geneticin (G418). Genomic DNA extraction and PCR amplification of *Oni-fads2* using primers

TiF6RY_F and TiF6RY_R were performed for confirmation. PCR was carried out using GoTaq in a 10 µl volume containing 0.2 mM of each dNTP, 1 pmol of each primer, 2.5 mM $MgCl_2$, 5× Green GoTaq Flexi buffer, and 0.25 U GoTaq (Promega). Reactions were carried out as described above. As an internal control, *Sc-actin* (*Sc-act*) was amplified using primers Actin-F and Actin-R to assess the quality of genomic DNA extraction in a 10 µl volume using GoTaq (Promega). Reactions were carried out at 95 °C for 3 min, followed by 40 reaction cycles of 30 s at 95 °C, 30 s at 59 °C, and 30 s at 72 °C, with a final elongation step at 72 °C for 5 min. PCR products were analysed on a 2% agarose gel with ethidium bromide staining.

Yeast cells (~50 mg) were collected for total RNA extraction and cDNA synthesis. Four transformants of *EvT-Δ6-C, EvA-Δ6-C* and *EvP-Δ6-C* were grown in 1 ml of YPD, cells were collected by centrifugation, and RNA was extracted. Approximately 2 µg of total RNA was used to synthesise cDNA using a First-Strand cDNA Synthesis Kit (GE Healthcare), and primers TiF6RY_F and TiF6RY_R (Table 3.1) were used to amplify the *Oni-fads2* fragment. *S. cerevisiae* β-actin (*Sc-act*) was also amplified with RT-PCR using a primers Actin-F and Actin-R (Table 3.1) to confirm the quality of the cDNA template.RT-PCR was carried out using GoTaq (Promega) as described earlier, and products were analysed on a 2% agarose gel with ethidium bromide staining.

3.4.4 Δ6 desaturation activity

To determine the Δ6 desaturation activity with n6-PUFA as substrate, non-transformed *S. cerevisiae* (NT) and *EvTC*, *EvAC*, *EvPC*, *EvT-Δ6-C*, *EvA-Δ6-C*, and *EvP-Δ6-C* transformants were grown in 1 l of YPD containing exogenous substrate C18:2n6 at 0.1, 0.2 or 0.4 mM at 30 °C with shaking at 200 rpm in Erlenmeyer flasks.

Since *S. cerevisiae EvT-Δ6-C* exerted the highest Δ6 desaturation activity, we further tested whether it could convert C18:3n3 to C18:4n3 with n3-PUFA as substrate. NT and *S. cerevisiae EvTC* and *EvT-Δ6-C* cells were grown in 1 l of YPD containing exogenous substrate C18:3n3 at 0.2 mM. After 24 h, *S. cerevisiae* cells were collected by centrifugation at 12,000 rpm for 5 min at 4 °C, washed three times with 0.85% NaCl, and stored at -20 °C until use.

To investigate the Δ6 desaturation activity of crude cell extracts, *S. cerevisiae EvT-Δ6-C* cells were grown in 1 l of YPD at 30 °C for 24 h. Subsequently, cells were harvested, and 10 g of cells were resuspended in 10 ml of lysis buffer (100 mM potassium phosphate pH 7.2, 0.33 M sucrose), frozen in liquid nitrogen, thawed, and homogenyzed by five cycles of freezing for 2 min in liquid nitrogen and thawing for 2 min at 25 °C. Lysis buffer was added to the cell homogenate to a volume of 40 ml. Reactions with or without substrate were performed in triplicate by incubating the cell homogenate in the absence and presence of substrate C18:2n6 at 0.2 mM, respectively, with shaking at 200 rpm at 28 °C. After 2 h, each reaction was subjected to fatty acid composition analysis.

Fatty acid extraction and composition analysis were performed as described previously (Tanomman et al., 2013). Each fatty acid peak in the chromate-gram was identified by comparing its retention time to those fatty acid methyl ester (FAME) standards (Supelco Component FAME mix; Supelco Bellefonte, USA; Cayman Chemical Company, MI, USA). The fatty acid composition was calculated based on the area of each peak, and the amount was determined by comparison with the methylheptadecanoate (C17:0) internal standard.

3.4.5 Statistical analysis

All data were analysed by one-way analysis of variance using SPSS for Windows (Release 10; SPSS Inc. Chicago, IL, USA). When significant differences were found among groups, Tukey's tests were used to rank the groups. Differences were considered significant at $P<0.05$.

3.5 Results

The expression vector DSY-5 containing the p*TEF* promoter and the *CYC1* terminator was assigned as *EvTC* and used as empty vector for the p*TEF* promoter (Figure 3.1A). *S. cerevisiae* actin (p*ACT*) and phosphoglycerate kinase (p*PGK*) promoters were used to replace the p*TEF* promoter to generate control empty vector *EvAC* (Figure 3.1B, 3.1C). All control empty vectors (*EvTC*, *EvAC*, *EvPC*) were transformed into *S. cerevisiae*. Meanwhile, the cDNA corresponding to *Oni-fads2* was cloned and placed under the control of p*TEF*, p*ACT* and p*PGK* in *EvTC*, *EvAC* and *EvPC* to generate expression vectors *EvT*-Δ6-*C*, *EvA*-Δ6-*C* and *EvP*-Δ6-*C*, respectively, (Figure 3.1D-F). All expression vectors were transformed into *S. cerevisiae*, and the transformation efficiency was determined by PCR amplification of *Oni-fads2* using genomic DNA (Figure 3.1G-I).

The insertion of *Oni-fads2* into *EvTC*, *EvAC* and *EvPC* generated *EvT-Δ6-C* (D), *EvA-Δ6-C* (E) and *EvP-Δ6-C* (F), respectively. Determination of *Oni-fads2* by PCR was performed using genomic DNA extracted from non-transformed (NT) and transformed *EvTC* and *EvT-Δ6-C* (G), transformed *EvAC* and *EvA-Δ6-C* (H) and transformed *EvPC* and *EvP-Δ6-C* (I). Note that PCR amplification of *S. cerevisiae* actin *(Sc-act)* was used as an internal control to verify the quality of genomic DNA extracted

from all experimental yeast. The *Oni-fads2* amplicon was detectable in transformed *S. cerevisiae EvT-Δ6-C*, *EvA-Δ6-C* and *EvP-Δ6-C* but not in NT nor transformants *EvTC, EvAC* or *EvPC*.

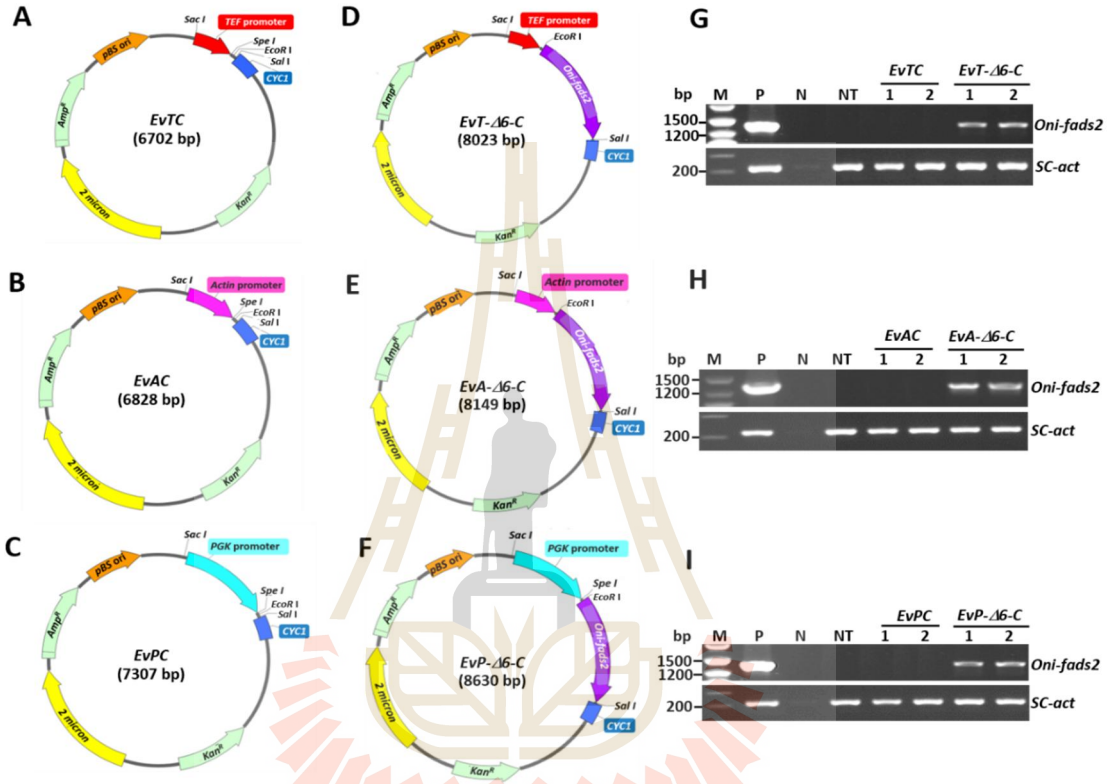

Figure 3.1 Maps of experimental expression vectors and PCR of *Oni-fads2* in transformants. Plasmid vectors *EvTC* (A), *EvAC* (B) and *EvPC* (C) containing p*TEF, pACT* and *pPGK* promoters, respectively, and the *CYC1* terminator (*CYC1*), were used as control empty vectors. M represents DNA markers. The negative control (N) included distilled water instead of DNA. Positive controls (P) for *Oni-fads2* or *Sc-act* used plasmid DNA containing *Oni-fads2* or *Sc-act*. p*BS* ori, pBluescript origin of replication; *Kan*^{R}, kanamycin resistance gene allowing selection of transformed *S. cerevisiae* in YPD containing G418; 2 *micron*, 2 micron origin of

replication allowing replication of plasmid in *S. cerevisiae* at high copy number; Amp^R, ampicillin resistance gene allowing selection of transformed *Escherichia coli*.

Expression in *S. cerevisiae* cells carrying *EvT*-Δ6-*C*, *EvA*-Δ6-*C* and *EvP*-Δ6-*C* was tested using RT-PCR, and the results showed that all strains produced *Oni-fads2* mRNA (Figure 3.2A). The Δ6 desaturation activity (conversion of C18:2n6 to C18:3n6) was then tested. Growth of transformants *EvT*-Δ6-*C*, *EvA*-Δ6-*C* and *EvP*-Δ6-*C* was scaled up in shake flasks in the presence of exogenously supplied fatty acid substrate C18:2n6. Before fatty acid analysis, genomic DNA was extracted from transformants *EvT*-Δ6-*C*, *EvA*-Δ6-*C* and *EvP*-Δ6-*C*, and PCR amplification of *Oni-fads2* was performed to validate the expression vectors (Figure 3.2B).

To evaluate promoter performance, NT cells carrying empty vector *EvTC*, *EvAC* and *EvPC*, and cells carrying expression vector *EvT*-Δ6-*C*, *EvA*-Δ6-*C* and *EvP*-Δ6-*C* were grown in shake flasks containing exogenous fatty acid C18:2n6. Table 3.2 shows the fatty acid composition of all experimental yeast strains. Transformants expressing *EvT*-Δ6-*C*, *EvA*-Δ6-*C* and *EvP*-Δ6-*C* yielded a peak identified as C18:3n6, but this was not observed with transformants *EvTC*, *EvAC* and *EvPC* (Figure 3.2C). These results revealed no detectable C18:3n6 fatty acid or other n6-PUFAs such as C20:3n6 or C20:4n6 in NT or *EvTC*, *EvAC* and *EvPC* cells (Table 3.2). By contrast, transformant *EvT*-Δ6-*C* produced a significant amount of the C18:3n6 product, followed by *EvA*-Δ6-*C* and *EvP*-Δ6-*C*. Similarly, the level of C20:3n6 produced by transformants *EvT*-Δ6-*C* and *EvA*-Δ6-*C* was higher than that of transformant *EvP*-Δ6-*C*, and C20:4n6 was observed only with transformant *EvT*-Δ6-*C*. The composition of other

detectable fatty acids appeared to be similar among experimental yeast, demonstrating that the Δ6 desaturation activity had no effect on general lipid metabolism in *S. cerevisiae* (Table 3.2).

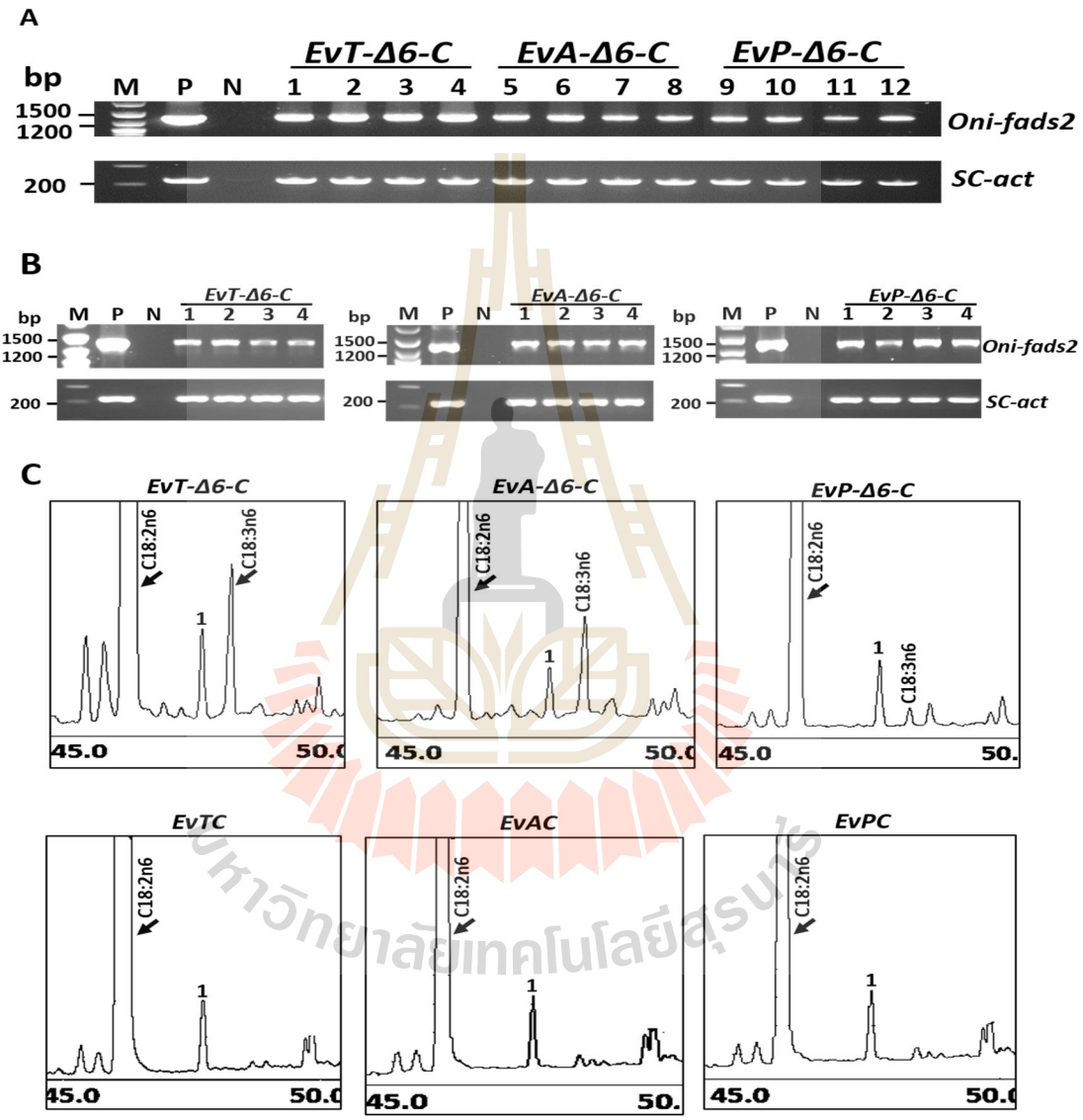

Figure 3.2 Expression of transformed *S. cerevisiae.* (A) RT-PCR of *Oni-fads2* in four transformants of *EvT-Δ6-C*, *EvA-Δ6-C* and *EvP-Δ6-C*. RT-PCR of *Sc-act* was used as an internal control to verify the quality of cDNA. In order to determine the Δ6 desaturation activity, four transformants of *EvT-Δ6-C*, *EvA-Δ6-C* and *EvP-Δ6-C* were cultured in YPD containing substrate

C18:2n6. Before fatty acid determination, transformed yeast were verified for the presence of *Oni-fads2*. (B) PCR of *Oni-fads2* and *Sc-act* from genomic DNA extracted from transformed yeast. *Sc-act* was used as an internal control to verify the quality of genomic DNA. M represents DNA markers. The negative control (N) included distilled water instead of DNA. Positive controls (P) for *Oni-fads2* or *Sc-act* used plasmid DNA containing *Oni-fads2* or *Sc-act* amplicon. (C) Determination of FA substrate C18:2n6 and product C18:3n6 using transformed *S. cerevisiae EvT-Δ6-C*, *EvA-Δ6-C*, *EvP-Δ6-C*, *EvTC*, *EvAC* and *EvPC*. Note that the peak corresponding to C18:3n6 was observed in transformed *S. cerevisiae EvT-Δ6-C*, *EvA-Δ6-C* and *EvP-Δ6-C*. See Table 3.2 for the mean amount of C18:2n6 and C18:3n6. 1 = C20:0.

To determine whether the concentration of exogenous substrate C18:2n6 had an effect on the Δ6 desaturation efficiency, NT and all transformants were grown in the presence substrate C18:2n6 at 0.1, 0.2 and 0.4 mM. Table 3.2 shows that the Δ6 desaturation product C18:3n6 was not observed in either NT nor cells carrying empty vectors (*EvTC*, *EvAC* or *EvPC*), and neither C20:3n6 nor C20:4n6 were detected. In addition, in all experimental yeasts, an increase in the concentration of exogenous substrate C18:2n6 led to an increase the amount of cellular C18:2n6 product, in a dose-dependent manner. In the presence of C18:2n6 substrate at 0.1 mM, the C18:3n6 product was observed in transformants expressing *EvT*-Δ6-*C*, *EvA*-Δ6-*C* or *EvP*-Δ6-*C*, and transformant *EvT*-Δ6-*C* accumulated the largest amount (Table 3.2). In the presence of C18:2n6 at 0.2 and 0.4 mM, C18:3n6, C20:3n6 and C20:4n6 were found in

transformant *EvT*-Δ6-*C*, *EvA*-Δ6-*C* and *EvP*-Δ6-*C*, and transformant *EvT*-Δ6-*C* displayed the highest Δ6 desaturation efficiency. The amount of product C18:3n6 in the transformant carrying *EvA*-Δ6-*C* or *EvP*-Δ6-*C* grown in media containing substrate C18:2n6 at 0.4 mM was higher than at a lower C18:2n6 concentration. However, the amount of product C18:3n6 in transformant *EvT*-Δ6-*C* grown in media containing substrate C18:2n6 at 0.2 mM was higher than in media containing C18:2n6 at 0.4 mM, demonstrating that an increase in the amount of substrate did not elevate the Δ6 desaturation efficiency. Taken together, our results suggest that p*TEF* was the strongest promoter for expressing *Oni-fads2*, hence *S. cerevisiae* cells carrying *EvT*-Δ6-*C* were used for further experiments.

To investigate the Δ6 desaturation activity with n3-PUFA substrate, we grew NT, *EvTC* and *EvT*-Δ6-*C* cells in media containing exogenous substrate C18:3n3, and the fatty acid C18:4n3 was detectable only in transformant *EvT*-Δ6-*C* (Table 3.3, Figure 3.3). In addition, other n3-PUFAs such as C20:4n3 and C20:5n3 were only detectable in transformant *EvT*-Δ6-*C*. Comparison of NT and *EvTC* revealed a higher C22:6n3 content in the latter, but the difference was not significant (Table 3.3).

Table 3.2 Fatty acid composition (mg/100 g lipid) of NT and transformed *S. cerevisiae* cells grown in YPD containing different concentrations of substrate C18:2n6 (mean±sd, n=4).

Fatty acids	C18:2n6C	C18:3n6	C20:3n6	C20:4n6
Substrate C18:2n6 0.4 mM				
NT	2,351.69±1.16^{a}	nd	nd	nd
EvTC	2,313.86±1.70^{a}	nd	nd	nd
EvT-Δ6-C	2,150.96±69.23^{b}	19.72±1.35^{a}	7.03±1.69^{a}	5.97±0.15^{a}
EvAC	2,340.61±35.43^{a}	nd	nd	nd
EvA-Δ6-C	2,189.51±7.00^{b}	14.36±0.89^{b}	6.33±0.14^{a}	3.13±0.10^{b}
EvPC	2,337.56±8.49^{a}	nd	nd	nd
EvP-Δ6-C	2,269.01±24.67^{ab}	8.43±0.08^{c}	3.15±0.24^{b}	1.99±0.01^{c}
Substrate C18:2n6 0.2 mM				
NT	750.49±25.07	nd	nd	nd
EvTC	743.29±33.16	nd	nd	nd
EvT-Δ6-C	833.82±12.02	42.15±3.39^{a}	1.92±0.53^{a}	0.96±0.01
EvAC	794.62±40.96	nd	nd	nd
EvA-Δ6-C	738.1±24.07	8.85±1.58^{b}	1.54±0.28^{a}	nd
EvPC	686.90±36.90	nd	nd	nd
EvP-Δ6-C	720.99±16.48	2.39±0.51^{c}	0.58±0.09^{b}	nd
Substrate C18:2n6 0.1 mM				
NT	374.85±2.61^{bc}	nd	nd	nd
EvTC	384.85±2.61^{ab}	nd	nd	nd
EvT-Δ6-C	355.53±0.95^{d}	8.15±0.02^{a}	nd	nd
EvAC	388.39±1.38^{a}	nd	nd	nd
EvA-Δ6-C	366.08±0.04^{c}	4.47±0.47^{b}	nd	nd
EvPC	389.97±5.00^{a}	nd	nd	nd
EvP-Δ6-C	388.64±1.79^{a}	1.79±0.57^{c}	nd	nd

Means with different superscripts in each column differ significantly from each other ($P<0.05$).

Table 3.3 Fatty acid composition (mg/100 g lipid) of NT and transformed *S. cerevisiae* grown in YPD containing different substrate C18:3n3 (mean±sd, n=4).

Fatty acids	**NT**	***EvTC***	***EvT-Δ6-C***
C8:0	3.53±1.29	3.45±0.36	3.62±0.24
C10:0	61.98±4.76	59.84±2.39	58.11±5.29
C12:0	61.81±9.47	60.07±2.66	58.59±3.04
C14:0	52.57±3.83	51.12±4.23	53.51±9.44
C16:0	733.29±64.18	706.12±36.73	703.01±15.09
C18:0	263.66±46.16	277.99±30.87	267.41±38.00
C20:0	10.59±3.07	10.41±3.88	11.05±3.03
C22:0	4.84±1.04	4.64±0.30	4.28±0.54
C14:1	2.08±0.99	2.18±0.48	2.16±0.29
C16:1	297.24±8.59	302.10±35.70	306.77±28.38
C18:1n9T	2.61±0.47	2.06±0.30	2.13±0.25
C18:1n9C	123.00±55.31	119.94±26.92	120.99±24.25
C22:1n9	4.69±0.54	5.04±0.59	4.76±0.45
C24:1	1.61±0.59	1.78±0.51	1.63±0.41
C20:2	4.15±0.52	4.10±0.55	4.25±0.11
C18:2n6T	2.24±0.64	2.38±0.23	2.50±0.39
C18:2n6C	29.79±2.96	29.58±5.16	26.20±7.26
C18:3n6	nd	nd	nd
C20:3n6	nd	nd	nd
C20:4n6	nd	nd	nd
C18:3n3	808.24±16.56[a]	822.56±58.03[a]	715.41±50.21[b]
C18:4n3	nd	nd	27.56±7.35
C20:3n3	nd	nd	9.20±4.85
C20:5n3	nd	nd	0.85±0.47
C22:6n3	19.72±2.45	19.77±2.00	24.94±3.71
ΣSFA	1,192.25±65.30	1,173.62±23.07	1,159.58±54.47
ΣMUFA	431.21±55.54	433.10±28.72	438.43±10.25
Σn6 PUFA	32.02±2.43	31.96±5.16	28.70±7.56
Σn3 PUFA	827.97±17.79	842.33±58.61	777.95±63.59

Means with different superscripts in each row differ significantly from each other ($P<0.05$).

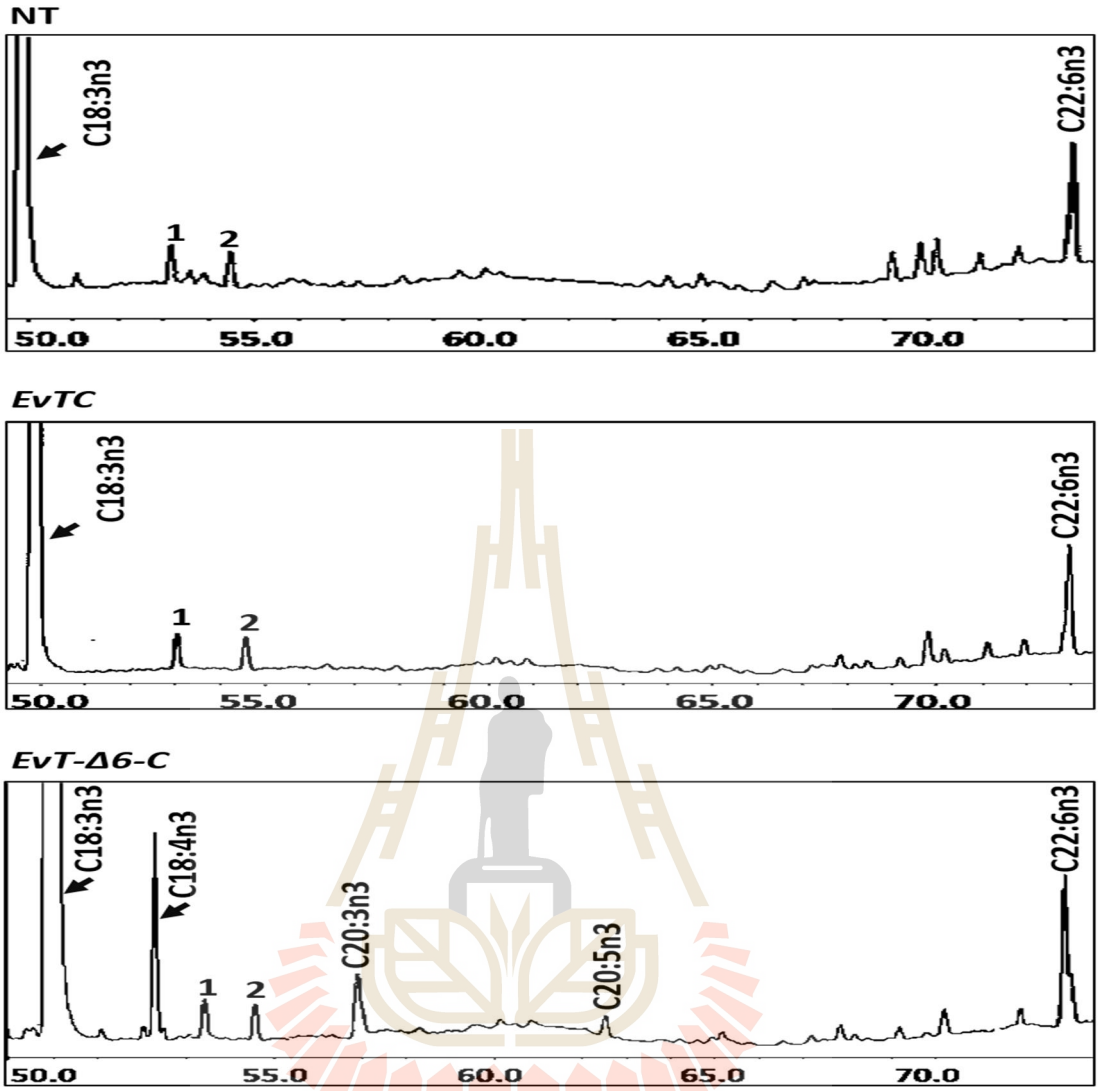

Figure 3.3 Determination of fatty acid substrate C18:3n3 and product C18:4n3 with NT and transformed *S. cerevisiae EvTC* and *EvT-Δ6-C*. Note that C20:3n3 and C20:5n3 were observed in transformed *S. cerevisiae EvT-Δ6-C* but not in NT or transformed *S. cerevisiae EvTC*. Transformed *S. cerevisiae EvT-Δ6-C* has a higher C22:6n3 content, but the difference is not significant. See Table 3 for the mean amount of C18:3n3, C18:4n3, C20:3n3, C20:5n3 and C22:6n3. 1=C20:2 and 2=C22:0.

The crude extract from transformant *EvT*-Δ6-*C* was analysed for Δ6 desaturation activity in the absence or presence of exogenously supplied C18:2n6 at 0.2 mM. Fatty acids C18:2n6, C18:3n6, C20:3n6 and C20:4n6 were detectable in the crude extract reaction in the presence of substrate C18:2n6 (Table 3.4, Figure 3.4). Additionally, a small amount of C18:2n6 was detectable in the crude extract reaction without adding exogenous C18:2n6.

Table 3.4 Fatty acid composition (mg/ 100 g lipid) achieved with a crude extract of *S. cerevisiae EvT-Δ6-C* cultured in the presence or absence of substrate C18:2n6 (mean±sd, n=3).

Fatty acids	**C18:2n6**	
	0 mM	**0.2 mM**
C18:2n6	73.95±3.60[b]	892.03±2.07[a]
C18:3n6	nd	9.35±0.15
C20:3n6	nd	6.42±0.39
C20:4n6	nd	1.55±0.04

Means with different superscripts in each row differ significantly from each other ($P<0.05$).

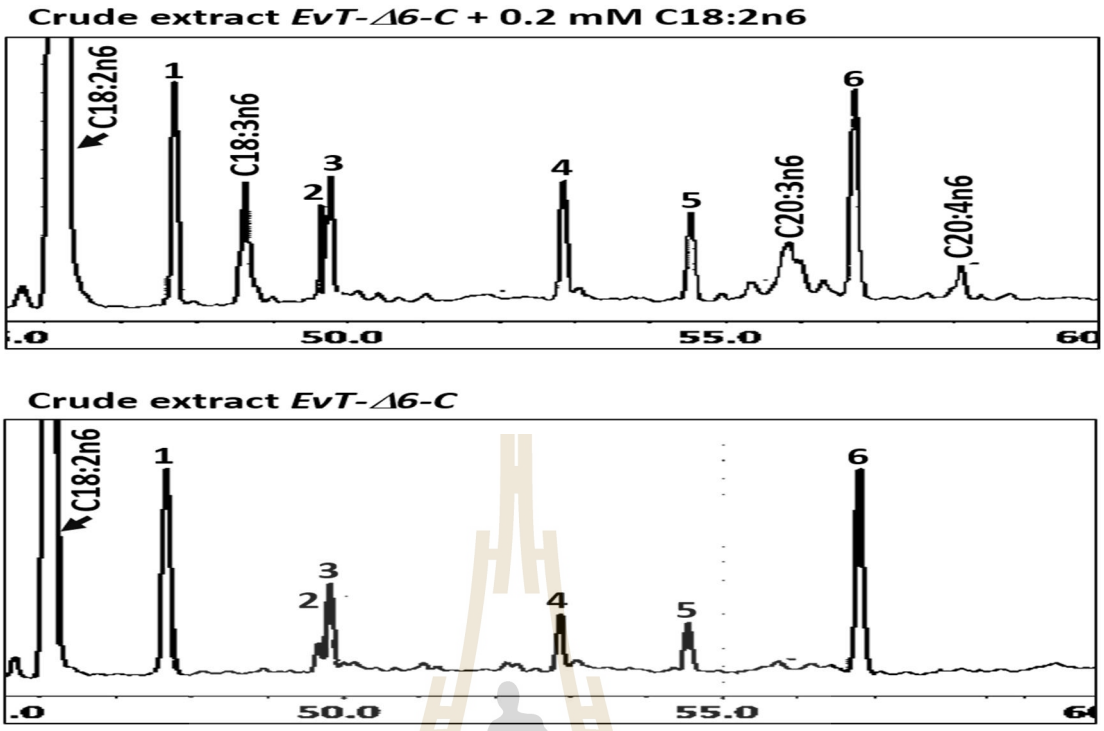

Figure 3.4 Crude extract of transformed *S. cerevisiae EvT-Δ6-C* showing Δ6 desaturation activity for the conversion of C18:2n6 into C18:3n6. When crude extract of transformed *S. cerevisiae EvT-Δ6-C* was incubated with C18:2n6, C18:3n6, C20:3n6 and C20:4n6 were detectable, but these were not observed in crude extract without substrate C18:2n6. See Table 4 for the mean amount of C18:2n6, C18:3n6, C20:3n6 and C20:4n6. 1=C20:0, 2=C20:1n9, 3=C18:3n3, 4=C20:2, 5=C22:0, 6=C22:1n9.

3.6 Discussion

Yeast, particularly, *S. cerevisiae*, have been used as microbial cell factories for the production of a wide range of food products, and are applied widely in science, food, feed, medicine and agriculture industries (Kim et al., 2015; Vieira et al., 2018). Recently, LC-PUFA production for functional foods and animal feeds has received significant attention (Simopoulos, 2000; Abedi and Sahari, 2014; Li and Hu, 2009).

Enzymes involved in LC-PUFA biosynthesis pathways have been cloned and characterised from various fish species including Nile tilapia (Tanomman et al., 2013; Zheng et al., 2005; Gonzalez-Rovira et al., 2009; Monroig et al., 2010). Heterologous expression of these enzymes has been conducted using an inducible galactose-based promoter. However, galactose-inducible promoter-mediated metabolic engineering of LC-PUFA production cannot be applied in *S. cerevisiae* because galactose is not the preferred carbon source for growth, and its use as inducer is not commercially viable. Instead, alternative glucose-based promoters driving the expression of enzymes related to LC-PUFA biosynthesis are required. In the present study, we developed constitutive glucose-based promoters to drive expression of *Oni-fads2* from Nile tilapia, which encodes the Δ6 enzyme that catalyses the first and rate-limiting step, generating a double bond in the LC-PUFA substrate. Our findings expand the applicability of *S. cerevisiae* for use as a microbial cell factory for the sustainable production of LC-PUFAs.

Heterologous expression of *Oni-fads2* in *S. cerevisiae* driven by the strong galactose-inducible *GAL*1 promoter (p*GAL*) demonstrated that Δ6 desaturation activity converts C18:2n6 into C18:3n6 when exogenous substrate C18:2n6 is supplied in media containing galactose (Tannoman et al., 2013). In addition, the galactose-inducible p*GAL*, *fads2* has been cloned and characterised from a number of plant, algae and fish species (Qiu et al., 2002; Zheng et al., 2005; Hsiao et al., 2007; Gonzalez-Rovira et al., 2009; Monroig et al., 2010). These findings suggest that p*GAL* can drive *fads2* expression to achieve high Δ6 activity, providing a useful tool analysis of *fads2* for activity. Since the preferred carbon sources for *S. cerevisiae* are generally glucose and fructose, we constructed an expression vector using the well-defined endogenous and glucose-based

promoters p*ACT*, p*PGK* and p*TEF*. Actin is an abundant protein and highly conserved among eukaryotic taxa, explaining why the actin gene is constitutively highly expressed (Gallwitz and Seidel, 1980; Ng and Abelson, 1980). As a result, p*ACT* has been used for the construction of expression vectors to produce heterologous proteins in *S. cerevisiae*. For example, p*ACT* was used to drive expression of *Aspergillus oryzae* α-amylase and *A. nidulans endoxylanase* in *S. cerevisiae* to improve the quality of breadmaking (Randez-Gil et al., 1995; Monfort et al., 1996). The glycolytic phosphoglycerate kinase (*PGK*) gene has been highly expressed in *S. cerevisiae*, and p*PGK* has been employed in yeast for direct heterologous protein production in media containing glucose (Dobson et al., 1982; Tuite et al., 1982; Mellor et al., 1985; Kingman et al., 1990; Peng et al., 2015).

Promoters of genes encoding the cellular translation machinery are also strong and constitutive, including that of *TEF*. The p*TEF* promoter has been engineered and employed for heterologous expression of proteins in yeasts including *S. cerevisiae* and *Yarrowia lipolytica* (Schirmaier and Philippsen, 1984; Nevoigt et al., 2006; Xie et al., 2015; Shabbir et al., 2015). For example, to produce engineered *S. cerevisiae* for the production of poly-3-D-hydrozybutyrate from xylose, p*TEF* was employed to drive acetyl-CoA acetyltransferase, an enzyme involved in the pathway for production of 3-D-hydrozybutyrate (Sandstrom et al., 2015). Thus, herein, three constitutive promoters, p*ACT,* p*PGK* and p*TEF*, were employed to drive the expression of *Oni-fads2*. Promoter strength was compared using shake flasks with nutrient-rich and glucose-rich media supplemented with exogenous substrate C18:2n6. The accumulation of product C18:3n6 was evaluated over 24 h of cultivation indicating that glucose was available over the entire cultivation period. Our results showed that *Oni-fads2* driven by the p*TEF*

promoter yielded the strongest Δ6 activity, followed by p*ACT*, and the p*PGK* promoter was the weakest. Similarly, using *lacZ* as a reporter gene, p*TEF* was demonstrated to be the strongest among seven promoters. Although expression driven by p*TEF* and p*PGK* was similar when glucose was abundant (8 h), p*TEF* appeared to drive higher expression than p*PGK* when glucose was exhausted (24 h) (Partow et al., 2010). In addition, using the *gfp* reporter gene, among 14 promoters including p*TEF* and p*PGK*, p*TEF* drove highest expression levels when both glucose and oxygen were abundant. Furthermore, p*TEF* also showed highest yielded highest expression under glucose-limited conditions (Sun et al., 2012). The p*TEF* and p*PGK* strength was similar for *gfp* expression in *S. cerevisiae* in flask batch cultivation in the presence of abundant glucose. However, following a diauxic shift in glucose batch cultures, p*TEF* performed less well than p*PGK* (Peng et al., 2015). Therefore, p*TEF* appears to be strongest among the three constitutive promoters, and therefore most suitable for driving *fads2* expression in simple nutrient-rich media at laboratory-scale, and possibly at larger scale in microbial cell factories for LC-PUFA synthesis.

In the present study, the ability of promoters to drive *Oni-fads2* expression for maximal Δ6 activity was evaluated by quantitative determination of fatty acid composition in cultured cells grown in media supplemented with exogenous fatty acid substrate. Although the incorporation of exogenous fatty acid substrates was not efficient and requires improvement, their uptake was sufficient to use as useful tools for functional analysis (Tripodi et al., 2018). The main fatty acids in *S. cerevisiae* are saturated, and the dominant fatty acids in all experimental *S. cerevisiae* strains were C16:0, C16:1, C18:0 and C18:1, consistent with the fatty acids content demonstrated previously in recombinant *S. cerevisiae* (Napier et al., 1998; Laoteng et al., 2000; Hsiao

et al., 2007; Gonzelez-Rovira et al., 2009; Tanomman et al., 2013). Our findings showed that increasing or decreasing the exogenous substrate C18:2n6 led to a corresponding increase or decrease in C18:2n6 product. Consistently, in heterologous expression of *fads2* from *Mortierella alpine and Micromonas pusilla* in *S. cerevisiae* in media containing 0.5 mM of C18:2n6 yielded higher cellular C18:2n6 than in media containing C18:2n6 and C18:3n3 at 0.25 mM. Also, accumulation of C18:3n3 was dose-dependent regarding supplementation of C18:3n3 in the culture medium (Shi et al., 2015). Our results showed that the amount product C18:3n6 in transformant *S. cerevisiae EvT-Δ6-C* grown in media containing C18:2n6 at 0.2 mM was higher than in media containing C18:2n6 at higher or lower concentrations. These findings suggest that the uptake mechanism of exogenous substrate fatty acids depended on the concentration of fatty acid supplementation. Nevertheless, substrate concentration had a minor effect on the performance of promoters in the conversion of substrate fatty acids into products.

Since p*TEF* was the strongest promoter, transformant *S. cerevisiae EvT-Δ6-C* was used for further study to evaluate the Δ6 activity in the conversion of C18:3n3 into C18:4n3. The Δ6 observed desaturation activity with n3-LC-PUFA substrate was similar to previous reports (Tanomman et al., 2013; Zheng et al., 2005; Gonzalez-Rovira et al., 2009; Monroig et al., 2010). Moreover, other n6- and n3- PUFAs including C20:3n6, C20:4n6, C20:3n3, C20:5n3 and C22:6n3 were detectable, but not in non-transformed *S. cerevisiae* and *S. cerevisiae EvTC* carrying empty vectors. Indeed, heterologous expression of *Oni-fads2* also resulted in Δ5 activity with n6-LC-PUFA substrate, suggesting that *Oni-fads2* expression produced the bifunctional enzyme (Tanomman et al., 2013). Mitochondria elongase activity leading to elongation

of endogenous fatty acids has been demonstrated in *S. cerevisiae* to (Bessoule et al., 1987). Therefore, whether *Oni-fads2* could possess other enzyme activities, increase the amount of substrate C18:2n6 or C18:3n3 or their respective products, or induce endogenous desaturase and elongase activities remained to be investigated.

Crude cell extracts can be used to measure enzyme activity and hence functional expression of heterologous enzymes in yeast and *E. coli* (Kalscheuer et al., 2004; Cardillo et al., 2008). This approach can be used to investigate the biochemical functions of recombinant proteins in yeast (DiRusso et al., 2005). In the present work, to validate Δ6 activity, crude extracts of recombinant *S. cerevisiae EvT-Δ6-C* carrying *Oni-fads2* driven by p*TEF* were tested for Δ6 activity with n6-LC-PUFA substrate. A small amount of C18:2n6 was detectable in crude extracts without exogenous substrate C18:2n6, but no C18:3n6 product was detectable, demonstrating that low levels of substrate were insufficient for Δ6 activity assays. However, when the *EvT-Δ6-C* cell extract was supplied with exogenous substrate C18:2n6, the C18:3n6 product and other n6-PUFAs such as C20:3n6 and C20:4n6 were detected. These findings suggest that recombinant *S. cerevisiae EvT-Δ6-C* could be applicable for use in a microbial cell factory expressing recombinant Δ6.

3.7 Conclusion

In conclusion, we tested the performance of three promoters for heterologous expression of *Oni-fads2*, and p*TEF* exhibited the strongest promoter activity to drive Δ6 activity with both n6- and n3-PUFA substrates. Additionally, a crude extract of recombinant *S. cerevisiae* carrying *Oni-fads2* driven by p*TEF* could be applicable for

use in a microbial cell factory to express recombinant Δ6 and thereby produce LC-PUFAs in an efficient, affordable and sustainable manner.

3.8 References

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CHAPTER IV

PRODUCTION OF RECOMBINANT *S. CEREVISIAE* THAT STABLY EXPRESS THE DELTA 6 DESATURASE (*ONI-FADS2*) FROM NILE TILAPIA (*O. NILOTICUS*)

4.1 Abstract

This study aimed to produce recombinant *Saccharomyces cerevisiae* (RY-Δ6) stably expressing fatty acid delta 6 desaturase from Nile tilapia (*Oni-fads2*). The expression cassette of *Oni-fads2* driven by Translation elongation factor I (TEF I) promoter which was ligated with ribosomal RNA gene was constructed. These expression cassettes employed their integration to *S. cerevisiae* genome. The integration of the recombinant gene construct in the genome was confirmed by DNA sequencing. The heterogenous expression *Oni-fads2* in RY-Δ6 at transcription and translation levels were detectable by RT-PCR and western blot analysis, respectively. RY-Δ6 exhibited delta 6 desaturase (Δ6) activity by converting C18:2n6 and C18:3n3 to C18:3n6 and C18:4n3, respectively, when it was cultured in the presence of exogenous substrate C18:2n6 and C18:3n3. The efficiency of Δ6 activity in RY-Δ6 depends on amount of concentration of substrate and temperature and period of reaction. The efficiency of Δ6 activity in RY-Δ6 was highest when the reaction was performed at 28 °C for 2 hours. In addition, crude extract of RY-Δ6 displayed Δ6 activity. Crude extract of RY-Δ6 were capable to change the fatty acid composition by

increasing the fatty acids of n6- and n3-PUFA when it was incubated in soybean oil and linseed oil.

4.2 Introduction

Recombinant protein technology has become a powerful tool to produce functional protein that overcome the limitations imposed by extraction of natural sources. Yeast *S. cerevisiae* is generally recognized as safe (GRAS) organism and widely used as host for heterologous protein expression (Martinez et al., 2012). Recently, functional protein production by recombinant *S. cerevisiae* has been successfully applied for production of a number of functional proteins used in modern industries for functional food, feed, biopharmaceutical and chemical (Kim et al., 2015; Milton et al., 2018).

Metabolic engineering in *S. cerevisiae* by expressing enzymes that are involved in LC-PUFA synthesis would enable its used as microbial cell factory to produce sustainable LC-PUFA source for global food safety and food security. From previous study, the promoters performances including *Act*, *PGK* and *TEF* promoters were compared their promoter performances to express *Oni-fads2*. The previous results showed that p*TEF* promoter was the strongest promoter to drive *Oni-fads2* to produce recombinant *S.cerevisiae* expressing delta 6 desaturase activity (Δ6) which was designated *EvT-Δ6-C*. The recombinant *S. cerevisiae* carried extrachromosomal plasmid *EvT-Δ6-C* (non-integrative plasmid) exerted the ability to convert C18:2n6 and C18:3n3 into C18:3n6 and C18:4n3, respectively, providing a potential use as yeast cell factory for LC-PUFA. However, the non-integrative plasmids are instability due to plasmid loss (Kilonzo et al., 2009; Aherm et al., 1988), segregational instability and/or allele

segregation (Impoolsup et al., 1989; Patnaik, 2000; Friehs, 2004). Consequently, there would be a problem in gene expression within the population of recombinant yeast (Da Silva and Srikrishnan, 2012; Karim et al., 2013), especially during long term and large-scale industrial cultivations.

Due to these limitations, stable expression of recombinant yeast which carry the recombinant DNA cassette in its chromosome would be preferred for industrial production. The genome integration of foreign DNA could be accomplished via homologous recombination. Mostly, expression vector was engineered using *E. coli* as host (Anathy et al., 2001; Funkenstein et al., 2005). Subsequently, the expression vector which contained the desired promoter, target gene and selection marker was linearized and introduced into the competent *S. cerevisiae* (Sikorski and Hieter, 1989). However, the site-specific integration of linear DNA fragments was flanked by homologous arms (Storici et al., 2003). The location of integration is one of the main factor for integration efficiency. For instance, with delta locus integration, multi-copy integration of heterologous genes were observed at the Ty retrotransposon delta sites in the yeast genome (Da Silva and Srikrishnan, 2012; Sakai et al., 1990; Shi et al., 2014; Yamada et al., 2010; Yuan and Ching, 2013; Yuan and Ching, 2014).

Since yeast genome contained multiple copies of ribosomal genes, ribosomal gene loci were demonstrated as the appropriate position for target gene recombination. The homologous recombination of foreign gene into ribosomal RNA genes would enable to increase the copy number of recombinant DNA transferred into yeast genome. Gene transfer by targeting ribosomal RNA position was demonstrated in *S. cerevisiae*. High copy number of recombinant gene in chromosome of yeast were observed with high expression protein level (Fuji et al., 1990; Klabunde et al. 2003; Liu et al., 2012).

In this study, therefore, recombinant *S. cerevisiae* (RY-Δ6) that carrying the expression cassette of *EvT-Δ6-C* was produced. The RY-Δ6 showed stable expression of the delta 6 desaturase. The Δ6 desaturation activity were evaluated using C18:2n6, C18:3n3 and vegetable oil as substrate.

4.3 Objective

To produce recombinant *S. cerevisiae* that stably expressed *Oni-fads2* which exerted Δ6 desaturation activity and evaluate the Δ6 activity in several substrate fatty acids including C18:2n6, C18:3n3 and vegetable oil.

4.4 Materials and methods

4.4.1 Yeast strain and culture media

S. cerevisiae strain DSY-5 (*MATalpha leu 2 trp1 ura3-52 his3 PGAL1-GAL4pep4 prb1-1122*) were grown in YPD rich medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose) at 30 °C with shaking at 200 rpm in Erlenmeyer flasks. After 24 h, *S. cerevisiae* cells were collected by centrifugation at 12,000 rpm for 5 min at 4 °C, washed three times with 0.85% NaCl, frozen and stored at -80 °C for genomic DNA and total RNA extraction, or at -20 °C for fatty acid extraction.

4.4.2 Production of expression cassette

The expression cassette (*R45-EvT-Δ6-C-URA3-R67*) (Figure 4.3) was constructed for transformation into yeast to generate RY-Δ6. The resulted DNA cassette was integrated at ribosomal RNA loci in yeast chromosome.

Genomic DNA extraction and cloning of DNA for ribosomal RNA

Genomic DNA extraction from *S. cerevisiae* DSY-5 (*MATalpha leu 2 trp1 ura3-52 his3 PGAL1-GAL4pep4 prb1-1122*) was performed for cloning of DNA for ribosomal RNA. Yeast cells (50 mg) were suspend in lysis buffer (40 mM TRIS-acetate, 20 mM sodium acetate, 1% w/v sodium dodecyl sulphate [SDS], pH 7.8) containing 0.04 g of glass beads (450-600 µm) and disrupted using two cycles of bead-beating for 5 min. After bead-beating, 0.5 µl of 100 mg/ml proteinase K (Promega, Madison, WI, USA) was added, and the mixture was incubated at 37 °C for 30 min. Cell lysis was performed phenol-chloroform extraction and isopropanol precipitation. After centrifugation at 12,500 rpm for 30 min at 4 °C, the DNA pellet was resuspended in 300 µl of 1× TE buffer (10 mM TRIS-HCl, pH 8.0, 1 mM EDTA) containing 30 µg of RNase A (Life Technologies Inc., Carlsbad, CA, USA). The DNA solution was treated with RNase at 37 °C for 30 min, and phenol-chloroform purification and ethanol precipitation were carried out, followed by centrifugation, and the DNA pellet was resuspended in nuclease-free water, adjusted a concentration of 50 ng/µl and used as template for PCR throughout of this study.

Cloning of Uracil gene (*URA3*)

The plasmid pYES2.1/V5-His-TOPO (Invitrogen) was used as template for cloning of the Uracil gene (*URA3*) using two gene-specific primers (URA3-F and URA3-R; Table 4.1). The PCR was performed in a 50 µl volume containing 50 ng of pYES2.1, 400 µM of each dNTP, 5 pmol of each primer, 2.5 mM $MgCl_2$, 1.0x LA Taq buffer, and 2.5 U LA TAq (Takara Shuzo, Shiga, Japan). Reactions were carried out at 95 ºC for 3 min, followed by 40 cycles of 45 s at 95 ºC, 45 s at 60 ºC and 90 s at 72 ºC, and a final elongation step at 72 ºC for 5 min.

PCR products of the expected size were isolated and purified using the UltraClean™ 15 (MO Bio Laboratories, Solana Beach, CA, USA). The amplicon was ligated into pGEM®-T Easy (Promega) and subsequently transformed into *E. coli*. The plasmid containing *URA3* (p*URA3*) was verified by sequencing Macrogen, Inc. (Korea). Subsequently, p*URA3* was digested with *Kpn*I, performed alkaline phosphatase and kept for further use.

Cloning of ribosomal RNA (rRNA67)

The fragment of ribosomal RNA gene (rRNA67) was amplified by PCR using genomic DNA as template and two gene-specific primers (R6-F and R7-R; Table 4.1). The PCR reaction was carried out in a 10 µL total volume containing 200 µM of each dNTP, 1 pmol of each primer, 1.0× buffer EX Taq™ (within Mg^{2+}) and 0.25 U EX Taq™ (Takara Shuzo, Shiga, Japan). The PCR reaction was carried out at 95 °C initial denaturation for 3 min, and then 40 reaction cycles were run, each consisting of 45 s at 95 °C for denaturation, 30 s at 60 °C for annealing, and 45 s at 72 °C for extension. The final elongation step was 72 °C for 15 min. The PCR products of the expected size were isolated and purified using the UltraClean™ 15 (MO Bio Laboratories). The amplicon was ligated into pGEM®-T Easy (Promega) and subsequently transformed into *E. coli*. The plasmid ribosomal RNA67 (prRNA67) was verified by sequencing Macrogen, Inc. (Korea). The plasmid prRNA67 were digested by *Kpn I*. The DNA fragment of rRNA67 was purified and kept for further use.

Construction of p*URA3*-R67

The DNA fragment of rRNA67 was inserted in the p*URA3* to generate p*URA3*-R67 (Figure 4.1). The p*URA3*-R67 was transformed into *E. coli*. The plasmid p*URA3*-R67 was extracted, purified and verified by sequencing at Macrogen, Inc. (Korea).

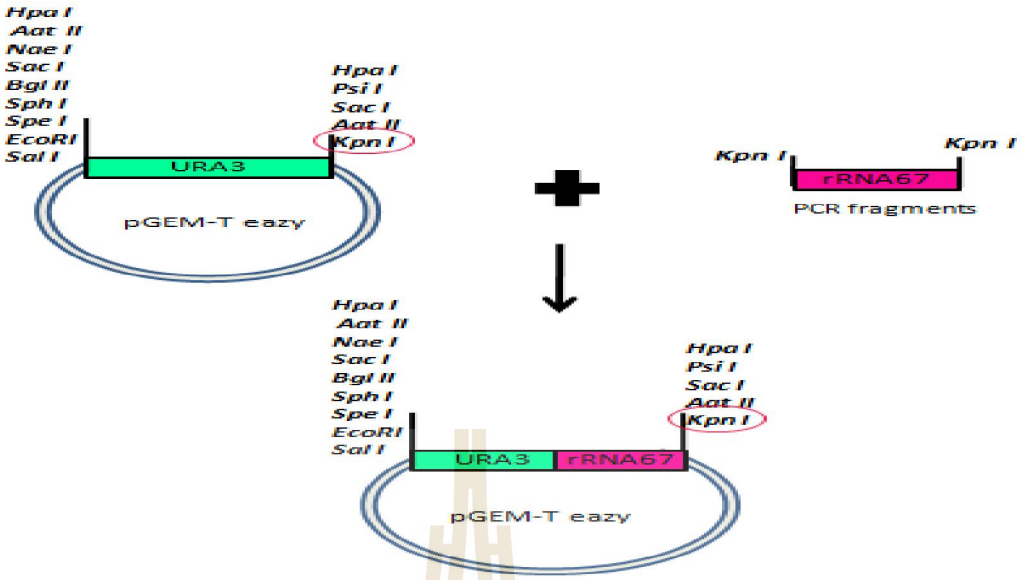


Figure 4.1 Construction of p*URA3*-R67.

Cloning of expression vector *EvT-Δ6-C* (pGEM-EvT-*Δ6-C*)

The plasmid p*EvT-Δ6-C* (Chapter 3; Figure 3.1D) was used as template for cloning of pGEM-*EvT-Δ6-C*. The expression cassette *EvT-Δ6-C* was amplified by PCR with p*EvT-Δ6-C* template using two gene-specific primers (TEF-F and CYC-R; Table 4.1). The PCR was performed using LA Taq™ (Takara) following the conditions described above. PCR product of the expected size was isolated and purified using the UltraClean™ 15 (MO Bio Laboratories). The amplicon was ligated into pGEM®-T Easy (Promega) and subsequently transformed into *E. coli*. The plasmid pGEM-*EvT-Δ6-C* was verified by sequencing at Macrogen, Inc. (Korea). Later, pGEM-*EvT-Δ6-C* was digested with *Bgl II*, performing alkaline phosphatase and purified.

Table 4.1 Primer in experiment.

Primer	Sequence	Remark
TiF6RY_F	5'-ACTAGTGAATCCGAATTCATGGGAGGT GGAAGCCAGCAGACGG-3'	Clone FAD6
TiF6RY-R	5'-AGATCTAAGCTTGTCGACTCTAGATCAT TTATGGAGATATGCATCCAGCCAG-3'	Clone FAD6
TEF_F	5'-GTTAACGCATGCGAGCTCGCATGCAGAT CTATAGCTTCAAAATGTTTCTAC-3'	PCR
CYC_R	5'-GTTAACGCATGCGGTACCGTACCGGCC GCAAATTAAAGC-3'	PCR
NiFAD6-F1	5'-ATGGGAGGTGGAAGCCAGCAGACGG-3'	RT-PCR
NiFAD6-R1	5'-TCATTTATGGAGATATGCATCC-3'	RT-PCR
FADd6_1	5'-TGCAGCATGACTTTGGCCACCTGTC-3'	PCR
D6F1	5'-CGAGCAGTCCTTCTTCAACGACTGG-3'	PCR
D6R1	5'-CTCTCCGATCAGCAGCGGCTTCAGA-3'	PCR
ActinSC-F	5'-GACGACGCTCCTCGTGCTGTCTTCC-3'	RT-PCR
ActinSC-R	5'-GGGGCAACTCTCAATTCGTTGTAGA-3'	RT-PCR
URA3-F	5'-GTTAACGACGTCGCCGGCGAGCTCAGAT CTGCATGCACTAGTGAATTCGTCGACGGGT AATAACTGATATAATTAAATT-3'	Clone URA3
URA3-R	5'-GTTAACTTATAAGAGCTCGACGTCGGTAC CTAGCTTTTCAATTCAATTCATCATT-3'	Clone URA3
R4_F	5'-AGATCTTATTGAGACCATGAGAGTAGC-3'	Clone rRNA45
R5_R	5'-AGATCTGTGACAGGTGCCCCGGGTAAC-3'	Clone rRNA45
R6_F	5'-GGTACCGTTACCCGGGGCACCTGTCAC-3'	Clone rRNA67
R7_R	5'-GGTACCTTTCCTCTAATCAGGTTCCAC-3'	Clone rRNA67

Cloning ribosomal RNA45 (rRNA45) and construction of pGEM-*R45-EvT-Δ6-C*

The DNA of ribosomal fragment (rRNA45) was amplified by PCR using genomic DNA with two gene-specific primers (R4-F and R5-R; Table 4.1). The PCR was carried out in a 10 µL total volume containing 200 µM of each dNTP, 1 pmol of each primer, 1.0x buffer EX Taq™ (within Mg^{2+}) and 0.25 U EX Taq™ (Takara Shuzo, Shiga, Japan). The PCR reaction and condition was carried out as described as the above. The PCR products of the expected size were isolated and purified using the UltraClean™ 15 (MO Bio Laboratories). The amplicon was ligated into pGEM®-T Easy (Promega) and subsequently transformed into *E. coli*. The plasmid ribosomal RNA45 (rRNA45) was verified by sequencing at Macrogen, Inc. (Korea). In order to generate pGEM-*R45-EvT-Δ6-C*, DNA of rRNA45 was digested with *Bgl II*, purified and ligated into linearized pGEM-*EvT-Δ6-C*. The pGEM-*R45-EvT-Δ6-C* was subsequently transformed into *E. coli*. The plasmid pGEM-*R45-EvT-Δ6-C* (Figure 4.2) was extracted, purified and verified by sequencing at Macrogen, Inc. (Korea).

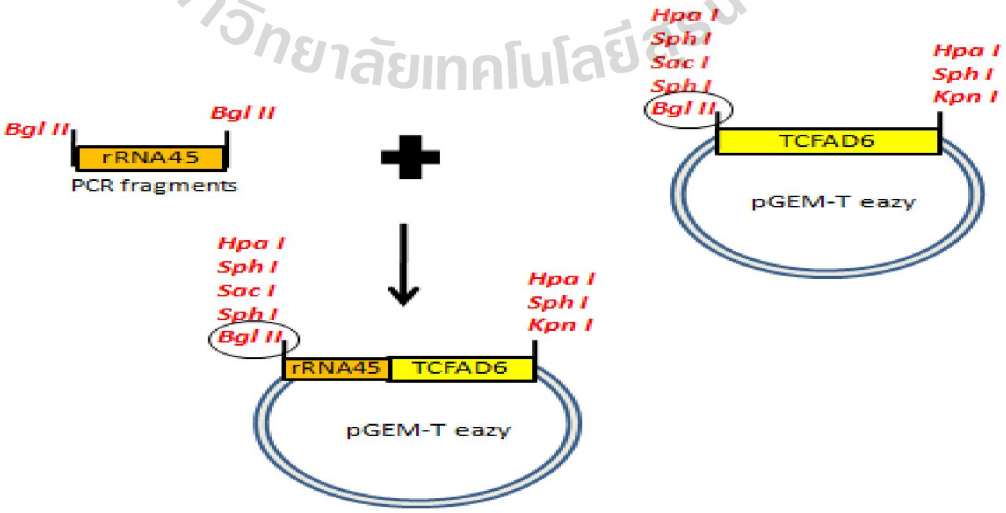


Figure 4.2 Construction of pGEM- *R45-EvT-Δ6-C*.

Construction of the expression cassette (*R45-EvT-Δ6-C-URA3-R67*)

The plasmid pGEM-*R45-EvT-Δ6-C* was digested with *Sph* I and performed alkaline phosphatase. The DNA fragment of *URA3*-R67 was obtained by digesting the p*URA3*-R67 with *Sph* I. The digested DNA fragment of *URA3*-R67 was purified and ligated into linearized pGEM-*R45-EvT-Δ6-C* to generate p*R45-EvT-Δ6-C-URA3-R67*. The plasmid p*R45-EvT-Δ6-C-URA3-R67* was transformed into *E. coli*. The plasmid p*R45-EvT-Δ6-C-URA3-R67* (Figure 4.3) was extracted, purified and verified by sequencing at Macrogen, Inc. (Korea).

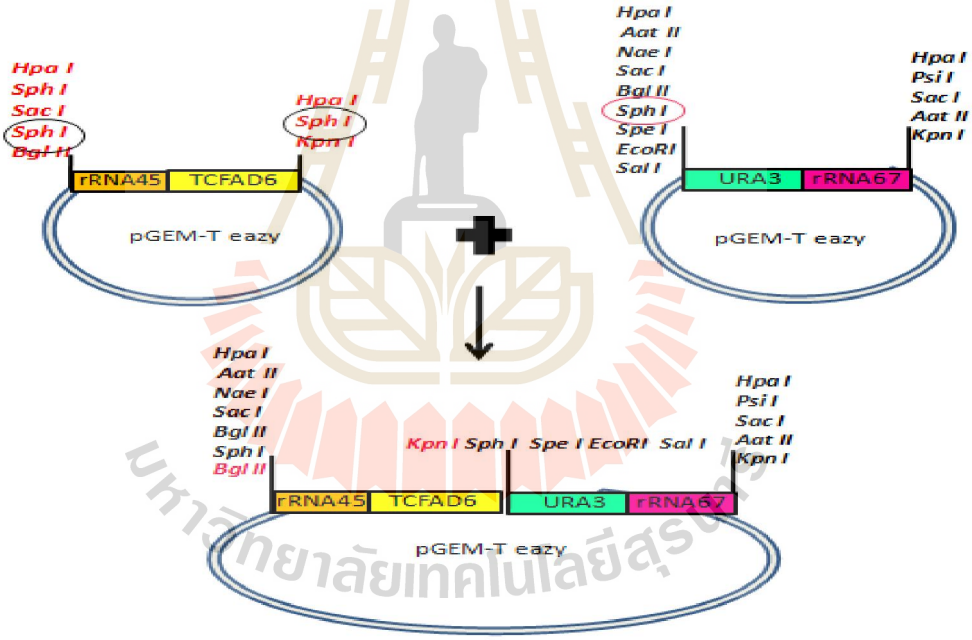


Figure 4.3 Construction of the expression cassette (*R45-EvT-Δ6-C-URA3-R67*).

The expression cassette *R45-EvT-Δ6-C-URA3-R67* which contained ribosomal RNA (*rRNA45*), TEF promoter, *Oni-fads2*, CYC terminal, URA3 gene and ribosomal RNA (*rRNA67*) was used as PCR template. Subsequently, the DNA fragment of *R45-EvT-Δ6-C-URA3-R67* was amplified using two gene-specific primers (R4-F and

R7-R; Table 4.1). The PCR was performed using LA Taq™ (Takara) following the conditions described above. The PCR reaction was carried out at 95 °C initial denaturation for 3 min, and then 40 reaction cycles were run, each consisting of 45 s at 95 °C for denaturation, 45 s at 62 °C for annealing, and 90 s at 72 °C for extension. The final elongation step was 72 °C for 15 min. The PCR products of the expected size were analyzed by agarose gel electrophoresis, performed phenol/chloroform purification and kept for further use for transformation.

4.4.3 Yeast transformation

The amplicon of expression cassette *R45-EvT-Δ6-C-URA3-R67* (Figure 4.4) was transformed into *S. cerevisiae* DSY-5 using the S. c. EasyComp™ Transformation Kit (Invitrogen). Transformed *S. cerevisiae* was selected on 2% glucose with minimal medium plate lacking uracil (SC-Uracil) incubated on 30 °C for 3-4 days. Four colonies of transformed yeast expressing Δ6 desaturation (RY-Δ6) were selected to determine the presence of transformed expression cassette.

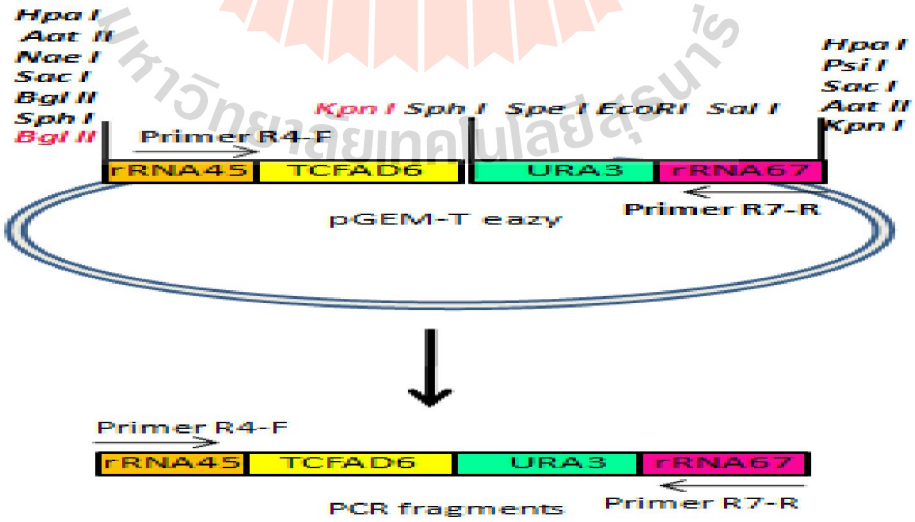


Figure 4.4 The amplicon of expression cassette *R45-EvT-Δ6-C-URA3-R67* was amplified by PCR.

Determination of the presence of transformed expression cassette in RY-Δ6 was conducted by PCR. The genomic DNA was extracted in lyticase buffer and 10 mM EDTA and performed using 0.04 g of glass bead (size 450-600 μm) and Bead beater instrument (5 min × 2 time). Centrifugation at 12,000 rpm for 5 minutes at 4 °C and transferred the upper phase to new tube. Subsequently, upper phase were diluted in nuclease-free water (1:10) for using DNA template PCR. The PCR genomic DNA of RY-Δ6 using 6 gene-specific primers (ActinSC-F and ActinSC-R, NiFAD6-F1 and NiFAD6-R1, TEF-F and D6R1, FADd6_1 and NiFAD6-R1, D6F1 and NiFAD6-R1, URA3-F and URA3-R; Table 4.1). The PCR was carried out in a 10 μL total volume containing 0.2 mM of each dNTP, 1 pmol of each primer, 2.5 mM $MgCl_2$, 5x Green GoTaq® Flexi buffer, and 0.25 U GoTaq® (Promega). The PCR reaction was carried out at 95 °C initial denaturation for 3 min, and then 40 reaction cycles were run, each consisting of 45 s at 95 °C for denaturation, 45 s at 59 °C for annealing, and 90 s at 72 °C for extension. The final elongation step was 72 °C for 5 min. The PCR products were analyzed on 2% agarose gels with ethidium bromide staining.

4.4.4 Analysis of the expression cassette in RY-Δ6

The recombinant yeast (RY-Δ6) was extracted genomics DNA following the method described above. The Cloning 8 fragment (subcloning) were amplified by PCR genomics DNA of RY-Δ6 using gene-specific primers were included fragment 1 (R4-F and TEF-R2 primer; 815 bp), fragment 2 (TEF-F and D6R1 primer; 729 bp), fragment 3 (NiFAD6-F1 and B6R1 primer; 886 bp), fragment 4 (FADd6-1 and NiFAD6-R1 primer; 803 bp), fragment 5 (D6F1 and CYC-R primer; 498 bp), fragment 6 (CYC-F and URA3-R2 primer; 803 bp), fragment 7 (URA-F2 and URA-R1 primer; 860 bp) and fragment 8 (URA-F3 and R7-R primer; 871 bp) (Figure 4.5). The PCR

was performed using EX Taq™ (Takara) and PCR reaction was carried out at 95 °C initial denaturation for 3 min, and then 40 reaction cycles were run, each consisting of 45 s at 95 °C for denaturation, 45 s at 59 °C for annealing, and 90 s at 72 °C for extension. The final elongation step was 72 °C for 5 min. PCR products of the expected size were isolated and purified using the UltraClean™ 15 (MO Bio Laboratories, Solana Beach, CA, USA). The amplicon was ligated into pGEM®-T Easy (Promega) and subsequently transformed into *E. coli*. The plasmid DNA was verified by sequencing at Macrogen, Inc. (Korea) and analyzed the sequence of nucleotides.

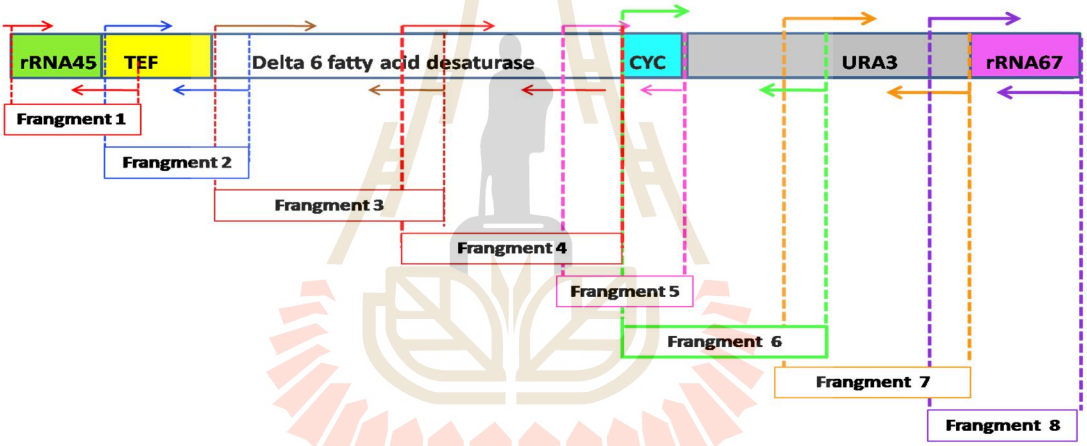


Figure 4.5 The Cloning 8 fragment (subcloning) were amplified by PCR genomics DNA of RY-Δ6.

4.4.5 Heterologous expression of *fads2* gene in RY-Δ6

The RY-Δ6 was grown in 2% glucose SC-Uracil at 30 °C for 18 h. The RY-Δ6 approximate 50 mg (two replicates) was collected for total RNA extraction and subsequent cDNA synthesis. The RT-PCR for *Oni-fads2* was performed using GoTaq® (Promega) with two gene specific primers (NiFAD6-F1 and NiFAD6-R1; Table 4.1). In addition, RT-PCR for the β-actin of *S. cerevisiae* (SC-actin) was also performed

using a pair of primers (ActinSC-F and ActinSC-R; Table 4.1) to assure the quality of the cDNA template. Both RT-PCR assays were carried out with GoTaq® (Promega), following the conditions described above.

The expression of the recombinant yeast (RY) was evaluated at different time periods of cultivation: at 4, 8, 12, 16, 24, 48 and 72 hour. The RY was grown in 2% glucose SC-Ura and YPD broth at 30 °C for 18 hour. The RY was approximate 50 mg (two replicates) was collected for total RNA extraction and subsequent cDNA synthesis. The RT-PCR for *Oni-fads2* was performed using GoTaq® (Promega) with two gene specific primers (NiFAD6-F1 and NiFAD6-R1; Table 4.1). In addition, RT-PCR for the β-actin of *S. cerevisiae* (SC-actin) was also performed using a pair of primers (ActinSC-F and ActinSC-R; Table 4.1) to assure the quality of the cDNA template. Both RT-PCR assays were carried out with GoTaq® (Promega), following the conditions described above. The PCR products were analyzed on 2% agarose gels with ethidium bromide staining.

4.4.6 Analyze delta 6 desaturase in RY-Δ6 by western blot analysis

The RY-Δ6 and wild type (NT) yeast were grown in YPD both at 30 °C for 18 h at shaking speed of 200 rpm. The RY-Δ6 and NT yeast approximate 800 mg (two replicates) was collected for protein extraction and western blot analysis. Yeast pellets were resuspended in 800 µl Lysis buffer (20mM Tris-Cl pH 7.9, 1 mM EDTA and 5% glycerol) with an equal volume of 0.5 mm glass bead and put on bead beater instrument was vibrated for 10 min. Mitochondria were removed by centrifugation at 500 g for 10 min at 4 °C and transferred supernatant to new tube. After that the microsomes were pelleted at 10,000 g for 10 min at 4 °C. Microsomes were resuspended in solubilization buffer (20mM Tris-Cl pH 7.9, 0.1mM EDTA and 10 % glycerol, 0.5 mM

NaCl, 1 % Fos-choline 16) and incubated at 4 °C for 2 hour. Centrifigation at 25,000 g for 30 min at 4 °C and transferred microsomal membrane protein to new tube for Western blotting analysis (Chen et al., 2013). In addition, the liver of pig were used as a positive control for this experiment. The liver were homogenized in 0.25 M sucrose dissolved in 50 mM phosphate buffer solution (pH 7.4) at 4 °C and then following the extraction described above.

The total protein concentration was determined with protein assay kit (BIOTEH). About 60 µg of cell lysates (RY-Δ6, NT yeast and liver of pig) were mixed with 6x SDS sample buffer (1:1) and incubated at 95 °C for 5 min, then set on ice. The samples were loaded onto 10% SDS-PAGE running gel ran at 10 mA and adjusted to 20 mA when the protein was moving to the running gel. Then, the SDS-PAGE gels were used for Western blot.

For western blot analysis, protein gels were transferred onto a Hybond-N^+ membrane (GE Healthcare Life Sciences) by electroblotting (80 mA, 3 hr) using Mini Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories). The membrane was blocked with 10 % skim milk in PBST (13.7 mM NaCl, 0.27 mM KCl, 0.1 mM Na_2HPO_4, 0.2 mM KH_2PO_4, 0.1% Tween 20) and incubated for 16-18 h at 4 °C. then washed with PBST (3 times) for 5 min. The protein membrane was probed with 2:10000 FAD6 primary antibody (FADS2 antibody-N-terminal region, Host Rabbit, AVIVA system biology®) and incubated for 1 h then washed with PBST (3 times) for 5 min, followed by 1:10000 secondary antibody (1:10000) (Goat Anti-Rabbit IgG Antibody (HRP-Conjugated), Host goat, AVIVA system biology®) and incubated for 1 h then washed with PBST (3 times) for 5 min. Peroxidase activity was revealed by following

the procedure provided for the ECL Plus detection system kit (Amersham ECL plus western blotting detection reagents, GE Healthcare®)

4.4.7 Analysis of delta 6 desaturase in recombinant yeast (RY-Δ6)

The fatty acid composition was determined for RY-Δ6 and NT grown in YPD both was cultured at 30 °C for 24 h. Each culture was added each PUFA substrates; C18:3n3 (0.2 mM), and C18:2n6 (0.8 mM). Subsequently, the RY-Δ6 and NT cells were harvested and homogenized in chloroform/methanol (2:1, v/v) containing antioxidant (0.01% butylated hydroxytoluene; BHT) as described by Folch et al. (1957). The fatty acid methyl ester (FAME) technique was performed with modifications according to Morrison and Smith (1964). Extracted lipids were saponified with 0.5 N NaOH in 1.5 ml of methanol and heated for 7 min at 100 °C. The extracted lipids were esterified using 14% of BF3 in methanol and heated for 5 min at 100 °C. FAME was analyzed using a gas chromatograph (HP7890 ; Agilent, United States) equipped with a flame ionization detector with a SP™-2560 Capillary GC Column (100 m × 0.25 mm ID × 0.20 µm; Sigma-Aldrich Pte Ltd). Condition for injected was 70 °C (Hold time 4.00 min), 175 °C (rate 13, Hold time 27 min), 215 °C (rate 4, Hold time 17 min) and 240 °C (rate 4, Hole time 10 min) (Tanomman et al., 2013). Identification of each fatty acid peak in the chromatogram was performed by comparing its retention time with FAME mix standards (Supelco™ Component FAME mix, Supelco Bellefonte, USA; Cayman Chemical Company, MI, USA). The fatty acid composition was calculated based on the area of each peak, and the amount was determined by comparison with the methyl heptadecanoate internal standard. The percentage of substrate conversion was determined as 100 × [product amount / (substrate amount + product amount)]

Additionally, the fatty acyl ethyl esters of RY-Δ6 was analyzed by GC-MS/MS in full scan mode using Agilent 7890 GC with an Agilent 7000 Triple Quadrupole GC/MS system. The column used was Agilent J&W DB-WAX Ultra Inert Capillary GC columns (60 m × 250 μm × 0.25 μm) (Agilent Technologies) and the method included injection volume as 1 μl without split mode and oven temperature ramped from 70 to 240 °C (initial ramp at the rate of 13°C/min from 70 to 175 °C and then 3 °C/min until 240 °C) and the total run time was 90 min. Data analysis was performed using MassHunter (Agilent) and fatty acid species were identified and quantified by comparison to authentic standards.

4.4.8 Analyze delta 6 desaturase in crude extract of recombinant yeast (RY-Δ6)

The effect of level of C18:2n6 substrates on delta 6 desaturase activity

The RY-Δ6 yeast were grown in YPD both at 30 °C for 18 h at shaking speed of 200 rpm and were collected by centrifuge 3000 rpm for 20 min at 4 °C. The RY-Δ6 yeast approximate 10 g (three replicates) were resuspended in lysis buffer (100mM potassium phosphate pH 7.2, 0.33 M sucrose) (1:1) and freezed in liquid nitrogen (LN_2). The yeast cell was homogenized at 12,500 rpm for 2 min and freezed in LN_2 (repeat 5 times). Delta 6 desaturase activity was assayed in mixture of cell homogenate and Lysis buffer (1:1) and was added C18:2n6 substrates; 0 mM, 0.2 mM (55.98 mg/ml), and 0.02 mM (5.598 mg/ml) and incubated at 200 rpm for 2 h at 28 °C. Then fatty acid analysis was determined following fatty acid extraction method (Tanomman et al., 2013) and at the indicated fatty acid composition determined using GC.

The effect of temperature and incubation time on delta 6 desaturase activity

Cell homogenates were prepared as described above. Delta 6 desaturase activity was assayed in mixture of cell homogenate and Lysis buffer (1:1) and was added 0.02 mM (5.598 mg/ml) of C18:2n6 substrates and incubated at 200 rpm for 2 h and 24 h at 15 °C and 28 °C. Then fatty acid analysis was determined following fatty acid extraction method (Tanomman et al., 2013) and at the indicated fatty acid composition determined using GC.

The effect of level of C18:3n3 substrates on delta 6 desaturase activity

Cell homogenates were prepared as described above. Delta 6 desaturase activity was assayed in mixture of cell homogenate and Lysis buffer (1:1) and was added 0.045 mg/ml and 0.09 mg/ml of C18:3n3 substrates and incubated at 200 rpm for 2 h at 28 °C. Then fatty acid analysis was determined following fatty acid extraction method (Tanomman et al., 2013) and at the indicated fatty acid composition determined using GC.

The effect of soybean oil and linseed oil on delta 6 desaturase activity

Cell homogenates were prepared as described above. Delta 6 desaturase activity was assayed in mixture of cell homogenate and Lysis buffer (1:1) and was added 0.18 mg/ml of soybean oil (soybean oil has C18:2N6=288.7 mg/ml, added 0.18 mg of C18:2N6 in soybean oil) and 0.18 mg/ml of linseed oil (linseed oil has C18:3N3=575 mg/ml, C18:2N6=175 mg/ml and oleic acid=175 mg/ml, added 0.18 mg of C18:3N3 in linseed oil) and incubated at 200 rpm for 2 h at 28 °C. Then fatty acid analysis was determined following fatty acid extraction method (Tanomman et al., 2013) and at the indicated fatty acid composition determined using GC and GC-MS.

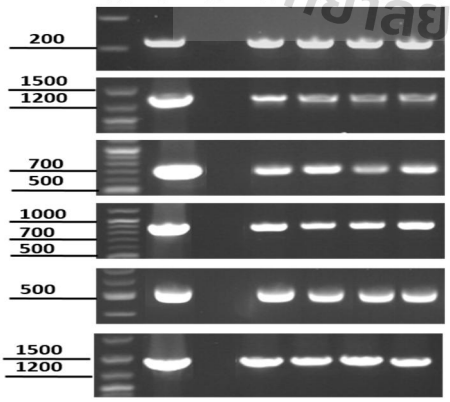
4.4.9 Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA) using SPSS for windows (Release 10) (SPSS Inc., Chicago, IL, USA). When significant differences were found among the groups, Turkey's tests were used to rank the groups. Throughout the experiment, effects and differences were declared to be significant at $P<0.05$.

4.5 Results

4.5.1 Production of recombinant *S. cerevisiae* stably expressing *Oni-fads2*

After transformation, four recombinant *S. cerevisiae* RY-Δ6 was selected on 2% glucose SC-Uracil incubated on 30 °C for 3-4 days. The RY-Δ6 which stably expressed *Oni-fads2* contained a cassette of gene including TEF promoter, *Oni-fads2* cDNA, *CYC1* transcription terminator, *URA3* gene (*URA3*; marker gene) in its genome. The integrated genes were determined by PCR of genomic DNA (Figure 4.6). In addition, the integration of the recombinant gene construct in the genome was confirmed by DNA sequencing (Figure 4.7).



Forward primer	Reverse primer	PCR product (bp)
Actin SC-F	Actin SC-R	220
NiFAD6-F1	NiFAD6-R1	1338
TEF-F	D6R1	700
FADd6-F1	NiFAD6-R1	803
D6F1	CYC-R	500
URA3-F	URA3-R	1200

Figure 4.6 The recombinant yeast (RY-Δ6) were selected at least 4 colony for verified by colony lysate and PCR. M represents the DNA marker. The positive

controls (P) used the plasmid DNA R45-TCFAD6-URA3-R67. The negative control (N) used distilled water. The positive control (P) for *β-actin* of *S. cerevisiae* (*sc-actin*) used the genomic DNA of *S. cerevisiae*. 1, 2, 3 and 4 represents the colony lysate of RY-Δ6 clone 1, 2, 3 and 4.

T7 promoter — *Apa I* — *Aat II* — *Sph I* — *Bgl II* — **rRNA-45**

```
1     TAATACGACTCACTATAGGGCGAATTGGGCCCGACGTCGCATGCAGATCTTATTGAGACC   60
61    ATGAGAGTAGCAAACGTAAGTCTAAAGGTTGTTTTATAGTAGTTAGGATGTAGAAAATGT  120
121   ATTCTGATAGGCCATTTTACATTTGGAGGGACGGTTGAAAGTGGACAGAGGAAAAGGTGC  180
181   GGAAATGGCTGATTTTGATTGTTTATGTTTTGTGTGATGATTTTACATTTTTGCATAGTA  240
241   TTAGGTAGTCAGATGAAAGATGAATAGACATAGGAGTAAGAAAACATAGAATAGTTACCG  300
301   TTATTGGTAGGAGTGTGGTGGGGTGGTATAGTCCGCATTGGGATGTTACTTTCCTGTTAT  361
361   GGCATGGATTTCCCTTTAGGGTCTCTGAAGCGTATTTCCGTCACCGAAAAAGGCAGAAAA  420
421   AGGGAAACTGAAGGGAGGATAGTAGTAAAGTTTGAATGGTGGTAGTGTAATGTATGATAT  480
481   CCGTTGGTTTTGGTTTCGGTTGTGAAAAGTTTTTTGGTATGATATTTTGCAAGTAGCATA  540
541   TATTTCTTGTGTGAGAAAGGTATATTTTGTATGTTTTGTATGTTCCCGCGCGTTTCCGTA  600
601   TTTTCCGCTTCCGCTTCCGCAGTAAAAAATAGTGAGGAACTGGGTTACCCGGGGCACCTG  660
```

Bgl II — **TEF promoter**

```
661   TCACAGATCTATAGCTTCAAAATGTTTCTACTCCTTTTTTACTCTTCCAGATTTTCTCGG  720
721   ACTCCGCGCATCGCCGTACCACTTCAAAACACCCAAGCACAGCATACTAAATTTCCCCTC  780
781   TTTCTTCCTCTAGGGTGTCGTTAATTACCCGTACTAAAGGTTTGGAAAAGAAAAAAGAGA  840
841   CCGCCTCGTTTCTTTTTCTTCGTCGAAAAAGGCAATAAAAATTTTTATCACGTTTCTTTT  900
901   TCTTGAAAATTTTTTTTTTGATTTTTTTCTCTTTCGATGACCTCCCATTGATATTTAAGT  960
961   TAATAAACGGTCTTCAATTTCTCAAGTTTCAGTTTCATTTTTCTTGTTCTATTACAACTT 1020
```

Xba I *Spe I*

```
1021  TTTTTACTTCTTGCTCATTAGAAAGAAAGCATAGCAATCTAATCTAAGTTTTCTAGAACT 1080
```

Bam HI *Sma I* *Pst I* *Eco RI* **Start codon delta 6 fatty acid desaturase**

```
1081  AGTGGATCCCCCGGGCTGCAGGAATTCATGGGAGGTGGAAGCCAGCAGACGGTGCCGGGA 1140
                                  M  G  G  G  S  Q  Q  T  V  P  G
1141  GAACCGGACAGCGGGAAAGCTAAAGGTGTTTACACCTGGGAGGAGGTGCAGAGCCACTGC 1200
      E  P  D  S  G  K  A  K  G  V  Y  T  W  E  E  V  Q  S  H  C
1201  AGCAGGAATGATCAATGGCTGGTCATCGATCGAAAGGTTTACAACATCACTCAGTGGGCC 1260
      S  R  N  D  Q  W  L  V  I  D  R  K  V  Y  N  I  T  Q  W  A
1261  AAAAGGCATCCAGGAGGGTTTCAAGTCATCAGCTACTATGCTGGAGAGGATGCCACGGAG 1320
      K  R  H  P  G  G  F  Q  V  I  S  Y  Y  A  G  E  D  A  T  E
1321  GTATTCACTGCTTTTCATCCTGATCCTAAGTTTGTGCAAAAGTTTCTGAAGCCGCTGCTG 1380
      V  F  T  A  F  H  P  D  P  K  F  V  Q  K  F  L  K  P  L  L
1381  ATCGGAGAGTTGGCAACGACAGAGCCGAGCCAGGACCGGGACAAAAATGCAGCCATCGTG 1440
      I  G  E  L  A  T  T  E  P  S  Q  D  R  D  K  N  A  A  I  V
1441  CAGGATTTCGAAACATTACGAGCTCAGGTGGAGAAAAGGGGTCTGTTTCGAGCTCAGCCT 1500
      Q  D  F  E  T  L  R  A  Q  V  E  K  R  G  L  F  R  A  Q  P
1510  TTGTTTTTCTTCCTCCACCTCAGTCACATCCTGCTGCTAGAAGCCCTTGGATGGCTGACC 1560
      L  F  F  F  L  H  L  S  H  I  L  L  L  E  A  L  G  W  L  T
1570  GTCTGGATGTGGGGCACAGGCTGGATACAAACACTTGTGTGCTCTGTGTTTCTCGCAACC 1620
      V  W  M  W  G  T  G  W  I  Q  T  L  V  C  S  V  F  L  A  T
1630  GCTCAGGCACAGGCTGGATGGCTGCAGCATGACTTTGGTCACCTGTCTGTCTTTAAAAAG 1680
      A  Q  A  Q  A  G  W  L  Q  H  D  F  G  H  L  S  V  F  K  K
1680  TCCAGCTGGAATCACCTCGCCCACAAGTTTGTCATTGGTCATTTAAAGGGAGCTTCTTCC 1740
      S  S  W  N  H  L  A  H  K  F  V  I  G  H  L  K  G  A  S  S
1741  AACTGGTGGAATCACCGACATTTCAGGCATCACGCTAAACCCAACATTTTCATTAAGGAC 1800
      N  W  W  N  H  R  H  F  R  H  H  A  K  P  N  I  F  I  K  D
1801  CCAGATATCAACACGTTGCACCTCTTCGTACTTGGCCGAACTCAACCAGTGGAATACGGG 1860
      P  D  I  N  T  L  H  L  F  V  L  G  R  T  Q  P  V  E  Y  G
1861  ATAAAGAAGATCAAACACATGCCTTACAATCGTCAGCACCATTACTTCTTTCTCGTGGGA 1920
      I  K  K  I  K  H  M  P  Y  N  R  Q  H  H  Y  F  F  L  V  G
1921  CCACCGCTGATCATTCCAGTTTTCTTCAACATTCATGTAATGCAGACCATGGTATCCCGG 1980
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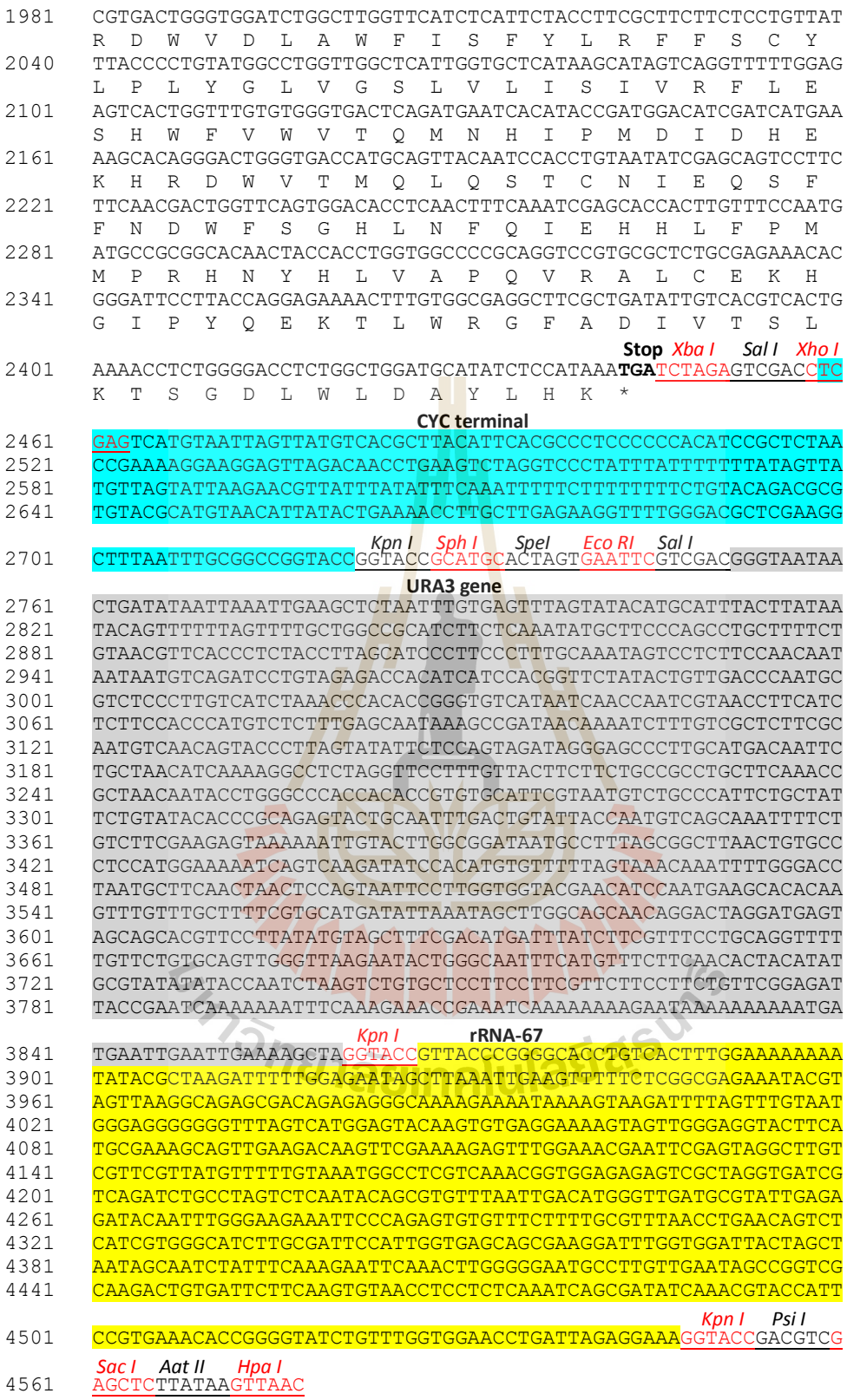


Figure 4.7 Sequence nucleotides of gene construction (*R45-EvT-Δ6-C-URA3-R67*)

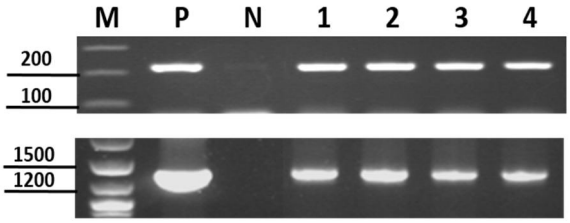
were transformed to recombinant yeast (RY-Δ6) and included ribosomal

RNA (rRNA-45), TEF promoter, *Oni-fads2* gene, CYC terminal, URA3 gene and ribosomal RNA (rRNA-67).

4.5.2 Expression analysis of *Oni-fads2*

The transcription and translation of *Oni-fads2* in the RY-Δ6 was analyzed by RT-PCR and western blot analysis, respectively. The *Oni-fads2* mRNA was detected in four colonies of RY-Δ6 with expected size at 1338 bp (Figure 4.8). In addition, the expression of the RY-Δ6 was evaluated at different time periods of cultivation: at 4, 8, 12, 16, 24, 48 and 72 hour using different culture media. The RY-Δ6 was grown in 2% glucose SC-minimal medium without Uracil (SC-Ura) and YPD broth at 30 °C for 18 hour. The RT-PCR for *Oni-fads2* was performed, the amplicon of *Oni-fads2* was detectable at each and every time tested. The RY-Δ6 which was cultured in both SC-Ura and YPD broth gave the similar results (Figure 4.9). As internal control, RT-PCR for the β-actin of *S. cerevisiae* (*sc-actin*) was also performed, and the amplicon of 220 bp in size was detectable.

The translation of the expression of *Oni-fads2* in the RY-Δ6 was evaluated by western blot analysis. The RY-Δ6 and non-transformed yeast (NT) yeast were grown in YPD at 30 °C for 18 h for protein extraction. Using western blot analysis, the protein extracted from liver of pig gave the positive signals at the expected size 50 kDA which was used as the positive control. Figure 4.10 showed that the signal of *Oni-fads2* was detectable in the RY-Δ6 but not in NT, suggesting that the RY-Δ6 could produce Δ6 protein.



Forward primer	Reverse primer	PCR product (bp)
Actin SC-F	Actin SC-R	220
NiFAD6-F1	NiFAD6-R1	1338

Figure 4.8 RT-PCR of *Oni-fads2* of RY-Δ6 were grown in 2% glucose SC-minimal medium without Uracil (SC–Ura) at 30 °C for 18 h. M represents the DNA marker. The positive controls (P) for *Oni-fads2* used the plasmid DNA R45-TCFAD6-URA3-R67. The negative control (N) used distilled water. The positive control (P) for *β-actin* of *S. cerevisiae* (*sc-actin*) used the genomic DNA of *S. cerevisiae*. 1, 2, 3 and 4 represents the colony lysate of RY-Δ6 clone 1, 2, 3 and 4.

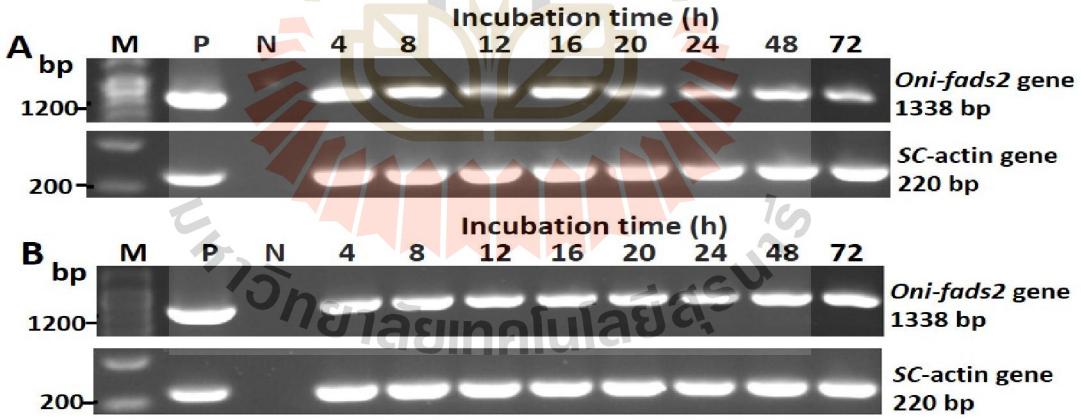


Figure 4.9 RT-PCR of *Oni-fads2* of RY-Δ6 were grown in 2% glucose SC-Ura (A) and YPD both (B) at 4, 8,12,16,20, 24, 48 and 72 h of cultivation at 30 °C. M represents the DNA marker. The positive controls (P) for *Oni-fads2* used the plasmid DNA R45-TCFAD6-URA3-R67. The negative control (N) used distilled water. The positive control (P) for *β-actin* of *S. cerevisiae* (*sc-actin*) used the genomic DNA of *S. cerevisiae*.

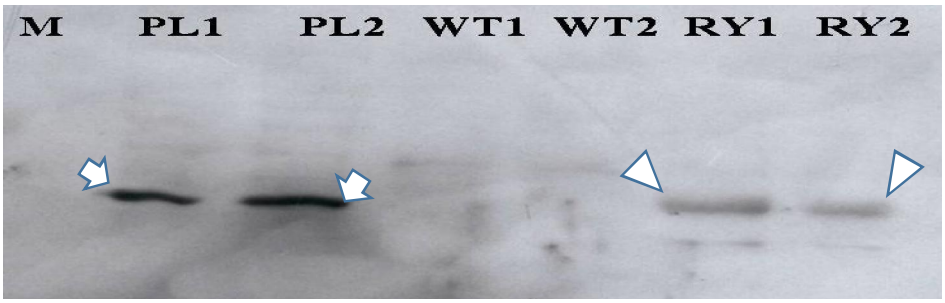


Figure 4.10 Delta 6 desaturase expression of recombinant yeast (RY-Δ6) was determined by western blotting using using FADS2 antibody-N-terminal region from rabbit. The arrow indicates the protein expression of liver of pig. The triangles indicate the delta 6 desaturase expressed recombinant proteins of RY-Δ6. M represents protein marker. The positive controls (PL1 and PL2) for delta 6 desaturase used the protein extracted from liver of pig. The negative control (WT1 and WT2) used the protein extracted from wild type yeast (WT). RY1 and RY2 represents the protein extracted from RY-Δ6.

4.5.3 Functional analysis of Δ6 in yeast RY-Δ6

The fatty acid composition was determined for RY-Δ6 and NT grown in YPD both in the presence of substrate C18:2n6 (0.8 mM) and C18:3n3 (0.2 mM). The results showed that the RY-Δ6 and NT contained crude lipid at 1-1.5 % by wet weight. In the presence of exogenous C18:2n6, the chromatogram of C18:2n6 was distinctly observed in both the RY-Δ6 and NT. While chromatogram of C18:3n6, C20:3n6 and C20:4n6 were detectable in the RY-Δ6, they were not observed in the NT (Figure 4.11). Identification of the FAME of RY-Δ6 was confirmed by GC-MS, and the result found that fatty acid C18:2n6C18:3N6, C20:3N6 and C20:4N6 have comparable retention

time with known standards fatty acid Figure 4.11. Indeed, the results of GC-MS confirmed the amount and mass spectra of C18:3n6 in RY-Δ6 (Figure 4.12). In addition, GC-MS was identified the mass-spectra of C20:3n6 and C20:4n6 in RY-Δ6 (Figure 4.13-4.14). Identification of GC-MS demonstrated the mass peak of each product fatty acid γ-Linolenic acid (C18:3n6) (mass peak m/z 292, RMF=871) (Figure 4.13), 8,11,14-Eicosatrienoic acid, methyl ester (C20:3n6) (mass peak m/z 320, RMF=918) (Figure 4.14) and 5, 8, 11, 14-Eicosatetraenoic acid, methyl ester (C20:4n6) (mass peak m/z 318, RMF=883) (Figure 4.15). Table 4.2 showed the amount of the fatty acid composition of RY-Δ6 and NT. The results showed that fatty acid C18:3n6, C20:3n6 and C20:4n6 were observed in RY-Δ6 while they were not detectable in NT. Note that there were no significant differences in other fatty acid composition ($P>0.05$).

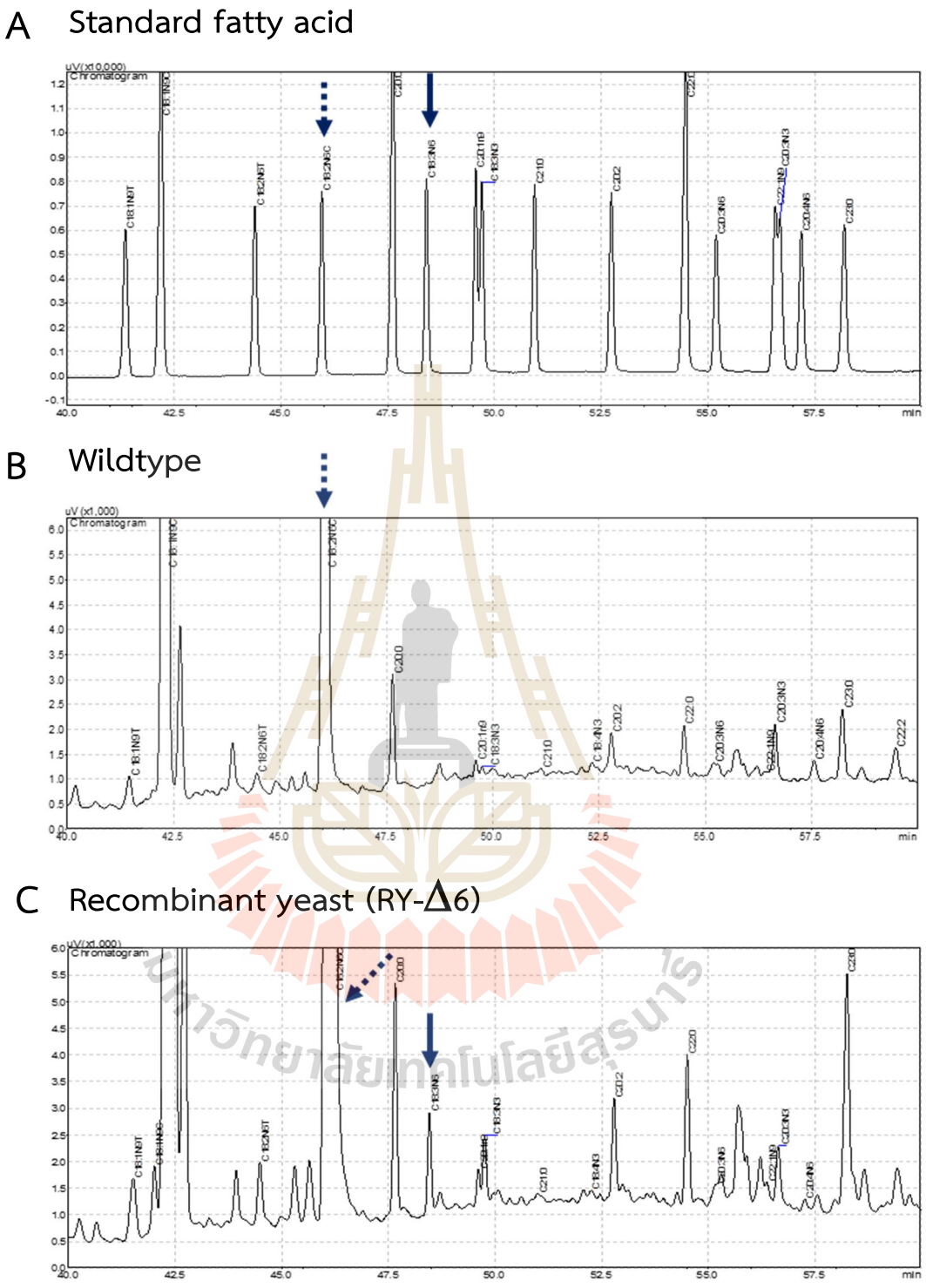


Figure 4.11 Determination of fatty acid substrates and products in NT and RY-Δ 6 yeast grown in YPD and added C18:2n6 (0.8 mM) substrate (B and C). The arrow indicates peaks were identified as C18:2n6 and C18:3n6, respectively.

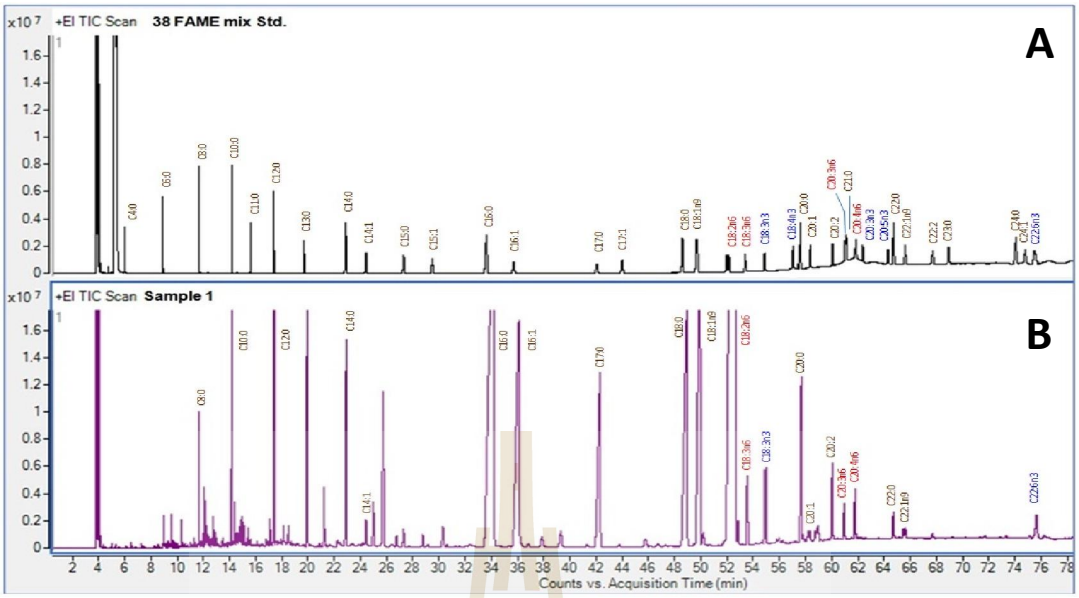


Figure 4.12 Identification of fatty acid in RY-Δ6 grown in YPD grown in the presence of C18:2n6 (0.8 mM) substrate by GC-MS which compared the retention time of standard fatty acid (A) and unknown peaks of fatty acid of RY-Δ6 (B).

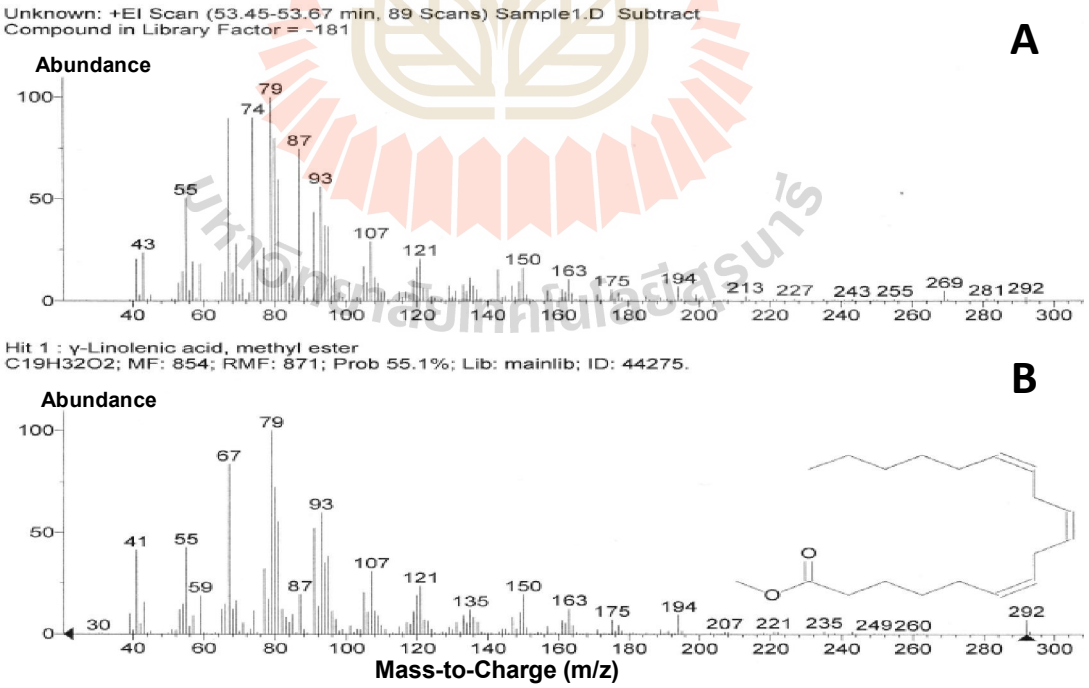


Figure 4.13 GC-MS analysis fatty acid in RY-Δ6 grown in YPD the presence of C18:2n6 (0.8 mM) substrate. The retention times of fatty acid extracted

from RY-Δ6 were compared to standards fatty acid and were identified compound mass spectra (mass peak m/z) by comparison to authentic standards (A) which corresponding to γ-Linolenic acid (C18:3n6) (B).

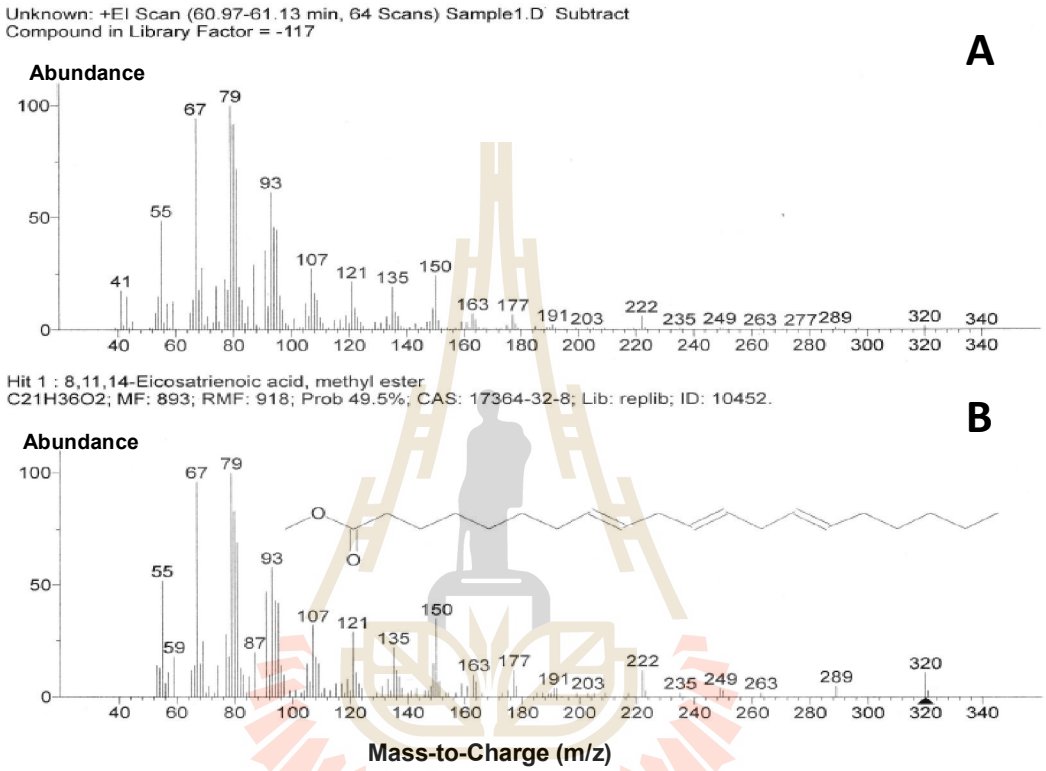


Figure 4.14 GC-MS analysis fatty acid in RY-Δ6 grown in YPD the presence of C18:2n6 (0.8 mM) substrate. The retention times of fatty acid extracted from RY-Δ6 were compared to standards fatty acid and were identified compound mass spectra (mass peak m/z) by comparison to authentic standards (A) which corresponding to 8,11,14-Eicosatrienoic acid, methyl ester (C20:3n6) (B).

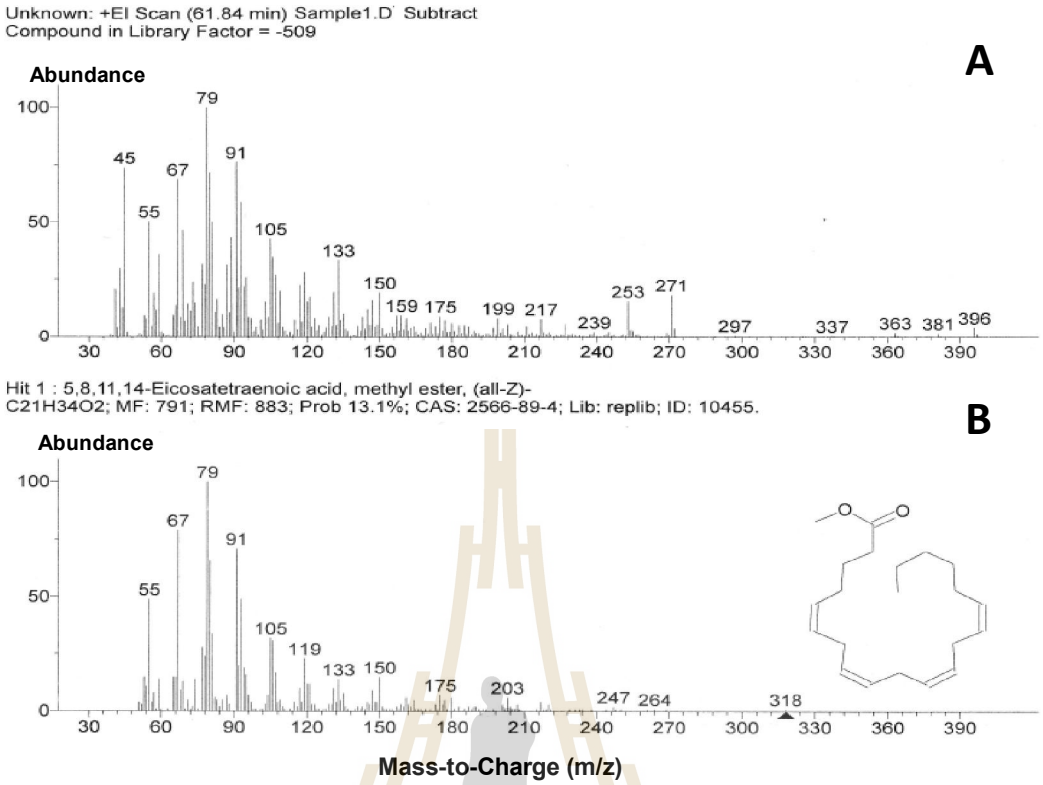


Figure 4.15 GC-MS analysis fatty acid in RY-Δ6 grown in YPD the presence of C18:2n6 (0.8 mM) substrate. The retention times of fatty acid extracted from RY-Δ6 were compared to standards fatty acid and were identified compound mass spectra (mass peak m/z) by comparison to authentic standards (A) which corresponding to 5, 8, 11, 14-Eicosatetraenoic acid, methyl ester (C20:4n6) (B).

Table 4.2 Fatty acid composition (mg/100 g lipid) of NT and RY-Δ6 were grown in YPD and added C18:2n6 substrate (0.8 mM) (mean±sd, n=4) (nd, not detected).

Fatty acids	**NT**	**RY-Δ6**
C8:0	29.49±0.61	29.15±1.30
C10:0	364.05±16.17	362.01±29.13
C12:0	554.67±22.53	550.94±14.49
C14:0	578.61±20.38	570.43±135.73
C14:1	51.47±4.89	51.68±6.23
C16:0	5,780.08±131.13	5,865.61±129.29
C16:1	2,392.07±233.20	2,130.54±259.71
C18:0	2,808.66±245.22	2,853.06±100.98
C18:1n9T	13.46±1.11	13.83±1.07
C18:1n9C	1,281.11±36.57	1,290.29±52.73
C18:2n6T	20.87±0.32	19.04±1.47
C18:2n6C	4,831.66±83.53	4,781.96±74.19
C20:0	79.10±4.51	79.33±10.54
C18:3n6	nd	16.47±1.33
C20:1n9	9.49±2.74	10.40±2.67
C18:3n3	17.43±1.28	18.13±1.45
C20:2	28.82±1.23	29.95±0.81
C22:0	28.61±1.65	29.50±3.46
C20:3n6	nd	4.89±0.17
C22:1n9	9.49±2.74	11.15±0.98
C20:3n3	nd	nd
C20:4n6	nd	19.70±2.05
C20:5N3	nd	nd
C24:1	nd	nd
C22:6n3	64.71±1.63	63.91±2.35
∑SFA	10,223.27±277.07	10,340.02±155.30
∑MUFA	3,757.08±194.92	3,507.91±227.70
∑PUFA	4,963.49±82.92	4,954.06±73.38
∑n3-PUFA	82.14±1.24	82.04±2.90
∑n6-PUFA	4,852.53±83.81	4,842.06±71.79

The RY-Δ6 and NT grown in YPD both was cultured at 30 °C for 24 h and added C18:3n3 (0.2 mM) substrate. Found that RY-Δ6 exhibited the distinct peaks of corresponding fatty acid product (C18:4n3) (Figure 4.16C) and NT exhibited indistinct peaks of corresponding fatty acid products (C18:4n3) (Figure 4.16B). Identification of the FAME of RY-Δ6 was confirmed by GC-MS, found that fatty acid C18:4n3, 20:3n3 and 20:5n3 and C22:6n3 were compared the retention time to known standards fatty acid (Figure 4.17) and GC-MS identified compounds found that fatty acid Methyl stearidonate (C18:4n3) (mass peak m/z 290, RMF=818) (Figure 4.18), 11,14,17-Eicosatrienoic acid, methyl ester (C20:3n3) (mass peak m/z 320, RMF=895) (Figure 4.19), 5, 8, 11, 14, 17-Eicosapentaenoic acid, methyl ester (C20:5n3) (mass peak m/z 316, RMF=863) (Figure 4.20) and Methyl 4, 7, 10, 13, 16, 19-docosahexaenoate (C22:6n3) (RMF=815) (Figure 4.21). The fatty acid C18:4n3, 20:3n3 and 20:5n3 were detected in RY-Δ6 but not detected in NT. In addition the RY-Δ6 has the level of fatty acid (C22:6n3) higher than NT (Table 4.3), however other fatty acid of RY-Δ6 and NT were not significant difference ($P>0.05$).

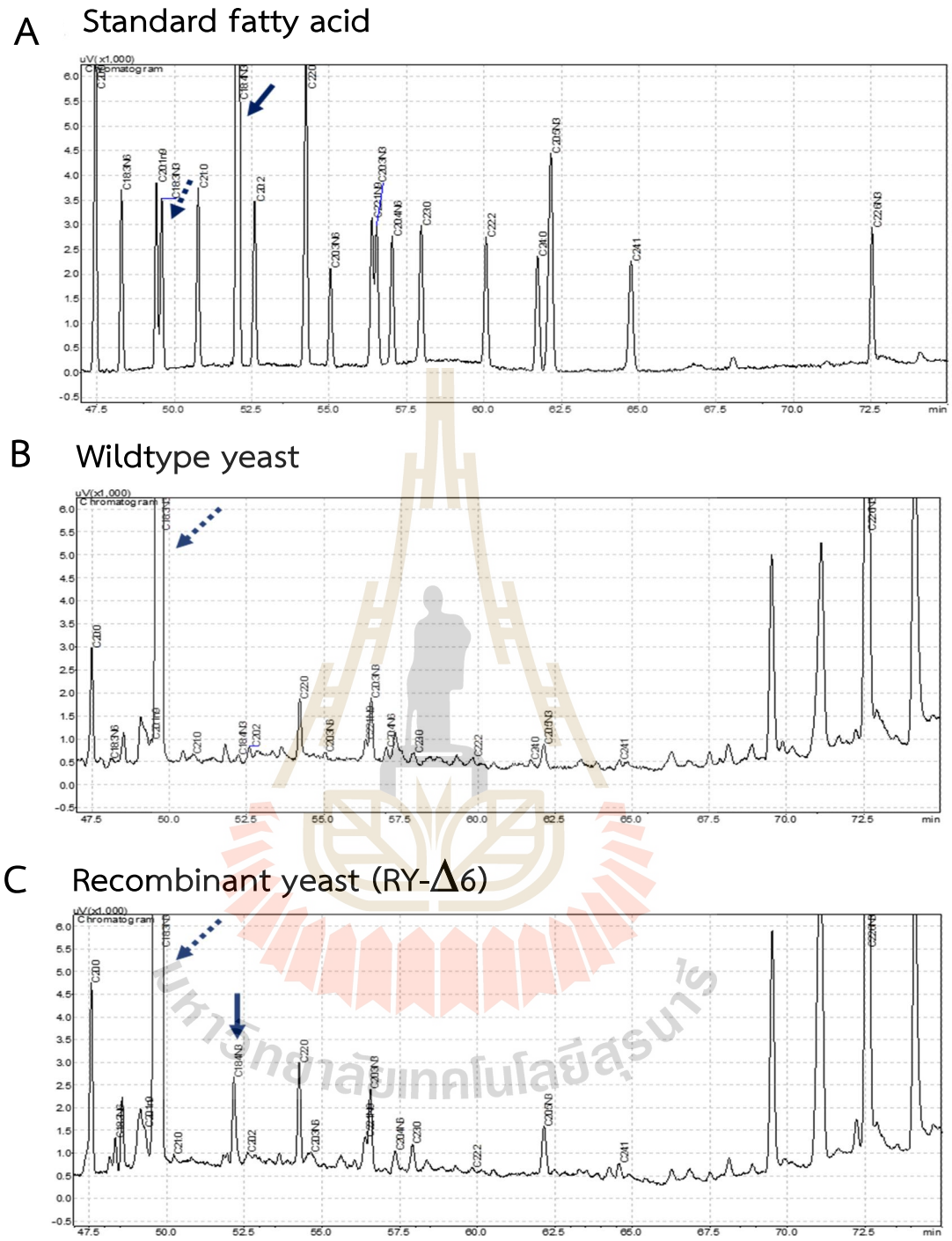


Figure 4.16 Determination of fatty acid substrates and products in NT and RY-Δ6 grown in YPD and added C18:3n3 (0.2 mM) substrate (B and C). The arrow indicates peaks were identified as C18:3n3 and C18:4n3, respectively.

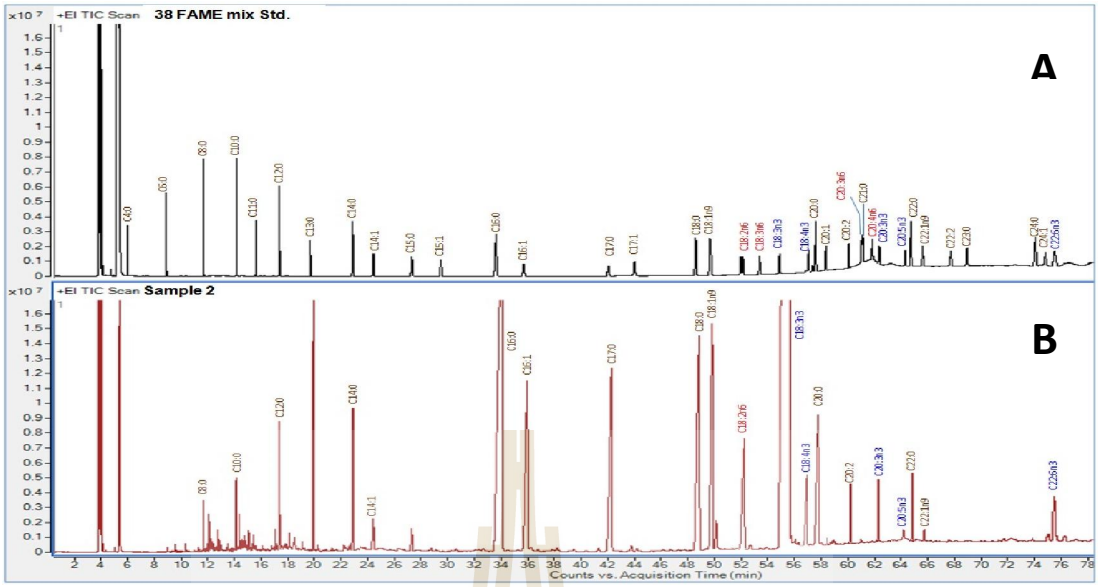


Figure 4.17 Identification of fatty acid in RY-Δ6 grown in YPD grown in the presence of C18:3n3 (0.2 mM) substrate by GC-MS which compared the retention time of standard fatty acid (A) and unknown peaks of fatty acid of RY-Δ6 (B).

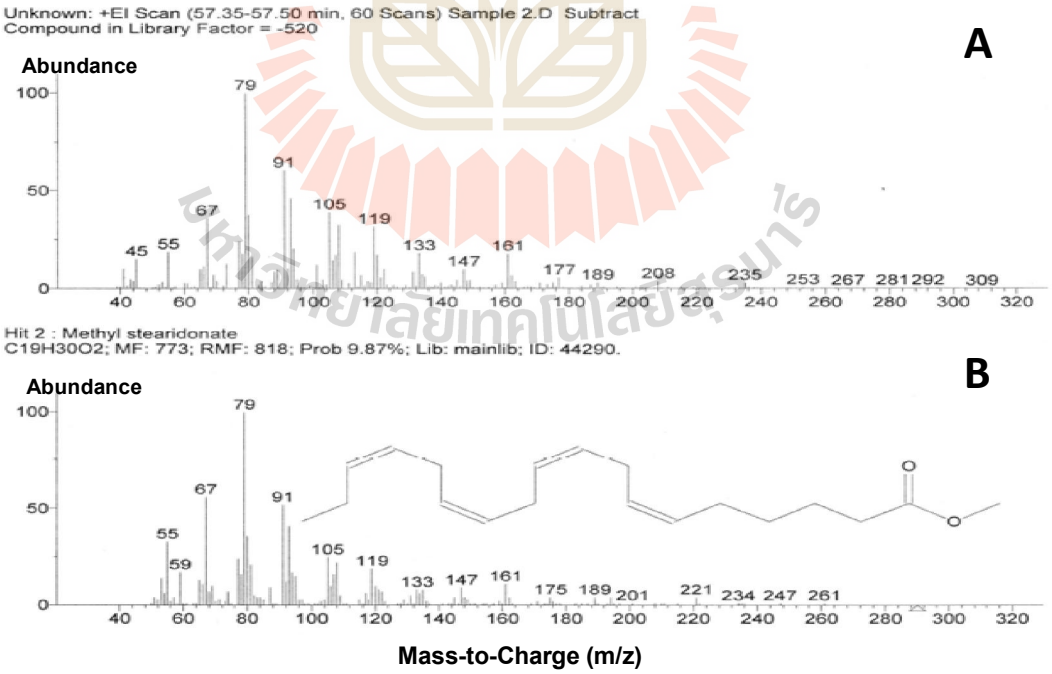


Figure 4.18 GC-MS analysis fatty acid in RY-Δ6 grown in YPD the presence of C18:3n3 (0.2 mM) substrate. The retention times of fatty acid extracted

from RY-Δ6 were compared to standards fatty acid and were identified compound mass spectra (mass peak m/z) by comparison to authentic standards (A) which corresponding to Methyl stearidonate (C18:4n3) (B).

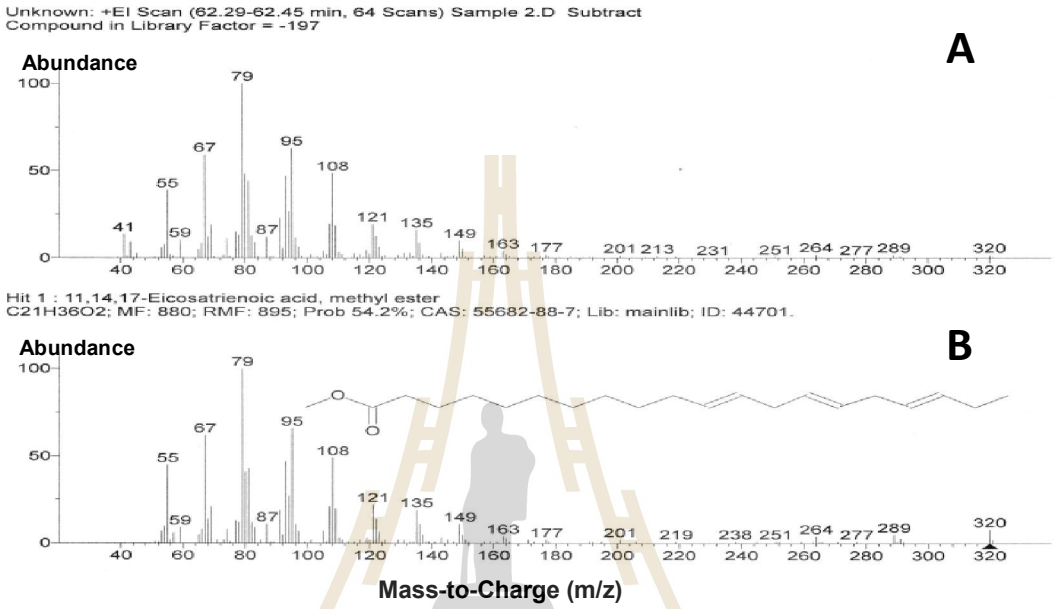


Figure 4.19 GC-MS analysis fatty acid in RY-Δ6 grown in YPD the presence of C18:3n3 (0.2 mM) substrate. The retention times of fatty acid extracted from RY-Δ6 were compared to standards fatty acid and were identified compound mass spectra (mass peak m/z) by comparison to authentic standards (A) which correspondingto 11,14,17-Eicosatrienoic acid, methyl ester (C20:3n3) (B).

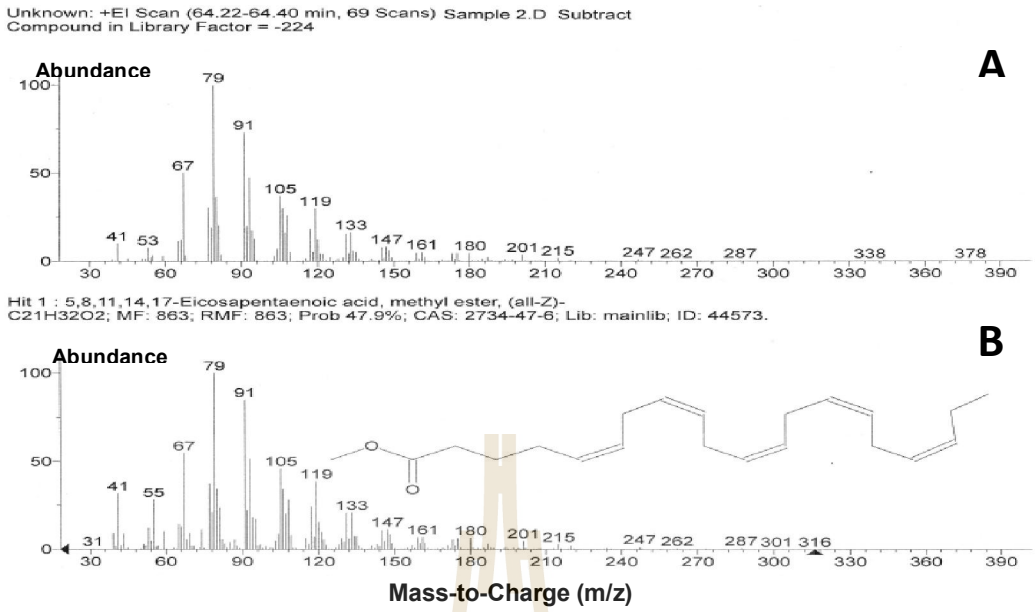


Figure 4.20 GC-MS analysis fatty acid in RY-Δ6 grown in YPD the presence of C18:3n3 (0.2 mM) substrate. The retention times of fatty acid extracted from RY-Δ6 were compared to standards fatty acid and were identified compound mass spectra (mass peak m/z) by comparison to authentic standards (A) which corresponding to 5, 8, 11, 14, 17-Eicosapentaenoic acid, methyl ester (C20:5n3) (B).

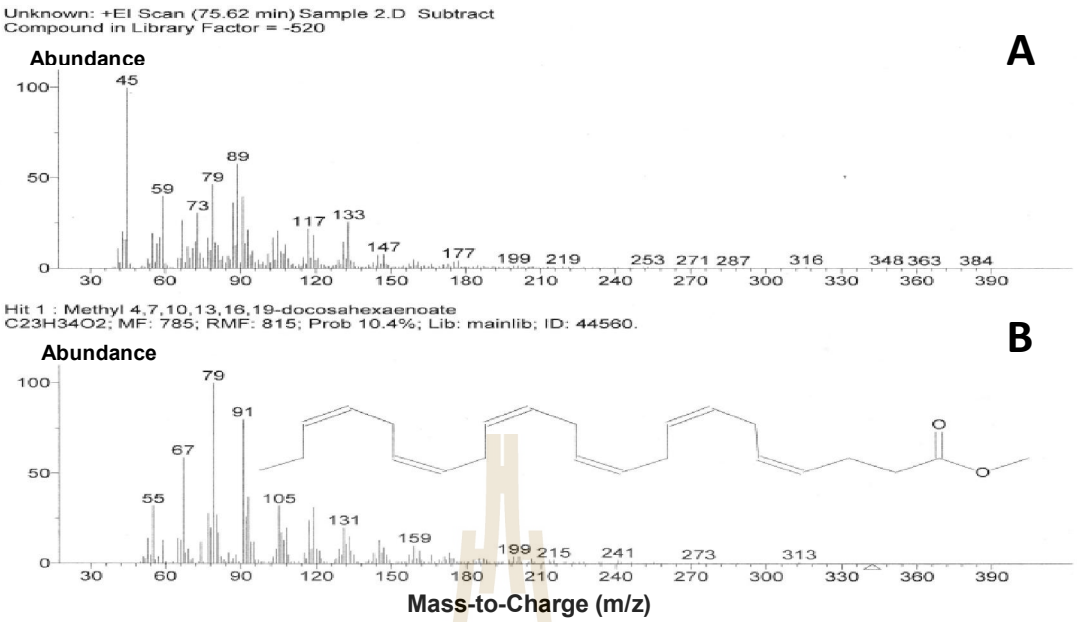


Figure 4.21 GC-MS analysis fatty acid in RY-Δ6 grown in YPD the presence of C18:3n3 (0.2 mM) substrate. The retention times of fatty acid extracted from RY-Δ6 were compared to standards fatty acid and were identified compound mass spectra (mass peak m/z) by comparison to authentic standards (A) which corresponding to Methyl 4, 7, 10, 13, 16, 19-docosahexaenoate (C22:6n3) (B).

Table 4.3 Fatty acid composition (mg/100 g lipid) of NT and RY-Δ6 were grown in YPD and added C18:3n3 substrate (0.2 mM) (mean±sd, n=4).

Fatty acids	NT	RY-Δ6
C8:0	27.23±0.64	27.99±1.22
C10:0	358.74±27.81	362.50±29.43
C12:0	562.32±19.96	565.96±26.17
C14:0	569.64±34.96	568.95±19.14
C14:1	53.24±2.33	54.51±2.87
C16:0	5,667.16±226.12	5,660.13±145.26
C16:1	2,382.69±177.31	2,355.75±208.49
C18:0	2,338.02±144.49	2,385.69±155.77
C18:1n9T	12.21±0.77	13.40±0.80
C18:1n9C	1,310.68±74.63	1,293.15±52.88
C18:2n6T	18.03±0.56	18.15±1.30
C18:2n6C	172.79±4.65	171.39±22.26
C20:0	78.35±3.65	73.89±6.05
C18:3n6	nd	nd
C18:3n3	4,784.36±109.56	4,676.98±188.78
C18:4n3	nd	23.86±1.17
C20:2	26.56±2.04	27.91±1.86
C22:0	28.78±0.91	30.41±3.81
C20:3n6	nd	nd
C22:1n9	9.04±0.63	10.63±1.60
C20:3n3	nd	25.95±4.70
C20:4n6	nd	nd
C20:5n3	nd	8.72±1.84
C22:6n3	67.89±4.47[b]	113.64±15.00[a]
ΣSFA	9,630.25±170.03	9,675.53±306.19
ΣMUFA	3,767.86±220.47	3,727.44±251.73
ΣPUFA	5,069.62±106.21	5,066.61±175.75
Σn3-PUFA	4,852.25±110.59	4,849.16±186.34
Σn6-PUFA	190.81±4.12	189.54±21.20

Means with different superscripts in each row differ significantly from each other (*P<0.05*).

4.5.3 Delta 6 desaturase in crude extract of recombinant yeast (RY-Δ6)

In order to determine whether the crude extract of RY-Δ6 could have delta 6 desaturase, the crude extract of RY-Δ6 was incubated with the presence of C18:2n6 substrate at 0 mM, 0.02 mM (5.598 mg/ml) and 0.2 mM (55.98 mg/ml) for 2 h at 28 °C. Table 4.4 showed that only the small amount of substrate C18:2n6 was detected in the crude extract incubated with the absence of exogenous increase substrate C18:2n6, and the amount of substrate C18:2n6 increased with increasing the amount of substrate C18:2n6 supplementation. In addition, the product fatty acids including C18:3n6, C20:3n6 and C20:4n6 were detected, suggesting that the crude extract of RY-Δ6 could exert delta 6 desaturase. Note that increase the amount of substrate C18:2n6 did not increase the amount of C18:3n6, C20:3n6 and C20:4n6 (Table 4.4). The effects of the lower temperature and longer incubation time on delta 6 desaturation of crude extract of RY-Δ6 were investigated (Table 4.5). The results demonstrated that lower temperature incubation at 15 °C led to decrease the amount of product C18:3n6 and C20:4n6, and no detectable was observed for C20:3n6. In addition, longer incubation period appeared to decrease the amount of product C18:3n6, C20:3n6 and C20:4n6.

Table 4.4 Delta 6 desaturase activity from crude extract of RY-Δ6 were incubated in the presence of C18:2n6 (0, 0.02 and 0.2 mM) on fatty acid composition (mean±sd) (nd=not detected).

Fatty acids	**Level of C18:2n6 substrate**		
(mg/100 g lipid)	**0 mM**	**0.02 mM**	**0.2 mM**
C18:2n6	73.95±3.60^{c}	1848.92±28.00^{b}	8,806.23±120.48^{a}
C18:3n6	nd	14.85±1.03^{b}	16.68±0.15^{a}
C20:3n6	nd	5.60±0.70^{b}	6.21±0.15^{a}
C20:4n6	nd	6.12±0.19^{b}	8.20±0.16^{a}

Means with different superscripts in each row differ significantly from each other (*P<0.05*).

Table 4.5 Effects of temperature and incubation time on delta 6 desaturase activity from crude extract of RY-Δ6 were incubated in the presence of C18:2n6 on fatty acid composition (mg/100 g lipid) (mean±sd).

Fatty acids	**2 hour**		**24 hour**	
	15 °C	**28 °C**	**15 °C**	**28 °C**
C18:2n6	1,932.70±52.76	1,893.92±7.36	1,867.96±19.37	1,877.96±27.76
C18:3n6	5.92±0.39^{c}	14.75±0.06 ^{a}	5.70±0.46^{c}	13.88±0.08^{b}
C20:3n6	nd	5.00±0.02	nd	4.88±0.14
C20:4n6	2.86±0.19^{b}	5.35±0.89^{a}	2.35±0.26^{b}	4.86±0.14^{a}

Means with different superscripts in each row differ significantly from each other (*P<0.05*).

This study also investigated the delta 6 desaturation on n3-PUFA of the crude extract RY-Δ6 in the presence of C18:3n3 at 0.045 mg/ml and 0.09 mg/ml for 2 h at 28 °C. (Table 4.6). The amount of substrate C18:3n3 increased with increasing the amount of substrate C18:3n3 supplementation. The product fatty acid C18:4n3 and

fatty acid C20:3n3 and C20:5n3 were observed only in the crude extract RY-Δ6 but not in the crude extract of NT. Although C22: 6n3 was detectable in the crude extract NT, higher amount of C22:6n3 was observed in the crude extract RY-Δ6.

Table 4.6 Delta 6 desaturase activity from crude extract of NT and RY-Δ6 were incubated in the presence of C18:3n3 (0.045 and 0.09 mg/ml) on fatty acid composition (mg/100 g lipid) (mean±sd) (nd, not detected).

Fatty acids	0.045 mg/ml		0.09 mg/ml	
	NT	RY-Δ6	NT	RY-Δ6
C18:3n3	397.70±3.18^{c}	368.85±14.44^{d}	851.83±25.99^{a}	814.61±4.37^{b}
C18:4n3	nd	5.75±0.31^{b}	nd	10.30±1.20^{a}
C20:3n3	nd	11.00±0.13^{b}	nd	15.16±1.80^{a}
C20:5n3	nd	22.54±1.05^{b}	nd	36.56±4.09^{a}
C22:6n3	66.48±1.33^{c}	130.48±16.47^{b}	66.68±1.50^{c}	155.68±5.65^{a}

Means with different superscripts in each row differ significantly from each other (*P<0.05*).

This study investigated the delta 6 desaturase activity of crude extract RY-Δ6 incubated with soybean oil (0. 18 mg/ ml) compared with that of crude extract NT (Table 4.7). The fatty acid composition of crude extract RY-Δ6 in soybean oil, C18:3n6, C20: 3n6 and C20: 4n6 were detectable. In addition, C18: 4n3 and C20: 3n3 were found in the crude extract RY- Δ6 while they were not detectable in crude extract NT. Note that other fatty acid composition were significant different in crude extract between NT and RY- Δ6. GC-MS was carried out to confirm the amount of fatty acid composition (Figure 4.22). Moreover, the mass spectra of C18:3N6 (mass peak m/z 292, RMF=935), C20: 3N6 (mass peak m/ z 320, RMF= 938) and C20: 4N6 (mass peak m/ z 318, RMF=823) were confirmed (Figure 4.23-4.25).

Table 4.7 Delta 6 desaturase activity from crude extract of NT and RY-Δ6 were incubated in the presence of soybean oil on fatty acid composition (mg/100 g lipid) (mean±sd) (nd, not detected).

Fatty acids	NT	RY-Δ6
C8:0	26.20±0.44	26.62±0.09
C10:0	368.47±1.68	367.65±7.47
C12:0	562.43±20.44	554.66±8.15
C14:0	678.43±4.01	682.33±16.71
C14:1	200.29±10.09	187.52±10.41
C16:0	5,648.65±42.86	5,680.99±113.87
C16:1	7,985.16±75.33	8,054.36±191.54
C18:0	878.24±3.40[b]	891.89±3.68[a]
C18:1N9T	22.21±0.51	22.95±0.91
C18:1N9C	4,996.45±115.94	4,933.86±7.82
C18:2N6T	20.62±0.88	20.57±0.67
C18:2N6C	1,307.00±16.01[a]	1,262.51±0.42[b]
C20:0	72.14±0.85	71.02±1.36
C18:3N6	nd	9.07±0.42
C20:1n9	11.68±0.65	11.19±0.04
C18:3N3	47.47±0.34[a]	46.09±0.26[b]
C18:4N3	nd	1.91±0.07
C20:2	30.19±1.49	29.69±0.79
C22:0	28.73±0.81	29.32±0.69
C20:3N6	nd	2.21±0.09
C22:1N9	9.02±1.40	9.57±0.63
C20:3N3	nd	2.38±0.29
C20:4N6	nd	2.80±0.10
C20:5N3	nd	nd
C22:6N3	66.62±1.69	66.52±1.29
∑SFA	8,263.29±67.69	8,304.46±117.03
∑MUFA	13,224.80±199.81	13,219.±46±210.10
∑PUFA	1,471.90±14.98[a]	1,443.76±2.08[b]
∑n3-PUFA	114.09±1.34	116.91±1.34
∑n6-PUFA	1,327.62±15.13[a]	1,297.16±1.53[b]

Means with different superscripts in each row differ significantly from each other (*P<0.05*).

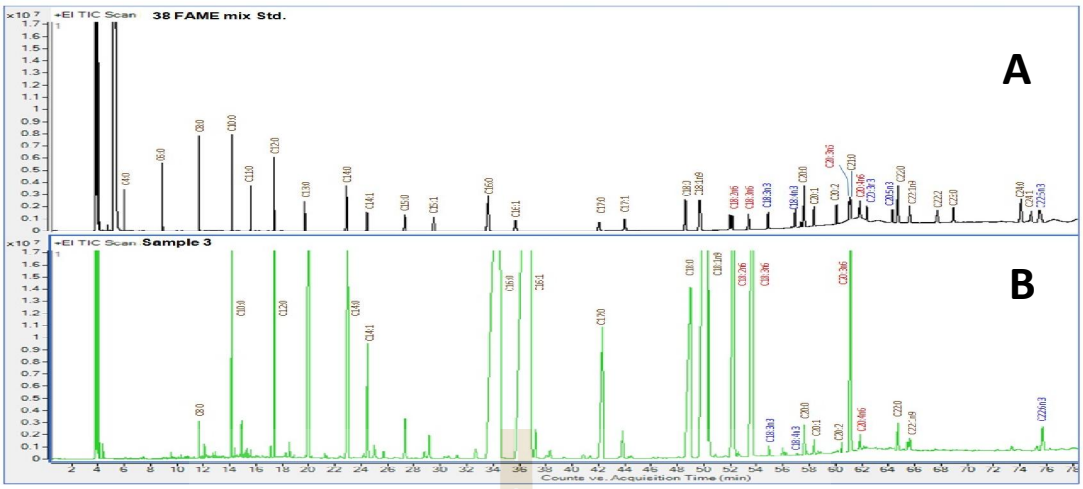


Figure 4.22 Identification of fatty acid in RY-Δ6 grown in YPD grown and were added soybean oil by GC-MS which compared the retention time of standard fatty acid (A) and unknown peaks of fatty acid of RY-Δ6 (B).

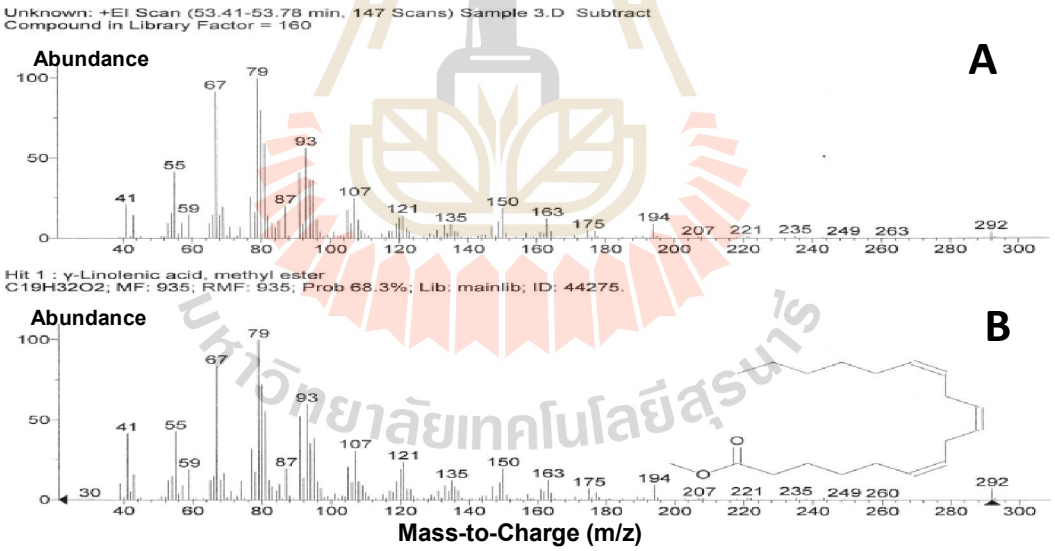


Figure 4.23 GC-MS analysis fatty acid in RY-Δ6 grown in YPD grown and were added soybean oil. The retention times of fatty acid extracted from RY-Δ6 were compared to standards fatty acid and were identified compound mass spectra (mass peak m/z) by comparison to authentic standards (A) which corresponding to γ-Linolenic acid (C18:3n6) (B).

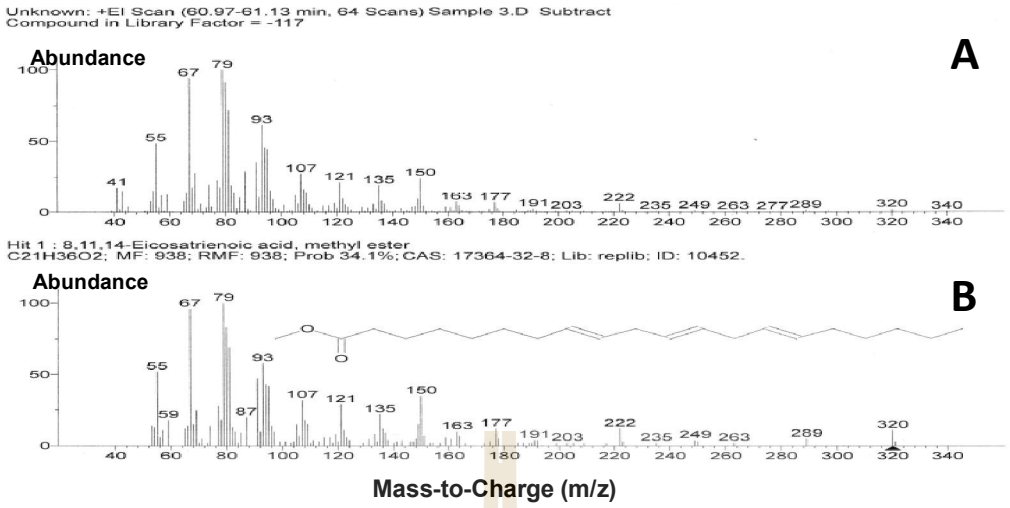


Figure 4.24 GC-MS analysis fatty acid in RY-Δ6 grown in YPD and were added soybean oil. The retention times of fatty acid extracted from RY-Δ6 were compared to standards fatty acid and were identified compound mass spectra (mass peak m/z) by comparison to authentic standards (A) which corresponding to 8,11,14-Eicosatrienoic acid, methyl ester (C20:3n6) (B).

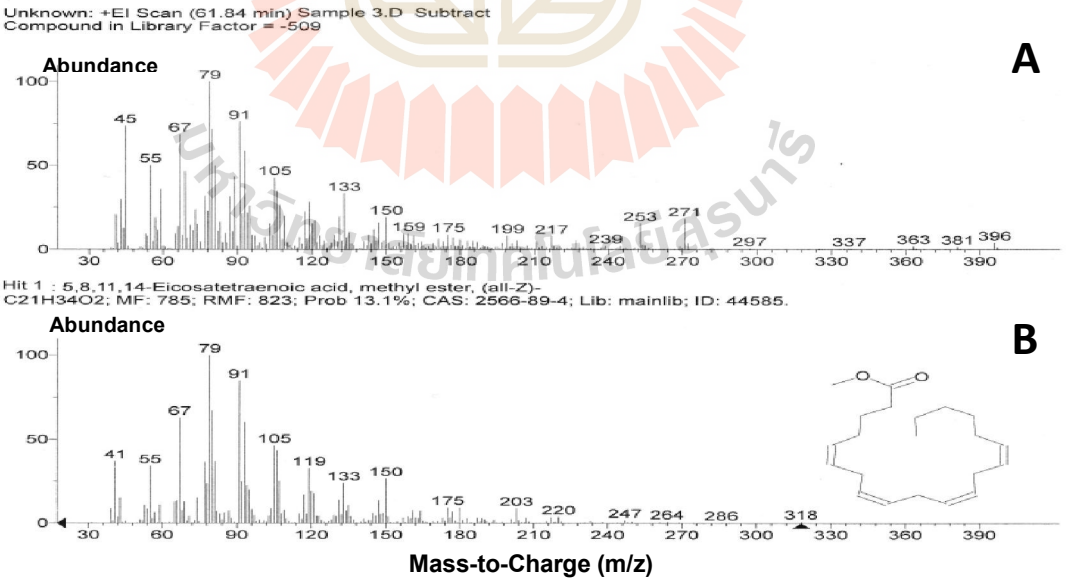


Figure 4.25 GC-MS analysis fatty acid in RY-Δ6 grown in YPD and were added soybean oil. The retention times of fatty acid extracted from RY-Δ6 were

compared to standards fatty acid and were identified compound mass spectra (mass peak m/z) by comparison to authentic standards (A) which corresponding to 5, 8, 11, 14-Eicosatetraenoic acid, methyl ester (C20:4n6) (B).

The delta 6 desaturase activity of crude extract RY-Δ6 incubated with linseed oil (0.18 mg/ml) compared with that of crude extract NT were showed in Table 4.8. While C18:4n3, C20:3n3 and C20:5n3 were detectable in crude extract RY-Δ6 incubating with flax seed oil, they were not observed in crude extract NT. In addition, crude extract RY-Δ6 has higher amount of C22:6n3 higher than that of crude extract NT. Moreover, C18:3n6 and C20:3n6 were detectable in crude extract RY-Δ6 but not in crude extract NT. Figure 4.26 showed the chromatogram of GC-MS which confirmed the amount of fatty acid composition. Furthermore, the mass spectrum of C18:4N3 (mass peak m/z 290, RMF=802), C20:3N3 (mass peak m/z 320, RMF=877), C20:5N3 (mass peak m/z 316, RMF=802) and C22:6N3 (mass peak m/z 316, RMF=802) were confirmed (Fig 4.27, 4.28, 4.29 and 4.30, respectively).

Table 4.8 Delta 6 desaturase activity from crude extract of NT and RY-Δ6 were incubated in the presence of linseed oil on fatty acid composition (mg/100 g lipid) (mean±sd) (nd, not detected).

Fatty acids	NT	RY-Δ6
C8:0	26.88±0.72	25.74±0.65
C10:0	360.72±3.90	366.19±3.35
C12:0	571.77±10.56	568.27±9.94
C14:0	687.73±11.20	689.12±11.06
C14:1	193.13±1.32	190.14±6.96
C16:0	5,656.77±9.96	5,576.82±97.04
C16:1	8,031.88±120.65	8,192.05±75.86
C18:0	858.72±26.07	865.44±7.68
C18:1N9T	24.24±0.95	25.14±0.56
C18:1N9C	4,693.10±2.35	4,621.13±90.66
C18:2N6T	21.17±1.11	20.90±0.94
C18:2N6C	477.23±0.17[a]	466.73±1.49[b]
C20:0	70.92±1.24	72.25±0.88
C18:3N6	nd	4.93±0.39
C20:1n9	11.11±0.04	10.64±0.72
C18:3N3	1,300.64±1.06[a]	1,247.63±1.73[b]
C18:4N3	nd	8.01±0.44
C20:2	30.30±0.60	31.23±0.60
C22:0	29.48±0.53	28.42±1.54
C20:3N6	nd	2.04±0.08
C22:1N9	10.54±0.03[a]	10.39±0.01[b]
C20:3N3	nd	5.43±0.38
C20:4N6	nd	nd
C20:5N3	nd	14.27±0.64
C22:6N3	66.65±1.69[b]	114.26±3.16[a]
∑SFA	8,262.99±32.54	8,192.24±129.08
∑MUFA	12,963.99±118.67	13,049.49±160.83
∑PUFA	1,895.99±1.21[b]	1,915.43±6.98[a]
∑n3-PUFA	1,367.28±2.74[b]	1,389.60±5.46[a]
∑n6-PUFA	498.40±0.93[a]	494.61±2.12[b]

Means with different superscripts in each row differ significantly from each other (*P<0.05*).

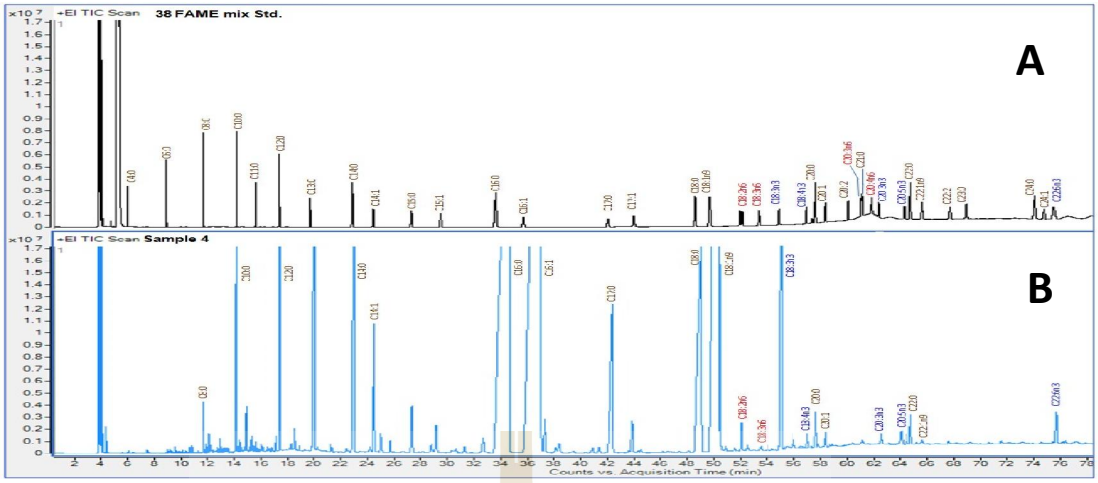


Figure 4.26 Identification of fatty acid in RY-Δ6 grown in YPD and were added linseed oil by GC-MS which compared the retention time of standard fatty acid (A) and unknown peaks of fatty acid of RY-Δ6 (B).

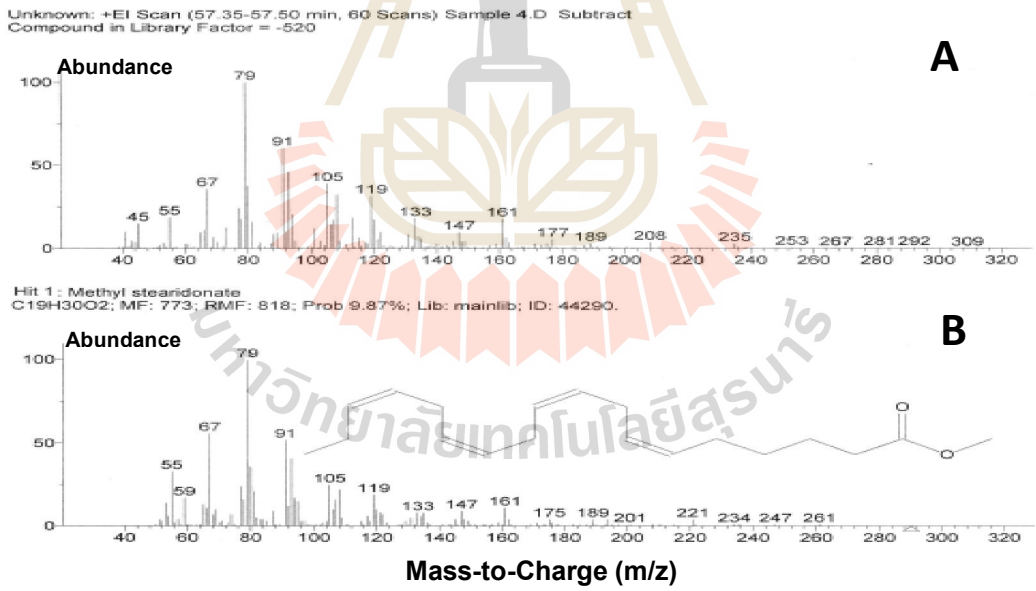


Figure 4.27 GC-MS analysis fatty acid in RY-Δ6 grown in YPD and were added linseed oil. The retention times of fatty acid extracted from RY-Δ6 were compared to standards fatty acid and were identified compound mass spectra (mass peak m/z) by comparison to authentic standards (A) which corresponding to methyl stearidonate (C18:4n3) (B).

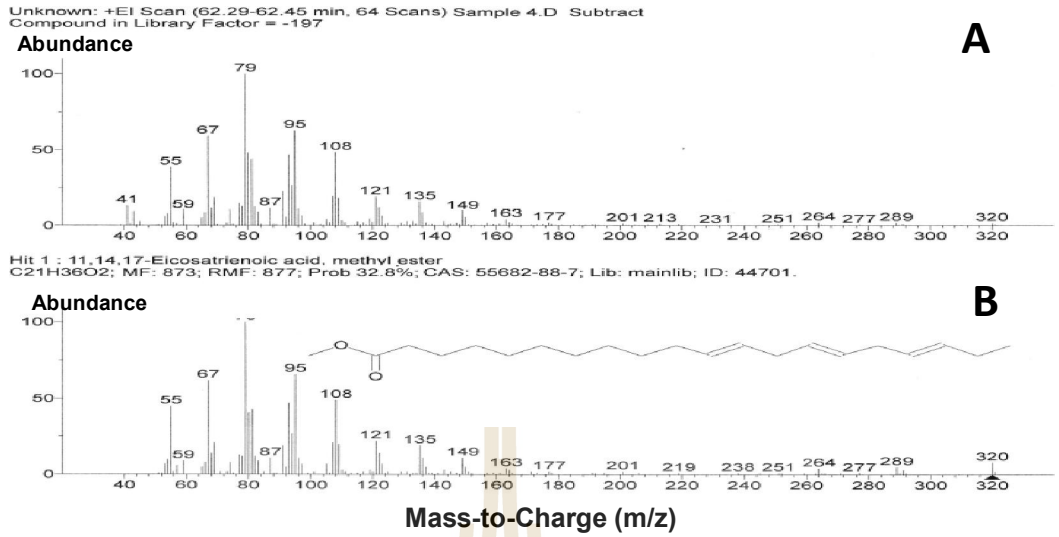


Figure 4.28 GC-MS analysis fatty acid in RY-Δ6 grown in YPD and were added linseed oil. The retention times of fatty acid extracted from RY-Δ6 were compared to standards fatty acid and were identified compound mass spectra (mass peak m/z) by comparison to authentic standards (A) which corresponding to 11,14,17-Eicosatrienoic acid, methyl ester (C20:3n3) (B).

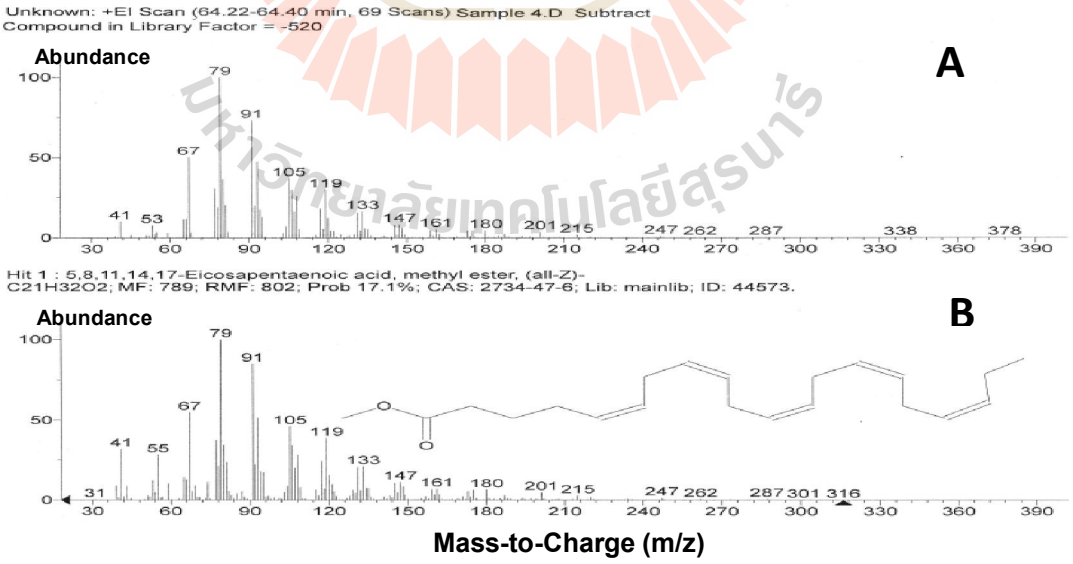


Figure 4.29 GC-MS analysis fatty acid in RY-Δ6 grown in YPD and were added linseed oil. The retention times of fatty acid extracted from RY-Δ6 were

compared to standards fatty acid and were identified compound mass spectra (mass peak m/z) by comparison to authentic standards (A) which corresponding to 5, 8, 11, 14, 17-Eicosapentaenoic acid, methyl ester (C20:5n3) (B).

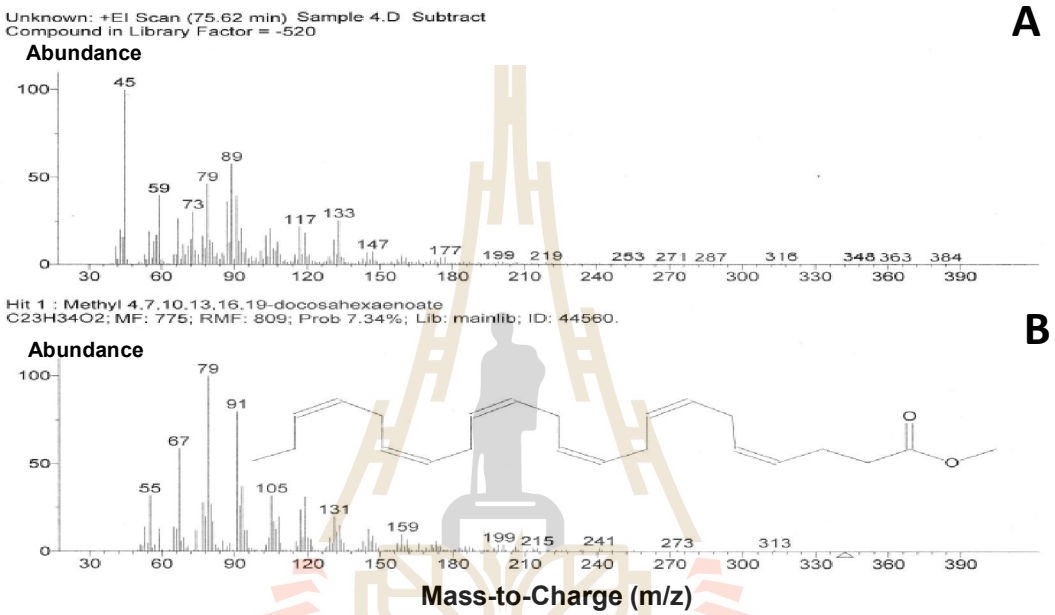


Figure 4.30 GC-MS analysis fatty acid in RY-Δ6 grown in YPD and were added linseed oil. The retention times of fatty acid extracted from RY-Δ6 were compared to standards fatty acid and were identified compound mass spectra (mass peak m/z) by comparison to authentic standards (A) which corresponding to Methyl 4, 7, 10, 13, 16, 19- docosahexaenoate (C22:6n3) (B).

4.6 Discussion

The RY-Δ6 which stably expressed *Oni-fads2* contained a cassette of gene including pTEF promoter, *Oni-fads2* cDNA, *CYC1* transcription terminator, *URA3* gene (*URA3*; marker gene) in its genome. TEF1 promoter has been widely used as a promoter for the efficient expression of heterologous proteins in *S. ceveresiae*. For example, TEF1 promoter could efficiently expressed and produced poly-3-D-hydroxybutyrate from xylose in flask culture system (Sandstrom et al., 2015). In addition, the TEF1 promoter could express fatty acid synthase gene including acetyl-CoA carboxylase (ACC1), fatty acid synthase 1 (FAS 1) and fatty acid synthase 2 (FAS 2). As a results, the recombinant yeast had high amount of fat (17% of dry weight) which was 4 times higher than that of control group (Runguphan and Keasling, 2013). Moreover, TEF1 promoter was demonstrated to express recombinant protein in *Yarrowia lipolytica* yeast, which is an oleaginous yeast containing more than 20% fat content (Hussian et al., 2015).

To overcome the limitation of extrachromosomal expression plasmid vector in *S. cerevisiae*, stable expression of recombinant yeast was established by making the construct that could integrated into yeast genome at gene for ribosomal RNA loci. The ribosomal RNA gene are multicopy genes which employed and increase the chance of the expression cassetted to integrate in the yeast genome. It was revealed that the location of integration is one of the main factor for integration efficiency. For instance, with delta locus integration, multi-copy integration of heterologous genes were observed at the Ty retrotransposon delta sites in the yeast genome (Da Silva and Srikrishnan, 2012; Sakai et al., 1990; Shi et al., 2014; Yamada et al., 2010; Yuan and Ching, 2013; Yuan and Ching, 2014).

The heterogenous expression of RY-Δ6 was detectable by RT-PCR and western blot analysis. The *Oni-fads2* mRNA was detected in RY-Δ6 with expected amplicon at 1338 bp. The transcript of the *fads2* was detectable RY-Δ6 at various culture times in both 2% glucose SC-minimal medium without Uracil (SC-Ura) and YPD broth. Likewise, as reported for recombinant *S. cerevisiae* expressing green fluorescent gene (GFP) by using TEF 1 promoter, the recombinant yeast produced GFP protein throughout the study period from 2 to 50 hours (Peng et al., 2015).

Western blot analysis was demonstrated to detect protein Δ6 in liver and intestine of European sea bass (Geay et al., 2010) and cell adipocytes (Ralston et al., 2015). In this study, western blot analysis was carried out to confirm the recombinant Δ6 of tilapia compared to the positive signal in the protein extract of pig liver. The *Oni-fads2* gene of tilapia consisting nucleotide 1338 bp which encoded the protein of 445 amino acids (molecular weight of 51.49 kDa) (Tanomman et al., 2013). This study investigated the gene expression of RY-Δ6 at translation level using western blot analysis. The result showed that the recombinant delta 6 desaturase was detected in RY-Δ6 protein with the size about 50 kDa.

Heterogeneous expression of *Oni-fads2* using GAL 1 promoter was demonstrated to exert delta 6 desaturase activity by converting C18:2n6 and C18:3n3 into C18:3n6 and C18:4n3, respectively, (Tanomman et al., 2003). The GAL 1 promoter is an inducible promoter and express in the present of galactose (as an inducer) and the absent glucose. Indeed, characterization and the function studies of *fads2* gene in a number of fish including Atlantic salmon (*S. salar*), European sea bass (*D. labrax*), black seabream (*A. schlegeli*), gilthead seabream (*S. aurata*), Atlantic bluefin tuna (*T. thynnus* L.), Atlantic cod (*G. morhua*), turbot (*P. maxima*) and rabbitfish (*Siganus*

canaliculatus), rainbow trout (*Oncoryhnchus mykiss*), common carp (*C. carpio*) and zebrafish (*Danio rerio*) were revealed to exert delta 6 desaturase activity to convert to C18:2n6 and C18:3n3 in to C18:3n6 and C18:4n3, respectively, (Hastings et al., 2001; Zheng et al., 2004; Hasting et al., 2005; Tocher et al., 2006; Gonzalez-Rovira et al., 2009; Zheng et al., 2009; Monroig et al., 2010; Li et al., 2010; Morais et al., 2011; Kim et al., 2011; Tanomman et al., 2013; Xie et al., 2014).

It was found that the RY-Δ6 contained main fatty acid including C16:0, C16:1, C18:0, C18:1n9. These fatty acids were commonly detectable in *S. cerevisiae* (Gonzalez-Rovira et al., 2009; Li et al., 2010; Tanomman et al., 2013). Additionally, the fatty acid C20:3n6 and C20:4n6 could be detected in RY-Δ6 which was added C18:2n6 substrate. Additionally, the fatty acid 20:3n3 and 20:5n3 and C22:6n3 could detected in RY-Δ6 which was cultured in the presence of C18:3n3 substrate. Indeed, *Oni-fads2* had bifunctional destaruase activities including Δ5 and Δ6. Heterologous expression of the *Oni-fads2* gene in recombinant yeast *S. cerevisiae* using GAL 1 promoter showed Δ5 activity to convert C20:3n6 to C20:4n6 (Tanomman et al., 2013). Likewise, as reported in Atlantic salmon and Cobia, *fads2* gene were capable to convert fatty acid C20:3n6 to C20:4n6 and C20:4n3 to C20:5n3 (Hastings et al., 2005; Zheng et al., 2009; Li et al., 2010). These findings would suggest that whether *Oni-fads2* could exert other desaturase function which remained to be further determined.

Development of recombinant proteins for use in agriculture need simple extraction process. Therefore, in this study, the delta 6 desaturase activity from crude extract of RY-Δ6 was investigated. The minimum amount of C18:2n6 substrates (5.598 mg/ml) was enough to determine the delta 6 desaturase activity in crude extract of RY-Δ6. The delta 6 desaturase activity of crude extract of RY-Δ6 had efficacy of

conversion C18:2n6 to C18:3n6 at 28 °C higher than that at 15 °C. The explanation of this finding would be because of the optimum temperature of recombinant delta 6 desaturase also depended on the optimum temperature of Nile tilapia. In addition, the incubation time at 28 °C for 2 h was enough, and it did not need to extend the period of incubation at 28 °C to 24 h. Taken together, crude extract of RY-Δ6 could exert delta 6 desaturase activity, and the optimum condition would be the incubating of substrate fatty acid at 28 °C for 2 h. In addition, with this condition, crude extract of RY-Δ6 could exert delta 6 desaturase activity for C18:3n3 substrate as well as alteration of fatty acid profile of vegetable oils such as soybean oil and linseed oil. Similarly, the crude extracts of recombinant proteins from *S. ceveresiae* were revealed to use for evaluation their functions. For example, the recombinant yeast *S. ceveresiae* that produce β-D-N-acetylglucosaminide-β-1, 4-galactosyl transferase (gal-T) showed that the crude extract from recombinant yeast exhibited gal-T activity (Krezdorn et al., 1993). The crude extract of recombinant yeast *Pichia pastoris* was capable to produce β-1, 4-xylanase of *Thermomyces lanuginosus* and exert efficacy on digestion of β-1, 4-xylose. In addition, the efficacy of recombinant protein depended on temperature and period of reaction. Variable activities of crude extract of recombinant yeast *P. pastoris and* purified β-1,4-xylanase were 20%, 60% and 95% when the reactions were performed at 50 ^{o}C for 4 h, 80 ^{o}C for 40 minute and 70 ^{o}C for 80 minute, respectively. The authors suggested that the loss of β-1, 4-xylanase activity in crude extract of recombinant yeast *P. pastoris* might be due to proteolysis and protein aggregation (Damaso et al., 2003). Moreover, the efficiency of xylanase activity of crude extract from recombinant yeast *P. pastoris* was demonstrated to depend on its substrate (Gaffney et al., 2009). Combined together,

the efficacy of the crude extract of recombinant protein would depend on level of substrate, type of substrate, temperature of reaction and reaction time.

4.7 Conclusion

This study produced recombinant *S. cerevisiae* RY-Δ6 which stably expressed *Oni-fads2* driven by Translation elongation factor I (TEF I) promoter. Using RT-PCR and western blot analysis, the transcription and the translation of *Oni-fads2* in RY-Δ6 was detectable. The RY-Δ6 exhibited delta 6 desaturase (Δ6) activity by converting exogenous substrate C18:2n6 and C18:3n3 to C18:3n6 and C18:4n3, respectively. In addition, crude extract of RY-Δ6 could convert exogenous substrate C18:2n6 and C18:3n3 to C18:3n6 and C18:4n3, respectively. Moreover, the crude extract RY-Δ6 altered the fatty composition of soybean and linseed oils.

4.8 References

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CHAPTER V

USE OF MICROENCAPSULATION RECOMBINANT *S. CEREVISIAE* (RY-Δ6) AS PROBIOTIC ON GROWTH PERFORMANCE, IMMUNE, WHOLE BODY COMPOSITION AND FATTY ACID PROFILE IN NILE TILAPIA (*O. NILOTICUS*)

5.1 Abstract

This study aimed to investigate the nutritive value of the RY-Δ6. In addition, the effects of the use of RY-Δ6 as dietary probiotic were demonstrated. Microencapsulation of RY-Δ6 was carried out, and it showed to have ability to pass through and colonize in the gastrointestinal tract of Nile tilapia. The effects of dietary supplementation of RY-Δ6 as probiotic on growth performance, body composition, hematological parameters, intestinal microbiota and fatty acid contents in liver and meat of Nile tilapia were evaluated. Seven treatment diets (each diet replicated four times) were as follows: (1) Basal diet with 2% linseed oil; (2) and (3) are Basal diet with 2% linseed oil and supplemented with wild type yeast 10^6 CFU/g feed (Li+NT10^6) and 10^8 CFU/g feed (Li+NT10^8), respectively; (4) and (5) are Basal diet with 2% linseed oil and supplemented with recombinant yeast 10^6 CFU/g feed (Li+RY10^6) and 10^8 CFU/g feed (Li+RY10^8), respectively; (6) Basal diet with 2% fish oil and (7) commercial diet. Fish were reared in concrete ponds 90 days, and growth performance, hematological indices,

blood chemical, chemical composition, intestinal microbiota, intestinal morphology and fatty acid deposition were eveluated. The results showed dietary RY-Δ6 for 90 days improve growth performance. Survival rate did not significance difference in among experimental diets. There were not significant differences in chemical composition in body and meat, blood chemistry and hematological indices among experimental treatments ($P>0.05$). Dietary RY-Δ6 led to increase immune (Ig, lysozyme and ACH50), villus height intestine and decrease intestinal *Vibrio* spp. ($P<0.05$). The significant increases in C18:3n6 and C18:4n3 in liver and meat were found in Nile tilapia fed dietary RY-Δ6 for 90 day ($P<0.05$), suggesting that RY-Δ6 could exerted delta 6 desaturase to increase product fatty acid C18:3n6 and C18:4n3. Taken together, microencapsulated RY-Δ6 could be used as dietary probiotics to exert positive effects on growth performance, immune parameters, intestinal morphology and intestinal microbiota. Additionally, dietary RY-Δ6 could exert delta-6 desaturation activity which changed the fatty profile in meat and liver of Nile tilapia. Therefore, RY-Δ6 could be provide application to develop to be the recombinant probiotic to produce long chain-polyunsaturated fatty acids.

5.2 Introduction

Probiotics are live microorganisms that exert beneficial effects on animals. In general, probiotics pass all the way through gastrointestinal tract and colonize in intestine. Some probiotics produce bacteriocin or inhibitory compounds to prevent the adhesion of pathogenic bacteria, therefore modifying the intestinal microbiota. Consequently, probiotics have the potential to be a biotherapeutic approach for the improvement of host health (Fuller, 1989; Verschuere et al., 2000; FAO/WHO, 2016;

Salinas et al., 2005; Balcazar et al., 2007; Wongsasak et al., 2015; Boonanuntanasarn et al., 2016). In addition, probiotics improve the efficiency of feed utilization by improving digestive enzyme activity and the growth response of host, resulting in increase feed utilization, and growth performance in animal (Tovar-Ramírez et al., 2002; Wang and Xu, 2006; Askarian et al., 2011).

Most probiotics have been commonly used in aquaculture such as prokaryotic microorganisms including *Lactobacillus* spp., *Bacillus* spp. and *Pediococcus* spp. (Balcazar et al., 2007; Wongsasak et al., 2015; Boonanuntanasarn et al., 2016) and eukaryotic microorganisms including microalgae, fungi, and yeast which have been used as single-cell protein sources, feed additives and probiotics in aquatic animals (Barnes et al., 2006; Reyes-Becerri et al., 2008; Nayak, 2011 for review, see Gatesoupe, 2007). In fact, a number of live yeasts were found to be a part of microbiota in freshwater and marine fish gut (for review, see Blackburn and Avery, 2003). Eukaryotic and prokaryotic probiotics would exert different effects in several properties (for review, see Gatesoupe, 2007). For example, eukaryotic probiotics such as yeast are resistant to antibiotics (Blackburn and Avery, 2003). Therefore, dietary supplementation with eukaryotic probiotics could be a practical way to contribute to the balance of intestinal microbiota during antibiotic treatment. Several prokaryotic probiotics were demonstrated to have the ability to transfer antibiotic resistant genes to pathogens in the gastrointestinal tract of the host (Verraes et al., 2013). However, the transmission of plasmid-encoded genes was not detectable from probiotic yeast (Kourelis et al., 2010). *S. cerevisiae*, which is generally recognized as safe by the Food and Drug Administration (USA), has been widely used in human food and the animal feed industry. Indeed, *S. cerevisiae* was demonstrated to constitute as normal microflora in

gastrointestinal tract in rainbow trout (for review, see Blackburn and Avery, 2003). Since *S. cerevisiae* is capable of using as host for recombinant protein production, *S. cerevisiae* would provide practical applicability for recombinant probiotic. There have been reports describing the use of *S. cerevisiae* as a probiotic dietary supplement in aquatic feeds in tilapia (*O. mossambicus*), Japanese flounder (*Paralichthys olivaceus*) and striped catfish (*Pangasianodon hypophthalmus*) (Lim et al., 2005; Liu et al., 2012; Boonanuntanasarn et al., 2018; Taoka et al., 2006; Wache et al., 2006; Essa et al., 2011)

Nile tilapia (*O. niloticus*) is an important economic fish of Thailand. Global tilapia production has increased year by year to provide a sustainable fish food (FAO, 2013). In tilapia feed, vegetable oil has been utilized as a fat source for both essential fatty acids and energy producing nutrients (Lim et al., 2011). The global demand for biofuels has led to increased demand for oil-based energy, thereby affecting the use of common plant-based oils in aquaculture feed. Thus, the investigation of LC-PUFA biosynthetic pathways, especially Δ6 desaturation, at molecular level in tilapia has attracted great interest. It would provide biological information of fatty acid bioconversion process in fish. Recombinant protein producing delta 6 desaturase would provide an useful approach to produce sustainable LC-PUFA sources.

Survival, colonization, and multiplication of probiotic in host gastrointestinal tract are the most important factors influencing the probiotic benefits. Micro-encapsulation of probiotic for the use as dietary supplementation has been one of the powerful techniques to attribute the survival of probiotic passage through the adverse conditions of host gut (Shori, 2017). Microencapsulation is a process in which cells are incorporated within an encapsulating matrix, which are generally food grade biopolymers such as guar gum, alginate, and pectin. Immobilization of probiotic in an

encapsulating matrix would contribute the reduction of cell death when exposure to gastric solution during stomach passage. Additionally, the polymer matrix could control the release of entrapped probiotics in intestine. Currently, a number of microencapsulated innovations have been applied for industrial probiotics (Cook et al., 2012; Ditthab and Boonanuntanasarn, 2016). Microencapsulation of *S. cerevisiae* using guar gum as encapsulation materials following freeze drying was revealed to extend the shelf life of *S. cerevisiae* up to 16 weeks (Brinker and Reiter, 2011). In this study, therefore, we investigated the effects of supplementation of microencapsulation recombinant *S. cerevisiae* expressing delta 6 desaturase as probiotic on growth performance, whole body composition, meat composition, health status (such as hematological, blood chemical and immune parameters), microbial populations in intestinal tract and fatty acid profile in Nile tilapia.

5.3 Objective

To study the effects of the use of dietary microencapsulation recombinant *S. cerevisiae* (RY-Δ6) as probiotic on growth performance, whole body composition, meat composition, health status (such as hematological, blood chemical and immune parameters), microbial populations in intestinal tract and accumulation of fatty acid profile in meat and liver of Nile tilapia.

5.4 Materials and methods

5.4.1 Recombinant *S. cerevisiae* (RY-Δ6) and its chemical composition

The *S. cerevisiae* strain DSY-5 (*MATalphaleu 2 trp1 ura3-52 his3 PGAL1-GAL4pep4 prb1-1122*) (RY-Δ6) carrying *Oni-fads2* driven by Translation

elongation factor promoter (p*TEF*). The RY-Δ6 were grown in YPD at 200 rpm for 18 h at 30 °C. The recombinant yeast was collected by centrifugation at 3000 rpm for 20 min at 4 °C for chemical composition analysis. Chemical composition analysis were conducted including proximal chemical analysis, fatty acid composition, yeast glucan composition and amino acid composition. Proximate chemical composition including crude protein, crude fat, crude fiber, ash, and moisture of RY-Δ6 were analyzed according to AOAC (1990). Fatty acid composition of RY-Δ6 was determined following fatty acid extraction method (Tanomman et al., 2013). Yeast glucan composition was included total glucan, α-glucan and β-glucan was analyzed by following the procedure provided for Megazyme Mushroom and yeast beta-glucan assay (Megazyme International Ireland). Amino acid composition of RY-Δ6 was determined following method of In house method based on Official Journal of the European Communities, L257/16.

5.4.2 Encapsulation and observation the RY-Δ6

The RY-Δ6 were grown in YPD both (1% yeast extract, 2% peptone and 2% D-glucose) at 30 °C for 24 h at shaking speed of 200 rpm and collected by centrifugation at 3000 rpm for 20 min at 4 °C. Yeast cells were washed by peptone water and added 1% guar gum in YPD as cryopreservation for RY-Δ6. Then, the yeast cells were conducted freeze drying by Freeze-dryer (Lyostar 11, FTS Kinetics USA) and kept at 4 °C until use.

Scanning electron microscope (SEM) observations were used to analyze morphology of microencapsulated RY-Δ6 without guar gum, with guar gum and non freeze dried RY-Δ6. The samples were prepared as described previously by Oliviera et al. (2010). Briefly, yeast was fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer

(pH 7.2) for 18 h at a low temperature (5-6 °C). Then, the samples were carefully washed with 0.1 M phosphate buffer (pH 7.2). Post-fixation was carried out for 2 h at room temperature with 2% osmium tetroxide. Initial dehydration was accomplished by placing specimens in the following series of ethanol gradients: 50% and 70% (two times for 10 min), 95% (two times for 5 min) and 100% (two times for 1 min), respectively. Then, samples were dehydrated with acetone (two times for 30 s) until dried by the critical point method in liquid CO_2. Subsequently, the specimens of the RY-Δ6 were coated with gold in a vacuum evaporator and examined with a scanning electron microscope (SEM Quanta-200, FEI, Czech Republic). Images were assembled with Photoshop (Adobe Photoshop CS3 Extended). Cell dimensions were determined by using bars. Morphology index (Mi).

5.4.3 Experimental design and diet preparation

The experimental design was completely randomized with seven treatment diets, each of which was replicated four times to test the validity of the conclusions. The seven treatment diets were as follows: (1) Basal diet with 2% linseed oil; (2) and (3) are Basal diet with 2% linseed oil and supplemented with wild type yeast 10^6 CFU/g feed (Li+NT10^6) and 10^8 CFU/g feed (Li+NT10^8), respectively; (4) and (5) are Basal diet with 2% linseed oil and supplemented with recombinant yeast 10^6 CFU/g feed (Li+RY10^6)and 10^8 CFU/g feed (Li+RY10^8), respectively; (6) Basal diet with 2% fish oil and (7) commercial diet (Table 5.1, 5.2).

The proximate composition (moisture, crude protein, crude fat and ash content) of the experimental diets was determined following standard AOAC methods (AOAC, 1990) and fatty acid analysis was determined following fatty acid extraction method (Tanomman et al., 2013) (Table 5.3).

Table 5.1 Group of experimental.

Treatment	Diet	Oil (%/g feed)	Yeast (CFU/g feed)
Linseed oil	Basal diet	Linseed oil 2%	-
Li+NT10^6	Basal diet	Linseed oil 2%	Wild type 10^6
Li+NT10^8	Basal diet	Linseed oil 2%	Wild type 10^8
Li+RY10^6	Basal diet	Linseed oil 2%	Recombinant yeast 10^6
Li+RY10^8	Basal diet	Linseed oil 2%	Recombinant yeast 10^8
Fish oil	Basal diet	Fish oil 2%	-
Commercial diet	Commercial diet	-	-

Table 5.2 Composition in basal diet for Nile tilapia.

Ingredients	Percentage (%)
Fish meal	18
Soybean meal	27
Rice bran	14.5
Broken rice	15
Cassava	20
Vegetable oil	2
Binder	2
Premix[a]	1
Vitamin C	0.5

[a]Vitamin and trace mineral mix provided the following (IU kg^{-1} or g kg^{-1}diet): biotin, 0.25 g; folic acid, 0.003 g; inositol, 0.25 mg; niacin, 0.0215 g; pantothenic acid, 0.03 g; vitamin A, 5,000 IU; vitamin B1, 0.0025 g; vitamin B2, 0.0012 g; vitamin B6, 0.0075 g; vitamin B12 0.00005 mg; vitamin C, 1 g; vitamin D3, 1,000 IU; vitamin E, 100 IU; vitamin K, 0.008 g; copper, 0.02 g; iron, 0.2 g; selenium, 0.3 mg; zinc, 0.32 g

5.4.4 The experimental fish and fish culture

All male Nile tilapia, *O. niloticus* strain Chitralada 3 (body weight at 350-400 grams) were used in this study. Nile tilapia were reared in earthen ponds at the Suranaree University of Technology Farm (SUT Farm; Nakhon Ratchasima, Thailand). Ten tilapia were randomly distributed in the experimental hapas. Thirty two hapas (cages; 0.7×0.7×1.5 m) were maintained in an concrete pond. Before the experiment began, the experimental fish was fed basal diet (25% protein) for 2 weeks for adaptation to the experimental condition. The experimental fish were fed experimental diet *ad libitum* for 2 times daily (9.00 and 15.00) for 90 days. The water temperature, pH, and dissolved oxygen in the culture pond ranged from 27.5 to 29.0 ºC, 7.48 to 7.73, and 5.09 to 5.41 mg/L, respectively.

Table 5.3 Chemical composition and fatty acid profile in experimental diet (mean±sd).

Treatment	Linseed oil	Li+NT10^{6}	Li+NT10^{8}	Li+RY10^{6}	Li+RY10^{8}	Fish oil	Commercial diet
Proximate composition (%)							
%DM	87.57±0.29	87.69±0.17	87.61±0.12	87.54±0.37	87.56±0.17	87.52±0.37	87.65±0.40
% Ash	6.82±0.47	6.95±0.27	6.75±0.24	6.82±0.44	6.87±0.37	6.79±0.32	6.83±0.56
%CP	25.66±0.05	25.66±0.10	25.68±0.12	25.64±0.04	25.65±0.06	25.67±0.05	25.66±0.05
%CF	7.08±0.10	7.07±0.08	7.06±0.02	7.05±0.04	7.06±0.02	7.09±0.09	7.05±0.15
%Fiber	5.47±0.12	5.48±0.12	5.45±0.04	5.44±0.29	5.43±0.30	5.48±0.32	5.41±0.15
Fatty acid (mg/100g lipid)							
C8:0	0.82±0.01	0.86±0.04	0.80±0.03	0.75±0.07	0.81±0.08	0.79±0.02	0.89±0.04
C10:0	0.36±0.01	0.39±0.03	0.38±0.02	0.39±0.01	0.41±0.01	0.37±0.01	5.49±0.50
C12:0	3.36±0.44	3.49±0.84	3.13±0.36	3.22±0.48	3.27±0.55	3.42±0.47	114.96±6.13
C13:0	0.76±0.03	0.76±0.10	0.76±0.02	0.77±0.08	0.78±0.08	0.79±0.08	0.99±0.04
C14:0	50.13±1.36	51.07±2.52	50.92±2.66	50.17±1.34	51.60±1.95	54.59±0.76	61.65±3.14
C14:1	0.43±0.06	0.46±0.08	0.44±0.02	0.46±0.03	0.48±0.02	0.44±0.03	1.06±0.05
C15:0	13.04±0.19	13.48±0.41	13.33±0.57	13.37±0.26	13.78±0.65	13.09±0.39	8.82±0.54
C16:0	634.06±16.56	669.46±36.07	644.81±28.08	633.59±35.98	642.08±34.11	645.63±21.92	890.96±42.86
C16:1	61.20±2.92	64.11±4.05	63.93±2.44	64.37±3.80	65.20±2.87	63.03±0.83	75.60±3.97
C18:0	170.82±19.27	181.39±17.21	171.54±12.14	169.86±5.48	178.65±9.10	172.64±9.73	252.20±11.94
C18:1n9T	nd	nd	nd	nd	nd	nd	nd
C18:1n9C	741.72±14.85	793.27±46.20	773.23±39.74	768.93±34.06	794.89±26.02	750.70±32.95	890.17±33.90

Table 5.3 Chemical composition and fatty acid profile in experimental diet (mean±sd) (Continuoued).

	Linseed oil	Li+WT10^{6}	Li+WT10^{8}	Li+RY10^{6}	Li+RY10^{8}	Fish oil	Commercial diet
Fatty acid (mg/100g lipid)							
C18:2n6T	5.66±0.13	6.09±0.39	5.70±0.14	5.68±0.52	6.11±0.39	3.84±0.19	2.63±0.11
C18:2n6C	600.05±7.64	617.33±29.87	610.14±12.27	611.86±8.05	610.50±5.64	501.55±13.73	578.36±23.70
C20:0	19.22±0.34	19.64±1.38	19.58±0.78	19.24±1.48	19.70±0.54	18.98±0.06	15.67±0.58
C18:3n6	1.23±0.02	1.28±0.07	1.24±0.04	1.26±0.05	1.30±0.03	1.08±0.03	1.25±0.08
C20:1n9	3.16±0.15	3.28±0.12	3.17±0.13	3.18±0.07	3.30±0.05	3.41±0.15	1.97±0.08
C18:3n3	135.80±1.88	135.42±3.56	132.39±5.09	132.68±2.76	134.74±2.17	89.78±2.88	100.25±5.24
C21:0	1.83±0.18	1.89±0.22	1.91±0.13	1.83±0.10	1.90±0.07	1.80±0.08	1.76±0.15
C18:4n3	4.17±0.18	4.13±0.31	4.28±0.29	4.19±0.09	4.27±0.14	4.19±0.17	4.17±0.11
C20:2	5.89±0.04	6.17±0.33	5.90±0.18	5.94±0.18	6.14±0.18	5.88±0.19	8.18±0.49
C22:0	9.84±0.18	9.90±0.46	9.80±0.39	9.88±0.32	10.02±0.42	9.80±0.08	8.30±0.34
C20:3n6	2.16±0.08	2.29±0.18	2.18±0.21	2.10±0.07	2.23±0.06	2.26±0.07	2.06±0.34
C22:1n9	1.64±0.03	1.71±0.09	1.72±0.09	1.71±0.08	1.72±0.44	1.65±0.11	4.88±0.21
C20:3n3	2.50±0.08	2.55±0.22	2.59±0.23	2.54±0.12	2.52±0.08	2.83±0.21	2.53±0.10
C20:4n6	23.12±0.61	23.58±0.77	23.94±0.62	23.63±0.86	23.76±0.75	23.58±0.39	12.11±0.59
C23:0	31.58±0.66	33.14±1.71	32.20±1.14	33.05±1.49	33.39±0.89	32.06±1.09	11.87±0.10
C20:5n3	31.64±0.41	31.42±1.08	31.71±1.42	31.46±1.00	31.53±1.01	49.36±0.91	12.44±0.63
C24:0	14.37±0.49	14.63±0.64	14.22±0.44	14.58±0.48	14.43±0.60	14.43±0.06	8.33±0.29
C24:1	5.02±0.13	5.20±0.25	4.47±1.42	5.01±0.20	5.18±0.13	4.93±0.10	1.84±0.04

Table 5.3 Chemical composition and fatty acid profile in experimental diet (mean±sd) (Continuoued).

	Linseed oil	Li+WT10^{6}	Li+WT10^{8}	Li+RY10^{6}	Li+RY10^{8}	Fish oil	Commercial diet
Fatty acid (mg/100g lipid)							
C22:6n3	114.85±1.80	115.76±2.73	116.71±3.60	115.91±3.07	116.55±3.23	256.50±3.33	25.95±1.16
∑SFA	950.18±27.98	1000.10±52.67	963.37±44.78	950.69±44.77	970.80±38.53	968.38±26.99	1381.88±59.22
∑MUFA	813.18±14.64	868.03±50.32	846.97±41.69	843.65±36.10	870.77±25.45	824.16±32.46	975.52±37.22
∑PUFA	927.04±8.48	946.01±32.42	936.75±12.47	937.24±7.37	939.64±6.49	940.85±12.05	749.97±30.44
∑n3-PUFA	288.96±1.90	289.28±4.36	287.66±1.02	286.78±2.08	289.61±1.29	402.65±2.91	145.35±6.78
∑n6-PUFA	632.20±7.96	650.56±31.00	643.19±12.75	644.52±7.95	643.89±5.86	532.31±14.01	596.44±24.10

5.4.5 Determination growth performance

Growth performance and fish sampling were carried out after 45 days and 90 days for feeding period. Fish were fasted for 18 h, sampled and anesthetized with 2-phenoxyethanol (0.2%). Growth performance were determined as the following:

Weight gain (WG) = Final weight - Initial weight

Feed intake (FI) = Total feed intake/ experimental days

Average daily gain (ADG)

= (Final mean body weight - Initial mean body weight) / Experimental days

Specific growth rate (SGR)

= 100 × [(*ln* final body weight - *ln* initial body weight)/ Experimental days]

Feed conversion ratio (FCR)

= Dry feed fed/Wet weight gain

Survival rate (%) = 100 × (Initial number of fish/Final number of fish)

5.4.6 Fish sampling

After 45 days and 90 days of the experiment period, fish were not fed for 18 h before sampling. Two fish from each hapa were removed and anesthetized with 2-phenoxyethanol (0.2%). A blood sample was collected from the caudal vein using a 21-gauge needle. The collected blood samples were divided into two sets. One set was mixed with K_2EDTA (at 1.5 mg/mL blood) as an anticoagulant for hematological examination and plasma collection. Another set was left to clot by being kept on ice for 3 h, followed by 1 h at room temperature. Serum was collected by centrifuging the

clotted blood at 9000×g for 10 min at room temperature. In addition, plasma was collected by centrifuging K_2EDTA-blood at 9000×g for 10 min at 4 ^oC and was stored at -80 ^oC for further analysis. The specimens from each replication hapa were pooled for chemical composition analysis in whole body and meat, for fatty acid profile analysis in liver and meat of tilapia and collected the intestinal tract for intestinal microbiota analysis and histological analysis**.**

5.4.7 Proximate chemical composition

Proximate chemical composition including crude protein, crude fat, crude fiber, ash, and moisture of experimental diets will be analyzed according to AOAC (1990). In addition, whole body and meat will be determined the chemical composition such as crude protein, crude fat, ash, and moisture according to AOAC (1990).

5.4.8 Hematological analysis

Analysis of the hematological parameters was conducted using K_2EDTA blood. The red blood cell (RBC) number was counted in duplicate using a Neubauer hemocytometer after dilution with Grower's solution (Voigt, 2000). The hemoglobin content was determined using the photometrical cyanohemoglobin method. Hematocrit values was evaluated in duplicate by placing blood into glass capillary tubes and subjecting them to microhematocrit centrifugation 15,000×g for 5 min.

5.4.9 Blood chemistry analysis

The blood chemistry were analyzed including glucose, cholesterol, triglyceride, calcium, Iron, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), direct bilirubin (BID), total bilirubin (BIT), albumin and total protein. Immediately after blood sampling, the K_2EDTA-treated blood was used to measure glucose in plasma will be quantitatively analyzed using

GOD-PAP method (Trinder, 1969). Plasma cholesterol was quantitatively analyzed using the cholesterol oxidase phenol aminophenazone (CHOD-PAP) technique described by Flegg (1973). Plasma triglyceride content was measured using the glycerol-3-phosphate oxidase-sodium N-ethyl-N-(3-sulfopropyl) m-anisidine (GPO-ESPAS) method described by Bucolo and David (1973). Plasma calcium contained was determined using the o-cresolphthalein direct method (Moorehead and Biggs, 1974). Iron in the plasma or serum was measured using an iron ferene quantitative determination (IDS, Liege, Belgium). SGOT and SGPT were analyzed using Reitman and Frankel's colorimetric method (Reitman and Frankel, 1957). BID and BIT contents were measured using the new diazo-DMSO method (Winsten and Cehelyk, 1969). Plasma albumin content was quantitatively estimated using the bromocresol green method (Doumas et al., 1971). Plasma protein contents were determined using the Biuret method (Gornall et al., 1949).

5.4.10 Immune assay

Immune parameters, including total immunoglobulin, lysozyme activity and alternative complement haemolytic 50 (ACH50) activity, were measured. Total immunoglobulin was measured according to Siwicki et al. (1994). Using fish serum, lysozyme activity was estimated as described by Pitaksong et al. (2013), and ACH50 activity was measured according to Sunyer and Tort (1995).

5.4.11 Intestinal microbiota analysis

At the end of the experimental period (45 and 90 days), two fish from each replication of each treatment were sampled for microbiological studies. The fish were then dissected under sterile condition, and the gut was remove, weighed, homogenized and suspended in sterile 0.9% NaCl. The suspension, serially diluted to 10^{-4} and

0.15 ml of the solution was spread onto plate count agar (PCA) culture medium (total bacteria), de Man, Rogosa and Sharpe (MRS) agar (lactic acid bacteria), bifidobacterium agar (Bifidobacteria), thiosulfate citrate bile salts sucrose (TCBS) agar (Vibrio) and Sabouraud agar (yeast and fungi). All of the plates were incubated at 37 ºC for 2 days. After incubation, the total numbers of colony-forming units (CFU)/g were calculated from statistically viable plates (i.e., plates containing 30-300 colonies) (Rawling et al., 2009).

5.4.12 Histological analysis

At the end of the experimental period (45 and 90 days), two fish from each replicate of each treatment were sampled and prepared for histological analysis as described previously (Phumyu et al., 2012). Portions of the anterior, middle and posterior parts of the intestine were dissected and preserved in 10% phosphate-buffered formalin (pH 7.2). After dehydration, the tissue was embedded in paraffin wax, cut into slices 5 µm thick, and mounted on glass slides. After deparaffinization, the slides were dehydrated and stained with hematoxylin and eosin. Villus height was measured on stained sections under a microscope using an ocular micrometer at 100x magnification. The five longest intact villi in each intestinal position were measured in two cross-sections from each sample. In addition, the number of goblet cells along the selected intact villi were counted.

5.4.13 Fatty acid analysis

The fatty acid profiles in liver and meat of tilapia will be determined using gas chromatography (GC). Tissue (2 g) was homogenized in chloroform/methanol (2:1, v/v) containing antioxidant (0.01% butylatedhydroxytoluene; BHT) as described by Folch et al. (1957). The fatty acid methyl ester (FAME) technique was performed with

modifications according to Morrison and Smith (1964). Extracted lipids were saponified with 0.5 N NaOH in 1.5 ml of methanol and heated for 7 min at 100 °C. The extracted lipids were esterified using 14% of BF_3 in methanol and heated for 5 min at 100 °C. FAME was analyzed using a gas chromatograph (HP7890; Agilent, United States) equipped with a flame ionization detector with a SP™-2560 Capillary GC Column (100 m × 0.25 mmID × 0.20 μm; Sigma-Aldrich Pte Ltd). Condition for injected was 70 ºC (Hold time 4.00 min), 175 ºC (rate 13, Hold time 27 min), 215 ºC (rate 4, Hold time 17 min) and 240 ºC (rate 4, Hole time 10 min) (Tanomman et al., 2013). Identification of each fatty acid peak in the chromatogram was performed by comparing its retention time with FAME mix standards (Supelco™ Component FAME mix, Supelco Bellefonte, USA; Cayman Chemical Company, MI, USA). The fatty acid composition was calculated based on the area of each peak, and the amount was determined by comparison with the methyl heptadecanoate internal standard. The percentage of substrate conversion was determined as 100 × [product amount / (substrate amount + product amount)]

5.4.14 Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA) using SPSS for Windows (Release 10) (SPSS Inc., Chicago, IL, USA). When significant differences were found among the groups, Tukey's test was used to rank the groups. Throughout the experiment, effects and differences were declared to be significant at $P<0.05$.

5.5 Results

5.5.1 Chemical composition of RY-Δ6

Proximate chemical composition including dry matter, crude protein, crude fat, crude fiber and ash of RY-Δ6 was demonstrated in Table 5.4. Fatty acid composition of RY-Δ6 was analyzed and showed Table 5.5. The glucan composition of yeast included 94.5±0.01 mg/g dry yeast of total glucan, 5.59±0.07 mg/g dry yeast of α-glucan and 88.87±0.20 mg/g dry yeast of β-glucan (Table 5.6). Amino acid composition of RY-Δ6 was showed in Table 5.7.

Table 5.4 Chemical composition (g/kg dry weight) of recombinant yeast (RY-Δ6) (mean ±sd).

Chemical composition	**NT**	**RY-Δ6**
Dry matter	690.9±0.4	690.2±1.1
Crude protein	471.7±4.8	473.2±6.7
Crude fat	11.4±0.8	11.1±0.6
Crude fibre	14.2±1.6	13.1±0.3
Ash	61.5±0.7	61.9±0.4

Table 5.5 Fatty acid composition of recombinant yeast (RY-Δ6) (mean ±sd).

Fatty acid	**mg/100g lipid**
C8:0	22.86±0.83
C10:0	354.72±3.38
C12:0	633.82±3.62
C14:0	650.37±8.55
C16:0	2367.21±36.02
C18:0	1374.81±23.53
C20:0	85.61±2.03
C21:0	6.80±0.45
C22:0	99.04±2.61
C23:0	35.76±0.86
C24:0	57.20±1.64
C14:1	34.03±0.72
C16:1	207.35±3.94
C18:1n9C	4506.89±61.50
C20:1n9	43.63±1.14
C18:2n6C	12502.61±62.53
C18:3n6	42.22±2.26
C18:3n3	1100.41±13.21
C18:4n3	14.14±0.45
C20:3n3	3.56±0.12
C22:6n3	62.27±2.26
∑SFA	5688.19±57.05
∑MUFA	4791.90±58.00
∑PUFA	13725.22±74.43

Table 5.6 Glucan composition of recombinant yeast (RY-Δ6) (mean ±sd).

Glucan composition (mg/g dry yeast)	**RY-Δ6**
Total glucan	94.5±0.01
α-glucan	5.59±0.07
β-glucan	88.87±0.20

Table 5.7 Amino acid composition of recombinant yeast (RY-Δ6) (mean ±sd).

Amino acid (mg/100g)	**RY-Δ6 yeast**
Alanine	653.22
Arginine	541.32
Aspartic acid	874.11
Cystine	128.43
Glutamic acid	1910.68
Glycine	537.49
Histidine	250.79
Hydroxylysine	Not detected
Hydroxyproline	50.51
Isoleucine	428.72
Leucine	714.47
Lysine	868.32
Methionine	92.05
Phenylalanine	397.43
Proline	380.45
Serine	501.98
Threonine	563.30
Tryptophan	119.44
Tyrosine	357.67
Valine	561.56

5.5.2 Observation the RY-Δ6 by Scanning electron microscope (SEM)

Figure 5.1 showed the result of SEM of fresh RY-Δ6 (Figure 5.1A) and RY-Δ6 which was frozen drying without 1% guar gum (cryoprotectant) (Figure 5.1B) and with 1% guar gum (RY-Δ6 with guar gum) (Figure 5.1C). The RY-Δ6 (Figure 5.1C) was freeze drying with 1% guar gum (RY-Δ6 with guar gum) showed a normal cell surface as same as the morphology of fresh RY-Δ6. The RY-Δ6 which was freeze

drying without 1% guar gum (RY-Δ6 without guar gum) showed damage of cell surface (Figure 5.1 B). Additionally, survival rate of RY-Δ6 with guar gum were 95-97% and higher than that of RY-Δ6 without guar gum (survival=15-20%). Figure 5.2 showed the SEM observation of diet (Figure 5.2 A), diet supplemented with guar gum alone (Figure 5.2 B) and diet supplemented with microencapsulated of RY-Δ6 with guar gum. The microencapsulated yeast cells were observed on the experimental diet supplemented with probiotic RY-Δ6 at $1x10^8$ CFU.

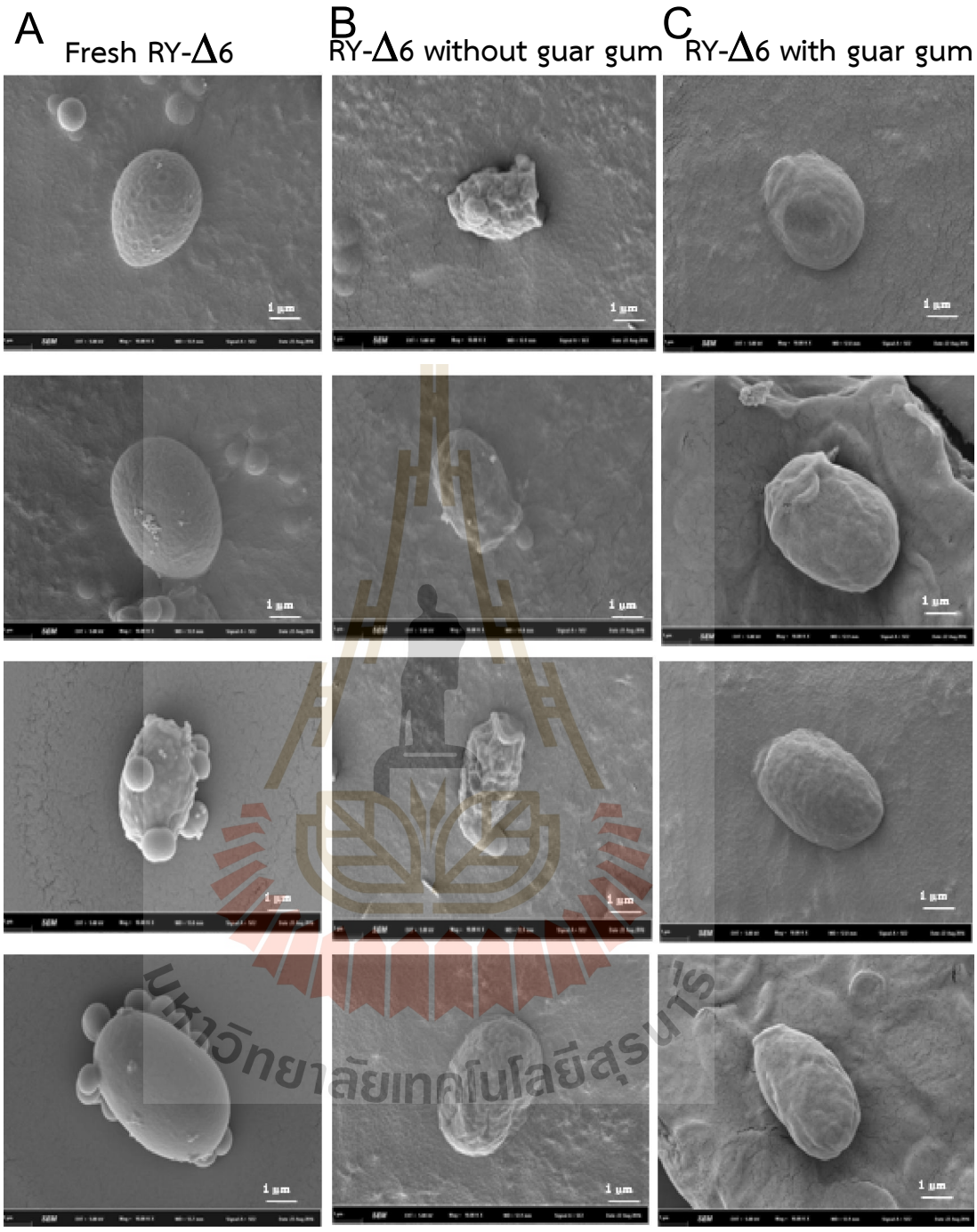

Figure 5.1 Cell morphologies of recombinant yeast (RY-Δ6) are 3 conditions including RY-Δ6 was non freeze dry (A), RY-Δ6 was freeze drying without 1% guar gum (RY-Δ6 without guar gum) (B) and RY-Δ6 was freeze drying with 1% guar gum (RY-Δ6 with guar gum) (C) were observed by scanning electron microscope (SEM). Scale bars represent 1 μm.

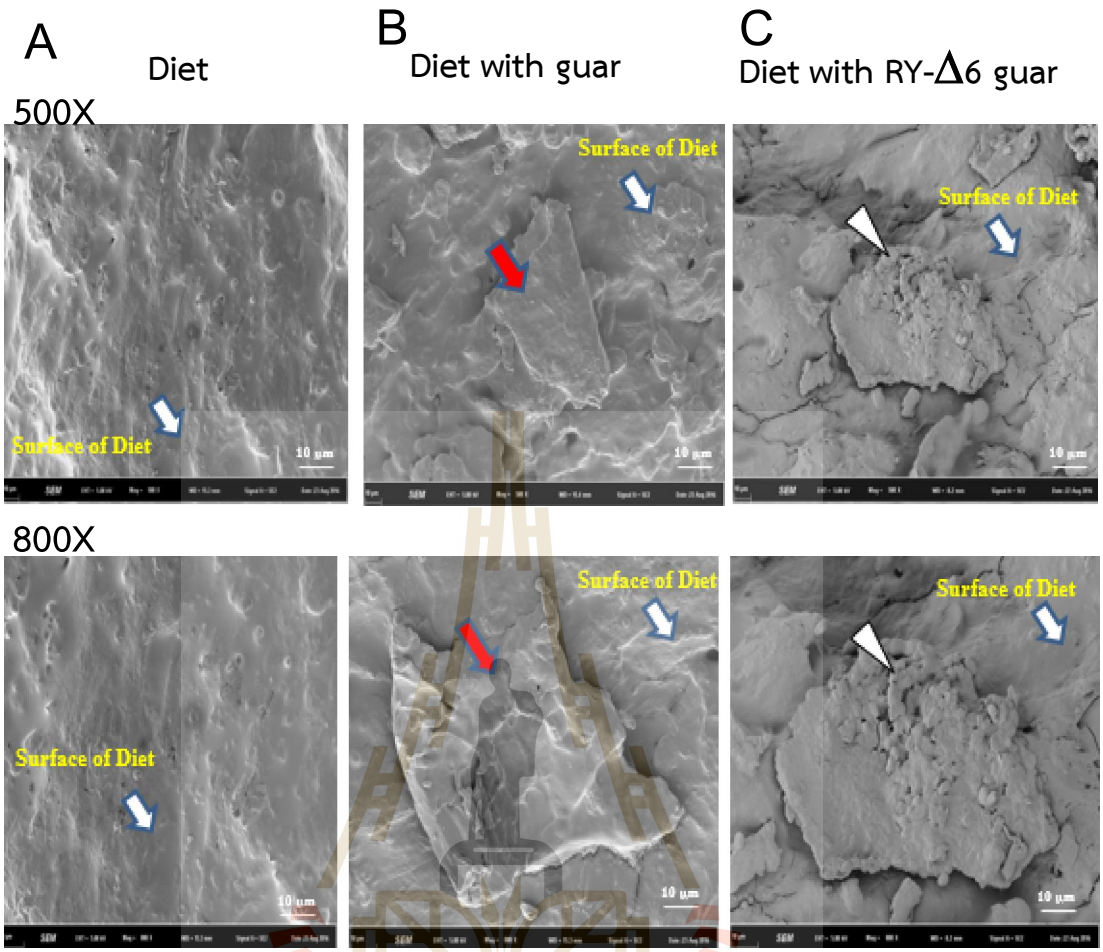

Figure 5.2 Surface of diet (A), diet with guar gum freeze dried (B) and diet with RY-Δ6 was freeze drying with 1% guar gum (RY-Δ6 with guar gum) (C) were observed by scanning electron microscope (SEM). Scale bars represent 10 µm. The white arrow indicates the surface of diet. The red arrow indicates guar gum freeze dried. The triangles indicate the RY-Δ6 with guar gum freeze dried.

5.5.3 The effects of the use of RY-Δ6 as probiotic on growth performance of Nile tilapia

The effects of the use of RY-Δ6 as probiotic on growth performance of Nile tilapia for 45 days and 90 days are shown in Table 5.8. The growth performance includes weight gain, specific growth rate, feed intake (FI), average daily weight gain (ADG), survival rate and feed conversion ratio (FCR) of fish were fed the experimental diet for 45 days were no significant differences among the experimental groups (*P>0.05*). For 90 days of experimental period, Nile tilapia fed basal diet with 2% linseed oil supplemented with recombinant yeast at 10^6 CFU/g feed (Li+RY10^6) and 10^8 CFU/g feed (Li+RY10^8) had highest growth performances including final weight, weigh gain, ADG and SGR (*P<0.05*) when compare with Nile tilapia fed basal diet with 2% linseed oil, basal diet with 2% fish oil and fed commercial diet (Table 5.8) FCR was not significant different among experimental diets. Survival rate were not significance difference in among group of experiment through the experimental diets (*P>0.05*) (Table 5.8).

Table 5.8 Growth performance of Nile tilapia fed experimental diets for 45 days and 90 days (mean±sd, n=4).

45 Days	Initial weigh (g)	Final weigh (g)	Feed intake (g/day)	Weight gain (g)	ADG (g/day)	SGR (%)	FCR	Survival rate (%)
Linseed oil	235.12±2.37	331.84±5.71	2.32±0.11	96.71±5.09	2.15±0.11	0.77±0.03	1.08±0.01	95.00±10.00
Li+NT10^{6}	236.46±1.94	331.24±8.35	2.24±0.18	94.78±9.14	2.11±0.21	0.75±0.06	1.07±0.03	97.50±5.00
Li+NT10^{8}	235.07±2.03	329.81±5.47	2.17±0.15	94.75±7.42	2.11±0.16	0.75±0.06	1.03±0.03	95.00±5.77
Li+RY10^{6}	236.46±2.59	331.24±11.25	2.18±0.19	94.78±10.07	2.11±0.22	0.75±0.06	1.04±0.02	97.50±5.00
Li+RY10^{8}	235.67±2.99	334.04±8.08	2.27±0.13	98.37±7.44	2.19±0.16	0.78±0.05	1.04±0.03	95.00±5.77
Fish oil	235.09±3.82	332.16±4.25	2.24±0.09	97.07±3.64	2.16±0.08	0.77±0.03	1.04±0.03	95.00±10.00
Commercial diet	235.63±2.19	332.68±7.65	2.24±0.21	97.06±6.93	2.16±0.15	0.76±0.05	1.03±0.04	95.00±5.77
90 Days	**Initial weigh (g)**	**Final weigh (g)**	**Feed intake (g/day)**	**Weight gain (g)**	**ADG (g/Day)**	**SGR (%)**	**FCR**	**Survival rate (%)**
Linseed oil	235.12±2.37	428.36±7.36^{c}	2.81±0.14	193.23±7.30^{bc}	2.15±0.08^{bc}	0.67±0.02^{bc}	1.32±0.11	93.75±7.22
Li+NT10^{6}	236.46±1.94	441.99±4.22^{ab}	2.78±0.09	205.54±5.25^{ab}	2.28±0.06^{ab}	0.69±0.02^{abc}	1.22±0.04	93.30±7.77
Li+NT10^{8}	235.07±2.03	438.81±2.36^{abc}	2.77±0.16	203.75±2.99^{abc}	2.26±0.03^{abc}	0.69±0.01^{abc}	1.22±0.06	93.30±7.77
Li+RY10^{6}	236.46±2.59	445.73±3.71^{a}	2.71±0.14	209.27±6.02^{a}	2.32±0.07^{a}	0.71±0.02^{ab}	1.17±0.06	93.75±7.22
Li+RY10^{8}	235.67±2.99	445.32±6.33^{a}	2.84±0.10	209.65±4.44^{a}	2.33±0.05^{a}	0.71±0.01^{a}	1.22±0.06	93.30±7.77
Fish oil	235.09±3.82	427.10±4.79^{c}	2.79±0.19	192.01±1.41^{c}	2.14±0.02^{c}	0.66±0.01^{c}	1.31±0.09	93.75±7.22
Commercial diet	235.63±2.19	430.99±8.05^{bc}	2.86±0.15	195.37±8.73^{bc}	2.17±0.10^{bc}	0.67±0.03^{abc}	1.32±0.09	93.30±7.77

Means with different superscripts in each column differ significantly from each other ($P<0.05$).

5.5.4 Effects of the use of RY-Δ6 as probiotic on chemical composition in whole body and meat of Nile tilapia

Effects of supplement RY-Δ6 on chemical composition in whole body and meat of Nile tilapia for 45 days and 90 days are shown in Table 5.9 and 5.10, respectively. The proximate chemical composition of the experimental Nile tilapia were no significant differences in ash, dry matter, crude protein, crude lipid among the experimental groups (*P>0.05*), suggesting that the supplementation of RY-Δ6 in diet had no effects on chemical composition in whole body and meat of Nile tilapia.

Table 5.9 Whole body composition (%) of Nile tilapia fed experimental diet for 45 days and 90 days (mean±sd, n=4).

45 Days	**Ash (%)**	**Dry matter (%)**	**Crude protein (%)**	**Crude lipid (%)**
Linseed oil	3.79±0.15	27.44±1.26	15.98±0.36	3.85±0.28
Li+WT10^{6}	3.87±0.10	27.75±1.04	16.19±0.60	3.90±0.25
Li+WT10^{8}	3.83±0.25	27.62±1.08	16.13±0.53	3.87±0.19
Li+RY10^{6}	3.87±0.19	27.34±0.77	16.18±0.64	3.91±0.13
Li+RY10^{8}	3.89±0.13	27.60±0.86	16.10±0.98	3.88±0.34
Fish oil	3.86±0.24	27.25±0.77	16.00±0.60	3.86±0.17
Commercial diet	3.84±0.24	27.75±1.15	16.08±0.43	3.92±0.16
90 Days	**Ash (%)**	**Dry matter (%)**	**Crude protein (%)**	**Crude lipid (%)**
Linseed oil	4.10±0.22	26.91±0.65	16.14±0.82	4.15±0.22
Li+WT10^{6}	4.13±0.19	26.69±0.84	16.28±0.81	4.13±0.16
Li+WT10^{8}	4.16±0.12	26.98±0.80	16.34±0.91	4.09±0.18
Li+RY10^{6}	4.13±0.20	26.79±0.84	16.27±0.79	4.07±0.16
Li+RY10^{8}	4.13±0.11	26.49±1.05	16.35±0.48	4.13±0.32
Fish oil	4.14±0.10	26.76±1.15	16.11±0.18	4.14±0.16
Commercial diet	4.11±0.39	26.50±1.03	16.40±0.67	4.11±0.22

Table 5.10 Meat composition (%) of Nile tilapia fed experimental diet for 45 days and 90 days (mean ±sd, n=4).

45 Days	**Ash (%)**	**Dry matter (%)**	**Crude protein (%)**	**Crude lipid (%)**
Linseed oil	1.66±0.07	22.88±0.36	18.01±1.14	2.10±0.17
Li+WT10^{6}	1.60±0.19	22.42±0.59	18.01±0.94	2.12±0.19
Li+WT10^{8}	1.59±0.12	22.46±1.39	17.75±1.14	2.10±0.22
Li+RY10^{6}	1.61±0.12	22.39±1.14	17.94±0.81	2.15±0.38
Li+RY10^{8}	1.61±0.15	22.14±0.41	17.76±0.57	2.14±0.12
Fish oil	1.66±0.10	22.40±1.41	17.75±0.78	2.11±0.13
Commercial diet	1.63±0.13	22.30±1.02	17.90±0.50	2.13±0.11
90 Days	**Ash (%)**	**Dry matter (%)**	**Crude protein (%)**	**Crude lipid (%)**
Linseed oil	1.68±0.06	21.77±0.66	18.48±1.14	2.29±0.28
Li+WT10^{6}	1.69±0.09	21.71±0.91	18.37±0.52	2.31±0.36
Li+WT10^{8}	1.70±0.07	21.64±1.01	18.37±1.26	2.30±0.40
Li+RY10^{6}	1.71±0.06	21.11±0.38	18.15±0.71	2.29±0.19
Li+RY10^{8}	1.69±0.11	21.39±0.49	18.05±0.95	2.33±0.28
Fish oil	1.70±0.08	21.19±0.80	17.92±0.86	2.30±0.28
Commercial diet	1.69±0.06	21.44±1.17	18.17±0.85	2.30±0.16

5.5.5 The effects of the use of RY-Δ6 as probiotic on health status such as hematological, blood chemical and immune parameters of Nile tilapia

Effects of supplement RY-Δ6 on hematological parameters of Nile tilapia for 45 days and 90 days are shown in Table 5.11. The hematological parameters including blood cell (RBC) number hemoglobin content and hematocrit values were not significant differences among experimental diet *(P>0.05)*.

Table 5.11 Hematological parameters of Nile tilapia fed experimental diet for 45 days and 90 days (mean±sd, n=4).

45 Days	**Hematocrit (%)**	**RBC (10^6 cell/mm^3)**	**Hemoglobin (g/dl)**
Linseed oil	31.50±3.24	1.90±0.41	6.58±0.96
Li+WT10^6	31.50±2.68	1.88±0.06	6.56±0.59
Li+WT10^8	30.50±2.38	1.91±0.19	6.72±1.22
Li+RY10^6	30.38±1.70	1.86±0.42	6.72±0.34
Li+RY10^8	31.00±2.48	1.83±0.16	6.45±0.52
Fish oil	30.75±3.07	2.00±0.13	6.69±0.70
Commercial diet	30.88±2.10	1.97±0.13	6.79±0.50
90 Days	**Hematocrit (%)**	**RBC (10^6 cell/mm^3)**	**Hemoglobin (g/dl)**
Linseed oil	31.88±1.31	2.18±0.26	7.16±0.81
Li+WT10^6	32.75±1.32	2.08±0.06	7.17±0.74
Li+WT10^8	31.75±1.26	2.12±0.45	7.07±0.65
Li+RY10^6	32.63±0.85	2.17±0.16	7.03±0.65
Li+RY10^8	32.50±1.08	2.13±0.19	7.07±0.64
Fish oil	32.50±1.29	2.16±0.19	7.10±1.15
Commercial diet	32.13±0.63	2.13±0.05	7.14±0.70

The blood chemical parameters, including glucose, cholesterol, triglyceride, calcium, Iron, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), albumin and total protein (TP) of Nile tilapia which were fed experimental diet for 45 days and 90 days were shown in Table 5.12. There were no significant differences in these blood chemical parameters among the experimental groups ($P>0.05$).

Table 5.12 Blood chemical of Nile tilapia fed experimental diet for 45 days and 90 days (mean±sd, n=4).

45 Days	Glucose (mmol/L)	CHOL (mmol/L)	TG (mmol/L)	TP (g/L)	Albumin (g/L)	Ca (mmol/L)	Iron (µmol/L)	SGOT (Unit/L)	SGPT (Unit/L)
Linseed oil	3.16±0.31	5.18±0.22	2.19±0.07	45.61±0.87	20.64±0.10	2.80±0.16	13.38±0.52	24.27±1.86	8.17±0.72
Li+WT10^{6}	3.16±0.26	5.17±0.37	2.13±0.05	45.38±0.37	20.86±0.29	2.80±0.21	13.44±0.58	24.94±1.83	8.22±1.36
Li+WT10^{8}	3.11±0.21	5.13±0.11	2.18±0.16	45.94±0.87	20.81±0.14	2.81±0.31	13.54±0.59	25.10±1.63	8.25±0.56
Li+RY10^{6}	3.09±0.36	5.16±0.23	2.19±0.20	45.80±0.81	20.63±0.12	2.79±0.26	13.47±0.71	25.07±1.62	8.22±1.36
Li+RY10^{8}	3.14±0.11	5.12±0.20	2.18±0.13	45.88±0.38	20.87±0.31	2.81±0.41	13.39±0.69	25.10±1.63	8.19±0.95
Fish oil	3.11±0.37	5.18±0.14	2.16±0.17	45.97±0.72	20.76±0.20	2.82±0.28	13.55±0.56	24.88±1.71	8.21±0.98
Commercial diet	3.11±0.42	5.16±0.22	2.17±0.19	46.02±0.83	20.80±0.20	2.82±0.23	13.37±0.29	24.61±1.72	8.18±0.96
90 Days	**Glucose (mmol/L)**	**CHOL (mmol/L)**	**TG (mmol/L)**	**TP (g/L)**	**Albumin (g/L)**	**Ca (mmol/L)**	**Iron (µmol/L)**	**SGOT (Unit/L)**	**SGPT (Unit/L)**
Linseed oil	3.24±0.46	5.20±0.11	2.24±0.14	47.50±0.12	21.79±0.49	3.11±0.50	13.81±0.67	25.61±1.62	8.24±0.99
Li+WT10^{6}	3.22±0.43	5.22±0.09	2.28±0.19	47.83±0.60	22.02±0.25	3.12±0.32	13.63±0.55	25.29±1.93	8.21±0.94
Li+WT10^{8}	3.26±0.21	5.20±0.13	2.24±0.12	47.60±0.11	21.88±0.83	3.12±0.47	13.72±0.70	25.53±0.97	8.25±1.20
Li+RY10^{6}	3.28±0.32	5.23±0.12	2.28±0.24	48.01±0.78	22.03±0.28	3.13±0.49	13.74±0.59	25.64±1.22	8.22±0.98
Li+RY10^{8}	3.29±0.39	5.24±0.09	2.29±0.07	47.57±0.10	21.93±0.21	3.10±0.45	13.82±0.35	25.85±0.93	8.26±0.97
Fish oil	3.24±0.15	5.22±0.15	2.25±0.15	47.92±0.84	22.26±0.11	3.13±0.47	13.77±0.56	25.99±0.52	8.20±0.36
Commercial diet	3.23±0.11	5.19±0.16	2.26±0.28	47.59±0.14	21.89±0.57	3.10±0.29	13.75±0.58	25.67±1.05	8.26±1.20

The effects of supplement RY-Δ6 in diet on immune parameters including total immunoglobulin, lysozyme activity and alternative complement haemolytic 50 (ACH50) activity of Nile tilapia for 45 days and 90 days. Nile tilapia fed diet supplemented with Li+RY10^6 and Li+RY10^8 had significant higher total immunoglobulin, lysozyme activity and ACH50 when compared to that fed diet with 2% linseed oil, diet with 2% fish oil and fed commercial diet ($P<0.05$) (Table 5.13). These results suggested that the supplementation RY-Δ6 and NT in diet cloud increase these immune parameters in Nile tilapia.

Table 5.13 Immune parameter of Nile tilapia fed experimental diet for 45 days and 90 days (mean±sd, n=4).

45 Days	**Immunoglobulin (g/L)**	**Lysozyme (µg/ml)**	**ACH50 (Unit/ml)**
Linseed oil	29.97±0.58^b	16.99 ±0.72^b	30.07±1.09^b
Li+WT10^6	33.01±0.20^a	18.06±0.68^{ab}	33.68±0.90^a
Li+WT10^8	33.62 ±0.87^a	18.35±0.62^a	33.89±0.84^a
Li+RY10^6	33.27 ±0.77^a	18.07 ±0.68^{ab}	33.57±0.92^a
Li+RY10^8	33.41 ±0.91^a	18.28±0.46^{ab}	34.50±0.50^a
Fish oil	29.77 ±0.51^b	17.02±0.37^b	30.12±0.73^b
Commercial diet	29.72 ±0.72^b	17.00±0.26^b	30.40±1.01^b
90 Days	**Immunoglobulin (g/L)**	**Lysozyme (µg/ml)**	**ACH50 (Unit/ml)**
Linseed oil	31.22 ±0.20^b	19.22±0.96^b	30.23±0.53^b
Li+WT10^6	34.86 ±0.25^a	22.01±0.66^a	33.75±0.88^a
Li+WT10^8	35.13±0.94^a	22.69±0.73^a	33.77±1.02^a
Li+RY10^6	35.06 ±0.91^a	22.04±0.65^a	33.70±0.77^a
Li+RY10^8	34.62 ±0.30^a	22.41±0.78^a	34.09±0.84^a
Fish oil	31.54 ±0.71^b	19.78±0.84^b	30.26±0.26^b
Commercial diet	31.07 ±0.41^b	19.96±0.45^b	30.14±0.47^b

Means with different superscripts in each column differ significantly from each other ($P<0.05$).

5.5.6 The effects of the use of RY-Δ6 as probiotic on microbial populations in intestinal tract of Nile tilapia

The effects of supplement RY-Δ6in diet on microbial populations in intestinal tract of Nile tilapia for 45 days and 90 days are shown in Table 5.14. Total bacteria, *Bifidobacterium* spp. and *Lactobacilus* spp. of Nile tilapia fed experimental diet for 45 days were no significant differences among the experimental groups *(P>0.05)*. Nile tilapia fed diet supplemented Li+RY10^6 and diet supplemented Li+RY10^8 for 45 days exhibited increase the number of yeast and fungi and decrease the number of *Vibrio* spp. *(P<0.05)* compared with fish fed diet with 2% linseed oil, diet with 2% fish oil and fed commercial diet. Note that the RY-Δ6 was detectable in the intestinal tract of experimental fish.

The effects of supplement RY-Δ6in diet on microbial populations in intestinal tract of Nile tilapia for 90 days. There were no significant differences in the number of intestinal total bacteria among the experimental groups *(P>0.05)* except for *Vibrio* spp. Fish fed diet supplemented Li+RY10^6 and diet supplemented Li+RY10^8 for 90 days had lower number of *Vibrio* spp. *(P<0.05)* compared with fed diet with 2% linseed oil, diet with 2% fish oil and fed commercial diet (Table 5.14) In addition, RY-Δ6 could be isolated from the intestine of Nile tilapia, demonstrating that RY-Δ6 can survive and colonized in the intestinal environment of Nile tilapia.

Table 5.14 Microbial populations in intestinal tract of Nile tilapia fed experimental diet for 45 days and 90 days (mean±sd, n=4).

45 Days	**Total Bacteria (log CFU/g)**	**Yeast & Fungi (log CFU/g)**	***Bifidobacterium* spp. (log CFU/g)**	***Lactobacilus* spp. (log CFU/g)**	***Vibrio* spp. (log CFU/g)**	**Recombinant yeast (log CFU/g)**
Linseed oil	6.19±0.02	3.84±0.10^{b}	5.13±0.06	4.15±0.04	5.30±0.03^{a}	0.00
Li+WT10^{6}	6.18±0.01	4.21±0.05^{a}	4.88±0.12	4.07±0.13	4.66±0.09^{b}	0.00
Li+WT10^{8}	6.22±0.05	4.11±0.08^{a}	4.86±0.41	3.99±0.57	4.70±0.08^{b}	0.00
Li+RY10^{6}	6.24±0.08	4.19±0.06^{a}	4.92±0.22	4.00±0.21	4.69±0.08^{b}	3.83±0.03^{b}
Li+RY10^{8}	6.19±0.02	4.26±0.07^{a}	5.17±0.16	4.02±0.08	4.70±0.03^{b}	4.20±0.15^{a}
Fish oil	6.18±0.01	3.81±0.14^{b}	4.90±0.03	4.01±0.20	5.21±0.15^{a}	0.00
Commercial diet	6.21±0.06	3.86±0.06^{b}	5.00±0.10	4.09±0.10	5.29±0.03^{a}	0.00
90 Days	**Total Bacteria (log CFU/g)**	**Yeast & Fungi (log CFU/g)**	***Bifidobacterium* spp. (log CFU/g)**	***Lactobacilus* spp. (log CFU/g)**	***Vibrio* spp. (log CFU/g)**	**Recombinant yeast (log CFU/g)**
Linseed oil	6.23±0.06	4.01±0.23	5.47±0.22	4.00±0.13	5.38±0.20^{a}	0.00
Li+WT10^{6}	6.20±0.05	4.42±0.18	5.55±0.25	4.08±0.09	4.77±0.08^{b}	0.00
Li+WT10^{8}	6.20±0.04	4.38±0.14	5.55±0.41	4.15±0.03	4.70±0.42^{b}	0.00
Li+RY10^{6}	6.23±0.08	4.44±0.14	5.85±0.21	4.09±0.04	4.67±0.17^{b}	4.09±0.05^{b}
Li+RY10^{8}	6.20±0.06	4.45±0.23	5.76±0.15	4.20±0.10	4.59±0.07^{b}	4.35±0.04^{a}
Fish oil	6.18±0.12	4.11±0.22	5.48±0.20	4.00±0.14	5.29±0.17^{a}	0.00
Commercial diet	6.18±0.03	4.08±0.31	5. 42±0.19	4.01±0.14	5.28±0.07^{a}	0.00

Means with different superscripts in each column differ significantly from each other ($P<0.05$).

5.5.7 The effects of the use of RY-Δ6 as probiotic on intestinal villus height and number of goblet cells in intestinal tract of Nile tilapia

Effects of dietary supplement with RY-Δ6 on intestinal villi height and number of goblet cells in intestinal tract of Nile tilapia at 45 days and 90 days were show in the Table 5.15. The supplementation with RY-Δ6 and NT had significantly increase villus height in the anterior intestine ($P<0.05$) but not in middle and posterior portion of intestine. There were no significant differences in the number of goblet cells in intestinal tract ($P>0.05$).

5.5.8 Effects of the use of RY-Δ6 as probiotic on accumulation of fatty acid profile in meat and liver of Nile tilapia

Effect of supplement RY-Δ6 on deposition of fatty acid profile in meat of Nile tilapia for 45 days are shown in Table 5.16. The deposition of fatty acid in meat of Nile tilapia were not significant differences among the experimental groups *(P>0.05)*. When extend the experimental period to 90 days Table 5.17, the fatty acid C14:0, C14:1, C16:1, C20:0, C20:1n9, C20:2, C22:0, C20:3n6, C22:1n9, C20:3n3, C22:2, C24:0 and C24:1 in meat of Nile tilapia were not significant differences among the experimental groups *(P>0.05)*. Nile tilapia which were fed diet supplemented RY-Δ6 (Li+RY10^6 and Li+RY10^8 group) had higher C18:3n6 andC18:4n3 in meat ($P<0.05$). Both fatty acids are products of delta 6 desaturase, suggesting that probiotic RY-Δ6 would exert delta 6 desaturase activity. Fish fed dietary supplementary with fish oil had highest C20:5n3 and C22:6n3. Note that, fish fed dietary Li+RY10^6 and Li+RY10^8 had higher C22:6n3 when compared to fish fed dietary linseed oil alone; however, the amount of C22:6n3 did not increase as the similar amount observed in meat of fish fed dietary fish oil. Similar effect of supplement RY-Δ6 on deposition of fatty acid profile

in liver of Nile tilapia for 45 days are shown in Table 5.18. The deposition of fatty acid liver of Nile tilapia were not significant differences among the experimental groups *(P>0.05)*.

Effect of supplement RY-Δ6 on accumulation of fatty acid profile in liver of Nile tilapia for 90 days are shown in Table 5.19. The fatty acid C14:0, C14:1, C16:1, C20:0, C20:1n9, C20:2, C22:0, C20:3n6, C22:1n9, C20:3n3, C20:4n6, C22:2, C24:0 and C24:1 in liver of Nile tilapia were not significant differences among the experimental groups *(P>0.05)*. Dietary Li+RY10^{6} and Li+RY10^{8} group could increase C18:3n6 and C18:4n3 in liver (*P<0.05*). Accumulation of C18:3n6 and C18:4n3 were increased in liver of fish when increase the level of supplementation of RY-Δ6 (*P<0.05*). Fish fed dietary supplementary with fish oil had highest hepatic C20:5n3 and C22:6n3. Note that, fish fed dietary Li+RY10^{6} and Li+RY10^{8} had higher C22:6n3 when compared to fish fed dietary linseed oil alone.

Table 5.15 Intestinal villus height and no. goblet cells in different regions of the intestine of Nile tilapia fed experimental diet for 45 days and 90 days (mean±sd, n=4).

45 Days	Anterior		Middle		Posterior	
	Villus height (µm)	No. goblet cells	Villus height (µm)	No. goblet cells	Villus height (µm)	No. goblet cells
Linseed oil	782.08±81.65^{b}	21.33±1.90	538.47±20.22	9.08±0.32	318.00±35.23	6.00±1.12
Li+WT10^{6}	980.62±14.33^{a}	21.50±1.26	536.69±46.30	8.92±0.50	310.31±82.17	5.92±0.92
Li+WT10^{8}	982.27±15.34^{a}	21.84±0.19	582.43±65.21	8.92±0.57	322.92±17.84	6.09±1.03
Li+RY10^{6}	979.81±53.84^{a}	21.75±2.10	586.88±73.76	9.00±0.98	321.84±44.72	5.92±1.13
Li+RY10^{8}	983.90±20.98^{a}	21.75±3.47	567.90±69.21	9.08±0.99	321.48±48.54	5.92±0.32
Fish oil	779.84±60.65^{b}	21.42±0.88	589.76±79.00	9.08±1.34	312.26±15.98	5.92±0.42
Commercial diet	775.31±52.49^{b}	21.09±1.83	585.15±48.92	9.00±1.36	329.55±46.27	5.92±1.23
90 Days						
Linseed oil	903.81±46.17^{b}	27.67±3.08	741.62±39.50	12.00±3.89	567.23±28.88	6.58±1.53
Li+WT10^{6}	1,315.87±90.42^{a}	27.33±1.96	733.16±32.96	11.34±1.46	530.78±11.68	6.42±0.84
Li+WT10^{8}	1,337.92±106.68^{a}	28.00±2.13	738.41±10.79	12.17±3.32	556.29±79.30	6.08±1.64
Li+RY10^{6}	1,329.16±60.56^{a}	28.17±2.29	743.80±19.30	12.17±3.32	551.64±24.63	6.08±0.88
Li+RY10^{8}	1,321.53±88.97^{a}	28.25±0.16	733.56±7.96	12.67±2.45	564.52±64.91	6.25±0.50
Fish oil	900.81±122.01^{b}	28.25±0.88	738.38±9.80	12.42±0.17	552.56±42.69	6.17±0.79
Commercial diet	904.66±137.29^{b}	28.50±1.00	737.42±13.93	12.59±1.40	531.07±57.54	6.09±0.69

Means with different superscripts in each column differ significantly from each other ($P<0.05$).

Table 5.16 Accumulation of fatty acid profile (mg/g lipid) in meat of Nile tilapia fed experimental diet for 45 days (mean±sd, n=4).

Fatty acid	Linseed oil	Li+WT10^{6}	Li+WT10^{8}	Li+RY10^{6}	Li+RY10^{8}	Fish oil	Commercial diet
C10:0	0.53±0.12	0.52±0.13	0.55±0.15	0.52±0.10	0.56±0.08	0.53±0.07	0.56±0.09
C12:0	0.20±0.05	0.19±0.05	0.22±0.05	0.20±0.04	0.21±0.06	0.20±0.07	0.21±0.04
C14:0	2.57±0.77	2.53±0.53	2.58±0.59	2.57±0.37	2.55±0.72	2.53±0.67	2.54±0.59
C14:1	0.09±0.03	0.10±0.02	0.11±0.05	0.09±0.03	0.09±0.02	0.11±0.02	0.09±0.02
C16:0	37.95±8.60	36.06±5.78	37.36±6.94	36.39±9.21	38.08±7.68	37.40±6.58	36.50±6.61
C16:1	3.74±0.84	3.66±0.80	3.83±0.47	3.83±0.63	3.64±0.71	3.84±0.49	3.84±0.58
C18:0	10.69±3.32	10.34±3.15	11.51±2.74	10.58±2.17	10.26±2.78	11.44±2.94	11.50±2.24
C18:1N9T	nd	nd	nd	nd	nd	nd	nd
C18:1N9C	30.73±6.56	30.39±3.69	31.26±3.14	30.31±5.17	31.09±7.97	30.77±4.72	31.11±5.31
C18:2N6T	nd	nd	nd	nd	nd	nd	nd
C18:2N6C	11.92±3.79	11.49±2.63	11.27±2.07	11.22±2.23	11.93±3.24	10.21±2.96	10.82±2.95
C20:0	0.51±0.09	0.53±0.07	0.55±0.08	0.54±0.08	0.53±0.12	0.54±0.09	0.53±0.10
C18:3N6	0.43±0.10	0.38±0.11	0.43±0.06	0.39±0.09	0.41±0.09	0.41±0.06	0.43±0.07
C20:1n9	1.84±0.53	1.82±0.70	1.83±0.18	1.83±0.58	1.82±0.48	1.83±0.26	1.85±0.30
C18:3N3	1.29±0.32	1.28±0.40	1.28±0.17	1.29±0.21	1.28±0.44	1.28±0.49	1.26±0.15
C18:4N3	0.29±0.07	0.27±0.06	0.30±0.07	0.28±0.09	0.28±0.05	0.27±0.09	0.29±0.06
C20:2	1.11±0.27	1.11±0.15	1.18±0.28	1.17±0.28	1.16±0.31	1.19±0.09	1.20±0.15
C22:0	0.12±0.03	0.12±0.02	0.12±0.01	0.12±0.03	0.12±0.03	0.13±0.02	0.13±0.02

Means with different superscripts in each row differ significantly from each other ($P<0.05$).

Table 5.16 Accumulation of fatty acid profile (mg/g lipid) in meat of Nile tilapia fed experimental diet for 45 days (mean±sd, n=4) (Continued).

Fatty acid	Linseed oil	Li+WT10^{6}	Li+WT10^{8}	Li+RY10^{6}	Li+RY10^{8}	Fish oil	Commercial diet
C20:3N6	1.23±0.33	1.22±0.33	1.24±0.24	1.26±0.33	1.24±0.46	1.22±0.16	1.24±0.18
C22:1N9	0.31±0.07	0.29±0.05	0.30±0.03	0.31±0.05	0.32±0.10	0.30±0.06	0.31±0.04
C20:3N3	0.24±0.10	0.23±0.07	0.25±0.09	0.24±0.06	0.25±0.09	0.23±0.05	0.24±0.06
C20:4N6	3.14±0.71	3.11±0.34	3.16±0.44	3.11±0.55	3.16±0.80	3.18±0.35	3.15±0.53
C22:2	0.10±0.03	0.10±0.04	0.11±0.03	0.10±0.02	0.10±0.03	0.11±0.01	0.110.02
C20:5N3	0.23±0.07	0.23±0.08	0.23±0.05	0.22±0.06	0.23±0.06	0.23±0.09	0.24±0.04
C24:0	0.10±0.03	0.10±0.03	0.10±0.03	0.10±0.04	0.10±0.02	0.10±0.02	0.10±0.02
C24:1	0.10±0.02	0.09±0.02	0.10±0.01	0.10±0.02	0.10±0.02	0.10±0.02	0.10±0.02
C22:6N3	3.62±0.89	3.23±0.49	3.53±0.53	3.46±0.84	3.63±0.45	3.27±0.54	3.65±0.62
∑SFA	52.69±9.64	51.60±9.92	52.85±11.31	51.04±9.67	52.41±10.21	52.86±7.62	52.06±6.03
∑MUFA	37.21±7.85	36.10±5.37	37.79±4.98	36.86±5.84	37.45±9.33	37.34±5.02	37.75±5.83
∑PUFA	23.61±6.25	22.54±3.88	23.30±3.99	22.75±3.82	23.67±5.54	21.60±3.11	22.62±4.09
∑n3-PUFA	5.67±1.21	5.25±0.92	5.88±0.95	5.49±1.06	5.67±0.95	5.28±0.98	5.68±0.83
∑n6-PUFA	16.73±4.84	16.02±2.91	16.11±3.04	15.98±2.87	16.73±4.44	15.01±2.95	15.64±3.38

Means with different superscripts in each row differ significantly from each other ($P<0.05$).

Table 5.17 Accumulation of fatty acid profile (mg/g lipid) in meat of Nile tilapia fed experimental diet for 90 days (mean±sd, n=4).

Fatty acid	Linseed oil	Li+WT10^{6}	Li+WT10^{8}	Li+RY10^{6}	Li+RY10^{8}	Fish oil	Commercial diet
C10:0	0.58±0.09^{b}	0.59±0.07^{b}	0.57±0.06^{b}	0.60±0.04^{b}	0.59±0.08^{b}	0.60±0.07^{b}	0.72±0.06^{a}
C12:0	0.24±0.02^{b}	0.25±0.02^{b}	0.24±0.04^{b}	0.25±0.03^{b}	0.25±0.03^{b}	0.26±0.05^{b}	0.41±0.06^{a}
C14:0	3.27±0.44	3.31±0.49	3.46±0.58	3.38±0.51	3.47±0.33	3.33±0.47	3.36±0.50
C14:1	0.14±0.01	0.15±0.05	0.14±0.04	0.15±0.03	0.15±0.02	0.16±0.03	0.14±0.03
C16:0	45.51±6.65^{b}	45.88±4.35^{b}	45.09±7.78^{b}	44.61±3.28^{b}	45.74±5.93^{b}	44.97±2.86^{b}	56.10±4.50^{a}
C16:1	4.60±0.50	4.51±0.40	4.61±0.56	4.60±0.50	4.63±0.28	4.55±0.72	4.58±0.78
C18:0	13.22±1.30^{b}	13.30±2.24^{b}	13.55±2.01^{b}	13.13±1.47^{b}	13.31±1.33^{b}	13.56±1.71^{b}	16.93±1.41^{a}
C18:1N9T	nd	nd	nd	nd	nd	nd	nd
C18:1N9C	48.34±4.43^{b}	47.67±5.41^{b}	48.24±5.35^{b}	47.38±4.20^{b}	49.72±4.85^{b}	49.16±4.17^{b}	74.12±7.55^{a}
C18:2N6T	nd	nd	nd	nd	nd	nd	nd
C18:2N6C	22.59±3.5^{ab}	23.13±1.20^{a}	22.46±1.18^{ab}	22.56±2.50^{ab}	23.14±3.58^{a}	18.63±1.22^{c}	19.04±1.55^{bc}
C20:0	0.66±0.03	0.68±0.10	0.64±0.15	0.67±0.07	0.66±0.11	0.68±0.13	0.65±0.12
C18:3N6	0.53±0.05^{c}	0.55±0.12^{c}	0.54±0.11^{c}	0.69±0.07^{b}	0.93±0.10^{a}	0.49±0.05^{c}	0.47±0.07^{c}
C20:1n9	2.71±0.40	2.52±0.70	2.74±0.85	2.64±0.58	2.65±0.56	2.75±0.79	2.67±0.25
C18:3N3	2.25±0.26^{a}	2.24±0.18^{a}	2.23±0.25^{a}	2.20±0.26^{ab}	2.29±0.32^{a}	1.73±0.15^{c}	1.85±0.21^{bc}
C18:4N3	0.34±0.09^{c}	0.34±0.05^{c}	0.34±0.09^{c}	0.52±0.06^{b}	0.72±0.12^{a}	0.32±0.07^{c}	0.31±0.06^{c}
C20:2	1.47±0.15	1.46±0.46	1.43±0.19	1.44±0.15	1.47±0.14	1.48±0.21	1.45±0.22
C22:0	0.20±0.07	0.21±0.06	0.19±0.05	0.21±0.03	0.20±0.03	0.20±0.04	0.18±0.04

Means with different superscripts in each row differ significantly from each other ($P<0.05$).

Table 5.17 Accumulation of fatty acid profile (mg/g lipid) in meat of Nile tilapia fed experimental diet for 90 days (mean±sd, n=4) (Continued).

Fatty acid	Linseed oil	Li+WT10^{6}	Li+WT10^{8}	Li+RY10^{6}	Li+RY10^{8}	Fish oil	Commercial diet
C20:3N6	1.43±0.10	1.45±0.11	1.47±0.15	1.47±0.09	1.48±0.09	1.43±0.15	1.38±0.12
C22:1N9	0.39±0.04	0.38±0.08	0.37±0.10	0.40±0.11	0.40±0.06	0.40±0.10	0.39±0.06
C20:3N3	0.31±0.10	0.32±0.07	0.31±0.05	0.30±0.05	0.33±0.04	0.29±0.07	0.30±0.09
C20:4N6	3.58±0.45	3.61±0.56	3.67±0.35	3.71±0.33	3.71±0.22	3.57±0.39	3.18±0.29
C22:2	0.13±0.03	0.15±0.03	0.14±0.02	0.14±0.01	0.14±0.04	0.14±0.01	0.14±0.01
C20:5N3	0.41±0.06^{b}	0.40±0.06^{b}	0.42±0.04^{b}	0.43±0.08^{b}	0.41±0.06^{b}	0.63±0.11^{a}	0.22±0.04^{c}
C24:0	0.21±0.03	0.19±0.04	0.21±0.05	0.19±0.07	0.19±0.04	0.19±0.03	0.19±0.03
C24:1	0.14±0.01	0.15±0.03	0.14±0.04	0.13±0.03	0.14±0.03	0.13±0.02	0.13±0.03
C22:6N3	8.50±0.48^{c}	8.28±0.40^{c}	8.27±0.74^{c}	9.56±0.89^{b}	9.99±0.44^{b}	11.31±0.78^{a}	5.97±0.61^{d}
∑SFA	63.90±8.49^{b}	64.41±4.35^{b}	63.95±6.95^{b}	63.03±4.16^{b}	64.42±6.47^{b}	63.79±3.62^{b}	78.54±6.21^{a}
∑MUFA	56.78±4.75^{b}	55.86±6.02^{b}	56.71±6.56^{b}	55.76±4.15^{b}	58.16±5.25^{b}	57.63±4.81^{b}	82.53±7.72^{a}
∑PUFA	41.54±4.40^{ab}	41.93±1.27^{ab}	41.28±1.96^{ab}	43.15±3.07^{ab}	44.78±3.80^{a}	40.01±2.46^{b}	34.31±2.23^{c}
∑n3-PUFA	11.81±0.67^{cd}	11.59±0.34^{d}	11.57±0.86^{d}	13.01±1.15^{bc}	13.73±0.58^{ab}	14.27±0.98^{a}	8.64±0.63^{e}
∑n6-PUFA	28.13±3.82^{a}	28.74±1.23^{a}	28.15±1.41^{a}	28.56±2.55^{a}	29.43±3.82^{a}	24.12±1.49^{b}	24.08±1.56^{b}

Means with different superscripts in each row differ significantly from each other ($P<0.05$).

Table 5.18 Accumulation of fatty acid profile (mg/g lipid) in liver of Nile tilapia fed experimental diet for 45 days (mean±sd, n=4).

Fatty acid	Linseed oil	Li+WT10^{6}	Li+WT10^{8}	Li+RY10^{6}	Li+RY10^{8}	Fish oil	Commercial diet
C10:0	0.78±0.06	0.81±0.03	0.80±0.04	0.81±0.04	0.79±0.07	0.79±0.03	0.79±0.04
C12:0	0.23±0.06	0.23±0.08	0.23±0.05	0.25±0.07	0.22±0.06	0.22±0.05	0.25±0.07
C14:0	13.39±3.50	13.32±2.67	13.81±2.03	13.65±5.61	13.65±2.63	13.83±2.25	13.29±4.69
C14:1	0.42±0.09	0.38±0.01	0.40±0.05	0.40±0.06	0.39±0.11	0.38±0.14	0.40±0.11
C16:0	83.38±8.94	82.84±7.91	83.18±5.28	82.90±7.94	83.12±8.86	82.32±7.19	83.23±3.94
C16:1	10.89±0.87	10.44±1.50	10.52±0.67	10.24±1.43	10.86±1.24	10.41±1.98	10.90±0.56
C18:0	38.65±9.63	38.84±4.04	38.74±6.68	38.41±7.61	38.74±5.48	38.06±5.17	38.47±3.87
C18:1N9T	nd	nd	nd	nd	nd	nd	nd
C18:1N9C	130.84±9.84	131.68±8.69	131.99±7.86	131.08±8.03	131.33±7.33	132.34±6.97	131.96±5.37
C18:2N6T	nd	nd	nd	nd	nd	nd	nd
C18:2N6C	25.25±4.02	24.97±3.63	25.74±4.35	25.14±2.05	25.33±3.43	25.83±3.10	25.31±3.74
C20:0	1.12±0.29	1.13±0.15	1.09±0.21	1.19±0.06	1.20±0.04	1.17±0.07	1.14±0.14
C18:3N6	2.03±0.26	2.01±0.62	2.01±0.48	2.15±0.24	2.29±0.10	2.08±0.41	1.91±0.47
C20:1n9	1.40±0.14	1.39±0.07	1.38±0.17	1.43±0.34	1.40±0.31	1.42±0.32	1.39±0.32
C18:3N3	8.09±0.80	8.11±0.96	8.13±0.45	8.02±0.62	8.15±0.39	8.11±0.67	8.07±0.32
C18:4N3	0.85±0.16	0.84±0.11	0.80±0.20	0.80±0.21	0.82±0.14	0.81±0.17	0.81±0.05
C20:2	1.28±0.55	1.25±0.22	1.26±0.57	1.24±0.09	1.23±0.22	1.27±0.51	1.28±0.22
C22:0	2.13±0.44	2.23±0.51	2.22±0.79	2.20±0.75	2.19±0.76	2.29±0.69	2.18±0.85

Means with different superscripts in each row differ significantly from each other ($P<0.05$).

Table 5.18 Accumulation of fatty acid profile (mg/g lipid) in liver of Nile tilapia fed experimental diet for 45 days (mean±sd, n=4) (Continued).

Fatty acid	Linseed oil	Li+WT10^{6}	Li+WT10^{8}	Li+RY10^{6}	Li+RY10^{8}	Fish oil	Commercial diet
C20:3N6	2.81±0.21	2.74±0.61	2.72±0.26	2.77±0.54	2.78±0.42	2.76±0.45	2.76±0.41
C22:1N9	0.05±0.02	0.05±0.02	0.05±0.02	0.05±0.02	0.04±0.01	0.05±0.02	0.05±0.02
C20:3N3	0.49±0.17	0.47±0.13	0.47±0.23	0.49±0.25	0.50±0.16	0.49±0.16	0.49±0.16
C20:4N6	8.49±0.99	8.55±0.56	8.41±0.61	8.42±0.91	8.51±0.37	8.51±0.43	8.58±0.57
C22:2	0.17±0.03	0.19±0.03	0.19±0.05	0.18±0.04	0.19±0.02	0.20±0.04	0.19±0.04
C20:5N3	2.51±0.24	2.44±0.39	2.39±0.39	2.38±0.53	2.47±0.35	2.76±0.83	2.310.40
C24:0	0.27±0.04	0.30±0.02	0.27±0.03	0.29±0.05	0.30±0.05	0.29±0.06	0.29±0.04
C24:1	3.70±0.96	3.62±0.82	3.67±0.74	3.70±0.61	3.67±0.89	3.63±0.88	3.70±0.59
C22:6N3	11.38±1.89	11.03±2.79	10.91±1.58	11.11±2.60	10.44±2.62	12.25±2.45	10.87±2.30
∑SFA	139.96±15.48	139.70±11.92	140.35±8.40	139.71±13.59	140.21±7.27	138.97±1.91	139.62±1.63
∑MUFA	147.29±8.90	147.57±8.47	148.02±7.11	146.89±8.33	147.69±6.71	148.23±7.28	148.39±4.16
∑PUFA	63.35±6.46	62.58±4.86	63.05±4.28	62.69±4.73	65.05±4.34	65.05±4.02	62.56±4.16
∑n3-PUFA	23.31±2.59	22.88±2.02	22.70±2.13	22.80±2.71	22.38±2.90	24.41±2.43	22.54±1.67
∑n6-PUFA	38.58±4.41	38.26±4.71	38.88±4.35	38.48±3.54	41.24±4.33	39.18±3.21	38.55±3.23

Means with different superscripts in each row differ significantly from each other ($P<0.05$).

Table 5.19 Accumulation of fatty acid profile (mg/g lipid) in liver of Nile tilapia fed experimental diet for 90 days (mean±sd, n=4).

Fatty acid	Linseed oil	Li+WT10^{6}	Li+WT10^{8}	Li+RY10^{6}	Li+RY10^{8}	Fish oil	Commercial diet
C10:0	1.00±0.14	0.96±0.29	1.01±0.09	0.96±0.20	1.02±0.19	0.97±0.23	1.35±0.15
C12:0	0.53±0.12^{b}	0.53±0.15^{b}	0.49±0.06^{b}	0.50±0.17^{b}	0.51±0.14^{b}	0.52±0.17^{b}	1.34±0.38^{a}
C14:0	21.19±3.84	22.12±2.97	21.58±2.46	21.48±2.71	22.49±2.44	21.51±3.73	21.53±4.39
C14:1	0.69±0.04	0.64±0.11	0.68±0.17	0.66±0.06	0.65±0.12	0.61±0.07	0.62±0.18
C16:0	109.14±6.66^{ab}	107.32±6.77^{b}	109.81±5.04^{ab}	107.63±8.62^{ab}	107.87±7.96^{ab}	109.79±4.69^{ab}	122.08±3.40^{a}
C16:1	31.49±7.82	32.88±4.91	32.29±6.38	31.66±3.86	31.33±6.05	31.98±4.98	32.39±5.05
C18:0	44.49±3.63	43.22±5.91	45.18±6.14	44.35±6.01	45.03±3.96	43.23±8.22	53.95±2.39
C18:1N9T	nd	nd	nd	nd	nd	nd	nd
C18:1N9C	150.78±6.68^{b}	149.43±5.68^{b}	150.28±10.58^{b}	148.22±8.92^{b}	150.36±5.94^{b}	150.13±9.81^{b}	171.88±7.29^{a}
C18:2N6T	nd	nd	nd	nd	nd	nd	nd
C18:2N6C	38.32±5.04	39.05±3.91	38.24±5.90	38.43±3.82	38.96±3.27	30.32±1.89	32.61±2.01
C20:0	1.31±0.42	1.33±0.24	1.32±0.14	1.30±0.32	1.31±0.18	1.31±0.29	1.30±0.26
C18:3N6	2.83±0.36^{b}	2.88±0.56^{b}	2.85±0.31^{b}	3.74±0.59^{ab}	4.63±0.33^{a}	2.75±0.28^{b}	2.82±0.51^{b}
C20:1n9	1.69±0.22	1.56±0.17	1.61±0.13	1.58±0.18	1.63±0.29	1.60±0.16	1.61±0.41
C18:3N3	15.48±1.16^{a}	15.46±1.80^{a}	15.16±1.66^{a}	15.79±2.25^{a}	15.07±1.36^{a}	9.50±1.03^{b}	11.24±1.59^{b}
C18:4N3	1.21±0.05^{b}	1.23±0.04^{b}	1.27±0.14^{b}	1.58±0.21^{a}	1.77±0.25^{a}	1.22±0.13^{b}	1.21±0.18^{b}
C20:2	1.35±0.42	1.40±0.12	1.37±0.25	1.33±0.15	1.35±0.13	1.35±0.14	1.39±0.17
C22:0	2.91±0.26	2.95±0.57	2.97±0.23	2.84±0.30	2.98±0.31	2.95±0.26	2.87±0.34

Means with different superscripts in each row differ significantly from each other ($P<0.05$).

Table 5.19 Accumulation of fatty acid profile (mg/g lipid) in liver of Nile tilapia fed experimental diet for 90 days (mean±sd, n=4) (Continued).

Fatty acid	Linseed oil	Li+WT10^{6}	Li+WT10^{8}	Li+RY10^{6}	Li+RY10^{8}	Fish oil	Commercial diet
C20:3N6	3.19±0.14	3.22±0.71	3.25±0.28	3.30±0.10	3.28±0.13	3.17±0.10	3.13±0.74
C22:1N9	0.05±0.01	0.06±0.01	0.06±0.01	0.05±0.02	0.06±0.01	0.06±0.01	0.05±0.01
C20:3N3	0.60±0.08	0.60±0.08	0.60±0.09	0.61±0.09	0.61±0.10	0.64±0.07	0.59±0.08
C20:4N6	10.32±2.78	10.22±3.16	10.49±3.21	10.98±2.32	11.21±1.91	10.12±2.17	9.83±2.34
C22:2	0.21±0.07	0.22±0.01	0.21±0.01	0.19±0.04	0.21±0.01	0.21±0.01	0.20±0.02
C20:5N3	3.84±0.52^{b}	3.84±0.44^{b}	3.80±0.54^{b}	3.86±0.59^{b}	3.89±0.28^{b}	5.44±0.36^{a}	2.94±0.24^{b}
C24:0	0.39±0.01	0.41±0.04	0.38±0.04	0.40±0.07	0.38±0.01	0.40±0.04	0.39±0.05
C24:1	4.20±0.65	4.18±0.59	4.22±0.47	4.20±0.98	4.16±0.59	4.28±0.82	4.08±0.84
C22:6N3	12.32±0.34^{d}	12.56±0.53^{cd}	12.44±0.37^{cd}	15.56±1.74^{b}	15.32±0.54^{bc}	23.37±2.62^{a}	10.51±0.61^{d}
∑SFA	180.96±9.14	178.83±8.49	182.75±9.19	179.46±16.98	181.60±13.21	180.67±12.50	204.80±7.59
∑MUFA	188.89±4.96^{b}	188.76±8.37^{b}	189.14±11.55^{b}	186.37±8.82^{b}	188.19±11.26^{b}	188.65±6.17^{b}	210.63±5.58^{a}
∑PUFA	89.66±2.94^{a}	90.66±6.56^{a}	89.67±8.51^{a}	95.36±6.58^{a}	96.31±2.72^{a}	88.08±0.22^{ab}	76.48±4.62^{b}
∑n3-PUFA	33.44±1.02^{c}	33.68±1.20^{bc}	33.27±1.87^{c}	37.40±2.25^{ab}	36.66±1.60^{abc}	40.16±2.16^{a}	26.49±1.46^{d}
∑n6-PUFA	54.66±2.01^{ab}	55.36±5.77^{ab}	54.82±7.42^{ab}	56.45±5.09^{ab}	58.08±1.65^{a}	46.36±2.39^{b}	48.39±3.73^{ab}

Means with different superscripts in each row differ significantly from each other ($P<0.05$).

5.6 Discussion

The NT Yeast and RY-Δ6 had the proximate chemical compositions include protein, fat, fiber and ash as well as brewer's yeast as similar to the composition of yeast demonstrated previously. The chemical composition of brewer's yeast including protein at 442.3 g/kg, fat at 29.3 g/kg, fiber at 3.0 g/kg, and ash at 64.5 g/kg. (Pongpet et al., 2015). Dried yeast contained 42.03% protein, 6.52% fat and 6.15% ash (Ebrahim and Abou-sief, 2008). Additionally, the investigated chemical composition of *S. cerevisiae* for feed ingredients were composed protein 38.0-46.1%, fat 0.83-2.9%, fiber 2.9-8.0% and ash 5.0-8.1% (Schulze, 1995; Dobrzanski et al., 2007; Tacon et al., 2009; Yalcam et al., 2011).

Fatty acid C16:0, C16:1, C18:0 and C18:1n9 were the major fatty acids detected in RY-Δ6 which were reported in other recombinant *S. cerevisiae* (Napier et al., 1998; Laoteng et al., 2000; Hsiao et al., 2007; Gonzalez-Rovira Li et al., 2010; Tanomman et al., 2013). Recombinant yeast RY-Δ6 had amino acids in high quantity which included glutamic acid, lysine and aspartic acid. The low quantity of amino acids included methionine, cysteine and tryptophan. These findings were similar to the study of the amino acid composition of *S. cerevisiae* yeast (Albers et al., 1996; Dobrzanski et al., 2007). It could be noted that RY-Δ6 has lysine in high quantity, which is the essential amino acid in fish and in most animals. However, methionine is low. Therefore, it could be in caution in the amount of methionine when using RY-Δ6 as feed ingredients in animal diet.

Beta glucan (β-glucan) is important substances in the animal feed industry. It was used as an additive in animal feeds that affected growth performance and health of animals (Bernardeau and Vernous, 2013; Song et al., 2014; Wongsasak et al., 2014).

Yeast is an important source of glucan. In this study, the level of glucan in yeast cells was analyzed found that recombinant yeast RY-Δ6 had glucan 94.5 mg/ dry yeast, and the most of glucan are β-glucan form. This result was similar to the study of the levels of glucan in *S. cerevisiae* yeast (Manmers et al., 1973; Lesage and Bussey, 2006; Waszkiewicz-Robak, 2013). Additionally, yeast freeze dried (RY-Δ6 and NT) were microencapsulated with 1% guar gum in YPD which could attribute to protect the viability of cells, demonstrating that microencapsulation of *S. cerevisiae* using guar gum as encapsulation materials could increase the viability of freeze drying process (Ding and Shah, 2009). Additionally, the investigated microencapsulation of *S. cerevisiae* using guar gum as encapsulation materials following freeze drying was revealed to extend the shelf life of *S. cerevisiae* up to 16 weeks (Brinker and Reiter, 2011). Therefore, our results suggested that RY-Δ6 could be used as recombinant probiotic *S. cerevisiae* in aquatic feeds.

Although dietary RY-Δ6 had no positive effect on growth performance of Nile tilapia when feeding for 45 days, dietary RY-Δ6 for 90 days could increase growth performance including final weight, weigh gain, ADG and SGR and decrease FCR. However, growth performance of fish fed diet supplemented with RY-Δ6 (Li+RY10^6) was not significant different when compared with fish fed diet supplement with wild type yeast (Li+NT10^6 and Li+NT10^8). Survival rates were not significance different among experimental groups. These findings suggested that the supplement RY-Δ6 as probiotic yeast for long term could increase growth performance of fish which were similar to the resulted reported previously. For example, Abu-Elala et al. (2013) demonstrated that the effect of supplement yeast as probiotic, prebiotic and synbiotics (probiotic+prebiotic) in diet of Nile tilapia for 2 month. The supplementation of

probiotic yeast, prebiotic and synbiotics improved growth performance when compared with control fish. The supplementation of *S. cerevisiae* as probiotic in diet of convict cichlid (*Amatitlania nigrofasciata*) and three spot cichlid (*Cichlasoma trimaculatum*) for 8 weeks found that it increased growth performance and decreased FCR (Mohammadi et al., 2015; Mohammadi et al., 2016). In addition, supplementation with *L. acidophilus*, *B. subtilis* and *P. acidilactici* as probiotic in diet of rainbow trout for 4 weeks did not affect growth performance among experimental groups ($P>0.05$) while the supplementation for 8 weeks increased growth performance including final mean body weight, daily growth index and protein efficiency ratio and decreased FCR of fish ($P<0.05$) (Ramos et al., 2015). Additionally, in orange-spotted grouper (*Epinephelus coioides*), fish fed diet supplemented with yeast at 10^5-10^7 CFU/g of diet for 4 weeks had improvement of growth performance (Chiu et al., 2010). The effect of supplementation of *S. cerevisiae* in diet of African catfish *(Clarias gariepinus)* for 186 days and supplementation of *Lactobacillus acidophilus* as probiotic in diet of snakehead (*Channa striata*) for 12 weeks found that dietary probiotics led to improve growth performance ($P<0.05$) (Essa et al., 2011; Talpur et al., 2014). Likewise, as reported for tilapia (Abdel-Tawwa et al., 2008; AbdelsameeGoda et al., 2002), rainbow trout (Pooramini et al., 2009), common carp (Abdelnaby et al., 2013) and beluga (Hoseinifar et al., 2011), the supplement yeast in diet cloud increase growth performance and decrease FCR when compare with control dietary without yeast supplementation. However, dietary supplementation of probiotics in diet did not affect growth performance in several fish species. For example, supplementation of *S. cerevisiae* in diet of hybrid striped bass (*Morone chrysops x M. saxatilis*) for 45 days and guppy (*Poecilia reticulata*) for 30 days did not have effects on growth performance

(*P>0.05*) (Li and Gatlin, 2003; Sahandi et al., 2013). Seabass fed diet with dietary supplementation with *Debaryomyces hansenii* 23 days had not effects on weight gain of fish (*P>0.05*) (Tovar-Ramirez et al., 2002). Additionally, the supplementation of probiotic *Lactobacillus plantarum*in diet of basa fish *(Pangasius bocourti)* for 28 days did not affect growth performance including SGR and FCR when comparing to the control group (*P>0.05*) (Doan et al., 2014). Therefore, whether supplementation of Yeast *S. cerevisiae*as probiotic in dietary could improve growth performance of fish would depend on fish species, optimum level and period of supplementation.

Through the experiment periods, the survival rate did not significant differences among experimental diet (*P>0.05*). This finding was similar to the reports in several studies. For instant, supplementation of *D. hansenii*in die did not affect survival rate of fish seabass (Tovar-Ramirez et al., 2002), Nile tilapia (Abdel-Tawwab et al., 2008; AbdelsameeGoda et al., 2002), hybrid striped bass (Li and Gatlin, 2003), African catfish (Essa et al., 2011) and beluga (Hoseinifar et al., 2011).

This study found that deitary supplementation of RY-Δ6 as probiotic in diet of Nile tilapia had no effects on chemical composition including ash, dry matter, crude protein and crude fat in whole body and meat of fish for 45 days and 90 days (*P>0.05*). Our findings were similar to that demonstrated in convict cichlid (Mohammadi et al., 2015). In addition, African catfish fed with dietary supplementation with dried yeast at 1-2% had similar chemical composition in whole body when comparing with the fish fed diet without yeast supplemenation (Essa et al., 2011). Other probiotics including *B. subtilis*, *P. acidilactici* and *L. acidophilus* also had no effects on chemical composition of rainbow trout (Merifield et al, 2011; Ramos et al., 2015). However, Abdel-Tawwab (2012) found that the supplement live yeast in diet had increased ash, and the

supplementation of live yeast at 2-5 g/kg of diet increased crude protein and decreased crude fat in whole body of fry Nile tilapia (Abdel-Tawwab et al., 2008). In juvenile rainbow trout and three spot cichlid, dietary supplementation with yeast altered chemical composition in whole body of fish (Pooramini et al., 2009; Mohammadi et al., 2016). Therefore, the effect of supplement yeast on chemical composition depended on fish species, growth stage of fish, and it appeared that supplementation of probiotic yeast affected the chemical composition of juvenile fish more than that of adult fish.

In this study, supplement RY-Δ6 had no effect on hematological parameters of Nile tilapia for 45 days and 90 days including blood cell (RBC) number, hemoglobin content and hematocrit values *(P>0.05)*. Our findings were similar to that observed for beluga and juvenile leopard grouper (Hoseinifar et al., 2011; Reyes-Becerrill et al., 2008, 2011). However, Taoka et al. (2006) revealed that the supplementation of live yeast and dead yeast as probiotic in diet of Nile tilapia at 1% in diet for 15 days had no effects on hemoglobin of fish (*P>0.05*), however, the supplementation of probiotic at 1% in diet for 30 days led to increase hemoglobin (*P<0.05*) (Taoka et al., 2006). Supplementation of live yeast at 1-5% in diet of fry Nile tilapia for 12 weeks increased RBC, hemoglobin and haematocrit (Abdel-Tawwab et al., 2008). Additionally, supplementation of yeast probiotic in diet of snakehead for 8 weeks and 12 weeks increased RBC and hemoglobin of fish (Talpur et al., 2014). Combined together, the effect of supplementation of yeast probiotic on hematological would depend on growth stage of fish and time of feeding.

Blood biochemical parameter could be used as index to measure health, physiological responses, nutritional status, and environmental conditions in fish (Cnaani et al., 2004, Hoseinifar et al. 2011). In this study, blood glucose in Nile tilapia

was not significantly different among the experimental groups which were similar to the observation in striped catfish (Boonanuntanasarn et al., 2018) and seabass (Piccolo et al., 2015). In contrast, increase levels of *S. cerevisiae* resulted in elevation of blood glucose of fry Nile tilapia (Abdel-Tawwab et al., 2008). Nevertheless, yeast supplementation increased blood glucose in parasite infected olive flounder (Harikrishnan et al., 2011). Probiotic *S. cerevisiae* had no significant effect on cholesterol and triglyceride levels in the current study. Similarly, supplementation with probiotics *S. cerevisiae* had no effect on cholesterol and triglyceride in striped catfish (Boonanuntanasarn et al., 2018). In contrast, probiotic LAB increased cholesterol and triglyceride concentrations in seabass (Piccolo et al., 2015).

The blood chemistry parameters such as total protein and albumin can be used as indicators of protein metabolism. In this study, no significant differences in total protein, and albumin were observed among the experimental groups which were in agreement with the result observed in striped catfish (Boonanuntanasarn et al., 2018) and leopard grouper (Becerril et al., 2011). In contrast, supplementation with *S. cerevisiae* at 1.0-5.0 g/kg diet resulted in significant increases in blood albumin in fry Nile tilapia, with the highest blood albumin observed at 1 g/kg diet, and no changes in those fed 0-0.5 g/kg diet (Abdel-Tawwab et al., 2008), Both live and dead *S. cerevisiae* increased plasma protein in juvenile Nile tilapia (Taoka et al., 2006). Different probiotic species had different effects on total protein in the blood of infected olive flounder (Harikrishnan et al., 2011). Therefore, the variable effects of probiotics on protein metabolic blood chemistry parameters appear to depend on the probiotic species, the level of supplementation, the duration of intake, age of fish and fish species.

Dietary RY-Δ6 had no significant differences in SGOT and SGPT. In contrast, the supplementation probiotic had increased SGOT and SGPT in the blood of infected olive flounder and parrot fish (Harikrishnan et al., 2011; Kim et al., 2012). The microbial probiotics might affect mineral metabolism via intestinal metabolite fermentation, enzyme activity, or supplying vitamins that are involved in mineral absorption (Scholz-Ahrens et al. (2007) for review, see Dimitroglou et al. (2011)). The concentration of blood minerals, including calcium and iron, were not significant difference among the experimental groups in the current study, suggesting that probiotic *S. cerevisiae* had no effect on mineral absorption. Similarly, the supplementation *S. cerevisiae* in diet had no effects on blood mineral in striped catfish (Boonanuntanasarn et al., 2018). There is limited information available regarding the effects of dietary probiotic yeast on mineral metabolism in fish. Further studies are needed to investigate the effects of probiotic *S. cerevisiae* on intestinal mineral metabolism in fish. Taken together, the effects of dietary probiotics on blood metabolic parameters appear to depend upon the probiotic species, the duration of supplementation, and the fish species.

Non-specific immune system is an important mechanism on preventing aquatic pathogens. Supplementation of *S. cerevisiae* as probiotics could stimulate immunity in Nile tilapia. From this study, the effects of supplement RY-Δ6 increased immune parameters including total immunoglobulin, lysozyme activity and alternative complement haemolytic 50 (ACH50) activity. Total Ig is a protein in the blood for serving to prevent infection. When supplementing *S. cerevisiae* as probiotic in diet of Nile tilapia, total Ig increased significantly ($P<0.05$). These findings were similar to the observation in rainbow trout (Brunt et al., 2007; Newaj-Fyzul et al., 2007;

Merrifield et al., 2010). The positive effect of probiotic RY-Δ6 on lysozyme activity were consistent with the findings in rainbow trout (Brunt et al., 2007; Newaj-Fyzul et al., 2007; Merrifield et al., 2010, 2011), grouper (Sun et al., 2010) and channel catfish (Shelby et al., 2007). The supplementation of yeast as probiotic in diet of Nile tilapia increased ACH50 which were similar the results of gilthead seabeam ($P<0.05$) (Reyes-Becerril et al., 2008).

Probiotic yeast *S. cerevisiae* is a probiotic that can colonize in the intestine which modulate microbial population in intestinal tract (Tovar et al., 2002). Our results showed that the supplementation of RY-Δ6and NT yeast in diet increased the number of yeast, fungi, *Bifidobacterium* spp. and *Lactobacilus* spp. *(P<0.05)*. These finding were similar to the observation for red tilapia fed with *Pediococcus acidilactici* (Ferguson et al., 2010). In addition, in the Pacific white shrimp (*Litopeneaus vannamei*), shrimp fed dietary supplementation *P. acidilactici* increased number of *Lactobacilus* spp. in intestinal tract of shrimp (Boonanuntanasarn et al., 2015). Furthermore, the supplement RY-Δ6 and NT yeast in diet of Nile tilapia decreased the number of *Vibrio* spp. in intestinal tract ($P<0.05$). However, there were no significant differences in the number of intestinal total bacteria among the experimental groups *(P>0.05)*. Additionally, RY-Δ6 could be isolated from the intestine of Nile tilapia, demonstrating that RY-Δ6 can survive and colonized in the intestinal environment of Nile tilapia.

In this study, dietary supplementation with RY-Δ6 and NT could increase the villus height in intestine of Nile tilapia, particularly in the anterior intestine where the majority of digestive and absorptive take place. Similarly, dietary supplementation of *S. cerevisiae* increase villus height of striped catfish (Boonanuntanasarn et al., 2018).

The supplementation mix probiotic in diet of tilapia could increase villus height in intestinal tract (Ramos et al., 2017). The increased villus height suggests and increase in the intestinal nutrient absorption area, which would contribute to improving the dietary nutrient utilization and growth performance. Dietary supplementation RY-Δ6 and NT did not affect number of goblet cells in intestine of fish. In contrast, the supplementation of mix probiotic in diet increase number of goblet cells in intestinal tract of tilapia for 8 weeks (Ramos et al., 2017). Goblet cells produce mucus that prevents dehydration, lubricates and protects the gastrointestinal tract against physical damage and injurious agents. (Mc Guckin et al., 2011).

Effect of supplement RY-Δ6 on accumulation of fatty acid profile in meat and liver of Nile tilapia for 45 days after feeding found that the supplementation of RY-Δ6 in diet did not affect fatty acid compostion in muscle and liver ($P>0.05$). Supplementation of RY-Δ6 for 90 days increased product C18:3n6 and C18:4n3 delta 6 desaturase in meat and liver ($P<0.05$), suggesting that RY-Δ6 could exert delta 6 desaturase and attribute the fatty profile of meat and liver of Nile tilapia. Indeed, recombinant yeast expressing delta 6 desaturase of Nile tilapia (Tanomman et al., 2013), borage *(Borago officinalis)* (Qiu et al., 2002), cobia (*Rachycentron canadum*) (Zheng et al., 2009), common carp, rainbow trout, gilthead seabream, turbot (*Psetta maximus)* (Zheng et al., 2004) and Atlantic bluefin tuna (*Thunnus thynnus*) (Morais et al., 2011) were found exert delta 6 desaturase to convert fatty acid substrate C18:3n3 to C18:4n3 and fatty acid substrate C18:2n6 to C18:3n6. Additionally, fish fed dietary supplement with RY-Δ6 had fatty acid C22:6n3, ∑n3-PUFA and ∑n6-PUFA content in meat and liver higher than fish fed dietary without RY-Δ6, demonstrating that the

effect of RY-Δ6 expressing delta 6 desaturase would contribute to increase LC-PUFA content in muscle and liver.

5.7 Conclusion

This study demonstrated the nutritive value of recombinant RY-Δ6 expressing delta 6 desaturase. Microencapsulation of RY-Δ6 could protect; therefore, it could be used as probiotic to pass through gastrointestinal tract of Nile tilapia. Dietary supplementation of encapsulation RY-Δ6 increase growth performance of Nile tilapia at 90 days of feeding. Dietary supplementation of encapsulation RY-Δ6 had no effects on survival rate and the chemical composition of whole body and meat composition. RY-Δ6 had no effects on hematological indices and blood chemistry. However, RY-Δ6 led to increase immune parameters (Ig, lysozyme and ACH50), villus height of intestine and alteration intestinal microbiota (P<0.05). Dietary RY-Δ6 expressing delta 6 desaturase significant increase C18:3n6 and C18:4n3 in liver and meat, suggesting that RY-Δ6 could exert delta 6 desaturase and lead to alter the fatty profile in meat and liver.

5.8 References

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CHAPTER VI

USE OF RECOMBINANT *S. CEREVISIAE* (RY-Δ6) TO ENRICH *ARTEMIA* AS DIETARY PROBIOTIC FOR SEABASS (*LATES CALCARIFER)* LARVAE

6.1 Abstract

This study aimed to investigate the effects of the use of recombinant *S. cerevisiae* (RY-Δ6) to enrich *Artemia* as dietary probiotic on growth performance, fatty acid content, intestinal microbiota in seabass (*Lates calcarifer)* larvae. In addition, the effects of RY-Δ6 on stress resistance to ammonia was examined. Eight treatment diets (each diet replicated four times) were as follows: (1) Non-enrich *Artemia*, (2) *Artemia* enrichment with 3% fish oil (positive control), (3) *Artemia* enrichment with 3% soybean oil,(4) and (5) *Artemia* co-enrichment with 3% soybean oil and RY-Δ6 at 10^5 and 10^7 CFU/g, respectively, (6) *Artemia* enrichment with 3% linseed oil, (7) and (8) *Artemia* co-enrichment with 3% linseed oil and RY-Δ6 at 10^5 and 10^7 CFU/g, respectively. Fish were reared in tank for 45 days, and growth performance, survival rate, intestinal microbiota, fatty acid deposition and stress resistance to ammonia were evaluated. The results showed that *Artemia* co-enriched with soybean oil or linseed oil and RY-Δ6 improved growth performance and survival rate. The RY-Δ6 decreased intestinal *Vibrio* spp ($P<0.05$) of seabass larvae. RY-Δ6 modulated fatty acid profile in whole body of seabass larvae. *Artemia* co-enriched with soybean oil or linseed oil and RY-Δ6 led to

increase C18:3n6 or C18:4n3, respectively, in whole body, suggesting that probiotic RY-Δ6 would exert delta 6 desaturase activity. Additionally, seabass fed co-enriched *Artemia* with linseed oil and RY-Δ6 had increased in fatty acid C20:4n6, ∑n3-PUFA and ∑n6-PUFA content in whole body, suggesting that RY-Δ6 expressing delta 6 desaturase would contribute to increase LC-PUFA content in whole body of fish. Furthermore, co-enriched with *Artemia* and RY-Δ6 lower accumulative mortality when fish were challenged with ammonia, demonstrating that yeast could improve stress resistance.

6.2 Introduction

Long chain polyunsaturated fatty acids (LC-PUFAs) particularly eicosopentaenoic acid (EPA; C20:5n3) and decosahexaenoic acid (DHA; C22:6n3) were demonstrated to be essential fatty acids in a number of marine fish (Tocher et al., 2001; Tocher et al., 2004). As a result, dietary EPA and DHA are essential fatty acids in marine fish, especially in marine fish larvae. Enriched *Artemia* with fish oil as a source of LC-PUFAs particularly EPA and DHA were conducted for the use as live feed for marine fish larvae (Villaita et al., 2005; Martins et al., 2006). However, fish oil was not sustainable LC-PUFA source in aquatic feed. Therefore, an alternative of LC-PUFAs are needed in order to provide sustainable fatty acids for aquatic feed particularly marine fish.

The *Saccharomyces cerevisiae* have been demonstrated to be probiotic in aquatic feed (Jafaryan et al., 2009). In addition, *S. cerevisiae* was widely used as host for recombinant protein technology. Combined together, *S. cerevisiae* expressing enzymes which are involved in the LC-PUFAs biosynthesis would be used as

recombinant probiotic. The recombinant probiotic expressing delta 6 desaturase would have potential to be used as an alternative source for LC-PUFAs sources. The RY-Δ6 could be used as dietary probiotics to exert positive effects on growth performance, immune parameters, intestinal morphology and intestinal microbiota in Nile tilapia. Additionally, dietary RY-Δ6 could exert delta-6 desaturation activity which changed the fatty profile in meat and liver of Nile tilapia. Therefore, the RY-Δ6 could be provide application to develop to be the recombinant probiotic to produce LC-PUFAs.

Recently, the production of fish larvae relies heavily on the brine shrimp *Artemia* as the main live food organism used in commercial hatcheries (Sorgeloos et al., 1998; Wouters et al., 2009; Cobo et al., 2015). *Artemia* contains high protein and fatty acid; however, the amount n3-PUFAs were not enough to be used as live feed for marine fish (Figueiredo et al., 2009; Lèger and Sorgeloos, 1992). *Artemia* is a non-selective filter feeding organism. Therefore, enhancement of n-3 LCPUFA levels could be achieved through enrichment techniques. Consequently, enriched *Artemia* was improved their nutritional value which therefore were be able to use for feeding fish larvae (Sorgeloos et al., 1998; Wouters et al., 2009). The larvae which were fed enriched *Artemia* had high growth performance and survival rate (Leger and Sorgeloos, 1994; Xu et al., 1993; Rees et al., 1994; Kyuungin et al., 2000; Immanuel et al., 2001). Additionally, the probiotics were enriched to *Artemia* and fed to fish larvae found. *Artemia* enriched with probiotics led to improve growth performances and immune systems in silver carp, Persian sturgeon, *Catla catla* and pejerrey (Adineh et al., 2011; Iranshahi et al., 2012; Divya et al., 2014; Fuhr et al., 2016). These findings suggested that, the enrichment *Artemia* with oil (rich HUFA) and probiotic which increase the nutritional contents improve growth performance, survival rate and immune of fish

larvae. The researcher attempt to study to using the RY-Δ6 to enrich *Artemia* for cultured seabass larvae which important in commercial of aquaculture.

Seabass (*Lates calcarifer*) is an important economic fish in tropical countries. It grows to comparatively large size with delicate flavoured flesh and commands premium price in the market (FAO, 2012). In the nursing of seabass larvae very important because seabass larvae sensitive on water quality especially ammonia, its led to stress on fish larvae. The stress condition can be due to environmental and anthropogenic stressors (Dederen et al., 1986; Modesto and Martinez, 2010). Ammonia toxicity is one of the most common environmental stressors during fish culture, especially in aquaculture intensive systems due to high stocking densities. Ammonia stress causes several physiological changes, including oxygen consumption, homeostasis, and immune suppression, which impair normal growth, health status, and survival rate (de Lourdes Cobo et al., 2014; Racotta and Hernandez-Herrera, 2000). Therefore, the enrichment *Artemia* with RY-Δ6 would be a practical way to improve health under stressful conditions. Therefore, in this study to use of RY-Δ6 to enrich *Artemia* on growth performance, survival rate, fatty acid profile and stress resistance of seabass larvae.

6.3 Objective

To study the effects of the use of RY-Δ6 to enrich *Artemia* on growth performance, survival rate, fatty acid profile and stress resistance of seabass larvae.

6.4 Materials and methods

6.4.1 Preparation recombinant *S. cerevisiae* (RY-Δ6) and wild type yeast (NT)

The RY-Δ6 and NT were grown in 1 l of YPDat 30 °C with shaking at 200 rpm in Erlenmeyer flasks. After 24 h, *S. cerevisiae* cells were collected by centrifugation at 12,000 rpm for 5 min at 4 °C, washed three times with 0.85% NaCl, and stored at -20 °C until use.

6.4.2 Hatching *Artemia*

The *Artemia franciscana* cysts (5 g l^{-1}) were allowed to hatch under optimum haching condition (salinity 35 ppt, temperature 25 °C, pH 7.5-8). After 24 h for hatching, the *Artemia* nauplii were separated from the empty cyst shells and washed with salt water, then transferred in to the enrichment tank.

6.4.3 Experimental design and enrichment *Artemia* and fatty acid analysis

The completely randomized design was employed to investigate the fatty acid profile of enrichment *Artemia*. Sixteen treatments were as follows: (1) *Artemia* without enrichment; (2) *Artemia* enrichment with 3% fish oil; (3) and (4) *Artemia* enrichment with non-transformed yeast (NT) 10^5 and 10^7 CFU/g, respectively; (5) and (6) *Artemia* enrichment with recombinant yeast (RY-Δ6) 10^5 and 10^7 CFU/g, respectively; (7) *Artemia* enrichment with 3% soybean; (8) and (9) *Artemia* enrichment with 3% soybean oil and supplements NT 10^5 and 10^7 CFU/g, respectively; (10) and (11) *Artemia* enrichment with 3% soybean oil and supplements RY-Δ6 10^5 and 10^7 CFU/g, respectively; (12) *Artemia* enrichment with 3% linseed oil; (13) and (14) *Artemia* enrichment with 3% linseed oil and supplements NT 10^5 and 10^7 CFU/g; (15) and (16) *Artemia* enrichment with 3% linseed oil and supplements RY-Δ6 10^5 and 10^7 CFU/g, respectively, (Table 6.1). The *Artemia* nauplii (300 nauplii/ml) were incubated in

enrichment solution. Each enrichment were replicated three times to test the validity of the conclusions. After 8 h incubation with enrichment, the *Artemia* were washed with salt water to discard non absorbed nutrition and performed fatty acid analysis using GC (Tanomman et al., 2013).

Table 6.1 Enrichment *Artemia* in experimental solution.

Treatment	***Artemia* enrichment**	
	Oil (%/g *Artemia*)	**Yeast (CFU/g *Artemia*)**
Non enrich	Non enriched *Artemia*	
Fish oil	Fish oil	-
NT 10^5	-	NT 10^5
NT 10^7	-	NT 10^7
RY-Δ6 10^5	-	RY-Δ6 10^5
RY-Δ6 10^7	-	RY-Δ6 10^7
Soybean oil (SB)	Soybean oil	-
SB+NT 10^5	Soybean oil	NT 10^5
SB+NT 10^7	Soybean oil	NT 10^7
SB+RY-Δ6 10^5	Soybean oil	RY-Δ6 10^5
SB+RY-Δ6 10^7	Soybean oil	RY-Δ6 10^7
Linseed oil (LO)	Linseed oil	-
LO+NT 10^5	Linseed oil	NT 10^5
LO+NT 10^7	Linseed oil	NT 10^7
LO+RY-Δ6 10^5	Linseed oil	RY-Δ6 10^5
LO+RY-Δ6 10^7	Linseed oil	RY-Δ6 10^7

6.4.4 Effect of the use of *Artemia* enriched with RY-Δ6 on growth performance, survival rate, fatty acid profile and stress resistance of seabass larvae

Experimental fish and fish sampling

In this experiment, seabass larvae (age 8 day; 25-26 mg body weight) were obtained from the commercial farm in Chachersao province. Seabass were reared in 32 tanks (14×60×30 cm.) at the Suranaree University of Technology Farm (SUT Farm; Nakhon Ratchasima, Thailand). Two hundred and fifty fish were randomly distributed in the experimental tank. The experimental fish were fed diet *ad libitum* for 2 times a day (9.00 and 15.00). The salinity of water, water temperature, pH, and dissolved oxygen in the culture tank ranged from 4 to 5 ppt, 27.5 to 29.0 ºC, 7.5 to 8.0, and 5.09 to 5.41 mg/L, respectively.

Experimental design

The experimental design was completely randomized with seven treatment diets, each of which was replicated four times to test the validity of the conclusions. The eight treatment diets were as follows: (1) Non-enrich *Artemia*; (2) *Artemia* enrichment with 3% fish oil; (3) *Artemia* enrichment with 3% soybean oil; (4) and (5) *Artemia* enrichment with 3% soybean oil and supplements recombinant yeast 10^5 and 10^7 CFU/g, respectively; (6) *Artemia* enrichment with 3% linseed oil; (7) and (8) *Artemia* enrichment with 3% linseed oil and supplements recombinant yeast 10^5 and 10^7 CFU/g, respectively, (Table 6.2).

Table 6.2 Group of experimental.

Treatment	Oil (%/g feed)	Yeast (CFU/g)
Non enrichment	Non-enrichment	
Fish oil	Fish oil 3%	-
Soybean oil	Soybean oil 3%	-
Soy-RY 10^5	Soybean oil 3%	RY-Δ6 10^5
Soy-RY 10^7	Soybean oil 3%	RY-Δ6 10^7
Linseed oil	Linseed oil 3%	-
Lin-RY 10^5	Linseed oil 3%	RY-Δ6 10^5
Lin-RY 10^7	Linseed oil 3%	RY-Δ6 10^7

Determination growth performance

Fish in each tank were anesthetized with 2-phenoxyethanol (0.2%), counted and weighed at 15, 30 and 45 days of the experiment period, following 18 h of feed deprivation. Weight measurements and fish counts were used for estimation of weight gain, condition factor, specific growth rate and survival rate.

Weight gain (WG) = Final weight - Initial weight

Condition factor (CF) = (Weight / length) x 100

Specific growth rate (SGR)

= 100 × [(*ln* final body weight - *ln* initial body weight)/ Experimental days]

Survival rate (%) = 100 × (Final no. of fish/Initial no. of fish)

Intestinal microbiota analysis

At the end of the experimental period (45 days), ten fish from each replication of each treatment were sampled for microbiological studies. The fish were then dissected under sterile condition, and the intestine was removed, weighed,

homogenized and suspended in sterile 0.9% NaCl. The suspension, serially diluted to 10^{-4} and 0.15 ml of the solution was spread onto plate count agar (PCA) culture medium (total bacteria), de Man, Rogosa and Sharpe (MRS) agar (lactic acid bacteria), bifidobacterium agar (Bifidobacteria), thiosulfate citrate bile salts sucrose (TCBS) agar (*Vibrio* spp.) and Sabouraud agar (yeast and fungi). All of the plates were incubated at 37 ^{o}C for 2 days. After incubation, the total numbers of colony-forming units (CFU)/g were calculated from statistically viable plates (i.e., plates containing 30-300 colonies) (Rawling et al., 2009).

Fatty acid analysis

At the end of the experimental period (45 days), the fatty acid profiles in whole body of seabass larvae was determined using gas chromatography (GC) as described elsewhere (Tanomman et al., 2013).

Ammonia stress challenge

After 45 days of the feeding trial, seabass in each treatment were exposed to ammonia by adding NH_4Cl to each tank to obtain an NH_4^+ concentration of 1.3 mg/l. The accumulation of mortality was recorded after ammonia exposure for 8 h, 12 h, 24 h, 48 h, 72 h and 96 h show in Table 6.3.

Cumulative mortality (%) = 100 × (Summary the mortality of fish at each time interval/Initial no. of fish)

Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA) using SPSS for windows (Release 10) (SPSS Inc., Chicago, IL, USA). When significant differences were found among the groups, Tukey's test was used to rank the groups. Throughout the experiment, effects and differences were declared to be significant at $P<0.05$.

6.5 Results

6.5.1 Fatty acid profile of enrichment *Artemia*

The fatty acids composition of enriched *Artemia* was analyzed and showed in Table 6.4. The *Artemia* co-enriched with soybean oil and RY-Δ6, linseed oil and RY-Δ6 and *Artemia* enriched with soybean oil, linseed oil and fish oil had fatty acids composition higher than non-enriched *Artemia* and enriched *Artemia* with NT and RY-Δ6. The result show that the fatty acid C10:0 and C22:1n9 were not significant differences among the experimental groups *(P>0.05)*. Among experimental groups, the *Artemia* co-enrichment with 3% soybean oil and RY-Δ6 at 10^5 and 10^7 CFU/g had C18:3n6 highest. Also, the co-enriched *Artemia* with 3% linseed oil with RY-Δ6 at 10^5 and 10^7 CFU/g had highest C18:4n3. In addition, these co-enriched *Artemia* with had higher C20:4n6 when compare with other experimental groups. Additionally, the fatty acid of *Artemia* enrichment with NT and RY-Δ6 did not different with the fatty acid composition of non-enrich *Artemia*.

Therefore, we selected the enrichment solution including fish oil (as positive control), soybean oil, soybean oil co-supplemented with RY-Δ6 at 10^5 and 10^7 CFU/g, linseed oil, and linseed oil co-supplemented with RY-Δ6 at 10^5 and 10^7 CFU/g, respectively, for investigation the effect of RY-Δ6 for *Artemia* enrichment on growth performance and survival rate of seabass larvae.

Table 6.3 Fatty acid profile (mg/100 g lipid) in enriched *Artemia* (mean±sd, n=3).

Treatment	C8:0	C10:0	C12:0	C14:0	C14:1	C16:0	C16:1
Non enrich	2.45±0.10^{b}	6.37±0.94	7.09±2.20^{c}	62.50±2.11^{c}	2.80±0.28^{b}	932.90±13.65^{cd}	154.09±9.11^{c}
Fish oil	2.53±0.04^{b}	7.09±0.87	23.29±3.97^{a}	117.36±20.11^{a}	6.88±0.26^{a}	4,726.01±251.89^{a}	263.88±53.09^{ab}
NT 10^5	2.52±0.05^{b}	6.20±0.67	6.18±0.60^{c}	62.48±4.54^{c}	2.69±0.17^{bc}	863.09±100.34^{d}	150.90±29.41^{c}
NT 10^7	2.41±0.05^{b}	6.44±0.69	6.91±0.32^{c}	64.57±3.52^{bc}	2.66±0.32^{bc}	976.90±10.40^{cd}	156.41±6.92^{c}
RY-Δ6 10^5	2.42±0.06^{b}	6.92±0.77	8.02±1.11^{c}	62.92±4.64^{c}	2.42±0.22^{bc}	909.55±40.38^{cd}	154.33±7.36^{c}
RY-Δ6 10^7	2.44±0.10^{b}	6.36±0.46	6.89±0.58^{c}	61.55±3.23^{c}	2.56±0.21^{bc}	904.68±72.53^{cd}	153.88±11.40^{c}
Soybean oil (SB)	2.50±0.09^{b}	6.63±1.74	7.15±0.94^{c}	90.58±8.14^{b}	2.16±0.06^{c}	4,451.68±639.47^{a}	261.68±22.11^{ab}
SB+NT 10^5	2.59±0.18^{b}	5.75±0.39	6.86±1.95^{bc}	81.44±9.66^{bc}	2.28±0.15^{bc}	4,419.36±885.35^{a}	255.81±19.91^{ab}
SB+NT 10^7	2.61±0.09^{b}	6.25±1.03	7.73±1.31^{bc}	83.28±8.42^{bc}	2.69±0.3^{bc}	5,011.54±666.14^{a}	287.71±17.83^{ab}
SB+RY-Δ6 10^5	2.47±0.06^{b}	6.57±1.47	7.15±1.01^{c}	85.58±3.79^{bc}	2.39±0.07^{bc}	4,715.67±992.81^{a}	282.76±18.26^{ab}
SB+RY-Δ6 10^7	2.46±0.05^{b}	7.27±1.28	7.05±1.60^{c}	77.89±11.68^{bc}	2.66±0.19^{bc}	5,066.31±643.38^{a}	275.86±14.21^{ab}
Linseed oil (LO)	7.13±0.21^{b}	7.29±1.10	12.33±1.19^{b}	81.20±3.20^{bc}	2.67±0.17^{bc}	2,377.54±679.93^{bcd}	268.65±8.10^{ab}
LO+NT 10^5	6.85±0.05^{a}	8.66±±0.85	9.79±2.18^{bc}	79.21±7.74^{bc}	2.66±0.10^{bc}	2,166.17±586.80^{bcd}	265.16±16.77^{ab}
LO+NT 10^7	7.06±0.14^{a}	7.86±1.09	9.98±2.21^{bc}	80.89±15.49^{bc}	2.51±0.17^{bc}	2,389.90±540.56^{bcd}	319.40±21.77^{a}
LO+RY-Δ6 10^5	6.57±0.24^{a}	7.38±0.97	10.55±0.98^{bc}	78.99±3.12^{bc}	2.47±0.10^{bc}	2,436.25±158.18^{bc}	237.34±43.75^{b}
LO+RY-Δ6 10^7	7.28±1.84^{a}	7.04±0.32	10.15±0.70^{bc}	81.60±6.29^{bc}	2.47±0.21^{bc}	2,650.36±231.18^{b}	264.69±16.87^{ab}

Means with different superscripts in each column differ significantly from each other ($P<0.05$).

Table 6.3 Fatty acid profile (mg/100 g lipid) in enriched *Artemia* (mean±sd, n=3) (Continuoued).

Treatment	C18:0	C18:1N9T	C18:1N9C	C18:2N6T	C18:2N6C	C18:3N6
Non enrich	572.90±89.74^{b}	nd	1,269.20±118.28^{c}	nd	660.10±29.74^{c}	14.27±2.69^{e}
Fish oil	1,891.56±151.64^{a}	nd	5,737.40±248.15^{b}	nd	1,527.58±136.52^{c}	18.30±1.03^{e}
NT 10^{5}	550.51±77.88^{b}	nd	1,193.46±120.75^{c}	nd	651.86±6.94^{c}	13.08±1.29^{e}
NT 10^{7}	601.60±153.04^{b}	nd	1,363.22±285.04^{c}	nd	708.91±43.08^{c}	16.99±1.22^{e}
RY-Δ6 10^{5}	531.52±17.10^{b}	nd	1,140.49±30.79^{c}	nd	654.73±6.20^{c}	12.50±0.65^{e}
RY-Δ6 10^{7}	609.89±68.35^{b}	nd	1,394.96±195.99^{c}	nd	655.12±10.22^{c}	13.00±0.29^{e}
Soybean oil (SB)	2,021.43±252.50^{a}	nd	7,512.34±427.32^{ab}	nd	13,922.13±1,056.99^{a}	77.39±4.25^{d}
SB+NT 10^{5}	1,761.83±211.06^{a}	nd	7,915.43±541.55^{a}	nd	13,852.42±122.17^{a}	77.97±1.64^{d}
SB+NT 10^{7}	1,951.44±408.24^{a}	nd	8,239.77±357.13^{a}	nd	13,975.87±298.81^{a}	75.77±1.56^{d}
SB+RY-Δ6 10^{5}	1,933.47±58.45^{a}	nd	7,743.31±410.37^{ab}	nd	14,430.15±558.55^{a}	217.36±1.79^{a}
SB+RY-Δ6 10^{7}	1,841.75±235.64^{a}	nd	8,416.74±1,555.13^{a}	nd	13,695.16±1,130.43^{a}	219.99±4.45^{a}
Linseed oil (LO)	1,912.50±118.23^{a}	nd	7,882.20±357.90^{a}	nd	3,221.73±148.54^{b}	18.57±1.03^{e}
LO+NT 10^{5}	2,028.80±392.43^{a}	nd	8,193.43±499.26^{a}	nd	3,439.80±443.92^{b}	18.49±0.27^{e}
LO+NT 10^{7}	1,754.18±528.05^{a}	nd	7,781.05±802.94^{ab}	nd	3,560.79±354.63^{b}	18.76±1.13^{e}
LO+RY-Δ6 10^{5}	2,002.68±328.05^{a}	nd	7,678.22±995.60^{ab}	nd	3,383.09±478.85^{b}	155.52±10.62^{c}
LO+RY-Δ6 10^{7}	1,863.96±250.39^{a}	nd	8,183.66±1,510.14^{a}	nd	3,235.20±360.91^{b}	171.60±3.38^{b}

Means with different superscripts in each column differ significantly from each other ($P<0.05$).

Table 6.3 Fatty acid profile (mg/100 g lipid) in enriched *Artemia* (mean±sd, n=3) (Continuoued).

Treatment	C20:0	C20:1n9	C18:3N3	C18:4N3	C20:2	C22:0	C20:3N6
Non enrich	20.39±2.93^{f}	753.08±28.47^{b}	1,025.82±21.61^{b}	115.91±4.97^{d}	24.68±0.81^{b}	19.04±1.46^{d}	10.09±0.35^{b}
Fish oil	149.11±26.15^{a}	1,145.35±44.14^{b}	1,032.26±24.76^{b}	112.23±6.97^{d}	61.24±6.44^{a}	89.25±1.44^{b}	10.87±1.57^{b}
NT 10^5	19.48±0.99^{f}	769.54±22.32^{b}	961.26±114.47^{b}	91.20±55.96^{d}	22.10±1.68^{b}	18.56±3.34^{d}	9.71±0.84^{b}
NT 10^7	20.38±0.76^{f}	788.20±2.53^{b}	1,158.39±274.92^{b}	108.74±7.10^{d}	24.07±3.01^{b}	19.84±2.12^{d}	10.07±0.90^{b}
RY-Δ6 10^5	18.39±1.93^{f}	786.70±5.25^{b}	1,007.31±54.77^{b}	117.69±4.25^{d}	21.59±2.24^{b}	18.43±1.06^{d}	9.45±1.99^{b}
RY-Δ6 10^7	18.78±1.92^{f}	794.82±3.94^{b}	1,087.58±80.47^{b}	111.32±5.86^{d}	23.77±3.78^{b}	19.31±2.20^{d}	10.47±1.67^{b}
Soybean oil (SB)	109.87±3.51^{bcd}	2,186.69±246.29^{a}	1,150.70±16.02^{b}	113.49±7.78^{d}	51.23±6.53^{a}	126.51±11.01^{a}	16.54±0.29^{a}
SB+NT 10^5	121.92±14.66^{b}	2,238.85±226.34^{a}	1,160.03±32.21^{b}	117.58±6.76^{d}	52.78±8.65^{a}	124.92±10.35^{a}	16.53±1.09^{a}
SB+NT 10^7	109.23±0.68^{bcd}	2,459.93±424.70^{a}	1,155.85±95.40^{b}	107.16±3.76^{d}	50.49±3.43^{a}	118.52±6.51^{a}	16.78±2.63^{a}
SB+RY-Δ6 10^5	112.20±7.74^{bc}	2,166.78±201.71^{a}	1,133.57±3.26^{b}	174.88±6.37^{c}	53.37±4.07^{a}	126.10±13.62^{a}	19.76±0.86^{a}
SB+RY-Δ6 10^7	110.71±5.20^{bc}	2,097.89±188.55^{a}	1,130.54±33.42^{b}	180.62±1.44^{c}	48.79±6.58^{a}	122.75±11.56^{a}	19.72±2.71^{a}
Linseed oil (LO)	77.72±5.21^{e}	1,986.59±290.12^{a}	2,870.99±16.44^{a}	192.77±10.01^{c}	52.53±6.48^{a}	72.03±4.29^{bc}	16.13±0.05^{a}
LO+NT 10^5	86.38±1.34^{cde}	2,091.30±92.71^{a}	2,904.03±36.08^{a}	195.32±17.70^{c}	49.36±3.55^{a}	66.80±10.93^{bc}	16.45±0.60^{a}
LO+NT 10^7	83.75±4.35^{de}	1,907.02±124.78^{a}	2,834.95±2.45^{a}	186.50±5.48^{c}	54.99±0.98^{a}	68.87±8.72^{bc}	17.14±0.01^{a}
LO+RY-Δ6 10^5	86.50±7.09^{cde}	2,261.33±371.13^{a}	2,788.44±8.37^{a}	259.98±3.25^{b}	53.06±3.66^{a}	63.91±10.93^{bc}	20.83±2.31^{a}
LO+RY-Δ6 10^7	82.12±9.39^{e}	2,099.70±94.81^{a}	2,752.10±63.88^{a}	326.59±7.88^{a}	52.88±7.95^{a}	69.13±4.22^{bc}	20.74±3.28^{a}

Means with different superscripts in each column differ significantly from each other ($P<0.05$).

Table 6.3 Fatty acid profile (mg/100 g lipid) in enriched *Artemia* (mean±sd, n=3) (Continuoued).

Treatment	C22:1N9	C20:3N3	C20:4N6	C22:2	C20:5N3	C24:0	C24:1
Non enrich	73.07±3.05	10.81±0.04^{f}	66.70±4.28^{ef}	3.81±0.42^{b}	180.98±6.64^{b}	7.91±1.64^{b}	50.85±3.45
Fish oil	72.43±5.62	11.39±0.70^{ef}	69.58±1.71^{ef}	7.11±0.27^{a}	2,444.19±289.71^{a}	44.69±1.27^{a}	50.13±4.62
NT 10^5	78.69±2.42	11.14±0.49^{ef}	69.94±0.90^{ef}	3.87±0.22^{b}	201.16±20.36^{b}	6.72±1.04^{b}	53.00±3.53
NT 10^7	77.19±4.05	11.66±1.20^{ef}	69.58±1.44^{ef}	3.91±0.09^{b}	213.42±14.46^{b}	9.02±0.32^{b}	51.58±3.17
RY-Δ6 10^5	79.36±1.94	12.30±1.52^{ef}	69.29±2.97^{ef}	3.85±0.18^{b}	199.67±18.93^{b}	7.16±1.34^{b}	50.23±5.72
RY-Δ6 10^7	87.60±3.53	11.52±1.18^{ef}	69.24±2.18^{ef}	3.93±0.04^{b}	204.08±11.48^{b}	6.70±0.98^{b}	53.07±3.15
Soybean oil (SB)	80.67±9.46	12.29±0.90^{def}	84.52±3.45^{bcd}	3.98±0.12^{b}	182.75±0.63^{b}	47.73±2.92^{a}	43.25±3.94
SB+NT 10^5	82.42±6.61	12.61±1.58^{def}	86.19±2.12^{bcd}	3.92±0.02^{b}	190.31±5.46^{b}	47.96±0.17^{a}	50.05±5.45
SB+NT 10^7	86.18±2.25	13.93±0.88^{de}	85.57±2.47^{bcd}	3.86±0.02^{b}	182.30±4.18^{b}	45.93±3.75^{a}	53.03±5.66
SB+RY-Δ6 10^5	91.05±2.73	13.27±0.66^{def}	94.14±4.34^{ab}	3.79±0.03^{b}	201.92±21.76^{b}	54.40±4.62^{a}	47.31±2.52
SB+RY-Δ6 10^7	78.19±6.31	14.79±0.52^{d}	98.65±3.70^{a}	3.73±0.02^{b}	201.16±7.16^{b}	47.42±6.03^{a}	47.56±8.11
Linseed oil (LO)	87.48±12.08	18.52±0.16^{bc}	77.41±1.91^{de}	3.90±0.27^{b}	185.85±12.75^{b}	50.93±6.65^{a}	49.85±1.22
LO+NT 10^5	86.46±10.19	17.91±0.45^{c}	80.79±1.27^{cd}	3.64±0.14^{b}	206.36±18.27^{b}	49.17±8.13^{a}	47.03±2.21
LO+NT 10^7	84.06±3.34	18.07±0.52^{c}	80.50±2.06^{cd}	3.58±0.07^{b}	208.18±14.65^{b}	47.81±8.76^{a}	49.51±5.36
LO+RY-Δ6 10^5	88.28±7.80	21.37±1.61^{ab}	89.85±3.09^{abc}	3.79±0.20^{b}	204.65±32.47^{b}	53.90±7.70^{a}	48.74±4.23
LO+RY-Δ6 10^7	78.37±8.53	22.79±1.60^{a}	89.09±7.61^{abc}	3.97±0.11^{b}	207.03±10.05^{b}	48.63±2.18^{a}	48.30±2.02

Means with different superscripts in each column differ significantly from each other ($P<0.05$).

Table 6.3 Fatty acid profile (mg/100 g lipid) in enriched *Artemia* (mean±sd, n=3) (Continuoued).

Treatment	C22:6N3	SFA	MUFA	PUFA	n3-PUFA	n6-PUFA
Non enrich	72.34±2.23^{b}	1,631.54±92.57^{d}	2,303.09±151.20^{c}	2,197.63±36.21^{d}	1,405.86±20.48^{c}	763.29±26.93^{c}
Fish oil	3,936.23±80.91^{a}	7,050.89±201.00^{a}	7,276.08±312.20^{b}	9,239.68±528.95^{b}	7,536.31±399.56^{a}	1,635.02±135.48^{c}
NT 10^5	82.22±10.33^{b}	1,535.74±79.76^{d}	2,248.29±128.63^{c}	2,127.99±178.21^{d}	1,346.97±174.55^{c}	755.04±7.21^{c}
NT 10^7	81.04±4.93^{b}	1,708.08±146.38^{d}	2,439.27±288.16^{c}	2,415.99±285.59^{d}	1,573.25±263.45^{c}	814.77±40.41^{c}
RY-Δ6 10^5	83.45±1.16^{b}	1,565.33±34.18^{d}	2,213.51±29.46^{c}	2,200.73±49.52^{d}	1,420.42±45.60^{c}	754.87±4.03^{c}
RY-Δ6 10^7	78.82±7.07^{b}	1,636.60±115.41^{d}	2,486.88±196.28^{c}	2,277.65±103.03^{d}	1,493.32±93.23^{c}	756.64±10.91^{c}
Soybean oil	78.53±5.45^{b}	6,864.07±890.60^{a}	10,086.80±253.48^{a}	15,705.97±1,071.21^{a}	1,537.76±21.79^{c}	14,113.00±1,062.70^{a}
SB+NT 10^5	80.15±1.66^{b}	6,572.64±845.36^{ab}	10,544.84±769.60^{a}	15,662.10±149.18^{a}	1,560.67±22.84^{c}	14,044.74±121.03^{a}
SB+NT 10^7	76.05±3.93^{b}	7,336.52±711.63^{a}	11,129.31±299.02^{a}	15,756.78±254.43^{a}	1,535.30±90.39^{c}	14,167.14±299.29^{a}
SB+RY-Δ6 10^5	81.41±8.69^{b}	7,043.61±946.62^{a}	10,333.61±424.13^{a}	16,438.30±592.46^{a}	1,605.06±36.76^{c}	14,776.08±559.85^{a}
SB+RY-Δ6 10^7	82.08±4.00^{b}	7,283.60±720.32^{a}	10,918.90±1,715.96^{a}	15,709.69±1,133.91^{a}	1,609.20±29.78^{c}	14,047.97±1,136.38^{a}
Linseed oil	78.46±10.08^{b}	4,598.67±711.67^{c}	10,277.45±114.85^{a}	6,752.94±169.74^{c}	3,346.59±26.51^{b}	3,349.92±148.96^{b}
LO+NT 10^5	93.54±2.30^{b}	4,501.82±975.93^{c}	10,686.04±416.86^{a}	7,041.66±415.69^{c}	3,417.15±40.55^{b}	3,571.51±443.58^{b}
LO+NT 10^7	89.91±3.64^{b}	4,450.30±1,048.59^{c}	10,143.55±739.38^{a}	7,087.54±341.24^{c}	3,337.61±17.36^{b}	3,691.36±353.28^{b}
LO+RY-Δ6 10^5	102.07±7.32^{b}	4,746.74±200.20^{bc}	10,316.38±1,073.23^{a}	7,096.06±475.27^{c}	3,376.50±24.58^{b}	3,662.71±470.93^{b}
LO+RY-Δ6 10^7	104.30±6.24^{b}	4,820.27±460.58^{bc}	10,677.19±1,597.43^{a}	7,000.41±351.24^{c}	3,412.80±52.90^{b}	3,530.76±357.64^{b}

Means with different superscripts in each column differ significantly from each other ($P<0.05$).

6.5.2 Effects of the use of RY-Δ6 *Artemia* for enrichment on growth performance and survival rate of seabass larvae

The effects of the use of RY-Δ6 for *Artemia* enrichment on growth performance, condition factor and survival rate of seabass larvae for 15 days, 30 days and 45 days were shown in Table 6.5. The growth performance included final weight, weight gain and specific growth rate (SGR). For 15 days feeding, fish were fed enriched *Artemia* had higher weight gain and SGR higher than that were fed non-enriched *Artemia* (*P<0.05*). For 30 days and 45 days of experimental period, fish fed enriched *Artemia* with 3% linseed oil co-supplemented with RY-Δ6 at 10^7 CFU/g had highest growth performance including final weight, weigh gain and SGR (*P<0.05*) when compared with other experimental groups. Survival rate of fish which were fed with enriched *Artemia* higher than fish were fed non-enrich *Artemia* (*P<0.05*).

6.5.3 Effects of the use of RY-Δ6 *Artemia* for enrichment on microbrial population of seabass larvae

The effects of use of RY-Δ6 to enrich *Artemia* on microbial populations of seabass for 45 days after feeding are shown in Table 6.6. The population of *Bifidobacterium* spp. and *Lactobacilus* spp. were not significant different among the experimental groups *(P>0.05)*. Seabass larvae fed *Artemia* co-enriched with soybean oil or linseed oil and RY-Δ6 at 10^5 and 10^7 CFU/g had higher number of total bacteria, yeast and fungi and lower number of *Vibrio* spp. *(P<0.05)* compared with fish fed *Artemia* enriched with 3% fish oil, soybean oil, linseed oil and non-enriched *Artemia*.

Table 6.4 Growth performance and survival rate of seabass larvae fed the experimental diets for 15, 30 and 45 days (mean±sd, n=4).

Treatment	Initial weight (mg)	Final weight (mg)	Length (cm)	CF (%)	WG (mg)	SGR (%)
0-15 days						
Non enrichment	25.40±0.08	41.25±1.50^{b}	1.35±0.09	3.08±0.26	15.85±1.46^{b}	3.23±0.23^{b}
Fish oil	25.38±0.10	51.25±1.50^{a}	1.58±0.16	3.27±0.29	25.88±1.41^{a}	4.69±0.17^{a}
Soybean oil	25.53±0.10	51.25±4.79^{a}	1.57±0.07	3.29±0.41	25.73±4.73^{a}	4.62±0.62^{a}
Soy-RY 10^{5}	25.43±0.13	53.75±2.63^{a}	1.53±0.14	3.53±0.39	28.33±2.64^{a}	4.99±0.33^{a}
Soy-RY 10^{7}	25.45±0.13	53.00±0.82^{a}	1.55±0.05	3.42±0.14	27.55±0.74^{a}	4.89±0.09^{a}
Linseed oil	25.40±0.14	50.50±1.00^{a}	1.47±0.10	3.45±0.23	25.10±1.01^{a}	4.58±0.14^{a}
Lin-RY 10^{5}	25.40±0.08	53.25±0.50^{a}	1.52±0.08	3.52±0.22	27.85±0.44^{a}	4.93±0.05^{a}
Lin-RY 10^{7}	25.53±0.10	54.00±1.41^{a}	1.55±0.06	3.49±0.17	28.48±1.35^{a}	5.00±0.16^{a}
15-30 days						
Non enrichment	41.25±1.50^{b}	64.25±4.92^{c}	1.56±0.11	4.12±0.32^{b}	23.00±4.97^{ab}	2.94±0.54
Fish oil	51.25±1.50^{a}	73.30±3.98^{b}	1.70±0.07	4.33±0.30^{ab}	22.05±2.83^{ab}	2.38±0.22
Soybean oil	51.25±4.79^{a}	71.25±3.20^{bc}	1.66±0.08	4.30±0.36^{ab}	20.00±4.55^{b}	2.22±0.59
Soy-RY 10^{5}	53.75±2.63^{a}	76.35±1.58^{ab}	1.71±0.08	4.49±0.23^{ab}	22.60±2.73^{ab}	2.35±0.33
Soy-RY 10^{7}	53.00±0.82^{a}	81.75±2.36^{a}	1.71±0.09	4.79±0.40^{ab}	28.75±2.06^{a}	2.89±0.17
Linseed oil	50.50±1.00^{a}	70.75±0.96^{bc}	1.68±0.07	4.22±0.19^{ab}	20.25±1.71^{b}	2.25±0.19
Lin-RY 10^{5}	53.25±0.50^{a}	77.50±2.89^{ab}	1.75±0.12	4.44±0.16^{ab}	24.25±3.20^{ab}	2.50±0.29
Lin-RY 10^{7}	54.00±1.41^{a}	83.25±3.77^{a}	1.72±0.05	4.84±0.21^{a}	29.25±3.30^{a}	2.88±0.27

Means with different superscripts in each column differ significantly from each other ($P<0.05$).

Table 6.4 Growth performance and survival rate of seabass larvae fed the experimental diets for 15, 30 and 45 days (mean±sd, n=4) (Continuoued).

Treatment	Initial weight (mg)	Final weight (mg)	Length (cm)	CF (%)	WG (mg)	SGR (%)	Survival rate (%)
30-45 days							
Non enrichment	64.25±4.92^{c}	81.75±4.99^{d}	1.86±0.11	4.42±0.33^{c}	17.50±6.14^{d}	1.61±0.58^{d}	73.20±0.98^{b}
Fish oil	73.30±3.98^{b}	97.75±1.89^{cd}	2.00±0.19	4.93±0.52^{bc}	24.45±4.72^{d}	1.93±0.41^{d}	81.80±2.86^{a}
Soybean oil	71.25±3.20^{bc}	97.00±1.41^{cd}	1.88±0.17	5.20±0.39^{bc}	25.75±2.99^{d}	2.06±0.28^{cd}	81.50±1.91^{a}
Soy-RY 10^{5}	76.35±1.58^{ab}	108.98±6.05^{c}	1.83±0.19	5.98±0.55^{abc}	32.63±6.41^{cd}	2.37±0.42^{cd}	83.10±3.73^{a}
Soy-RY 10^{7}	81.75±2.36^{a}	128.75±6.65^{b}	2.16±0.32	6.03±0.53^{abc}	47.00±7.53^{bc}	3.02±0.42^{bc}	82.80±4.05^{a}
Linseed oil	70.75±0.96^{bc}	96.80±2.64^{cd}	1.92±0.27	5.11±0.60^{bc}	26.05±2.45^{d}	2.09±0.17^{cd}	82.00±2.40^{a}
Lin-RY 10^{5}	77.50±2.89^{ab}	128.50±11.27^{b}	1.99±0.22	6.50±0.58^{ab}	51.00±9.93^{b}	3.36±0.51^{ab}	82.80±2.71^{a}
Lin-RY 10^{7}	83.25±3.77^{a}	154.75±11.67^{a}	2.14±0.36	7.40±1.55^{a}	71.50±9.47^{a}	4.12±0.38^{a}	82.80±3.22^{a}

Means with different superscripts in each column differ significantly from each other ($P<0.05$).

Table 6.5 Microbial populations in intestinal tract of seabass fed enrichment *Artemia* for 45 days (mean±sd, n=4).

Treatment	Total Bacteria (log CFU/g)	*Bifidobacterium* spp. (log CFU/g)	Yeast & Fungi (log CFU/g)	*Lactobacilus* spp. (log CFU/g)	*Vibrio* spp. (log CFU/g)
Non-enrichment	5.74±0.14^{d}	3.92±0.27	3.69±0.20^{b}	3.97±0.27	3.31±0.14^{a}
Fish oil	5.84±0.06^{bcd}	3.98±0.14	3.67±0.06^{b}	3.95±0.14	3.27±0.14^{a}
Soybean oil	5.82±0.13^{bcd}	3.90±0.04	3.68±0.24^{b}	4.10±0.12	3.24±0.11^{a}
Soy-RY 10^{5}	6.08±0.12^{ab}	4.04±0.06	4.09±0.15^{ab}	4.20±0.14	2.46±0.22^{b}
Soy-RY 10^{7}	6.36±0.16^{a}	4.14±0.03	4.26±0.12^{a}	4.17±0.19	2.37±0.16^{b}
Linseed oil	5.78±0.12^{cd}	4.01±0.14	3.68±0.32^{b}	4.08±0.25	3.28±0.19^{a}
Lin-RY 10^{5}	6.04±0.09^{bc}	4.04±0.01	3.94±0.09^{ab}	4.18±0.16	2.58±0.18^{b}
Lin-RY 10^{7}	6.36±0.10^{a}	4.17±0.05	4.23±0.15^{a}	4.14±0.24	2.34±0.08^{b}

Means with different superscripts in each column differ significantly from each other ($P<0.05$).

6.5.4 Effects of the use of RY-Δ6 for enrichement of *Artemia* on accumulation of fatty acid profile of seabass larvae

Effect of use of RY-Δ6 to enrich *Artemia* on composition of fatty acid in whole body of seabass larvae for 45 days are shown in Table 6.6. The deposition of fatty acid in whole body of seabass larvae including C8:0, C10:0, C14:1, C16:1, C24:0 and C24:1 were not significant different among the experimental groups *(P>0.05)*. Seabass larvae were fed enriched *Artemia* with soybean oil, linseed oil and those co-supplemented with RY-Δ6 at 10^5 and 10^7 CFU/g had higher C18:3n6 and C18:4n3, respectively, in whole body (*P<0.05*). Both fatty acids are products of delta-6 desaturase, suggesting that probiotic RY-Δ6 would exerted delta 6 desaturase activity. Additionally, fatty acid C20:4n6 were highest in seabass fed co-enriched *Artemia* with linseed oil and RY-Δ6 when compared with other experimental groups (*P<0.05*). Seabass which were fed with *Artemia* enriched with fish oil had highest C20:5n3 and C22:6n3. Also, fish fed with *Artemia* co-enriched with linseed oil and RY-Δ6 at 10^5 and 10^7 CFU/g had higher C22:6n3 when compared to fish fed non-enriched *Artemia*, enriched *Artemia* with soybean oil and linseed oil, and *Artemia* co-enriched with soybean oil and RY-Δ6 at 10^5 and 10^7 CFU/g.

Table 6.6 Accumulation of fatty acid profile (mg/g lipid) in whole body of seabass fed experimental diet for 45 days (mean±sd, n=4).

Fatty acids	C8:0	C10:0	C12:0	C14:0	C14:1	C16:0	C16:1
Non enrichment	4.46±0.61	149.22±10.81	26.22±2.36^{b}	54.76±4.84^{c}	23.32±2.63	795.92±25.03^{cd}	62.11±5.80
Fish oil	4.16±0.26	144.59±24.89	35.70±3.59^{a}	112.75±8.06^{a}	22.88±1.54	748.76±88.73^{d}	63.04±5.67
Soybean oil	5.03±0.68	124.70±20.03	28.79±2.20^{ab}	76.82±4.11^{b}	25.98±5.30	1,883.20±126.59^{a}	63.94±4.50
Soy-RY 10^{5}	4.74±0.83	140.71±50.50	26.19±3.75^{b}	81.13±6.05^{b}	19.71±11.84	1,975.65±82.17^{a}	59.28±7.28
Soy-RY 10^{7}	4.52±0.55	145.03±19.42	28.73±6.80^{ab}	79.49±1.23^{b}	29.05±6.04	1,945.41±211.81^{a}	56.48±3.92
Linseed oil	4.30±0.28	144.25±27.33	24.75±3.61^{b}	78.35±6.15^{b}	22.55±5.74	1,099.09±68.78^{b}	59.49±3.87
Lin-RY 10^{5}	4.97±0.78	141.68±7.60	24.31±3.13^{b}	80.59±2.24^{b}	25.40±3.50	1,078.59±40.79^{bc}	60.17±3.32
Lin-RY 10^{7}	4.34±0.35	151.22±4.49	24.67±3.22^{b}	79.38±6.89^{b}	24.44±4.21	1,187.28±217.03^{b}	58.62±1.93

Means with different superscripts in each column differ significantly from each other ($P<0.05$).

Table 6.6 Accumulation of fatty acid profile (mg/g lipid) in whole body of seabass fed experimental diet for 45 days (mean±sd, n=4) (continued).

Fatty acids	C18:0	C18:1N9T	C18:1N9C	C18:2N6T	C18:2N6C	C20:0	C18:3N6
Non enrichment	667.25±150.40^{b}	nd	814.94±62.37^{b}	nd	273.33±43.77^{c}	18.12±1.13^{b}	4.85±1.55^{f}
Fish oil	657.89±167.64^{b}	nd	799.30±96.91^{b}	nd	235.34±16.15^{c}	45.61±3.93^{a}	4.47±0.48^{f}
Soybean oil	1,603.69±205.59^{a}	nd	1,161.93±105.67^{a}	nd	1,767.60±71.83^{a}	47.97±4.85^{a}	24.10±2.34^{e}
Soy-RY 10^{5}	1,743.73±103.37^{a}	nd	1,231.32±157.52^{a}	nd	1,857.04±102.87^{a}	51.68±5.18^{a}	71.06±4.16^{b}
Soy-RY 10^{7}	1,552.71±353.82^{a}	nd	1,201.85±99.44^{a}	nd	1,800.42±131.74^{a}	49.30±4.29^{a}	81.77±4.25^{a}
Linseed oil	1,781.14±278.08^{a}	nd	1,165.73±151.61^{a}	nd	1,035.46±64.11^{b}	51.09±1.85^{a}	17.83±1.34^{e}
Lin-RY 10^{5}	1,787.10±163.43^{a}	nd	1,147.21±74.25^{a}	nd	1,034.70±41.42^{b}	49.99±1.15^{a}	41.70±2.88^{d}
Lin-RY 10^{7}	1,525.78±277.13^{a}	nd	1,225.03±47.26^{a}	nd	1,100.97±117.13^{b}	51.00±5.18^{a}	53.76±2.48^{c}

Means with different superscripts in each column differ significantly from each other ($P<0.05$).

Table 6.6 Accumulation of fatty acid profile (mg/g lipid) in whole body of seabass fed experimental diet for 45 days (mean±sd, n=4) (Continued).

Fatty acids	C20:1n9	C18:3N3	C18:4N3	C20:2	C22:0	C20:3N6	C22:1N9
Non enrichment	5.46±0.31^{b}	587.12±114.54^{c}	28.84±1.11^{e}	18.81±1.79^{b}	9.70±1.77^{c}	19.66±1.83^{cd}	23.46±3.79
Fish oil	5.83±0.20^{b}	546.53±48.78^{c}	28.56±0.90^{e}	36.80±3.46^{a}	21.38±1.88^{b}	17.50±1.01^{d}	23.57±1.69
Soybean oil	15.83±1.95^{a}	1,007.70±57.95^{b}	29.88±1.59^{e}	36.99±3.36^{a}	68.18±5.11^{a}	19.50±1.97^{cd}	24.73±3.43
Soy-RY 10^{5}	15.66±0.68^{a}	1,022.44±71.02^{b}	72.85±4.76^{d}	39.78±1.35^{a}	68.99±3.99^{a}	27.49±3.44^{bc}	23.57±2.81
Soy-RY 10^{7}	15.88±0.75^{a}	1,064.72±83.15^{b}	90.87±7.11^{c}	38.28±2.40^{a}	67.90±3.45^{a}	30.68±2.30^{b}	24.11±1.43
Linseed oil	15.41±1.99^{a}	1,569.96±99.95^{a}	28.88±5.44^{e}	39.44±1.84^{a}	21.36±1.21^{b}	22.41±2.43^{bcd}	23.24±1.34
Lin-RY 10^{5}	15.69±0.96^{a}	1,584.99±86.77^{a}	122.00±8.84^{b}	38.86±2.36^{a}	21.01±1.46^{b}	29.81±5.59^{b}	22.98±1.86
Lin-RY 10^{7}	15.11±1.02^{a}	1,372.67±162.00^{a}	189.62±9.27^{a}	39.66±1.24^{a}	23.66±0.69^{b}	46.03±7.42^{a}	24.18±2.48

Means with different superscripts in each column differ significantly from each other ($P<0.05$).

Table 6.6 Accumulation of fatty acid profile (mg/g lipid) in whole body of seabass fed experimental diet for 45 days (mean±sd, n=4) (Continued).

Fatty acids	C20:3N3	C20:4N6	C22:2	C20:5N3	C24:0	C24:1	C22:6N3
Non enrichment	61.67±2.33^{c}	190.02±17.63^{b}	nd	53.52±4.75^{c}	81.43±5.10	45.08±1.43	69.26±4.34^{c}
Fish oil	61.28±3.91^{c}	191.61±10.76^{b}	nd	191.31±3.29^{a}	87.58±7.25	44.71±3.56	728.71±58.26^{a}
Soybean oil	62.27±7.12^{c}	186.36±5.18^{b}	nd	58.40±3.46^{bc}	83.69±4.94	46.36±2.15	59.19±6.40^{c}
Soy-RY 10^{5}	61.23±3.85^{c}	181.43±12.46^{b}	nd	61.06±0.57^{bc}	87.20±6.26	44.81±1.11	73.57±5.43^{c}
Soy-RY 10^{7}	58.10±5.48^{c}	195.10±8.22^{b}	nd	62.52±0.72^{b}	84.41±3.11	46.66±2.15	78.02±9.05^{c}
Linseed oil	63.72±4.29^{c}	182.83±5.65^{b}	nd	58.67±6.67^{bc}	80.66±7.18	44.98±3.06	63.67±7.80^{c}
Lin-RY 10^{5}	86.31±1.98^{b}	260.24±12.46^{a}	nd	55.81±2.48^{bc}	84.81±4.34	47.16±4.05	107.14±6.51^{c}
Lin-RY 10^{7}	104.96±1.17^{a}	270.97±15.26^{a}	nd	53.85±2.83^{c}	86.82±2.88	46.56±1.66	209.82±6.71^{b}

Means with different superscripts in each column differ significantly from each other ($P<0.05$).

Table 6.6 Accumulation of fatty acid profile (mg/g lipid) in whole body of seabass fed experimental diet for 45 days (mean±sd, n=4) (Continued).

Fatty acids	SFA	MUFA	PUFA	n3-PUFA	n6-PUFA
Non enrichment	1,807.07±162.98^{c}	974.37±65.19^{b}	1,307.06±177.24^{d}	800.38±116.22^{d}	487.87±60.39^{d}
Fish oil	1,858.42±242.54^{c}	959.32±95.83^{b}	2,042.10±74.43^{c}	1,556.38±51.97^{b}	448.92±26.61^{d}
Soybean oil	3,922.08±289.99^{a}	1,338.76±97.23^{a}	3,251.99±60.28^{ab}	1,217.43±51.85^{c}	1,997.57±75.78^{a}
Soy-RY 10^{5}	4,180.00±115.96^{a}	1,394.35±171.90^{a}	3,467.94±94.97^{a}	1,291.15±79.18^{c}	2,137.01±94.89^{a}
Soy-RY 10^{7}	3,957.47±359.85^{a}	1,374.03±93.69^{a}	3,500.47±163.41^{a}	1,354.23±88.72^{bc}	2,107.97±129.10^{a}
Linseed oil	3,284.98±379.09^{b}	1,331.40±145.58^{a}	3,082.86±118.47^{b}	1,784.90±88.30^{a}	1,258.53±60.44^{c}
Lin-RY 10^{5}	3,273.06±181.39^{b}	1,318.61±76.99^{a}	3,361.55±100.70^{ab}	1,956.24±73.76^{a}	1,366.45±42.30^{bc}
Lin-RY 10^{7}	3,134.14±90.30^{b}	1,393.92±44.35^{a}	3,442.30±224.24^{a}	1,930.92±160.90^{a}	1,471.74±109.91^{b}

Means with different superscripts in each column differ significantly from each other ($P<0.05$).

6.5.5 Effects of the use of RY-Δ6 for enrichment of *Artemia* on stress resistance to ammonia of seabass larvae

Effect of use of RY-Δ6 to enrich *Artemia* on stress resistance of seabass larvae are shown in Figure 6.1. After exposed with ammonia at 8, 12, 24, 48, 72 and 96 h, among experimental groups, seabass larvae which were fed non-enriched *Artemia* had highest accumulative mortality whereas fish fed co-enriched *Artemia* with linseed oil and RY-Δ6 10^5 and 10^7 CFU/g had lower accumulative mortality.

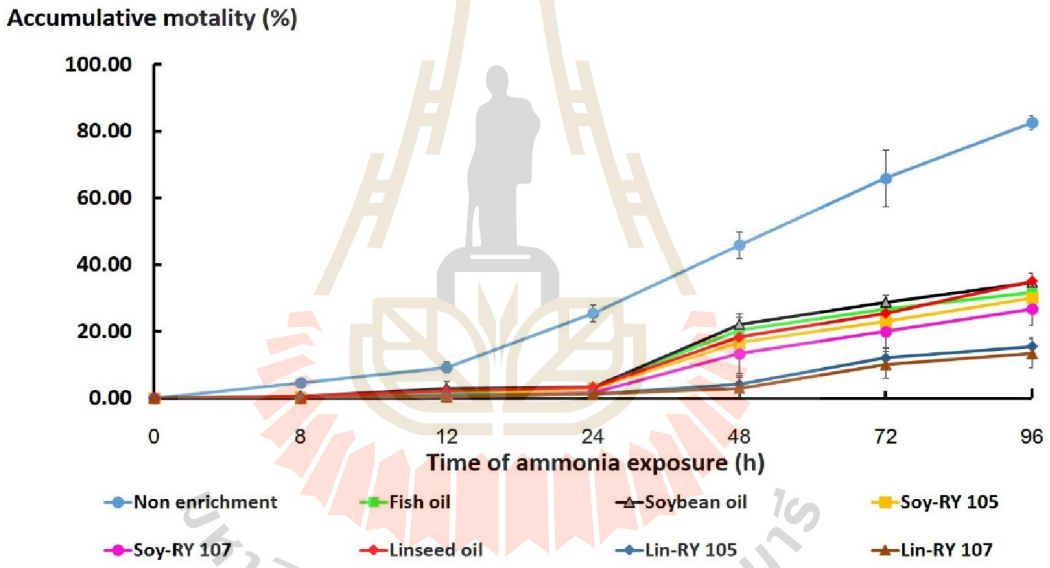

Figure 6.1 Accumulative mortality of seabass larvae fed on enriched (fish oil, soybean oil, soybean oil and supplements RYΔ6 10^5 and 10^7 CFU/g ml, respectively, linseed oil, linseed oil and supplements RYΔ6 10^5 and 10^7 CFU/g ml, respectively) and non-enriched *Artemia* were exposed with ammonia at 8, 12, 24, 48 72 and 96 h.

6.6 Discussion

The enriched *Artemia* with either soybean oil, linseed oil or fish oil had fatty acids composition higher than non-enriched *Artemia*. Similarly, enriched *Artemia* with fish oil, linseed oil and soybean oil had the fatty acid composition higher than non-enrich *Artemia* (Villaita et al., 2005; Martins et al., 2006; Benitez-Dorta et al., 2013; Abedain KenarI et al., 2016). Additionally, the *Artemia* which were co-enriched with 3% soybean oil or 3% linseed oil RY-Δ6 had high content of C18:3n6 or C18:4n3, respectively, ($P<0.05$), suggesting that, the RY-Δ6 would exert delta 6 desaturase activity by converting substrate C18:2n6 in soybean oil and C18:3n3 in linseed oil to C18:3n6 and C18:4n3, respectively. Therefore, our results suggested that RY-Δ6 could be used as recombinant probiotic *S. cerevisiae* in aquatic feeds.

The co-enriched *Artemia* with soybean oil or linseed oil with RY-Δ6 could promote growth performance, condition factor and survival rate of seabass larvae. Similarly, enriched *Artemia* with linseed oil, soybean oil and yeast led to improve the growth performance of freshwater fish including tiger barb, *Catla catla*, rainbow trout, common carp (Abolhasani et al., 2013; Divya et al., 2014; Matty and Smith, 1978; Radhakrishnan et al., 2008). In contrast, in marine fish, linseed oil enriched *Artemia* did not improved growth performance of turbot (Sargent et al., 2002). These findings suggested that in freshwater fish, dietary soybean oil or linseed oil would be effective to promote growth performance, however, it was ineffective in marine fish which required EPA and DHA. Our results indicated that the RY-Δ6 could promote the growth performances of seabass larvae which is a brackish water fish. These findings suggested that seabass could utilized C18:3n6, C18:4n3, C20:4n6 for growth and survival. Additionally, the supplement RY-Δ6 as probiotic yeast could increase growth

performance of Nile tilapia which were similar to the resulted reported previously. Enriched *Artemia* with yeast as probiotic were demonstrated to improve the growth performance and survival rate of marine pejerry, Persian sturgeon and *Litopenaeus vannamei* (Fuhr et al., 2016; Iranshahi et al., 2012; Ahmadi et al., 2017).

Probiotic yeast *S. cerevisiae* was demonstrated to colonize in the intestine which modulate microbial population in intestinal tract (Tovar et al., 2002). Although not all microbial population, seabass larvae fed co-enriched *Artemia* with soybean oil or linseed oil and RY-Δ6 had higher number of total bacteria, yeast and fungi and lower number of *Vibrio* spp. *(P<0.05)*. Our results demonstrated that enrichment of *Artemia* with RY-Δ6 could transfer RY-Δ6 into the intestinal tract of seabass; thereby, RY-Δ6 modulated intestinal microbial population by reducing pathogenic bacteria in intestinal tract of seabass larvae.

These finding were similar to the observation for the Pacific white shrimp. Dietary probiotic bacilli increased the number of total bacteria and probiotic bacilli in hepatopancreas of shrimp (Jamali et al., 2015). In addition, in *Penaeus monodon*, shrimp fed enriched *Artemi*a with *Lactobacillus* sp. and *Saccharomyces* sp. as probiotic could increase the number of *Lactobacillus* sp. and *Saccharomyces* sp. in hepatopancreas of shrimp. Furthermore, these dietary probiotics decreased pathogenic bacteria *Vibrio harveyi* in shrimp (Immanuel et al., 2007).

Effect of use of RY-Δ6 to enrich *Artemia* on composition of fatty acid profile in whole body of seabass larvae at 45 days feeding period. The deposition of fatty acid in whole body of seabass larvae including C8:0, C10:0, C14:1, C16:1, C24:0 and C24:1 were not significant differences among the experimental groups *(P>0.05)*, indicating that dietary RY-Δ6 did not affect saturated fatty acids and unsaturated fatty acids.

Seabass larvae which were co-enriched *Artemia* with soybean oil or linseed oil and RY-Δ6 had higher C18:3n6 or C18:4n3, respectively, in whole body ($P<0.05$). Both fatty acids are products of delta 6 desaturase, suggesting that probiotic RY-Δ6 would exert delta 6 desaturase activity. These results were similar to the findings of supplementation RY-Δ6 as probiotic in diet of tilapia (Chapter 5). In addition, recombinant yeast expressing delta 6 desaturase from Nile tilapia (Tanomman et al., 2013), borage *(Borago officinalis)* (Qiu et al., 2002), cobia (*Rachycentron canadum*) (Zheng et al., 2009), common carp, rainbow trout, gilthead seabream *(Sparus aurata)*, turbot (*Psetta maximus)* (Zheng et al., 2004) and Atlantic bluefin tuna (*Thunnusthynnus*) (Morais et al., 2011) were found to exert delta 6 desaturase to convert fatty acid substrate C18:3n3 to C18:4n3 and C18:2n6 to C18:3n6. Additionally, dietary co-enriched *Artemia* with linseed oil and RY-Δ6 increased the content of C20:4n6, ∑n3-PUFA and ∑n6-PUFA content in whole body, demonstrating that RY-Δ6 expressing delta 6 desaturase would contribute to increase LC-PUFA content in whole body of fish.

The PUFA are important for structural and functional integrity in membrane particularly in fish larvae (Brown, 1994). Therefore, this study further investigated the effect of use of RY-Δ6 to enrich *Artemia* on stress resistance of seabass larvae at 45 days feeding. After exposed with ammonia at 8, 12, 24, 48, 72 and 96 h, dietary *Artemia* co-enriched with linseed oil and RY-Δ6 had lowest accumulative mortality when compare with other experimental groups. Similarly, the tiger barb larvae were fed enriched artemia with linseed oil had mortality lower than that of fish fed non-enriched *Artemia* (Abolhasani et al., 2013). In addition, in *Farfantepenaeus paulensis*, enriched *Artemia* with HUFA relieved the ammonia stress with lethal concentrations (LC_{50})

when compare with shrimp fed non-enrich artemia (Martins et al., 2006). Additionally, Persian sturgeon and shrimp *L. vannamei* were fed enriched *Artemia* with *S. cerevisiae*. The enriched *Artemia* with *S. cerevisiae* led to improve the survival rate after stress when compared to that fed with non-enrich *Artemia* (Iranshahi et al., 2012; Ahmadi et al., 2017). These findings explained that yeast could improve stress resistance in fish and shrimp since whole cell yeast contained compounds such as β-glucans, nucleic acids, chitin, mannan oligonucleotides and other cell wall component.

6.7 Conclusion

This study demonstrated the fatty acid profile of enriched *Artemia* with soybean oil and linseed oil, and co-enriched *Artemia* with RY-Δ6 expressing delta 6 desaturase. The *Artemia* could carry RY-Δ6 pass through intestinal tract of fish and leading to improve growth performance and survival rate. The RY-Δ6 could reduce pathogenic bacteria in intestinal tract of seabass larvae. The *Artemia* enriched with RY-Δ6 led to increase C18:3n6 and C18:4n3 in whole body, suggesting that probiotic RY-Δ6 would exert delta 6 desaturase activity. Additionally, *Artemia* co-enriched with linseed oil and RY-Δ6 increased C20:4n6, ∑n3-PUFA and ∑n6-PUFA content in whole body, demonstrating RY-Δ6 expressing delta 6 desaturase would contribute to increase LC-PUFA content in whole body of fish. Furthermore, *Artemia* co-enriched with linseed oil and RY-Δ6 reduced accumulative mortality of seabass fry which were challenged with ammonia, demonstrating that yeast could improve stress resistance of seabass larvae.

6.8 References

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CHAPTER VII

OVERALL CONCLUSION

This study developed recombinant yeast *Saccharomyces cerevisiae* expressing *Oni-fads2* and tested its used as probiotic in fish. The first experiment was conducted to compare the performance of three promoters (p*ACT*, p*PGK* and p*TEF*) for the expression of *Oni-fads2*. *S. cerevisiae* expressing *Oni-fads2* showed the conversion of exogenous C18:2n6 and C18:3n3 substrates to C18:3n6 and C18:4n3, respectively. The results showed that p*TEF* was the strongest promoter for *Oni-fads2* expression. Crude extracts of recombinant yeast exhibited Δ6 activity.

Subsequently, recombinant *S. cerevisiae* RY-Δ6 stably expressing *Oni-fads2* driven by TEF I promoter was produced. The heterogenous expression of *Oni-fads2* in RY-Δ6 at transcription and translation levels were detectable by RT-PCR and western blot analysis, respectively. RY-Δ6 exhibited delta 6 desaturase (Δ6) activity by converting C18:2n6 and C18:3n3 to C18:3n6 and C18:4n3, respectively. The efficiency of Δ6 activity in RY-Δ6 depends on amount of concentration of substrate and temperature and period of reaction. The efficiency of Δ6 activity in RY-Δ6 was highest when the reaction was performed at 28 ºC for 2 hours. In addition, crude extract of RY-Δ6 displayed Δ6 activity. Crude extract of RY-Δ6 were capable to change the fatty acid composition by increasing the fatty acids of n6- and n3-PUFA when it was incubated in soybean oil and linseed oil.

The third experiment was investigated the effects of the use of RY-Δ6 as dietary probiotic. Microencapsulation of RY-Δ6 was produced, and it showed to have ability to pass through and colonize in the gastrointestinal tract of Nile tilapia. Dietary RY-Δ6 for 90 days improve growth performance. Survival rates were significance different among experimental diets. There were not significant differences in chemical composition in whole body and meat, blood chemistry and hematological indices among experimental treatments ($P>0.05$). Dietary RY-Δ6 led to increase immune (Ig, lysozyme and ACH50), villus height of intestine and decrease intestinal *Vibrio* spp. ($P<0.05$). The significant increases in C18:3n6 and C18:4n3 in liver and meat were found in Nile tilapia fed dietary RY-Δ6 for 90 day ($P<0.05$), suggesting that RY-Δ6 could exerted delta 6 desaturase to increase product fatty acid C18:3n6 and C18:4n3. Therefore, RY-Δ6 could be used as the recombinant probiotic to produce long chain-polyunsaturated fatty acids.

The fourth experiment was investigated the effects of the use of RY-Δ6 to enrich *Artemia* as dietary probiotic on growth performance, fatty acid content, intestinal microbiota in seabass (*Lates calcarifer*) larvae. In addition, the effects of RY-Δ6 on stress resistance to ammonia was examined. *Artemia* co-enriched with soybean oil or linseed oil and RY-Δ6 improved growth performance and survival rate. The RY-Δ6 decreased intestinal *Vibrio* spp. ($P<0.05$) of seabass larvae. *Artemia* co-enriched with soybean oil or linseed oil and RY-Δ6 led to increase C18:3n6 or C18:4n3, respectively, in whole body, suggesting that probiotic RY-Δ6 would exert delta 6 desaturase activity. Additionally, co-enriched *Artemia* with linseed oil and RY-Δ6 led to increase fatty acid C20:4n6, ∑n3-PUFA and ∑n6-PUFA content in whole body, suggesting that RY-Δ6 expressing delta 6 desaturase would contribute to increase LC-PUFA content in whole

body of fish. Furthermore, co-enriched *Artemia* with linseed oil and RY-Δ6 lowered accumulative mortality when fish were challenged with ammonia, demonstrating that yeast could improve stress resistance.

Combined together, the RY-Δ6 expressing *Oni-fads2* has potential to be used as dietary recombinant probiotics. Further investigations to examine their uses in other aquafeed or animal feed are required.

BIOGRAPHY

Miss Araya Jangprai was born on November 22, 1984 in Nakhon Ratchasima, Thailand. In 2003, she finished high school from Koratpittayakom School, Nakhon Ratchasima. In 2007, she graduated the Bachelor's degree of Animal production technology, Suranaree University of Technology, Nakhon Ratchasima. In 2011, she graduated the Master's degree of Animal production technology, Suranaree University of Technology, Nakhon Ratchasima. She began her Ph.D. studies in School of Animal Production Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima and received a scholarship from the External Grants and Scholarships for Graduate Students (OROG), Suranaree University of Technology. Her Ph.D thesis was the production and efficacy of recombinant probiotic yeast producing delta 6 desaturase in fish.