กระบวนการต่อสายยาวของกรดไขมันในเมทิลโลโทรฟิกยีสต์ *Hansenula polymorpha* CBS 1976 และสายพันธุ์กลายชนิดที่ต้องการกรดไขมัน

นางสาววนิดา วงศ์สัมพันธ์ชัย

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FATTY ACID ELONGATION IN METHYLOTROPHIC YEAST *Hansenula polymorpha* CBS 1976 AND FATTY ACID AUXOTROPHIC MUTANTS

Miss Wanida Wongsumpanchai

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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By Miss Wanida Wongsumpanchai

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Thesis Advisor Associate Professor Amorn Petsom, Ph.D.

Thesis Co-advisor Sarintip Anamnart, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctor's Degree

……………………………….Dean of the Faculty of Science
(Professor Piamsak Menasveta, Ph.D.)

Thesis Committee

……………………………….Chairman
(Associate Professor Sirirat Rengpipat, Ph.D.)

……………………………….Thesis Advisor
(Asssociate Professor Amorn Petsom, Ph.D.)

……………………………….Thesis Co-advisor
(Sarintip Anamnart, Ph.D.)

……………………………….Member
(Associate Professor Pairoh Pinphanichakarn, Ph.D.)

……………………………….Member
(Assistant Professor Nattaya Ngarmrojnavanich, Ph.D.)

……………………………….Member
(Kobkul Laoteng, Ph.D.)

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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ได้แยกสายพันธุ์กลายชนิดที่ต้องการกรดไขมัน (V1 ถึง V10) ของ ***Hansenula polymorpha*** จากการกลายพันธุ์ของ wild-type โดยใช้สารเคมี ethyl methanesulfonate (EMS) เมื่อทำการเพาะเลี้ยงสายพันธุ์กลาย พบว่า ทุกสายพันธุ์กลายที่คัดเลือกได้ไม่สามารถเจริญได้บนอาหาร YEPD หลังจากการปั๊มด้วยเพลทต้นแบบของ YEPD ที่เสริมด้วยกรดไขมันผสมของ C14:0 C16:0 และ C18:0 ที่มีความเข้มข้นอย่างละ 1 mM เพื่อที่จะจัดจำแนกกลุ่มของสายพันธุ์กลายจึงได้ทำการศึกษาการเจริญบนอาหารที่เสริมด้วย กรดไขมันชนิดต่างๆ ได้แก่ C14:0 C16:0 และ C18:0 ที่ความเข้มข้น 1 mM และ/ หรือ 2 mM ผลการศึกษาพบว่าสามารถจัดจำแนกสายพันธุ์กลายได้ 3 กลุ่ม ซึ่งมีการแสดงฟีโนไทป์ ที่ต่างกัน โดยกลุ่มที่หนึ่งมี 6 สายพันธุ์กลาย (V1 ถึง V6) ซึ่งไม่สามารถเจริญบนอาหารแข็งที่เสริมด้วย 1 mM ของ C16:0 อย่างไรก็ตาม 2 mM ของ C16:0 และ 1-2 mM ของ C18:0 สามารถช่วยเสริมการเจริญของสายพันธุ์กลายกลุ่มนี้ได้ จากผลของกลุ่มแรกที่ไม่สามารถเจริญได้ใน 1 mM ของ C16:0 แสดงว่าสายพันธุ์กลายกลุ่มนี้น่าจะมีความบกพร่องบางส่วนในขั้นตอนของการต่อสายยาวของคาร์บอนจาก C16 ถึง C18 สำหรับกลุ่มที่สอง พบว่ามีเพียง 1 สายพันธุ์กลาย (V7) ซึ่งไม่สามารถเจริญได้บนอาหารที่เสริมด้วย 1 mM ของ C14:0 และเมื่อเลี้ยงในอาหารที่เสริมด้วยกรดไขมันผสมของ C14:0 C16:0 และ C18:0 พบว่ามีการสะสมของกรดไขมัน C14:0 มากกว่า parental strain ที่เลี้ยงในอาหารสูตรเดียวกัน แสดงว่าสายพันธุ์กลายกลุ่มนี้ น่าจะมีความบกพร่องที่ขั้นตอนของการต่อสายยาวของคาร์บอนจาก C14:0 ถึง C16:0 ส่วนสายพันธุ์กลายกลุ่มสุดท้าย (V8 V9 และ V10) พบว่าไม่สามารถเจริญได้ บนอาหารที่เสริมด้วย 1 mM ของ C16:0 และ 1 mM ของ C18:0 แต่สามารถเจริญได้บนอาหารที่ต้องมี C14:0 เป็นองค์ประกอบ แสดงว่าสายพันธุ์กลายกลุ่มนี้น่าจะมีความบกพร่องที่ขั้นตอนการต่อสายยาวของคาร์บอนที่มีความยาวน้อยกว่า C14

จากการผสมสายพันธุ์ระหว่างสายพันธุ์กลายทั้งสามกลุ่มกับสายพันธุ์ที่ต้องการกรดไขมัน (***ura3-1***) และทำการคัดเลือกลูกผสมบนอาหาร minimal medium (MIN) ที่เสริมด้วยกรดไขมันผสมของ C14:0, C16:0 และ C18:0 แต่ไม่เติม uracil และบนอาหาร MIN ที่เติม uracil โดยลูกผสมที่ได้จากทั้งสามกลุ่มของสายพันธุ์กลายได้นำไปใช้เป็น recipient strain สำหรับขั้นตอนของการทดสอบการ complementation โดยยีสต์ลูกผสมได้ถูกทรานสฟอร์มด้วย plasmid YCpELO1.MOD ที่มีลำดับการถอดรหัสของ ***S-ELO1*** โดยมี ***URA3*** เป็น selective marker และ plasmid YCpGALELO2(U) ที่มีลำดับการถอดรหัสของ ***S-ELO2*** โดยมี ***URA3*** และ galactose-inducible GAL promoter เป็น selective marker เซลล์ยีสต์ถูกทรานสฟอร์ม โดยใช้ลิเทียม อะซิเตท และคัดเลือกบนอาหาร MIN ที่เสริมด้วยกรดไขมัน สำหรับประสิทธิภาพของการทรานสฟอร์มถูกวัดโดยการทำ plating aliquots ของทรานสฟอร์มแมนท์บนอาหารเลี้ยงเชื้อ จากสายพันธุ์กลายทั้งสามกลุ่ม พบว่ามีเพียง ***ELO2*** ทรานสฟอร์มแมนท์ กลุ่มเดียวที่สามารถเจริญได้บนอาหารที่ไม่เสริมกรดไขมัน แต่ใส่ galactose โดยประมาณ 15 จาก 150 ทรานสฟอร์มแมนท์ออกแบบเป็น VT1 (***URA3, FAE1***) ที่มี ***ELO2*** ถูกคัดเลือกแบบสุ่มเพื่อนำไปใช้ศึกษาลักษณะการเจริญของทรานสฟอร์มแมนท์ VT1 บนอาหารแข็งและอาหารเหลวที่เสริมด้วยกรดไขมันชนิดต่างๆ เปรียบเทียบกับลักษณะการเจริญของ wild-type ผลการศึกษาในทุกสภาวะของการทดลองพบว่า VT1 มีลักษณะของการเจริญที่พบทั้งในอาหารแข็งและอาหารเหลว คล้ายกับการเจริญของ wild-type นอกจากนี้ จากการเปรียบเทียบโครมาโตแกรมของ fatty acid methyl esters (FAMEs) ของ wild-type และ VT1 ที่เจริญบนอาหร YEPD และ YEPD ที่เสริมด้วยกรดไขมันชนิดต่างๆ จากการเลี้ยงที่อุณหภูมิ 37 องศาเซลเซียส เป็นเวลา 24 ชั่วโมง พบว่ามีปริมาณและสัดส่วนของกรดไขมันต่างๆคล้ายคลึงกัน

สำหรับผลการศึกษาที่กล่าวมาแสดงให้เห็นว่า ***ELO2*** ของ ***S. cerevisiae*** สามารถชดเชยส่วนที่บกพร่องของสายพันธุ์กลาย V1 ของ ***H. polymorpha*** ได้ ซึ่งแสดงให้เห็นว่ามีความบกพร่องของกระบวนการผลิตกรดไขมันในสายพันธุ์กลาย V1 โดยพบว่ามีความบกพร่องบางส่วนอย่างน้อยคือในขั้นตอนของการต่อสายยาวของคาร์บอน จาก C16 ถึง C18 นอกจากนี้ข้อมูลที่ได้จากการศึกษานี้สามารถนำไปใช้ในการศึกษาสายพันธุ์กลายชนิดที่มีความบกพร่องตรง elongase เพื่อมุ่งสู่ความรู้ความเข้าใจเกี่ยวกับวิถีเมตาโบลิซึมในยีสต์นี้

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WANIDA WONGSUMPANCHAI: FATTY ACID ELONGATION IN METHYLOTROPHIC YEAST *Hansenula polymorpha* CBS 1976 AND FATTY ACID AUXOTROPHIC MUTANTS. THESIS ADVISOR: ASSOC. PROF. AMORN PETSOM, Ph.D., THESIS CO-ADVISOR: SARINTIP ANAMNART, Ph.D., 124 pp. ISBN 974-17-5913-4

Fatty acid auxotrophic mutants (V1 to V10) of *Hansenula polymorpha* were isolated from the wild-type strain (*leu1-1*) of *H. polymorpha* CBS 1976 by mutagenization with ethyl methanesulfonate (EMS). All the mutants were screened as colonies which were unable to proliferate on YEPD after replication from initial YEPD containing a mixture of 1mM each of C14:0 C16:0 and C18:0 where the mutagenized cells were plated. In order to classify the mutants, their growth on media supplemented with various fatty acids of 1mM and/or 2mM C14:0 C16:0 and C18:0 were examined. Three groups of mutants possessing different phenotype were identified. The first group including six mutants (V1 to V6) did not grow on solid media supplemented with 1mM of C16:0, however, 2mM of C16:0 and 1-2mM of C18:0 could support the growth of this group. Ability of the first group which were unable to grow in 1mM C16:0 suggesting that this group of the mutants might have partially defect at the step of elongation of C16 to C18. The second group included only one mutant (V7) whose no growth when grown on media supplemented with 1mM of C14:0 and when cultivated on media supplemented with a mixture of C14:0 C16:0 and C18:0 noted that C14:0 was also increased when compared with those of the parental strain in the same media. Taken together, from these results suggesting that this mutant probably have the defect at the elongation of C14:0 to C16:0. Mutants in the third group (V8 V9 and V10) did not grow on medium cultivated with 1mM of C16:0 and 1mM of C18:0 supplementation but demonstrated their ability to proliferate on media with addition of C14:0 suggesting that this mutant might have the defect at the elongation before C14.

From crosses of all the three groups of the mutants with an auxotrophic strain (*ura3-1*), all diploid hybrids were selected on minimal medium containing a mixture of C14:0, C16:0 and C18:0 fatty acids without uracil and MIN with uracil. The segregants of all the three groups were chosen as the recipient strain for complementation test. The diploid yeast cells were transformed with a plasmid YCpELO1.MOD harboring the coding sequences of *S-ELO1* and containing the *URA3* gene as a selective marker, in addition to the YCpGALELO2(U) harboring the coding sequences of *S-ELO2* which containing the *URA3* gene and galactose-inducible GAL promoter as a selective marker. The transformation of the yeast cells were performed using the lithium acetate method and then collected on fatty acid supplemented MIN medium. Transformation efficiency was determined by plating aliquots of transformants on the medium. Among them, only the *ELO2* transformants could grow on the medium containing galactose without fatty acid supplementation. Fifteen of ~150 transformant cells designed as VT1 (*URA3, FAE1*) carring *ELO2* were randomly chosen to further analysed the growth characteristic of VT1 transformant on solid and liquid medium containing various fatty acids related to wild-type strain. The results revealed the similar growth phenotype as found in solid and liquid medium by the growth of VT1 could reach closely to that of wild-type strain under any conditions. Moreover, comparison of chromatograms of fatty acid methyl esters (FEMEs) from the wild-type strain and VT1 mutant grown in YEPD media and YEPD supplemented with various fatty acids at 37 ^{o}C for 24 h found that their fatty acids were similar in content and profile.

Taken together, these results demonstrated that the *ELO2* gene of *S. cerevisiae* could function in *H. polymorpha* V1, indicating a clear picture of the lesion in fatty acid biosynthesis in V1 mutant that the partial defect at least in the elongation step of C16 to C18. The information obtain from these studies could be exploited to create defective fatty acid elongase strains to be learnt about the mechanism of action of this yeast.

Department……–……………….. Student's signature……………………

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ABBREVIATIONS

ACC	=	acetyl-CoA carboxylase
ACP	=	acyl carrier peptide
ARA	=	arachidonic acid
AT	=	acetyl transferase
ATP	=	adenosine 5'-triphosphate
CoA	=	CoenzymeA
^{o}C	=	degree centigrade of celsius
d	=	day
DEGS	=	diethylene glycol succinate
Δ	=	delta
DH	=	dehydrase
DHA	=	docosahexanoic acid
EDTA	=	ethylenediaminetetra-acetic acid
EMS	=	ethyl methanesulfonate
EPA	=	eicosapentoenoic acid
ER*	=	enoyl reductase
ER**	=	endoplasmic reticulum
FAD	=	fatty acid desaturase
FAS	=	fatty acid synthase
g	=	gram
GLA	=	γ-linolenic acid
h	=	hour
KCS	=	ketoacyl-CoA synthase
LA	=	linoleic acid
mg	=	milligram
min	=	minute
ml	=	millilitre
mm	=	milimeter
M	=	molar
mM	=	milimolor
mono-UFAs	=	monounsaturated fatty acids
MOX-promoter	=	methanol oxidase-promoter
MT	=	malonyl transferase
NADH	=	nicotinamide-adenine dinucleotide reduced form
NADPH	=	nicotinamide-adenine dinucleotide phosphate reduced form
PCR	=	polymerase chain reaction
poly-UFAs	=	polyunsaturated fatty acids
PT	=	palmitoyl transferase
rpm	=	round per minute
SFAs	=	saturated fatty acids
μg	=	microgram
μl	=	microlitre
UV	=	ultraviolet
VLCFA	=	very-long-chain fatty acid
WT	=	wild-type

CHAPTER I

INTRODUCTION

1.1 Background

Fatty acids are known to be vital compounds for cellular organisms, produced by fatty acid synthesis such as elongation process. For the pathway of fatty acid synthesis, elongase is the key enzyme and plays an essential role in lipid metabolism. Methylotrophic yeast *H. polymorpha* is becoming an attractive model for the study of genes and regulatory mechanisms controlling elongation of fatty acids, and to use the obtained information of creation of the more advanced yeast species *H. polymorpha* (Hansen and Hollerberg, 1996; Anamnart *et al*., 1998; Gellissen, 2002). In *H. polymorpha*, a precise biochemical pathway of fatty acid elongation is still lacking. To understand how fatty acids are synthesized, what the key reactions of the pathway are, how fatty acid biosynthesis is regulated, and what roles the elongation play in *H. polymorpha* cells, the study of isolating and characterizing saturated fatty acid elongation defective mutants of *H. polymorpha* was carried out.

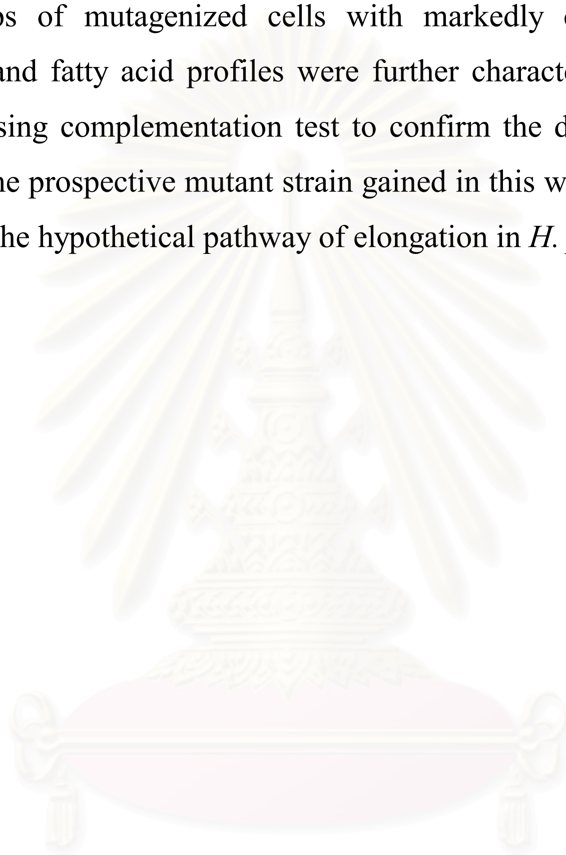
1.2 Objective

To isolate and characterize saturated fatty acid elongation defective mutants of *H. polymorpha* by direct analysis of their growth behavior and fatty acid composition.

1.3 Scopes

The *H. polymorpha* mutants obtained in this work will be used for understanding fatty acid elongation mechanism and regulation on biological processes. Fatty acid elongation defective mutants were isolated from the ethyl methanesulfonate (EMS) treated *H. polymorpha* based on the growth ability. The specific saturated fatty acid supplementation was employed for selection

of mutants and fatty acid composition analysis was used for studying the fatty acid profiles. Using C14:0, C16:0 and C18:0 supplemented medium, we hope to obtain mutants with defects at various steps of the elongation pathway. When determined the growth behavior and fatty acid compositions, we are able to classify the mutagenized cells into three groups according to the changes in their fatty acid profiles compared to that of parental strain. Mutants from the three groups of mutagenized cells with markedly change in the growth phenotype and fatty acid profiles were further characterized for their genetic lesion by using complementation test to confirm the defect in the elongation pathway. The prospective mutant strain gained in this work will be used for the unraveling the hypothetical pathway of elongation in *H. polymorpha*.



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CHAPTER II

LITERATURE REVIEW

2.1 Importance of *Hansenula polymorpha*

Hansenula polymorpha, a thermotolerant methylotrophic yeast, has extremely high biotechnological potential. It would be one of appropriate genetic and gene-engineering models for studying various unique metabolic pathways and cellular processes such as peroxisome biogenesis, methanol metabolism, fatty acid metabolism and for producing several useful bioproducts (Hansen and Hollerberg, 1996; Anamnart *et al*., 1998; Gellissen, 2002). Moreover its biotechnological potential was based on its executive methanol oxidase-promoter (MOX-promoter) and extensive fermentation processes (Escalante *et al*., 1990; de Roubin *et al*., 1991).

The thermotolerance of this yeast is a favorable characteristic for industrial application, especially in tropical countries. *H. polymorpha* in particular has become a preferred organism for the production of various proteins of scientific or commercial interest. The ability of these organisms to grow to high cell densities on relatively inexpensive carbon sources as well as the availability of strong, tightly regulated promotors renders them favorable cell factories. Recent year, advanced molecular genetic tools have become available for *H. polymorpha*, including an efficient electro-transformation procedure (Faber *et al*., 1994), efficient methods for homologous integration into genomic sequences (Faber *et al*., 1992; Sohn *et al*., 1996), as well as a method for the rapid selection of integrants with various copy numbers (Agaphonov *et al*., 1999).

Therefore, *H. polymorpha* has been successfully exploited as a cell factory for the large-scale production of such components. Stable, engineered strains can be obtained by site-directed integration of expression cassettes into the genome, for which various constitutive and inducible promoters are

available to control the expression of the foreign genes. In addition, the facultatively methylotrophic yeast *H. polymorpha* has become a preferred organisms for the production of recombinant proteins on an industrial scale (Gellissen, 2000; Gellissen, 2002). Product examples range from pharmaceuticals such as hepatitis B vaccines (Janowicz *et al*., 1991; Schaefer *et al*., 2001; Schaefer *et al*, 2002) to industrial enzymes like the feed additive phytase (Mayer *et al*., 1999; Papendieck *et al*., 2002). Despite its favorable characteristics, it is desirable to supplement a key system like this by microbial alternatives which could provide advantages for certain product and process developments. In these instances it would be useful to assess several selected organisms for criteria like appropriate protein processing and modification or secretion to identify the most suitable host.

For instances, it may be the organism of choice for reliable, large-scale production of heterologous membrane proteins, using inducible intracellular membranes and targeting sequences to specifically insert these proteins stably into these membranes. Furthermore, the use of *H. polymorpha* offers the possibility to accumulate the produced components into specific compartments, namely peroxisomes (van Dijk *et al*, 2000) Moreover, important advantages of methylotrophic systems are easy scale-up from shake-flask culture to high-density fermentor without loss of product yield and their ability to efficiently secrete heterologous proteins, which is a very important aspect for the biotechnological industry (Cregg *et al*., 1993; Mendoza-Vega *et al*., 1994; Romanos *et al*., 1992).

In addition, yeast would be the one of appropriated candidates for studying of fatty acid biosynthesis mechanisms because of their safety and ease of handling. Some non-conventional yeasts such as *H. polymorpha* (Wijeyaratne *et al*., 1986), *Candida* (*Yarrowia*) *lipolytica* (Kates and Paradis, 1973; Pugh and Kates, 1973), *Lipomyces starkeyi*, *Kluyveromyces thermotolerans* and *Pichia angusta* (Anamnart, 1998b) are known to be able to synthesize polyunsaturated fatty acids (poly-UFAs) such as linoleic acid

(18:2) and linolenic acid (18:3). Among these potential producers of poly-UFAs, a thermotolerant methylotrophic yeast *H. polymorpha*, has been repeatedly demonstrated to be a good prospective candidate for the production of several useful bioproducts (Gellissen, 2002). In addition, *H. polymorpha*, unlike *Saccharomyces cerevisiae*, is able to synthesize poly-UFAs in addition to mono-UFA (Anamnart *et al*., 1998). In previous study, it was demonstrated that *H. polymorpha* like higher plants. Taken together, these advantages make *H. polymorpha* a good model for the study of genes and regulatory mechanisms controlling biosynthesis of fatty acids, for deriving some information leading to create poly-UFA producing strains of this yeast or even *S. cerevisiae* in the future.

2.2 The parameters influencing fatty acid production by microorganisms

Fatty acids and unsaturated fatty acids (UFAs) are well known to be vital compounds for cellular organisms. Especially, poly-UFAs such as linoleic acid (18:2), linolenic acid (18:3) and eicosapentoenoic acid (EPA) are considered to be high value substances in various field including neutraceuticals and pharmaceuticals (Stoof *et al*., 1989; Prickett *et al*., 1983). Their natural sources for human supply include vegetables, fish and meat. Yeasts would be obvious further candidates for a microbial producer because of their safety and ease of handling. The microbial fatty acid productions have led to consider not only selecting the microorganisms but also studying the physiological factors influenced on cultivation processes, such as medium volume, gyration speed, pH , aeration rate, irradiance, culture temperature, cell concentration, microbial species, growth phase and the composition of the medium. For instance, to promote the EPA productivity in *Phaeodactylum tricornutum*, culture conditions such as nitrate, ammonia and urea concentrations, pH (Yongmanitchai and Ward, 1991), aeration rate and

irradiance (Sanchez Perez *et al*., 1994), culture temperature (Seto *et al*., 1984) and cell concentration (Cohen *et al*., 1988) have been investigated.

2.2.1 Growth phase

One of the most important parameters to control fatty acid production is the growth phase. Several changes in microbial lipid composition have been investigated during the growth phase of cells (Arneberg *et al*., 1992; Jostensen and Landfald, 1996). The variation of fatty acid compositions and the level of unsaturation of lipid was also influenced by the phase of growth cycle. In general, lipid accumulation occurs at the end of growth phase when depletion of nitrogen. For instance, high amounts of 16:1 and 18:1 fatty acids were found in exponentially growing cells of *Yersinia pestis*, while the proportion of cyclopropanoic acid increased corresponding to a decrease in the amount of olefinic acids in older cultures (Decallonne *et al*., 1991; Kaneda, 1991) In *Crytococus albido* (Hansson and Dostalek, 1986), its lipid content of cell culture during the exponential growth was low and a degree of unsaturation was high. In contrast, the culture entering the stationary phase contained high levels of lipid with a low degree of unsaturation (Hansson *et al*., 1989).

2.2.2 Growth temperature

Another crucial parameter is the growth temperature, which regulates the fatty acid composition and acts directly on the physical state and fluidity of the microbial membrane (Jantzen and Lassen, 1980; Nozawa and Kasai, 1978; Wijeyaratne *et al*., 1986). Generally, a number of alterations in cellular fatty acid components are induced when cell grown at changing temperature, including the extension of fatty acids unsaturation and alteration in proportion of lipid classes including alteration the ratio of lipid: protein and the modification of membrane lipid composition (Murata and Los, 1997; Vigh *et al*., 1998). The most pronounced effect of temperature on microbial lipid is

the relative increase of unsaturation of fatty acids of microorganisms cultivated under lower temperature conditions (Logue *et al*., 2000). This adaptation also enhances the expression of genes for desaturation (Vigh *et al*., 1998; Miquel *et al*., 1993) resulting from an increase of oxygen solubility (Chavant *et al*., 1981).

Moreover, an increase in temperature results in a higher proportion of saturated long-chain and cyclopropane fatty acids incorporated into the lipid membrane with a subsequent decrease in the proportions of unsaturated branched-chain and/or saturated short-chain fatty acids (Cronan and Vagelos, 1972; Toubiana and Asselineau, 1962). Bacteria change their fatty acid composition to maintain a degree of fluidity in their lipid membrane compatible with cellular growth and function (Moss, 1990). The thermotolerant yeast, *H. polymorpha* CK-1, was suitable for studying the lipid compositions of cell grown at different temperatures because of its wide growth temperature rage (15 to 50 ^{o}C). Cells of this yeast grown at the optimum temperature of 40 ^{o}C contained very high proportions of oleic (18:1), linoleic (18:2) and linolenic (18:3) acids, although psychrophilic yeasts are generally rich in these unsaturated fatty acids. There was a sharp decrease in the proportion of 18:3 acid with an increase in growth temperature from 20 to 50 ^{o}C, whereas large proportions of 18:1 and 18:2 acids were found in cells grown even at 40 and 50 ^{o}C (Kates and Baxter, 1962; Wijeyaratne *et al*., 1986; Watson *et al*., 1976).

2.2.3 Composition of the media

Nitrogen and carbon sources of culture media have a maked effect on growth and lipid production (Farag *et al*., 1983; Chesters and Peberdy, 1965). Individual organism has a capability to use a broad variety of carbon compounds. Several studies have been done in the culture media rich in carbohydrates such as glucose, sucrose, maltose, galactose and xylitol. Glucose, which is frequency used as a carbon source, supports the growth and

promotes the accumulation of high lipid content in several micro-organisms. The previous studies found that the maximum of lipid contents achieved when the carbon concentration in the medium is high and nitrogen level is relatively very low (Chesters and Peberdy, 1965; Hansson and Dostalek, 1988). Occasionally, the presence of mineral ions in the culture medium could affect cell growth, enzyme activity and lipid production i.e. Mg^{2+} or Mn^{2+} (Jernejc *et al*., 1989). In contrast, the presence of Zn^{2+} -ions was observed to inhibit lipid synthesis (Jambuhulkar and Shankhapal, 1992).

2.3 Biochemistry of fatty acid biosynthesis

2.3.1 Fatty acid biosynthesis

The general structure of a fatty acid is hydrocarbon chain with a carboxyl group at one end and a methyl group at the other. The most abundant fatty acids have straight-chains carbon acids usually with an even number of carbon atoms that may contain isolated-*cis* double bonds. Fatty acids without double bonds are called saturated fatty acids (SFAs). Those containing one double bonds are referred to as mono-UFAs and fatty acids with two or more double bonds is called a poly-UFAs. The generalized formula of saturated fatty acid and unsaturated fatty acid are illustrated in Table 2.1.

Table 2.1 Generalized formula of fatty acid (Hanekamp, 2003)

Fatty acids	Generalized formula	Example	Formula
Saturated	$CH_3(CH_2)_nCOOH$	butyric acid	4:0
Unsaturated (oil)	$CH_3(CH_2)_n(CH{=}CH)_mCOOH$	oleic acid	18:1Δ9
		linoleic acid	18:2Δ9,12

Examples for saturated fatty acids are myristic acid, palmitic acid and steric acid. The most common mono-UFA is oleic acid. poly-UFAs include linoleic acid, α- and γ-linolenic acids (ALA, GLA), arachidonic acid

(ARA), eicosapentanoic acid (EPA) and docosahexanoic acid (DHA). Fatty acids are characterized by the number of carbon atoms n and the number of double bonds m as (n:m) as shown in Fig. 2.1. Moreover, The number after the delta (Δ) specifies the position of double bond.

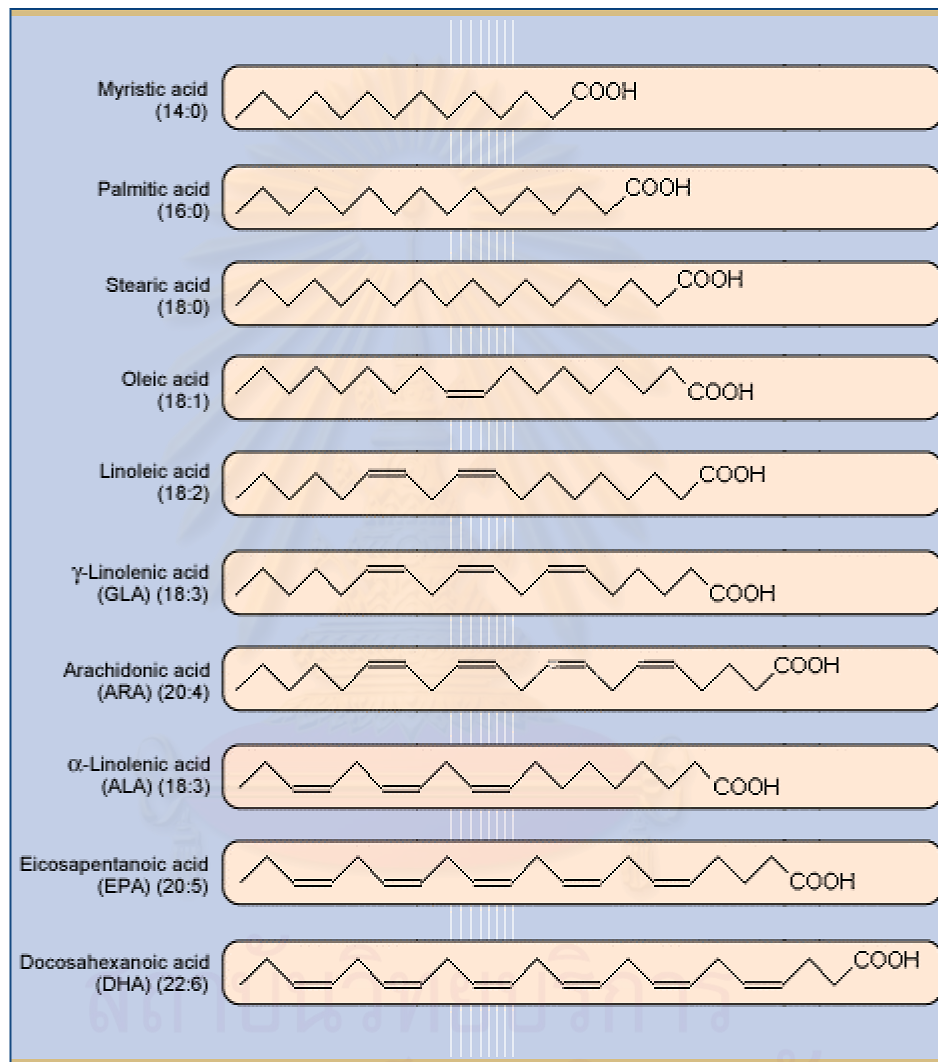

Fig. 2.1 Examples for saturated fatty acids and unsaturated fatty acids found in the diet and the body in eukaryotes (Bruckdorfer, 1997)

Unsaturated fatty acids are important components of cellular structure and function, being involved in roles ranging from membrane fluidity to acting as signal molecules (Gill and Valivety, 1997; Broun *et al*., 1999). In particular, the class of fatty acids known as the poly-UFAs has attracted considerable interest as structural component of membrane glycerolipids, as precursors of families of signaling molecules including prostaglandins, thromboxanes and leukotrienes in animal (Smith, 1986; Weller and Dvorak, 1994) and as pharmaceutical and nutraceutical compounds (Broun *et al*., 1999; Horrobin, 1990). The biosynthesis of fatty acids appears to be almost identical in all organisms. Fatty acid biosynthesis occurs similarly to beta-oxidation-acetyl groups are added to a growing chain (Hanekamp, 2003). One might predict that the pathway for the systhesis of fatty acids would be the reversal of the oxidation pathway. However, this would not allow distinct regulation of the two pathways to occur even given the fact that the pathways are separated within reverse of degradation. These are several key differences among them as show in Table 2.2.

Table 2.2 Fatty acid biosynthesis (Hanekamp, 2003)

	Synthesis	Beta-Oxidation
1.	In cytosol (chloroplasts of plants)	In mitochondria
2.	Uses NADPH as reductant	Uses NAD^+ and FAD as oxidants
3.	ATP is used to carboxylate acetyl-CoA to malonyl-CoA, which is used as an intermediate	Malonyl-CoA is not an intermediate
4.	Attached to ACP (acyl carrier peptide) during reaction cycles	Attached to CoA during reaction cycles
5.	D-β-hydroxybutyryl is intermediate in reduction	L-β-hydroxybutyryl is intermediate in oxidation

The biosynthesis of saturated fatty acids is catalyzed by acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). Fatty acid biosynthesis from acetyl-CoA to palmitate is carried out by fatty acid synthase. The active enzyme is a dimer of identical subunits. All of the reactions of the fatty acid synthesis are carried out by the multiple enzymatic activities of FAS as shown in Table 2.3.

Table 2.3 The various enzymatic activities of the fatty acid synthase complex in eukaryotes (Matthews *et al.*, 2000)

Step	Enzyme	Enzyme-catalyzed reaction
1	Acetyl-CoA-ACP Transacylase	Acetyl-CoA+ACP<=>Acetyl-ACP+ CoASH
2	Malonyl-CoA-ACP Transacylase	Malonyl-CoA+ACP<=>Malonyl-ACP +CoASH
3	β-Ketoacyl-ACP Synthase	Acetyl-ACP+Malonyl-ACP<=>β-Ketoacyl-ACP+ACP+CO2
4	β-ketoacyl-ACP reductase	β-Ketoacyl-ACP+NADPH+H$^+$<=> D-3-Hydroxyacyl-ACP+NADP$^+$
5	3-Hydroxylacyl-ACP Dehydrogenase	D-3-Hydroxyacyl-ACP<=>*Trans*-Δ2-enoyl-ACP+H2O
6	Enoyl-ACP Reductase	*Trans*-Δ2-enoyl-ACP+NADPH+H$^+$ <=>Acyl-ACP+NADP$^+$

Starting with acetyl-CoA, the process cycles between steps 1-6 seven times to yield palmitoyl-ACP which is hydrolyzed to give palmitate and ACP. Note that the CO_2 which was added to acetyl-CoA in the acetyl-CoA carboxylase-catalyzed step, is removed subsequently and not incorporated in to the final product (Fig. 2.2).

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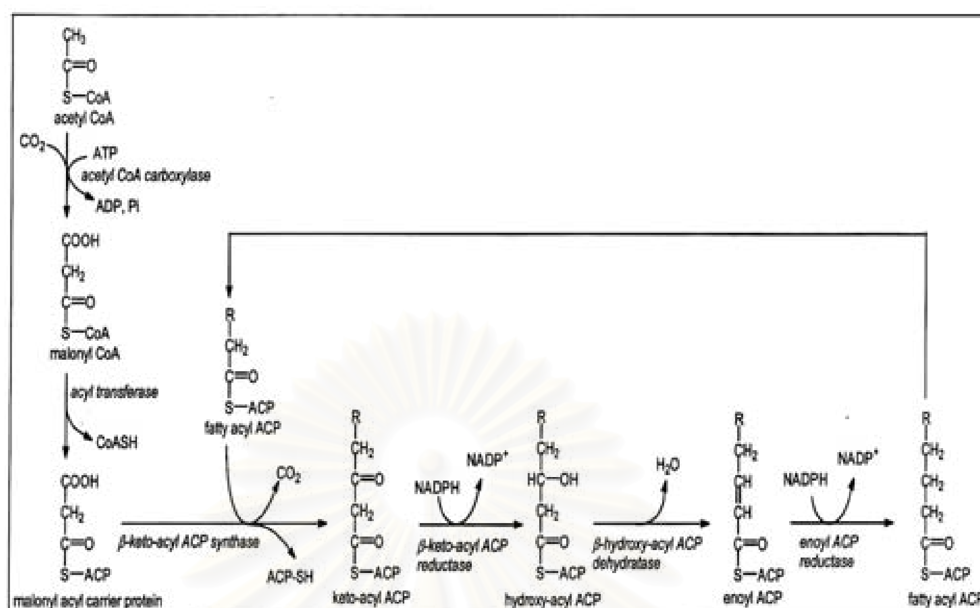


Fig. 2.2 The overall reaction for synthesis of palmitate, starting with malonyl-ACP and acetyl-ACP (Matthews *et al.*, 2000).

The first step in the synthesis of SFA is the conversion of acetyl-CoA to malonyl-CoA. In bacteria such as *E. coli*, the acetyl-CoA carboxylase in a biotin free molecule requires the presence of carrier protein with biotin, biotin carboxylase and transcarboxylase (Ohlrogge and Browse, 1995; Li and Cronan, 1992; Volpe and Vagelos, 1976). In eukaryotes such as animal and yeast, acetyl-CoA carboxylases are themselves biotin-containing multienzyme complexes. Studies of the enzymes from birds and mammals indicate that it is a heterotetrameric molecule consisting of two subunits, each subunit contains two identical polypeptide chains. Each chain has molecular weight of 129 kDa and 117 kDa and the last one is associated with biotin (Hardie *et al.*, 1981; Beaty and Lane, 1982). Acetyl-CoA carboxylase from yeast, *S. cerevisiae* (Sumper and Riepertinger, 1972) and *C. lipolytica* (Mishina *et al.*, 1976) have a homotetrameric structure with molecular weight of 190 kDa and 230 kDa, respectively, and contain one mol of covelently

bound biotin per subunit. Sequence analysis and comparison of the deduced amino acid sequences to protein databases unveiled 68 % similarity of a 374-amino acids peptide fragment to published C termini of chicken and rat acetyl-CoA carboxylases and almost 100% identity to the product of the FAS3 gene from yeast (Hasslacher *et al*., 1993). Comparison of the deduced amino acid sequences with those of chicken and yeast acetyl CoA carboxylases confirmed that both types encoded acetyl CoA carboxylase, corresponding to the c-terminal half of the enzyme. The overall identity of the maize and chicken sequences was 37% (58% similarity) but for some shorter regions was much higher (Ashton *et al*., 1994).

In some systems, the activities are present on separate enzyme units. In enzyme complex can exist as both a monomer and dimer. The individual monomers are generally inactive. This reaction requires ATP and NADPH (Fig. 2.2). Structural organization of FAS protein varies in different organisms (McCarthy and Hardie, 1983). In bacteria such as *E. coli* there are seven separate enzymes plus an ACP (Volpe and Vagelos, 1976). Plant also have individual proteins for the various activities which are associated in a quaternary complex (Shimakata and Stumpf, 1982a, b). The plant fatty acid synthase machinery is similar to prokaryotes in that the enzymatic components are separable polypeptides rather than large multifunctional polypeptides as found in animals and fungi (Schultz *et al*., 2000; Voelker and Kinney, 2001).

The FAS complex of animals and yeast is called type I FAS. The active form of the animal FAS is homodimer. Each subunit of the enzyme contains all catalytic sites required for palmitate synthesis. Unlike the animal FAS, the yeast FAS synthesizes palmitoyl-CoA instead of free palmitate. The fatty acid product released from FAS is palmitate (via the action of palmitoyl thioesterase) which is a 16:0 fatty acid, i.e. 16 carbons and no sites of unsaturation. Whereas palmitate synthesis occurs in the cytosol, elongation and unsaturation of fatty acids occurs in both the mitochondria and endoplasmic reticulum (ER) (microsomal membranes). The ER is the

dominant system. Elongation in the ER differs from cytosolic synthesis in employing coenzyme A (instead of acyl carrier protein) and separate enzymes (instead of a complex). Elongation involves condensation of acyl-CoA groups with malonyl-CoA. The resultant product is two carbons longer (CO_2 is released from malonyl-CoA as in the FAS reaction) which undergoes reduction, dehydration and reduction yielding a saturated fatty acid. Mitochondrial elongation using elongase in the mitochondria involves a mechanism that is essentially the reverse of beta-oxidation except substitution of NADPH for FADH2 in the last reaction (Fig. 2.3).

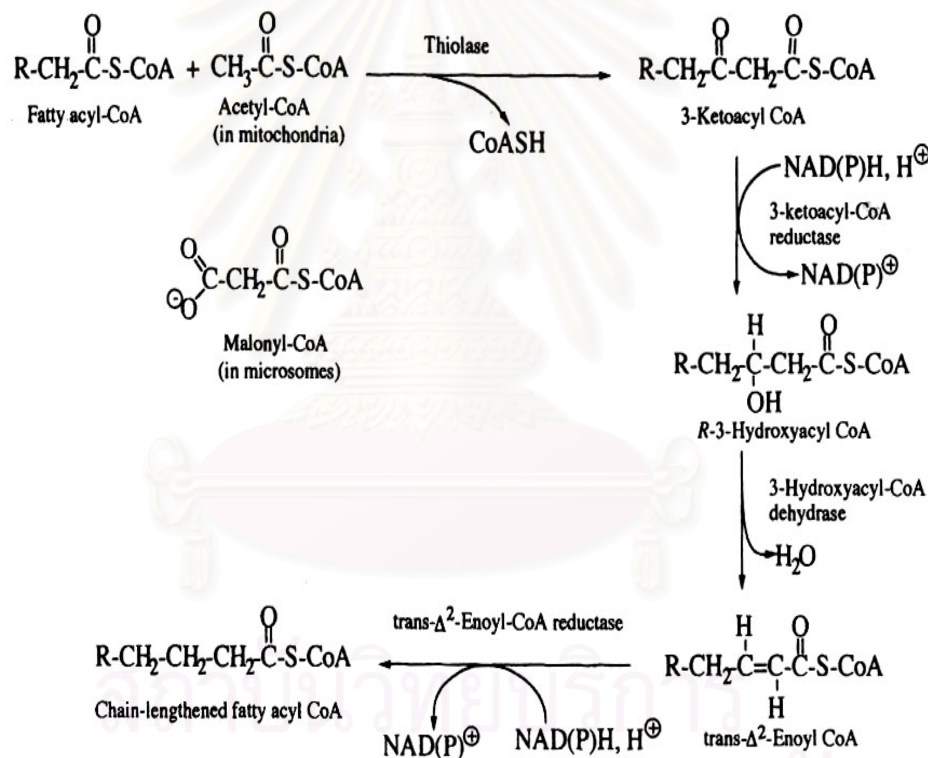


Fig. 2.3 Steps involved in elongation of fatty acid chains in eukaryotes (Rawn, 1989)

The bulk of cellular fatty acids originates from *de novo* biosynthesis, which is catalyzed by the fatty acid synthase (FAS) multienzyme system. The fatty acids thus produced are of 16-18 carbon atoms in length and thereby conform to the physiological requirements of biological membranes. Shorter-chain fatty acids are found in certain plant seeds (Slabas and Fawcett, 1992; Töpfer *et al*., 1995) and in the neutral lipids of specific animal glands (Libertini and Smith, 1978; Rogers *et al*.,1982).

2.3.2 Post-fatty acid biosynthesis of elongation

In eukaryotes, the production of very-long-chain saturated (C20-C26) and poly-UFAs takes place independently of FAS by membrane-bound elongases on the cytosolic face of the ER membrane. Fatty acid elongation has been investigated intensively in *S. cerevisiae*, although most eukaryotes are belived to have quite similar elongation enzyme systems, existing probably as multimeric complexes (Cinti *et al*., 1992; Dittrich *et al*., 1998). Very-long-chain fatty acids (VLCFAs) of up to 26 carbon atoms are used as precursors in the synthesis of sphingolipids (O' Brien *et al*., 1964; Smith and Lester, 1974), essential for lipid raft formation (Welch and Burlingame, 1973; Bagnat and Simons, 2002) and glycosyl phosphatidyl-inosital-anchor lipid fractions of eukaryotic cell membranes (Conzelmann *et al*., 1992).

In yeast, products of elongation are involved in the formation and trafficking of the secretory lipid vesicles (David *et al*., 1998). In mammals, the 20-carbon poly-UFA, arachidonic acid, is an intermediate of eicosanoid biosynthetic pathway. Very-long-chain fatty acids (> 18 carbons) are found in the glucocerebrosides of plant plasma membranes (Cahoon and Lynch, 1991) and as precursors of plant epicuticular waxes (Post-Beittenmiller, 1996). In all eukaryotic system, the production of VLCFA by elongation of the medium-chain or long-chain fatty acids takes place independently of *de novo* fatty acid synthesis (Welch and Burlingame, 1973;

James *et al*., 1995). The enzyme required for this process, long-chain fatty acid elongase, is an FAS-independent multienzyme system. Its activity has been identified and biochemically investigated in a variety of organisms such as fungi (Bessoule *et al*., 1988), plants (Bessoule *et al*., 1989; James *et al*., 1995) and animals (Anderson and Kolattukudy, 1985). Studies on acyl elongation in plants and animals indicate that, in general, the properties of acyl elongation in plants and animals are quit similar (Cinti *et al*., 1992; Cassagne *et al*., 1994). Both plant and animal elongases require malonyl-CoA and NAD(P)H and use a variety of primers with varied requirements for ATP. For animal mitochondria, though, elongation with acetyl-CoA or malonyl-CoA was reported (Bloch and Vancem, 1977; Bessoule *et al*., 1989). Malonyl-CoA-dependent fatty acid elongation is generally believed to be associated with the microsomal membrane fraction (Bessoule *et al*., 1989).

In principle, fatty acid elongation systems utilize similar mechanisms as fatty acid synthase to make C18 fatty acids. The main difference is the location (smooth ER in animals) and the fact that it takes place on acyl-CoA, rather than acyl-ACP. Malonyl-CoA is still used as the acetyl doner, followed by reduction, dehydration and reduction by NADPH. Hence, they should contain a minimum of four constituent enzyme; i.e. β-ketoacyl synthase, β-ketoacyl reductase, dehydratase and enoyl reductase in the most simple case of CoA rather than acyl-carrier-protein-bound phosphopantetheine, acting as a carrier of the reaction intermediates (Bessoule *et al*., 1989).

2.3.3 Post-fatty acid biosynthesis of desaturation

Fatty acid desaturation occurs in the ER membranes as well and in mammalian cells involves 4 broad specificity fatty acyl-CoA desaturases (non-heme iron containing enzymes). These enzymes introduce unsaturation at C4, C5, C6 or C9. The electrons transferred from the oxidized fatty acids during desaturation are transferred from the desaturases to cytochrome b5 and

then NADH-cytochrome b5 reductase. These electrons are uncoupled from mitochondrial oxidative-phosphorylation and therefore, do not yield ATP. Terminal desaturases produce unsaturated fatty acids. One such enzyme is fatty acyl-CoA desaturase. The reaction catalyzed by this enzyme on a stearoyl-CoA is shown in Fig. 2.4.

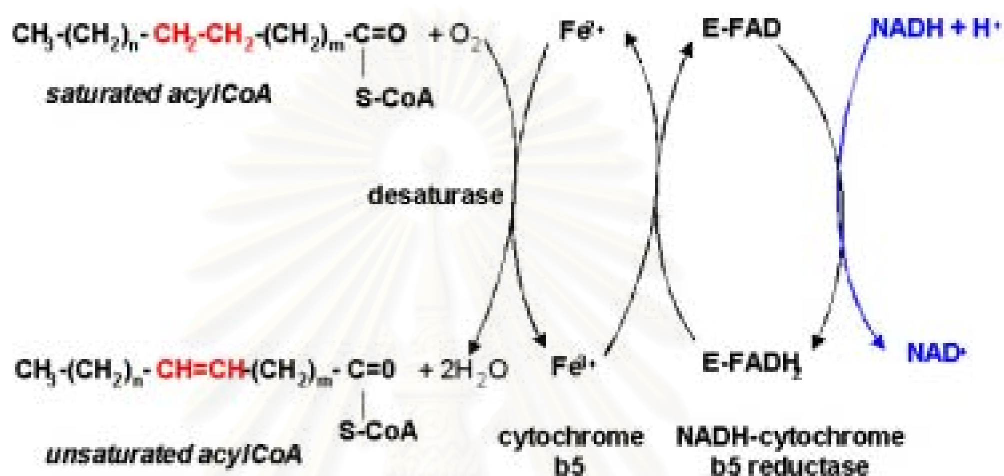
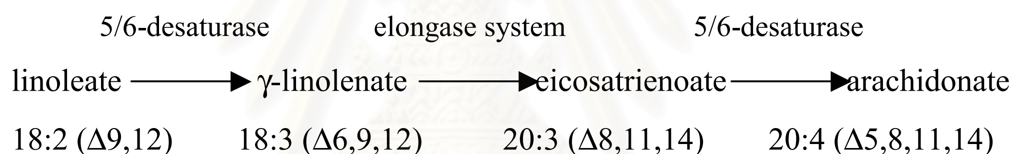


Fig. 2.4 The delta-9 fatty acyl-CoA desaturase complex (Bruckdorfer, 1997)

Note the unusual electron transferring pathway in which electrons from NADH are ultimately passed to oxygen, forming water. The energy released in this process drives oxidation of stearoyl-CoA to oleyl-CoA. From the free methyl end, mammals cannot make double bonds closer to the end than the delta-9 position (oleic acid is a delta-9 fatty acid). Thus, linoleic acid (delta-9,12 double bonds) and linolenic acid (delta-9,12,15 double bonds) must be provided in the diet of mammals and are called essential fatty acids. Linoleic is especially important in that it required for the synthesis of arachidonic acid. Arachidonate is a precursor for the eicosanoids (the prostaglandins and thromboxanes). This is a crucial role of fatty acids in eicosanoid synthesis involving in the proper growth, wound healing and dermatitis in human. Also, linoleic acid is a constituent of epidermal cell

sphingolipids that function as the skins water permeability barrier. Plants and animals differ in where double bonds are introduced into fatty acids. Plant add in delta-9 (a 9-10 double bond) and then can add in double bonds at three carbon intervals towards the tail (delta-9,12, delta-15). They can also add delta-6, but not common. Animals also start with delta-9 then can add at three carbon intervals toward carboxy end (delta-6 and delta-6). Animals cannot add towards the tail. Therefore animals cannot make linoleoyl-CoA (C18:2, delta-9,12) but can make oleoyl-CoA (C18:1, delta-9). Animals must therefore take in plant products (either directly as herbivores or indirectly by eating herbivores) to acquire essential unsaturated fatty acids such as linoleic and arachidonic acids (Tinoco, 1982; Holman, 1986). When animals consume linoleate, their own enzymes can convert it to arachidonate in stepwise manner.



Biosynthesis of oleoyl-CoA is the only reaction of desaturation of C18 fatty acids in animals including human and some yeasts, *S. cerevisiae* and *Schizosaccharomyces pombe.* Unlike animals, autotrophic micro-organisms and plants belonging to poly-UFA synthetics, which have abilities to synthesize and desaturase long-chain UFAs from short carbon molecules, are determined by their enzymatic structure (Fig. 2.5).

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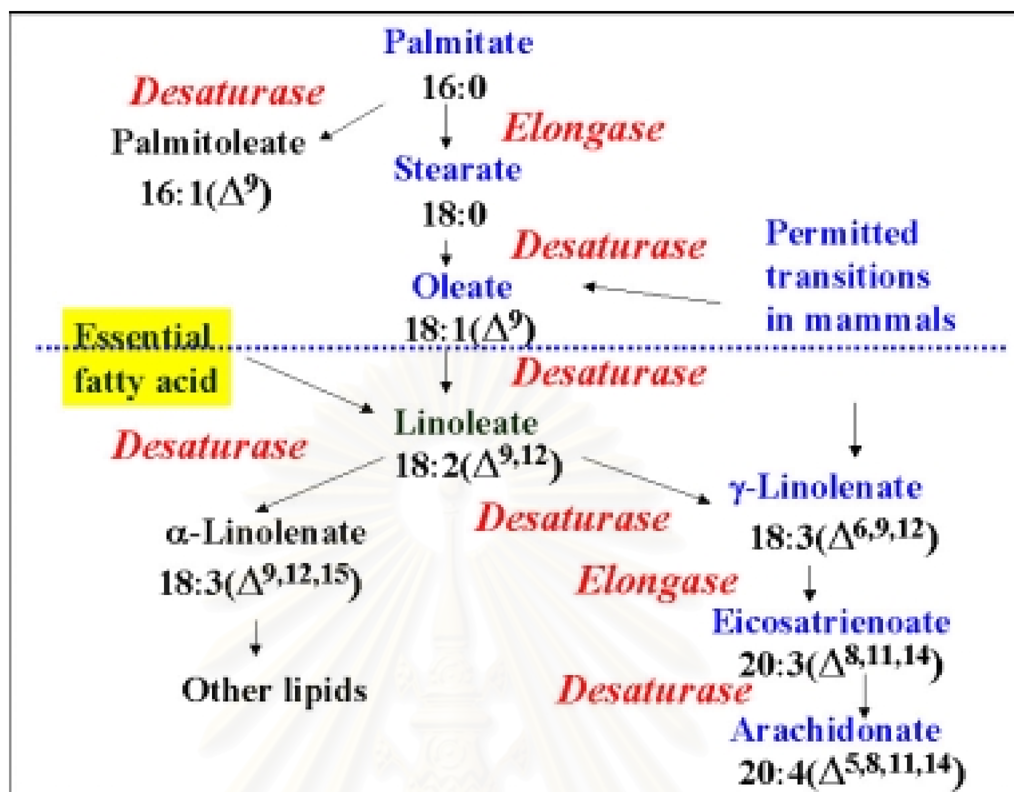

Fig. 2.5 Biosynthesis of polyunsaturated fatty acids in plants (Eharris, 2004)

Oleic acid (18:1, Δ9) is converted to linoleic acid (18:2, Δ9,12), α-linolenic acid (18:3, Δ9,12,15) and/or γ-linolenic acid (18:3, Δ6,9,12) by sequential desaturation reactions catalyzed by Δ6-, Δ9- and Δ12-desaturase, respectively (Fig. 2.5). Certainly, desaturases with different substrate utilization are position specificity, which play essential roles in the multicomponent enzymatic complex designating for UFAs biosynthesis.

The polyunsaturated fatty acids (PUFAs) can be classified into two groups, *n*-6 or *n*-3 depending on the position of the double bond nearest the methyl end of the fatty acid, where *n* is the chain-length of the fatty acid (Gill and Valivety, 1997; Broun *et al*., 1999; Napier *et al*., 1999). Thus, γ-linolenic acid (18:3, Δ6,9,12) is classified as an 18:3, *n*-6 PUFA whereas α-linolenic acid (18:3, Δ9,12,15) is an 18:3, *n*-3 PUFA. In animal tissue,

additional double bonds can only be inserted between an existing double bond and the carboxyl group. The linoleic acid, which is the primary precursor molecule for the (*n*-6) family of fatty acids, must comes from the diet. Biosynthesis of polyunsaturated fatty acids requires a sequence of chain elongation and desaturation steps, as illustrated in Fig. 2.6 and the various enzymes require the acyl-Coenzyme A esters as substrates not intact lipids (unlike plants). The liver is the main organ involved in the process.

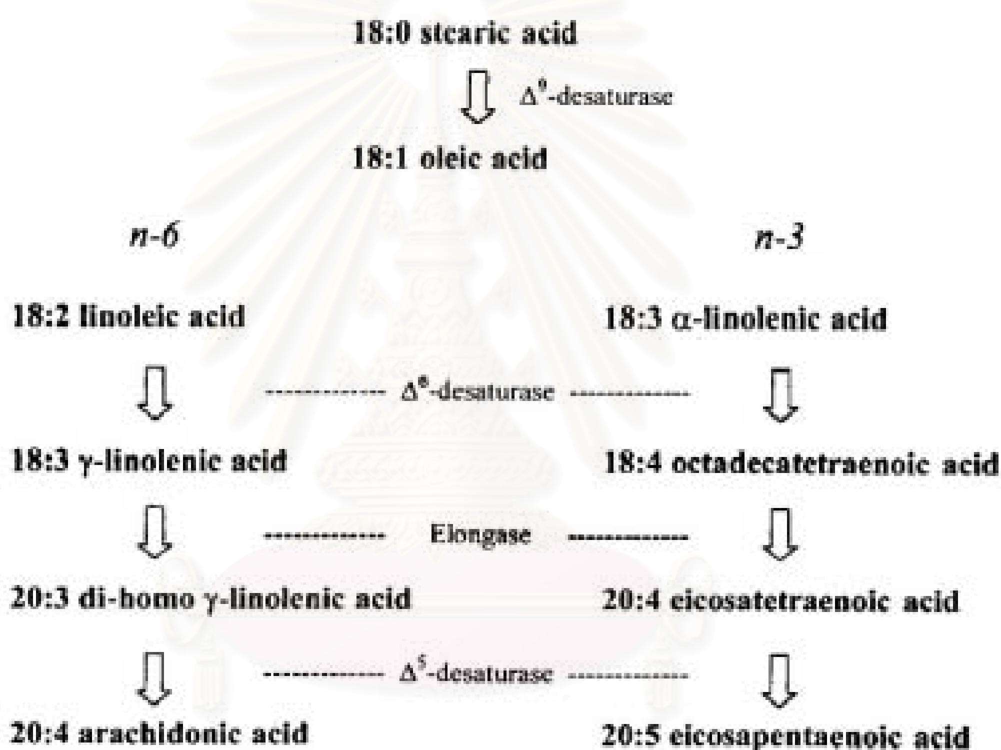

Fig. 2.6 Scheme representing the biosynthesis of PUFAs in animals. Primary *n-6* and *n-3* fatty acids (in the form of linoleic and α-linolenic acid) must be provided from dietary intake because of an inability to desaturate oleic acid further. The enzyme activities required for this pathway are shown (Beaudoin *et al*., 2002).

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C20 fatty acids such as 20:4, *n*-6 are synthesized by sequential desaturation, elongation and further desaturation of dietary 18:2, *n*-6 (Broun *et al*., 1999; Napier *et al*., 1999). The α-linolenic acid, which is the primary precursor molecule for the (*n*-3) family of fatty acids in animal tissues, must comes from the diet. The main pathway to the formation of eicosapentaenoic acid (20:5, *n*-3) requires a sequence of chain elongation and desaturation steps (Δ5 and Δ6 desaturases) with acyl-Coenzyme A esters as substrate as illustrated in Fig. 2.6.

In general, fatty acid desaturases, according to their substrate specificity, can be devided into three types: acyl-CoA desaturases, acyl-acyl carrier protein (ACP) desaturases and acyl-lipid desaturases. Acyl-CoA desaturases introduce double bonds into fatty acids, which bound to coenzyme A. These enzymes are bound to the endoplasmic reticulum in animal, yeast and fungal cells (Holloway, 1983). Acyl-ACP desaturases introduce double bonds into fatty acids that are bound to ACP, they are present in the stoma of plant plastids. The first desaturation step for fatty acids is catalyzed by a plastidial stearoyl-acyl carrier protein (ACP) desaturase (McKeon and Stumpf, 1982). Acyl-lipid desaturases interact with the esterified fatty acids of glycerolipids (Wada *et al*., 1993). The acyl-lipid desaturases are bound to the endoplasmic reticulum and chloroplast membrane in plant cells (Jaworski, 1987) but to the thylakoid membrane in cyanobacteria cells (Wada *et al*., 1993).

Higher plants biosynthesize many different UFAs, but in membrane lipids the major locations for double bonds are at the Δ9, Δ12 and Δ15 positions of 18-carbon acyl chains and the corresponding Δ7, Δ10 and Δ13 positions of 16-carbon acyl chains (Browse and Somerville, 1991). Interestingly, the plant membrane-bound desaturases use complex glycero-lipids rather than acyl-CoAs as substrates. In vertebrates, the pathway from 18:2 (*n*-6) to arachidonic acid and from 18:3 (*n*-3) to eicosapentaenoic acid involves desaturations at the Δ6 and Δ5 positions in the carbon backbone and

an intermediate 2-carbon chain elongation step (Sprecher, 1981). Considerable variation between animal species in their abilities to synthesize the C20 and C22 PUFAs from the plant-derived C18 precursors 18:2 (*n*-6) and 18:3 (*n*-3). Some animals, notably extreme carnivores, have a very limited ability to synthesize C20 and C22 PUFAs and consequently have a strict requirment for a dietary source of preformed C20 and C22 PUFAs (Sinclair *et al*., 1979).

2.4 Genetic control of fatty acid biosynthesis

2.4.1 Genetic control of saturated fatty acid biosynthesis

The biosynthesis of saturated fatty acids is catalyzed by acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). In eukaryote such as yeast and lower fungi, the FAS in the cytosol (so called type I FAS) is a heteromultimeric complex of two multifunctional protein ($\alpha_6\beta_6$), which are encoded by two unliked genes, *FAS1* (subunit β) and *FAS2* (subunit α) (Schweizer *et al*., 1978; Henry and Fogel, 1971). The α-subunit contains three catalytic activities, β-ketoacyl synthase, β-ketoacyl reductase and acyl carrier protein while the β subunit contains five catalytic functions, i.e., acetyl transferase (AT), enoyl reductase (ER), dehydrase (DH) and malonyl/ palmitoyl transferase (MT/PT) (Stoops *et al*., 1978; Schweizer *et al*., 1973). The *FAS* genes have been cloned from various organisms. It was shown that the sequential order of the catalytic domains is similar only among related species such as yeast and lower fungi. *ACC1/FAS3* codes for acetyl-CoA carboxylase (ACC) and it was found that sporulation of diploids with a single disruption of the *ACC1* gene produced spores that germinated but failed to enter vegetative growth even when supplemented with fatty acids. This result demonstrated that the ACC is an enzyme essential for functions other than fatty acid synthesis (Kajiwara *et al*., 2001; Kottig *et al*., 1991).

Eukaryotic organisms synthesize very long chain fatty acids (fatty acids longer than 18 carbons) using a membrane-bound elongase system. These fatty acids are often found, for example, in sphingolipids. In

plants, they are major components of wax and components of seed oil. The first gene isolated encoding an elongase condensing enzyme was *FAE1* (fatty acid elongation 1) from *Arabidopsis thaliana* (James *et al*., 1995). The enzyme studying, 3-ketoacyl-CoA synthase (KCS), is anchored to membranes by two membrane-spanning domains near the N-terminus. It has a low level of homology to two soluble condensing enzymes such as chalcone synthase and stibene synthase, a condensing enzyme involved in flavonoid biosynthesis. In both cases, the homology is towards the C-terminus, and alignments suggest residues that may be involved with catalysis.

2.4.2 Genetic control of post-fatty acid biosynthesis modifications

2.4.2.1 Fatty acid elongation

Biochemical characterization of the *FAE1* gene from both *Arabidopsis* and jojoba (Lassner *et al*., 1996) supports the conclusion that Fae1p is involved in VLCFA synthesis. Surprisingly, there has no gene in *S. cerevisiae* with significant homology to *FAE1*. On the other hand, the ELO homologs comprise a gene family conserved from yeast to humans that are candidates for a novel class of condensing enzymes. Two lines of experiments identified the *S. cerevisiae ELO2* and *ELO3* genes as being involved in the formation of the very long-chain saturated fatty acid (VLCFA). Based on their homology to *ELO1*, a gene encoding a protein required for medium-chain-length fatty acid elongation (Dittrich *et al*., 1998; Toke and Martin, 1996), the *ELO2* and *ELO3* genes were determined to be required for VLCFA synthesis (Oh *et al*., 1997). To identify a role for the *ELO1* gene in medium-chain-length fatty acid synthesis through the analysis of elongation-defective yeast mutants, ELO1p is involved in elongation of myristate to palmitate. Identification of a role for the *ELO2* and *ELO3* genes in VLCFA synthesis through the analysis of sphingolipid synthesis mutants. Two yeast genes (*ELO2* and *ELO3*) involved in the formation of the VLCFA moiety of sphingolipids have been characterized at the genetic and biochemical levels.

ELO2p appears to be involved in the elongation of saturated or monounsaturated fatty acids up to 24 carbons whereas ELO3p apparently has broader substrate specificity and is essential for the conversion of 24-carbon to 26-carbon saturated fatty acids species (Oh *et al*., 1997).

Using conserved motifs inserted in these *ELO* genes, The potential ORFs encoding enzymes involved in fatty acid elongation in the poly-UFA-accumulating organism *Caenorhabditis elegans,* which has also been the subject of a completed genome sequencing program, were identified (The *C. elegans* Sequencing consortium, 1998). These ORFs were characterized functionally by heterologous expression in yeast allowing the identification of activities involved in poly-UFA elongation. The hypothesis that the specificity of elongation is conferred by the condensing enzymes is further supported by the observation in heterologous expression in yeast. The plant Fae1p condensing enzyme successfully reconstituted a functional C20+mono-UFAs-specific elongase although yeast does not normally elongate mono-UFAs longer than C16 or C18 fatty acids (Millar and Kunst, 1999; Beaudoin *et al*., 2002). Similarly, the effective reconstitution of the poly-UFA-specific elongase (via by heterologous expression of the *C. elegans* Pea1p in yeast) was obtained even though yeast has no endogenous capacity for either of these particular biosynthetic reactions (Beaudoin *et al*., 2002).

The Elo enzymes are believed to display different substrate specificity. It may be that the other elongating enzymes (reductases and dehydratase) are commom components of the endogenous microsomal fatty acyl elongases that function to lengthen all of the fatty acid elongase substrates. In the case of yeast, the C20-C26 saturated fatty acid components of sphingolipids are primarily synthesized by endogenous microsomal elongases having heterologous elongating activities function (Beaudoin *et al*., 2000). Until very recently, little was known about the identity of these other three enzyme activities and the precise contribution (s) to the activity and specificity of microsomal fatty acid elongases. Characterization of the *TSC10*

gene, which is one of a number of genes identified in a screen for temperature-sensitive (ts) mutants with defects in sphingolipid synthesis (Beeler *et al*., 1998), provided evidence that the *TSC13* gene encodes the enoyl reductase component of the microsomal elongase. Furthermore, Tsc13p was shown to coimmunoprecipitate with the presumptive condensing enzymes Elo2p and Elo3p (Kohlwein *et al*., 2001). Interestingly, a precise ER location for Tsc13p, enriched at sites of vacuole-nuclear envelope interaction, was observed. *TSC13* is essential, and is expected for a gene encoding a non-redundant fatty acid elongating activity. In recent studies aimed at identifying other enzymatic components of the fatty acid elongase, the *YBR159w* gene was required for reconstituting heterologous elongase activity in yeast. Analysis of the mutants lacking Ybr159p was demonstrated that these mutant cells showed many phenotypes as previously described in the *elo2Δ, elo3Δ* and *tsc13-1* (elongase-defective) mutant cells. These phenotypes demonstrate that Ybr159p is the major 3-ketoreductase activity required for endogenous VLCFA synthesis in yeast. Taken together, identification of enoyl reductase, Tsc13p and β-ketoreductase, Ybr159p from yeast leaves dehydratase as the only unknown component in the elongation pathway (Kohlwein *et al*., 2001; Han *et al*., 2002)

In *Hansenula polymorpha*, the *HpELO1* gene was reconstructed by recovery of the remaining region using inverse PCR technique. An HpElo1p composes of 320 amino acids and has five presumptive membrane spanning regions. Phylogenetic analysis showed that HpElo1p is equivalent to *S. cerevisiae* ScElo3p elongase involved in sphingolipid biosynthesis. Furthermore, Southern blot analyses of PCR fragments, designated as EL106, revealed the existence of another fatty acid elongase gene. Phylogenetic analysis indicates that *HpELO2* codes for ScElo2p-like protein, which the expression of this gene involved in sphingolipid biosynthesis, designated as *HpELO2*. In *H. polymorpha* IFO0799, gel electrophoresis and Southern analysis revealed that *HpELO1*

and *HpELO2* are located on chromosome IV and VI, respectively. The *HpELO1* gene, disruption was performed by transformation of *H. polymorpha* haploid cell (*leu1-1, ura3-1*) with a DNA fragment of plasmid pEL117, which contained *HpURA3* gene flanked by a part of *HpELO1*. It has been found that *HpELO1* is non-essential gene as in the case of *ScELO3*. Their deduced amino acid sequences suggested that HpElo1p and HpElo2p seem to function like ScElo3p and ScElo2p, respectively, in *S. cerevisiae* (Phatthanon, 2004). Fatty acid elongation and desaturase (s) systems act jointly in generation of long- and very-long-chain unsaturated fatty acids. Starting from C16 and C18, desaturases generate *cis*-monounsaturated palmitoleic acid (C16:1, Δ9) and oleic acid (C18:1, Δ9) (Stukey *et al*., 1990). Both palmitoleic and oleic acid are then used for the synthesis of a variety of other long-chain unsaturated fatty acids (Cinti *et al*., 1992; Dittrich *et al*., 1998).

2.4.2.2 Fatty acid desaturation

Fatty acid desaturation was originally described by Bloomfield and Block (1960) in a study of the yeast Δ9 desaturase enzyme. The UFA requiring *ole1* mutant of *S. cerevisiae*, is in capable of desaturating exogenous saturated fatty acids or converting ^{14}C-labeled acetate into UFAs and is apparently defective in the Δ9 desaturase activity (Resnick and Mortimer, 1966; Esfahani *et al*., 1981). Gene encoding Δ9-fatty acid desaturase (Δ9-FAD) have been cloned from various organisms (Thiede *et al*., 1986; Ntambi *et al*., 1988; Stukey *et al*., 1989; Sakamoto *et al*., 1994; Gargano *et al*., 1995; Meesters and Eggink, 1996; Luo *et al*., 1997; Meesters *et al*., 1997; Itoh *et al*., 1998) including *Pichia angusta* IFO 1475 (=CBS 7003). The gene designated as *P-OLE1* encoding Δ9-fatty acid desaturase of *Pichia angusta* which was isolated by polymerase chain reaction (PCR) and DNA hybridization techniques (Anamnart *et al*., 1997). *S-OLE1*, a gene encoding Δ9-fatty acid desaturase in *S. cerevisiae* was also found to be

essential for cell growth. Cells of the fatty acid desaturase-defective strains (*ole1* mutants) were screened based upon their inability to proliferate on UFA free medium. In recent studies, it was noted that the defect of the Δ9-fatty acid desaturase activity of *S. cerevisiae* affected not only on the unsaturated fatty acid biosynthesis but also the organelle inheritance (Warren and Wickner, 1996). *S-OLE1* gene was cloned by complementation of *ole1* mutation. A putative 510-amino acid polypeptide was encoded by the gene. Only an 257 amino acid internal region of the gene demonstrated identity and similarity, 36 and 60%, respectively, to the rat stearoyl-CoA desaturase protein (Stukey et al., 1989; 1990). The open reading frame of the gene demonstrated high homology with both *S. cerevisiae* and rat desaturases. It was shown that both the native *P-OLE1* gene and the rat stearoyl-CoA desaturase gene complemented the mutation *ole1* for Δ9-FAD in *S. cerevisiae.*

Expression of genes in conjunction with lipid biosynthesis has been shown to be regulated by fatty acids, temperature, precursor of phospholipids and other factors (Chirala, 1992; Chirala et al., 1994). The *S-OLE1* gene is induced by SFAs and repressed by UFAs (Choi et al., 1996) correlating with the data showing the reduction of the *OLE1* mRNA level when cultivated cells supplemented with unsaturated fatty acid (Bossie and Martin, 1989). In recent studies of independent system regulating the *OLE1* mRNA level in correlated with exogenous fatty acids, one represses the *OLE1* transcription and another controls the stability of the *OLE1* mRNA (McDonough *et al*., 1992; Gonzalez and Martin, 1996). The level of mRNA for Δ12-desaturase in cyanobacterium, *Synechocystis* sp. PCC 6803, increases with a decrease in ambient temperature (Los *et al*., 1997). In plants, the mechanism for the regulation of fatty acid biosynthesis is different from that in other organisms because they acquire reduced carbon by the fixation of photosynthetic CO_2, for example, in leaves noting that fatty acid synthesis is induced by light. The biosynthesis of polyunsaturated fatty acids have been reported from various organisms including *H. polymorpha*. However, it has

been shown very recently (Anamnart *et al*., 1998) that *H. polymorpha,* like higher plants. This observation permitted a new approach to elucidating the biosynthetic pathways and membrane functions of poly-UFAs by applying the wide spectrum of genetic, molecular and biochemical techniques to this advanced microbial eukaryotic model.

2.5 Future prospect

In the present study, the substrate specificity, cofactor requirement and kinetic characteristics of yeast, *H. polymorpha* strain CBS 1976 (=NCYC 495) elongase (s) were determined systematically using wild-type and chemically modified yeast strains. We have analyzed the fatty acid composition of *H. polymorpha* wild-type and mutant strains by gas chromatography and found that the difference pattern of distribution of cellular fatty acids. In order to understand the system controlling elongation of fatty acids in *H. polymorpha*, we isolated and analysed ten mutants reguiring fatty acids for growth. All the mutants were able to classify into three physiological groups which possessed the different growth abilities on solid and liquid medium supplemented with various kind of saturated fatty acids. However, analysis of complementation test demonstrated that only the *ELO2* transformants could grow on the selective medium. It was suggested that the *S-ELO2* gene from *S. cerevisiae* (Oh et al., 1997) is able to complement the elongation defective mutant of *H. polymorpha*. Analysis of the cellular fatty acid composition both wild-type and transformant of *H. polymorpha* showed similar pattern, this result demonstrated that the *ELO2* gene of *S. cerevisiae* could gain function in *H. polymorpha* mutant. In this work, screening and classifying of elongation defective mutant from *H. polymorpha* and the gene functional analysis are described.

CHAPTER III

MATERIALS AND METHODS

3.1 Equipments

Agarose gel electrophoresis equipment, Advance Co. Ltd., Japan.

Autoclave, Hirayama, Japan.

Controlled environment incubator shaker: model G25, New Brunswick Scientific Co. Ltd., U.S.A.

Freezer –20 °C, Sanyo, Japan.

Freezer –70 °C, Sanyo, Japan.

Gas chromatography: model Hewlett-Packard HP 6890 Series, U.S.A.

Gas chromatographic column, HP-5 column (30 m by 0.32 mm by 0.25 μm in film thickness), U.S.A.

Gas chromatography: model Shimadzu 15A, Shimadzu Corporation, Japan.

Gas chromatographic column, 3 mm glass column of 15% Diethylene glycol succinate (DEGS) on 60/80 mesh Chromasorb W AW, Japan.

Hot plate stirrer, HS-115, U.S.A.

Incubator, Sanyo, Japan.

Laminar flow, International Scientific Supply Co. Ltd., Thailand.

Microcentrifuge, MC-15A, U.S.A.

Microscope, CHS, Olympus Optical Co. Ltd., Japan.

pH-meter, F-13, Horiba, Japan.

Power supply, BIO-RAD Laboratories, U.S.A.

Refrigerated centrifuge, KR-20000T, Kubota Corporation, Japan.

Spectrophotometer, Spectronic 21, Bausch & Lomb Corporation, U.S.A.

Syringe 5 ml, 10 ml, Nipro, Thailand.

Ultrasonic cleaner: model D200, D.S.C. group Corporation, U.S.A.

UV transiluminator, Macrovue 2011, Bromma, U.S.A.

UV-Visible Recording Spectrometer, UV-160, Shimadzu Corporation, Japan.

Vortex mixer, vortex-2 Genei, model G 560E, Scientific Industries, U.S.A.

Water bath, model aquatherm G-86, New Brunswick Scientific Co. Ltd., U.S.A.

Water bath, shaker, Memmert, Germany.

3.2 Chemicals

Absolute ethanol, Merck, U.S.A.

Absolute methanol, Merck, U.S.A.

Agarose, Sigma Chemical, U.S.A.

Bacto peptone, Difco, U.S.A.

Bacto tryptone, Difco, U.S.A.

Bacto yeast nitrogen base without amino acids, Difco, U.S.A.

Boric acid, Merck, U.S.A.

Boron trifluoride, Merck, U.S.A.

Calcium chloride, Merck, U.S.A.

Chloroform, Merck, U.S.A.

Dextrose, Difco, U.S.A.

Diethyl ether, Merck, U.S.A.

Dipotassium hydrogen phosphate, Merck, U.S.A.

Disodium hydrogen phosphate, Merck, U.S.A.

D-sorbitol, Difco, U.S.A.

Ethidium bromide, Fluka, Switzerland.

Ethylenediaminetetra-acetic acid (EDTA), Bio-Rad Laboratories., U.S.A.

Ethyl methanesulfonate, Fluka, Switzerland.

Galactose, Difco, U.S.A.

Glacial acetic acid, Merck, U.S.A.

Glucose anhydrous, Fluka, Switzerland.

Glycerol, Merck, U.S.A.

Hexane, Merck, U.S.A.

Hydrochloric acid, Merck, U.S.A.

Isoamyl alcohol, Merck, U.S.A.

Isopropanol, Merck, U.S.A.

Lithium acetate, Fluka, Switzerland.

Magnesium chloride, Merck, U.S.A.

Malt extract, Difco, U.S.A.

Maltose, Difco, U.S.A.

Petroleum eher, Merck, U.S.A.

Phenol, Merck, U.S.A.

Polyethylene glycol 4000, Sigma Chemical, U.S.A.

Potassium acetate, Merck, U.S.A.

Potassium dihydrogen phosphate, Merck, U.S.A.

Potassium hydroxide, Merck, U.S.A.

Sodium acetate, Merck, U.S.A.

Sodium chloride, Merck, U.S.A.

Sodium dihydrogen phosphate, Merck, U.S.A.

Sodium dodecyl sulfate, BDH Laboratory Supplies, England.

Sodium hydroxide, Merck, U.S.A.

Sodium thiosulfate, Merck, U.S.A.

Sucrose, Difco, U.S.A.

Trisma base, Sigma Chemical, U.S.A.

Triton X-100, Merck, U.S.A.

Yeast extract, Difco, U.S.A.

3.3 Supplies

Cellulose acetate filter, pore size 0.45 μm, Sartorius, Germany.

Polaroid film, Polaroid, U.S.A.

3.4 Kit

Wizard®*Plus* SV Minipreps DNA purification system kit, Promega, U.S.A.

3.5 Enzymes and restriction enzymes

*Bam*HI, Promega, U.S.A.

λDNA/*Hind* III marker, Promega, U.S.A.

Hind III, Promega, U.S.A.

Lyticase, Sigma Chemical, U.S.A.

Proteinase K, Life Technology, U.S.A.

Ribonuclease I, Sigma Chemical, U.S.A.

3.6 Organisms, plasmids and DNAs

Hansenula polymorpha SH 4329 (*leu1-1*)*

H. polymorpha SH 4330 (*ura3-1*)**

Escherichia coli ATCC 25922***

Plasmids

YCpELO1.MOD****

A plasmid containing a 2.2-kb DNA fragment of the *ELO1* gene resulting from a *Bst*EII/*Eco*NI digestion of YCpELO1 to remove adjacent open reading frame sequences, followed by religation (Toke and Martin, 1996)

YCpGALELO2(U)****

A *Bam*HI/*Sal*I digestion of the pCRELO2 released 1.2-kb DNA fragment containing the protein coding sequence of the *ELO2* gene. This was ligated into the *Bam*HI/*Sal*I sites on plasmid YCpGAL1 containing galactose-inducible GAL promotor and the *URA3* gene as a selectable marker (Oh *et al.*, 1997)

* kindly provided from Prof. J. A. K. W. Kiel (University of Groningen, The Netherlands)

** kindly provided from Prof. M.A. Gleeson (University of Sheffield, U.K.)

*** Gift from Prof. S. Harashima (Osaka University)

**** Kindly supplied from Prof. C. Martin (Rutgers University, Piscataway, N.J.)

3.7 Fatty acids

Myristic acid (C14:0), Aldrich, U.S.A.

Myristoleic acid (C14:1-Δ9), NuCheck, U.S.A.

Palmitic acid (C16:0), Aldrich, U.S.A.

Palmitoleic acid (C16:1-Δ9), NuCheck, U.S.A.

Stearic acid (C18:0), Aldrich, U.S.A.

Oleic acid (C18:1-Δ9), NuCheck, U.S.A.

Linoleic acid (C18:2-Δ9,12), NuCheck, U.S.A.

α-Linolenic acid (C18:3-Δ9,12,15), NuCheck, U.S.A.

3.8 Storage of microorganisms

The parental strain *leu1-1* of *H. polymorpha* was propagated on YEPD agar plate (Appendix I, No. 1) and incubated at 37 °C for 3 days. A colony was suspended in 15% glycerol. The mixture was aliquoted into eppendorf tube and then stored at –70 °C until used.

3.9 Media and cultivations

H. polymorpha auxotrophic strains *leu1-1* and *ura3-1* were derived from CBS 1976 (NCYC 495). In all experiments, strains were cultivated at 37 °C unless otherwise noted. The *leu1-1* strain was used as parental strain for generating and screening mutant strains. The *ura3-1* strain was employed for crossing the phenotype of interest. Standard yeast extract-peptone-dextrose

(YEPD) and minimal (MIN) (Appendix I, No. 2) medium (Gleeson and Sudbery, 1988) were used for growth of *H. polymorpha* wild-type and the mutant strains. To screen the mutants defective in elongation of medium-to long-chain fatty acids, saturated fatty acids C14:0, C16:0 and C18:0 emulsifying in 1% Triton X-100 were supplemented in the media at the concentration of 1 mM or 2 mM. Standard yeast genetics methods were subjected for mating and sporulation analysis (Anyatonwu *et al*., 2003; Sherman *et al*., 1986). Both, maltose-malt extract medium (MAE) and malt extract medium (ME) (Appendix I, No. 3 and 4, respectively) supplemented with saturated fatty acids were used for mating and induction of sporulation of diploid cells. To inducible GAL promoter, galactose was then added to 3% into the medium without fatty acid for transformation with plasmid YCpGALELO2(U). *E. coli* was cultured on LB medium (Appendix I, No. 5) for preparation of competent cells at 37 °C for 24 h (Sambrook *et al*., 1989).

3.10 Mutagenic treatment of *H. polymorpha* with ethyl methanesulfornate (EMS) (Anamnart *et al*., 1998)

Mutagenesis of *H. polymorpha* was performed by treatment with EMS. The wild-type of *H. polymorpha* (*leu1-1*) were incubated at 37 °C for 24 h to obtain good proliferation. Cells from an YEPD overnight (O/N) culture were washed in 0.2 M sodium phosphate buffer (pH 8.0) (Appendix II, No. 1) and resuspended in 10 ml of the same buffer containing 2% glucose. 0.3 ml of EMS was added to the suspension which was incubated in a water bath shaker for 45 min at 30 °C. After treatment cells were washed for several times and resuspended in sterile distilled water.

3.11 Selection and screening of the mutants

Mutagenized cells were plated onto YEPD supplemented with a mixture of 1 mM each of C14:0, C16:0 and C18:0 (14/16/18 YEPD) and were incubated for 2-3 d at 37 °C until colonies appeared. The master plates were

replica-plated onto YEPD plated for screening the mutants with a phenotype of interest. Determination of mutant growth on the media containing the individual fatty acids was further done to characterize the prospective elongation-defective mutants.

3.12 Determination of growth ability of the mutants in liquid and solid media

The mutant cells were suspended at the concentration of $2x10^4$ cells in 5 ml YEPD supplemented with different saturated fatty acids or a mixture of C14:0, C16:0 and C18:0 (1 mM each or 0.2/0.4/0.2 mM, depended on the appropriated concentration of the substrates that providing the clearly results of the fatty acid profiles (Toke and Martin, 1996 and Appendix III)) and then were cultivated to mid logarithmic phase on a reciprocal shaker (120 rpm). Cell density was determined by spectrophotometry with absorbance at 660 nm. In the case of solid media, cultures were streaked onto 14/16/18 YEPD plates and replicated after 3-5 d growth onto YEPD and YEPD supplemented with different saturated fatty acids.

3.13 Lipid extraction from whole cells of *H. polymorpha*

Yeast cultures were grown in medium to late logarithmic stage. Before harvesting by centrifugation and washing the cells for three times with sterile water to get rid of supplemented fatty acid in the media, samples of each culture were analyzed for their non-reverted genotype by appropriate replica plating. Fatty acids, free and incorporated into different cellular compounds, were extracted from whole cells by direct saponification: 0.8 ml of 10% KOH in methanol was added to 1 g of wet cells in screw-capped tube. The tube was standed at 80 °C for 2 h and then cool down to room temperature. After the removal of unsaponified materials by extracting the mixture with 1 ml of petroleum ether, the aqueous phase was acidified with 0.3 ml of 6 N HCl and fatty acids were recovered by diethyl ether extraction. The recovered fatty

acids solvent was evaporated by flushing with nitrogen gas. Fatty acid methyl esters (FAMEs) were prepared by transmethylation with boron trifluoride (Morrison and Smith, 1964) at 80 °C for 3 min and then cool down to room temperature. After incubation, FAMEs were extracted by the addition of 1 ml of saturated NaCl solution and 0.5 ml of hexane. The mixture was mixed vigorously and centrifuged at 3000 rpm for 5 min to promote phase separation. The top phase was removed carefully and the solvent evaporated under a stream of nitrogen gas.

3.14 Gas chromatographic analyses

The FAMEs were resuspended in hexane and then separated on 3 mm glass column of 15% DEGS on 60/80 mesh Chromasorb W AW at 180 °C with Shimadzu 15A Gas chromatography and analyzed by gas chromatography using a Hewlett-Packard HP 6890 Series, equipped with an HP-5 column, with a temperature gradient (20 min at 200 °C, 10 °C/min to 250 °C, and 10 min at 250 °C), depended on the concentration of sample. Helium gas was used as a carrier gas. Fatty acids were identified by comparison of their retention times to those of commercially available methyl ester standard: C14:0, C14:1, C16:0, C16:1, C18:0, C18:1, C18:2 and C18:3. The fatty acid (FA) compositions was expressed as percentages in total fatty acids (TFA) (Carroll, 1961) as follow:

$$\% \text{ FA/TFA} = (\text{area FA}*100) / (\text{total area})$$

3.15 Mating and sporulation of diploid cells

The yeast haploids of *H. polymorpha* mutants (*leu1-1*) and auxotrophic strain (*ura3-1*) were crossed and the resulting diploids were selected on 14/16/18 MIN and MIN plates. A colony from the selected diploids was sporulated in ME media for induction of sporulation. Ascal cell walls were treated with diethyl ether to release the spore products of meiosis and then spreaded on YEPD supplemented with a mixture of fatty acids (14/16/18

YEPD). Replica-plated on the MIN containing uracil (Appendix I, No. 3) and 14/16/18 MIN medium. The prospective hybrids should unable to grow on these selective medium but can grow on the master plate of 14/16/18 MIN (uracil). The steps involved in mating of haploid strains to produce diploid cells, sporulation of diploids and analysis of tetrads after meiosis are mentioned by Anyatonwu, 2003.

3.16 Preparation of yeast chromosomal DNA (Burke *et al.*, 2000)

Yeast cells were grown overnight at 37 °C in 5 ml of YEPD broth medium. Cells were collected by 2000 rpm centrifugation for 5 min. Cells were resuspended in 0.5 ml of 1 M sorbitol, 0.1 M Na_2EDTA (pH 7.5) and transferred to a 1.5 ml microfuge tube. The solution was added 0.02 ml of a 2.5 mg/ml solution of Lyticase followed by incubation 1 h at 37 °C. The cells were collected by 1 min centrifugation, discarded the supernatant and resuspened in 0.5 ml of 50 mM Tris-HCl (pH 7.4) (Appendix II, No. 2), 20 mM EDTA. Added 0.05 ml of 10% SDS (Appendix II, No. 3) and then vigorously mixed of reaction solution, incubated for 30 min at 65 °C. Then, the solution was added with 0.2 ml of 5 M potassium acetate, mixed by gentle inversion and place the microfuge tube in ice for 1 h. The lysated cells were collected again by 5 min of centrifugation. Then, supernatant was transferred to a new microfuge tube and added one volume of 100% isopropanol at room temperature, mixed by gentle inversion and allowed precipitation for 5 min. Pellet of DNA was collected by brif centrifugation, poured off the supernatant and allowed air-dry the pellet. Then pellet was resuspended in 0.3 ml of TE (pH 7.4) (Appendix II, No. 4). Non-require RNA was digested by supplemention of 15 μl of a 1 mg/ml solution of RNase A and incubated the reaction at 37 °C for 30 min. DNA and RNA were precipitated by 0.03 ml of 3 M sodium acetate and 0.2 ml of 100% isopropanol. Pellet of DNA was collected by brief centrifugation again and poured off the supernatant. Finally, DNA pellet was dissolved in 0.1-0.3 ml of TE (pH 7.4).

3.17 Preparation of *E. coli* plasmid DNA

Plasmid DNA preparation was performed using Wizard®*Plus* SV Minipreps DNA purification system kit. Pellet 1-10 ml of bacterial overnigt culture (LB supplementation of 100 µg/ml of ampicillin) by 5 min at 10000 rpm in a microcentrifuge. Supernatant was poured off as much as possible. Cell pellet was resuspened in 250 µl of solution I and completely resuspended the cell pellet by vortexing well. Cell walls were lysed by addition of 250 µl of solution II and mixed by inverting the tube 4 times and than added 10 µl of alkaline protease solution and mixed by inverting the tube 4 times. The reaction was incubated at room temperature for 5 min and then added 350 µl of neutralization solution and mixed by inverting the tube 4 times. The bacterial lysate was separated by 10 min of centrifugation at room temperature. Then, the cleared lysate (approximately 850 µl) was transferred by decanting into spin column inserted into a 2 ml collection tube. The cleared lysate was separated by 1 min of centrifugation at room temperature, removed the spin column from the tube and discarded the flow through from the collection tube. Pellet of DNA was washed with 750 µl of wash solution to the spin column, collected by 1 min of centrifugation at room temperature and discarded the flow through from the collection tube. Then pellet was washed with 250 µl of wash solution again. Spin column was transferred to a clean, sterile 1.5 ml microcentrifuge tube. Finally, plasmid DNA was eluted by 100 µl of nuclease-free water.

3.18 Yeast *H. polymorpha* transformation (Sambrook *et al.*, 1989)

The plasmid YCpELO1.MOD and YCpGALELO2(U) were transformed into *H. polymorpha* fatty acid auxotrophic mutants using the lithium acetate method. By, the 0.4 ml of fresh overnight mutant strain of *H. polymorpha* culture that cultivated in YEPD supplemented with 14/16/18 YEPD (1 mM each) emulsifying in 1% Triton X-100 was inoculated to 10 ml of the same media. For overexpression experiments, yeast cells transformed by

galactose-inducible plasmid were cultured in minimal medium containing 3% galactose (Appendix I, No. 4) (Oh *et al.*, 1997). This culture was grown by shaking at 30 °C for 5-6 h. Cells were harvested by 2000 rpm centrifugation for 5 min at 0-4 °C. After supernatant was poured off cell pellets were washed by 5 ml of TE buffer and harvested by 2000 rpm for 5 min at 0-4 °C. Cells were resuspended with 2 ml of LA solution (0.1 M LiAc in TE (pH 7.6)) and placed on a water bath shaker at 30 °C for 1 h. After harvested cell pellets, were resuspended with 0.6 ml of LAG (0.1 M LiAc, 15% Glycerol in TE (pH 7.2)). Cells were divided into two tubes of 0.3 ml aliquots and added plasmid DNA (<20 µl) while one tube as a negative control no plasmid DNA was added. Then each tube was added with 0.7 ml of polyethylene glycol 4000 solution (50% PEG 4000) and mixed well. Cells were incubated for 1 h at 30 °C and collected by 12000 rpm centrifugation for 5 sec. After each tube of cells was washed by 1 ml of TE buffer, cells were resuspended in 0.2 ml of YEPD medium and then spreaded over the surface of the MIN agar plates. Plates were incubated for 3-5 d at suitable temperature. The yeast transformants were cultivated to exponential phase and screened prospective transformants capable of growth on synthetic medium without fatty acid supplementation and uracil.

3.19 Bacterial transformation (Sambrook *et al.*, 1989)

The 1-5 µl required DNA was gently mixed with competent cell solution which thawed on ice. The mixed solution was incubated on ice for 30 min. The solution tube was transferred to a rack placed in a circulating water bath that has been preheated to 42 °C and leaved the tubes in the rack for exactly 90 seconds (do not shake the tubes). Then the tube was rapidly transferred to an ice bath and allowed the cells to chill for 1-2 min. LB medium was added to the tube and incubated the culture for 45 min in water bath set at 37 °C to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. The appropriate volume (up to 200 µl per 90 mm plate) of transformed competent cells were transferred onto LB medium

containing 20 mM $MgSO_4$ and the appropriate antibiotic (ampicillin). Then the transformed cells were gently spreaded over the surface of the agar plate and incubated at 37 °C for the colonies to grow.

3.20 Preparation of competent cell (Sambrook *et al.*, 1989)

Ten ml of fresh overnight *E. coli* culture was inoculated into 1 L of LB and then grown by vigorously shaking at 37 °C until OD 660 reaches 0.5-0.6. Cells were cooled down on ice for 10 min and harvested by 8000 rpm centrifugation for 5 min at 4 °C. After supernatant was poured off, cell pellets were washed by 250 ml of 0.1 M $MgCl_2$. Washed pellet was collected by centrifugation and suspended with 250 ml of 0.1 M $CaCl_2$. Cells were pulled down again at 4 °C. Pellet was resuspended again with competent buffer (42.5 ml of 0.1 M $CaCl_2$, 7.5 ml of Glycerol). Each 100 µl of competent cell solution was divided to 1.5 ml microfuge tube. The competent cells were cooled on ice for 1 d before frozen in –70 °C.

3.21 Agarose gel electrophoresis

0.7 % (w/v) of agarose was dissolved in 0.5X TBE buffer (Appendix II, No. 5), melted at 70-80 °C and formed solid state at room temperature. DNA solutions were mixed with 1/10 of 10X Loading buffer (Appendix II, No. 6). Gel was run at constant current, 100 V. After the running is finished, gel was soaked in ethidium bromide (5 µl/ml). DNA bands were visible under UV transilluminator. The standard sizes of DNA fragments in this work were utilized λ-*Hind* III (23.1, 9.4, 6.6, 4.4, 2.3, 2.2, 2.0, 0.6, 0.1 kb) for estimation of DNA size.

3.22 Reextraction of the plasmids from the yeast transformant

Yeast transformant cells were grown overnight at 30 °C in 5 ml of MIN broth medium. Cells were transferred to a 1.5 ml microfuge tube and collected by 10,000 rpm centrifugation for 5 sec. Then, cells were resuspended

in 0.2 ml of a mixture solution of 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl (pH 8) and 1 mM Na_2EDTA. The solution was added with 0.2 ml of phenol : chloroform : isoamyl alcohol (25:24:1). Then, the solution was added with 0.3 g of acid-washed glass beads sterile, mixed of reaction solution for 2 min. The cleared lysate of plasmid DNA was collected by centrifugation for 5 min.

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CHAPTER IV

RESULTS AND DISCUSSION

4.1 Mutagenic treatment of *H. polymorpha* and selection of the mutants

Cells of a parental strain of *H. polymorpha* CBS 1976 (*leu1-1*), were subjected to EMS mutagenesis. To identify mutants with incapability to elongate C14:0, C16:0 and C18:0, the mutagen-treated cells were plated on to the growth medium YEPD agar containing a mixture of 1 mM each of C14:0, C16:0 and C18:0. The plates were incubated at 37 °C for 2-3 d. Colonies were then replicated on the YEPD medium without fatty acids supplementation. All the mutants were screened from colonies which were unable to grow on YEPD plates. Therefore, cells defective in fatty acid synthesis or elongation would fail to grow on medium without fatty acid supplementation. After replication on the selective plates, all the mutants which were unable to grow on YEPD were further characterized. In addition to confirm the results of the screening analysis of mutagenesis cells, we then restreaked these cells onto the selective medium. The example of the confirming results was shown in Fig. 4.1.

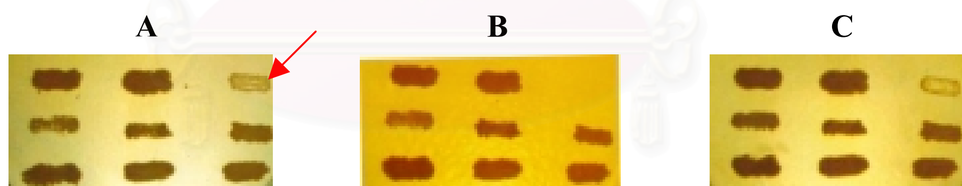

Fig. 4.1 Analysis of screening test of *H. polymorpha* mutagenesis cells in master plate of YEPD supplementation with a mixture of C14:0, C16:0 and C18:0 (1 mM each) (A). The master plate was then replicated onto selective medium of YEPD (B) and YEPD supplementation with a mixture of fatty acid (C). The arrow indicated the positively results in the selective medium for only one mutagenesis cell.

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Comparison of 95,000 colonies between the master and replica plates identified ten mutants (V1 to V10) which required fatty acids for growth. We further analysed the growth abilities of the mutants in solid media of YEPD containing various fatty acids of 1 mM and/or 2 mM C14:0, C16:0 and C18:0. The mutants were able to classify into three groups which possessed a phenotype of interest. In our analysis of the defective steps in fatty acid biosynthetic pathway of all three groups of the mutants, we determined their growth on solid media supplemented with individual saturated fatty acids, C14:0, C16:0, C18:0 (Fig. 4.2-4.3).

It was found that the mutants of the first group (V1 to V6) did not grow on media supplemented with 1 mM of C16:0. However, 2 mM of C16:0 and 1 mM and 2 mM of C18:0 could support the growth of the V1 mutant (representative mutant) as shown in Figures 4.2 and 4.3. Ability of the V1 mutant to grow in the presence of high concentration of C16:0 (2 mM) can be explained from the viewpoint by assuming either weak activity of the partially damaged elongase enzyme in the step of C16 to C18 extension or the presence of other enzymes involved in fatty acid biosynthesis. Nevertheless, the growth of V1 mutant on media supplemented with C16:0 was also less than the growth of parental strain and V1 supplemented with C18:0 (Fig. 4.2 and 4.3). This result showed that C18 fatty acid was required for proper growth of *H. polymorpha.*

The second group included only one mutant (V7) showing no growth when cultured on media supplemented with 1 mM of C14:0. However, 1 mM of C16:0 and 1 mM of C18:0 could support the growth of V7 mutant as shown in Fig. 4.2. Ability of the V7 mutant to grow in the presence of C16:0 and C18:0 can be explained from the viewpoint by assuming activity of the elongase enzyme in the level of C16 to C18 extension. Nevertheless, the growth of V7 mutant on media supplemented with C16:0 and C18:0 were also less than the growth of parental strain (Fig. 4.2). Mutants in the third group (V8, V9, V10) did not grow on medium cultivated with 1 mM of C16:0 and 1

mM of C18:0 supplementation. However, 1 mM of C14:0 could support the growth of the V8 mutant (representative mutant) as shown in Fig. 4.2. Ability of the V8 mutant to grow in the presence of C14:0 can be explained from the viewpoint by C14:0 biosynthesis was defective which myristate (C14:0) is essential for contribution to *N*-myristoyl-proteins. However, mutants in this group could not grow on YEPD supplemented with C16:0 and/or C18:0. These phenotypes showed as ntm1-181 mutant (*N*-myristoyl transferase mutant) that the ntm1-181 phenotype can be fully suppressed at non-permissive temperature (37 °C) by supplementing media with myristate (C14:0), but not shorter or longer chain saturated fatty acids (Johnson *et al*., 1994). Nevertheless, the growth of V8 mutant on media supplemented with C14:0 was also less than the growth of wild-type (Fig. 4.2). This total results of the three groups of the mutants (V1, V7 and V8 representative mutants) showed that those fatty acids were required for proper growth of *H. polymorpha*. Actually, most fatty acids in *H. polymorpha* are predominantly 16 and 18 carbons in length revealing that these fatty acids are critical for the normal development and cellular function (Table 4.1).

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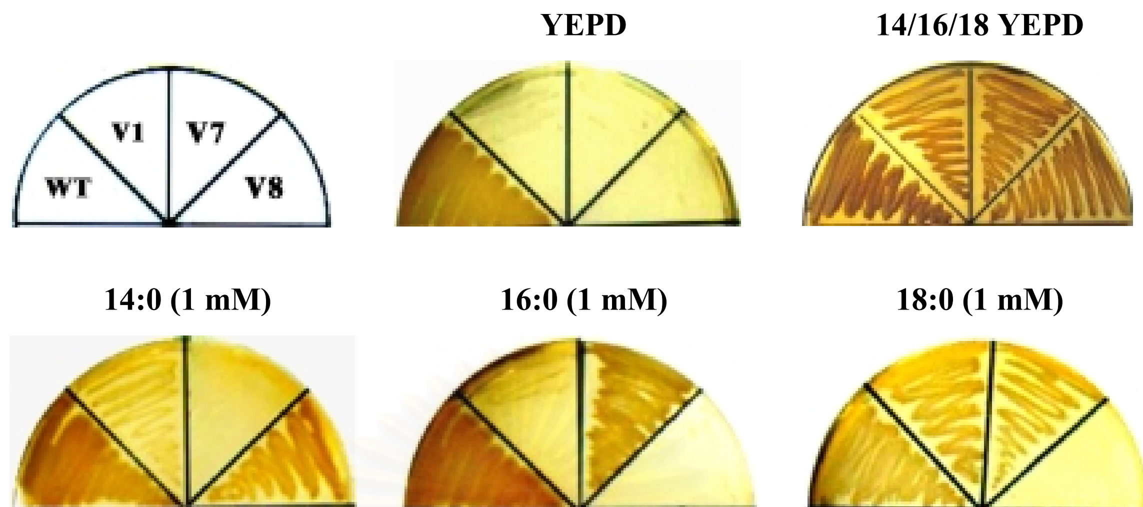

Fig. 4.2 Growth phenotype of mutants and wild-type of *H. polymorpha* CBS 1976 (*leu1-1*) on solid medium of YEPD and YEPD supplemented with different fatty acids. The growth phenotype of the three representative mutants (V1, V7 and V8) and wild-type (WT) strain are presented. Photographs were taken after 4 d incubation.

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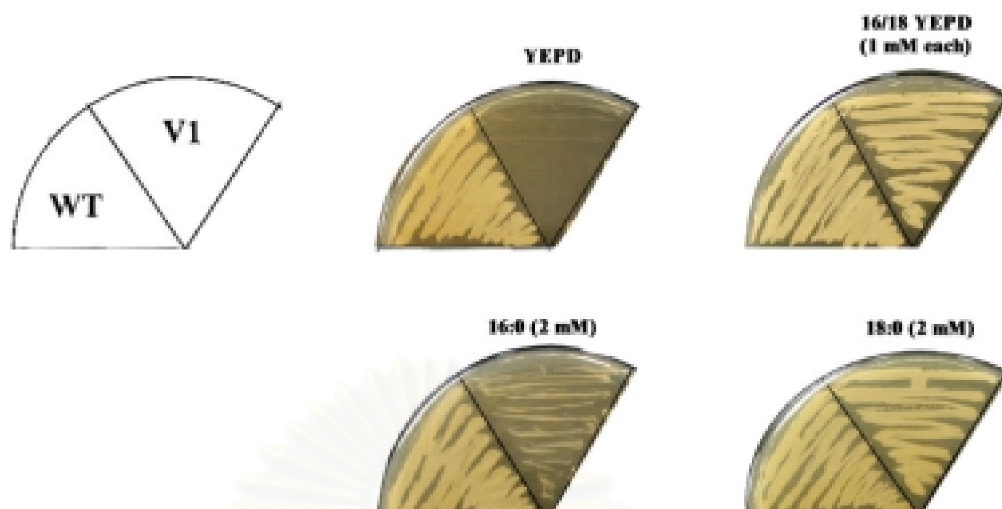

Fig. 4.3 Growth phenotype of V1 mutant and wild-type of *H. polymorpha* CBS 1976 (*leu1-1*) on solid medium of YEPD and YEPD supplemented with different fatty acids at the concentration of 2 mM. The growth phenotype of the representative mutant V1 and wild-type (WT) strain are presented. Photographs were taken after 4 d incubation.

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Table 4.1 Growth ability of auxotrophic mutants*

Strain	Supplement				
	None	14:0 (1 mM)	16:0 (1 mM)	18:0 (1 mM)	14:0/16:0/18:0 (0.2/0.4/0.2 mM)
WT	+	+	+	+	+
V1 to V6	−	∓	∓	+	+
V7	−	−	+	+	+
V8 to V10	−	±	−	−	+

* Cell were grown to the stationary phase (4 d) in YEPD and YEPD medium supplemented with fatty acids (as indicated) and incubated at 37 °C. The symbols +, ±, ∓, − indicate varying amounts of growth relative to the WT +, growth; ±, poor growth; ∓, very poor growth; −, none growth.

The results on liquid media showed that the growth behavior of all three groups of the mutants were similar to that of the results on solid media. WT and the mutants were grown on YEPD and YEPD supplemented with individual saturated fatty acids at the concentrations of 1-2 mM of C14:0, C16:0, C18:0 and a mixture of C14:0, C16:0 and C18:0 as shown in Fig. 4.4-4.9. The growth of V1 compared with those of WT from cultivated on YEPD with/without various fatty acids supplementation, found that the maximum growth of V1 when supplemented with C18:0 and could supported their growth of V1 closely to those of WT strain (Fig. 4.4C and 4.5). Moreover, the results of V1 from cultivated on liquid medium supplemented with C16:0 or the mixture of C14:0, C16:0 and C18:0 (0.2/0.4/0.2 mM each), demonstrated their similarly results of the maximum growth compared with those of V1 when cultivated on liquid media of YEPD. In addition, the growth of V1 cultivated on YEPD supplemented with 2 mM of C16:0 found that the shorter lag phase

when compared with those of WT with/without a mixture of fatty acid supplementation (Fig. 4.4-4.5). The results of V7 from cultivated on liquid medium of YEPD supplemented with C16:0, C18:0 and a mixture of fatty acids demonstrated that the lower growth when compared with those of WT (Fig. 4.6-4.7). For V8, the representative mutant of the third group demonstrated their ability to proliferate on YEPD with C14:0 supplementation. Moreover, V8 cultivated on YEPD supplemented with C14:0 or a mixture of fatty acids could provided the maximum growth closely to those of WT (Fig. 4.8-4.9).

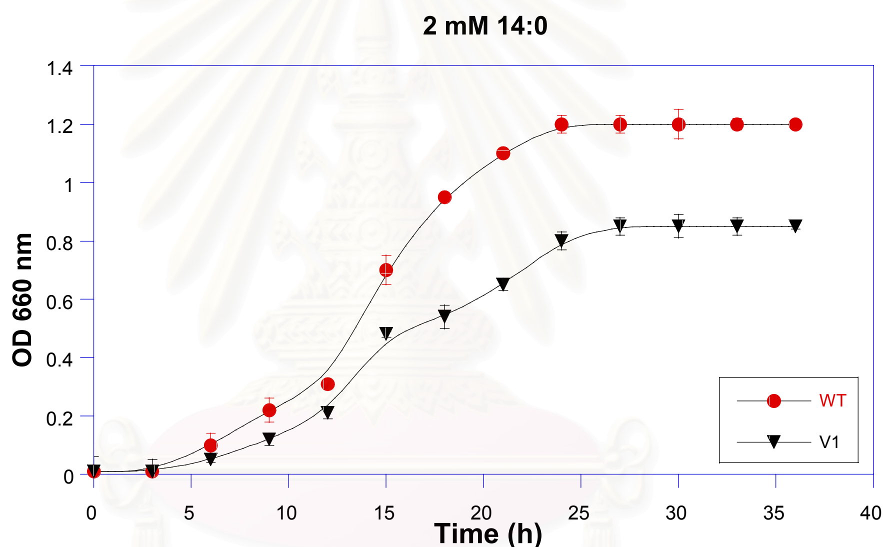

Fig. 4.4A Growth profiles of wild-type (WT) and V1 mutant of *H. polymorpha* cultivated on liquid medium of YEPD supplemented with 2 mM of 14:0 at 37 °C.

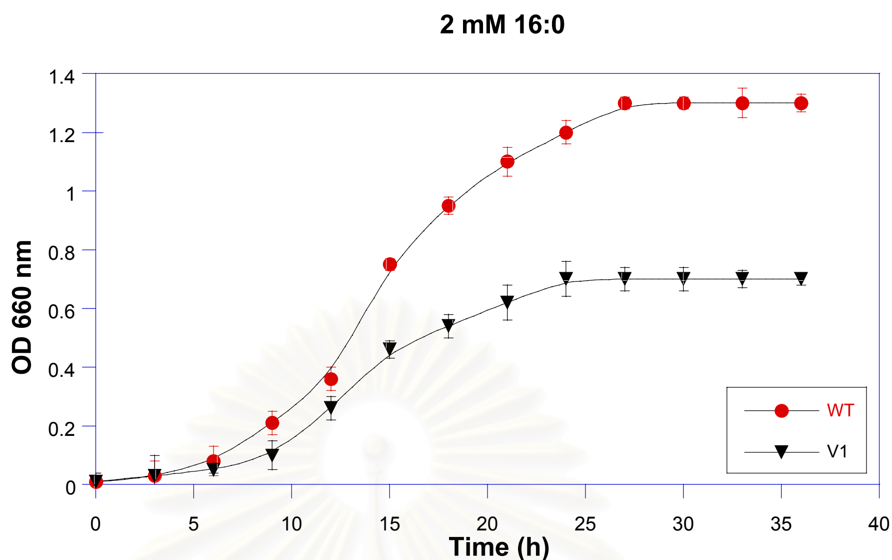

Fig. 4.4B Growth profiles of wild-type (WT) and V1 mutant of *H. polymorpha* cultivated on liquid medium of YEPD supplemented with 2 mM of 16:0 at 37 °C.

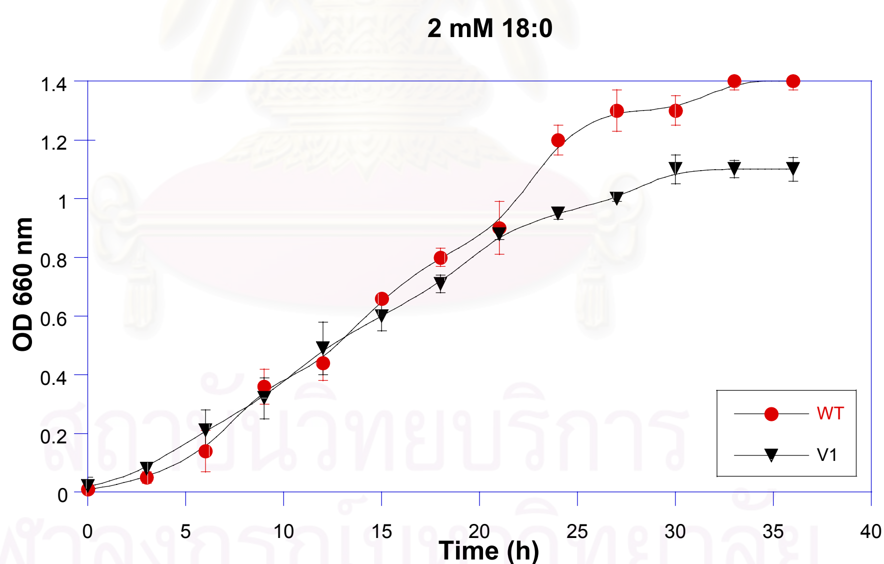

Fig. 4.4C Growth profiles of wild-type (WT) and V1 mutant of *H. polymorpha* cultivated on liquid medium of YEPD supplemented with 2 mM of 18:0 at 37 °C.

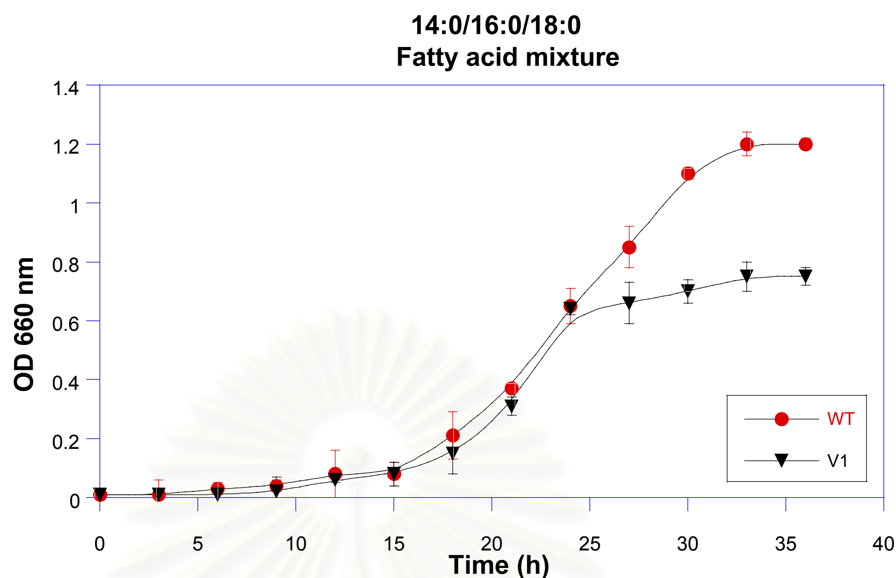

Fig. 4.4D Growth profiles of wild-type (WT) and V1 mutant of *H. polymorpha* cultivated on liquid medium of YEPD and YEPD supplemented with 14:0/16:0/18:0 (0.2/0.4/0.2 mM) at 37 °C.

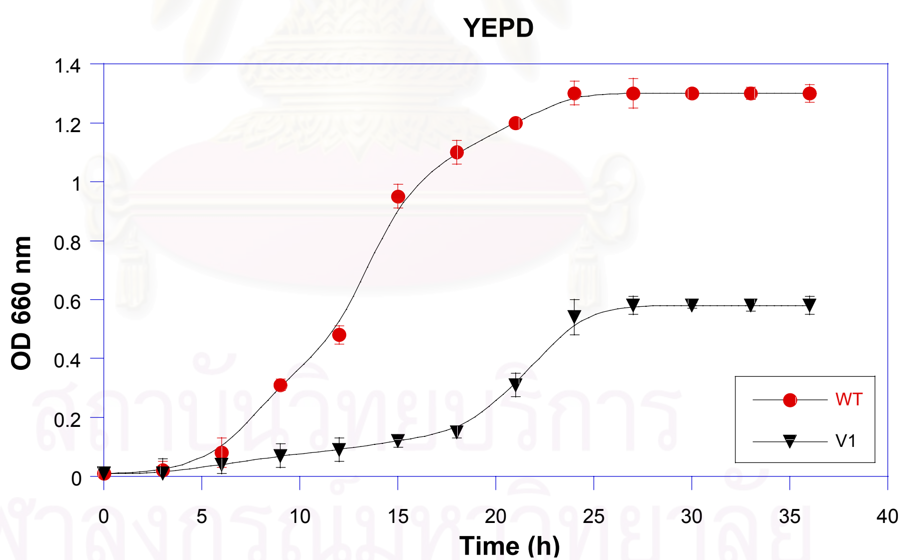

Fig. 4.4E Growth profiles of wild-type (WT) and V1 mutant of *H. polymorpha* cultivated on liquid medium of YEPD at 37 °C.

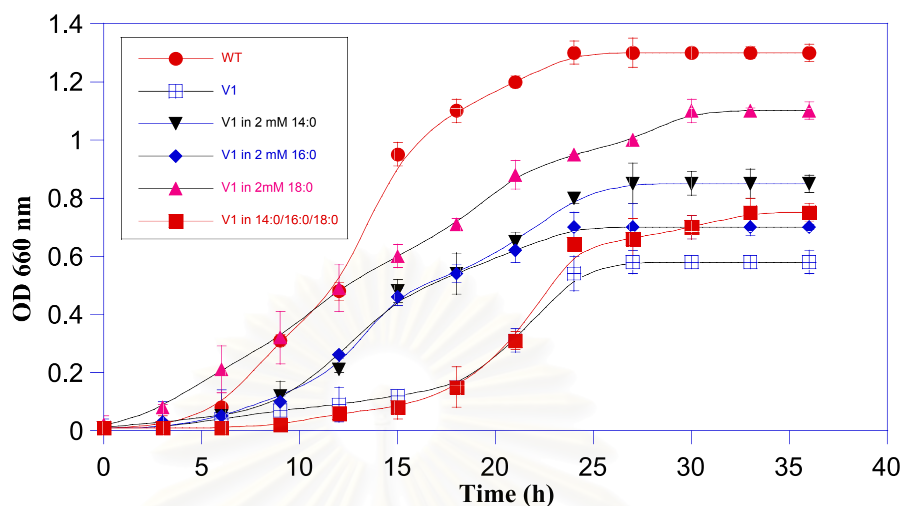

Fig. 4.5 Growth profiles of wild-type (WT) and V1 mutant of *H. polymorpha* cultivated on liquid medium of YEPD and YEPD supplemented with 2 mM of 14:0, 16:0, 18:0, a mixture of 14:0/16:0/18:0 (0.2/0.4/0.2 mM) at 37 °C.

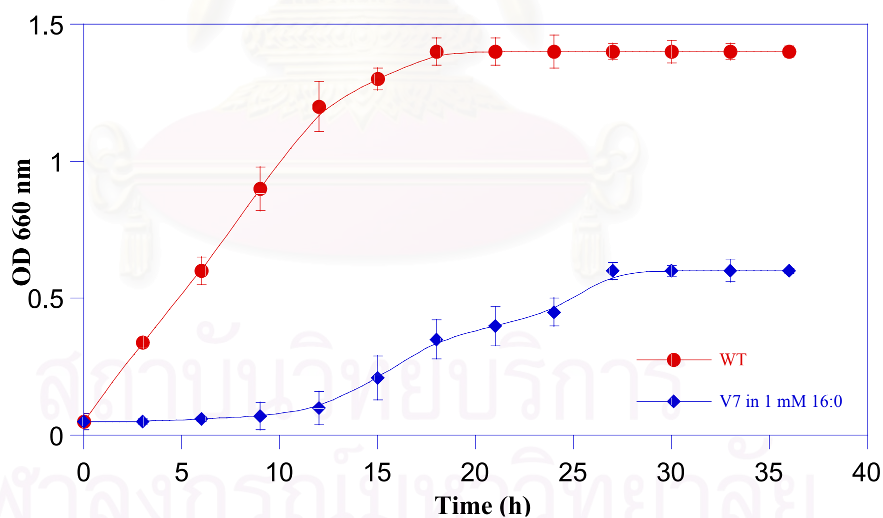

Fig. 4.6A Growth profiles of wild-type (WT) and V7 mutant of *H. polymorpha* cultivated on liquid medium of YEPD supplemented with 1 mM of 16:0 at 37 °C.

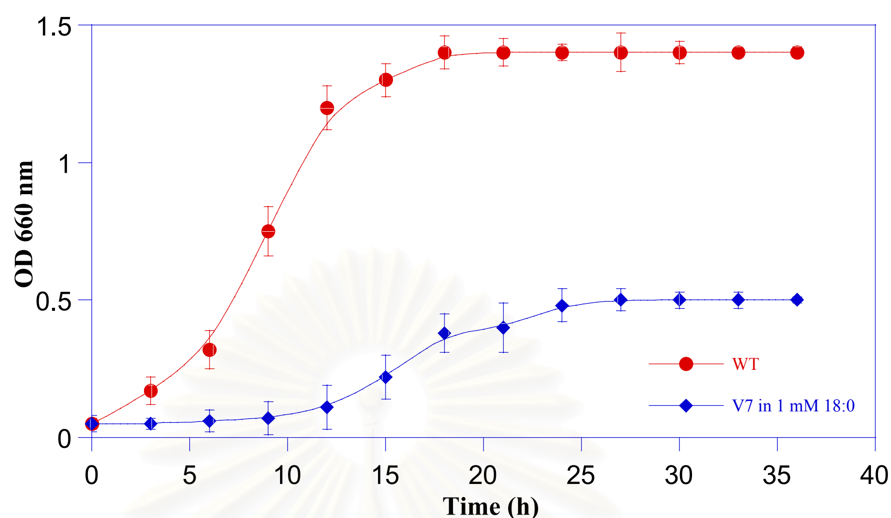

Fig. 4.6B Growth profiles of wild-type (WT) and V7 mutant of *H. polymorpha* cultivated on liquid medium of YEPD supplemented with 1 mM of 18:0 at 37 °C.

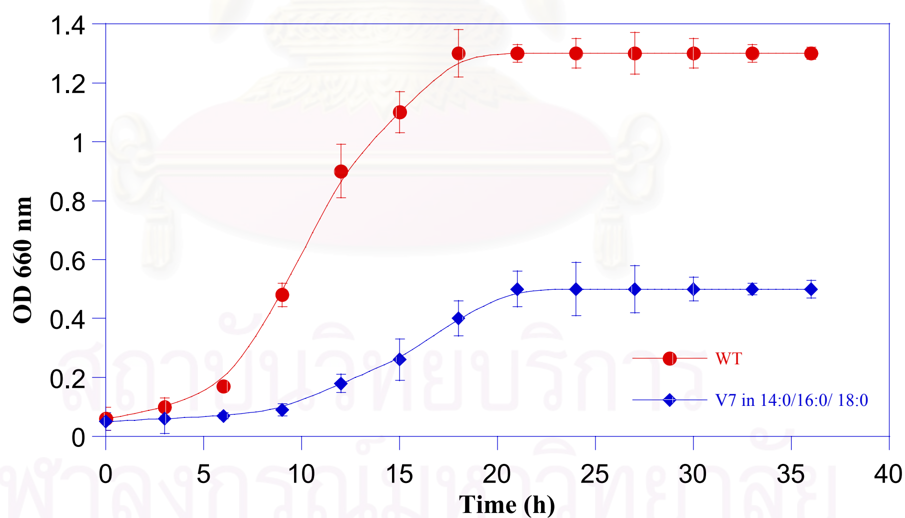

Fig. 4.6C Growth profiles of wild-type (WT) and V7 mutant of *H. polymorpha* cultivated on liquid medium of YEPD supplemented with 14:0/16:0/18:0 (0.2/0.4/0.2 mM) at 37 °C.

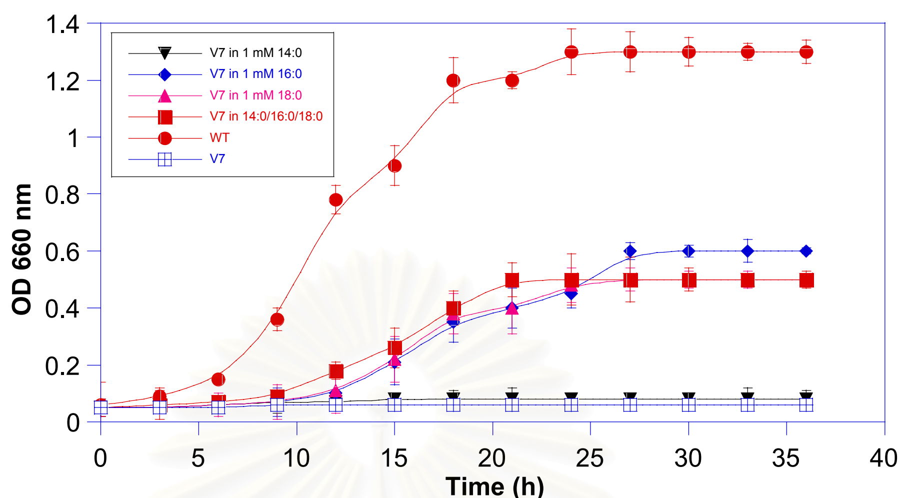

Fig. 4.7 Growth profiles of wild-type (WT) and V7 mutant of *H. polymorpha* cultivated on liquid medium of YEPD and YEPD supplemented with 1 mM of 14:0, 16:0, 18:0, a mixture of 14:0/16:0/18:0 (0.2/0.4/0.2 mM) at 37 °C.

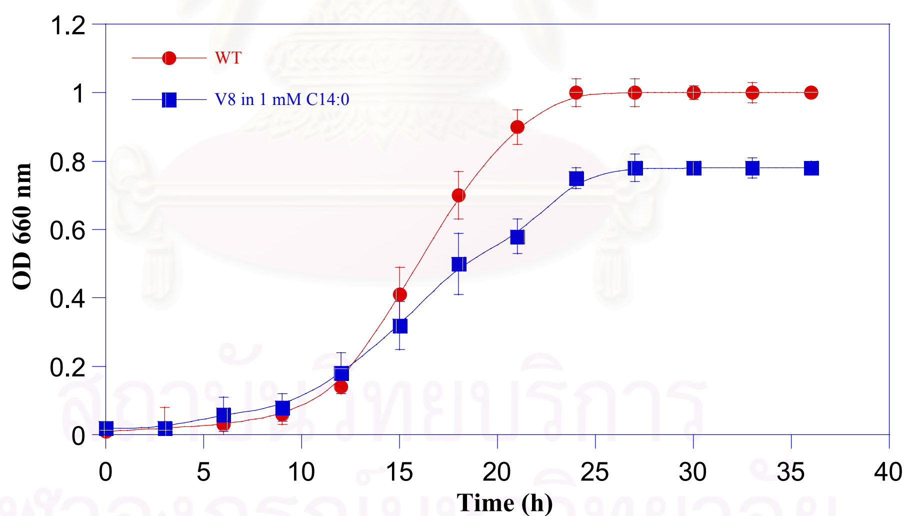

Fig. 4.8A Growth profiles of wild-type (WT) and V8 mutant of *H. polymorpha* cultivated on liquid medium of YEPD supplemented with 1 mM of 14:0 at 37 °C.

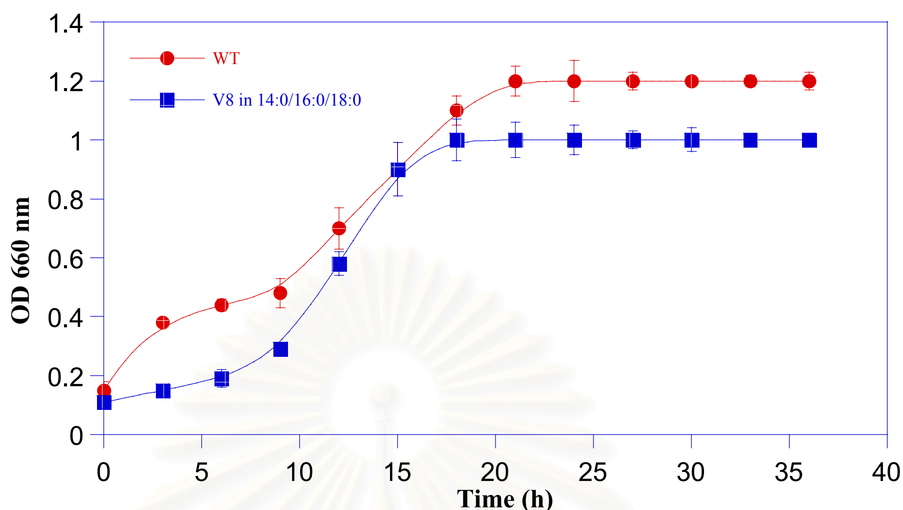

Fig. 4.8B Growth profiles of wild-type (WT) and V8 mutant of *H. polymorpha* cultivated on liquid medium of YEPD supplemented with 14:0/16:0/18:0 (0.2/0.4/0.2 mM) at 37 °C.

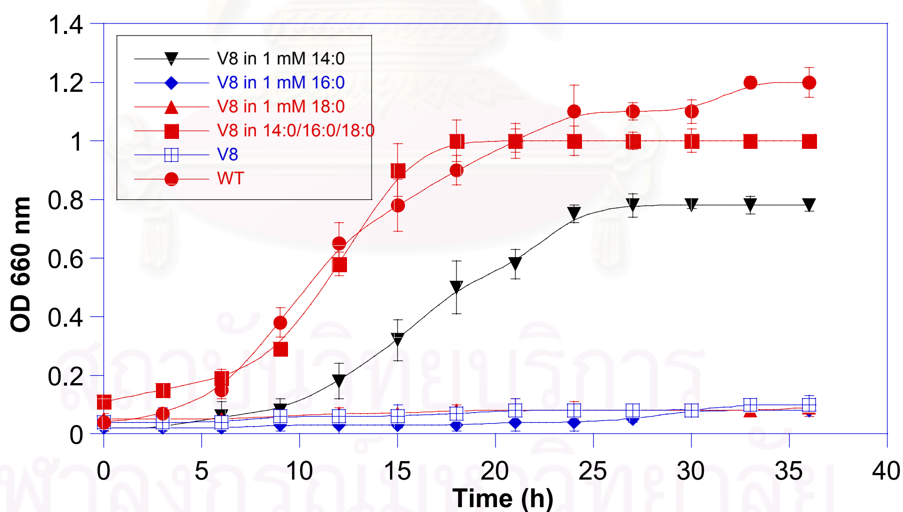

Fig. 4.9 Growth profiles of wild-type (WT) and V8 mutant of *H. polymorpha* cultivated on liquid medium of YEPD and YEPD supplemented with 1 mM of 14:0, 16:0, 18:0, a mixture of 14:0/16:0/18:0 (0.2/0.4/0.2 mM) at 37 °C.

Moreover, the analysis of fatty acid profiles of V1, V7 and V8 (representative mutants) cultivated in YEPD broth and YEPD broth supplemented with 1 mM of C14:0, C16:0, C18:0 and a mixture of C14/C16/C18 fatty acids (by using the concentration of 1 mM each or 0.2/0.4/0.2 mM, depended on a clearly result of the fatty acid profiles (Toke and Martin, 1996)) to late logarithmic stage (24 h) at 37 °C, were compared with those of the WT strain as shown in Fig. 4.10A-E, 4.11A-C and 4.12A,B. The difference pattern of distribution of cellular fatty acids were observed by gas chromatography using glass column of 15% DEGS or capillary column, depended on the concentration of sample. Because of the defect in the growth of the mutants, results in some conditions were not detected.

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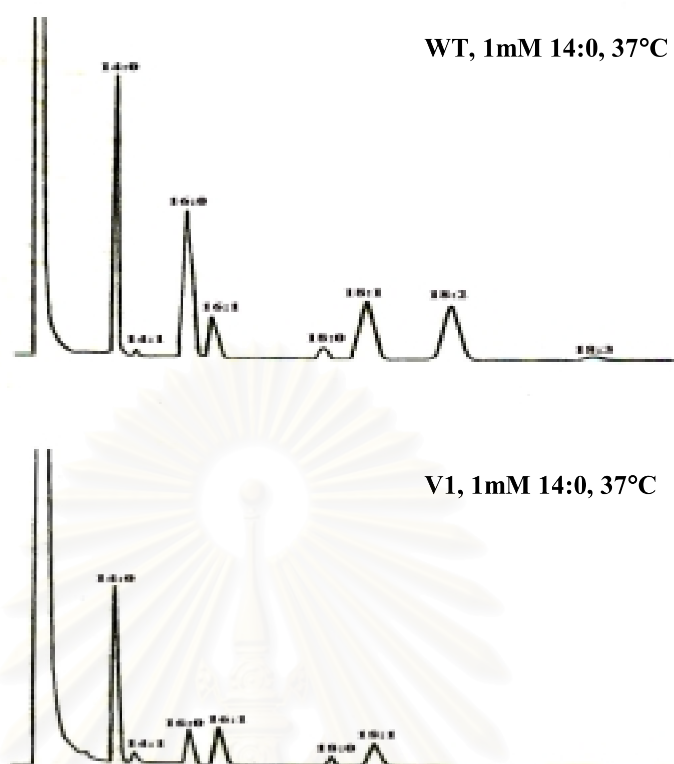

Fig. 4.10A Gas chromatographic analysis of fatty acid methyl esters derived from wild-type (WT) and V1 mutant of *H. polymorpha* grown on the media supplemented with 1 mM of 14:0 for 24 h. 14:0, 14:1, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3 (18:3, Δ9, Δ12, Δ15) indicate positions of the standard of the respective methyl esters of those fatty acids. (by using a glass column of 15% DEGS, as described in Materials and Methods 3.14)

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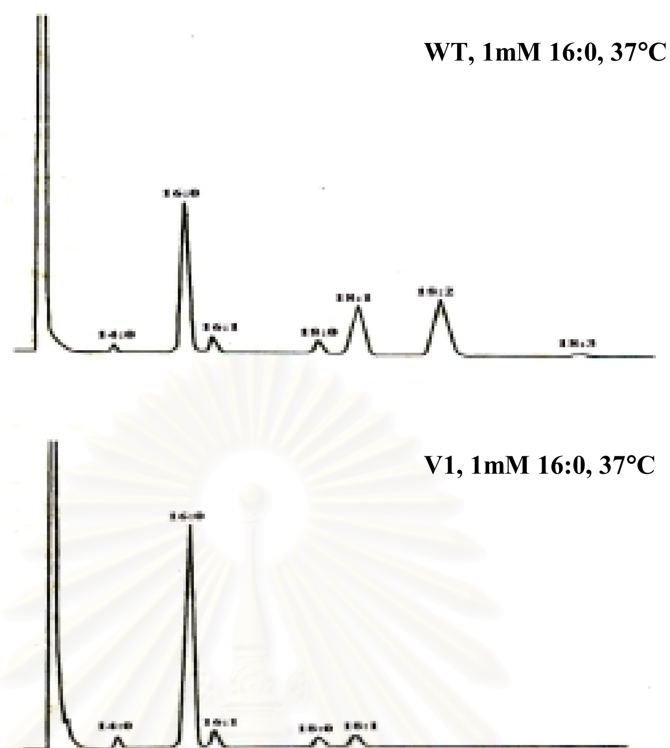

Fig. 4.10B Gas chromatographic analysis of fatty acid methyl esters derived from wild-type (WT) and V1 mutant of *H. polymorpha* grown on the media supplemented with 1 mM of 16:0 for 24 h. 14:0, 14:1, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3 (18:3, Δ9, Δ12, Δ15) indicate positions of the standard of the respective methyl esters of those fatty acids. (by using a glass column of 15% DEGS, as described in Materials and Methods 3.14)

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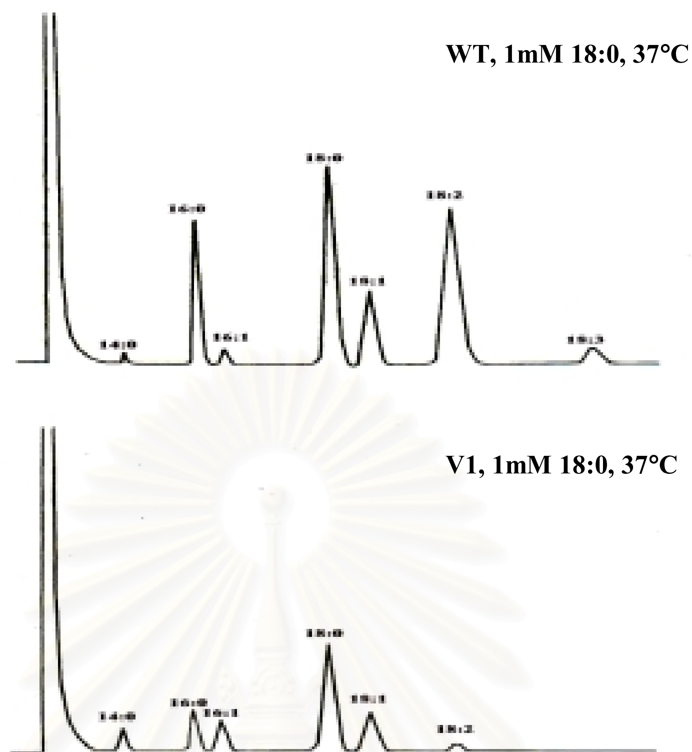

Fig. 4.10C Gas chromatographic analysis of fatty acid methyl esters derived from wild-type (WT) and V1 mutant of *H. polymorpha* grown on the media supplemented with 1 mM of 18:0 for 24 h. 14:0, 14:1, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3 (18:3, Δ9, Δ12, Δ15) indicate positions of the standard of the respective methyl esters of those fatty acids. (by using a glass column of 15% DEGS, as described in Materials and Methods 3.14)

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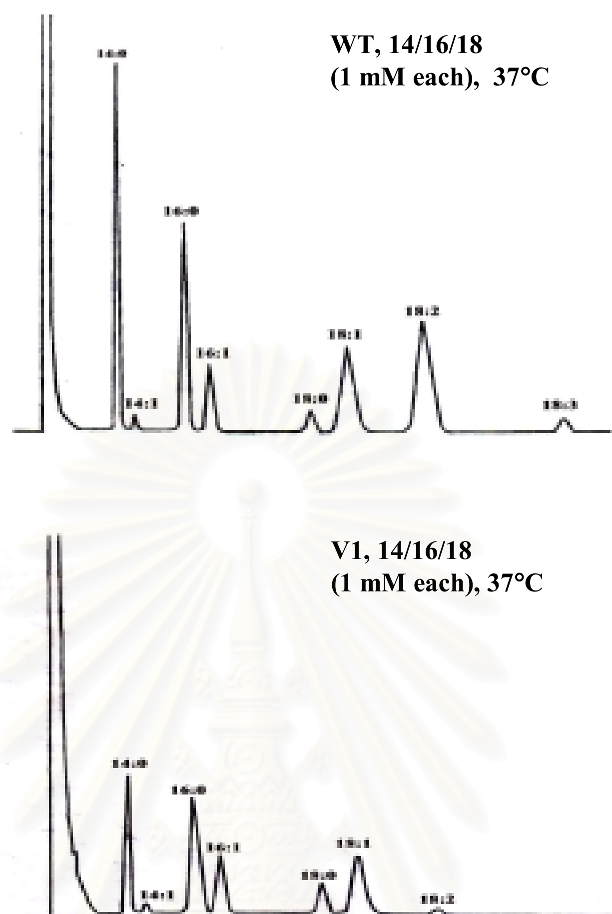

Fig. 4.10D Gas chromatographic analysis of fatty acid methyl esters derived from wild-type (WT) and V1 mutant of *H. polymorpha* grown on the media supplemented with 14:0/16:0/18:0 (1 mM each) for 24 h. 14:0, 14:1, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3 (18:3, Δ9, Δ12, Δ15) indicate positions of the standard of the respective methyl esters of those fatty acids. (by using a glass column of 15% DEGS, as described in Materials and Methods 3.14)

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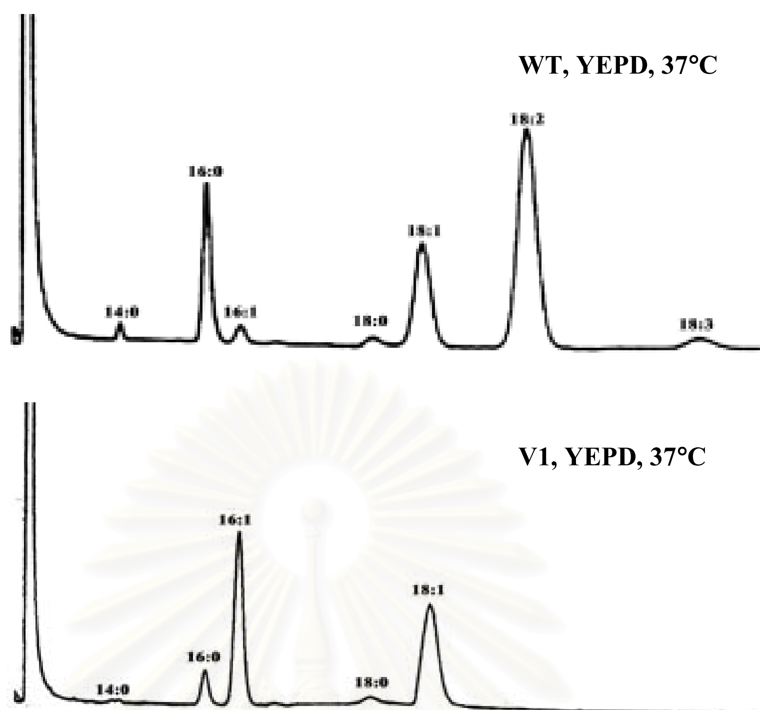

Fig. 4.10E Gas chromatographic analysis of fatty acid methyl esters derived from wild-type (WT) and V1 mutant of *H. polymorpha* grown on the media YEPD for 24 h. 14:0, 14:1, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3 (18:3, Δ9, Δ12, Δ15) indicate positions of the standard of the respective methyl esters of those fatty acids. (by using a glass column of 15% DEGS, as described in Materials and Methods 3.14)

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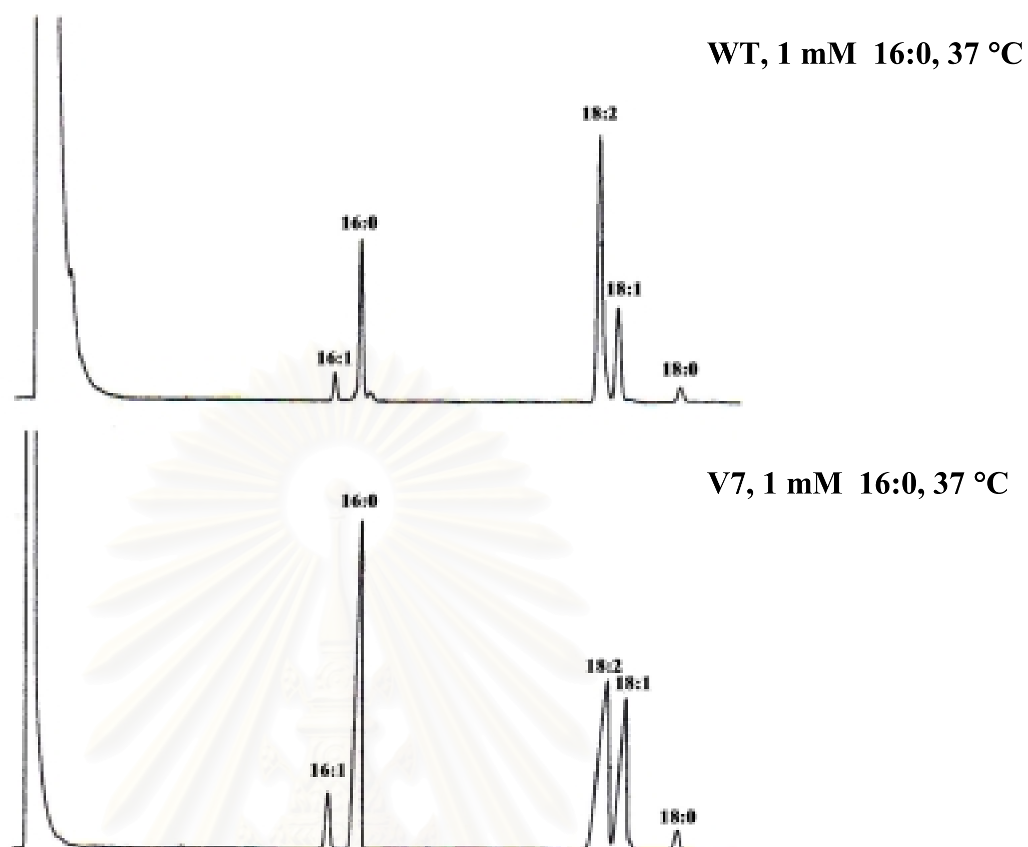

Fig. 4.11A Gas chromatographic analysis of fatty acid methyl esters derived from wild-type (WT) and V7 mutant of *H. polymorpha* grown on the media supplemented with 1 mM of 16:0 for 24 h. 14:0, 14:1, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3 (18:3, Δ9, Δ12, Δ15) indicate positions of the standard of the respective methyl esters of those fatty acids. (by using HP-5 column, as described in Materials and Methods 3.14)

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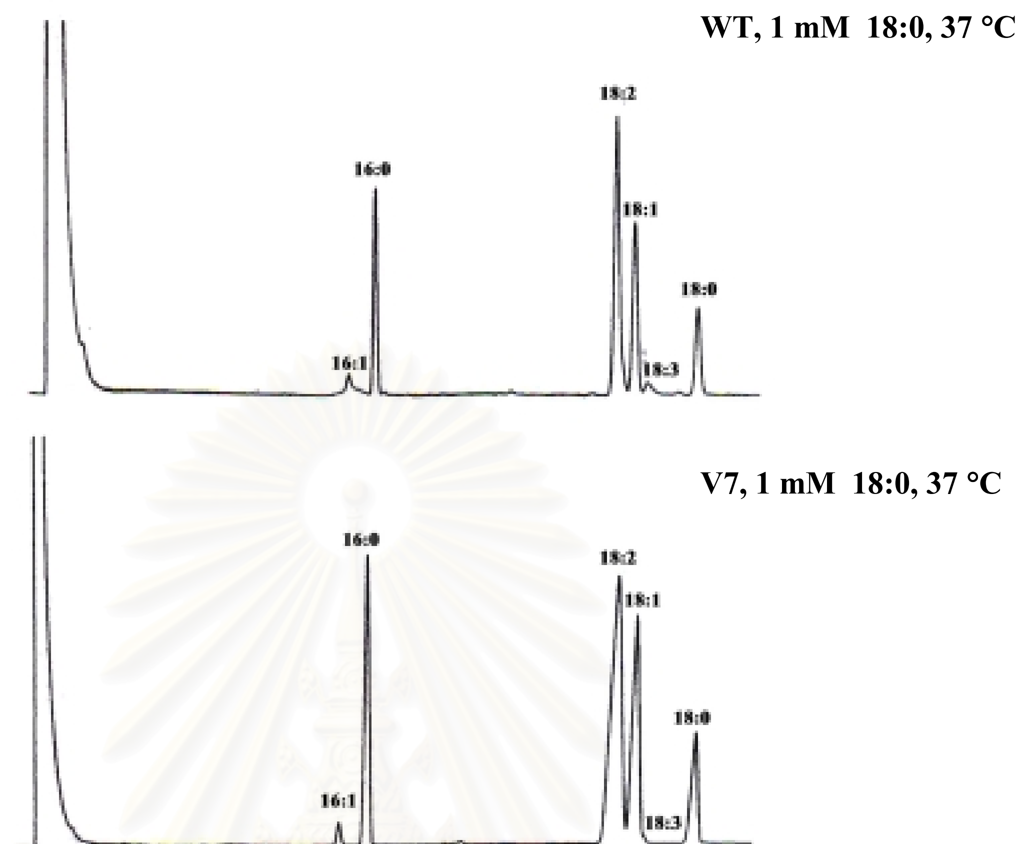

Fig. 4.11B Gas chromatographic analysis of fatty acid methyl esters derived from wild-type (WT) and V7 mutant of *H. polymorpha* grown on the media supplemented with 1 mM of 18:0 for 24 h. 14:0, 14:1, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3 (18:3, Δ9, Δ12, Δ15) indicate positions of the standard of the respective methyl esters of those fatty acids. (by using HP-5 column, as described in Materials and Methods 3.14)

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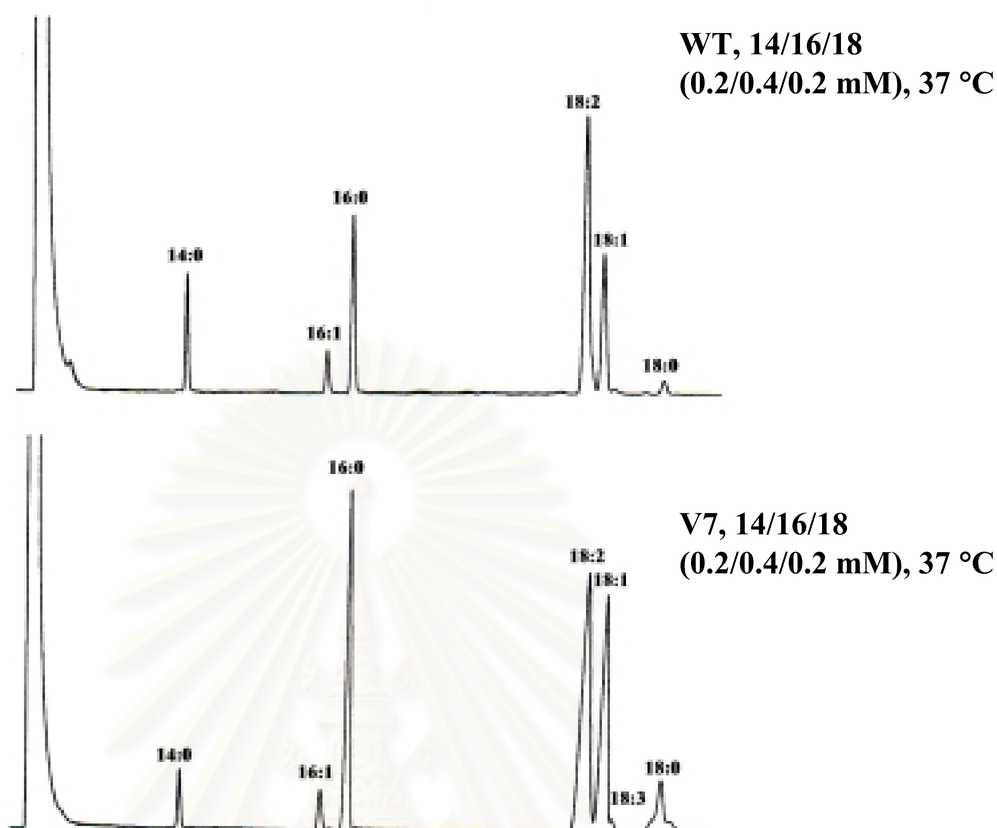

Fig. 4.11C Gas chromatographic analysis of fatty acid methyl esters derived from wild-type (WT) and V7 mutant of *H. polymorpha* grown on the media supplemented with 14:0/16:0/18:0 (0.2/0.4/0.2 mM) for 24 h. 14:0, 14:1, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3 (18:3, Δ9, Δ12, Δ15) indicate positions of the standard of the respective methyl esters of those fatty acids. (by using HP-5 column, as described in Materials and Methods 3.14)

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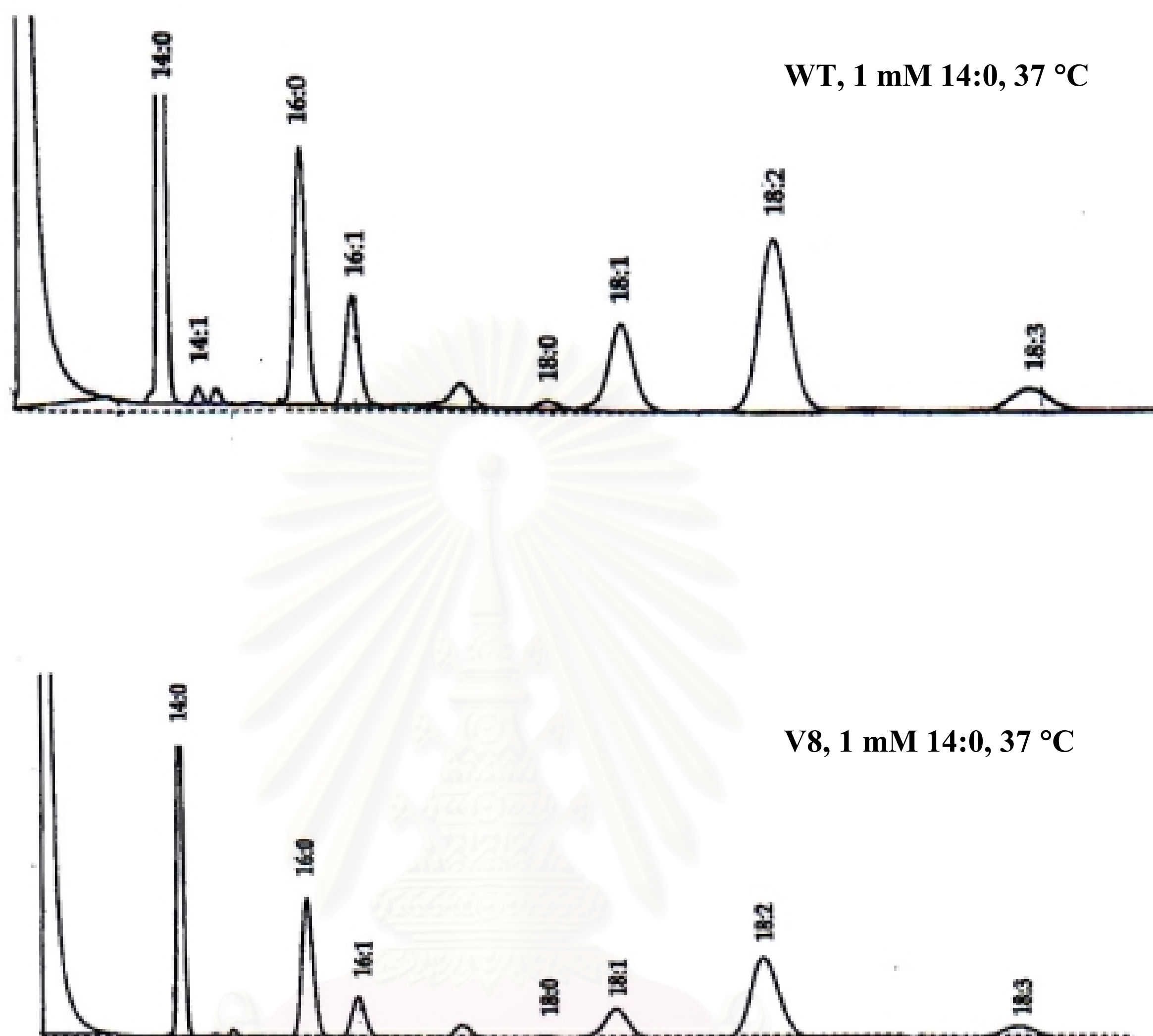

Fig. 4.12A Gas chromatographic analysis of fatty acid methyl esters derived from wild-type (WT) and V8 mutant of *H. polymorpha* grown on the media supplemented with 1 mM of 14:0 for 24 h. 14:0, 14:1, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3 (18:3, Δ9, Δ12, Δ15) indicate positions of the standard of the respective methyl esters of those fatty acids. (by using a glass column of 15% DEGS, as described in Materials and Methods 3.14)

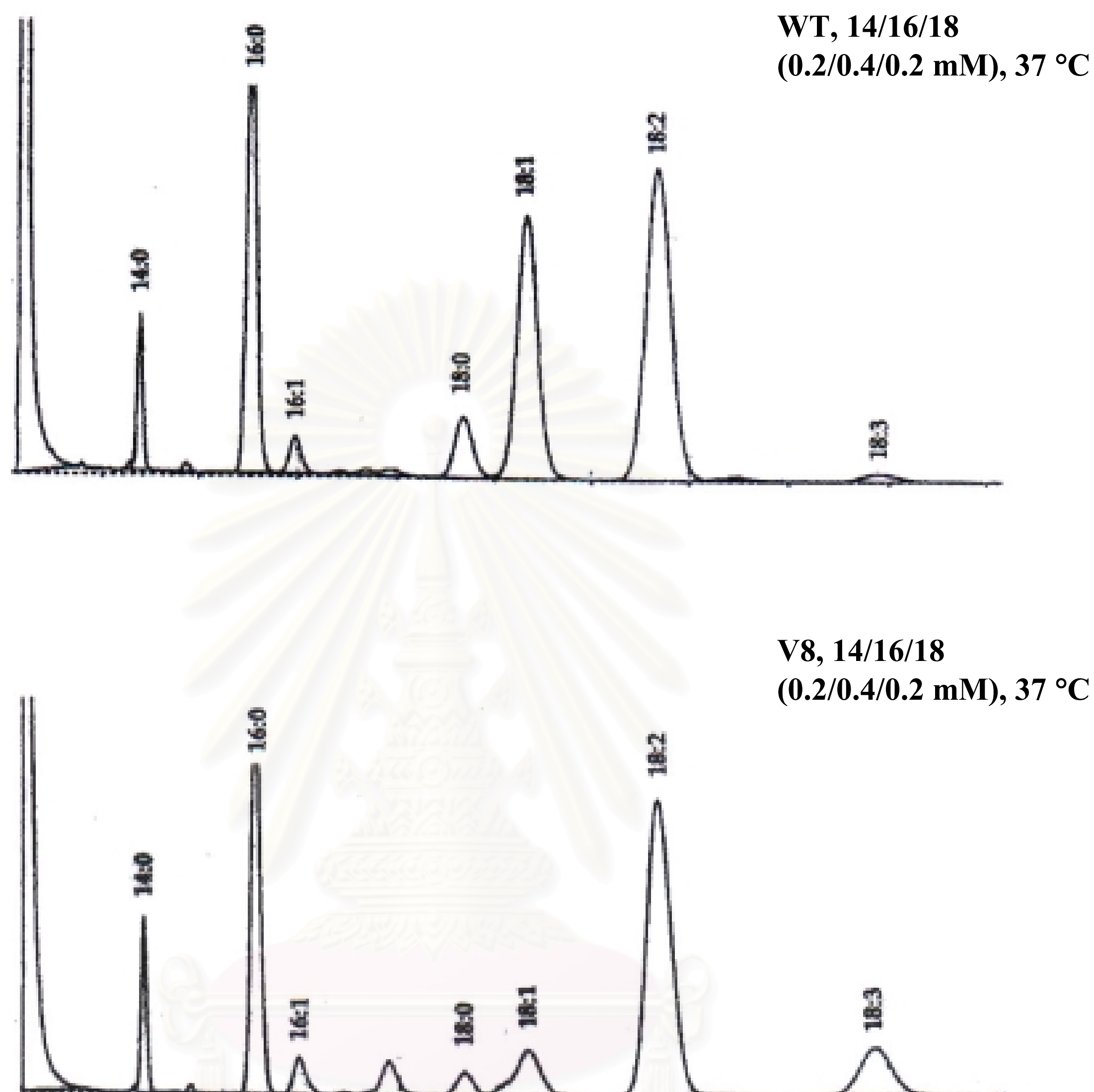

Fig. 4.12B Gas chromatographic analysis of fatty acid methyl esters derived from wild-type (WT) and V8 mutant of *H. polymorpha* grown on the media supplemented with 14:0/16:0/18:0 (0.2/0.4/0.2 mM) for 24 h. 14:0, 14:1, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3 (18:3, Δ9, Δ12, Δ15) indicate positions of the standard of the respective methyl esters of those fatty acids. (by using a glass column of 15% DEGS, as described in Materials and Methods 3.14)

The fatty acid composition in the wild-type strain, *H. polymorpha leu1-1,* and the representative mutant strains of the three groups (V1, V7 and V8 mutants) in late logarithmic stage (24 h) cultivated on YEPD and YEPD supplemented with various fatty acids are presented in Table 4.2 and Fig. 4.13 for V1, Table 4.3 and Fig. 4.14 for V7 and Table 4.4 and Fig. 4.15 for V8.

Table 4.2 Relative fatty acid composition of *H. polymorpha* WT and V1 mutant grown in YEPD broth and YEPD broth supplemented with 1 mM of 14:0, 16:0, 18:0 and a mixture of 14:0, 16:0 and 18:0 (1 mM each) for 24 h (late logarithmic phase) at 37 °C

Culture	Fatty acid composition (%) of total fatty acids in cells *									
	14:0	14:1	16:0	16:1	18:0	18:1	18:2	18:3	Others	C16/C18 raio
WT, 1 mM 14:0	25.5± 0.8	0.3± 0.4	22.0± 0.5	6.9± 0.7	2.8± 0.2	18.5± 0.5	20.9± 0.8	0.7± 0.3	2.4± 0.3	0.7
V1, 1 mM 14:0	43.9 ± 0.1	2.3 ± 0.7	14.3 ± 0.3	18.8 ± 0.4	5.0 ± 0.9	17.7 ± 0.8	0.0 ± 0.0	0.0 ± 0.0	2.8 ± 0.1	1.5
WT, 1 mM 16:0	0.4 ± 0.4	0.0 ± 0.0	31.6 ± 0.6	3.1 ± 0.3	5.9 ± 0.7	23.3 ± 0.2	32.2 ± 0.9	1.7 ± 0.8	2.4 ± 0.3	0.6
V1, 1 mM 16:0	0.6 ± 0.5	0.0 ± 0.0	82.5 ± 0.7	5.7 ± 1.1	1.6 ± 0.6	6.8 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	2.8 ± 0.4	10.5
WT, 1 mM 18:0	0.3 ± 0.1	0.0 ± 0.0	12.4 ± 0.1	1.3 ± 0.2	32.0 ± 0.9	13.9 ± 0.5	35.4 ± 0.2	3.8 ± 0.7	1.0 ± 0.1	0.2
V1, 1 mM 18:0	2.6 ± 0.1	0.0 ± 0.0	10.3 ± 0.3	7.9 ± 0.3	53.2 ± 0.5	22.3 ± 0.4	1.3 ± 0.1	0.0 ± 0.0	2.4 ± 0.2	0.2
WT, 14/16/18	20.9 ± 0.4	0.5 ± 0.0	20.0 ± 0.1	7.2 ± 0.1	3.2 ± 0.3	16.9 ± 0.3	26.7 ± 0.1	1.9 ± 0.7	2.6 ± 0.1	0.6
V1, 14/16/18	19.6 ± 0.3	0.9 ± 0.2	23.1 ± 0.5	13.5 ± 0.3	12.7 ± 0.4	26.4 ± 0.8	1.5 ± 0.7	0.0 ± 0.0	2.3 ± 0.4	0.9
WT, YEPD	1.0 ± 0.2	0.0 ± 0.0	22.0 ± 0.1	1.4 ± 0.4	2.6 ± 0.1	25.7 ± 0.3	44.0 ± 0.3	1.3 ± 0.5	1.8 ± 0.2	0.3
V1, YEPD	0.5 ± 0.4	0.0 ± 0.0	5.8 ± 0.7	38.6 ± 1.4	6.2 ± 0.9	45.2 ± 1.3	0.0 ± 0.0	0.0 ± 0.0	3.7 ± 0.7	0.9

* ± S. D. of at least three independent experiments. The fatty acid composition of each sample was analyzed twice.

Table 4.3 Relative fatty acid composition of *H. polymorpha* WT and V7 grown in YEPD broth supplemented with 1 mM of 16:0, 1 mM 18:0 and a mixture of 14:0, 16:0 and 18:0 (0.2/0.4/0.2 mM) for 24 h (late logarithmic phase) at 37 °C

Culture	Fatty acid composition (%) of total fatty acids in cells*									
	14:0	14:1	16:0	16:1	18:0	18:1	18:2	18:3	Others	C16/C18 ratio
WT, 1 mM 16:0	0.3 ± 0.3	0.0 ± 0.0	30.4 ± 1.1	3.9 ± 0.5	2.1 ± 0.3	27.1 ± 1.3	33.2 ± 0.8	1.2 ± 0.4	1.8 ± 0.5	0.5
V7, 1 mM 16:0	0.1 ± 0.2	0.0 ± 0.0	30.9 ± 0.9	3.3 ± 0.6	1.4 ± 0.4	17.2 ± 0.7	43.9 ± 1.5	0.0 ± 0.0	3.2 ± 0.4	0.6
WT, 1 mM 18:0	0.2 ± 0.1	0.0 ± 0.0	16.8 ± 0.7	1.4 ± 0.2	15.8 ± 1.1	20.1 ± 0.8	40.9 ± 0.9	0.9 ± 0.2	3.9 ± 0.6	0.2
V7, 1 mM 18:0	0.1 ± 0.2	0.0 ± 0.0	20.7 ± 0.6	1.1 ± 0.3	13.4 ± 0.5	22.9 ± 0.9	38.1 ± 1.2	1.3 ± 0.2	2.4 ± 0.3	0.3
WT, 14/16/18	3.9 ± 0.4	0.1 ± 0.1	22.1 ± 0.7	2.6 ± 0.5	5.3 ± 0.2	26.7 ± 0.6	36.9 ± 0.9	1.1 ± 0.5	1.3 ± 0.2	0.4
V7, 14/16/18	9.6 ± 0.5	0.0 ± 0.0	18.3 ± 0.8	4.3 ± 0.3	2.0 ± 0.1	19.4 ± 0.8	43.3 ± 1.3	0.3 ± 0.1	2.8 ± 0.5	0.4

* ± S. D. of at least three independent experiments. The fatty acid composition of each sample was analyzed twice.

Table 4.4 Relative fatty acid composition of *H. polymorpha* WT and V8 grown in YEPD broth supplemented with 1 mM of 14:0 and a mixture of 14:0, 16:0 and 18:0 (0.2/0.4/0.2 mM each) for 24 h (late logarithmic phase) at 37 °C

Culture	Fatty acid composition (%) of total fatty acids in cells*									C16/C18 ratio
	14:0	14:1	16:0	16:1	18:0	18:1	18:2	18:3	Others	
WT, 1 mM 14:0	32.6 ± 0.9	0.7 ± 0.8	14.3± 1.1	7.4± 0.8	1.5± 0.5	12.6± 0.7	23.9± 1.3	3.8± 0.8	3.2± 0.3	0.5
V8, 1 mM 14:0	27.0 ± 0.4	0.0 ± 0.0	18.7± 0.9	6.6± 0.5	0.8± 0.3	10.2± 0.9	27.5± 1.4	6.2± 0.7	3.2± 0.5	0.6
WT, 14/16/18	4.3 ± 0.5	0.2 ± 0.4	21.1± 1.3	2.0± 0.5	5.3± 0.8	26.2± 0.8	37.3± 0.7	1.4± 0.4	2.3± 0.3	0.3
V8, 14/16/18	5.7 ± 0.7	0.0 ± 0.0	22.0± 0.8	3.1± 0.4	2.8± 0.7	8.0± 0.5	43.7± 0.3	10.3± 0.2	4.2± 0.2	0.4

* ± S. D. of at least three independent experiments. The fatty acid composition of each sample was analyzed twice.

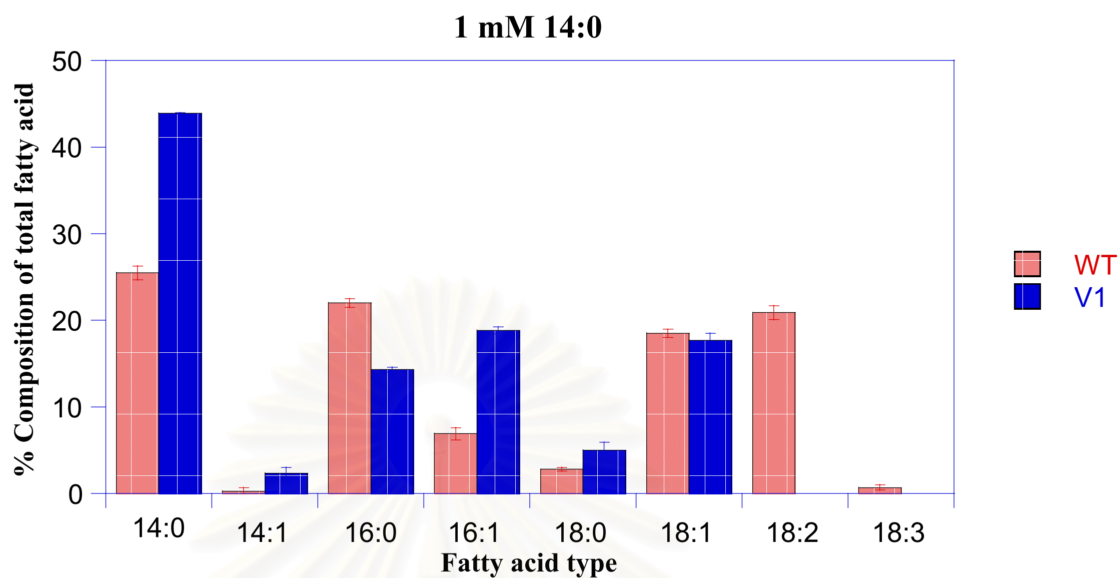

Fig. 4.13A Fatty acid composition of *H. polymorpha* WT and V1 mutant grown in YEPD broth supplemented with 1 mM of 14:0 for 24 h at 37°C.

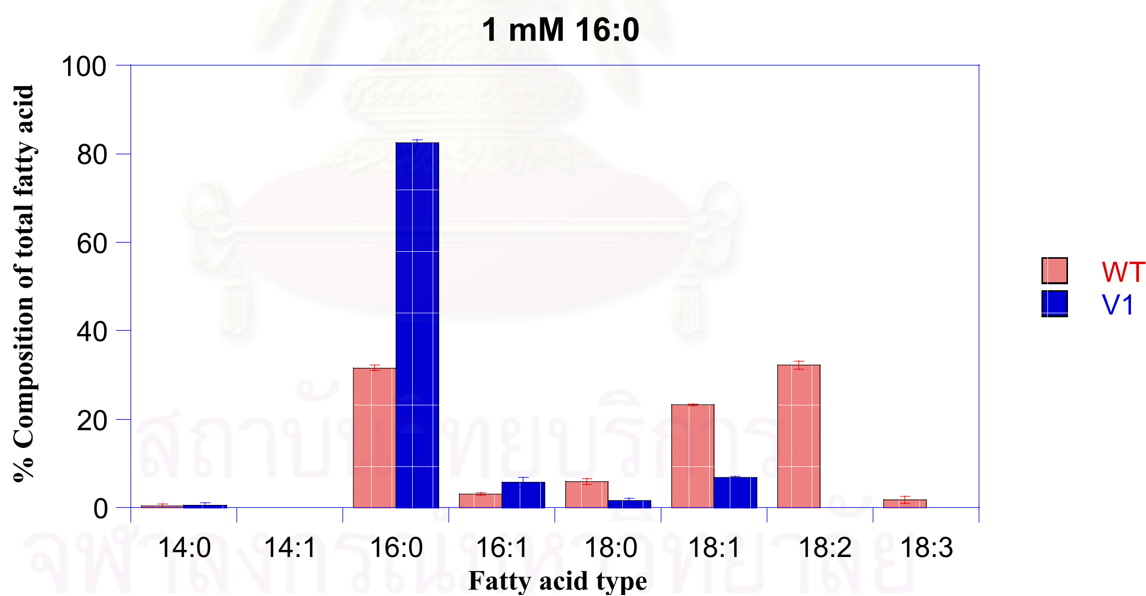

Fig. 4.13B Fatty acid composition of *H. polymorpha* WT and V1 mutant grown in YEPD broth supplemented with 1 mM of 16:0 for 24 h at 37°C.

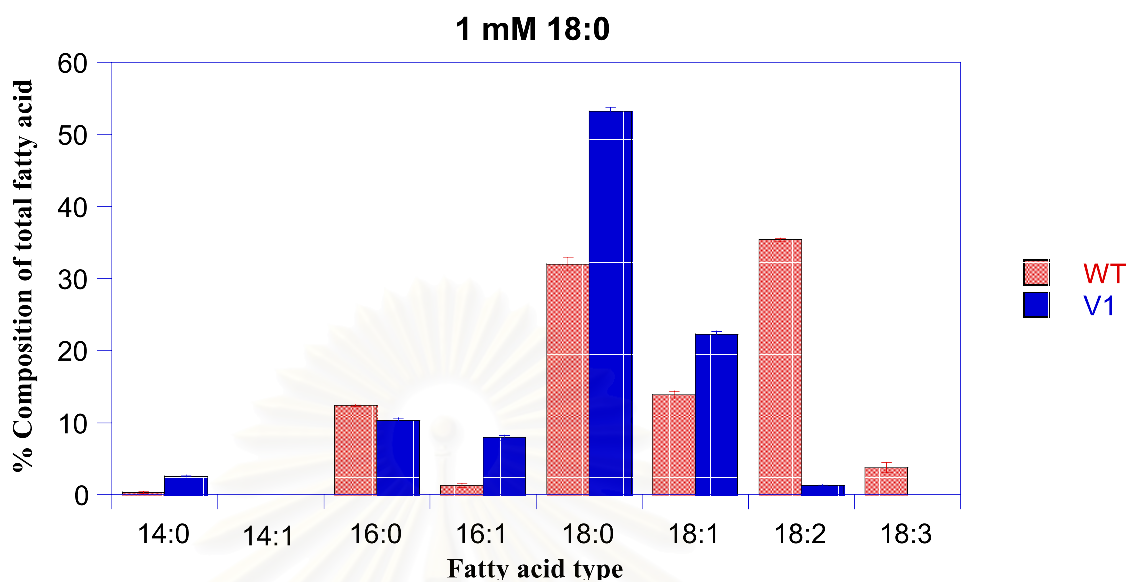

Fig. 4.13C Fatty acid composition of *H. polymorpha* WT and V1 mutant grown in YEPD broth supplemented with 1 mM of 18:0 for 24 h at 37°C.

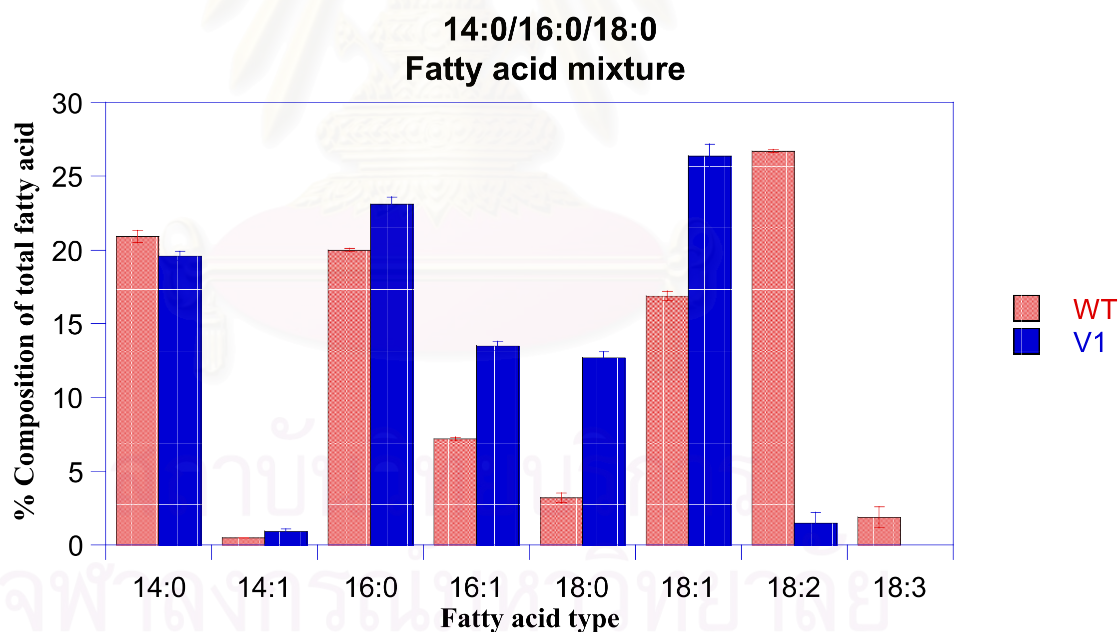

Fig. 4.13D Fatty acid composition of *H. polymorpha* WT and V1 mutant grown in YEPD broth supplemented with a mixture of 14:0, 16:0 and 18:0 (1 mM each) for 24 h at 37°C.

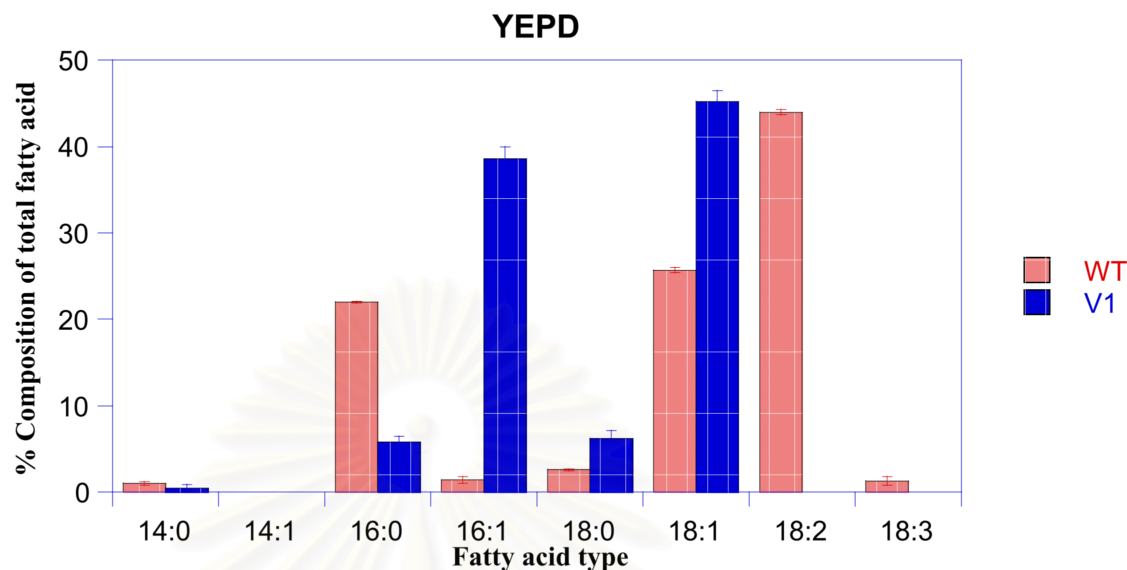

Fig. 4.13E Fatty acid composition of *H. polymorpha* WT and V1 mutant grown in YEPD broth for 24 h at 37°C.

The fatty acid profiles and compositions of V1 mutant cultivated in YEPD broth and YEPD broth supplemented with 1 mM of various fatty acids to late logarithmic stage (24 h) at 37 °C were different from those of the WT strain. The fatty acid analysis showed that linoleic (18:2, *cis*-Δ9,12) could not be detected or detected at very low relative level, and linolenic (18:3, *cis*-Δ9,12,15) could not be detected while mono-unsaturated fatty acids (C16:1 and C18:1) were also accumulated at high relative level in V1 grown at 37 °C (Table 4.2 and Fig. 4.13). Especially, C16:1, which was normally found in minor amounts in total fatty acids of WT strain, was increased significantly in V1 strain. The absence of the poly-unsaturated fatty acids and the increase of mono-unsaturated fatty acids in V1 seem to be the result of defect in desaturation processes. The result of V1 cultivating in YEPD broth at 37 °C was noted that C16:0 level was reduced and C18:0 was increased. However, when consider on the ratio of fatty acids of C16 and C18 in length, the

C16/C18 ratio of V1 cells grown in the medium of YEPD and YEPD supplemented with C14:0 demonstrated that the increase of the C16/C18 ratio of V1 was more significant than that of WT (Table 4.2). This finding provides the good explanation on the greater increase of the C16/C18 ratio of V1 was caused by the defective in elongation at the step of C16:0 to C18:0.

Taken together, the alteration of fatty acid phenotype in V1 might be caused from the defects in either elongation or desaturation processes. To further define the defective step in fatty acid biosynthesis of V1 mutant, in addition to the growth performance, fatty acid phenotype of V1 cultivated in the media containing C18:0 was determined as shown in Table 4.2. The result showed that the C18 saturated fatty acid was incorporated into the cells and subsequently converted to C18:1 and C18:2 respectively. Although the low amount of C18:2 was detected, the biotransformation of C18 fatty acids found in V1 indicated the presence of desaturation system.

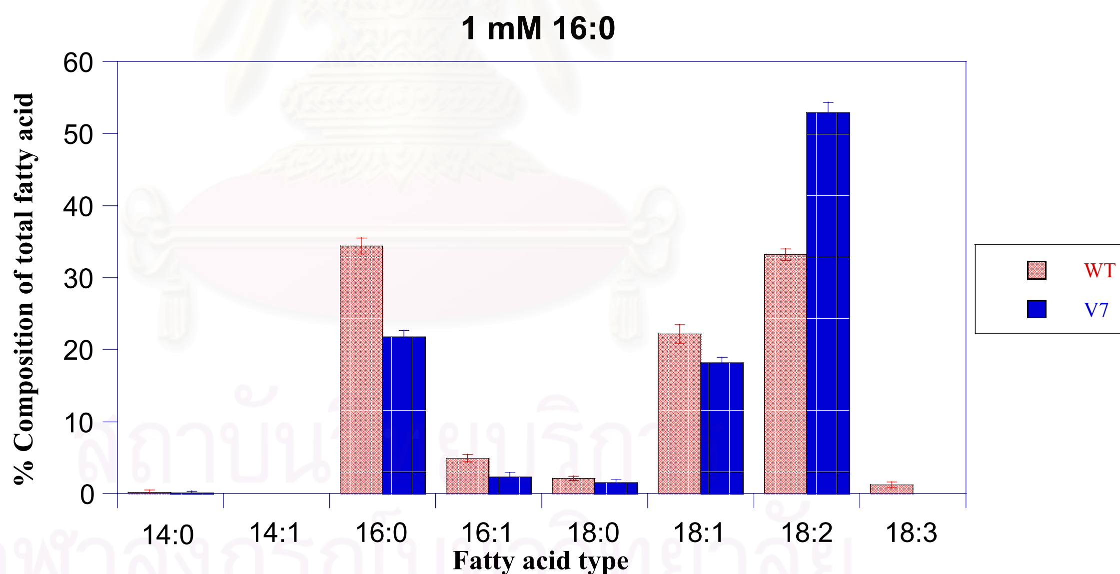

Fig. 4.14A Fatty acid composition of *H. polymorpha* WT and V7 mutant grown in YEPD broth supplemented with 1 mM of 16:0 for 24 h at 37°C.

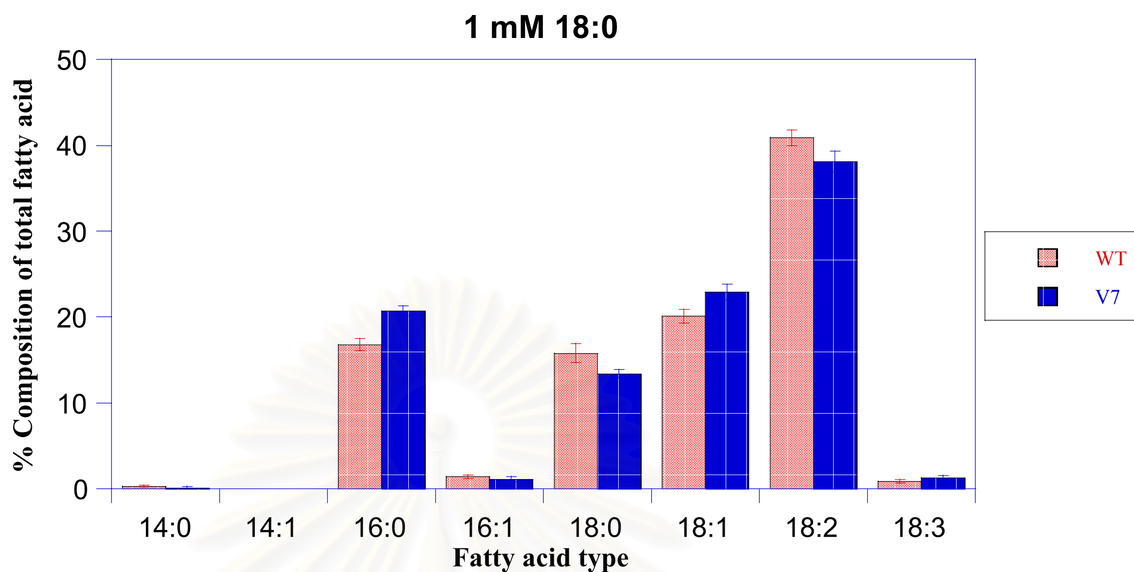

Fig. 4.14B Fatty acid composition of *H. polymorpha* WT and V7 mutant grown in YEPD broth supplemented with 1 mM of 18:0 for 24 h at 37°C.

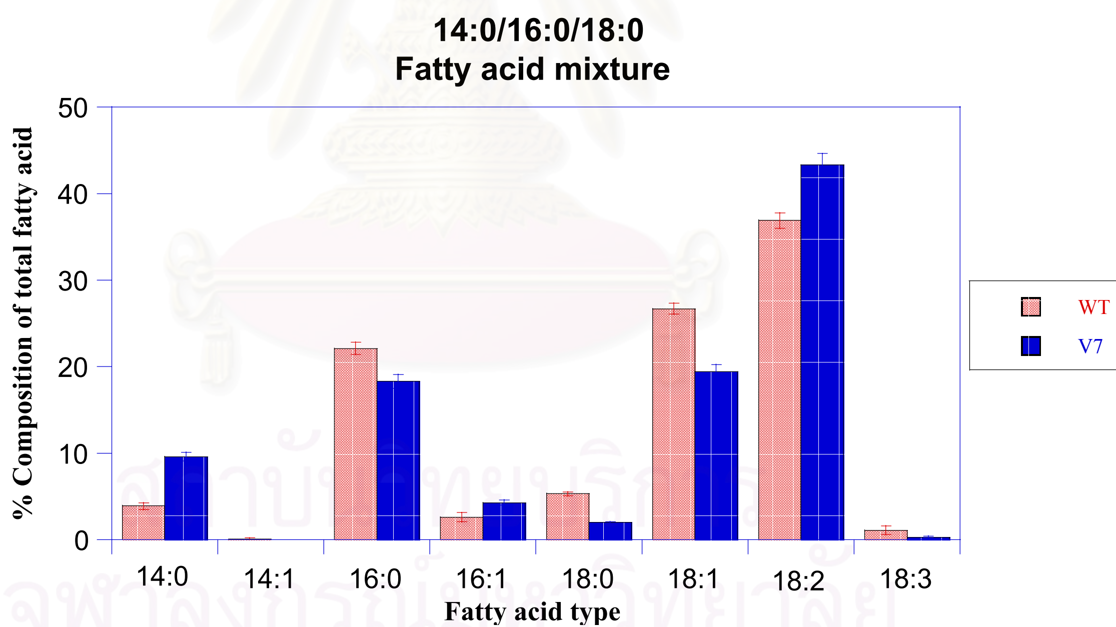

Fig. 4.14C Fatty acid composition of *H. polymorpha* WT and V7 mutant grown in YEPD broth supplemented with a mixture of 14:0, 16:0 and 18:0 (0.2/0.4/0.2 mM) for 24 h at 37°C.

The fatty acid profiles and compositions of V7 mutant cultivated in YEPD broth supplemented with C16:0, C18:0 and a mixture of various fatty acids (14/16/18 YEPD) to late logarithmic phase (24 h) at 37°C were different from those of the WT strain in some conditions. The fatty acid analysis showed that linoleic (18:2, *cis*-Δ9,12) could be detected both the V7 mutant and the WT strain at the closely relative level in any conditions as mentioned above. In addition to linolenic (18:3, *cis*-Δ9,12,15) could be detected both the V7 mutant and the WT strain excepting the cultivation of V7 in the media supplemented with 1mM of C16:0 at 37°C. Taken together, the fatty acid composition of V7 grown in the medium with the addition of C16:0, C18:0 and 14/16/18 YEPD were closely similar to the fatty acid composition of wild-type. The presence of the poly-unsaturated fatty acids demonstrated that these mutant not have the defect in desaturation process. Nevertheless, the V7 mutant mostly have the fatty acid composition and the ratio of C16/C18 similar to the wild-type but these mutant demonstrated their inability to proliferate on YEPD media and YEPD supplemented with 1 mM of C14:0. This result showed that the marked change in fatty acid composition and fatty acid phenotype in V7 might be caused from the defect in elongation or the pathway involved biosynthesis processes.

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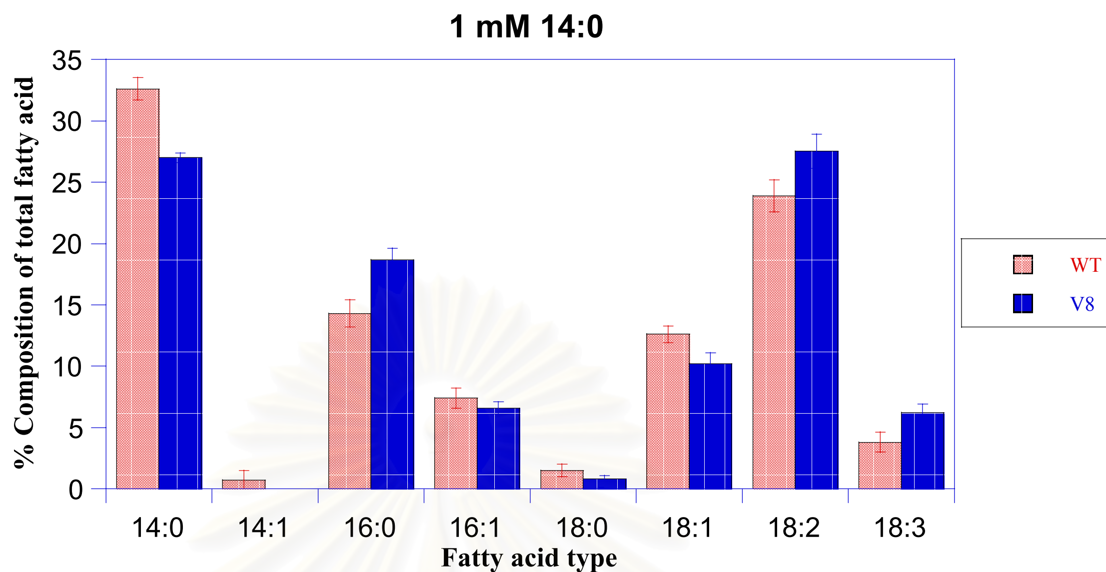

Fig. 4.15A Fatty acid composition of *H. polymorpha* WT and V8 mutant grown in YEPD broth supplemented with 1 mM of 14:0 for 24 h at 37°C.

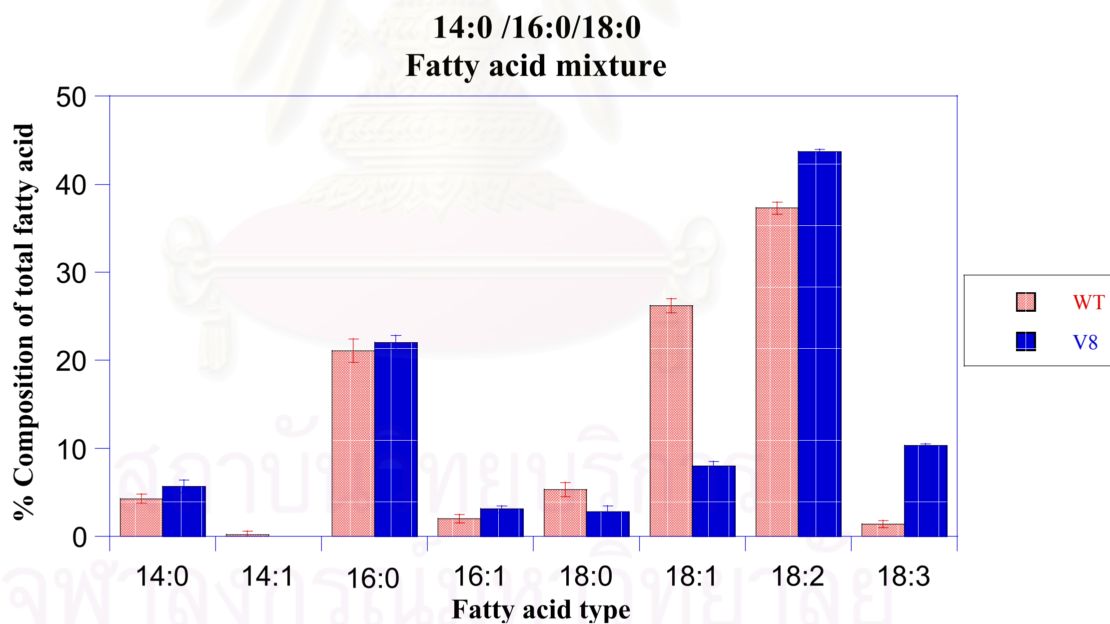

Fig. 4.15B Fatty acid composition of *H. polymorpha* WT and V8 mutant grown in YEPD broth supplemented with a mixture of 14:0, 16:0 and 18:0 (0.2/0.4/0.2 mM) for 24 h at 37°C.

The fatty acid profiles and compositions of V8 mutant cultivated in YEPD supplemented with C14:0 and a mixture of various fatty acids to late logarithmic phase (24 h) at 37°C were found that myristoleic (14:1, *cis*-Δ9) could not be detected in V8 while these fatty acid was accumulated at low relative level in WT (Table 4.4 and Fig. 4.15). Moreover, when consider on the accumulation of C18:0 of V8 mutant cultivated in YEPD supplemented with 1 mM of C14:0 and a mixture of C14:0, C16:0 and C18:0 were decreased about 2-folds when compared with those of the WT. The accumulation of C18:1 of V8 mutant grown in 14/16/18 YEPD supplementation was decreased about 3-folds when compared with those of the wild-type. In addition to C18:3 accumulation was increased about 2-7-folds when cultivated in 1 mM C14:0 and 14/16/18 (0.2/0.4/0.2 mM), respectively, both results compared with those of the wild-type.

The results of the accumulation of C18 demonstrated that desaturase enzyme in these mutant might be normally function and subsequently converted to C18:1, C18:2 and C18:3 by Δ9-, Δ12- and Δ15- desaturases, respectively. The C16/18 ratio of V8 was increased when compared with those of the WT. The growth ability demonstrated their inability to proliferate on solid and liquid YEPD medium without addition of exogenous fatty acids and YEPD with addition of C16:0 and C18:0. Taken together, the growth behavior and fatty acid composition of V8 might be cause from the defects in either elongation and/or biosynthesis processes while desaturation process might be normally occurred.

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4.2 Mating, sporulation and dissection and analysis of tetrads

The yeast hybrids between auxotrophic mutants, V1, designed as *leu1-1, fae1* and the recipient strain (*ura3-1*) were selected on 14/16/18 MIN and MIN plates. Diploid cells were then sporulated in malt extract medium (ME) for induction of sporulation as shown in Fig. 4.16A. The tetrads were analyzed the four spore products of meiosis (Fig. 4.16B). Eighty percent of the tetrads from the diploids produced four or three viable spores. Ascal cell walls were then treated with diethyl ether and released the four spores (Fig. 4.16C).

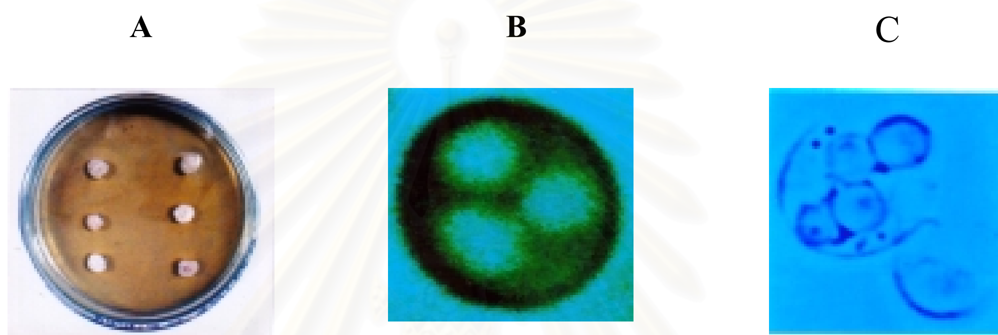

Fig. 4.16 Colony formation of diploid cells grown on ME media for 5-7 d at 37°C (A). The tetrads obtained after sporulation also have three-four spores in the ascus (B). Ascal cell walls were then treated with diethyl ether and released the four spores (C).

To confirm the hybrids designed as *ura3-1, fae1* that obtained from crossing of the recipient and the mutant strains, we selected hybrids on the selective MIN (uracil) and 14/16/18 MIN medium. The prospective hybrids should unable to grow on these selective medium but can grow on the master plate of 14/16/18 MIN (uracil). Analysis of tetrad segregation of the recipient strain (*ura3-1, FAE1*) x the mutants (*leu1-1, fae1*) demonstrated the percent of the prospective genotype (*ura3-1, fae1*) is approximately 25% of total genotype. Actually, the ratio of each genotype among the total spores is close

to 1:1:1:1, consistent with random assortment of each gene on its respective chromosome corresponding to the results by Anyatonwu, 2003. Following for the crossing results, we also got the hybrids designed as *ura3-1, fae7* from the crossing of V7 mutant (*leu1-1, fae7*) and the recipient (*ura3-1*) and the hybrids designed as *ura3-1, fae8* from crossing of V8 mutant (*leu1-1, fae8*) and the recipient (*ura3-1*).

4.3 Complementation of *H. polymorpha* fatty acid auxotrophic mutants

The marked change in fatty acid composition of V1, V7 and V8 exhibit the possibility that the defect were occurred at the elongation of C16:0 to C18:0 for the first group, C14:0 to C16:0 for the second group and before C14 for the third group. The result of fatty acid supplementation could not clearly clarify the genetic lesion in V1, V7 and V8 mutants of *H. polymorpha*. In earlier studies, at least three different yeast elongases have been defined. Elongase I extends C12-C16 fatty acyl-CoAs to C16-C18 fatty acids (Toke and Martin, 1996; Schneiter *et al*., 2000). Elongase II elongates palmitoyl-CoA and strearoyl-CoA up to C22 fatty acids and elongase III synthesizes 20-26 carbon fatty acids from C18-CoA primers (Oh *et al*., 1997; Rossler *et al*., 2003; Han *et al*., 2002).

Thus, *ELO1* and *ELO2* are apparently involved in the elongation of all the three groups of the mutants. In order to investigate the defect in the elongation step, the V1 mutant was transformed with *S. cerevisiae ELO1* and *ELO2* genes. The V7 and V8 mutants were transformed with *S. cerevisiae ELO1* gene. From crosses of the V1, V7 and V8 mutants (*fae* mutants) with an auxotrophic strain (*ura3-1*), the segregants (*ura3-1*, *fae1; ura3-1*, *fae7; ura3-1*, *fae8*) of all the three groups of the mutants were used for complementation test. The plasmids YCpELO1.MOD were harbored the coding sequences of *S. cerevisiae ELO1* and containing the *URA3* gene as a selective marker. In addition to the YCpGALELO2(U) harboring the coding sequences of *S. cerevisiae ELO2* which containing the *URA3* gene as a selective marker and galactose-inducible

GAL promoter. From the characteristic genotype of the appropriate plasmids, YCpELO1.MOD and YCpGALELO2(U) we preparated all the mutants to have the genotype of *ura3-1* from crossing between all the three groups of the *fae* mutants and the auxotrophic strain (*ura3-1*, *FAE1*) which allowed to screen the prospective transformants from the selective medium of MIN after complemented with the specific plasmid.

The transformation of the yeast cells were carried out using the lithium acetate method (Sambrook et al., 1986) and then plated onto the minimal medium supplemented with the fatty acids (14/16/18 MIN) and added 3% galactose. Transformation efficiency was determined by plating aliquots of transformed cells on the MIN medium. It was found that only the *ELO2* transformants could grow on the medium containing galactose without fatty acid supplementation as shown in Fig. 4.17. Therefore, fifteen of ~ 150 transformant cells designed as VT1 (*URA3, FAE1*) carrying *ELO2* were randomly chosen.

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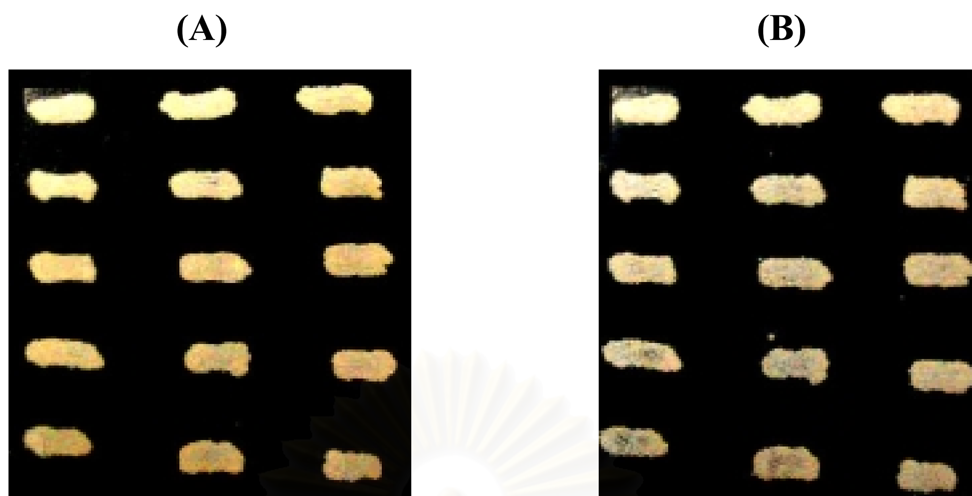

Fig. 4.17 Growth phenotype of the fifteen representative VT1 transformants (*URA3, FAE1*) which transformanted with the *S-ELO2* gene, streaked on the 14/16/18 MIN media containing galactose (A) and then replicated on MIN media containing galactose (B). Photographs were taken after 2 d incubation.

Following to confirm that the VT1 have defect at the step of elongation of C16 to C18 and really possed a mutation in the *ELO2* gene, we reextracted the plasmids from the VT1 transformant and then transformed to *Escherichia coli* in order to multify these specific plasmids. We further reextracted these plasmids from *E. coli* and carried out checking the DNA by electrophoresis. The results from Fig. 4.18 demonstrated that the DNA fragment of the VT1 transforment revealed the same size of DNA as found in the *S-ELO2* gene, indicating that the *ELO2* gene of *S. cerevisiae* was the similar gene that gain function in *H. polymorpha* V1.

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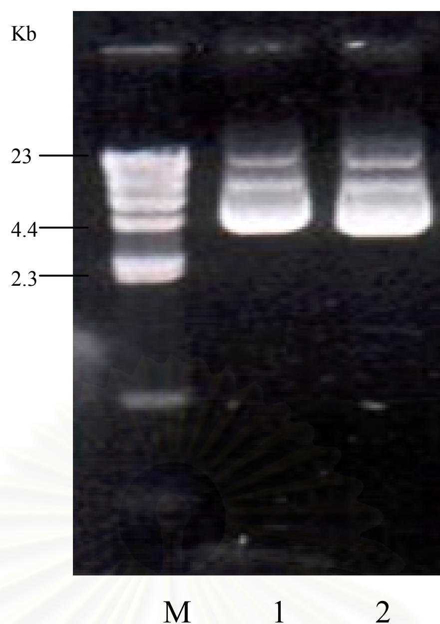

Fig. 4.18 Agarose gel electrophoresis of *Hind*III/lambda DNA marker (M), DNA fragment of the reextracted plasmids from the VT1 transforment (lane 2) and the band of the YCpGALELO2(U) (lane 3).

Therefore, *ELO2* is apparently involved in the defect at the elongation of C16:0 to C18:0. In order to investigate the marked change in the VT1, each single colony was used for phenotypic testing comparative with the recipient strain (*ura3-1*). Phenotypic analysis demonstrated that the growth abilities of the representative transformant designated as VT1 (*URA3, FAE1*) was similar to that of the *ura3-1* strain (Fig. 4.19). On the contrary of the V1 mutant, the VT1 strain could grow on both the media with or without exogenous fatty acids.

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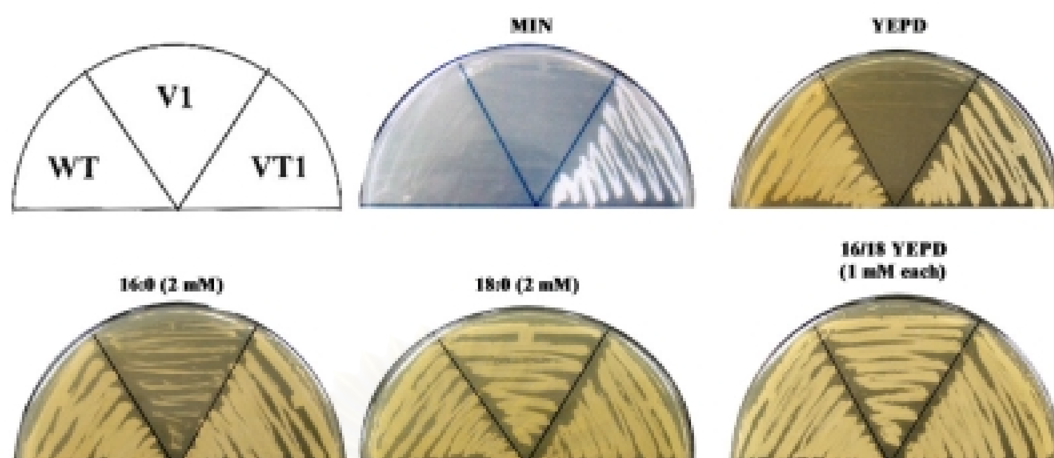

Fig. 4.19 Phenotypic analyses of mutant V1, transformant VT1 and WT strain of *H. polymorpha* on solid complete YEPD supplemented with different fatty acids or minimal medium. Photographs were taken after incubation at 37°C for 4 d.

We further analysed the growth characteristic of VT1 transformant in liquid medium containing different saturated fatty acids revealed the similar results as found in solid medium in which the growth of VT1 could reach closely to that of the WT strain under any conditions as showned in Fig. 4.20.

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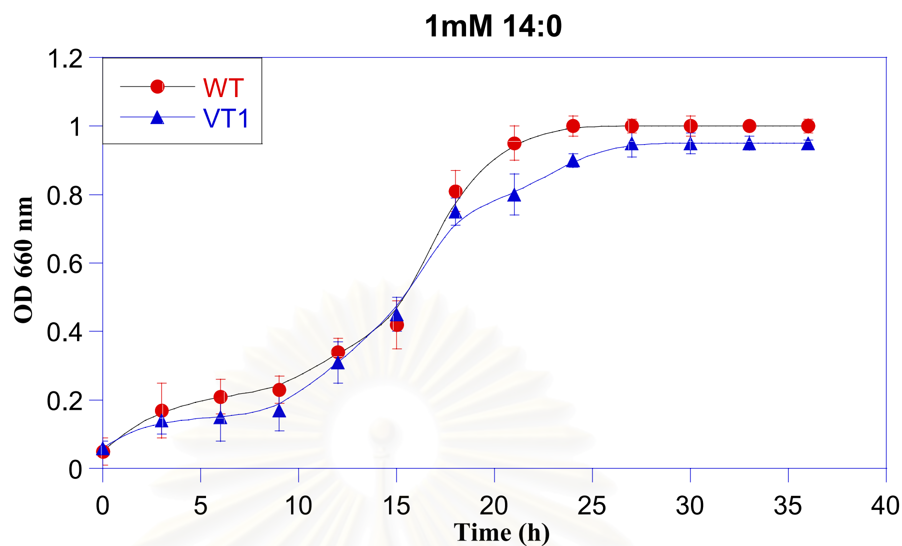

Fig. 4.20A Growth profiles of WT and VT1 mutant of *H. polymorpha* cultivated on liquid media of YEPD supplemented with 1 mM of 14:0 at 37 °C.

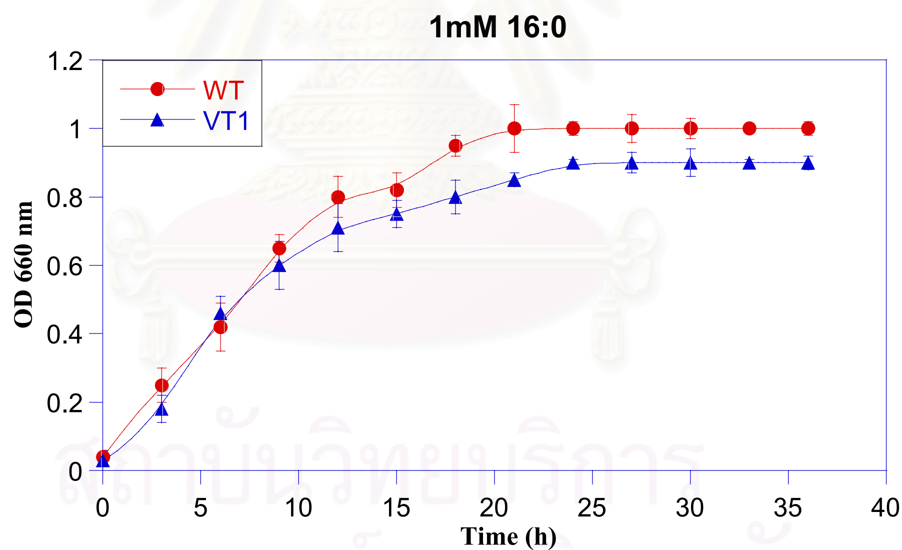

Fig. 4.20B Growth profiles of WT and VT1 mutant of *H. polymorpha* cultivated on liquid media of YEPD supplemented with 1 mM of 16:0 at 37 °C.

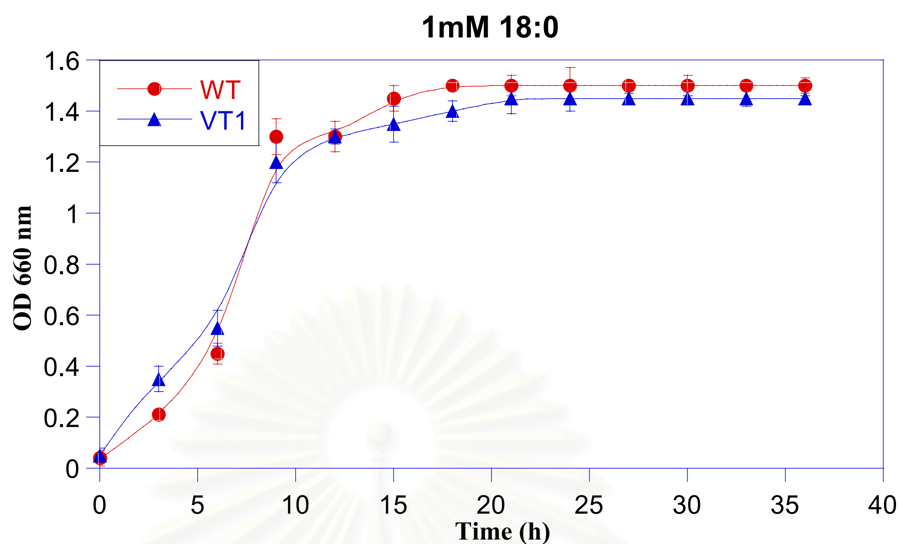

Fig. 4.20C Growth profiles of WT and VT1 mutant of *H. polymorpha* cultivated on liquid media of YEPD supplemented with 1 mM of 18:0 at 37 °C.

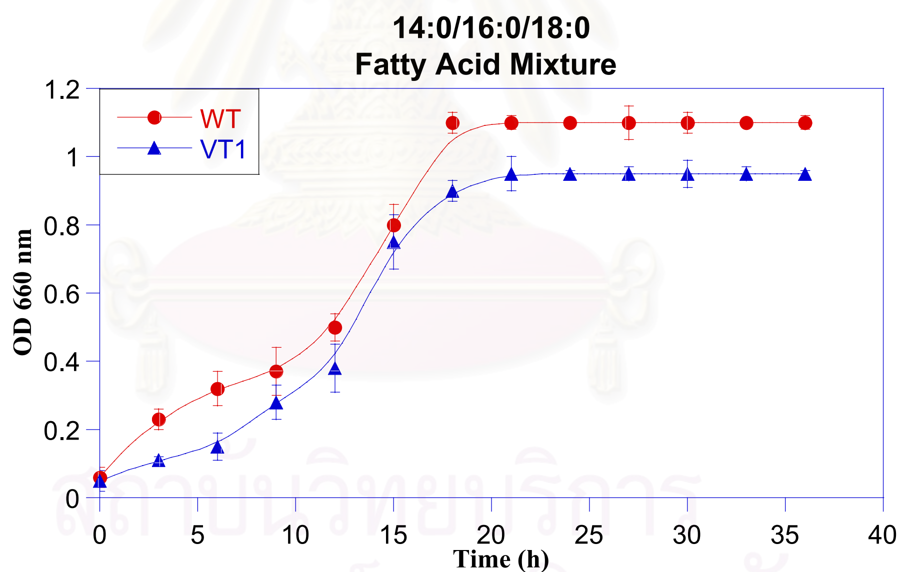

Fig. 4.20D Growth profiles of WT and VT1 mutant of *H. polymorpha* cultivated on liquid media of YEPD supplemented with 14:0/16:0/18:0 (0.2/0.4/0.2 mM) at 37 °C.

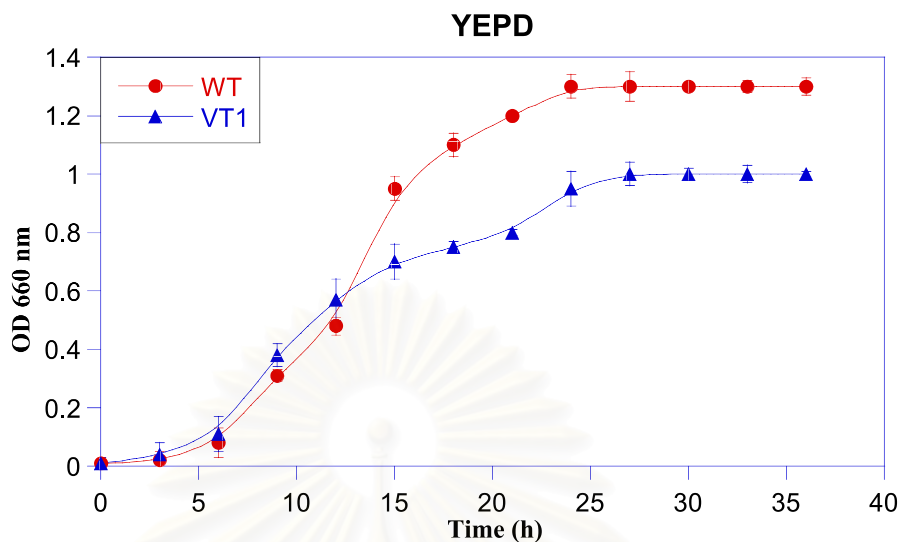

Fig. 4.20E Growth profiles of WT and VT1 mutant of *H. polymorpha* cultivated on liquid media of YEPD media at 37 °C.

Interestingly, comparison of chromatograms of fatty acid methyl esters from the WT strain and VT1 mutant grown in YEPD at 37 °C for late logarithmic phase noted that their fatty acids were similar in content and profile (Fig. 4.21). The fatty acid compositions and profiles of the VT1 mutant cultivated in any conditions were further analysed and compared with those of the WT strain as shown in Table 4.5 and Fig. 4.22 that is coincided with the characteristics of growth. In addition, the relative fatty acid composition of WT, V1 and VT1 cultivated in YEPD broth at 37 °C was shown in Table 4.6.

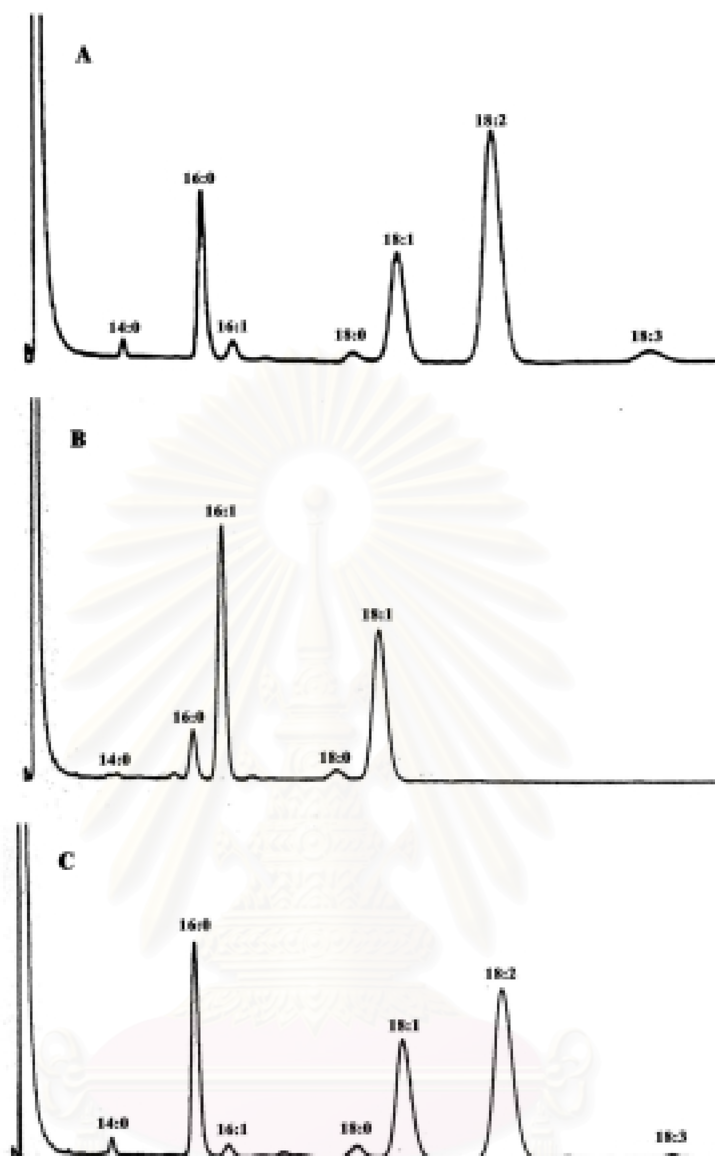

Fig. 4.21 Gas chromatographic analysis of fatty acid methyl esters derived from WT strain (A), V1 mutant (B) and VT1 (C) of *H. polymorpha* grown in YEPD at 37 °C, for 24 h (late logarithmic phase). 14:0, 16:0, 16:1, 18:0, 18:1, 18:2 and 18:3 indicate positions of the methyl esters of myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1-Δ9), stearic (C18:0), oleic (C18:1-Δ9), linoleic (C18:2-Δ9,12) and α-linolenic (C18:3-Δ9,12,15) acids, respectively. (by using a glass column of 15% DEGS, as described in Materials and Methods 3.14)

Table 4.5 Relative fatty acid composition of *H. polymorpha* WT and VT1 mutant grown in YEPD broth and YEPD broth supplemented with different fatty acids for 24 h at 37°C

Culture	Fatty acid composition (%) of total fatty acids in cells*								
	14:0	14:1	16:0	16:1	18:0	18:1	18:2	18:3	Others
WT, 1 mM 14:0	25.7 ± 0.5	0.0 ± 0.0	22.2 ± 0.1	7.1 ± 0.1	2.5 ± 0.2	18.3 ± 0.1	21.3 ± 0.9	0.6 ± 0.1	2.4 ± 0.1
VT1, 1 mM 14:0	27.5 ± 0.3	0.0 ± 0.0	18.1 ± 0.7	6.9 ± 1.0	1.4 ± 0.9	15.6 ± 0.8	25.4 ± 1.3	2.9 ± 1.5	2.1 ± 1.1
WT, 1 mM 16:0	0.4 ± 0.4	0.0 ± 0.0	31.6 ± 0.6	3.1 ± 0.3	5.9 ± 0.7	23.3 ± 0.2	32.2 ± 0.9	1.7 ± 0.8	2.4 ± 0.3
VT1, 1 mM 16:0	0.3 ± 0.3	0.0 ± 0.0	26.7 ± 0.9	3.6 ± 0.7	2.0 ± 1.5	23.3 ± 0.8	41.5 ± 1.8	1.2 ± 0.5	1.4 ± 0.4
WT, 1 mM 18:0	0.3 ± 0.1	0.0 ± 0.0	12.4 ± 0.1	1.3 ± 0.2	32.0 ± 0.9	13.9 ± 0.5	35.4 ± 0.2	3.8 ± 0.7	1.0 ± 0.1
VT1, 1 mM 18:0	0.3 ± 0.3	0.0 ± 0.0	18.2 ± 0.8	0.9 ± 1.0	16.8 ± 0.7	22.1 ± 0.2	40.9 ± 0.3	0.7 ± 0.7	0.3 ± 0.4
WT, 14/16/18	20.9 ± 0.4	0.5 ± 0.0	20.0 ± 0.1	7.2 ± 0.1	3.2 ± 0.3	16.9 ± 0.3	26.7 ± 0.1	1.9 ± 0.7	2.6 ± 0.1
VT1, 14/16/18	12.3 ± 0.1	0.2 ± 0.3	18.4 ± 0.2	4.1 ± 0.1	2.2 ± 0.2	19.9 ± 0.5	39.5 ± 0.9	2.1 ± 0.0	1.2 ± 0.2
WT, YEPD	1.0 ± 0.2	0.0 ± 0.0	22.0 ± 0.1	1.4 ± 0.4	2.6 ± 0.1	25.7 ± 0.3	44.0 ± 0.3	1.3 ± 0.5	1.8 ± 0.2
VT1, YEPD	0.6 ± 0.3	0.0 ± 0.0	27.4 ± 0.6	1.0 ± 0.1	4.1 ± 0.5	19.5 ± 0.4	42.3 ± 1.1	1.7 ± 0.8	3.5 ± 0.5

* ± S. D. of at least three independent experiments. The fatty acid composition of each sample was analyzed twice.

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Table 4.6 Relative fatty acid composition of parental strain (WT), V1 and VT1 of *H. polymorpha* grown in YEPD broth at 37 °C to late logarithmic phase. Stearic acid (C18:0) at the concentration of 1 mM was supplemented in V1 culture.

Culture	Fatty acid composition (%) of total fatty acids in cells[a]								C16/C18 ratio
	14:0	16:0	16:1	18:0	18:1	18:2	18:3	Others	
WT	1.0 ± 0.2	22.0 ± 0.1	1.4 ± 0.4	2.6 ± 0.1	25.7 ± 0.3	44.0 ± 0.3	1.3 ± 0.5	1.8 ± 0.2	0.3
V1	0.5 ± 0.4	5.8 ± 0.7	38.6 ± 1.4	6.2 ± 0.9	45.2 ± 1.3	0.0 ± 0.0	0.0 ± 0.0	3.7 ± 0.7	0.9
V1, 1 mM18:0	2.6 ± 0.1	10.3 ± 0.3	7.9 ± 0.3	53.2 ± 0.5	22.3 ± 0.4	1.3 ± 0.1	0.0 ± 0.0	2.4 ± 0.2	0.2
VT1	0.6 ± 0.3	27.4 ± 0.6	1.0 ± 0.1	4.1 ± 0.5	19.5 ± 0.4	42.3 ± 1.1	1.7 ± 0.8	3.5 ± 0.5	0.4

[a] ± S. D. of at least three independent experiments. The fatty acid composition of each sample was analyzed twice.

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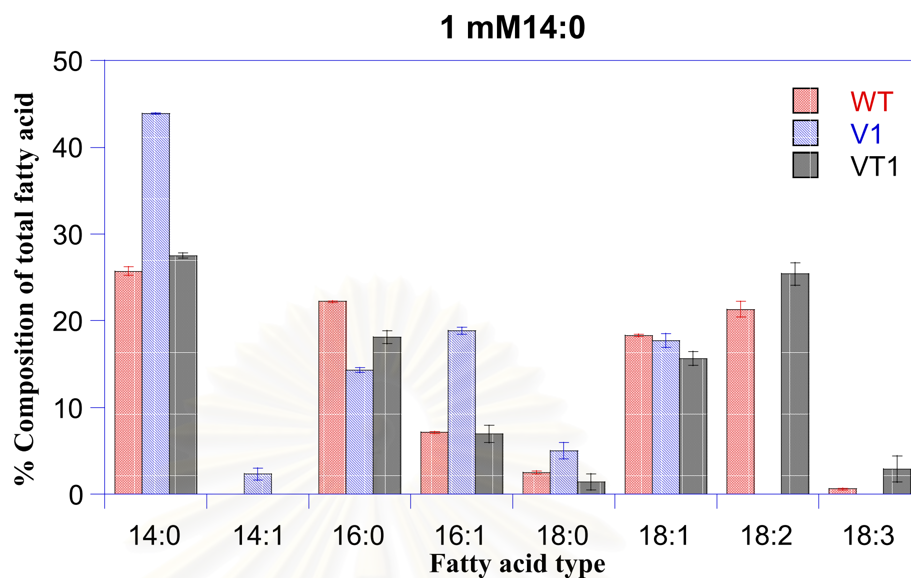

Fig. 4.22A Fatty acid composition of *H. polymorpha* WT, V1 mutant and VT1 mutant grown in YEPD broth supplemented with 1 mM of 14:0 for 24 h at 37°C.

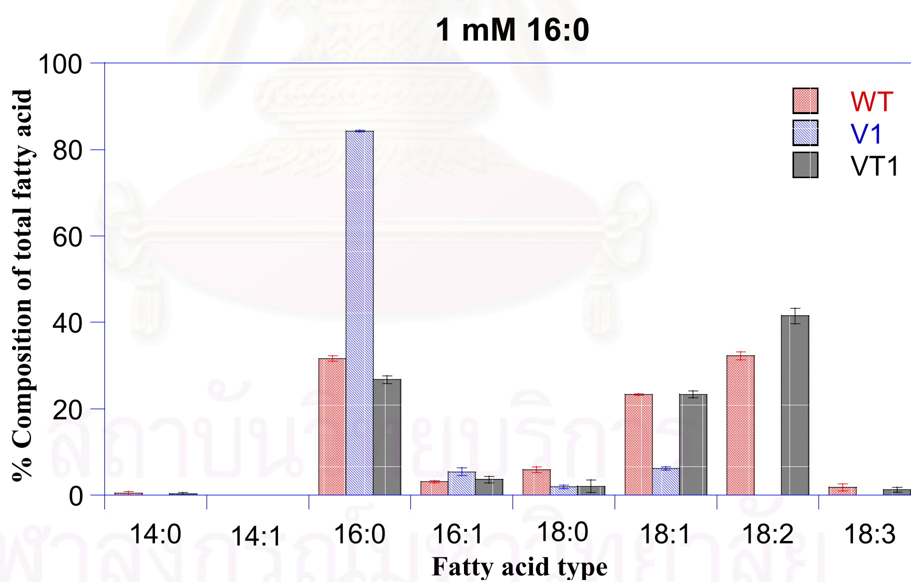

Fig. 4.22B Fatty acid composition of *H. polymorpha* WT, V1 mutant and VT1 mutant grown in YEPD broth supplemented with 1 mM of 16:0 for 24 h at 37°C.

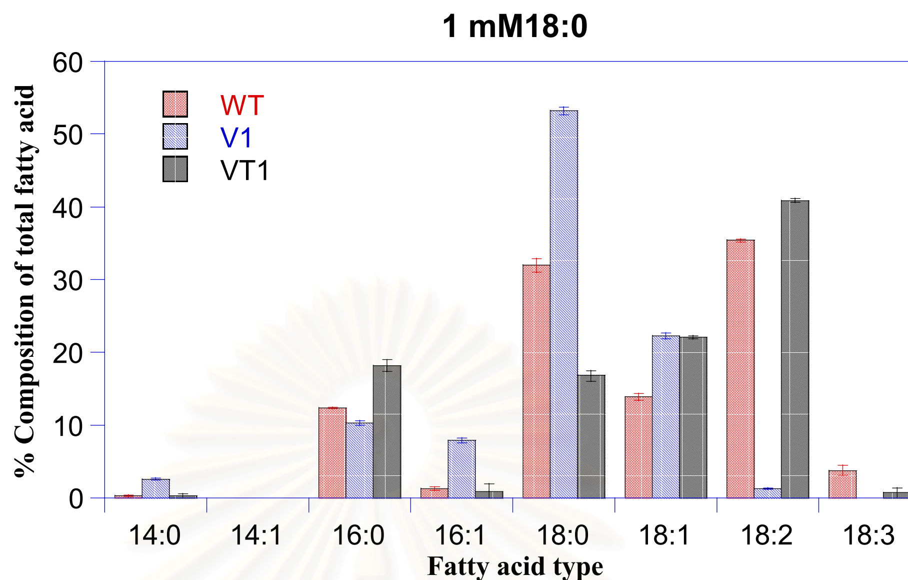

Fig. 4.22C Fatty acid composition of *H. polymorpha* WT, V1 mutant and VT1 mutant grown in YEPD broth supplemented with 1 mM of 18:0 for 24 h at 37°C.

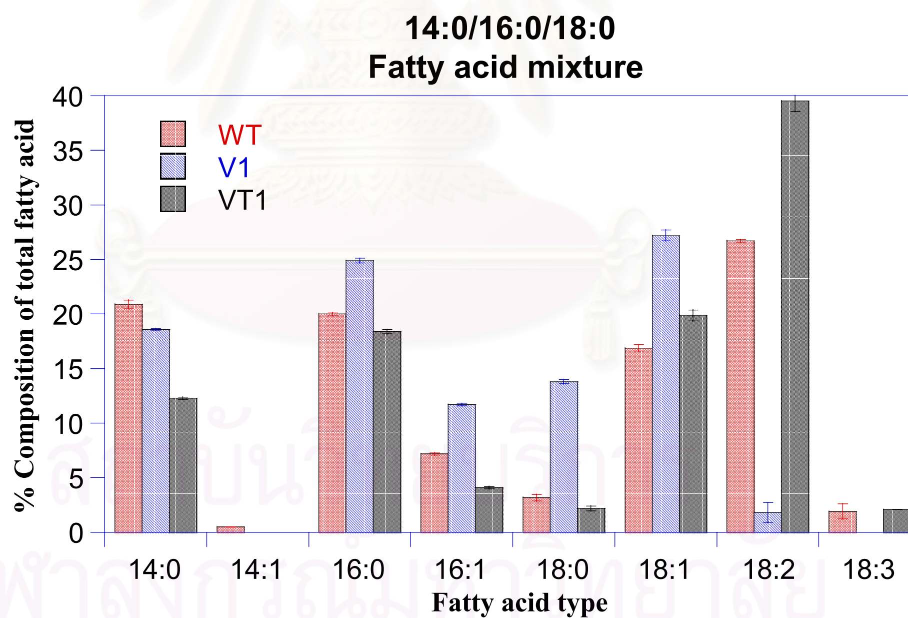

Fig. 4.22D Fatty acid composition of *H. polymorpha* WT, V1 mutant and VT1 mutant grown in YEPD broth supplemented with 1 mM each of 14:0, 16:0 and 18:0 for 24 h at 37°C.

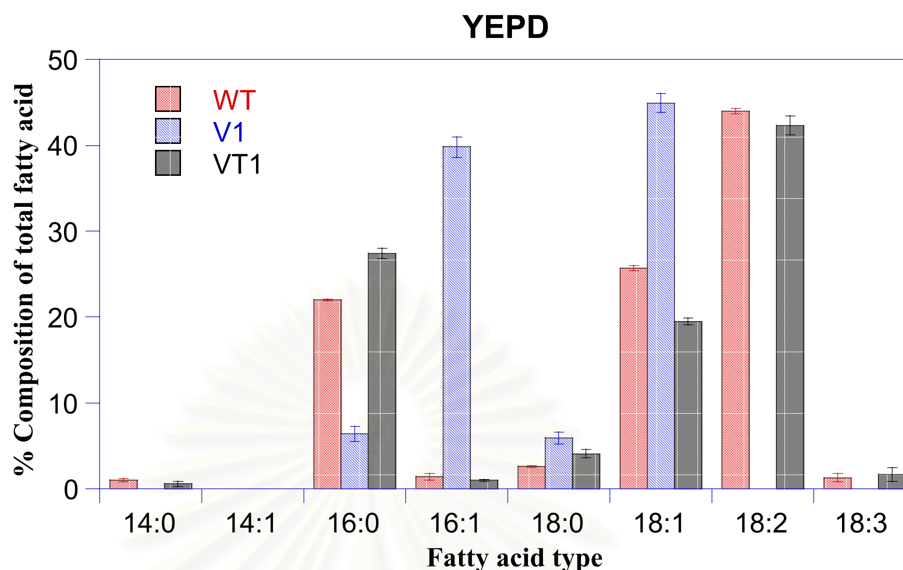

Fig. 4.22E Fatty acid composition of *H. polymorpha* WT, V1 mutant and VT1 mutant grown in YEPD broth for 24 h at 37°C.

This result demonstrated that the *ELO2* gene of *S. cerevisiae* could gain function in *H. polymorpha* V1. Taken together, our finding indicated a clear picture of the lesion in fatty acid biosynthesis in V1 mutant that the partial defect at least in the elongation step of C16 to C18 which is involved in ELO2 enzyme was found. The defect of this elongation system of the *H. polymorpha* mutant might be occurred in either transcription or translation levels.

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CHAPTER V

CONCLUSIONS

The identification of yeast *H. polymorpha* mutants defected in fatty acid elongation was performed. In previous study, selection of fatty acid auxotrophic mutants unable to grow in the absence of fatty acid has been performed on a few microorganisms (Resnick and Mortimer, 1966; Scott, 1977; Chattopadhyay *et al.*, 1985; Toke and Martin, 1996; Oh *et al*, 1998). Using C14:0, C16:0, C18:0 supplemented medium, mutants with defects at various steps of the elongation pathway were obtained. From the growth phenotype and fatty acid compositions, the mutagenized cells were classified into three groups according to the changes in their fatty acid profiles compared to that of WT grown at late logarithmic phase of cultivation time.

The first group of mutants (V1-V6) could not grow on 1 mM of C16:0 while 2 mM of C16:0 and 1mM and 2mM of 18:0 could support the growth of these mutants as shown in Fig. 4.2 and 4.3. From the ability of the first group of mutants to grow in the presence of high concentration of C16:0 (2 mM) can be suggested from the viewpoint by assuming either weak activity of the partially damaged elongase enzyme in the step of C16 to C18 extention or the presence of other enzyme in the step of C16 to C18 extention or the presence of other enzymes involved in fatty acid biosynthesis. In contrast, the growth of this group of mutants on the media supplemented with C16:0 was also less than the growth of WT strain and the first group mutants supplemented with C18:0 (Fig. 4.2 and 4.3). It was demonstrated that C18 fatty acid was essential for proper growth of this yeast.

The fatty acid compositions of the first group of mutants cultivated in YEPD broth and YEPD broth supplemented with 1 mM of various fatty acids to late logarithmic stage at 37 ^{o}C were different from those of the WT. This mutant displayed an accumulation of C18:2 (*cis*-Δ9,12) at very low relative

level and C18:3 (*cis*-Δ9,12,15) could not be accumulated while C16:1 (*cis*-Δ9) and C18:1 (*cis*-Δ9) were also accumulated at high relative level (Table 4.2 and Fig. 4.13). The absence of the poly-UFAs and the accumulation of mono-UFAs, especially C16:1 in the first group of mutants seem to be the results of defect in desaturation processes or the assumption that a two-carbon-atom shortening of the chain length of fatty acids (*i.e.*, C16:1) was on average equivalent to inserting one double bond (*i.e.*, C18:2) (Raclot and Groscolas, 1993). As we have demonstrated, C16:1 was accumulated at very high relative level while C18:2 could not be detected (Fig. 4.10E and Fig. 4.13E). When consider on the ratio of fatty acids of C16 and C18 in length, the C16/C18 ratio of this mutants was increased about 3-folds when compared with those of the parental strain. The accumulation of high level of C16 fatty acids (C16:0 + C16:1) in the first group might be explained by the lesion in the conversion of C16:0 to C18:0 by elongase.

Taken together, the alteration of fatty acid phenotype of the first group of mutants might be occurred from the defects in either elongation or desaturation processes. When this group grown in media containing C18:0 The C16/C18 ratio was similar to the ratio of the WT, providing the good explanation on the effect of C18 supplementation for promoting the growth of this group as mentioned in previous studies (van Roermund *et al.*, 1998; Lee and Ahlquist, 2003). However, the low amount of C18:2 and non-triene fatty acid might be resulting from the partial defective in desaturation.

The second group included only one mutant (V7) whose no growth when grown on YEPD medium supplemented with 1mM of C14:0 while 1mM of C16:0 and 1mM of C18:0 could support their growth (Fig. 4.2). Ability of this group to proliferate in the presence of C16:0 and C18:0 can be explained from the viewpoint by assuming activity of elongase enzyme in the level of C16 to C18 extension, nevertheless, their growth on those medium were also less than the growth of WT (Fig. 4.2). The differences in the fatty acid compositions of the second group cultivated in YEPD and YEPD

supplemented with various fatty acids compared with those of the WT were observed. This group also displayed the accumulation of C18:2 and C18:3 similar to the WT strain (excepting the cultivation in the media added with 1mM of C16:0 at 37 ^{o}C) suggesting that this mutant has no the defect in desaturation process. Moreover, the fatty acid composition of this mutant grown in a mixture of fatty acids supplementation and the ratio of C16/C18 were closely similar to those of WT strain. In contrast, the second group demonstrated their inability to proliferate on YEPD and YEPD added with 1 mM of C14:0, these observations suggested that this mutant might be caused from the defect in elongation process in the step of C14 to C16 or the pathway involved biosynthesis processes.

Mutants in the final group (V8,V9,V10) displayed the ability to grow on medium added with 1mM of C14:0, in contrast, these mutants did not grow on medium cultivated with 1 mM of C16:0 and 1 mM of C18:0 supplementation. Moreover, the growth of this group on media supplemented with C14:0 was also less than the growth of WT (Fig. 4.2). Taken together, from the total results suggested that these mutants might be defects in elongation process in the step before C14 extension or the defect of other enzymes involved in essential biosynthesis that need C14:0 for synthesis. Several essential proteins of *S. cerevisiae* such as G proteins require C14:0 to be covalently bound to their amino-terminal glycine for biological function (Russell *et al*., 1994). The addition of C14:0 can also stabilize protein-protein interactions and many acylated proteins require this modification for full expression of their biological function (Mcllhinney, 1998). In *Leishmania*, the myristate-containing glycophosphatidylinositol (GPI) anchors of the surface glycoinositolphospholipids are remodeled during synthesis by a system involving myristate exchange from a myristoyl-CoA donor (Ralton and McConville, 1998).

The results of the accumulation of C18 suggested that desaturase enzyme in these mutant might be normally function and subsequently

converted to C18:1, C18:2 and C18:3 by Δ9-, Δ12- and Δ15- desaturases respectively. Interestingly, the results of C16/C18 ratio of this mutant was increase when compared with those of the WT strain. Nevertheless, the pattern of distribution of cellular fatty acids that observed in the third group was closely similar to that of the WT but showing their inability to grow on solid and liquid medium without/ with addition of C16:0 and C18:0. Following with the total results of the growth behavior and fatty acid composition of the third group, they might be caused from the defects in either elongation and/or biosynthesis processes.

The marked change in fatty acid composition of all the three group of the mutants exhibit the possibility that the defect were occurred at the elongation of C16:0 to C18:0 for first group, C14:0 to C16:0 for the second group and before C14 for the third group. Therefore, *ELO1* and *ELO2* are apparently involved in the elongation of all the three groups of the mutants according to the previous studies (Oh *et al.*, 1997; Rossler *et al.*, 2003; Han *et al.*, 2002). After the preparation of all mutant to obtain the prospective genotype (*ura* minus) and then transformed with the appropriate gene, it was found that only the *ELO2* transformants could grow on the medium (containing galactose) without fatty acid addition. Following with the phenotypic analysis, the growth abilities and the fatty acid composition of the representative transformant designated as VT1 (*URA3*, *FAEI*) was similar to that of the *ura3-1* strain, the results suggesting that the *ELO2* gene of *S. cerevisiae* could gain function in *H. polymorpha* mutant (only the first group).

In conclusion, selection and characterization of the mutant deficient in the specific step of C16 to C18 elongation in this study was based on their inability to grow in the absence of exogenous fatty acids. This is the first report on the study of elongation of fatty acids in *H. polymorpha*. The mutant strain gained in this work will be used as a valuable tool for studying the metabolic pathway of fatty acid synthesis and the role of fatty acids in *H. polymorpha* as shown in Fig. 5.

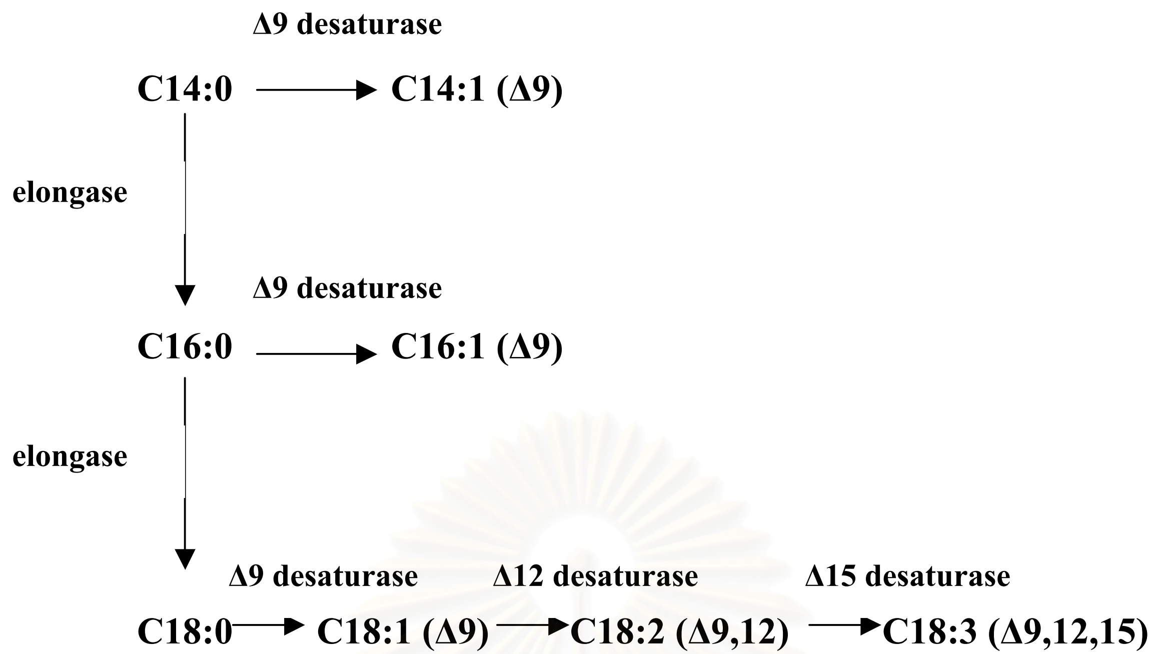

Fig. 5.1 Hypothetical scheme for the biosynthesis of fatty acids in *H. polymorpha*

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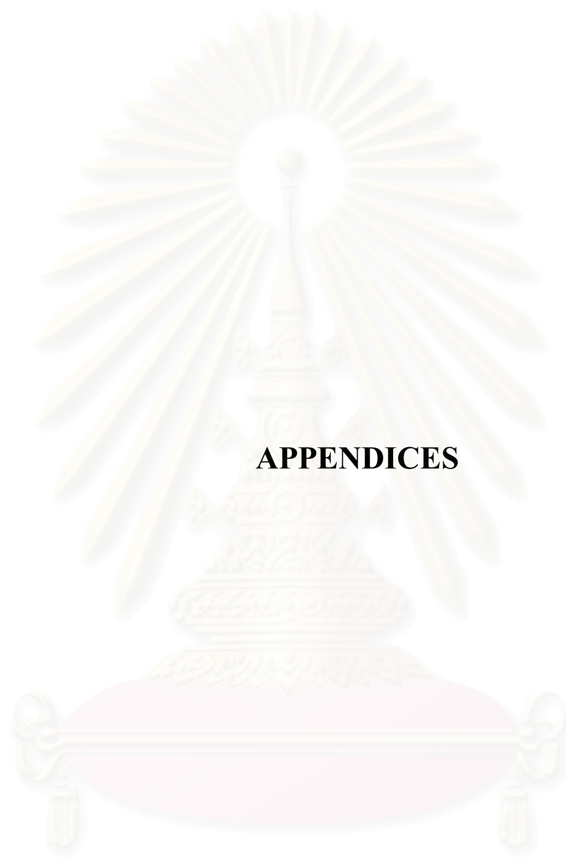

APPENDICES

สถาบันวิทยบริการ
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APPENDIX I

Media

1. Yeast extract peptone dextrose medium (YEPD)

Yeast extract	10	g
Bacto-peptone	20	g
Dextrose	20	g
Agar	20	g

Making up to a volume of 1 liter with deionized water and autoclave.

2. Minimal medium (MIN)

Bacto-yeast nitrogen base without amino acids	6.7	g
Glucose	20	g
Agar	20	g

Making up to volume of 1 liter with deionized water and autoclave.

3. Minimal medium (MIN) containing Uracil

Bacto-yeast nitrogen base without amino acids	6.7	g
Glucose	20	g
Agar	20	g
Uracil (1X working volumn)	10	ml

Making up to volume of 1 liter with deionized water and autoclave.

4. Minimal medium (MIN) containing 3% Galactose

Bacto-yeast nitrogen base without amino acids	6.7	g
Glucose	20	g
Agar	20	g
Galactose	30	g

Making up to volume of 1 liter with deionized water and autoclave.

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5. Maltose-malt extract medium (MAE)

Maltose	27	g
Malt extract	3	g
Agar	20	g

Making up to volume of 1 liter with deionized water and autoclave.

6. Malt extract medium (ME)

Malt extract	30	g
Agar	20	g

Making up to volume of 1 liter with deionized water and autoclave.

7. LB agar

Bacto-tryptone	10	g
Yeast extract	5	g
NaCl	10	g
Adjust to pH 7.0 and then added,		
Agar	13	g

Making up to a volume of 1 liter with deionized water and autoclave.

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APPENDIX II

Reagents

1. 0.2 M Sodium phosphate buffer, pH 8.0

$Na_2HPO_4.12H_2O$	186.4	ml
$Na_2HPO_4.2H_2O$	186.4	ml
Deionized water	500	ml

Adjust to pH 8.0 with 1 N NaOH. Making up to a volume of 1 liter with deionized water.

2. 50 mM Tris-HCl, pH 7.4

Tris base	1.21	g
Deionized water	150	ml

Adjust to pH 7.4 with 6N HCl. Making up to a volume 200 ml with deionized water and store at 4 ^oC.

3. 10% SDS

Sodium dodecyl sulfate (SDS)	100	g
Deionized water	500	ml

Making up to a volume of 1 liter with deionized water, autoclave and store at room temperature. Wear a face mask while weighing out SDS.

4. TE-buffer

Tris-HCl (pH 7.4) 10 mM	1.21	g
EDTA 1 mM	0.37	g

Adjust to pH 7.4. Making up to a volume 1 liter with deionized water and autoclave.

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5. 10x TBE buffer

Tris base	108	g
Baric acid	55	g
EDTA	9.5	g
Deionized water	750	ml

Adjust to pH 8.3 with 1N NaOH. Making up to a volume 1 liter with deionized water. For use: dilute to 0.5x by deionized water.

6. 10x Loading buffer

Bromophenol blue	2.5	ml
Xylenecyanol	2.5	ml
Glycerol	400	ml

Making up to volume 1 Liter with deionized water. Store at 4 ^{o}C. Warm to room temperature before use if precipitation occurs.

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APPENDIX III

Determination of fatty acid profiles of V1 mutant grown in different concentrations of individual fatty acids supplementation and different temperatures

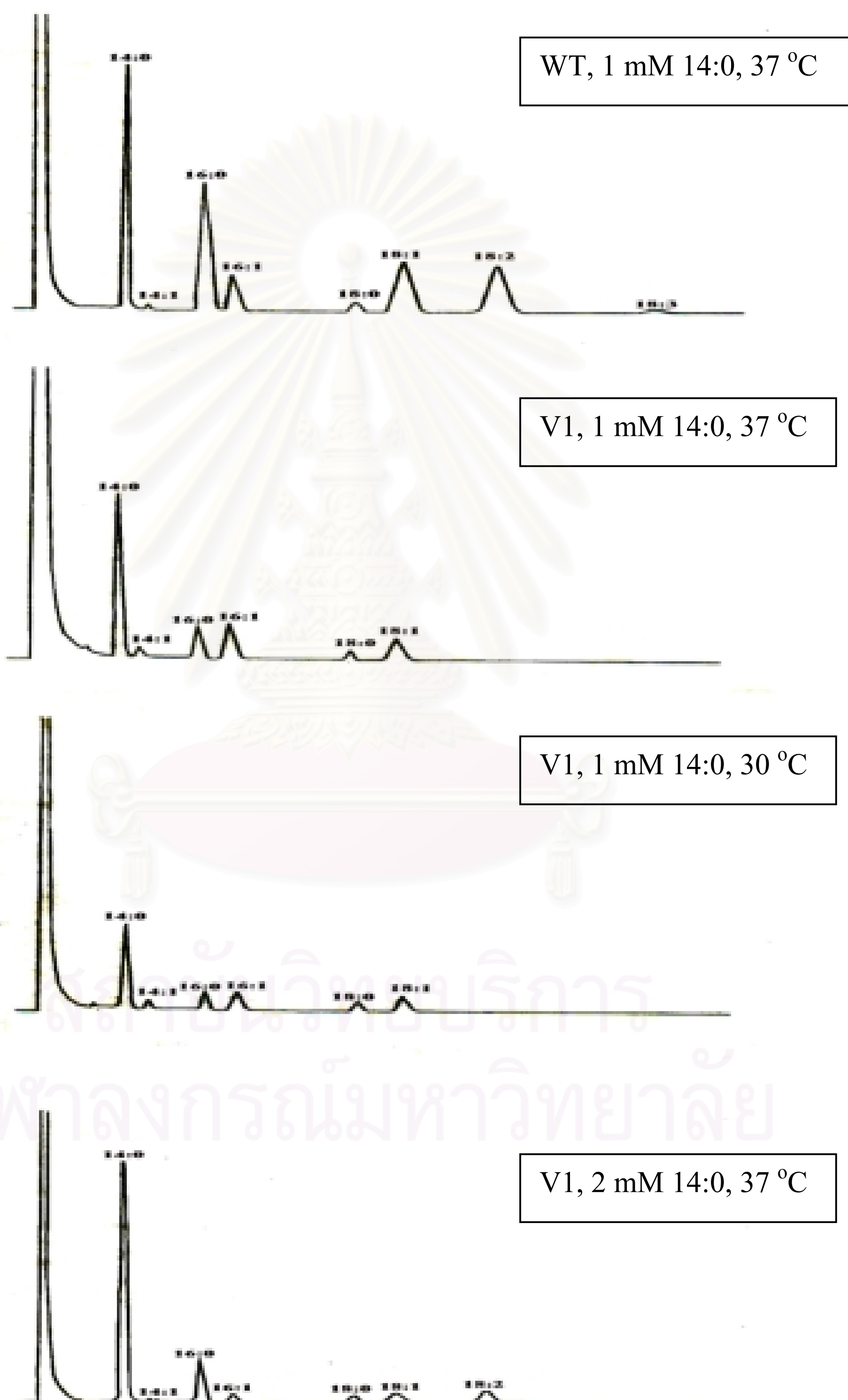

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Fig. 1 Gas chromatographic analysis of fatty acid methyl esters derived from V1 mutant of *H. polymorpha* grown on the media supplemented with different concentrations of C14:0 (1 and 2 mM) and different temperatures (30 °C and 37 °C).

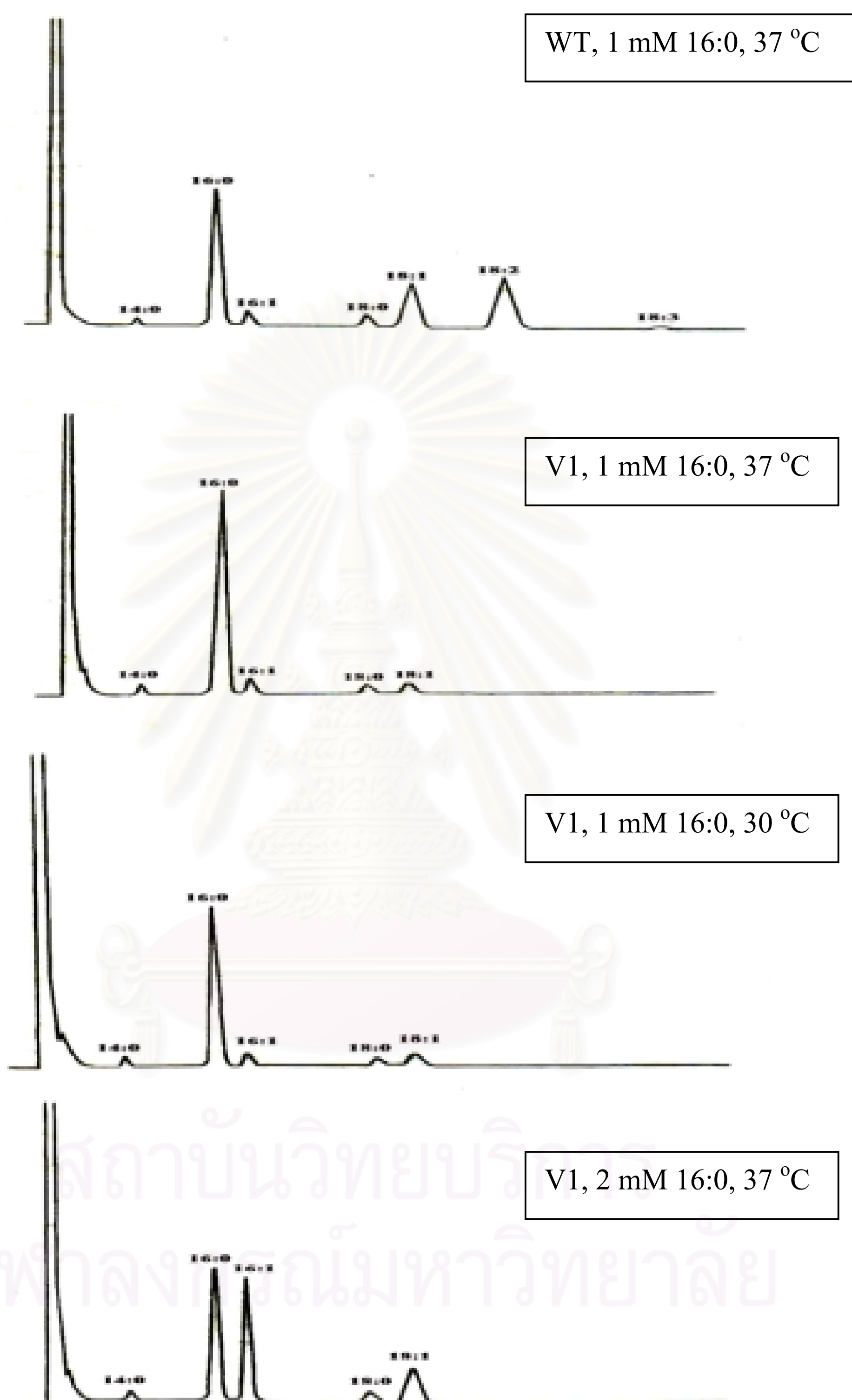

Fig. 2 Gas chromatographic analysis of fatty acid methyl esters derived from V1 mutant of *H. polymorpha* grown on the media supplemented with different concentrations of C16:0 (1 and 2 mM) and different temperatures (30 °C and 37 °C).

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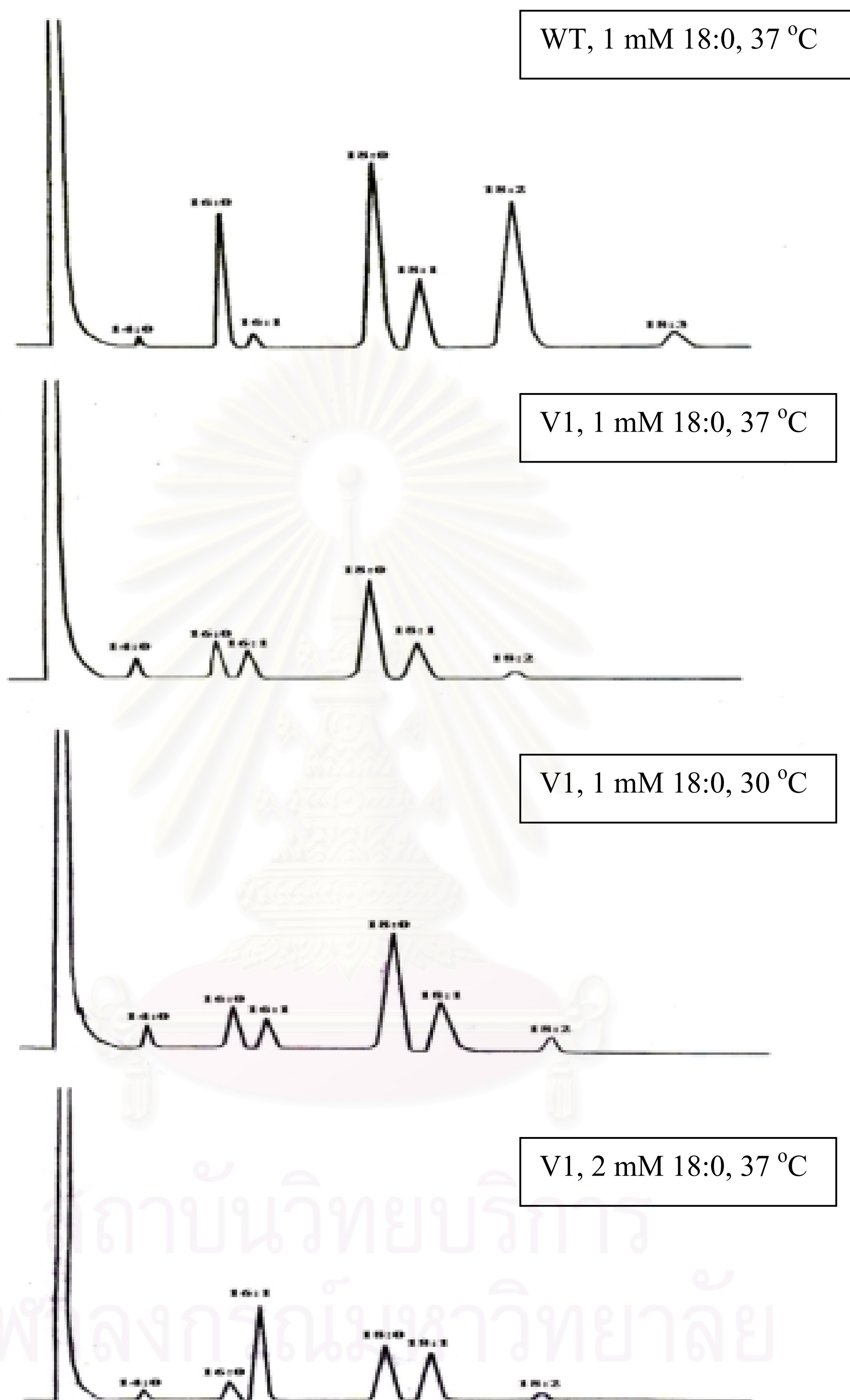

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Fig. 3 Gas chromatographic analysis of fatty acid methyl esters derived from V1 mutant of *H. polymorpha* grown on the media supplemented with different concentrations of C18:0 (1 and 2 mM) and different temperatures (30 °C and 37 °C).

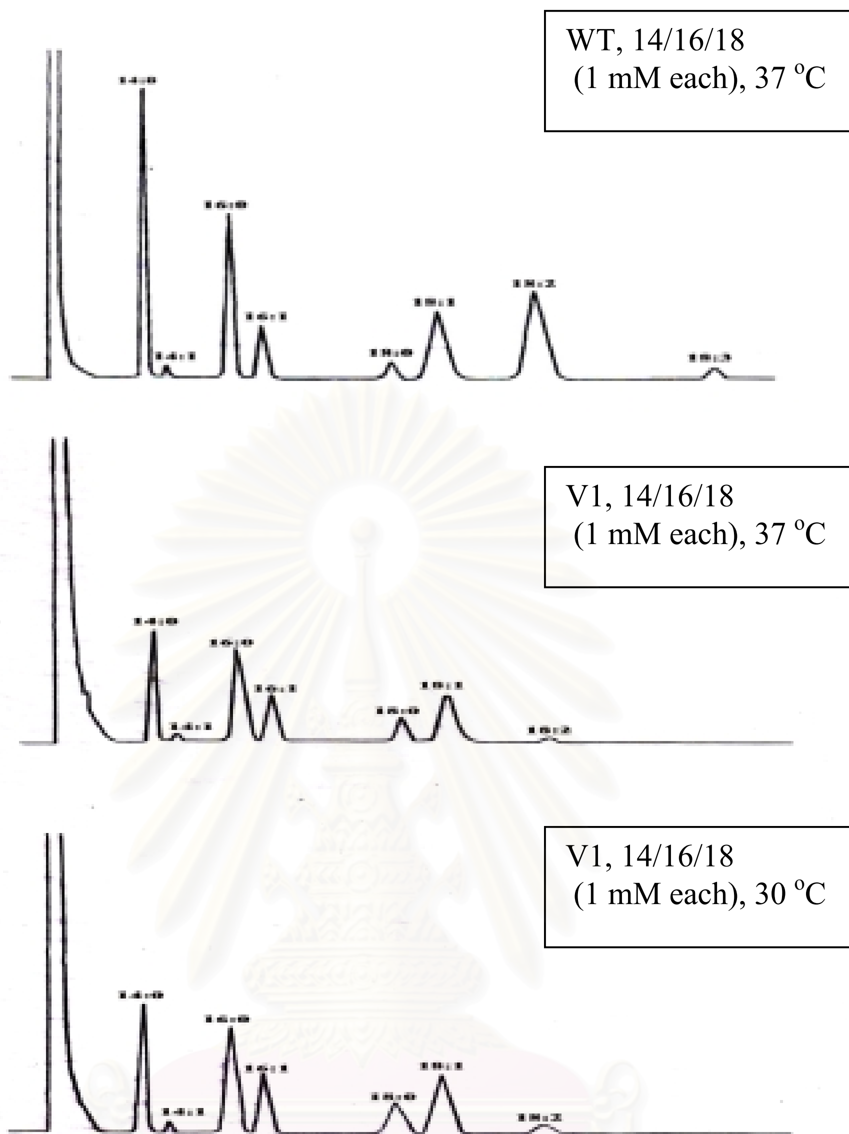

Fig. 4 Gas chromatographic analysis of fatty acid methyl esters derived from V1 mutant of *H. polymorpha* grown on the media supplemented with a mixture of fatty acids of C14:0, C16:0 and C18:0 (1 mM each) at different temperatures (30 °C and 37 °C).

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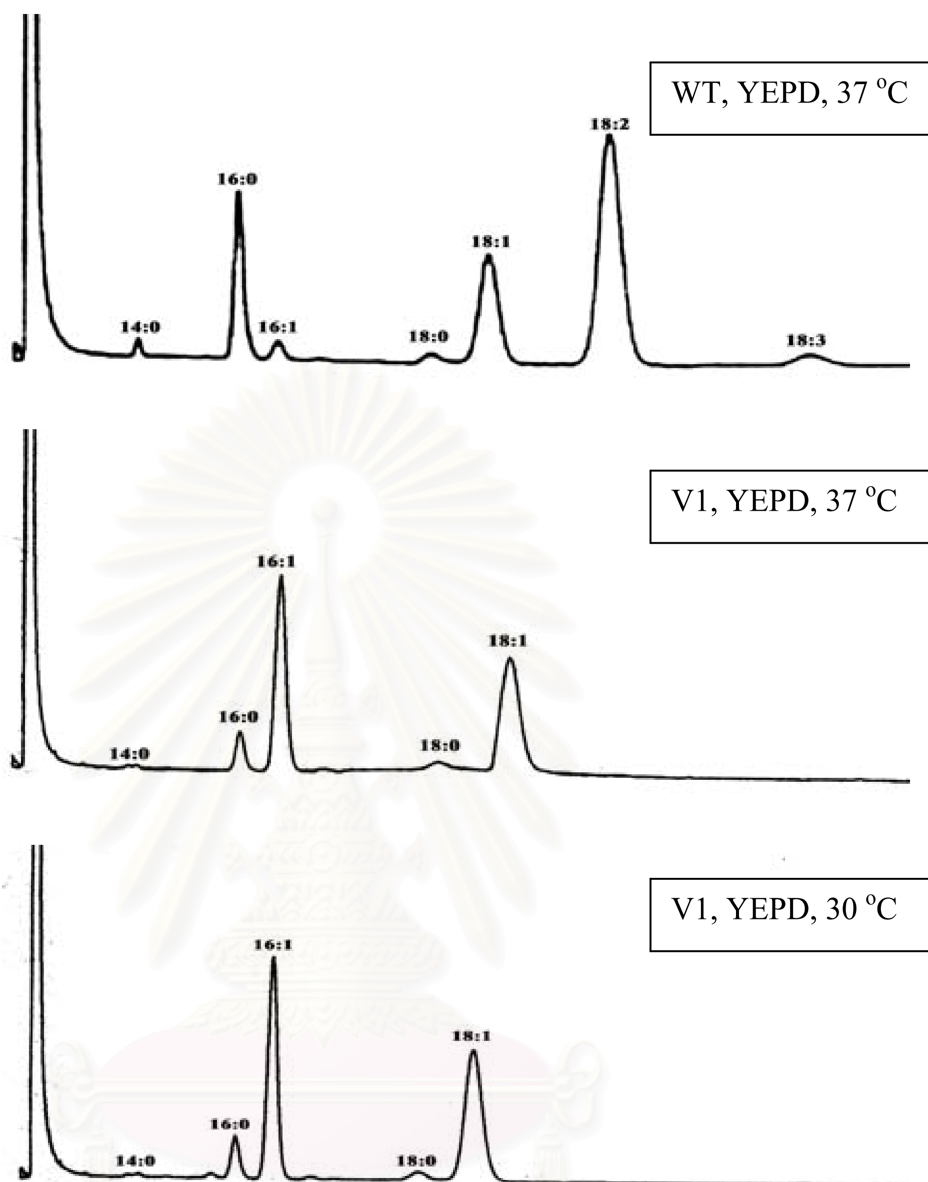

Fig. 5 Gas chromatographic analysis of fatty acid methyl esters derived from V1 mutant of *H. polymorpha* grown on the YEPD media without exogenous fatty acids at different temperatures (30 °C and 37 °C).

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BIOGRAPHY

Miss Wanida Wongsumpanchai was born on the 9 th of September 1972. She graduated with the Bachelor's Degree in Biology from Khonkaen University in 1994, Master's Degree in Biotechnology from King Mongkut's University of Technology Thonburi in 1999 and further her Ph.D. study in Biotechnology Program in the Faculty of Science, Chulalongkorn University.

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