



**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**EXPRESSION AND ACTIVITY CHARACTERIZATION
OF FATTY ACYL-COA REDUCTASES IN
*SACCHAROMYCES CEREVISIAE***

LIM PEI YU

SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

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School of Chemical and Biomedical Engineering

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List of Abbreviations

ACP	Acyl carrier protein
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
ADH	Alcohol dehydrogenase
AD	Aldehyde decarbonylase
ALD	Aldehyde dehydrogenase
BSA	Bovine serum albumin
CoA	Co-enzyme A
CAR	Carboxylic acid reductase
DNA	Deoxyribonucleic acid
FPLC	Fast protein liquid chromatography
FAME	Fatty acid methyl ester
FAS	Fatty acid synthase
FAR	Fatty acyl-CoA reductase
FDH	formate dehydrogenase
GC-MS	Gas chromatography - Mass spectrometer
HIS	Histidine
HRP	Horseradish peroxidase
IMAC	Immobilised metal ion affinity chromatography
NADPH/NADP ⁺	Nicotinamide adenine dinucleotide phosphate, reduced form/oxidised form
NAD/NAD ⁺	Nicotinamide adenine dinucleotide, reduced form/oxidised form

PCR	Polymerase chain reaction
PNT	Pyridine nucleotide transhydrogenase
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
STH	Soluble pyridine nucleotide transhydrogenase
TAG	Triacylglycerol
TBS	Tris buffer saline
TMB	3,3',5,5'-Tetramethylbenzidine

Abstract

Medium chain alkanes make up a huge fraction of gasoline that is widely used in land transportation today. Biosynthesis of alkanes from fatty acids was discovered in cyanobacteria and plants. Two enzymes, namely acyl-CoA reductase and aldehyde decarbonylase, catalyse this pathway with aldehyde as the intermediate. The aim of this project is to express and characterise acyl-CoA reductases. This would help to facilitate metabolic engineering of acyl-CoA reductases for fatty aldehyde production in *S. cerevisiae*. Six acyl-CoA reductase genes were selected for expression in *Saccharomyces cerevisiae* (*S. cerevisiae*). After cloning the reductase genes into *S. cerevisiae*, the yeast strain was analysed for fatty acyl-CoA reductase protein expression and activity. Gas chromatography-mass spectrometry (GC-MS) was used to detect the fatty aldehydes produced *in vivo* and *in vitro*. Fatty acids in the medium was shown to be readily uptaken and accumulated in *S. cerevisiae*. By comparing the respective peak areas, the ratio of C₁₂ saturated fatty acid to C₁₆ saturated fatty acid present in *S. cerevisiae* was increased more than 60 times from 0.09 to 6.39 through exogenous uptake of fatty acid. The expression of six FAR genes in *S. cerevisiae* was confirmed by Western Blot analysis. The activity of these expressed genes, however, was not detected *in vivo* and this could be due to competing pathways in *S. cerevisiae* for the consumption of fatty acids as *S. cerevisiae* contains aldehyde dehydrogenase (ALD) genes that catalyse the conversion of fatty aldehydes into fatty acids. *In vitro* studies were done by using crude lysate but fatty aldehyde was not detected. As a result, optimisation of purification process for FAR was carried out to remove

intracellular proteins for activity assays but insufficient amount of FAR was obtained for the assay as most of the protein was expressed in the insoluble form.

1 Introduction

1.1 Background

In recent years, biofuels have been the focus in the energy research field due to depleting non-renewable energy resources. Biofuels research has mainly focused on the production of ethanol and other light alcohols, which require new infrastructure for the usage of these fuels due to low combustion efficiency and high hygroscopy [1-3]. This project aims to produce medium chain (C_4 to C_{12}) alkanes that make up a huge fraction of gasoline [4] that is widely used in land transportation today. Medium chain alkanes are less miscible with water, and thus require lower recovery cost than ethanol [5]. In addition, medium chain alkanes are compatible with current transport infrastructure.

The overall aim of this research project is to metabolically engineer an industrially relevant yeast strain, i.e. *Saccharomyces cerevisiae* (*S. cerevisiae*), to produce high yields of medium chain alkane. Many organisms such as microalgae, cyanobacteria and plants have been reported to be able to synthesize long hydrocarbons chains naturally [6, 7], but production of short chain hydrocarbons necessitates extensive genetic manipulation of the hosts to channel precursor flux toward medium chain alkane synthesis. Some recent studies have shown that the conversion of fatty acids to alkanes in these microorganisms occurs in two steps catalysed by fatty acyl-CoA reductase (FAR) and aldehyde decarbonylase (AD) [8-10]. Unlike bacteria systems, which are often limited by plasmid instability problems, yeast cells are more stable for

genetic manipulation, where foreign genes can be integrated into the chromosomal DNA of yeast. Additionally, yeast production systems are capable of post-translation modifications of proteins and secretion of products into the medium, which could ease product recovery. This research thesis will focus on expression and characterization of fatty acyl-CoA reductases to facilitate genetic metabolic engineering of *S. cerevisiae* for medium chain aldehydes production which serve as precursors for medium chain alkanes production.

1.2 Objectives

The overall aim of this project is to characterise fatty acyl-CoA reductases (FARs) to aid metabolic engineering of *S. cerevisiae* to produce medium chain fatty aldehydes as alkane precursors. FAR genes *AcrI* [11] and *AcrM* [12] genes from a gram negative bacteria, *Acinetobacter*, and *Synpcc7942_1594* gene, hypothetical FAR *Ava_2534* and *AplaP_13983* genes [9] from cyanobacteria, and FAR *Maqu_2507* gene from *Marinobacter aquaeolei* were selected and cloned into *S. cerevisiae* for conversion of fatty acids to aldehydes. The FAR genes *AcrI*, *AcrM*, *Synpcc7942_1594*, *Ava_2534*, *AplaP_13983*, and *Maqu_2507* are renamed to *FAR1*, *FAR2*, *FAR3*, *FAR4*, *FAR5* and *FAR6* respectively, throughout this thesis.

The aims of this research are as follow:

- Molecular cloning of acyl-CoA reductase genes into *S. cerevisiae* BY4741 wild type and *poxI* β -oxidation knockout strains.
- Activity characterisation of heterologously expressed FAR genes – *FAR1*, *FAR2*, *FAR3*, *FAR4*, *FAR5* and *FAR6*.
- Optimisation of FAR protein expression level in *S. cerevisiae* BY4741.

1.3 Literature Review

Metabolic engineering of organism involves constructing new pathways to produce a target compound intracellularly in a given host. As these compounds are not endogenously produced in the host organism, it is important to identify and understand the possible effects of the construction of this new pathway on cell growth. Rational engineering of the strain to overcome identified constraints will then be performed. In this literature review section, (i) alkane producing pathways, (ii) considerations in host selection for alkane production and (iii) gene candidates for the production of fatty aldehydes are reviewed. Common limitations faced in cell engineering and approaches taken to resolve those limitations are also discussed.

1.3.1 Alkane production from fatty acids catalysed by fatty acyl-CoA reductase (FAR) and aldehyde dehydrogenase (AD)

Production of alkanes from fatty acids, by expressing acyl-ACP reductase and decarbonylase from cyanobacteria [9], has been successfully reported in *Escherichia coli* (*E. coli*) and the proposed pathway is shown in Figure 1 [8]. Fatty acids are first activated to fatty acyl-CoA by acyl-CoA synthetase, and reduced to fatty aldehyde by acyl-CoA reductase with nicotinamide adenine dinucleotide phosphate (NADPH) being oxidized to NADP⁺ at the same time. AD then catalysed the conversion of C_n fatty aldehydes into C_{n-1} alkanes with the reduction of 1 carbon chain length. In a study by Schirmer *et al.* (2010), C₁₃ to C₁₇ long chain alkanes were synthesized in *E. coli* through engineering of fatty acid biosynthesis pathways [9]. Besides *E. coli*, *S. cerevisiae* is another micro-organism that is widely used in

industries and laboratory due to its ease of genetic manipulation and its well-studied metabolism.

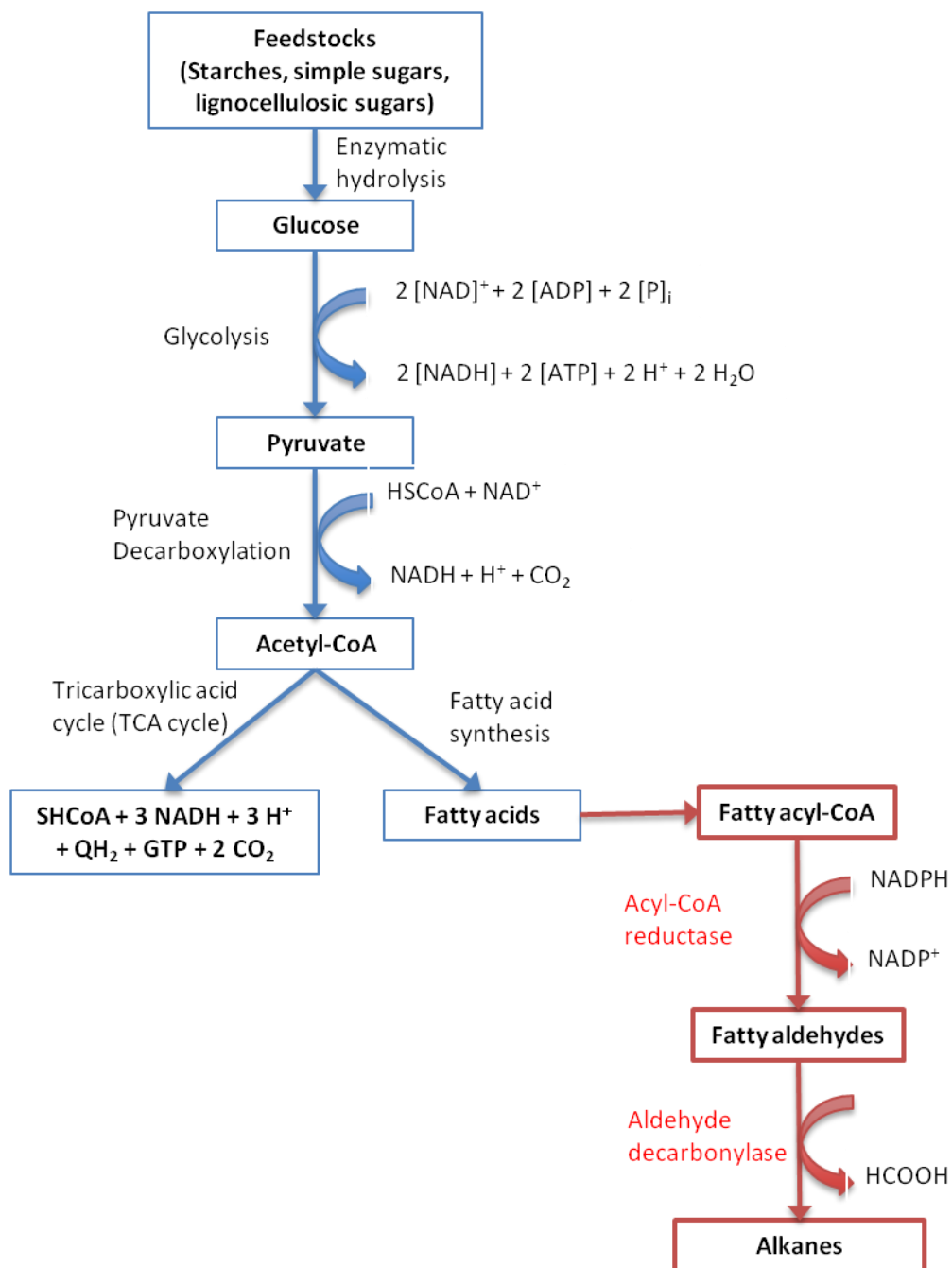


Figure 1 Pathways involved in the biosynthesis of alkanes from fatty acids [8]. Endogenous pathways are in blue whereas exogenous pathways are in red.

1.3.2 Advantages of *S. cerevisiae* as a host strain for metabolic engineering

Metabolic engineering of industrially available host strains such as *S. cerevisiae* has several advantages over the use of native organisms for production of desired products. Several microorganisms, such as cyanobacteria, are found to be capable of producing saturated n-alkanes naturally [13]. However, the yield of these products from native organisms may not be industrially viable and these native organisms lack molecular biology tools for genetic manipulation. Compared to those native organisms, industrially relevant strains, such as *S. cerevisiae* and *E. coli*, are well understood with respect to physiology, molecular biology, and their genetic manipulation using well-established genetic tools is commonly reported [14]. On the other hand, the disadvantage of using a recombinant strain is that imported genes may not perform as well in host strains compared to in native organisms. The activity of imported enzymes is often affected by genetic constraints (e.g. translation) and physiological factors and thus may act differently in different host strains. Therefore, genetic manipulations and protein engineering could be employed to improve the yields and enzyme activity [15].

Despite these possible limitations, the metabolic pathways in *S. cerevisiae* are well studied and its database is widely available [16] as compared to native alkane producing organism. Molecular biology tools for manipulation of relevant metabolic pathways in *S. cerevisiae* are also readily accessible. In this research, fatty acids will be used as a precursor for alkane production. Therefore, it is important that the fatty

acid metabolism in *S. cerevisiae* is well understood, which is reviewed in the following section.

1.3.3 Fatty acid synthesis and lipid accumulation in *S. cerevisiae*

Medium chain fatty acid is required as a precursor for the production of medium chain alkanes catalysed by FAR in *S. cerevisiae*. *S. cerevisiae* does not naturally produce and accumulate medium chain fatty acids. Therefore, methods to accumulate medium chain fatty acids were investigated. Lipid accumulation in yeast can be attained through two ways: *ex novo* pathway and *de novo* lipid synthesis as shown in Figure 2 [17, 18]. For *ex novo* pathway, lipids are uptaken by the cells, which may require the breakdown of the hydrophobic substrates *in vivo*. *De novo* lipid synthesis involves the elongation of fatty acids from malonyl-CoA through the addition of acetyl-CoA by two-carbon chain length. In yeast, most of the fatty acids are found to be C₁₆ and C₁₈ fatty acids [19].

There are two fatty acid synthesis pathways which are localized in the cytosolic and mitochondrial compartments, respectively, in yeast [18]. Mitochondrial fatty acid synthesis pathway is not responsible for the bulk of fatty acid synthesis though it is needed for respiration [17]. Majority of the fatty acid is synthesized through cytosolic fatty acid synthesis pathway involving type I fatty acid synthase [20]. Type I fatty acid synthase consists of two subunits: Fas1 (β subunit) and Fas2 (α subunit). Type I fatty acid synthetase acts mainly on acyl-CoA reductase as substrate. Mitochondrial fatty acid synthesis is carried out by type II fatty acid synthase and is known to involve fatty acyl-ACP [21]. In yeast, fatty acids are present in the form of free fatty acid,

acyl-CoA, or are converted to triacylglycerol (TAG), lipid body and sterol [17]. Understanding the flux of activated forms (i.e. acyl-CoA or acyl-ACP) of fatty acids present in the different cellular compartments (i.e. cytoplasm and mitochondrial) is crucial for the selection of FARs, where it has been reported that both acyl-CoA reductase and acyl-ACP reductase can be reduced to fatty aldehydes. Section 1.3.4 discusses the difference between these two reductases.

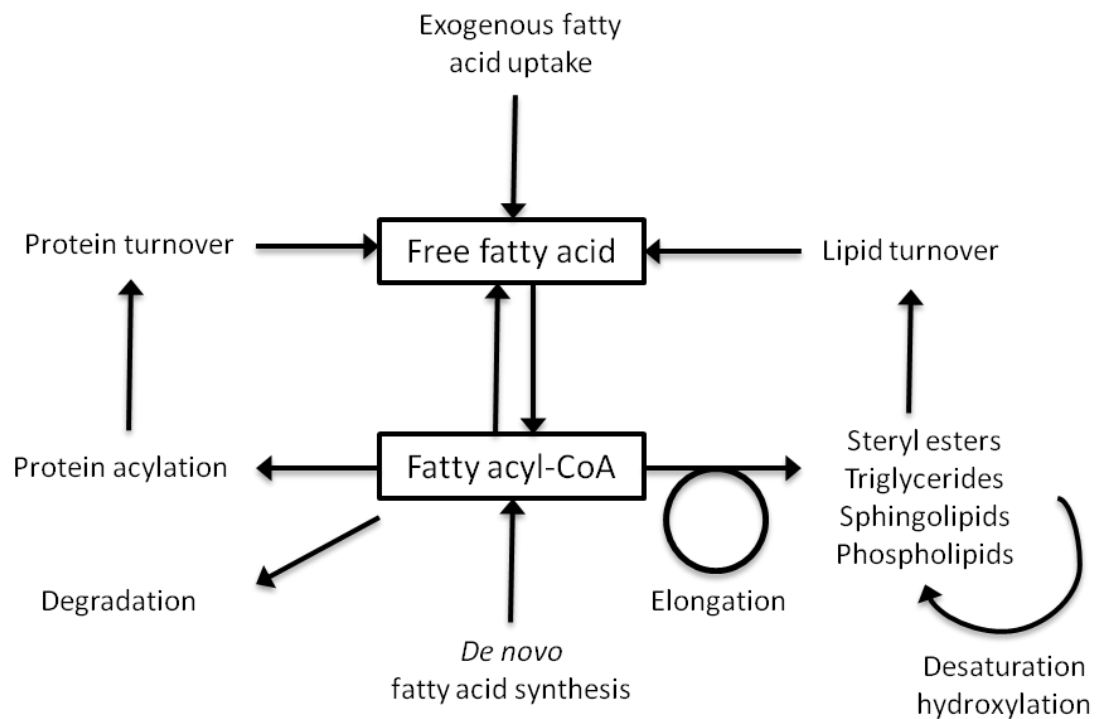


Figure 2 Schematic diagram of fatty acid metabolism in yeast [18].

1.3.4 Fatty acyl-CoA reductase (FAR)

FARs are enzymes with broad substrate specificity and catalyse the conversion of different carbon length of fatty acyl-CoA to fatty aldehydes. There are currently two types of reductases that have been reported [9, 11, 12], i.e. (i) acyl-CoA reductase and (ii) acyl-ACP (acyl-carrier protein) reductase, which catalyse the conversion of fatty acyl-CoA and acyl-ACP into fatty aldehyde, respectively. The substrate specificity of reductase is important, as most acyl-CoA reductase has been shown to have little or no activity towards acyl-ACP and vice versa. As discussed in Section 1.3.3, majority of the fatty acids is present as acyl-CoA in yeast and hence, a suitable reductase candidate would be one which has specificity for acyl-CoA over acyl-ACP.

FARs have previously been extracted from native organisms and protein expression of several FARs has also been reported in *E. coli* [11, 12]. Based on the literature review on the different FARs with published sequence and data, many of these enzymes do not catalyse the conversion of short to medium chain ($C_8 - C_{10}$) fatty acyl-CoA. Therefore, these FARs could not be chosen for our study as the aim of this research is to synthesise medium chain alkanes. Summary of chosen FAR candidates for this study and their reported characteristics is shown in Table 1. Both the FARs originating from the two *Actinetobacter* strains were reported to have zero or low activities towards short to medium chains acyl-CoA (shorter than C_{10}) but have a wide range of substrate specificity of fatty acyl-CoA from C_{10} to C_{22} chain length, which still falls within the range of medium alkanes found in gasoline.

Acinetobacter-derived reductases were reported to be over-expressed and localised in the membrane fraction in *E. coli* but still exhibit activities towards fatty acyl-CoA. However, expression and activity studies of these enzymes were not done in yeast, and membrane protein extraction methods could be used to extract these enzymes.

FAR3 which has been shown to successfully convert fatty acyl-ACP to fatty aldehyde was also cloned into *S. cerevisiae* to test its activity in a yeast system. Two other acyl-CoA gene candidates originating from alkane producing cyanobacteria *Anabaena variabilis* (*A. variabilis*) and *Athrospira platensis* (*A. platensis*) have been identified, and cloned into *S. cerevisiae* for activity determination. In a separate study done in our lab, aldehyde decarbonylase genes that were derived from *A. variabilis* and *A. platensis* showed good activity for the conversion of fatty aldehydes to alkanes. Moreover, cell free extract of *A. variabilis* was reported to convert stearate (C₁₈ fatty acid) to heptadecane (C₁₇ alkane) [22]. In addition, *A. variabilis* and *A. platensis* have high amino acid similarity of 72% and 68% respectively, to *FAR3* [9].

Even though *FAR6* converts fatty acyl-CoA to fatty alcohol, it was selected as another candidate as it has a wide range of substrate specificity (C8 to C20) which falls into the medium chain category [23]. The conversion of fatty acyl-CoA to fatty alcohol in a single step was because the intermediate product, fatty aldehyde, was not released after its formation [23]. Protein engineering could be used to alter *FAR6* such that it would release the fatty aldehyde intermediate while retaining its substrate specificity for medium chain fatty acyl-CoA.

Table 1 Summary of chosen FAR candidates for this study and their reported characteristics.

Gene	Organism	Expression host strain	Protein expression	Substrate specificity
<i>FAR1</i> [11]	<i>Acinetobacter baylyi</i>	<i>E. coli</i>	Localised in the insoluble fraction	C ₁₄ to C ₂₂ Acyl-CoA
<i>FAR2</i> [12]	<i>Acinetobacter sp. M-1</i>	<i>E. coli</i>	Localised in the membrane fraction	C ₁₀ to C ₂₀ Acyl-CoA
<i>FAR3</i> [9]	<i>Synechococcus elongatus</i> PCC7942	<i>E. coli</i>	N.A.	N.A.
<i>FAR4</i> [9]	<i>Anabaena variabilis</i> ATCC 29413	N.A.	N.A.	N.A.
<i>FAR5</i> [9]	<i>Athrospira platensis</i> str. Paraca	N.A.	N.A.	N.A.
<i>FAR6</i> [23]	<i>Marinobacter aquaeolei</i> VT8	<i>E. coli</i>	N.A.	N.A.

N.A. = information not available.

1.3.5 Membrane protein extraction and purification methods

FARs that were heterologously expressed in *E. coli* were reported to be localised in the membrane fraction [11, 12] and some are classified as membrane proteins. Expression of membrane protein is usually low [24, 25], and due to their hydrophobic nature, they have a high propensity to form aggregates when expressed heterologously in other host cells [26]. Difficulties in purification of membrane proteins are also often encountered.

To overcome this problem, detergents are commonly used to solubilise hydrophobic membrane protein in aqueous solution. Detergents are amphiphatic molecules that

mimic the lipid bilayer membrane environment. Commonly used detergents include Triton X-100, Tween 20, polyethylene glycol (PEG) [26]. However, there is no universal detergent that could entirely replicate the chemical and physical properties of membrane. In addition, high detergent concentration could interfere with protein stability and biochemical assays. This could be overcome by dialysis to remove excess detergent before performing activity assays on the recovered membrane proteins [27]. After solubilisation, membrane proteins could be purified in the same way as soluble proteins.

Membrane proteins could also be purified and concentrated by phase separation after being solubilised by detergents. Phase separation is a purification step which is used to separate the solubilised membrane proteins from hydrophilic components by adding salt such as ammonium sulphate [27]. Phase separation occurs when cloud point is reached by increasing the detergent concentration, concentration of salt (e.g. ammonium sulphate) or by temperature changes [26]. It is relatively easy to use and is readily amenable for scale up, making it an attractive method to recover large amounts of membrane proteins for detailed characterisation studies.

1.3.6 Protein Engineering

Proteins having the same function but derived from different organisms may have different substrate specificity and enzyme kinetics when introduced to another host cell due to dissimilarity in cells' innate environment. Given the limited target proteins available to perform a specific task in a given pathway, protein engineering could be used to improve the activity of enzymes. Enzyme substrate specificity and conversion

kinetics could be improved by using different protein engineering techniques such as directed evolution, rational design and semi-rational design [28]. In directed evolution, random mutagenesis [29] such as error prone PCR and DNA shuffling are applied to a protein, and mutants with the desired qualities are selected. In rational protein design, the protein is altered based on known structural and functional properties, and computational protein design algorithms [30] are also commonly used. However, the main disadvantage of rational design method is the lack of information as not all protein structure properties are being studied.

Directed evolution using random mutagenesis and site-directed mutagenesis, has recently been used to improve thermal stability of phosphate dehydrogenase that regenerates NADH and NADPH [31]. This resulted in an improvement in the catalytic efficiency as compared to the parent protein. To further increase protein yield, substrate specificity of the enzyme could be altered through rational protein design. In a reported study by Jung *et al.* (2012), a yeast reductase substrate specificity was modified using computer modelling to identify amino acids residues that directly interact with the substrate [32].

Besides protein engineering, engineering of metabolic pathways that involve fatty acid and fatty aldehyde consumption or synthesis should also be investigated to identify competing pathways that may interfere with the alkane-producing pathway.

1.3.7 Competing pathways which may decrease fatty aldehyde yield in yeast

To increase alkane yields, competing pathways (Figure 3) that may deplete the substrate and product yields should be repressed or eliminated, provided that their deletion is not detrimental to yeast growth.

Fatty acid can be used up in other pathways such as phospholipids production and β -oxidation where it is broken down to release acetyl-CoA for energy production. *POX1*, *FOX2 (POX2)*, *FOX3 (POX3)* genes are involved in the beta oxidation pathway as shown in Figure 4 [19]. By knocking out these genes, we could possibly improve lipid accumulation in yeast as demonstrated in *Yarrowia lipolytica* [33, 34].

In yeast, aldehydes can be converted to fatty acids by a $\text{NAD}^+/\text{NADP}^+$ -dependent fatty aldehyde dehydrogenase (ALD). There are five ALD genes known in yeast [35-37]. ALDs are conserved in many species and they usually play a role in metabolic pathways involving ethanol oxidation [37], conversion of acetaldehyde to acetate [37], and NADPH generation [38]. Other ALDs have been reported to be responsible for defence mechanism where they detoxify harmful substances [39].

Substrate specificity of *ALD2* from *S. cerevisiae* was tested and it was found that *ALD2* is able to catalyze the conversion of C_7 to C_{10} linear chain fatty aldehyde into fatty acids [40]. This chain length specificity for fatty aldehydes falls into the same range as that of FAR's substrate specificity. Therefore, *ALD2* might convert fatty

aldehyde produced by FAR back into fatty acid. *ALD2* gene could be knocked out to direct free fatty acids to the desired alkane-producing pathway.

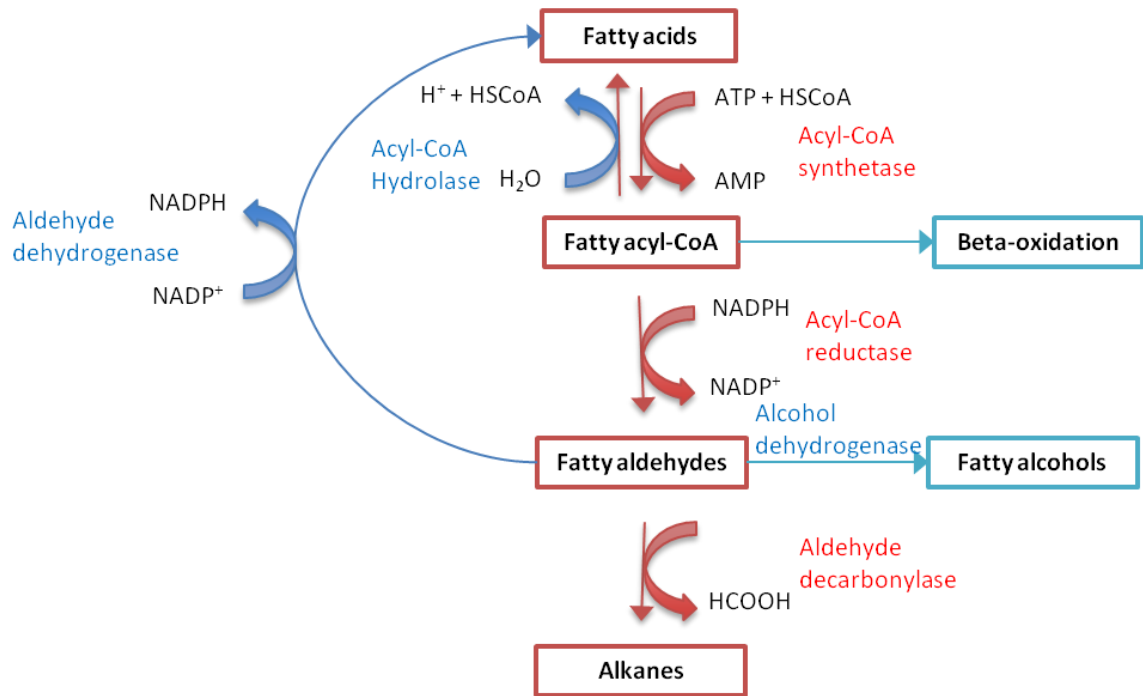


Figure 3 Competing pathways (highlighted in blue) in *S. cerevisiae* that utilises fatty aldehydes in yeast, which may reduce alkane production yield.

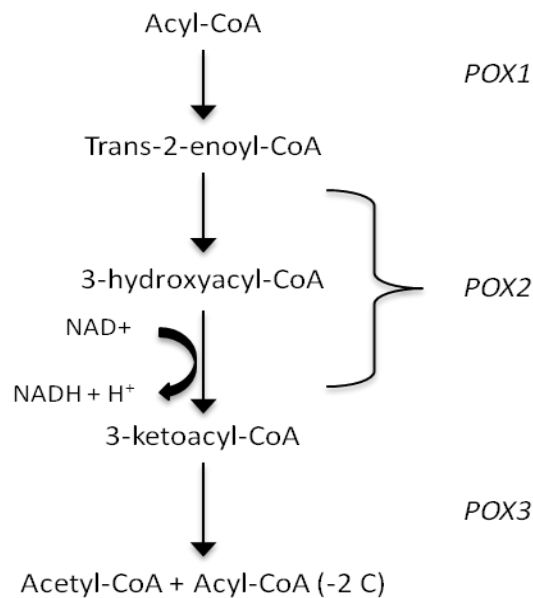


Figure 4 β -oxidation pathways involving *POX1*, *POX2* (*FOX2*) and *POX3* (*FOX2*) genes in *S. cerevisiae* [19].

2 Materials and Methods

2.1 Chemicals and Reagents

Expression plasmids pYES2/CT and pESC-URA plasmids were obtained from Invitrogen (Singapore) and Agilent Technologies (Singapore), respectively. Gene and oligonucleotides syntheses were carried out by GeneArt® (Germany) and 1st Base (Singapore), respectively. Restriction enzymes and Taq DNA polymerase were from Fermentas, New England Biolab (NEB) (Singapore) or Promega (Singapore). SeeBlue® Plus2 pre-stained protein standard and PageRuler™ prestained protein ladder plus used was purchased from Invitrogen (Singapore) and Fermentas (Singapore) respectively. Chemicals were mainly from Sigma-Aldrich (Singapore) unless otherwise stated.

2.2 Strains

Chemically competent *E. coli* cells were purchased from Invitrogen (Singapore). *Saccharomyces cerevisiae* BY4741 wild type, and *POX1* (Accession number Y04571), *POX2* (Accession number Y05080) and *POX3* (Accession number Y02319) knockout strains were bought from Euroscarf (Germany).

2.3 Plasmid Construction

2.3.1 Construction of pYES2/CT vectors

FAR1, *FAR2*, *FAR3*, *FAR4*, *FAR5*, and *FAR6* genes were synthesized from GeneArt® (Germany), Integrated DNA Technologies (Singapore), and Genscript (USA). All FARs except *FAR6* were designed with HindIII and XhoI restriction sites. HindIII and

SacI restriction sites were added to the 5' and 3' end of *FAR6* respectively. The vector was cut using the respective restriction enzymes at 37°C for 3 h and the gene fragments were ligated into pYES2/CT vector using T4 DNA ligase at 16°C overnight.

2.3.2 Construction of pESC-URA vectors

FAR1, *FAR2* and *FAR3* acyl-CoA reductase genes were amplified from pMA vectors using KOD Hotstart DNA polymerase (Novagen, Singapore) and primers pESC-FAR-F, pESC-FAR-R, and pESC-S-R with BamHI and XhoI restriction sites underlined, and 6xhis tag at the C-terminal using the following thermal cycling parameters: initial denaturation at 95°C for 2 min, followed by 25 cycles of 95°C for 20 s, 60°C for 15 s, and 70°C for 25 s. The amplified fragment was ligated into pESC-URA vector multiple cloning site 2 using T4 DNA ligase at 16°C, overnight. The ligation mix was then transformed into Top10 *E. coli* chemically competent cells. Clones were screened with Taq polymerase using the following thermo cycle: initial denaturation at 94°C for 5 min, 25 cycles of 94°C for 30 s, 56°C for 30 s, and 68°C for 1 min/kb, and final extension step at 68°C for 5 min.

2.4 Gene deletion of ALD2

Primers with ALD2 homologous recombination flanking sites were designed as such: ALD2-del-F and ALD2-del-R (**Table 2**). ALD2-del primers were used to amplified URA marker from pUG72 deletion cassette using the following following thermal cycling parameters: initial denaturation at 95°C for 2 min, followed by 25 cycles of 95°C for 20 s, 59°C for 15 s, and 70°C for 25 s. Successful deletion was checked

using four target specific primers: ALD2-A, ALD2-B, ALD2-C, and ALD2-D, and cassette specific primers: B-M and C-M (Table 2).

Table 2: Primer sequences used in this study.

Primer	Nucleotide sequence (5' to 3')
pESC-FAR-F	TACGGGATCCTTGTCCAGAGCTCAAGCTT
pESC-FAR-R	TACA <u>CTCGAGATGGT</u> GATGCTGATGATGCCAATGTTACCTGGAAATAATCTAGC
pESC-S-R	TACA <u>CTCGAGATGGT</u> GATGGTGAATGGCCAAAGCCAATGGTT
ALD2-del-F	TTGCATGTCCATCAAAAACAATCGTGAAAATAAGCCAAAA GAAAACAGCTGAAGCTTCGTACGC
ALD-del-R	TAGACTGTCAACATATTCATCCGTCGTCAGGCTAAGCTATT TAGGGCATAGGCCACTAGTGGATCTG
ALD2-A	GGGTTGCAAATGCGTGAG
ALD2-B	GTCTAAAGTCTCTAATGCG
ALD2-C	ACATCGAAACTGTTACAGG
ALD2-D	CAAGTCTTGGATTAGCAG
B-M	AAGAGAATCAGCGCTCCC
C-M	CTTCACGCAGGATGACAG

2.5 Protein extraction and purification for characterisation of FAR

Cells were resuspended in lysis buffer A (0.05 M Sodium phosphate 0.3 M NaCl, 5 mM imidazole, 1 mM PMSF, 2% (v/v) Triton X-100, pH 8.0) or lysis buffer B (50 mM Tris-HCl, 2.4 mM Triton X-100, 20 mM imidazole, pH 8.0) and lysed using Fastprep (4 m/s, 40 s, 6-8 times). The lysates were then centrifuged at 8,000 g for 10 min. The supernatant was further purified by 1 ml Histrap HP column (GE Healthcare Life Sciences, Singapore) using Fast Protein Liquid Chromatography (FPLC) (AKTA

Explorer, Amersham Pharmacia, GE Healthcare Life Sciences, Singapore) or Profinity™ IMAC resin (Biorad, Singapore). Binding buffer was used during the washing step. Elution buffer A (0.05 M sodium phosphate, 0.3 M NaCl, 250 mM imidazole, pH 8.0) or elution buffer B (50 mM Tris-HCl, 2.4 mM Triton X-100, 1 M imidazole, pH8.0) was used to elute the bound proteins.

2.6 SDS-PAGE analysis

SDS-PAGE was used for protein separation and analysis. Laemmli buffer was added to each samples and heated at 100°C for 10 min before loading into the gel. The electrophoresis was carried out at 200 V for 40 min using MOPS running buffer (250 mM MOPS, 250 mM Tris-HCl, 5 mM EDTA, and 0.5% (w/v) SDS). The gels were stained using Coomassie Brilliant Blue for 15 min and destained overnight using water.

2.7 Western blot analysis

For western blot analysis, the SDS-PAGE was transferred to a PVDF membrane at constant 1.3 A for 7 min according to manufacturer's protocol (Trans-Blot® Turbo™ Transfer System, Biorad, Singapore). The membrane was washed with Tris buffer saline (TBS) (50 mM Tris, 150 mM NaCl, pH 7.6) The membrane was blocked with 5% fat-free milk in a TBST buffer (blocking buffer) containing 25 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.5% (v/v) Tween 20 for 1 h at room temperature, and incubated overnight at 4°C with Anti-6X His tag® antibody (HRP) (Abcam®, Singapore) diluted at a ratio of 1:10 000 in blocking buffer, added into the same blocking buffer. The membrane was then washed 6 times for 5 min each with TBST

buffer to remove unbound antibodies. Colorimetric reagent 3,3',5,5'-Tetramethylbenzidine (TMB) Liquid Substrate System for Membranes (Sigma-Aldrich, Singapore) was used to detect his-tagged proteins on western blot.

2.8 Extraction of FARs using detergent and phase separation

Extraction of the membrane fraction of *S. cerevisiae* expressing FARs was carried out using Fricke's protocol [27]. Yeast cells were resuspended in Buffer A (50 mM Tris-HCl, pH 7.4) at a cell concentration of 100 mg wet cell / ml and disintegrated using glass beads. The lysate was then centrifuged at 4,000 g for 15 min to remove un-lysed cells. The crude cell envelopes and soluble cell components were separated by centrifugation at 60,000 g for 2 h. The soluble cell components were removed and the crude cell envelopes were washed with buffer A and centrifuged at 60,000 g for 2 h. The crude cell envelopes were then homogenized in an equal volume of Triton X-100 (8 g/100 ml, dissolved in buffer A) and mixed at room temperature. The sediment was separated from the supernatant by centrifugation at 60,000 g for 1 h. For phase separation, 40% (w/v) ammonium sulfate was added drop wise to a final concentration of 10% (w/v) in the solubilisate, and centrifuged at 20,000 g for 20 min. The upper phase was recovered for protein analysis by SDS-PAGE.

2.9 Analysis of metabolites produced *in vivo* by GC-MS

GC-MS analysis was used to detect fatty acids and aldehydes in *S. cerevisiae* expressing FARs. To prepare the samples for GC-MS analysis, overnight culture of yeast strains transformed with pESC-URA::FAR/AD or pYES2/CT::FAR grown in yeast nitrogenous base (YNB) medium lacking uracil, and supplemented with 2%

(w/v) dextrose (YNBD), were washed with sterile water and inoculated at an absorbance of 0.4 at 600 nm in YNB medium lacking uracil, supplemented with 2% (w/v) galactose and 1% (w/v) raffinose (YNBGR). The cultures were then washed twice with equal volumes of SB solution (9 g/L NaCl, 0.5% (w/v) bovine serum albumin (BSA)). Fatty acids, aldehydes, and alkanes were then extracted using Dyer and Bligh lipid extraction method [41]. Fatty acids were derivatized to fatty acid methyl esters (FAMES) using BF_3 and resuspended in hexane before GC-MS analysis. Aldehydes were extracted with ethyl acetate or derivatized using PFBHA.HCl to oxime and resuspended in hexane before GC-MS analysis.

2.10 *In vitro* activity assays of acyl-CoA reductases

To test for the activity of FAR, *in vitro* assays were done carried out using cell extracts, where the organic components of the cell were extracted and analysed using GC-MS. Fatty aldehydes was expected to be present in the organic phase if the enzyme was active. The enzyme reactions were also monitored at 340 nm at interval of 5 min up to 30 min, then at interval of 30 min up to 2 h, and overnight (16 h to 20 h). Controls without the addition of purified enzyme and acyl-CoA substrate were also included to take into consideration any background oxidation of NADPH. *In vitro* acyl-CoA reductase assays were done by adding 2 mM MgCl_2 , 0.2 mM NADPH, 0.1 mM acyl-CoA and crude cell lysate in 0.05 M Tris-HCl (pH 7.5) to a final volume of 100 μl . The reaction mixtures were then incubated at 37°C for different time intervals and 100 μl of ethyl acetate was added for extraction. The samples were then vortexed for 15 min and centrifuged at 13,000 g for 3 min for phase separation and the organic phase analysed by GC-MS.

3 Results and Discussion

FAR genes were cloned into *S. cerevisiae* BY4741 wild type and *pox1* knockout strains to investigate the activity of FARs for the production of fatty aldehydes *in vitro* and *in vivo*. *FAR3* that has been shown to have activity in *E. coli* in a recently reported study [9] was chosen as a benchmark for characterisation of *FAR1*, *FAR2*, *FAR4*, *FAR5*, and *FAR6* in *S. cerevisiae*. *S. cerevisiae* BY4741 *pox1* knockout strain (Y04571) was investigated in parallel with the wild type BY4741 strain to investigate the effect of β -oxidation on the production of fatty aldehydes. β -oxidation pathway is one of the competing metabolic pathways, which would break down acyl-CoA into acetyl-CoA and hence deplete the source of fatty acyl-CoA as substrate for FAR. Therefore, the use of a *pox1* knockout strain would allow the assessment of whether more fatty acids could accumulate and hence be directed to the fatty aldehyde-producing pathway.

3.1 Molecular cloning of FAR genes into co-expression plasmids and transformation into *S. cerevisiae* wild type (BY4741) and Δ *pox1* strains (Y04751)

FAR1, *FAR2*, *FAR3*, *FAR4*, *FAR5*, and *FAR6* were introduced into *S. cerevisiae* BY4741 and Y04571 strains by cloning them into commercially available pYES2/CT plasmid (Figure 5A). This plasmid was chosen because it encodes the sequence for a 6xHis tag, which facilitates protein detection by anti-his western blot and protein purification using immobilized metal ion affinity chromatography (IMAC).

The *FAR1*, *FAR2*, and *FAR3* were also cloned into co-expressing vector pESC-URA together with aldehyde decarbonylase *Synpcc7942_1593* which is renamed as *AD3* (Figure 5B). The expression of the FAR genes in this plasmid would yield the expressed protein which will contain 6x His tags at the C-terminus. The co-expression of the *AD3* gene was to test for the production of alkanes. In addition, medium chain aldehydes could be toxic to *S. cerevisiae* and by cloning in *AD3* gene, we hypothesize that the aldehydes could be rapidly converted to alkanes and thus alleviate aldehyde-induced toxicity problems, if any.

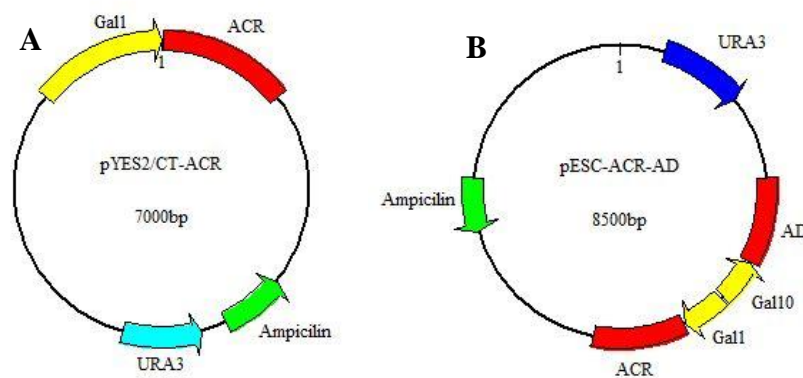


Figure 5 (A) Plasmid map of pYES2/CT with FAR as insert and (B) plasmid map of pESC-URA with FAR and AD as inserts. Both vectors contain *GAL* promoters and expression of proteins was induced by galactose.

3.2 Western blot analysis of FARs expression

Western blot analysis was carried out to confirm the expression of FAR in *S. cerevisiae*. *S. cerevisiae* cultures containing pYES2/CT-FAR plasmid were induced with galactose, and western blot analysis was performed using crude cell lysates and soluble fractions (Figure 6). A negative control consisting of *S. cerevisiae* BY4741 containing pYES2/CT plasmid without insert (crude lysate: lane 2 of Figure 6; and soluble fraction: lane 3 of Figure 6) was ran together with the strains containing FARs. Western blot analysis of the crude cell lysate fraction of *FAR1* (33 kDa), *FAR2* (33 kDa), *FAR3* (37.5 kDa), *FAR4* (37.5 kDa), *FAR5* (37.5 kDa), and *FAR6* (66 kDa) showed that all six FARs were expressed in *S. cerevisiae e* (*i.e.* Lanes 4, 6, and 8 of Figure 6(A), and lanes 1, 3, and 5 of Figure 6(B)), albeit at different expression levels. The soluble fractions of FAR were also analysed on the western blot. However, all the FARs were localised in the insoluble fraction as it was reported that some of the FARs are membrane-bound proteins [11, 12]. After the successful expression of FARs, the organic phase of *S. cerevisiae* expressing these FARs was extracted and analysed for aldehyde production using GC-MS.

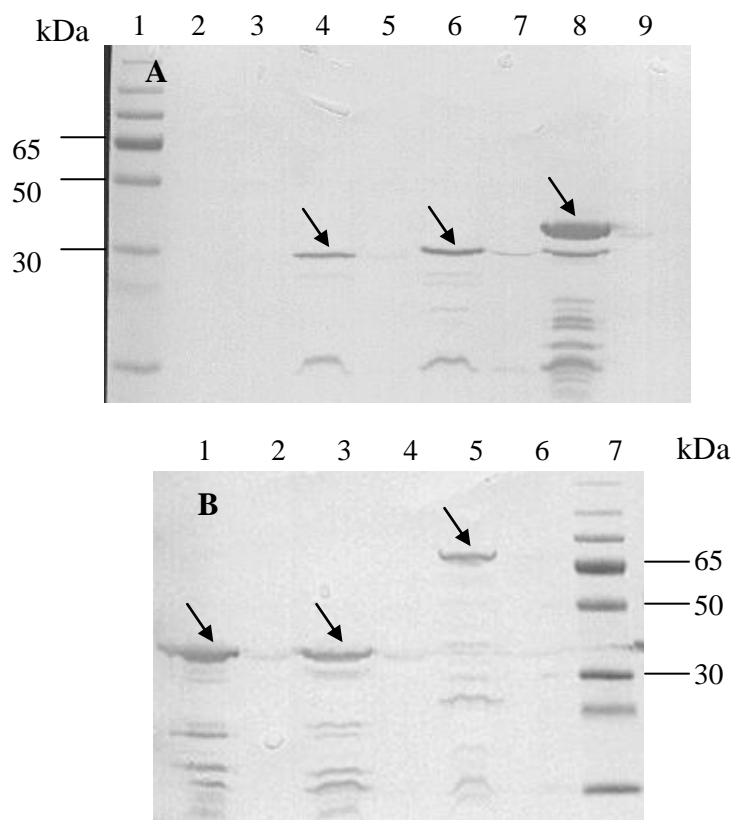


Figure 6 Western blot analysis of FAR expression in *S. cerevisiae* BY4741. (A) Lane 1: PageRuler prestained protein ladder plus; Lanes 2, 4, 6, and 8: whole cell lysate of plasmid without insert, with inserts encoding *FAR1* (33 kDa), *FAR2* (33 kDa), and *FAR3* (37 kDa) respectively. Lanes 3, 5, 7, and 9: supernatant of plasmid without insert, with inserts encoding *FAR1*, *FAR2*, and *FAR3* respectively. (B) Lanes 1, 3, and 5: whole cell lysate of plasmid without insert, *FAR4* (37 kDa), *FAR5* (37 kDa), and *FAR6* (66 kDa) respectively. Lanes 2, 4, and 6: supernatant of plasmid without insert, with inserts encoding *FAR4*, *FAR5*, and *FAR6* respectively. Lane 7: PageRuler prestained protein ladder plus (Fermentas). Arrows indicate the FARs.

3.3 *In vivo* characterisation of FARs

In vivo characterisation of the enzymatic activity of *FAR1*, *FAR2*, and *FAR3* in *S. cerevisiae* was performed. Strains of *S. cerevisiae* expressing FARs and AD3 were grown in minimal medium in the absence and presence of C₁₂ fatty acid. Culture growth was monitored for 48 h and the organic contents were then extracted using chloroform or ethyl acetate for lipid and aldehyde analysis on GC-MS, respectively.

3.3.1 Growth of *S. cerevisiae* in medium containing dodecanal (C₁₂ aldehyde)

Aldehyde toxicity toward *S. cerevisiae* were previously reported for C₁ to C₁₀ aldehydes [42, 43] but no studies on toxicity of C₁₂ fatty aldehyde (dodecanal) has been reported to date. To test the possible toxicity of C₁₂ fatty aldehydes to yeast, 0.1% (v/v) of C₁₂ fatty aldehyde was added into the growth medium. It was found that the addition of C₁₀ aldehyde (decanal) resulted in no cell growth (result not shown), while the addition of 0.1% (v/v) to 0.4% (v/v) of C₁₂ fatty aldehydes impeded the growth of yeast as compared to yeast culture medium not containing dodecanal (Figure 7). Therefore, dodecanal may be toxic to yeast cells at these concentrations. However, the amount of aldehydes produced from the expression of acyl-CoA reductase in yeast is yet to be determined based on activity characterisation.

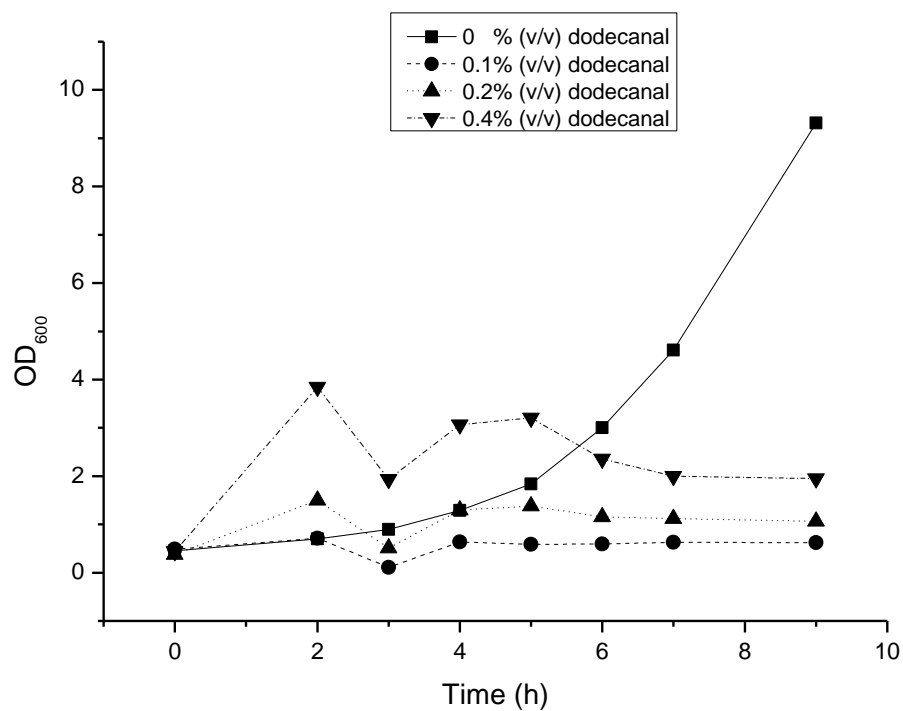


Figure 7 Growth profiles of *S. cerevisiae* BY4741 strain grown in YPD without and with the addition of varying concentrations of dodecanal.

3.3.2 Analysis of fatty acid uptake in *S. cerevisiae* by GC-MS

Accumulation of C_{12} fatty acids by yeast strains fed with C_{12} fatty acid was studied by GC-MS analysis. Accumulation of C_{12} fatty acid is important as it is the precursor for FAR to produce C_{12} (medium chain) aldehyde.

Lipid content was extracted using the procedures published by Bligh and Dyer (1959) [41] and analysed on GC-MS (Figure 8). No C_{12} fatty acid was detected in strains that were not fed with C_{12} fatty acids. For strains that were fed with C_{12} fatty acids, C_{12}

fatty acids were detected in amounts that greatly exceeded the intrinsic C₁₆ and C₁₈ saturated fatty acid content. By comparing the peak areas between C₁₂ FAME and C₁₆ FAME, there was a significant increase in the amount of C₁₂ fatty acid accumulated in *S. cerevisiae* cells (Figure 9). Therefore, it is clear that C₁₂ fatty acids could be readily uptaken by *S. cerevisiae*, and the accumulated C₁₂ fatty acid could serve as the precursor for FARs to produce C₁₂ aldehyde.

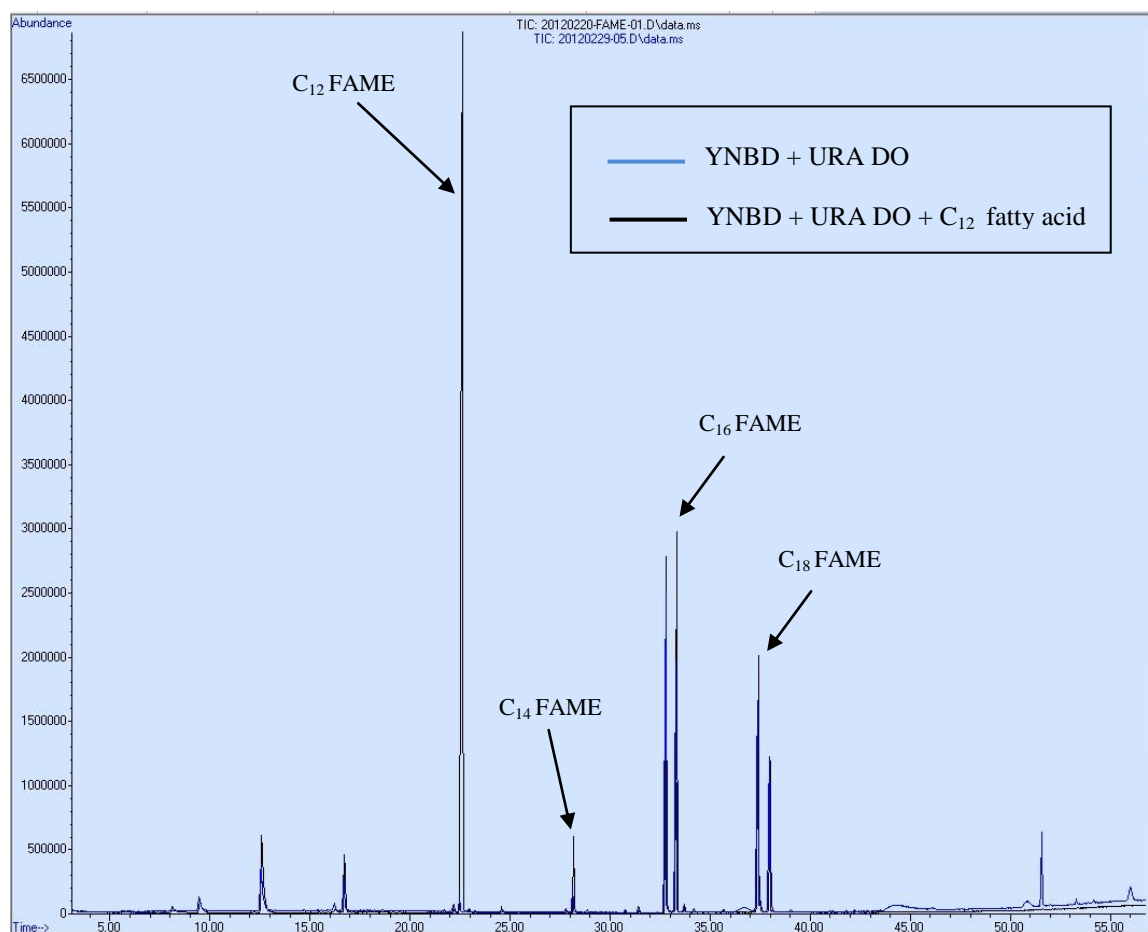


Figure 8 GC-MS analysis of fatty acids uptaken by *S. cerevisiae* BY4741 cells fed with 0.05% (w/v) of C₁₂ saturated fatty acid. Blue line corresponds to yeast cells grown in YNBD + URA DO; and black line corresponds to yeast cells grown in YNBD + URA DO + 0.05% (w/v) of C₁₂ saturated fatty acid.

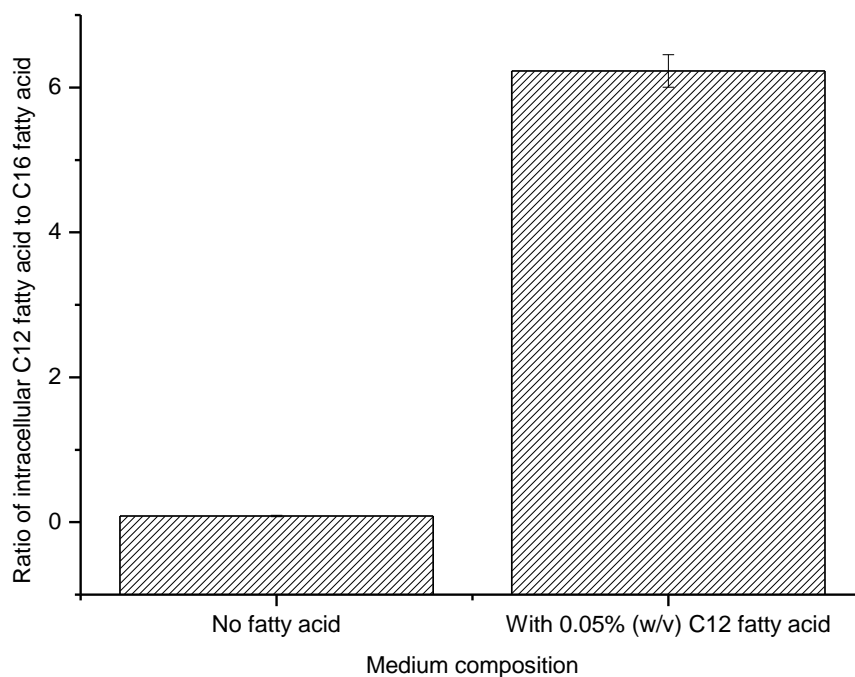


Figure 9 C₁₂ to C₁₆ saturated fatty acid peak area ratio of cultures grown without fatty acid and with C₁₂ fatty acid for 24 h.

3.3.3 Fatty aldehydes analysis in *S. cerevisiae* expressing FAR by GC-MS

GC-MS analysis of fatty aldehydes and alkanes were carried out on *S. cerevisiae* expressing FAR, and co-expressing FAR and AD, respectively. However, fatty aldehydes (Figure 10) and alkanes were not detected in the cells expressing FAR, and cells co-expressing FAR and AD, respectively. The presence of competing metabolic pathways, as discussed in Section 1.3.7, might have affected aldehyde production. Therefore, purification of FARs was carried out for *in vitro* characterisation to eliminate the possibility of interfering metabolic pathways which may have consumed majority of the fatty acids uptaken.

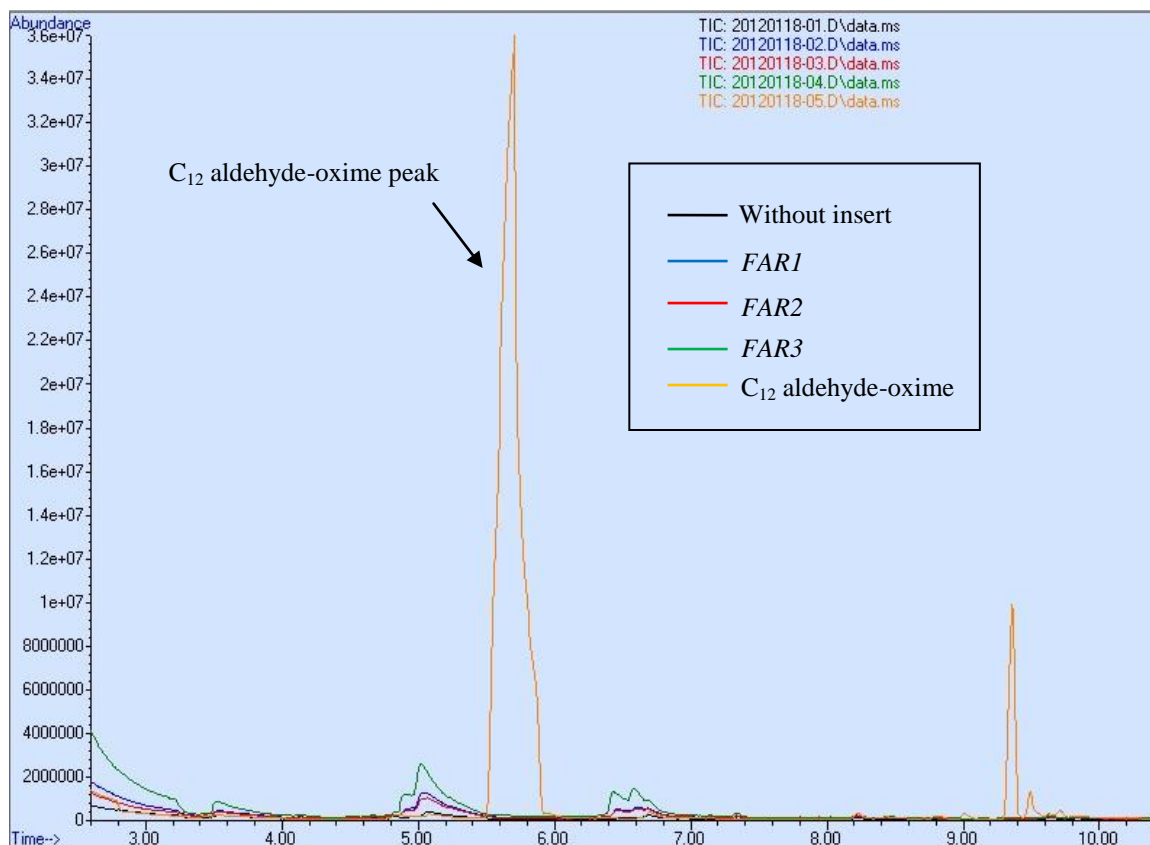


Figure 10 GC-MS analysis of aldehyde content of *S. cerevisiae* BY4741 co-expressing FAR and AD that is grown in YNBGR + URA DO medium containing C₁₂ fatty acid. *S. cerevisiae* BY4741 containing pESC-URA plasmid without insert, and expressing *FAR1*, *FAR2*, and *FAR3*, are represented by black, blue, red, and green line respectively. C₁₂ aldehyde-oxime standard is represented by orange line.

3.4 *In vitro* characterisation of FARs

3.4.1 Purification of FARs

Extraction and purification of FARs were attempted for *in vitro* characterisation. Without the addition of Triton X-100 to Tris-HCl lysis buffer, most of the FARs were not present in the soluble fraction (Figure 6) and were suspected to be localised to the membrane fraction as reported [11, 12]. Heterologous expression of membrane proteins is usually low [24] due to the hydrophobic nature of the proteins, where the

proteins are frequently found to be misfolded. Hence, Triton X-100 that was reported to be able to solubilise membrane protein was added to the lysis buffer to solubilise the FAR proteins. When the cells were lysed with lysis buffer containing 2% (v/v) Triton X-100, the FARs were found in the soluble fraction. The soluble fraction was subjected to IMAC purification as described in Section 2.4. The IMAC eluate fractions were then analysed by SDS-PAGE (Figure 11) and western blot (Figure 12; lanes 4, 7, and 10). The FAR proteins were detected by western blot but not detected on SDS-PAGE. In an attempt to increase the concentration of proteins to facilitate protein extraction for *in vitro* characterisation, the culture volume was increased by 30 times, but the FAR protein band was still undetected on SDS-PAGE analysis. Difficulties faced during expression and purification is not uncommon for membrane proteins due to their hydrophobic nature which bind them strongly to the lipid bilayer of the cell membrane [44]. In addition, the expression of *FAR1* and *FAR2* in *E. coli* resulted in the formation of the protein in the insoluble fraction. The use of a stronger detergent is not recommended as it might disrupt the structure of the protein, thus preventing these proteins to be recovered in the active form for *in vitro* characterisation.

Phase separation was also employed as another method to extract the FAR proteins. After solubilising the FAR proteins using Triton X-100, ammonium sulfate salt was added to decrease the cloud point of Triton X-100 to room temperature and thus separating the hydrophobic components (including membrane fraction) from the hydrophilic components [27]. The extraction of *FAR1*, *FAR2* and *FAR3* was, however,

unsuccessful using this method. *FAR3* having the highest expression level (Figure 6) out of the FARs was used to optimize the purification process.

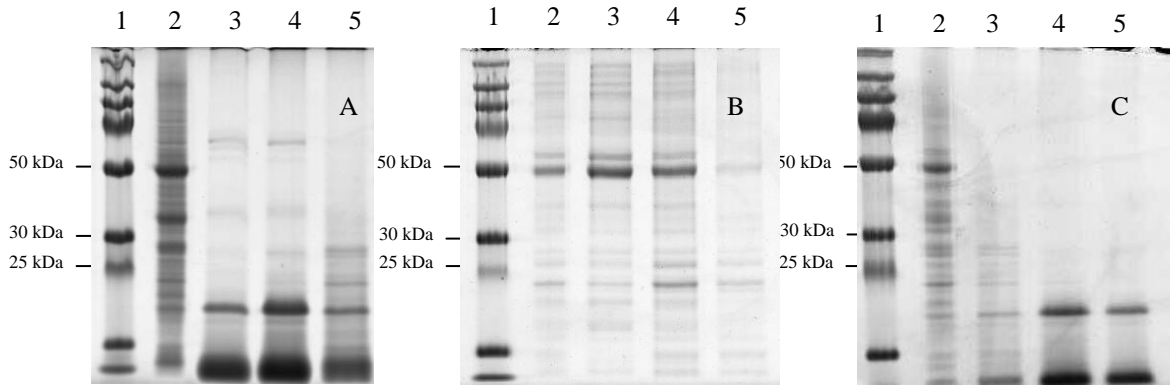


Figure 11 SDS-PAGE gel showing IMAC purification of (A) *FAR1*- 33 kDa, (B) *FAR2*- 33kDa and (C) *FAR3*- 38 kDa using HisTrap HP column (GE Healthcare). Lane 1: Fermentas Pageruler Plus pre-stained protein ladder, lane 2: proteins in reaction buffer after buffer exchange, lanes 3-5: eluate fraction.

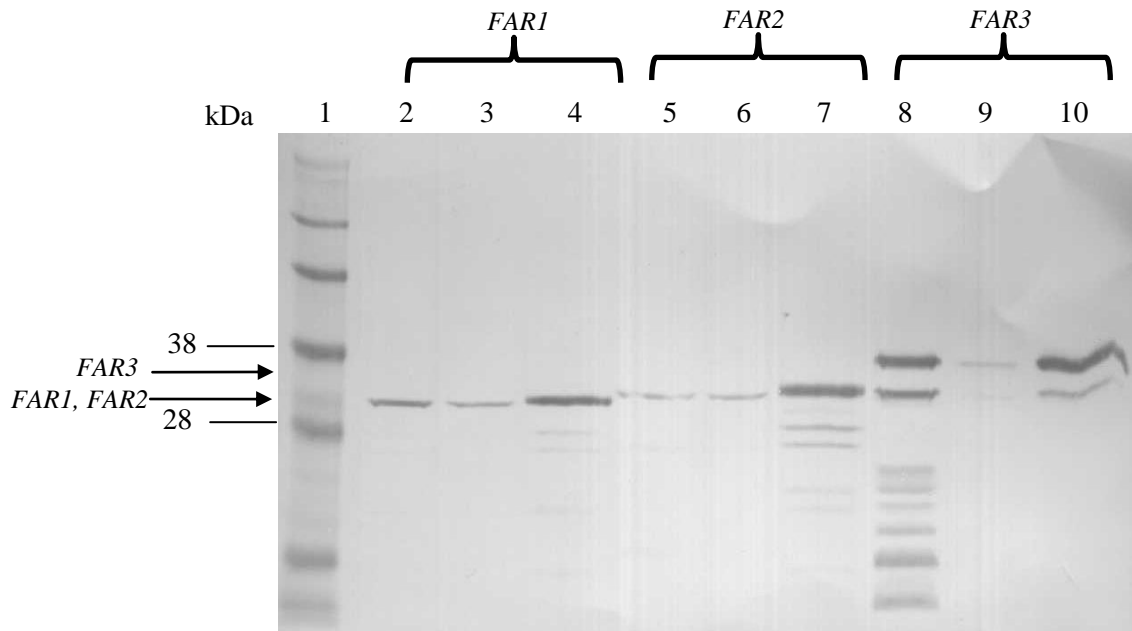


Figure 12 Western blot analysis of acyl-CoA reductases expression. Lane 1: SeeBlue Plus2 pre-stained protein standard; lanes 2-4: whole cell lysate, supernatant and elute fraction for *S. cerevisiae* BY4741 expressing *FAR1*, respectively; lanes 5-7: whole cell lysate, supernatant and elute fraction for *S. cerevisiae* BY4741 expressing *FAR2*, respectively; lanes 8-10: whole cell lysate, supernatant and elute fraction for *S. cerevisiae* BY4741 expressing *FAR3*, respectively.

3.5 Optimization of lysis and purification conditions for *FAR3*

To characterize the enzymatic activities of the FAR candidates, these enzymes need to be recovered and purified for *in vitro* activity assay. Several strategies such as using different detergents, and employing sonication after lysis, were employed to solubilise the FARs from the insoluble fraction for *in vitro* enzymatic activity assays, followed by Western blot analysis, using *FAR3* as the model candidate. Triton X-100 was found to be the most effective detergent compared to SDS, IGEPAL CA-630, Sodium lauroyl sarcosine, and Tween 20 in solubilising the membrane protein (Figure 13). The solubility of *FAR3* was improved in the presence of 3 times critical micelle concentration of Triton X-100 added to the lysis buffer.

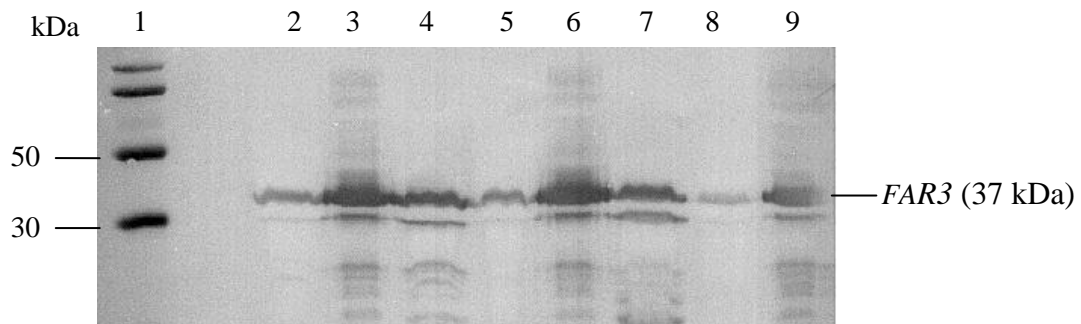


Figure 13 Western blot analysis of the effect of addition of Triton X-100 on *FAR3* solubility. Lane 1: PageRuler prestained protein ladder; lanes 2 and 3: soluble and insoluble fraction of *FAR3* lysed in 50 mM Tris-HCl buffer with 2.4 mM Triton X-100; lanes 4 and 7: whole cell lysate of *FAR3*; lanes 5 and 6: soluble and insoluble fraction of *FAR3* lysed and sonicated in 2.4 mM Triton X-100; lanes 8 and 9: soluble and insoluble fraction of *FAR3* lysed in 50 mM Tris-HCl buffer without Triton X-100.

The cell culture volume was increased to 1.5 L to increase the amount of *FAR3* for downstream purification for *in vitro* activity assays in the presence of Triton X-100. *FAR3* was expressed (Figure 14; lane 2) and the supernatant (Figure 14; lane 3) was added to IMAC resin for binding. The flow through fraction (Figure 14; lane 4) shows

that most if not all of the *FAR3* have bound to the resins. Eluate fractions 1 to 4 (Figure 14; lanes 5 to 8) showed that *FAR3* was mostly eluted in fractions 3 and 4, which were subsequently combined (Figure 14; lane 9) and buffer exchanged into reaction buffer (50 mM Tris-HCl, pH 7.4) (Figure 14; lane 10). Although *FAR3* was successfully purified and concentrated, the activity assay was impeded by insufficient protein concentration. An earlier study which characterized *FAR3* (Schirmer *et al.* (2010)) reported that a total purified protein concentration of 10 μ M (*i.e.* 37 mg/ml) was needed to perform the activity characterisation assay, which suggests a relatively poor enzyme conversion efficiency. It is clear that other alternative methods are needed to improve solubility of FARs for activity characterisation assays.

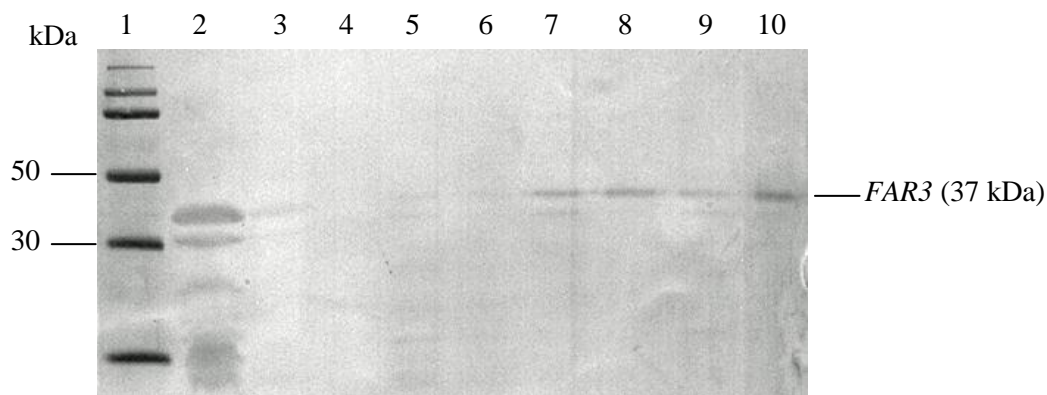


Figure 14 Western blot analysis of IMAC purification of *FAR3*. Lane 1: PageRuler prestained protein ladder plus; lane 2: whole cell lysate; lane 3: Supernatant; lane 4: flow through; lanes 5 to 8: eluate fractions 1 to 4; lane 9: protein sample before buffer exchange; lane 10: protein sample after buffer exchange.

In order to increase the amount of soluble FAR for downstream purification processes, the effect of the incubation time of cell lysate in the presence of Triton X-100 was investigated as studies have shown that prolonged incubation of protein in the presence of detergent could potentially increase their solubility [45, 46]. The result of

FAR3 incubated in the presence of Triton X-100 is shown in Figure 15 and Figure 16. *FAR3* was incubated at 4°C for a period of 0 to 3 h to determine the effect of incubation time has on the solubility of *FAR3* in the presence of Triton X-100. There was no improvement on the solubility of *FAR3* after incubating the cell lysate for 1 to 3 h after lysis by comparing the soluble fractions (Figure 15; lanes 2 to 5). Most of the *FAR3* was still found in the insoluble fraction (Figure 15; lanes 6 to 9).

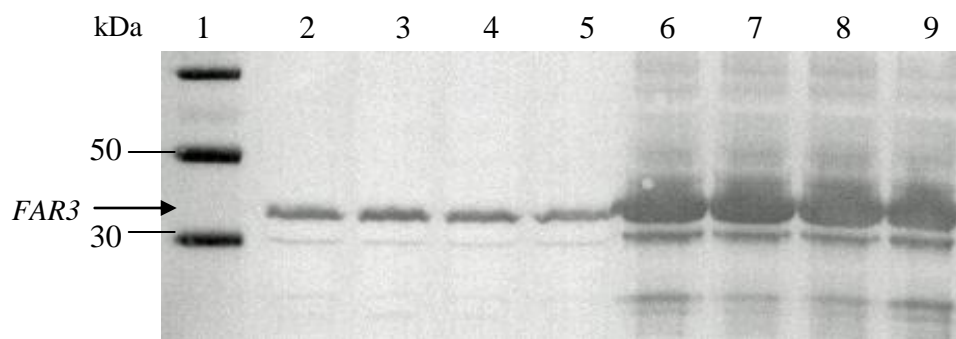


Figure 15 Western blot analysis of *FAR3* solubility at 4°C under different incubation time after lysis in the presence of 3 times CMC Triton X-100 in the lysis buffer. Lane 1: PageRuler prestained protein ladder; lanes 2 to 5: Soluble fraction of *FAR3* whole cell lysate incubated for 0, 1, 2, and 3 h, respectively; lanes 6 to 9: Insoluble fraction of *FAR3* whole cell lysate incubated for 0, 1, 2, and 3 h, respectively.

The incubation time was extended to overnight and the result is shown in Figure 16. The band in lane 4 (Figure 16) was thicker than lane 3 (Figure 16), indicating that there is more *FAR3* present in the soluble fraction after overnight incubation. This showed that overnight incubation improved the solubility of *FAR3* in the presence of Triton X-100.

In addition, as the incubation time was much longer, PMSF was also added to the cell lysate to prevent proteolysis and the effect was analysed (Figure 16). The addition of PMSF to the cell lysate seems to reduce protein degradation to a certain extent as the

smaller protein bands in lane 5 were weaker than those in lane 4 of Figure 16. It is interesting to note that there were proteins of larger size than *FAR3* after incubating the cell lysate overnight. This is likely due to the aggregation of membrane proteins. Even though the solubility of *FAR3* was improved through the addition detergent, optimization of Triton X-100 concentration and incubation time after lysis, the concentration of protein obtained was not sufficient for activity assays. Soluble expression of FARs could be improved upstream through varying culture conditions and medium composition to facilitate downstream purification process as downstream purification process has been optimized to purify and concentrate *FAR3*. This strategy could similarly be used to purify other FARs.

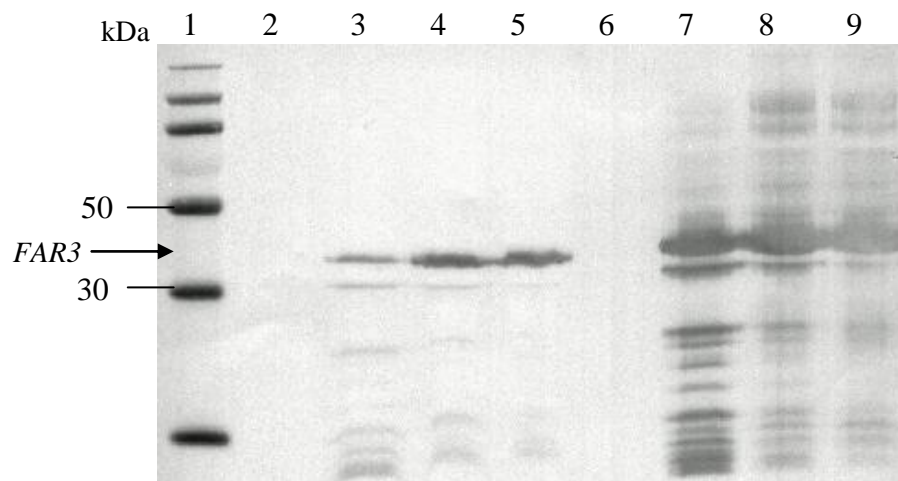


Figure 16 Western blot analysis of *FAR3* solubility under different condition. Lane 1: PageRuler prestained protein ladder; lanes 2 to 5: soluble fractions of negative control (plasmid without insert), lysate without incubation, lysate incubated overnight at 4°C, lysate incubated overnight at 4°C in the presence of PMSF; lanes 6 to 9: insoluble fractions of negative control (plasmid without insert), lysate without incubation, lysate incubated overnight at 4°C, lysate incubated overnight at 4°C in the presence of PMSF.

3.6 Deletion of *ALD2* gene from *S. cerevisiae* BY4741 and Y04571

The metabolic pathways in *S. cerevisiae* were studied, and pathways catalysed by alcohol dehydrogenase [47] and aldehyde dehydrogenase [40], were found to convert fatty aldehydes to fatty alcohols and fatty acids, respectively. Therefore, the presence of these enzymes in *S. cerevisiae* might hinder fatty aldehyde production through competing pathways for the substrate and product.

As no fatty alcohol has been detected in our metabolite analysis of *S. cerevisiae* BY4741 strain, the possibility of competing alcohol dehydrogenase activity was eliminated at this stage. However, the presence of aldehyde dehydrogenases that were reported to have substrate specificity on medium chain fatty aldehydes [40], could have converted the fatty aldehydes back to fatty acids (Figure 3). *ALD2* is an aldehyde dehydrogenase that was reported to catalyse the conversion of medium chain aldehydes to fatty acids [40]. This hypothesis was verified when C₁₂ fatty acids were detected in the strains that were fed solely with C₁₂ aldehydes (Figure 17), suggesting that the aldehyde dehydrogenase played a key role in fatty aldehyde oxidation to fatty acid. Therefore, to test the hypothesis, *ALD2* was knocked out in *S. cerevisiae* BY4741 and BY4741 *pox1* knockout strains using homologous recombination (Figure 18). Similar *in vitro* assays were performed using BY4714 Δ *POX1* Δ *ALD2* strains containing *FAR1*, *FAR2*, and *FAR3* with C₁₂ and C₁₈ acyl-CoA as substrates. However, no aldehyde was detected using GC-MS.

With the lack of information on the substrate specificity of ALD in *S. cerevisiae*, there is a possibility that other ALD may also be able to convert medium chain fatty aldehydes to fatty acids. In order to test this hypothesis further, extensive work to delete the other ALD genes may be carried out.

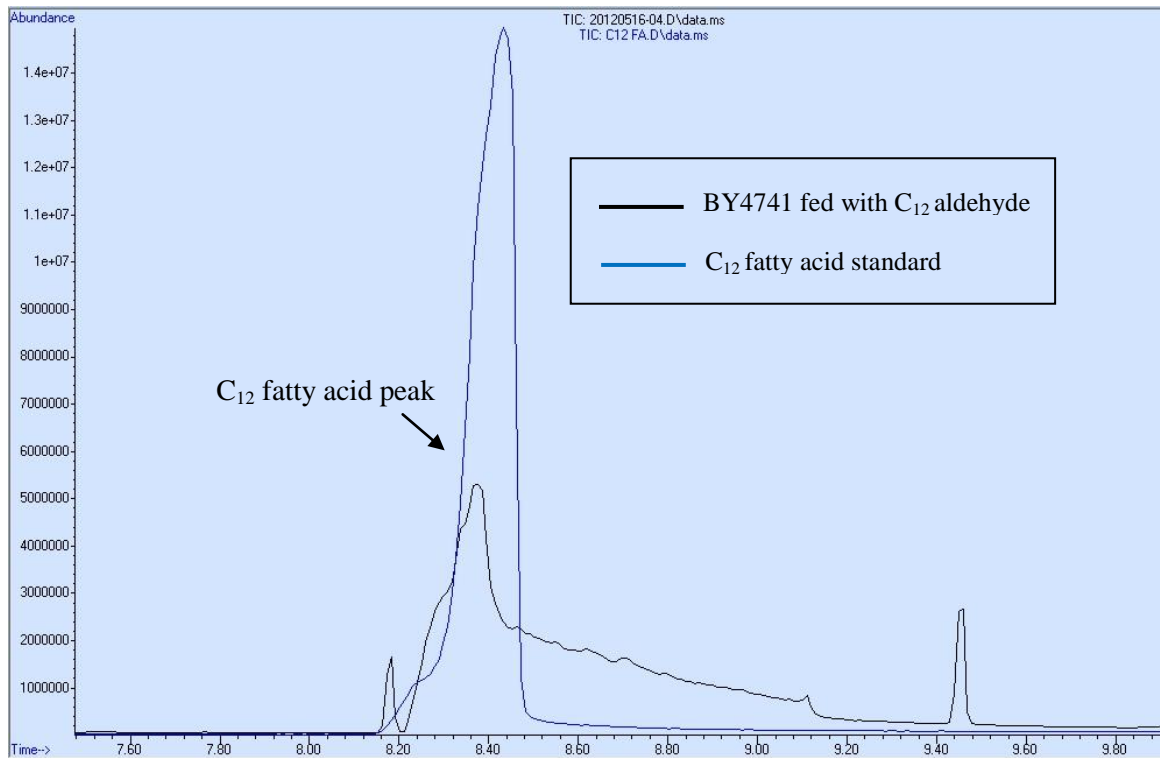


Figure 17 GC-MS analysis of *S. cerevisiae* BY4741 grown in minimal medium containing dodecanal. Blue line corresponds to C₁₂ fatty acid standard in ethyl acetate and black line corresponds to yeast cells grown in YNBD + URA DO + C₁₂ aldehyde.

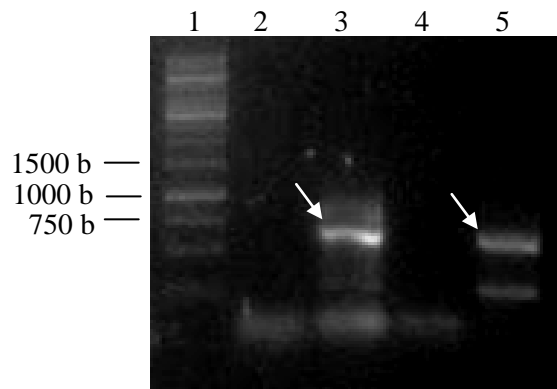


Figure 18 Gel electrophoresis image of colony PCR of *ALD2* knockout in *S. cerevisiae* using homologous recombination. Lane 1: 1 kb DNA ladder; lane 2 and 4: negative control; lane 3 and 5: successful *ALD2* knockout (indicated by white arrows).

4 Conclusion

Six fatty acyl-CoA reductase genes were successfully cloned and expressed in *S. cerevisiae*. However, due to their low expression and purification difficulties, the enzymes were unable to be characterized *in vitro*. The negative activity detection of the FARs *in vivo* suggests the need to engineer the yeast strain for fatty aldehyde production. The absence of FAR activity is hypothesised to be due to several reasons: (i) competing reactions for substrate or product consumption, (ii) protein insolubility, and (iii) balancing of redox reactions. Elimination of competing reaction could be important for *in vivo* activity assays as FARs could have lower enzymatic turnover rate than aldehyde dehydrogenase. Further study would be needed to confirm this. In addition, there are more than one aldehyde dehydrogenase genes in *S. cerevisiae*, more work is needed to eliminate competing reactions by knocking out an aldehyde dehydrogenase gene which has substrate specificity for medium chain fatty aldehydes. *In vitro* activity assays could also be useful to better understand the substrate specificity and enzyme kinetics of FARs. While the purification process was shown to successfully purify and concentrate *FAR3*, the amount obtained was not sufficient for fatty aldehyde detection. Solubility of FARs could be improved through upstream optimization such as modifying culture medium and conditions. In conclusion, the results achieved from this work have given some insights to understanding FARs characteristics when expressed in *S. cerevisiae* and also helped to identify some challenges that need to be overcome for metabolic engineering of *S. cerevisiae* for aldehyde production.

5 Future work

More work could be done to engineer *S. cerevisiae* to address the lack of activity in the heterologously expressed FARs, as discussed in the previous section. The reasons hypothesized for this outcome are: (i) presence of competing metabolic pathways, (ii) insoluble expression of FAR (iii) low turnover rate and/or (iv) insufficient cofactors (NADPH). Some work may be carried out in the future to address each of these possibilities is as described below.

5.1 Elimination of competing metabolic pathways in *S. cerevisiae*

Competing metabolic pathways such as β -oxidation, and the presence of aldehyde dehydrogenase that catalyses the conversion of aldehydes to fatty acids, could potentially hinder aldehyde production.

5.1.1 Deletion of genes involved in β -oxidation pathways in *S. cerevisiae*

β -oxidation breaks down fatty acids to acetyl-CoA in wild type *S. cerevisiae* strains and thus, might reduce the substrate concentration for FAR. Therefore, deletion of enzymes (i.e. *POX1*, *POX2*, and *POX3*) involved in the β -oxidation pathways is currently going to generate libraries of multiple copies of POX deletion strains to address fatty acid availability for conversion into fatty aldehydes.

5.1.2 Deletion of ALD genes in *S. cerevisiae*

ALD oxidises fatty aldehydes to fatty acids, which is undesirable as it reduces fatty aldehyde yields. There are currently five copies of ALD known in yeast and *ALD2*

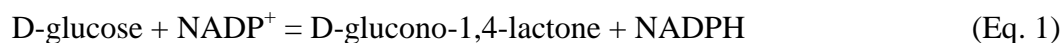
was chosen to be knocked out at this stage because *ALD2* has been reported to have high activity specificity for linear medium chain fatty aldehydes [40]. Work is currently ongoing to knockout *ALD2* in the wild type and *pox1*, *pox2* and *pox3* knockout strains, with the aim to remove competing metabolic pathways that affect aldehyde production.

5.2 Redox optimisation of intracellular compartment for increased NADPH generation

The conversion of fatty acyl-CoA into fatty aldehyde by acyl-CoA reductase involves the cofactor NADPH as shown in Figure 1. By manipulating the ratio of NADPH/NADP⁺: NADH/NAD⁺ ratio in the cells, fatty aldehyde yield could be improved [48]. When yeast is fed on glucose, NADH is predominantly involved in catabolic reactions such as glycolysis and Krebs cycle, whereas NADPH is used in anabolic reactions such as fatty acid synthesis pathway. Therefore, the balance of these cofactors, i.e. the rate of formation and consumption of these cofactors, is important to ensure that the cofactors are available for normal cell function and growth.

5.2.1 Increase NADPH concentration by over-expressing glucose dehydrogenase (GDH)

One way to increase NADPH concentration in *S. cerevisiae* is to overexpress glucose dehydrogenase (GDH). NADPH is produced by GDH in the pentose phosphate pathway [49]. GDH catalyses the following reaction in *S. cerevisiae*:



The over-expression of GDH in several studies has shown to improve production yield of NADPH-dependent reactions such as production of alcohols in *S. cerevisiae* [50-52]. In addition, Parachin *et al.* (2009) compared the production of keto alcohol, which is produced by NADPH-dependent aldo-keto reductase *YPR1*, in *S. cerevisiae* and *E. coli*. Keto alcohol conversion was higher in *S. cerevisiae* than in *E. coli* as a result of GDH overexpression.

5.2.2 Increase NADPH concentration by over-expressing pyridine nucleotide transhydrogenase (PNT)

NADPH could be regenerated *in situ* by over expression of PNT. The transfer of protons between NADPH and NAD^+ or NADP^+ and NADH depends on the physiological conditions [53] and the location of PNT [54, 55]. PNT catalyses the following reaction:



There are two types of PNT depending on the location of the transhydrogenase in the cell: (i) cytoplasmic and (ii) membrane-bound PNT. It is hypothesized that cytoplasmic or otherwise known as soluble PNT (STH), which do not depend on protein translocation (i.e. proton electrochemical potential across membrane) [55], consumes NADPH and NAD^+ , and generates NADP^+ and NADH (i.e. backward reaction of Eq. 2) [55]. Membrane-bound PNT, which are located in the inner mitochondrial membrane of mammalian cells and cytoplasmic membrane of bacteria, on the other hand catalyses the formation of NADPH from NADP^+ and NADH (i.e. forward reaction of Eq. 2). Membrane-bound PNT activity is suspected to supply up to

35- 45% of total NADPH in *E. coli* grown in medium containing glucose [56]. Therefore, in this work, membrane-bound PNT should be selected over STH for overexpression in *S. cerevisiae* to generate NADPH.

PNT is not naturally present in yeast; the expression of PNT derived from *E. coli* was attempted by Anderlund *et al.* (1999) in *S. cerevisiae*. PNT was found localized in the endoplasmic reticulum membrane of *S. cerevisiae* unlike in *E. coli* where it was found in the plasma membrane. *In vitro* testing of PNT expressed in *S. cerevisiae* showed that it was active and increased the amount of NADH and NADP⁺ in the cells, which suggested that the “backward” reaction (Eq. 2) was dominant. Therefore, it seems that the location in which PNT is expressed is important as the proton potential (ΔP) across the membrane affects the equilibrium of the reaction.

In the study by Weckbecker and Hummel (2004), membrane-bound pyridine nucleotide transhydrogenase (PNT) from *E. coli* and formate dehydrogenase (FDH) from *Candida boidinii* were cloned into *E. coli* to improve synthesis of chiral alcohols catalyzed by alcohol dehydrogenase (ADH) from *Lactobacillus kefir*. Production of (R)-phenylethanol was increased from 19% to 66% in the presence of PNT [57].

For this study, the overexpression of GDH and recombinant PNT to increase NADPH regeneration in *S. cerevisiae* will be studied. Overexpressing GDH involves fewer steps and enzymes than PNT but generates a side product, i.e. gluconic acid, which may decrease the physiological pH condition in cells [32] Changes in intracellular pH could affect protein activity by changing the charge of amino acids, which could

modify the active binding site [58]. A similar strategy used by Weckbecker and Hummel (2004) could also be employed for this study as shown in Figure 19. Expression of PNT also involves balance of NAD^+/NADH , where NADH is required for the conversion of fatty aldehyde to alkanes by decarbonylase. Therefore, there could also be a need to overexpress formate dehydrogenase (FDH) that would regenerate NADH for the decarbonylation of fatty aldehyde and regeneration of NADPH.

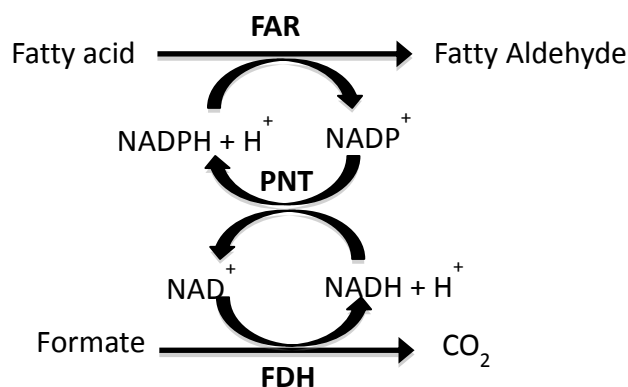


Figure 19 Schematic diagram of proposed NADPH regenerating system in *S. cerevisiae*.

5.2.3 NADPH regeneration by over-expressing malic enzyme

NADPH is also produced by malic enzyme in *S. cerevisiae*. Malic enzyme is involved in sugar metabolism and is responsible for catalysing the following reaction [59]:



In a study by Moreira Dos Santos *et al.* (2004), wild type and mutant strains of malic enzyme were overexpressed in the mitochondrial and cytosol respectively, together with pyruvate carboxylase in *S. cerevisiae* [60]. This decreased the pentose phosphate pathway flux, which could be due a decrease in the need for the production of

NADPH via this pathway. Even though there was no significant improvement on the ratio of $\text{NADP}^+/\text{NADPH}$, there was an increase in the activity of NADPH-dependent glutamate dehydrogenases [60], which resulted in a net PNT effect.

As demonstrated in the work by Moreira Dos Santos *et al.* (2004), overexpressing malic enzyme has shown to improve redox balances in *S. cerevisiae*, and this additional NADPH-generating pathway could be implemented into our work.

5.3 Protein engineering of FAR for improved activity

Besides introducing additional enzymes into the whole cell biocatalyst and altering the metabolic pathways, FAR could also be protein engineered to alter the enzyme's preference for NADH as cofactor instead of NADPH by rationally designing the protein structure [29, 32]. This could benefit the downstream reaction catalysed by AD which has been reported to have increased activity when NADH is used as a cofactor instead of NADPH [61]. Random mutagenesis could also be carried out to improve substrate specificity (e.g. to alter the substrate specificity of FAR to act on medium chain fatty acyl-CoA).

5.4 Alternative pathway involving Carboxylic acid reductase (CAR)

There are other enzymes which could be considered to be used in *S. cerevisiae* to produce aldehydes. Carboxylic acid reductase (CAR) was recently reported to be able to convert a wide range of fatty acids to fatty aldehydes (C_6 to C_{18}) (Figure 20) *in vitro* when expressed in *E. coli* [62]. When expressed *in vivo* with aldehyde decarbonylase

or aldehyde reductase, alkanes or alcohols were produced correspondingly and the yield achieved was much higher than that reported by Schirmer *et al.* (2010).

From this study, *S. cerevisiae* was shown to be able to accumulate fatty acids by introducing fatty acids into the culture medium. Failure to detect alkanes or aldehydes could be due several issues such as low expression level of FAR and solubility issue leading to inactivity of FAR *in vivo*, low turnover rate or substrate availability.

CAR could possibly be a better candidate than FAR as it does not require the substrate to be activated to fatty acyl-CoA, hence eliminating one potential limiting step.

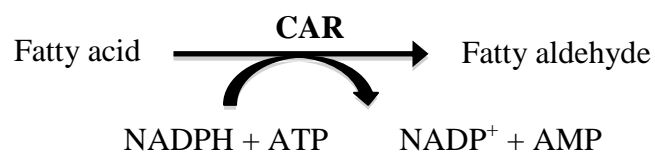


Figure 20 Conversion of fatty acid to fatty aldehyde catalyzed by CAR.

6 References

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