LIPID UTILIZATION OF THE YEAST *SACCHAROMYCES CEREVISIAE* ZINC CLUSTER DELETION STRAINS

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
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Abstract

Saccharomyces cerevisiae is a model organism widely used to study eukaryotic transcription. It is able to utilize various carbon substrates such as glycerol, ethanol and oils as well as fatty acids for growth and energy generation. In *S. cerevisiae*, zinc cluster proteins form a major class of transcription regulators. They play important roles in controlling expression of genes in various cellular processes, including sugar metabolism, amino acid synthesis, lipid metabolism and stress response. However, function of some zinc cluster proteins remains unknown. This study examined the involvement of some zinc cluster regulators of unknown function in lipid utilization. The phenotypes of zinc cluster deletion strains during growth on fatty acids or oils were examined. We found that five zinc cluster deletion strains (FZH, YZS, FZG, FZP and FZT) showed impaired growth on media, containing lipid or oil as a sole carbon source. The lipid class profiles of these zinc cluster deletion strains were analyzed and found that, using oleic acid as a sole carbon source, free fatty acid content of the FZG strain is increased by approximately 3-fold when compared with glucose grown condition. The sensitivity of these zinc cluster deletion strains in the presence of an oxidative agent menadione was also determined. The result showed that these zinc cluster deletion strains show defective growth upon the exposure to menadione, especially for the FZG strain. Then, the FZG strain (also called the Δ*yil130w* strain) was examined for the expression of genes required in lipid utilization by quantitative real time-polymerase chain reaction (qRT-PCR) analysis. The results showed that Yil130w is involved in the

activation of genes in lipid utilization, including triacylglycerol lipase (*TGL3*), peroxisomal transporter (*PXA1*), β-oxidation (*POX1*, *FOX2* and *POT1*), gluconeogenesis and glyoxylate cycle (*ICL1* and *PCK1*). Moreover, neutral lipid profiles of the FZG strain, grown at the exponential growth phase, showed high triacylglycerol (TAG) content at a similar level as found for the stationary phase cells. This result suggested that the Δ*yil130w* strain is unable to break down TAG. In summary, our results indicated a new set of zinc cluster proteins that are involved in lipid utilization. Importantly, the zinc cluster protein Yil130w is shown to function as a new transcriptional regulator of lipid utilization genes, including *TGL3*, *PXA1*, *POX1*, *FOX2*, *POT1*, *MDH2*, *ICL1* and *PCK1* genes.

Keywords: Lipid utilization/ Menadione/ Oxidative stress/ *Saccharomyces cerevisiae*/ Zinc cluster protein

หัวข้อวิทยานิพนธ์	กระบวนการใช้ลิปิดของสายพันธุ์ยีสต์ *Saccharomyces cerevisiae* ที่ขาดยีนในกลุ่ม zinc cluster
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ปีการศึกษา	2556

บทคัดย่อ

Saccharomyces cerevisiae เป็นแม่แบบของสิ่งมีชีวิตที่ถูกนำไปใช้อย่างแพร่หลายในการศึกษากระบวนการถอดรหัสทางพันธุกรรมของสิ่งมีชีวิตจำพวกยูคาริโอต เนื่องจากยีสต์ *S. cerevisiae* สามารถใช้แหล่งคาร์บอนได้หลากหลายชนิดในการเจริญเติบโตและสร้างพลังงานแก่เซลล์ อาทิเช่น กลีเซอรอล เอทานอล ไตรเอซิลกลีเซอรอล และกรดไขมัน การควบคุมการถอดรหัสทางพันธุกรรมของยีสต์ *S. cerevisiae* มีกลุ่มโปรตีนที่สำคัญเรียกว่า zinc cluster ซึ่งทำหน้าที่เกี่ยวข้องกับการควบคุมการแสดงออกของยีนในกระบวนการต่างๆภายในเซลล์ เช่น การสันดาปน้ำตาล การสังเคราะห์กรดอะมิโน เมตาบอลิซึมของลิปิด และการตอบสนองต่อสภาวะเครียด อย่างไรก็ตามยังมีโปรตีน zinc cluster บางตัวที่ยังไม่ทราบบทบาทที่ชัดเจน งานวิจัยนี้จึงได้ศึกษาบทบาทของโปรตีน zinc cluster เหล่านี้ โดยเน้นในส่วนที่เกี่ยวข้องกับการควบคุมกระบวนการใช้ลิปิด โดยการตรวจสอบลักษณะทางฟีโนไทป์ของยีสต์ที่ขาดยีนในกลุ่ม zinc cluster ภายใต้สภาวะที่เลี้ยงในอาหารที่ประกอบด้วยไตรเอซิล กลีเซอรอลหรือกรดไขมันเป็นแหล่งคาร์บอนเพียงชนิดเดียว ผลการทดลองพบว่ายีสต์สายพันธุ์ที่ขาดยีนในกลุ่ม zinc cluster 5 สายพันธุ์ได้แก่ สายพันธุ์ FZH YZS FZG FZP และ FZT มีการเจริญเติบโตที่ผิดปกติในแหล่งอาหารเหล่านี้ซึ่ง เมื่อใช้กลูโคสหรือกรดโอเลอิกเป็นแหล่งคาร์บอนเพียงชนิดเดียว ผลการวิเคราะห์ลิปิดโปรไฟล์แสดงให้เห็นว่ายีสต์สายพันธุ์ FZG ที่ถูกเลี้ยงในอาหารที่มีกรดโอเลอิก เซลล์มีปริมาณของกรดไขมันอิสระที่สูงกว่าในสภาวะที่มีกลูโคสเป็นแหล่งคาร์บอนประมาณ 3 เท่า ผู้วิจัยยังได้ทำการทดสอบการตอบสนองของยีสต์ทั้ง 5 สายพันธุ์ข้างต้นต่อสภาวะที่มีสารก่ออนุมูลอิสระ menadione และพบว่ายีสต์สายพันธุ์ที่ขาดยีนในกลุ่ม zinc cluster เหล่านี้มีการเจริญเติบโตที่ลดลงเมื่อเปรียบเทียบกับยีสต์สายพันธุ์ปกติ โดยเฉพาะสายพันธุ์ FZG มีการเจริญเติบโตลดลงมากที่สุด จากการตรวจสอบการแสดงออกของยีนที่เกี่ยวข้อง

กับการใช้ลิปิดในสายพันธุ์ FZG (ที่เรียกอีกชื่อว่า Δ*yil130w*) ด้วยเทคนิค quantitative real time-polymerase chain reaction (qRT-PCR) ผลการทดลองแสดงให้เห็นว่าโปรตีน Yil130w ทำหน้าที่เกี่ยวข้องกับการกระตุ้นการแสดงออกของยีนในกระบวนการใช้ ลิปิดได้แก่ triacylglycerol lipase (*TGL3*) peroxisomal transporter (*PXA1*) กระบวนการ β-oxidation (*POX1 FOX2* และ *POT1*)กระบวนการ gluconeogenesis และ กระบวนการ glyoxylate (*ICL1* และ *PCK1*) นอกจากนี้การวิเคราะห์ลิปิดโปรไฟล์ของยีสต์สายพันธุ์ FZG พบว่าในระยะที่ยีสต์มีการแบ่งตัวแบบทวีคูณ เซลล์มีปริมาณของไตรเอซิลกลีเซอรอล (TAG) สูงในระดับเดียวกับเซลล์ที่เจริญเติบโตในระยะคงจำนวนเซลล์ ซึ่งคาดว่าสายพันธุ์ FZG ไม่สามารถสลายไตรเอซิลกลีเซอรอลได้

จากผลการทดลองข้างต้นที่กล่าวมาแสดงให้เห็นว่ามีกลุ่มของโปรตีน zinc cluster ใหม่ ที่เกี่ยวข้องกับกระบวนการใช้ลิปิดเป็นแหล่งคาร์บอน โดยเฉพาะโปรตีน Yil130w ทำหน้าเป็นโปรตีนตัวใหม่ที่ควบคุมกระบวนการถอดรหัสของยีนที่เกี่ยวข้องในกระบวนการใช้ลิปิดได้แก่ ยีน *TGL3 PXA1 POX1 FOX2 POT1 MDH2 ICL1* และ *PCK1* เป็นต้น

คำสำคัญ: กระบวนการใช้ลิปิด/ โปรตีน zinc cluster/ สภาวะเครียดจากปฏิกิริยาออกซิเดชัน/ menadione/ *Saccharomyces cerevisiae*

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ABBREVIATIONS

ATP	=	Adenosine triphosphate
$^{\circ}C$	=	Degrees Celsius
cDNA	=	Complementary deoxyribonucleic acid
C_t	=	Threshold cycle
DBD	=	DNA binding domain
DNA	=	Deoxyribonucleic acid
FFA	=	Free fatty acid
g	=	Gram
h	=	Hour
H_2O_2	=	Hydrogen peroxide
HPLC	=	High performance liquid chromatography
l	=	Litre
µl	=	Microliter
mg	=	Milligram
mM	=	Millimolar
min	=	Minute
M	=	Molar
OD	=	Optical density
PDR	=	Pleiotropic drug resistance
qRT-PCR	=	Quantitative real-time polymerase chain reaction
RNA	=	Ribonucleic acid
ROS	=	Reactive oxygen species
rpm	=	Revolution per minute
S. cerevisiae	=	*Saccharomyces cerevisiae*
SE	=	Steryl ester
sec	=	Second
TAG	=	Triacylglycerol
TCA	=	Tricarboxylic acid cycle
v/v	=	Volume by volume
WT	=	Wild-type

CHAPTER 1 INTRODUCTION

1.1 Background

Biofuel is an alternative energy which has gained much recent interest in research due to it being clean, renewable, biodegradable and having less toxic properties. Generally, biodiesel is produced by the tranesterification reaction of vegetable or animal oil. However, vegetable and animal oil is mainly used for human consumption. Therefore, they are considered as a food source and the raw or starting materials have high cost for biodiesel production (Li et al., 2008). Thus, new oil sources for biofuel production are a highly interesting subject of research. Oil obtained from microorganisms such as yeasts is now gaining more attention. Microbial oil has many advantages when compared with plant oil as the production has a short-life cycle and is independent of climate and season. From past studies, lipid production of the oleaginous yeast *Yarrowia lipolytica* showed that it can be used to produce oil which is composed of fatty acids such as oleic acid, palmitic acid, palmitoleic acid and stearic acid. Its similarity in composition to vegetable oil indicates that it has potential for use as raw material to replace plant oils for biofuel production. Moreover, oils rich in essential polyunsaturated fatty acid have potential to complement nutrition (Tsigie et al., 2012).

Saccharomyces cerevisiae is an important yeast model, widely used to study yeast physiology, metabolism and gene regulation. *S. cerevisiae* is able to grow on a variety of carbon sources, including fatty acid and oil, and it is a useful model to gain insight into lipid metabolism. When yeast cells are grown in medium with fatty acids, it results in the up-regulation of genes encode for enzymes of β-oxidation, which are involved in the transformation of fatty acid to acetyl-CoA (Turcotte et al., 2010). Moreover, fatty acids also induce the proliferation of peroxisome to a sole site of fatty acid breakdown in yeasts. Many genes and gene products are ass^{oc}iated with fatty acid degradation—examples are Pox1, Fox2 and Pot3. These are specialized enzymes in β-oxidation pathway (Hiltunen et al., 2003). When those genes are disrupted, yeast cannot use lipids for growth, but they accumulate lipids in cells in the form of lipid bodies. Main storage lipids contain triacylglycerol and steryl esters. Fatty acid β-oxidation generates hydrogen peroxide as a by-product. The form of reactive oxygen species (ROS) including superoxide radicals, hydroxyl radicals and hydrogen peroxide are able to damage cellular components. When ROS in cells exceed the antioxidants capacity,

oxidative stress occurs. The yeast cells are regulated by the expression of genes in the oxidant defense system (Costa et al., 2001, Jamieson, 1998).

Zinc cluster proteins form a major class of transcription regulators in yeast *S. cerevisiae*. They contain the $Zn(II)_2Cys_6$ binuclear cluster DNA-binding motif with the consensus sequence of Cys-X_2-Cys-X_6-Cys-$X_{5–12}$-Cys-X_2-Cys-$X_{6–8}$-Cys. Zinc cluster proteins associated with many important cellular processes, including sugar metabolism, gluconeogenesis, respiration, amino acid metabolism, vitamin synthesis, nitrogen utilization, stress response and pleiotropic drug resistance (PDR). Some Zinc cluster proteins have been shown to be involved in lipid metabolism. For example, Upc2 is the activator of ergosterol biosynthetic genes. Sut1 and Sut2 associate with sterol uptake, while Oaf1 and Pip2 activate genes involved in peroxisome proliferation (MacPherson et al., 2006, Turcotte et al., 2010). However, functional of several zinc cluster proteins are still unknown.

The objective of this study is to characterize the roles of some unknown zinc cluster proteins and their involvement in lipid utilization via phenotypic analysis. The growth phenotypes of yeast zinc cluster deletion strains on fatty acids and oils as a sole carbon source was observed. The hypothesis is that the zinc cluster deletion strains with impaired growth on lipid-containing media may function in the regulation of lipid utilization genes. In addition, high performance liquid chromatography (HPLC) was performed to identify lipid classes of yeast cells cultured under glucose or oleic acid conditions. Since, the abnormal growth of zinc cluster deletion strains that are cultured in media containing lipid may be due to the effect of oxidative stress, a phenomenon which generally associates with non-fermentative metabolism, the sensitivity of yeast zinc cluster deletion strains to an oxidative agents was also examined via phenotypic analysis and cell viability assay. To identify gene targets of zinc cluster proteins that are involved in oleic acid utilization, the expression levels of some selected genes were tested for one zinc cluster deletion strains via qRT-PCR.

1.2 Objectives

1.2.1 Characterize new zinc cluster regulators that play roles in lipid utilization

1.2.2 Identify the function and selected gene targets of a selected zinc cluster protein in oleic acid utilization

1.3 Scope of work

1.3.1 Characterize zinc cluster genes of unknown role and implicate them in lipid utilization via phenotypic analysis

1.3.2 Analyze neutral lipid class and amount of accumulated lipids of some selected yeast zinc cluster deletion strains, grown in glucose or oleic acid conditions using high performance liquid chromatography (HPLC)

1.3.3 Examine the sensitivity of some yeast deletion strains to an oxidative agent menadione using phenotypic analysis and cell viability assay

1.3.4 Identify genes targets of a selected zinc cluster protein (Yil130w) in lipid metabolism during oleic acid utilization via qRT-PCR analysis

1.4 Benefits

1.4.1 This thesis study identifies new zinc cluster proteins that play role in lipid utilization in the yeast *S. cerevisiae*

1.4.2 Yeast deletion strains with accumulated high lipid content may be used as a model for study of transcriptional regulation of lipid utilization genes in other important industrial yeasts and fungi for possible applications in biofuel, bio-refinery, bio-plastic, food and pharmaceutical industries

CHAPTER 2 LITERATURE REVIEW

2.1 Model organism: *Saccharomyces cerevisiae*

Baker's yeast *S. cerevisiae* is a unicellular organism used in various industrial processes to produce biofuels, pharmaceutical components, beverages and food products. Moreover, it is often used as a model organism for studying eukaryotic molecular and cell biology such as lipid metabolism (Lushchak, 2006, Nevoigt, 2008). *S. cerevisiae* has various advantages over other organisms because it can be grown easily in a laboratory and also has the ability to utilize a wide variety of carbon sources, including non-fermentable carbons such as glycerol, ethanol and lipid (Turcotte et al., 2010). In addition, the *S. cerevisiae* genome has been completely documented and the metabolic pathway has been well described (Veen et al., 2004). These properties make *S. cerevisiae* a good model organism for the strain development via metabolic and genetic engineering. It is also used as the model yeast for studying lipid metabolism, utilization, production and accumulation (Dyer et al., 2002, Kamisaka et al., 2007).

2.2 Lipid metabolism of *Saccharomyces cerevisiae*

Eukaryotic organisms such as yeasts can store neutral lipids as a lipid droplets or lipid bodies (Czabany et al., 2007). In *S. cerevisiae,* triacylglycerols (TAG) are major neutral lipid storage that plays an important role as an energy storage in the cell. TAG is a suitable form of free fatty acid (FFA) storage because biological toxicity of TAG is lower than FFA (Sorger et al., 2003). TAG is not only used as the energy source, but also as a fatty acid donor for membrane biogenesis. The neutral lipids are accumulated as lipid droplets, and the amount of neutral lipids is increased when the cells enter into the stationary phase (Mullner et al., 2004). As an example of a response to changing environmental conditions, stationary phase cells are transferred to fresh glucose containing media, and lipid storage are hydrolyzed to degradation products that are used as building blocks for membrane synthesis (Czabany et al., 2007, Kohlwein et al., 2013).

2.2.1 Synthesis of triacylglycerols

Triacylglycerols (TAG) are the main lipid storage in yeast cells. They are synthesized through two pathways comprising of glycerol-3-phosphate (G-3-P) and

dihydroxyacetone phosphate pathway (DHAP) (Figure 2.1). For first, G-3-P is acylated by a G-3-P acyltransferase (GAT) to form lyso-phosphatidic acid (LPA). Then, LPA is converted to phosphatidic acid (PA) by 1-acyl G-3-P acyltransferase (AGAT). In addition, PA is also formed via the DHAP pathway. DHAP is acylated by DHAP acyltransferase (DHAPAT) and produces 1-acyl-DHAP. 1-acyl-DHAP reductase (ADR) converts 1-acyl-DHAP to yield LPA. Then, LPA is further acylated to phosphatidic acid by AGAT. PA can be produced from phospholipids by phosphorylation reaction of phospholipase. PA is dephosphorylated by a phosphatidate phosphatase (PAP) to yield DAG. In the final step of TAG synthesis, DAG is converted to TAG by diacylglycerol acyltransferase (DAGAT) (Czabany et al., 2007, Sorger et al., 2003).

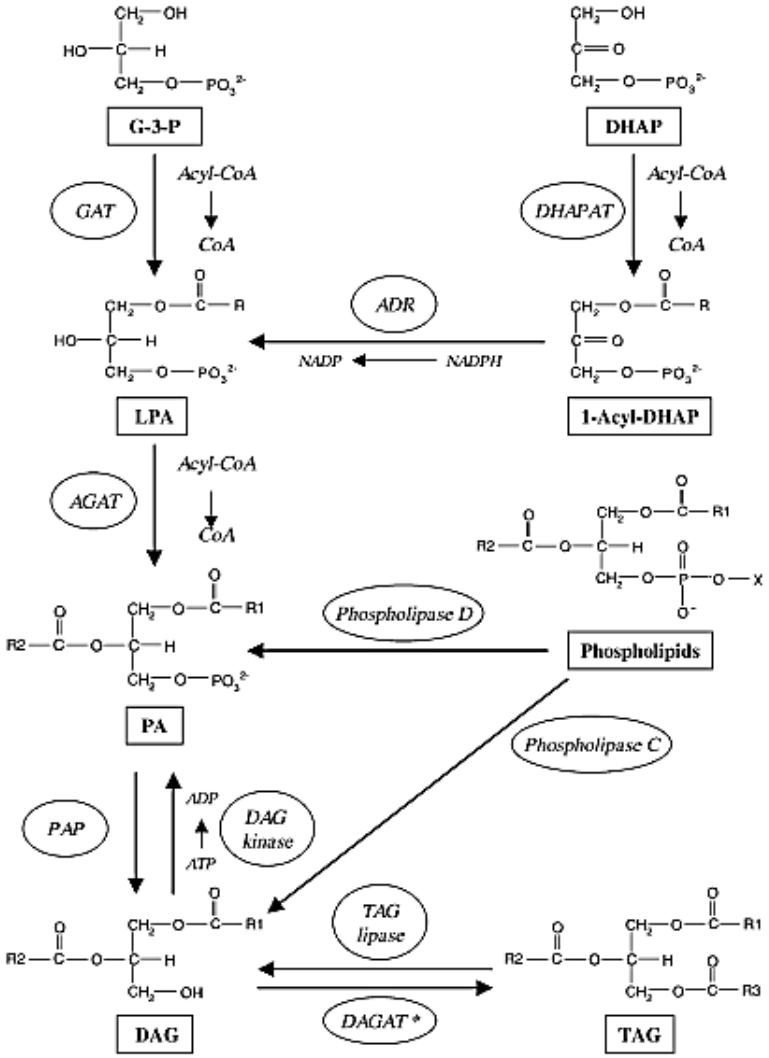

Figure 2.1 Pathway of triacylglycerol synthesis in *S. cerevisiae* (Sorger et al., 2003).

2.2.2 Utilization of triacylglycerols

The mobilization of neutral lipids occurs to fulfill the cell requirement for fatty acid. In yeast cells, TGA is broken down by triacylglycerol lipases, including Tgl3, Tgl4 and Tgl5. These TGA lipases have different substrate specificity (Beopoulos et al., 2011). Tgl3 is unspecific to the chain length of the fatty acid, whereas Tgl4 and Tgl5 are specific with C14:0 or C16:0 acid and C26:0 acid, respectively (Czabany et al., 2007). The hydrolysis of TAG provides diacylglycerol (DAG) and FFA, an important metabolite for membrane lipid biosynthesis and also for energy production. Fatty acids from triacylglycerol breakdown are used for the synthesis of phospholipid or further degraded through the β-oxidation pathway, located in the peroxisome (Beopoulos et al., 2011).

2.2.3 Fatty acid β-oxidation

S. cerevisiae can utilize a wide variety of carbon substrate including fatty acids (Hiltunen et al., 2003). The β-oxidation pathway is the process for breaking down fatty acids to generate acetyl-CoA. This process contains four steps, including CoA activation, oxidation, hydratation/ dehydrogenation and thiolytic cleavage (Kohlwein et al., 2013). The major breakdown routes of fatty acids are shown in Figure 2.2. Many genes are involved in fatty acid degradation (Table 2.1). Genes and gene products, which are involved in degradation of fatty acids, work in a variety of functions in the degradation. For example, transporters allow fatty acids and their metabolites to cross cell membranes, generating a cross-talk between peroxisomes and other subcellular compartments. Moreover, some genes are involved in regulation of gene expression in peroxisomal β-oxidation. Long-chain fatty acids are activated by fatty acyl-CoA synthetase Faa1 and Faa4. Then, they are transported to the peroxisome through ATP-binding cassette transports, Pxa1 and Pxa2. The deletion of *PXA1* and *PXA2* genes result in impaired fatty acid oxidation and cell growth is decreased on the medium containing long-chain fatty acid as a sole carbon source (Beopoulos et al., 2011). Activation of medium-chain fatty acid occurred in peroxisome via the peroxisomal acyl-CoA synthetase Faa2 (Beopoulos et al., 2011). Acyl-CoA substrates are oxidized to *trans*-2-enoyl-CoA by Pox1/Fox1, acyl-CoA oxidase in peroxisomes. Moreover, Pox1/Fox1 passes electrons directly to oxygen to generate hydrogen peroxide (H_2O_2). The next step of the β-oxidation pathway is the conversion of *trans*-2-enoyl-CoA to

3-ketocyl-CoA. This step depends on Mfe2/Fox2, (3R) hydroxide hydrogenase and 2-enoly-CoA hydratase 2, which is a homodimeric multifunction enzyme. The products of this last step are acetyl-CoA and C_2-shortened acyl CoA, the latter acting as substrate for Pox1/Fox1 (Hiltunen et al., 2003). The process can be repeated until complete breakdown of the fatty acid to acetyl-CoA (Beopoulos et al., 2011). Acetyl-CoA is transported to glyoxylate cycle and TCA cycle for the generation of ATP.

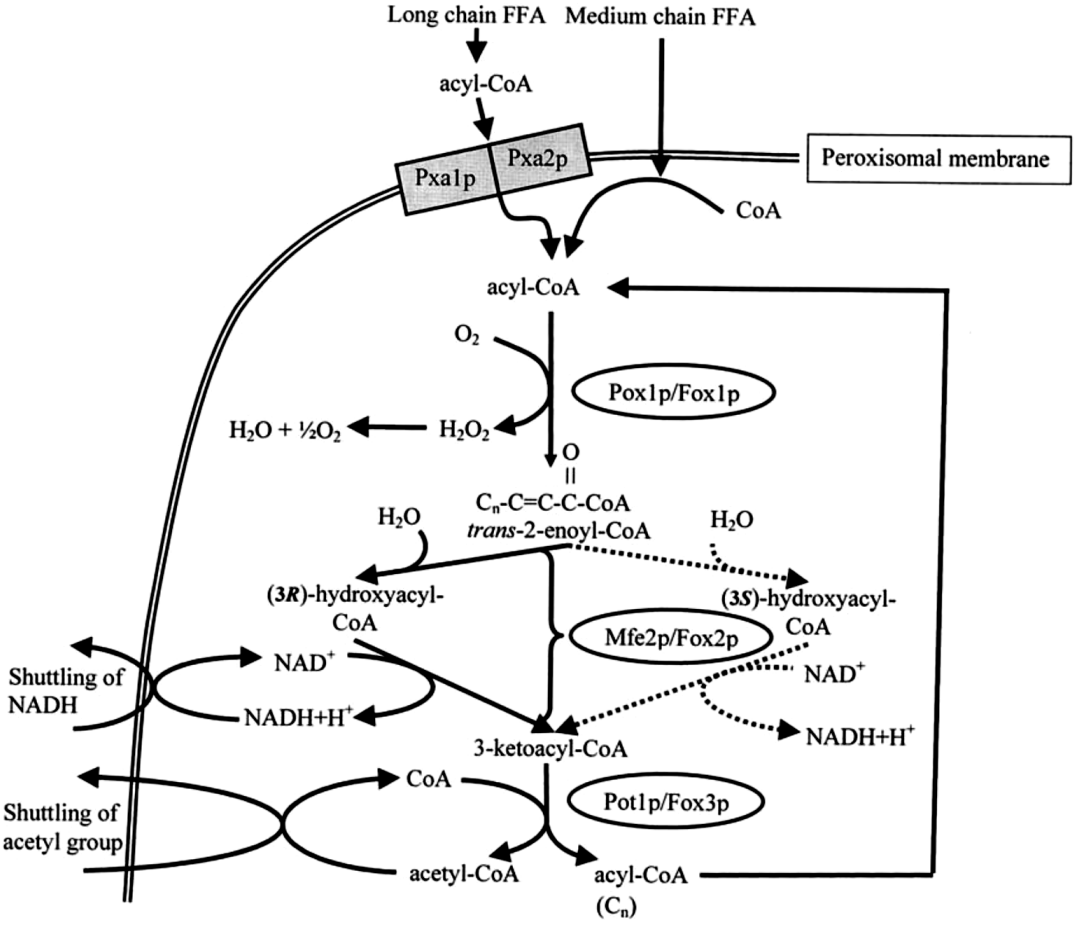

Figure 2.2 Yeast peroxisomal β-oxidation and fatty acid degradation. The broken arrows show the (3S)-hydroxy acyl-CoA-dependent route that occurs in β-oxidation system of other organisms (Hiltunen et al., 2003).

2.2.4 Reactive oxygen specie generation

In the first step of β-oxidation, fatty acyl-CoA is converted to *trans*-3-enoyl-CoA by acyl-CoA oxidase (Figure 2.2). This enzyme also passes electrons directly to oxygen to generate hydrogen peroxide (H_2O_2). It is one form of the reactive oxygen species (ROS). ROS are generated as a by-product of cellular metabolisms. The form of ROS, including superoxide anion, hydroxyl radical and hydrogen peroxide highly damage cellular components such as lipids, proteins and nucleic acids (Costa et al., 2001). Under normal conditions, the cells protect themselves from ROS by antioxidant defense systems. Hydrogen peroxide is broken into H_20 and O_2 by catalase A, which is encoded from *CTA1* in peroxisome (Hiltunen et al., 2003). Antioxidant systems play a role to neutralize the ROS and also repair molecular damage. However, when the concentration of ROS is more than the antioxidant buffering capacity, the cells are confronted with oxidative stress which may lead to cell death (Costa et al., 2001). Oxidative stress can be generated by the aerobic metabolism and also exogenously by being exposed to drug or oxidative agents such as hydrogen peroxide, diamide and menadione.

Menadione (2-methyl-1,4-napthaquinone: vitamin K_3) is a multivitamin component wildly used as a therapeutic agent for hypothrombinemia and cancer. It has been widely used as a model for studies of oxidative stress. Menadione is a quinone compound. Quinone is changed to the semiquinone by various flavone enzymes such as cytochrome P450 reeducates. In the presence of oxygen, semiquinone is converted into parent quinone and leads to the generation of ROS, including superoxide anion ($O_2\cdot$) and hydrogen peroxide and hydroxyl radical through redox cycling (Castro et al., 2007, Castro et al., 2008).

Table 2.1 Genes and gene products involved in lipid metabolism (Hiltunen et al., 2003).

Gene	ORF	Function	Regulation	Localization
1. Transporter				
ANT1	*YPR128C*	ATP carrier	Pip2-Oaf1	peroxisomal membrane
PXA1(SSH2, PAL1, PAL2, LPI1)	*YPL147W*	ABC transporter	Pip2-Oaf1	peroxisomal membrane

Gene	ORF	Function	Regulation	Localization
PXA2 (PAT1)	*YKL188C*	ABC transporter	Pip2-Oaf1	peroxisomal membrane
CRC1	*YOR100C*	carnitine/ acylcarnitine carrier	Pip2-Oaf1	mitochondrial inner membrane
SFC1 (ACR1)	*YJR095W*	succinate/fumarate antiporter	Pip2-Oaf1	mitochondrial inner membrane
AGP2	*YBR132C*	carnitine carrier	Pip2-Oaf1	plasma membrane
2. Enzymes involved in lipid utilization				
2.1 β-oxidation				
DCI1 (EHD2, ECI2)	*YOR180C*	$\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl CoA isomerase	Pip2-Oaf1	peroxisomal matrix
ECI1 (EHD1)	*YLR284C*	Δ^{3}-Δ^{2}-enoyl-CoA isomerase	Pip2-Oaf1	peroxisomal matrix
FOX2 (POX2), MFE2	*YKR009C*	(3R)-hydroxyacyl-CoA dehydrogenase, 2-enoyl-CoA hydratase 2	Pip2-Oaf1 Adr1	peroxisomal matrix
POT1 (FOX3, POX3)	*YIL160C*	L-ketoacyl-CoA thiolase	Pip2-Oaf1 Adr1	peroxisomal matrix
POX1 (FOX1)	*YGL205W*	acyl-CoA oxidase	Pip2-Oaf1 Adr1	peroxisomal matrix
SPS19 (SPX19)	*YNL202W*	2,4-dienoyl-CoA reductase	Pip2-Oaf1 Adr1	peroxisomal matrix
2.2 Glyoxylate shunt				
CIT2	*YCR005C*	citrate synthase	Rtg1, Rtg2	peroxisome
ACO1	*YLR304C*	aconitase		cytosol
ICL1	*YER065C*	isocitrate lyase	Cat8, Ino80	cytosol
MDH2	*YOL126C*	NAD^{+} regeneration	Cat8, Sir4	cytosol

Gene	ORF	Function	Regulation	Localization
MLS1	*YNL117W*	malate synthase	Cat8, Rds2	cytosol and peroxisomal
2.3 TCA cycle				
CIT1	*YNR001C*	citrate synthase		mitochondria
ACO1	*YLR304C*	aconitase		Cytosol
FUM1	*YPL262W*	fumarase		cytosol mitochondria
MDH1	*YKL085W*	mitochondrial malate dehydrogenase		mitochondria
IDH1	*YNL037C*	isocitrate dehydrogenase		mitochondria
IDH2	*YOR136W*	isocitrate dehydrogenase		mitochondria
KGD1	*YIL125W*	alpha-ketoGlutarate dehydrogenase		mitochondria
KGD2	*YDR148C*	alpha-ketoGlutarate dehydrogenase		mitochondria
LPD1	*YFL018C*	dihydrolipoamide dehydrogenase		mitochondria
LSC1	*YOR142W*	alpha subunit of succinyl-CoA ligase		mitochondria
LSC2	*YGR244C*	beta subunit of succinyl-CoA ligase		mitochondria
SDH1	*YKL148C*	succinate dehydrogenase		mitochondria
SDH2	*YLL041C*	succinate dehydrogenase		mitochondria
SDH3	*YKL141W*	succinate dehydrogenase		mitochondria

Gene	ORF	Function	Regulation	Localization
SDH4	*YDR178W*	succinate dehydrogenase		mitochondria
2.4 Gluconeogenesis				
ICL1	*YER065C*	isocitrate lyase	Cat8, Sip4	cytosol
PCK1	*YKR097W*	phosphoenolpyruvate carboxykinase	Cat8, Ert1, Gsm1, Rds2, Sip4	cytosol
FBP1	*YLR377C*	fructose-1,6-bisphosphatase	Cat8, Gsm1, Rds2	cytosol
MLS1	*YNL117W*	malate synthase	Cat8, Sip4, Rds2	cytosol peroxisome

2.2.5 Glyoxylate cycle

The glyoxylate cycle is essential for cell growth on non-fermentable carbon sources including fatty acids, ethanol and acetate. This process converts acetyl-CoA to succinate to replenish the TCA cycle intermediates and synthesis carbohydrate from acetyl units. The glyoxylate cycle contains five reactions catalyzed by isocitrate lyase (Icl1), malate synthase (Mls1), citrate synthase (Cit2), Aconitase (Aco1) and malate dehydrogenases (Mdh2) (Figure 2.3) (Lee et al., 2011). Isocitrate lyase (Icl1) and malate synthase (Mls1) are key enzymes in the glyoxylate cycle. Isocitrate lyase functions divide 6 units of carbon atoms into succinate and glyoxylate which is further condensed with acetyl-CoA by malate synthase to generate free CoA-SH and malate. Then, malate is used to continue the cycle by malate dehydrogenase (Mdh2). Succinate is released as net product that enters to the TCA cycle. When *ICL1* and *MLS1* are disrupted, the growth of cells are shown to impair growth on non-fermentable carbon sources (Strijbis et al., 2010). The enzymes involved in the glyoxylate cycle are shown in Table 2.1.

2.2.6 Tricarboxylic acid (TCA) cycle

The TCA cycle plays an important role in non-fermentable carbon utilization via oxidative generation of reducing equivalents (NADH), driving aerobic respiration to yield ATP. Moreover, this pathway is also an important source of biosynthetic building

blocks, such as α-ketoglutarate, succinyl-CoA and oxaloacetate, which are required for the synthesis of glucose and amino acids (Lee et al., 2011). The schematic of the TCA cycle is shown in Figure 2.3. Enzymes of the TCA cycle are important for biosynthetic processes such as gluconeogenesis, heme biosynthesis and amino acid synthesis. In *S. cerevisiae*, this pathway contains eight enzymes that are encoded by 15 different nuclear genes. Four of the enzymes, including citrate synthase, aconitase, fumarase and malate dehydrogenase are encoded by the single genes, *CIT1*, *ACO1*, *FUM1* and *MDH1*, respectively. The other four enzymes are composed of subunits encoded by more than one gene. Isocitrate dehydrogenase is comprised of Idh1 and Idh2 subunits that are encoded by *IDH1* and *IDH2* genes, respectively. The α-ketoglutarate dehydrogenase complex contains three subunits encoded by *KGD1*, *KGD2* and *LPD1* genes. Succinyl-CoA ligase is heterodimer that encoded by *LSC1* and *LSC2* genes. Succinate dehydrogenase is a protein that contains four subunits encoded by *SDH1-SDH4* genes. Deletion of TCA cycle genes leads to the cells not being able to suitably oxidize the acetyl-CoA generated from the β-oxidation of fatty acid in the developing peroxisome (Przybyla-Zawislak et al., 1999). The TCA cycle also contributes to gluconeogenesis in conjunction with the glyoxylate cycle (McCammon et al., 2003).

2.2.7 Gluconeogenesis

In *S. cerevisiae*, the utilization of non-fermentable carbon sources requires gluconeogenic enzyme activity. Gluconeogenesis is a process that is opposite to glycolysis (Figure 2.3). The enzymes involved in gluconeogenic pathway are those of the glyoxylate cycle and gluconeogenesis, including isocitrate lyase (*ICL1*), malate synthase (*MLS1*), phosphoenolpyruvte carboxykinase (*PCK1*) and fructose-1,6-bisphosphatase (*FBP1*) (Foy et al., 1977). The expression of the gluconeogenic genes, *PCK1*, *FBP1*, *ICL1* and *MLS1* are up-regulated during glucose depletion or in the presence of non-fermentable carbon (Turcotte et al., 2010).

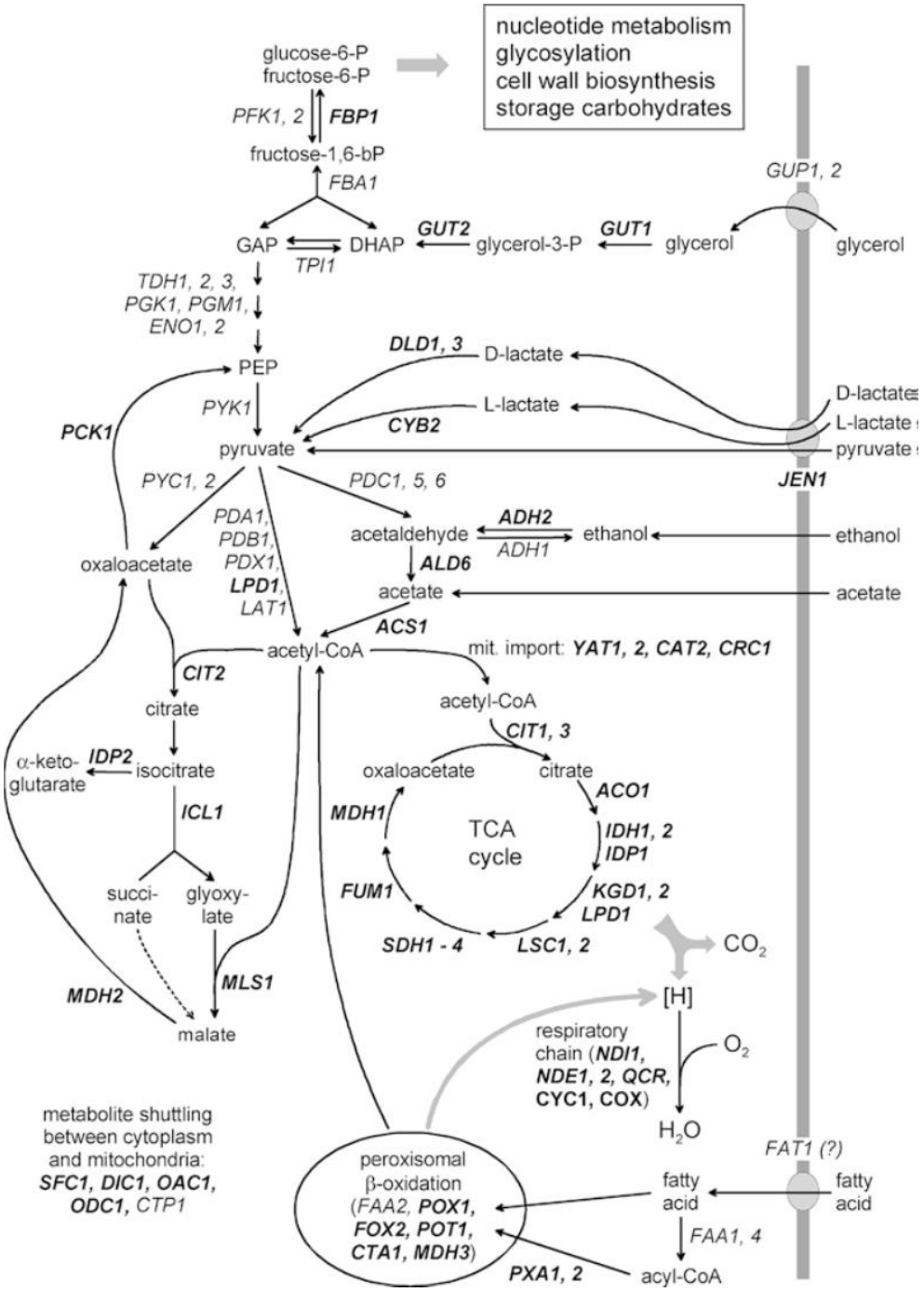

Figure 2.3 Schematic representations of the glyoxylate cycle, TCA-cycle and gluconeogenesis in yeast *S. cerevisiae* (Schuller, 2003)

2.3 Zinc cluster proteins

In eukaryotic cells, the zinc finger proteins are members of a large family of transcription regulators. They have various structures and display various functions. Zinc finger protein can bind to DNA and plays an important role in transcription and

translation processes. Moreover, they are involved in other physiological roles, such as protein-protein interaction, chromatin remodeling, lipid binding and zinc sensing. Zinc cluster proteins can be divided into three major classes based on their unique and highly conserved consensus amino acid sequences as shown in Table 2.2. Class I is the classical zinc finger protein which is one of the most common types of transcription factors found in eukaryotes. These proteins bind to nucleic acid as monomers. Class II represents the Cys_4 (C_4) zinc finger. Proteins in this class bind to DNA as homodimer or heterodimer. Class III is the class of zinc finger proteins that contains a DNA binding domain (DBD) that consists of cysteine residues bound to zinc atoms. These transcription factors are called zinc cluster, zinc binuclear cluster, or $Zn(II)_2Cys_6$ (Zn_2C_6) proteins. These proteins may interact with DNA as monomers, homodimers, or heterodimer. Transcription factor Gal4 in *S. cerevisiae* is a well-known and commonly studied zinc cluster protein (MacPherson et al., 2006).

Table 2.2 Three major classes of eukaryotic zinc finger proteins (MacPherson et al., 2006)

Zinc finger class	Subclass(es)	Consensus amino acid sequence	Example
I (C_2H_2)	FOG (C_2HC)	Cys-$X_{2–4}$-Cys-X_{12}-His-$X_{3–5}$-His	*Xenopus* TFIIIA
II (C_4)	GATA, nuclear receptors, LIM (C_3H)	Cys-X_2-Cys-X*n*-Cys-X_2-Cys-X*n*-Cys-X2-Cys-X*n*-Cys-X_2-Cys	Glucocorticoid receptor
III (C_6)		Cys-X_2-Cys-X_6-Cys-$X_{5–12}$-Cys-X_2-Cys-$X_{6–8}$-Cys	*S. cerevisiae* Gal4p

The zinc cluster proteins are Class III zinc finger proteins. They are a major class of transcription regulators in fungi such as *Saccharomyces*, *Aspergillus* and *Candida* species. The *S. cerevisiae* genome contains 55 genes, encoding for putative zinc cluster proteins. These proteins are associated with many important cellular processes. They can act as a repressor, activator, or both activator and repressor. For example, Rgt1 and Ume6 are both activators and repressors of glucose transportation and early meiotic genes, respectively (Ozcan et al., 1996). Moreover, Stb5 acts as an activator and a

repressor in the presence of oxidative stress (Akache et al., 2001, MacPherson et al., 2006).

2.3.1 The structure of zinc cluster proteins

The structure of zinc cluster proteins contains several functional domains, including DNA binding (DBD), regulatory and activation domains. The DBD is divided into three regions comprised of the zinc finger, linkers and dimerization regions (MacPherson et al., 2006). The DBD of zinc cluster proteins contain cysteine-rich regions located at the N-terminus, while the acidic activating domain is located at the C-terminus (Akache et al., 2001). A model of functional domains is shown in Figure 2.4. Cysteine mediated the binding of two zinc atoms, which are necessary for the zinc finger to bind to DNA (Figure 2.4). The DBD of zinc cluster protein contains a consensus amino acid sequence of Cys-X_2-Cys-X_6-Cys-X_5–12-Cys-X_2-Cys-$X_{6–8}$-Cys (Akache et al., 2001). Zinc cluster proteins play a role as a transcription regulator that bind to specific sequences of DNA. Zinc cluster proteins recognize trineucleotide sequences in specific CGG triplets in single or repeated forms, symmetrically and asymmetrically in the major groove of DNA. Zinc cluster proteins can bind as monomers, homodimers and heterodimers to CGG triplets, which can be oriented in everted, inverted, or direct repeats (Figure 2.5).

Figure 2.4 Structure of the functional domain of zinc cluster proteins. Structure of zinc cluster protein contains the DNA binding domain, regulatory domain and acidic region. Moreover, the DNA binding domain can be divided into three regions: the zinc finger, the linker and the dimerization (MacPherson et al., 2006).

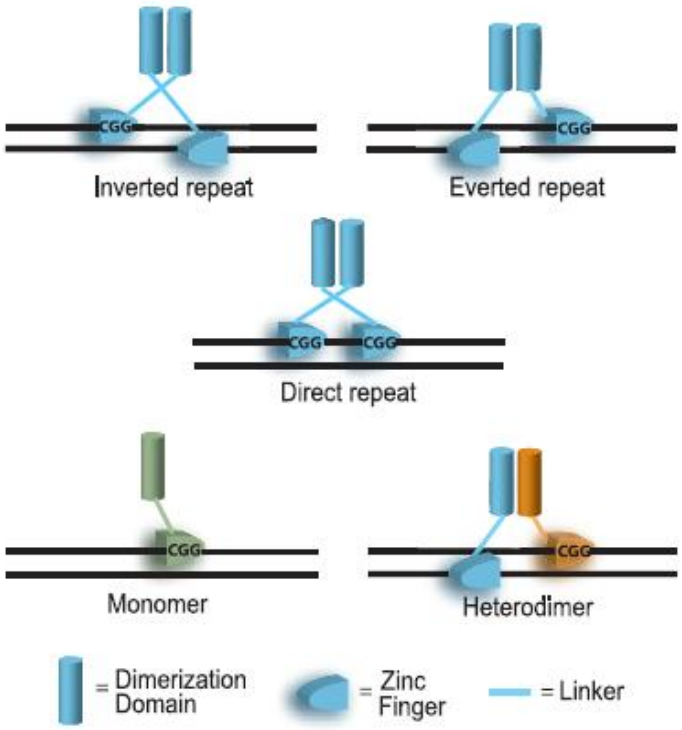

Figure 2.5 A model for zinc cluster proteins: DNA recognition. Zinc cluster protein can bind to the GCC triplet of DNA as monomers, homodimers and heterodimers with different configurations including everted, inverted, or direct repeats (MacPherson et al., 2006).

2.3.2 Role of Zinc cluster proteins

Zinc cluster proteins have been shown to be involved in a wide variety of cellular processes, including sugar metabolism, gluconeogenesis, respiration, amino acid metabolism, vitamin synthesis, nitrogen utilization, peroxisome proliferations, stress response and pleiotropic drug resistance (PDR). However, the functions of several zinc cluster proteins are unknown. Functions of zinc cluster proteins are shown in Table 2.3. Many of these transcriptional regulators not only have more than one distinct role, but they also have overlapping functions. For example, Upc2 plays a primary role in activating ergosterol biosynthesis genes, but it also plays a secondary role in anaerobic sterol uptake and expression of *DNA/TIR* mannoprotein genes (MacPherson et al., 2006)

Table 2.3 Classification of zinc cluster proteins in *S. cerevisiae* (MacPherson et al., 2006).

Role	Gene name	Localization	Functions
Sugar metabolism	*GAL4*	C, N	Activates genes involved in galactose metabolism (*GAL1*, *GAL10*)
	RGT1	C, N	Activator/repressor of hexose transport genes
	MAL13	U	Part of *MAL1* complex locus
	MAL33	U	Activator of maltose genes in maltose metabolism, forms part of *MAL3* complex locus
	MAL63	U	Activator of maltose genes
Amino acid, vitamin, and uracil metabolism	*ARO80*	N	Activator of aromatic acid catabolic genes
	LEU3	N	Activator/repressor of lucine biosynthesis genes
	LYS14	C, N	Activator of lycine metabolic enzyme
	PUT3	C, N	Induction of proline utilization genes
	THI2 (PHO6)	U	Activator of thiamine biosynthetic genes
	ARG81	C, N	Activator/repressor of arginine metabolism enzyme
	CHA4	C, N	Induction and basal expression of serine and threonine utilization, activates *CHA1*
	PPR1	N	Activate *URA1* and *URA3*
Chromatin remodeling	*RSC3*	N	Essential component of RSC chromatin-remodeling
	RSC30	N	Subunit of the RSC chromatin-remodeling complex
Meiosis and mitosis	*UME6*	N	Repress early meiotic genes
	CEP3	N	Essential kinetochore component, chromosome segregation
Nitrogen utilization	*UGA3*	N	Activate GABA genes
	DAL81	N	Activator of nitrogen catabolic genes, including allantoin and GABA genes
PDR/stress response	*PDR1*	N	Activator of PDR genes
	PDR3	N	Activator of PDR genes
	PDR8	C, N	Involved in PDR
	YRM1	C, N	Activator of PDR genes
	YRR1	C, N	Activator of PDR genes
	HAL9	C, N	Involved in salt tolerance

Role	Gene name	Localization	Functions
	STB5	C, N	Interact with Sin3, is an activator of PDR genes, and is involved in oxidative stress resistance
	RDR1	U	Repressor of PDR genes
	RDS1	U	Regulator of drug sensitivity
	RDS2	U	Regulator of drug sensitivity
	WAR1	N	Activator of *PDR12* in response to weak acid stress
	ASG (YIL130W)	N	Activator of stress response genes
Peroxisome proliferation	*OAF1*	C, N	Activate gene involved in peroxisome proliferation
	PIP2	C, N	Activate gene involved in peroxisome proliferation
Ergosterol biosynthesis	*UPC2*	C, N	Anaerobic sterol uptake, activator of ergosterol biosynthesis genes
	ECM22	C, N	Activator of ergosterol biosynthetic genes
	SUT1 (YGL162W)	N	Overexpression increases sterol
	SUT2	U	Overexpression increases sterol uptake, multicopy suppressor of low activity of cyclic AMP/proteinase kinase A pathway
Gluconeogenesis and respiration	*CAT8*	C, N	Activates genes needed for gluconeogenesis
	SIP4	U	
	HAP1	U	Snf1 kinase-dependent activator of gluconeogenesis gene Activate respiration genes
Miscellaneous	*SEF1*	U	Compensate for the essential function of RPM2 in cell growth
	TEA1	C, N	Activates transcription of Ty1 retrotransposon
	STB4	N	Interaction with Sin3p in yeast two-hybrid system
Unknown	*EDS1 (YBR033W)*	U	Expression is dependent on Rpb2
		C, N	Δ*ybr150c* is sensitive to thiabendazole
	TBA1(YER150c)	C, N	Interact with Rds2 in yeast two-hybrid system
	YBR239C	C, N	Δ*ydr520* is slightly sensitive to
	YDR520C	U	caffeine
	YER184C	U	Δ*yfl052* is hypersensitive to heat

Role	Gene name	Localization	Functions
	YFL052W		shock at 37^{o}C
	YJL103C	U	May be involved in oxidative
	YJL206C	U	phosphorylation
	YKL222C	U	Δ*ykl222c* is sensitive to caffeine
	YKR064W	C, N	
	YLL054C	C	
	YLR278C	N	Δ*ylr278c* is sensitive to caffeine
	YNR063W	U	

N, nucleus; C, cytoplasm; M, microtubules; U, unknown.

2.3.3 Zinc cluster proteins involved in non-fermentable carbon utilization

S. cerevisiae can utilize a wide variety of carbon sources, including fatty acids (Hiltunen et al., 2003). The utilization of these carbon sources requires an enzymatic pathway. Normally, enzymes needed for a specific pathway are produced only when required. This regulation is mainly exerted at the transcriptional level. A number of transcriptional regulators involved in alternate carbon source utilization have been identified and are shown in Table 2.4 (Turcotte et al., 2010).

Table 2.4 Major transcription regulators of non-fermentable carbon utilization and their targets (Turcotte et al., 2009).

Transcriptional regulator	Type of DNA binding domain	Target genes
Adr1 (Alcohol dehydrogenase regulator)	Cys_2His_2 zinc Finger protein	Non-fermentable carbon metabolism (e.g. *ADH2, ACS1, GUT1*) Peroxisome biogenesis and fatty acids utilization (e.g. *POX1, PAX 1*)
Cat8 (Catabolite repression)	Zinc cluster protein	Gluconeogenic genes (e.g. *PCK1, FBP1*) Glyoxylate cycle genes Transcription factor (*SIP4*)
Ert1(Ethanol regulator of translation)	Zinc cluster protein	*PCK1* Other target unknown
Gsm1 (glucose starvation modulator)	Zinc cluster protein	Gluconeogenesis (*PCK1, FBP1)*

Transcriptional regulator	Type of DNA binding domain	Target genes
Hap1 (heme activator protein)	Zinc cluster protein	Respiration gene (e.g. *CYC1*,*CYC7)*
Hap2/3/4/5 (heme activator domain)	CCAAT-binding complex	Respiration gene (e.g. *CYC1*), TCA cycle
Oaf1 (oleate-activted transcription factor)	Zinc cluster protein	Weak repressor of oleate-responsive genes)
Pip2 (peroxisome induction pathway)	Zinc cluster protein	Fatty acid utilization (e.g. *POX1, FOX1)* Peroxisome biogenesis
Rds2 (regulator of drug sensitivity)	Zinc cluster protein	Gluconeogenic genes (*PCK1, FBP1*) Glyoxylate cycle genes (*MLS1*,TCA cycle genes) Transcription factors (*HAP4, SIP4*)
Sip4 (Snf1-interacting protein)	Zinc cluster protein	Gluconeogenic genes (e.g. *PCK1*)

2.3.3.1 Transcriptional regulation of fatty acid β-oxidation

Many genes are associated with the breakdown of fatty acid, which occurs inside the peroxisome via the β-oxidation pathway. The expression of genes is regulated by specific proteins acting as regulators. The major players in the regulation of fatty acid-induced gene expression are Pip2 and Oaf1, as well as Adr1 (Hiltunen et al., 2003).

Oaf1 and Pip2 (Oaf2) are the zinc cluster regulators that are involved in the oleate response by binding as homodimers to oleate response elements found in the promoters of β-oxidation genes. Although *OAF1* and *PIP2* co-regulate the same genes, their expression is differentially regulated. *OAF1* expression is constitutive whereas the expression of *PIP2* is positively auto-regulated (Rottensteiner et al., 1996). Oaf1 and Pip2 are found at common promoters. The presence of Pip2 is also required for Oaf1 binding in most promoters. Oaf1 and Pip2 heterodimers are mainly responsible for the activation of target genes. Activation of the Ofa1/Pip2 heterodimer is mediated by the

direct binding of oleate to Oaf1. Moreover, the presence of oleate results in hyperphosphorylation of Oaf1 and correlates with its transcriptional activity (Turcotte et al., 2010).

Adr1 is a transcription factor of the Cys_2His_2 class of zinc figure that binds DNA as a monomer (Turcotte et al., 2010). Adr1 was originally identified as a regulator of the alcohol dehydrogenase gene *ADH2*. Moreover, Adr1 is also related to the transcription of the *CTA1* gene, whose gene encodes enzyme peroxisomal catalase A, Cta1. Cta1 is involved in hydrogen peroxide decomposition to water and oxygen (Hiltunen et al., 2003). Moreover, Adr1 is also important for yeast growth on fatty acid. (Gurvitz et al., 2000). Adr1 is involved in the regulation of gene encoding for peroxisome proteins, including the gene for the β-oxidation enzyme such as *FOX2* and *FOX3* genes (Gurvitz et al., 2000). The cells lacking Adr1 leads to no growth on media containing fatty acid due to the genes encoding for peroxisomal proteins are being impaired (Gurvitz et al., 2000).

2.3.3.2 Transcriptional regulation of gluconeogenesis

ICL1 that encoded isocitrate lyase contained a carbon source-responsive element (CSRE) in its promoter (Schuller, 2003). CSRE is also found in upstream of *FBP1*, *PCK1*, *MLS1*, *MDH2*, *ASC1*, *SFC1 and ACR1*. The function of Cat8 and Sip4 are specifically required for activation of CSRE-dependent structural gene. Cat8 and Sip4 bind to CSRE in the promoter of gluconeogenesis genes to play a role as an activator (Turcotte et al., 2010). Cat8 is required for maximal expression of *SIP4*. When *CAT8* is disrupted, transcription of *SIP4* is reduced. In addition, the cells lacking Cat8, but not Sip4 are unable to grow on a non-fermentable carbon source (Turcotte et al., 2010).

Previous research studies showed that Rds2 is a transcriptional regulator involved in gluconeogenesis. Phenotypes of deletion of *RDS2* include sensitivity to calcofluor white, antifungal drug ketoconazole and also impaired growth on non-fermentable carbon glycerol or lactate (Akache et al., 2001). Rds2 is a major regulator in gluconeogenesis and regulates genes in related pathways such as glyoxylate and the TCA cycle (Soontorngun et al., 2007). Rds2 regulates key structural genes in gluconeogenesis including *PCK1* and *FBP1*. The expressions of *PCK1* encoding for PEP carboxykinase and *FBP1* encoding for fructose bisphosphatase are strongly

repressed in the presence of glucose. The expression of gluconeogenic genes is induced in the presence of non-fermentable carbon (Soontorngun et al., 2012, Turcotte et al., 2010).

2.3.3.3 Regulation of oxidative stress

Stb5 is characterized as a regulator of drug resistance similar to Pdr1 and Pdr3. Deletion of *STB5* showed various phenotypes, such as sensitivity to cold, caffeine and translation inhibitor cycloheximide. Moreover, previous research showed that Stb5 regulates genes of the pentose phosphate pathway that are important for the resistance of cells to oxidative stress. In addition, deletion of *STB5* showed sensitivity to the oxidative agents, hydrogen peroxide and diamide. Stb5 is required for resistance to oxidative stress and NADPH regulation. It plays a role both as an activator and repressor in the presence of oxidative stress (Larochelle et al., 2006).

In summary, numerous studies have shown that zinc cluster proteins contribute to non-fermentable metabolism by regulating of the expression of a different set of genes in the above pathways.

CHAPTER 3 MATERIALS AND METHODS

3.1 Yeast strains

The *S. cerevisiae* strains used in this study were the wild-type strains FY73 (MATa *his3*-Δ200 *ura3*-52) and the zinc cluster deletion strains (Table 3.1). These deletion strains were obtained by disrupting the open reading frame (ORF) of genes encoding for zinc cluster proteins. Disruption was performed by the PCR method using *HIS3* as a marker for selection (Baudin et al., 1993).

Table 3.1 List of *S. cerevisiae* strains used in this study.

Strain name	Systematic name of deleted gene	Deletion
FI	*YBR033W*	aa 27-200
FA	*YBR150C*	aa 24-167
FC	*YCR106W*	aa 23-206
FZT	*YER184C*	aa 26-225
YZS	*YFL052W*	aa 24-173
FZG	*YIL130W*	aa 24-246
FZQ	*YJL206C*	aa 28-149
FZI	*YLL054C*	aa 25-220
FZO	*YNR063W*	aa 25-166
FZJ	*YOL089C*	aa 115-319
FZP	*YOR380W*	aa 25-196
FZH	*YPL133C*	aa 25-266

3.2 Phenotypic analysis of the yeast strains on lipid-containing media

To screen for the zinc cluster genes that are involved in the utilization of lipid as a sole carbon source, phenotypic analysis of zinc cluster deletion strains was performed. The yeast cell cultures were grown in YPD medium containing 1% yeast extract (Himedia Laboratories, India), 2% peptone (Himedia Laboratories, India) and 2% dextrose (Himedia Laboratories, India). Cells were incubated at 30^{o}C and 130 rpm overnight. Then, the cells were serially diluted in distilled water to an optical density (OD_{600}) of 0.1, 0.04, 0.0062 and 0.0015. After that, 10 µl of each dilution was spotted onto YP plates containing different carbon sources. The fatty acids and oils used as a sole carbon

source were at a final concentration of 0.125% oleic acid, 0.125% linoleic acid, 0.125% palmitic acid, 0.125% stearic acid, 10 mg/ml soybean oil, 10 mg/ml sunflower oil, 10 mg/ml palm oil, 10 mg/ml rice bran oil and 10 mg/ml waste cooking oil. Fatty acids and oils were emulsified by 0.5% of Tween 80 (LabChem, Australia). The plates were incubated at 30^{o}C for 2-3 days to monitor growth phenotypes. The growth of yeast strains on fatty acids or oil plates were monitored compared with growth on glucose.

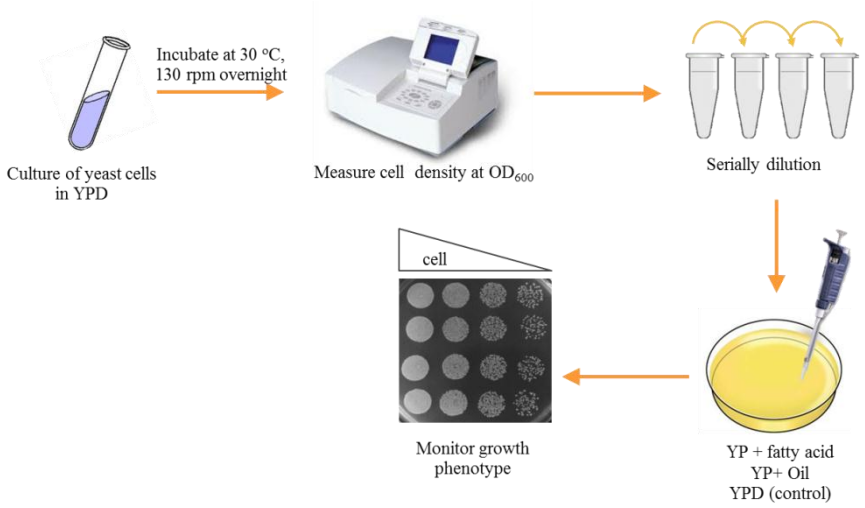

Figure 3.1 Phenotypic analysis of *S. cerevisiae* on media containing different fatty acids and oils.

3.3 Lipid analysis

3.3.1 Culture conditions for lipid analysis

The yeast cell cultures were grown in YPD media at 30 ^{o}C and 130 rpm overnight. The yeast cells with an initial OD_{600} of 0.1 were inoculated on the YPD medium for 5-7 h and regrown until reaching the exponential phase (OD_{600} of 0.6). Then, the culture was divided into half, spun down, and washed twice with autoclaved distilled water. The cells were transferred to YP media, containing 0.125% oleic acid, and emulsified with Tween 80. The cells were continually grown for an additional 3 h. For the stationary phase, the yeast cells initially at OD_{600} of 0.1were inoculated on the YPD medium and incubated for 96 h. Then, the cells were harvested by centrifugation at 4000 rpm for

5 min. Supernatant was poured off and discarded. The cells were washed twice with deionized water. After that, lipids in the cells were extracted.

3.3.1 Lipid extraction

The harvested cells were mixed with 10 ml methanol and the cells were disrupted with glass beads by vortex. Chloroform was added to the suspension (chloroform/methanol 2:1 (v/v)) and the suspension was stirred for 1 h. The extract was filtrated and 10 ml of 0.345% $MgCl_2$ solution was added. The extract was centrifuged at 3000 rpm for 5 min and allowed to aspirate off the upper aqueous layer before washing the organic phase with 10 ml of 2M KCl/methanol (4:1; v/v). It was centrifuged again at 3000 rpm for 5 min. Then, the upper aqueous layer was allowed to aspirate, including the protein layer that formed at the phase boundary. The organic phase was washed twice with 10 ml of artificial upper phase (chloroform/methanol/water, 3:48:47 per vol.). The solvent was evaporated in a rotary evaporator at 55^{o}C and 200 mbar. The lipid film was weighed and dissolved in 5-6 ml toluene.

3.3.2 High performance liquid chromatography (HPLC) analysis

HPLC was performed to analyze lipid class of lipid extract from the yeast cells. Lipid extract was dissolved in toluene, and 50 µl of the sample was injected into HPLC. The HPLC was equipped with a Sedex 80 Evaporative Light Scattering Detector (ELSD). Detector temperature was set at 30^{o}C and N_2 gas was set at 2 bar. The sample was analyzed on a 100 Å Phenogel column (300 mm × 7.8 mm ID, 5 µm). The column and injector were put in an oven set at 65^{o}C. The mobile phase that was used to analyze comprised of 100% toluene and 0.25% acetic acid. The flow rate of mobile phase was 1.0 ml/min.

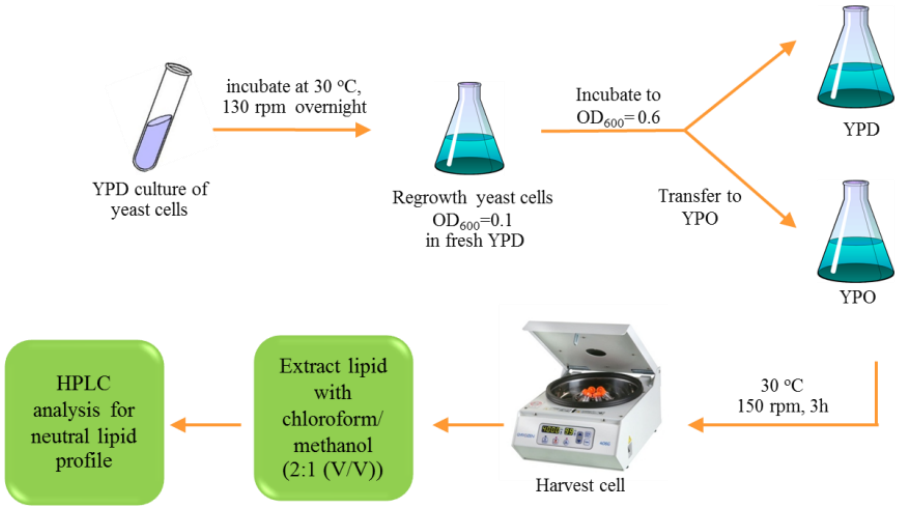

Figure 3.2 Diagram steps of culture conditions and for lipid class analysis via HPLC.

3.4 Oxidative stress tests

3.4.1 Phenotypic analysis

Phenotypic analysis was used to examine the sensitivity of yeast zinc cluster deletion strains under oxidative stress conditions. Menadione was used as an oxidative agent. The yeast cells were grown in YPD medium at 30^{o}C and 130 rpm overnight. The culture was inoculated into fresh media for an initial OD_{600} of 0.1. Mid-log phase cells (OD_{600} of 0.8) were exposed to 0.4 and 0.5 mM menadione soluble in DMSO, for 1 h at 30^{o}C and 100 rpm. The cells were serially diluted to OD_{600} of 0.1, 0.04, 0.00625 and 0.0015. After that, 10 µL of each dilution was spotted on appropriate YPD plates. Then, the plate was incubated at 30 ^{o}C for 2-3 days to monitor growth phenotypes. The growth of yeast strains was compared between non-treated and treated cells.

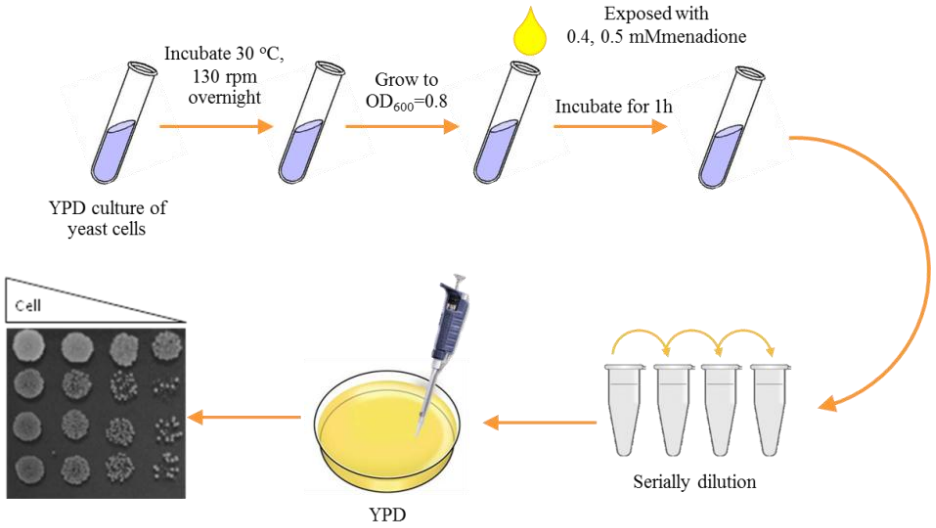

Figure 3.3 Methodology of phenotypic analysis under oxidative stress conditions. Mid-log phase cells were exposed to different concentrations of menadione and spotted on appropriate plates to observe growth phenotypes.

3.4.2 Cell viability assay

Cell viability of the wild-type and the zinc cluster deletion strains were determined by colony counting on YPD agar plates upon exposure with menadione. First, the yeast cell cultures were grown in YPD medium at 30°C and 130 rpm overnight. The culture was inoculated into fresh media for an initial OD_{600} of 0.1. Mid-log phase cells were exposed to 0.4 mM menadione soluble in DMSO, for 1 h at 30 °C and 130 rpm. The cells were serially diluted. After that, 50 µL of appropriate dilution was spotted and spread on YPD agar plates. Then, the cells were incubated at 30°C for 2-3 days to measure the colony number. The viability of cells was calculated by comparing the cells survival between non-exposed and exposed cells. Then, the viability of each zinc cluster deletion strain was compared with the wild-type strain as percentages of viability cells.

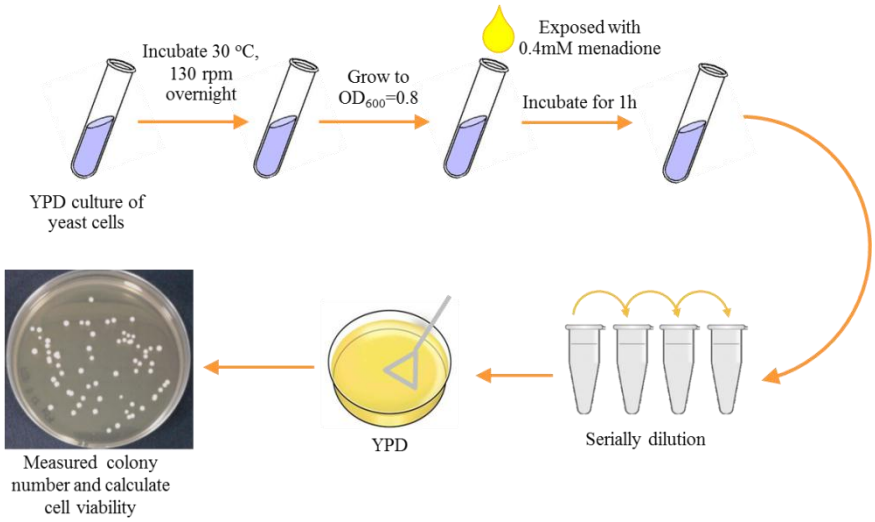

Figure 3.4 Determination of the sensitivity to oxidative stress of yeast *S. cerevisiae* via cell viability assay. The colony number was measured and calculated for cell viability percentage value.

3.5 RNA isolation and purification

3.5.1 Gene induction

Yeast cells were cultured in 5 ml of YPD medium at 30^{o}C and 130 rpm overnight. The cell cultures initial OD_{600} of 0.1 were inoculated in 40 ml YPD medium until reaching OD_{600} of 0.6. The cells were then divided in half for oleic acid induction. Then, the cells were spun at 3500 rpm for 5 min and washed twice with DI water. After, yeast cells were transferred to YP medium containing oleic acid. Then, the cell cultures were incubated for 3 h. The cultures were harvest by centrifugation at 3500 rpm, and washed with diethylpyrocarbonate (DEPC) water. Cell pallets were transferred to a new Eppendorf tube and kept at -80^{o}C for RNA extraction.

3.5.2 RNA extraction

The yeast cells were taken out of the -80^{o}C freezer and thawed on ice. Then 300 µl of Acid Phenol-Chloroform (5:1) (pre-warm for 10 min at 65^{o}C) and 300 µl of TES were added to the samples. Then, the cell mixtures were disrupted with glass beads by vortexing at the highest setting to re-suspend the cell pallets. The tubes were incubated at 65^{o}C in the water bath for 1 h and vortexed for 20 sec every 10 min. After that, the samples were vortexed for 20 sec and the solution was aliquot into a 1.5 ml Eppendorf

tube and spun for 20 min at 14000 rpm at 4^{o}C. The supernatant was transferred to a new Eppendorf tube, extracted with 750 µl of Acid Phenol-Chloroform per tube, vortexed for 20 sec and spun for 10 min at 4^{o}C. Then, the aqueous phase was transferred to a new Eppendorf tube, extracted with 750 µl of Choloroform-Isoamyl (25:1) per tube, vortexed for 20 sec and spun for 10 min at 4^{o}C. Then, the aqueous phase was transferred to a new Eppendorf tube with 50 µl of 3 M sodium acetate (NaAc) pH 5.2. Then 1 ml of 100% Ethanol (pre-cooled to -20^{o}C) was added. The samples were storageed at -20^{o}C overnight. The samples were spun down for 20 min at 4^{o}C. Then, RNAs were spun down for 1 min and aspirated to remove the last bit. RNAs were then allowed to air dried for 30 min and dissolved in DEPC-treated water. The RNAs were kept at -20^{o}C.

3.5.3 RNA purification

RNAs were purified by using the RNeasy Mini kit (QIAGEN). The RNA samples were adjusted to a volume of 100 µl with RNase-free water. Then, 350 µl Buffer RLT and 250 µl of ethanol were added and mixed well. The solution was transferred to RNeasy Mini spin column and then centrifuged for 15 sec at 14000 rpm. 350 µl of Buffer RW1 were added to the RNeasy spin column and centrifuged for 15 sec at 14000 rpm. A mixed solution of DNase I and Buffer RDD was applied to the column and centrifuged briefly twice. Then, columns were placed for 15 min, 350 µl of Buffer RW1 was added to the RNeasy spin column, and centrifuged for 15 sec at 14000 rpm. After that, 500 µl of Buffer RPE was added and centrifuged for 15 sec at 14000 rpm twice. RNeasy spin columns were removed to a new collection tube; 30 µl of RNase-free water were added to the column membrane and the sample was placed for 10 min, and for 15 sec at 14000 rpm. Finally, 30 µl of RNase-free water were added to each collection tube.

3.5.4 RNA gel and electrophoresis

Gel preparation: The final volume of gel was prepared as 150 ml. 1.5 g of agarose was weighed and 15 ml of 10X MOPS was added and completed with 104 ml of DEPC-treated water. Then, it was melted using a microwave, cooled down and followed by an addition of 30 ml of formaldehyde and 2.5 µl of 10 mg/ml ethidium bromide. Then, the gel was poured into the gel box.

RNA sample gel preparation: The RNA sample contained 5.5 µl of RNA, 1.0 µl of 10X MOPS, 3.5 µl of formaldehyde, 10 µl of formamide, and 2 µl of 5X loading dye. The final volume was 22 µl. The sample was heated at 65^{o}C for 15 min and quickly spun. The sample was kept on ice until loading. The gel was electrophoresed under the chemical hood. After, the gel was washed with distilled water overnight and a picture of the gel was recorded.

3.6 Quantitative Real Time–Polymerase Chain Reaction (qRT-PCR)

3.6.1 First-Strand cDNA synthesis

The cDNA synthesis was started by combining 5 µg of total RNA, Primer 1 µl (50 µM of oligo(dT)20, or 2 M gene-specific primer (GSP), or 50 ng/µl random hexamers), 10 mM dNTP mix, and up to 10 µl DEPC-treated water. All tubes were incubated at 65^{o}C for 5 min and then placed on ice for at least 1 min. Preparation of the following cDNA Synthesis Mix was done by adding each component in the indicated order (for 1 reaction): 2 µl of 10X RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl of RNaseOUTTM (40 U/µl), and 1 µl of SuperscriptTMIII RT (200 U/µl) (for the control reaction, add DEPC-treated water instead of SuperscriptTMIII RT (200 U/µl)). 10 µl of cDNA Synthesis Mix was added to each RNA/primer mixture, mixed gently, and collected by brief centrifugation. All solutions were incubated as follows: 50 min at 50^{o}C for oligo(dT)20, GSP primed, and for Random hexamer primed, incubated for 10 min at 25^{o}C, followed by 50 min at 50^{o}C. After incubation, the reactions were terminated at 85^{o}C for 5 min and chilled on ice. The reactions were collected by brief centrifugation. Next, 1 µl of RNase H was added to each tube and incubated for 20 min at 37^{o}C. cDNA synthesis reaction was stored at -20^{o}C or used for PCR immediately.

3.6.2 PCR

The interested genes and *ACT1* were amplified by quantitative real time-PCR (qRT-PCR) using the MxPro QPCR machine and MxPro QPCR software for analysis. The reaction mixtures contained 4 µl of the appropriate dilution of cDNA, 6 µl of 2X Brilliant SYBR green master mix (Kapabiosystem), and 10 µl of primer master mix (PMM). Then, the reaction mixtures were placed in a preheated (94^oC) real-time thermal cycler. The initial denaturation step was then performed (94^oC for 2 min.). This was followed by a performance of 40 cycles of PCR: this entailed denaturing at 94^oC for 15 sec; annealing at 55^oC for 30 sec; and extending at 68–72^oC for 1 min. Upon completion, reactions were maintained at 4^oC. An internal control, the *S. cerevisiae* actin gene *ACT1* was amplified. The relative mRNA level of the target gene was calculated by the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta C_t = (C_{t\ gene\ target} - C_{t\ ACT1})_\Delta - (C_{t\ gene\ target} - C_{t\ ACT1})_{WT}$. Primer sequences used in qRT-PCR are given in Table 3.2.

Table 3.2 Sequence of primers used in qRT-PCR analysis

Oligonucleotide	DNA Sequence (5'→ 3')
TGL3	CATACTTGGCACCATTGGTG and CTCATTAAAGGCTCTGGCTC
TGL4	TGGATGACCTCACTGGGAAA and CCTACATGGGAGTGCCTATA
PXA1	GTGATAATTTGGGAACTGTG and TCTTCCAACATATTGTAACC
PXA2	ATGGCAAGCGCATCTTTGGA and ATGACA AATGCAGTAGCAAC
FOX1	GCGAAGATGGGTCGTGACG and GCGAATTGCTGACGACCAA
FOX2	GGAAGAAGGGTTATTGGCCA and CTTTGCTTGTGCATCCAAGTCC
FOX3	TCAATGTTGGAGCCGGTGCT and GCAGCTCTTCAGAGGAGATC
CTA1	CACAGGTGGAACAAGCTGC and GGTGTGTCCTGT TGGATAT

Oligonucleotide	DNA Sequence (5'→ 3')
PCK1	GAAGTCACCACCATCAGAGC and ATGCATCGACAATATAAATG
MDH2	CCGACTTGTCTCATATAGA and GTTAGAAACCATCACTGGG
ICL1	TTCCCTGACCAATGGCTAG and TCCCATCCTTGGCAAACTC
ACT1	ATTATATGTTTAGAGGTTGCTGCTTTGG and CAATTCGTTGTAGAAGGTATGATGCC

CHAPTER 4 RESULTS AND DISCUSSION

4.1 Impaired growth of zinc cluster deletion strains on media containing fatty acid or oil as a sole carbon source

In previous studies, zinc cluster protein in yeast *S. cerevisiae* strains carrying deletion of genes encoding for zinc cluster proteins with unknown function were used to perform phenotypic analysis under various conditions, including growth on glycerol or lactate media and in the presence of membrane perturbing agent calcofluor white or caffeine. Even though many phenotypes are found for these zinc cluster proteins, their functions remain unknown. In this study, we focused on the involvement of these zinc cluster proteins in lipid utilization.

To examine the roles of these proteins, the effects of zinc cluster gene deletion on cell growth when lipids were used as an alternative carbon source was tested via spot tests. Cells of wild-type and zinc cluster deletion strains were serially diluted and spotted on plates, containing fatty acid or oils as a sole carbon source. The results showed that the FZT, YZS, FZH, FZP and FZG strains display impaired growth when fatty acids or oils were used as a sole carbon source when compared to the wild-type strain (Table 4.1 and Figure 4.1). In contrast, the FI, FA, FC, FZQ, FZI, FZO and FZJ strains grew normally on all carbon sources tested (Table 4.1 and Figure 4.1). The zinc cluster deletion strains showed impaired growth on the plates containing oleic acid, linoleic acid, palmitic acid and stearic acid, indicating their inability to utilize both saturated and unsaturated fatty acid as a sole carbon source. Thus, the function of these deleted zinc cluster genes may be involved in the regulation of lipid utilization genes. These include genes that are encoded for enzymes in triacylglycerol breakdown, peroxisomal transporter (Pxa1 and Pxa2), β-oxidation pathway (Pox1, Fox2 and Pot1), TCA cycle (Cit1 and Mdh1), glyoxylate cycle (Mls1 and Mdh2) or gluconeogenesis (Pck1 and Icl1). These processes are composed of several gene products such as peroxisomal transporter proteins (Pxa1 and Pxa2), acyl- CoA oxidase (Pox1), malate synthase (Mdh2), citrate synthase (Cit1) and phosphoenolpyruvate carboxykinase (Pck1).

In support, previous research on the oleaginous yeast *Y. lipolytica* also showed that this yeast can accumulate large amount of lipids in the cells. Importantly, disruption of *POX1-6* genes that encode for peroxisomal acyl-CoA oxidases resulted in the inability to utilize fatty acids as a sole carbon source which led to increased cellular lipid accumulation (Beopoulos et al., 2008). Since oils are also composed of different types of fatty acids for example major fatty acid in soy bean oil are palmitic acid, linoleic acid and oleic acid, yeasts with defected β-oxidation pathways which lack the enzymes , such as acyl-CoA oxidase, hydroxyacyl-CoA dehydrogenase, 2-enoyl-CoA hydratase 2 and ketoacyl CoA thiolase are unable to grow on lipid media (Hiltunen et al., 2003). Thus, FZT, YZS, FZH, FZP and FZG strains are likely lacking in or have attenuated β-oxidation activity.

Table 4.1 Phenotypes of some selected yeast zinc cluster deletion strains during growth on alternative carbon sources.

			Carbon source								
Strain name	**Systematic name of deleted genes**	**Deleted gene name**	**Oleic acid**	**Linoleic acid**	**Palmitic acid**	**Stearic acid**	**Palm oil**	**Rice bran oil**	**Soy bean oil**	**Sunflower oil**	**Waste cooking oil**
FY73 (Wild-type)			+	+	+	+	+	+	+	+	+
FI	*YBR033W*	-	+	+	+	+	+	+	+	+	+
FA	*YBR150C*	*TBS1*	+	+	+	+	+	+	+	+	+
FC	*YCR106W*	-	+	+	+	+	+	+	+	+	+
FZT	*YER184C*	-	x	x	x	x	x	x	x	x	x
YZS	*YFL052W*	-	x	x	x	x	x	x	x	x	x
FZG	*YIL130W*	*ASG1*	x	x	x	x	x	x	x	x	x
FZQ	*YJL206C*	-	+	+	+	+	+	+	+	+	+
FZI	*YLL054C*	-	+	+	+	+	+	+	+	+	+
FZO	*YNR063W*	-	+	+	+	+	+	+	+	+	+
FZJ	*YOL089C*	*HAL9*	+	+	+	+	+	+	+	+	+
FZP	*YOR380W*	*RDR1*	x	x	x	x	x	x	x	x	x
FZH	*YPL133C*	*RDS2*	x	x	x	x	x	x	x	x	x

+, Normal growth; x, Impaired growth

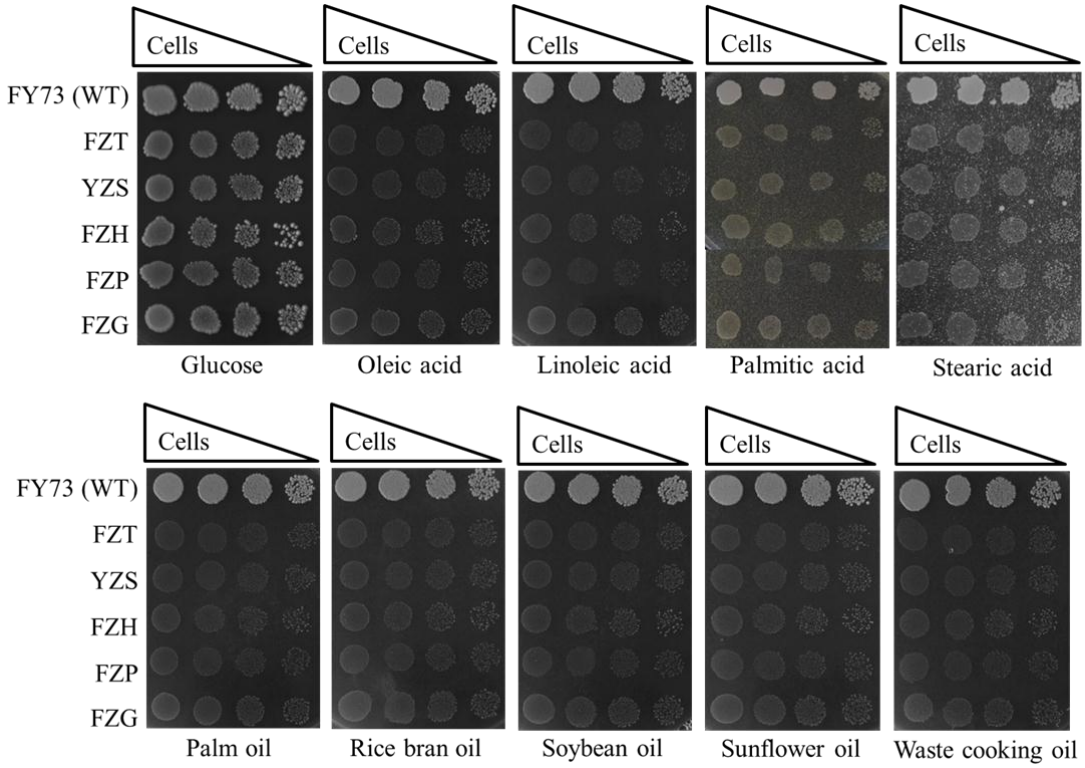

Figure 4.1 Phenotypic analysis of *S. cerevisiae* FY73 wild-type (WT) and zinc cluster deletion strains. Wild-type, FZT, YZS, FZH, FZP and FZG strains were grown on YP media, containing glucose, oleic acid, linoleic acid, palmitic acid, stearic acid, palm oil, rice bran oil, soybean oil, sunflower oil or waste cooking oil as a sole carbon source. See Materials and Methods for details.

4.2 Zinc cluster deletion strains FZT, YZS, FZH, FZP and FZG with inability to grow on oleic acid accumulate high free fatty acid content during glucose-oleic shift

S. cerevisiae are able to utilize various non-fermentable carbons including fatty acids for growth and energy generation. In the presence of fatty acids, the expression of genes *POX1*, *FOX2* and *POT1* encoding for enzymes in the β-oxidation has been shown to be up-regulated (Hiltunen et al., 2003). Long-chain fatty acids such as oleic acid are known to be imported to the peroxisome, a site of β-oxidation, by transporters, Pxa1 and Pxa2. Disruption of *PXA1* and *PXA2* results in impaired growth of cells on a medium containing long-chain fatty acid and a decrease in the β-oxidation level (Hettema et al.,

1996). We hypothesized that yeast cells that cannot utilize fatty acid as a sole carbon source would display compromised β-oxidation activity and accumulate fatty acids in the cells. To analyze classes of lipids that are accumulated in yeast cells, the yeast strains that were unable to grow on media containing lipids as a sole carbon source, including FZT, YZS, FZH, FZP and FZG strains were examined for lipid contents. First, cells were cultured in glucose media (YPD) and cell cultures were split in half prior to being transferred to media containing oleic acid or glucose for addition of 3 h. Then, cellular lipids were extracted with chloroform/methanol (2:1 v/v) and identified by HPLC analysis. Lipid profiles of individual yeast strains were shown (Table 4.2). The condition of HPLC analysis used in this study identified neutral lipids, including triacylglycerol, fatty acid methyl ester, free fatty acid, diacylglycerol and monoacylglycerol.

From HPLC analysis, the results showed that triglyceride and steryl ester were the major neutral lipids that were found in all strains. Free fatty acids were also identified. For the wild-type strain, free fatty acid content was found to be similar for cells cultured in glucose or glucose-oleic acid shift. Interestingly, free fatty acid content of the yeast deletion FZG strain that was cultured in glucose-oleic acid shift was found to be higher than when it was cultured in glucose media with an increase in free fatty acid content more than any other strains by approximately 3 fold (Table 4.2). This result indicated that deltion of zinc cluster *YIL130W* gene in FZG results in high free fatty acid accumulation. This may be due to the defects of fatty acid β-oxidation pathway, thereby preventing a conversion of fatty acid to acetyl-CoA. Moreover, the abnormal growth of these zinc cluster deletion strains on fatty acids may be due to toxicity of accumulated free fatty acid. In fact, free fatty acids have been reported to show high biological toxicity as compared to triglyceride. The toxicity mechanism of fatty acids are involved in reactive intermediates (ROS) and ceramide generation (Listenberger et al., 2003).

Table 4.2 Profiles of neutral lipid and free fatty acid contents of the wild-type and the zinc cluster deletion strains during growth in glucose or glucose-oleic shift. The wild-type and zinc cluster deletion strains were grown in glucose media and then shifted to oleic acid media. After the incubation, the cells grown during the exponential phase were collected and extracted for cellular lipids. The neutral lipids were then identified by HPLC analysis.

Strain name	Glucose		Glucose-Oleic acid Shift	
	%TAG+SE	%FFA	%TAG+SE	%FFA
FY73	90.5±1.6	9.6±1.6	92.3±3.3	7.8±3.3
FZH	96.2±0.5	3.9±0.5	90.6±7.3	9.4±7.4
YZS	95.9±2.6	4.1±2.6	92.6±3.2	7.4±3.2
FZG	94.2±1.6	5.9±1.6	82.9±4.1	17.1±4.1
FZP	93.9±4.0	6.1±4.0	92.4±4.5	7.7±4.5
FZT	95.3±4.1	4.7±4.1	92.2±0.3	7.9±0.4

%TG+SE refers to triacylglycerol and steryl ester contents of total lipids

%FFA refers to free fatty acid content of total lipids

4.3 Zinc cluster deletion strains FZH, YZS, FZG, FZP and FZT were sensitive to oxidative stress, generated from menadione

To explain why the five zinc cluster deletion strains including FZH, YZS, FZG, FZP and FZT strains showed impaired growth in medium containing fatty acid or oil as a sole carbon source, the sensitivity to oxidative stress of these strains was examined. Excess fatty acids are shown to be toxic to the cells and the mechanisms of fatty acid toxicity are involved in reactive intermediates or ROS generation (Listenberger et al., 2003). ROS are also generated in the first step of the β-oxidation. This step of fatty acyl-CoA is converted to *trans*-2-enoly CoA and generates H_2O_2 by acyl-CoA oxidase (Pox1) (Hiltunen et al., 2003). However, ROS are generated as a by-product of other cellular metabolisms, including the mitochondria respiratory chain which is the major source of ROS (Costa et al., 2001).

The result from HPLC analysis showed that the yeast zinc cluster deletion strain FZG had increased free fatty acid content during oleic acid shift (Table 4.2). The impaired growth on fatty acids and oils of the FZG strain may be due to the toxicity of free fatty acid, which is caused by the generation of H_2O_2 during fatty acid breakdown. Therefore, phenotypic analysis was used to examine sensitivity of the yeast wild-type and zinc cluster deletion strains when grown under oxidative stress conditions such as in the presence of the oxidative agent menadione. Menadione was used as a source of ROS because menadione is a quinone compound that can be reduced to semiquinone and formatted various ROS such as superoxide ion, hydrogen peroxide and hydroxyl radical. Briefly, the wild-type and FZH, YZS, FZG, FZP and FZT strains were grown over night in YPD medium. Mid log phase yeast cells were exposed to 0.4 and 0.5mM menadione, and then they were serially diluted and spotted on YPD agar plates. The result showed that, the wild-type has normal growth. On the other hand, zinc cluster deletion strains FZH, YZS, FZG, FZP and FZT strains showed impaired growth in the presence of menadione at final concentrations of 0.4 and 0.5 mM (Figure 4.2). From the results, we found that zinc cluster deletion of FZH, YZS, FZG, FZP and FZT strains showed impaired growth when exposed to menadione at various concentrations (Figure 4.2). The FZG strain showed more impaired growth than other strains when exposed to menadione while the YZS strain showed least impaired growth.

To confirm the results of spot tests (Figure 4.2), cell viability assay was performed. Yeast cells were again exposed to various concentrations of menadione. Then, the viability of yeast cells was determined by colonies counting. Cell survival was calculated as a viability percentage value of zinc cluster deletion strains compared with the wild-type strain that was given the 100% viability. From the results, we found that FZH, YZS, FZG, FZP and FZT strains have different sensitivity to menadione stress with %viability of 21.6%, 63.0%, 18.4%, 38.1% and 51.0%, respectively (Figure 4.3). Cell viability of FZG strain was lower than other zinc cluster deletion strains, which was consistent with phenotypic analysis obtained previously (Figure 4.2).

Our results implied a correlation between increased toxicity of high free fatty acid observed for yeast zinc cluster deletion FZG and increased sensitivity of these strain to oxidative stress generated by menadione.

In the wild-type cells, yeasts are able to protect themselves from molecular damage, including oxidative stress. Cells adapt in response to the increased ROS accumulation by activating transcription regulators to promote the expression of genes required for synthesis of antioxidants. The activation of an antioxidant response leads to a rapid elimination of ROS, reparation of oxidized biomolecules and restoration of cellular redox balance. Antioxidant enzymes thus play a role to protect the cells from ROS accumulation, including superoxide dismutase, peroxidase and catalase encoded by *SOD1*, *GTT1* and *CTA1*, respectively. (Farrugia et al., 2012). Hydrogen peroxide that is generated in the first step of β-oxidation is broken by the catalase A in the peroxisome. We questioned whether these zinc cluster deletion strains may have a defect in the expression of some important genes in the oxidant defense system, such as *CTA1*, which encodes catalase A (Cta1).

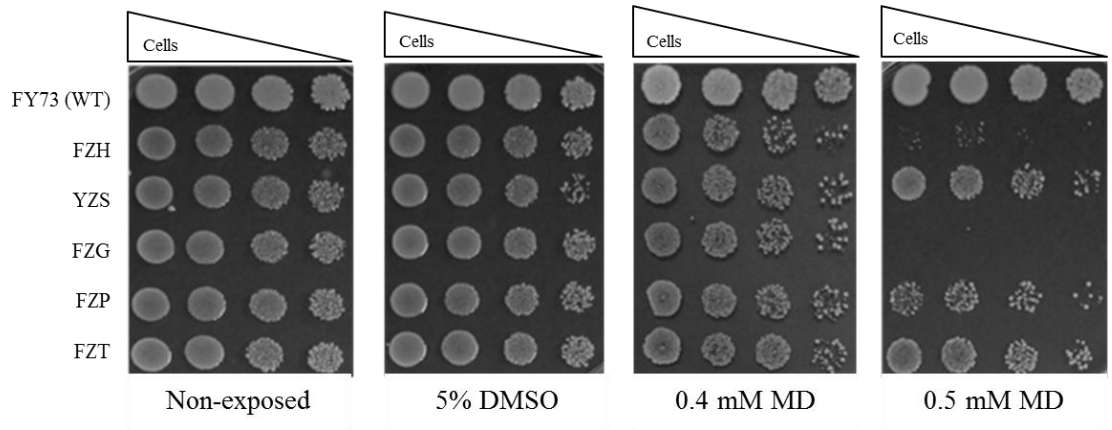

Figure 4.2 Phenotypes of the wild-type and zinc cluster deletion strains during grown in the presence of an oxidative agent menadione. The yeast cells were grown until mid-log phase of growth and then exposed to 0.4 and 0.5 mM final concentrations of menadione for 1 h. After the treatment, the cell cultures were serially diluted and, each dilution was then spotted onto appropriated YPD plates and incubated at 30^{o}C for 2-3 days to monitor the growth.

In the yeast *S. cerevisiae*, some transcription factors are associated with adaptation to oxidative stress response. For example, Yap1 plays a key role in this process. The lack of *YAP1* gene leads to cell hypersensitivity and impaired adaptive response to ROS,

produced by H_2O_2 (Farrugia et al., 2012). Previous research studies in yeast showed that the yeast Mn*SOD* mutant strains, which are unable to activate the Mn*SOD* gene expression, showed hypersensitivity to oxidative stress (Zhu et al., 1992). In this experiment, we hypothesized that the lack of zinc cluster genes impairs proper regulation of genes, associated with oxidative stress response, leading to abnormal growth exposed to menadione. From spot assays, the results showed that FZH, YZS, FZG, FZP and FZT strains display impaired growth in the presence of both concentrations of menadione tested, when compared to the wild-type strain (Figure 4.2). Moreover, cell viability also decreased when the cells were exposed to menadione (Figure 4.3). Menadione is known to generate various forms of ROS, including superoxide radical, and hydrogen peroxide. Therefore, the impaired growth of these zinc cluster deletion strains may due to the lack of regulatory proteins that function to regulate the expression of genes such as *SOD1* and *SOD2* in the oxidative stress defense system.

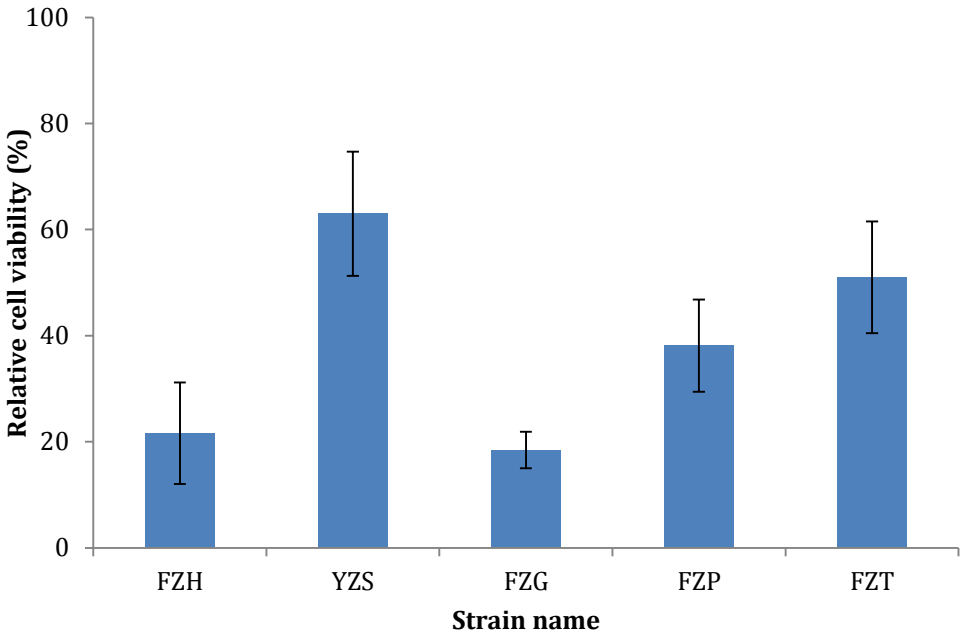

Figure 4.3 Viability of yeast zinc cluster deletion strains upon exposed to 0.4 mM of menadione for 1h. Viability of cells was calculated as survival percentage values compared to that of the wild-type strain which was given as 100% cell viability.

4.4 Yil130w a transcriptional regulator regulates lipid utilization genes

Utilization of alternative carbons such as lipid in yeast cells is involved in a wide variety of cellular processes, including β-oxidation, glyoxylate cycle, the TCA cycle and gluconeogenesis. These processes are required for lipid degradation and generation of important metabolites as well as ATPs (Schuller, 2003, Turcotte et al., 2010). From phenotypic analysis, we showed that some yeast strains, including FZH, YZS, FZG, FZP and FZT are unable to grow on media, containing lipid as a sole carbon source (Figure 4.1 and Table 4.1), suggesting impaired regulation of genes involved in lipid utilization. The results of neutral lipid content via HPLC analysis showed that these strains have increased free fatty acid accumulation when grown in oleic acid as a sole carbon source, especially for the FZG strain (Table 4.2), which accumulated approximately 3 folds more fatty acid content when compared to that found for glucose grown cells (Table 4.2). Moreover, this FZG strain or the Δ*yil130w* was unable to breakdown triacylglycerol. Since, deleted gene of *YIL130W* encode for a putative transcriptional regulator in the zinc cluster protein family, we hypothesized that the zinc cluster Yil130w may play a role as a regulator of genes involved in lipid utilization.

To test this hypothesis, qRT-PCR analysis was performed to determine the expression of genes that are involved in lipid utilization, including genes that encode triacylglycerol lipase, peroxisomal transporter, and enzymes in the β-oxidation pathway, glyoxylate cycle and gluconeogenesis (see detail of experiment in Material and Method). The expression levels of triacylglycerol lipase gene (*TGL3*), peroxisomal transporter gene (*PXA1*), β-oxidation gene (*POX1, FOX2* and *POT1*), glyoxylate cycle and gluconeogenic gene (*ICL1* and *PCK1*) were examined with wild type and Δ*yil130w* cells grown in glucose-oleic acid shift. Expression levels of these genes were normalized, using an *ACT1* as housekeeping gene. The mRNA fold enrichments were calculated, using the comparative $\Delta\Delta C_t$ method to compare the mRNA levels of gene for the Δ*yil130w* strain versus that for the wild-type strain (Livak et al., 2001). Our qRT-PCR results showed during under oleic acid shift, the expression levels of these genes were enhanced when compare to that in glucose condition. Importantly, the

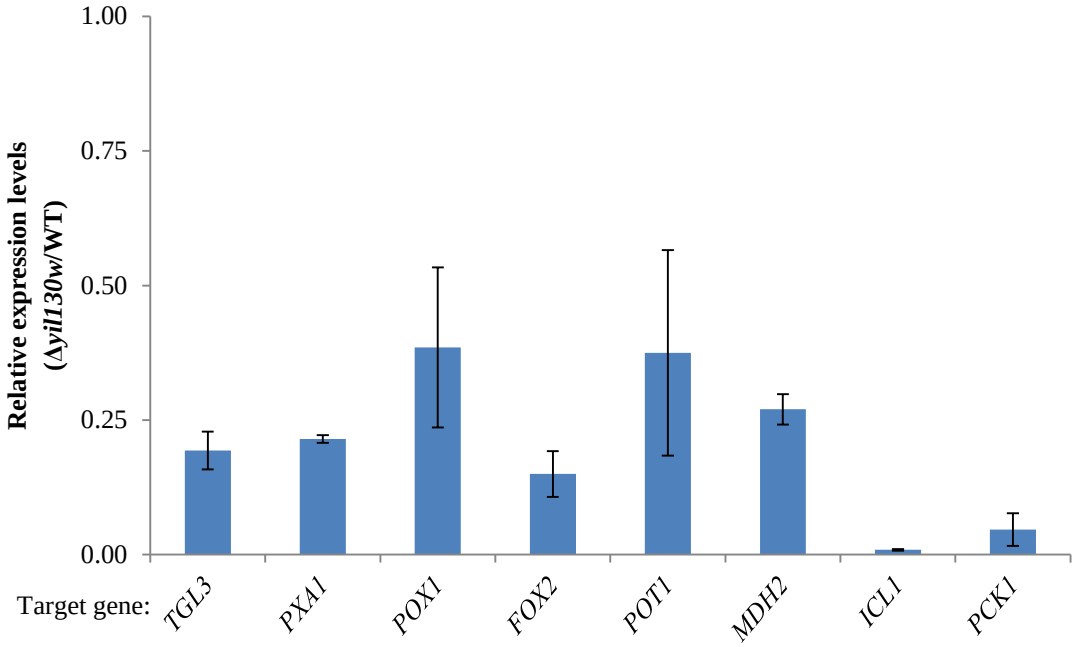

Figure 4.4 Expression of target genes of Yil130w during oleic acid induction. The mRNA levels were measured by qRT-PCR analysis. The relative expression levels of each gene were calculated, using the comparative $\Delta\Delta C_t$ method, normalized to the *ACT1* gene and expressed as a fold change of the mRNA levels of the Δ*yil130w* strain versus that of the wild-type strain.

expression of genes involved in lipid utilization was decreased in the Δ*yil130w* strain as compared to that of the wild-type (Figure 4.4 and Table 4.3). The expression level of *POX1* and *POT1* in the FZG (Δ*yil130w*) strain were decreased by approximately 2.6-fold, *MDH2* were decreased by approximately 3.7-fold, *TGL3*, *PXA1* and *FOX2* were decreased by approximately 5.2, 4.7 and 6.7-fold, respectively (Figure 4.4 and Table4.3). Importantly, the results show that, *ICL1* and *PCK1* were rarely expressed in the FZG (Δ*yil130w*) strain when compared to the wild-type (Figure 4.4 and Table 4.3). Therefore, *YIL130W* plays a key role in the activation of genes in lipid utilization, including *TGL3*, *PXA1*, *POX1*, *FOX2*, *POT1*, *ICL1* and *PCK1*. The model for gene target of zinc cluster protein Yil130w were shown in Figure 4.5.

Table 4.3 Relative expression levels of Yil130w target genes (mRNA levels of the Δ*yil130w* versus the wild-type strain) during glucose-oleic acid shift as measured by qRT-PCR analysis.

ORF	**Target genes**	**Description**	**Relative Expression (Δ*yil130w*/WT) (Fold Change)**	
			Glucose	**Oleic acid**
1. Triacylglycerol and fatty acid degradation				
YMR313C	*TGL3*	Triacylglycerol lipase	1.3	0.2
YPL147W	*PXA1*	Transporter for import of long-chain fatty acids into peroxisomes	0.8	0.2
YGL205W	*POX1*	Fatty acyl-coenzyme A oxidase	0.9	0.4
YKR009C	*FOX2*	Multifunctional enzyme with hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase activities	0.4	0.2
YIL160C	*POT1*	3-Ketoacyl-CoA thiolase	0.6	0.4
2. Gluconeogenesis and glyoxylate cycle				
YOL126C	*MDH2*	Cytoplasmic malate dehydrogenase	1.1	0.3
YER065C	*ICL1*	Isocitrate lyase	0.3	0.01
YKR097W	*PCK1*	Phosphoenolpyruvate carboxykinase	0.4	0.05

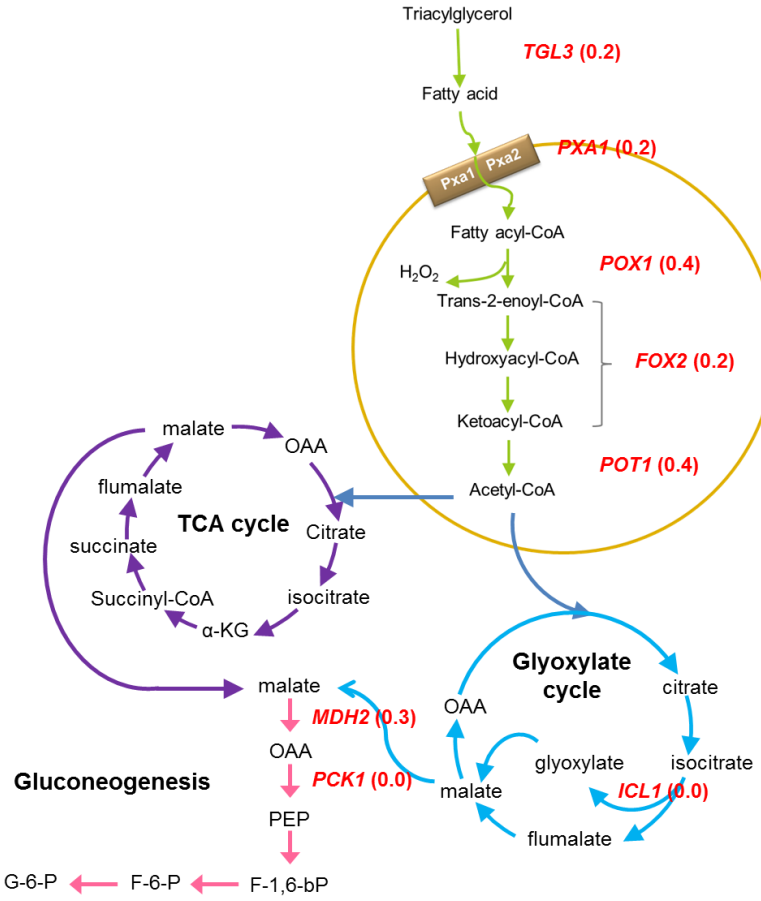

Figure 4.5 Model for role of zinc cluster protein Yil130w during lipid utilization and the relative expression levels of Yil130w target genes (as give in the bracelet). Zinc cluster protein Yil130w regulates genes involved in lipid utilization, including triacylglycerol lipase (*TGL3*), peroxisomal transporter (*PXA1*), β-oxidation (*POX1*, *FOX2* and *POT1*), glyoxylate cycle and gluconeogenesis (*ICL1*and *PCK1*)**.**

4.5 Zinc cluster deletion strain Δ*yil130w* (FZG) is unable to breakdown triacylglycerol

The results of qRT-PCR analysis indicated that Yil130w is involved in the regulation of triacylglycerol lipase gene, *TGL3* (Figure 4.4 and table 4.3) but not *TGL4* (data not shown). We hypothesized that the FZG strain may not be able to breakdown triacylglycerol efficiently and, as a result, accumulate high amounts of lipid contents in the cells. Therefore, we analyzed the neutral lipids, accumulated during growth under both on exponential and stationary phases of growth. Yeast cells are known to accumulate neutral lipids in the stationary growth phase of the cell cycle (Sandager et

al., 2002). Therefore, in this study, the yeast cells were grown in YPD medium for 9 h (log phase) and 96 h (stationary phase). After, the cells were harvested and extracted for lipids with chloroform and methanol. Then, HPLC analysis was performed to identify the classes of the extract lipids. Our results showed that the triacylglycerol and the steryl ester content of the wild-type glucose-grown cells at the exponential growth phase was lower than when growing at the stationary phase. However, the triacylglycerol and the steryl ester content of FZG strain were found to be almost unchanged for cells under both phases of growth (Figure 4.6). Triacylglycerol is an essential lipid for maintaining energy homeostasis and also a precursor or a buliding block for the synthesis of structural lipids and important components of membranes. It is also used to support rapid growth and division of cells (Czabany et al., 2007, Kurat et al., 2006).

During the exponential phase of growth, cells divide rapidly; therefore, triglycerides are continuously used. However, in stationary phase, cell division is decreased and triacylglycerol is stored (Sandager et al., 2002). These are consistent with our results, showing that the triglyceride content of exponential cells was lower than that in the stationary phase for the wild-type strain (Figure 4.6). However, the FZG strain showed similar triacylglycerol content for both phases of growth, perhaps because this strain was unable to breakdown triglyceride as well as compared to the wild-type. The deletion zinc cluster gene in the FZG strain may encode for the zinc cluster regulator that plays an important role in the regulation of gene in triacylglycerol breakdown such as *TGL3*, *TGL4* and *TGL5*. However, high triacylglycerol content of the FZG strain in exponential phase may also be other possibilities its ability to accumulate lipid more than the wild type strain.

We hypothesized that the zinc cluster deltion strain FZG (Δ*yil130w*) may be unable to break down triglyceride, thus accumulate lipids in the cells. Lipid accumulation of yeast wild-type (FY73) and Δ*yil130w* deletion strains was measued to determine lipid concentration and lipid content (Table 4.4). From the results, the wild-type strain accumulated lipid 0.011 g/g of cell dry weight wheareas the Δ*yil130w* strain contained 0.043 g/g of cell dry weight at 96 h of cultivation in glucose (Table 4.4). Lipid content of the Δ*yil130w* strain was found to be higher than that of the wild-type strain by approximately 4-fold (Table 4.4), confirming that the Δ*yil130w* strain accumulates more lipid than the wild-type strain. One explanation for the observed high total lipid

content was because the Δ*yil130w* strain unable to breakdown triacylglycerols and accumulate them in the cells. However, higher lipid concentration in Δ*yil130w* strain may be because this strain can produce lipids more than the wild-type strain; however it remains to be proven.

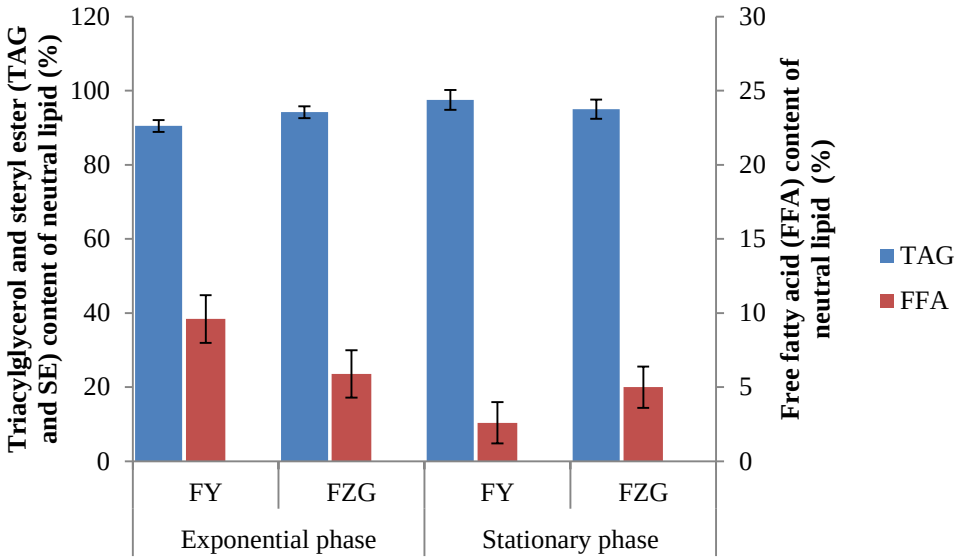

Figure 4.6 Neutral lipids (triacylglycerol and steryl ester) and free fatty acid contents of the yeast cells cultured in glucose medium during the exponential and stationary phase of growth. The glucose grown cells of the wild-type and the Δ*yil130w* (FZG) strains at the exponential and the stationary phase of growth were extracted for cellular lipids to measure neutral lipids by HPLC analysis.

Table 4.4 Lipid contents of the wild-type (FY73) and the Δ*yil130w* (FZG) cells grown in YP-glucose medium during the stationary phase of growth. Cells dry weight and lipid lipid weight were measured to obtain biomass and lipid concentration (Papanikolaou et al., 2002). Then lipid yield were calculated from lipid concentration divided by the biomass of the cells.

Strain name	Biomass(g/l)	Lipid concentration (g/l)	Lipid yield (g/g)
FY73 (wild-type)	4.820±0.715	0.053±0.003	0.011±0.002
FZG (Δ*yil130w*)	1.655±0.087	0.071±0.003	0.043±0.004

CHAPTER 5 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

In the first part, growth phenotypes of zinc cluster deletion strains, comprising of the FI, FA, FC, FZT, YZS, FZG, FZQ, FZI, FZO, FZJ, FZP and FZH strains, were investigated when the cells were grown in media, containing fatty acids or oils as a sole carbon source. The results showed that five zinc cluster deletion strains, including FZH, YZS, FZG, FZP and FZT strains which also called Δ*ypl133c*, Δ*yfl052w*, Δ*yil130w*, Δ*yor380w* and Δ*yer184c* display impaired growth on all fatty acids and oils tested (Table 4.1 and Figure 4.1). Our results showed that the deletion of these zinc cluster genes impairs lipid utilization in *S. cerevisiae*.

The effect of deleted zinc cluster genes on cellular lipid content was then investigated via HPLC analysis. The results showed that neutral lipids class from the wild-type and all deletion strains are triglyceride and steryl ester contents as well as free fatty acids (Table 4.2). Interestingly, the free fatty acid content of zinc cluster deletion strains (FZH, YZS, FZG, FZP and FZT) when cultured in oleic acid was higher than in glucose (Table 4.2). Especially, the FZG (Δ*yil130w*) strain showed an increased in free fatty acid content by approximately 3 folds as compared to the wild-type strain. The results showed that these strains with the inability to utilize fatty acid as a sole carbon source display the increase in cellular lipid accumulation.

Examination of increased free fatty acid contents of the FZH, YZS, FZG, FZP and FZT strains was also found to be correlated with their sensitivity to oxidative stress generated by menadione as shown by phenotypic analysis and cell viability assay. These yeast zinc cluster deletion strains were indeed involved in yeast oxidative stress response (Figure 4.3). The FZG (Δ*yil130w*) strain which displayed the highest free fatty acid content during growth on oleic acid was highly sensitive to menadione and showed lower percentage in cell viability 18% when compared with wild-type strain (Figure 4.3).

Next, lipid content of the wild-type and FZG (Δ*yil130w*) strains of cells grown in exponential or stationary phases was compared. In agreement with previous reports, the results indicated that triacylglycerol and steryl ester content of the wild-type strain

culture in stationary phase glucose-grown cells was higher than in exponential phase (Figure 4.6). However, triacylglycerol and steryl ester content of the FZG (Δ*yil130w*) strain found to be similar for both phases of cell culture. It is suggested that FZG (Δ*yil130w*) strain may be unable to breakdown triglyceride during the exponential phase and, therefore, accumulate high triacylglycerol at the early growth phase. The deleted zinc cluster gene in the FZG (Δ*yil130w*) strain may encode for a putative regulator that plays important role in the regulation of fatty acid utilization genes.

To prove the hypothesis that the zinc cluster protein FZG strain or Yil130w (FZG) regulates expression of genes involved in lipid utilization, qRT-PCR analysis was performed to quantify the mRNA levels of some putative target genes. Our results showed that in fact deletion of *YIL130W* results in the down-regulation of the expression of genes involved in lipid utilization, including triacylglycerol lipase (*TGL3*), peroxisomal transporter (*PXA1*), β-oxidation (*POX1, FOX2* and *POT1*), glyoxylate cycle and gluconeogenesis (*ICL1* and *PCK1*) genes (Figure 4.4 and Table 4.3).

Overall results identified new zinc cluster proteins that are involved in lipid utilization and adaptation to oxidative stress generated by excess free fatty acid and menadione. Importantly, the results showed that the zinc cluster Yil130w activates the expression of *TGL3*, *PXA1*, *POX1*, *FOX2*, *POT1*, *ICL1* and *PCK1* genes in response to utilization of oleic acid as a sole carbon source in *S. cerevisiae*.

5.2 Recommendations

Recently, green alternative energy has gained increased research and industrial interests. In particular, bio-oil produced from microorganisms such as yeasts because they have many advantages over the use of plant material, including a short-life cycle and processes that are independent to climate and seasonal change. Yeasts that can accumulate high amounts of oil in the cells such as *Yarrowia lipolytica* have made an important contribution in bio-oil production. In this study, *S. cerevisiae* is used as a model organism to gain insight into lipid metabolism of yeast. Our results showed that Yil130w regulates genes involved in lipid utilization and disruption of *YIL130W* leads to increased lipid production by 3-fold when cultured in glucose. This new data

obtained from the model yeast *S. cerevisiae* can be applied to metabolically engineer and to develop strains with higher lipid accumulation ability.

Lastly, we also found that Yil130w also plays a role in the oxidative stress defense system. It will be interesting to identify gene targets for the Yil130w regulator through gene expression analysis or other methods. The obtained information can be useful for construction of yeast strains with improved oxidative stress tolerance which is one of desired properties of many industrial yeast strains.

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APPENDIX

APPENDIX A

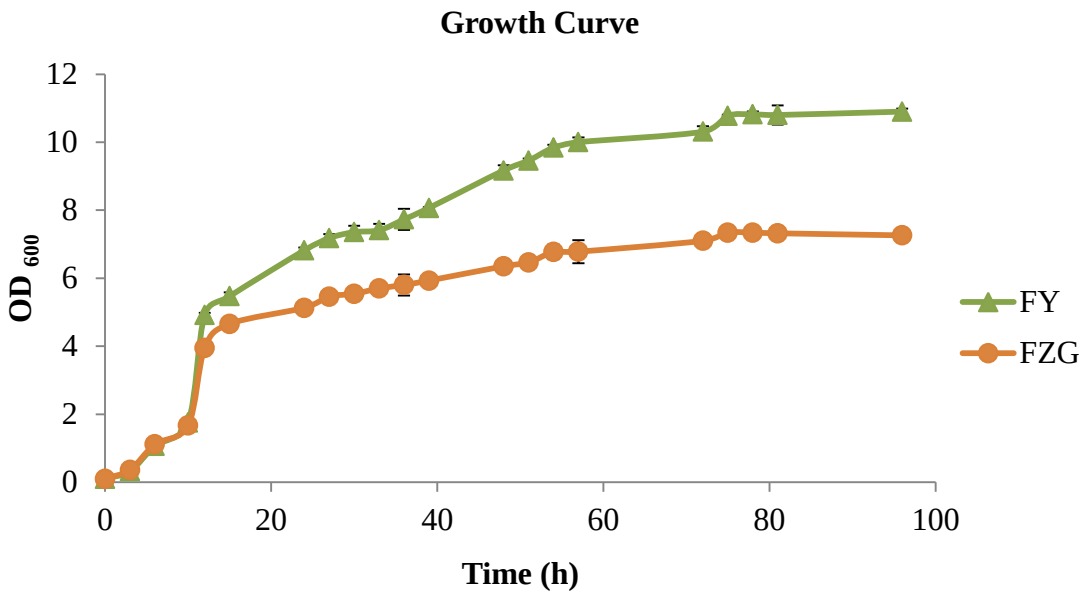


Figure A.1 Growth curves of the wild-type (FY73) and the zinc cluster deletion strain FZG (Δ*yil130w*) for analysis of lipid contents. The wild-type and FZG strains initial OD_{600} of 0.1 were grown in YP-glucose media. Then, the cells were aliquot and measured OD_{600}.

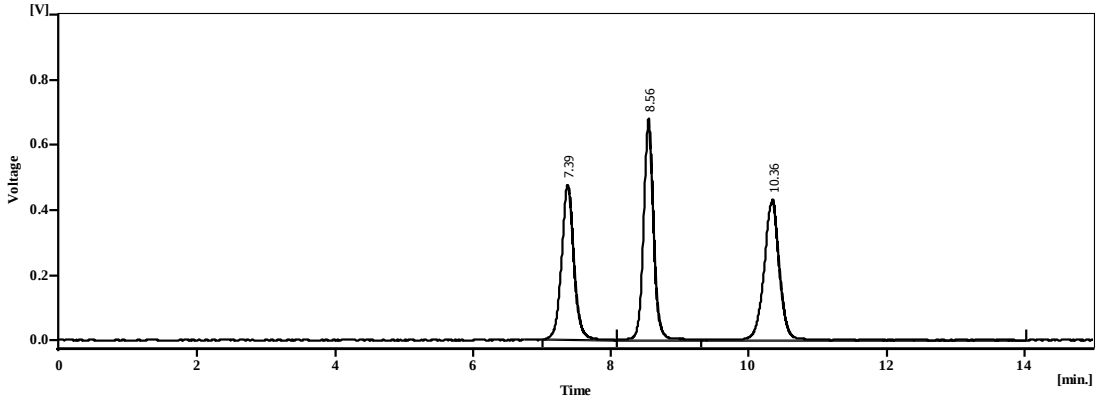
Table A.1 List of target genes and C_t values for qRT-PCR analysis during glucose and oleic acid induction of the wild-type and FZG strains. Ct values were obtain via qRT-PCR analysis and were used to calculate $2^{-\Delta\Delta Ct}$ values when $\Delta\Delta C_t = (C_{t\ \text{gene target}} - C_{t\ ACT1})_{\Delta} - (C_{t\ \text{gene target}} - C_{t\ ACT1})$. The $2^{-\Delta\Delta Ct}$ represents the relative mRNA level of target genes.

Gene	Glucose			Oleic acid		
	C_t		$2^{-\Delta\Delta Ct}$	C_t		$2^{-\Delta\Delta Ct}$
	Wild-type	FZG (Δ*yil130w*)		Wild-type	FZG (Δ*yil130w*)	
ACT1	16.80	16.75	-	17.43	17.43	-
TGL3	25.21	24.79	1.3	24.77	27.15	0.2
PXA1	22.29	22.58	0.8	21.09	23.27	0.2
POX1	25.10	25.25	0.9	16.55	18.10	0.4
FOX2	19.00	21.49	0.4	17.58	20.39	0.2
POT1	14.39	16.37	0.6	15.51	16.24	0.4
MDH2	21.03	20.82	1.1	17.36	19.38	0.3
ICL1	17.59	19.38	0.3	16.82	23.58	0.0
PCK1	24.03	25.38	0.4	19.22	26.98	0.0

Table A.2 Retention time (min) and Area (%) obtained from standard chromatogram of HPLC analysis as showed.

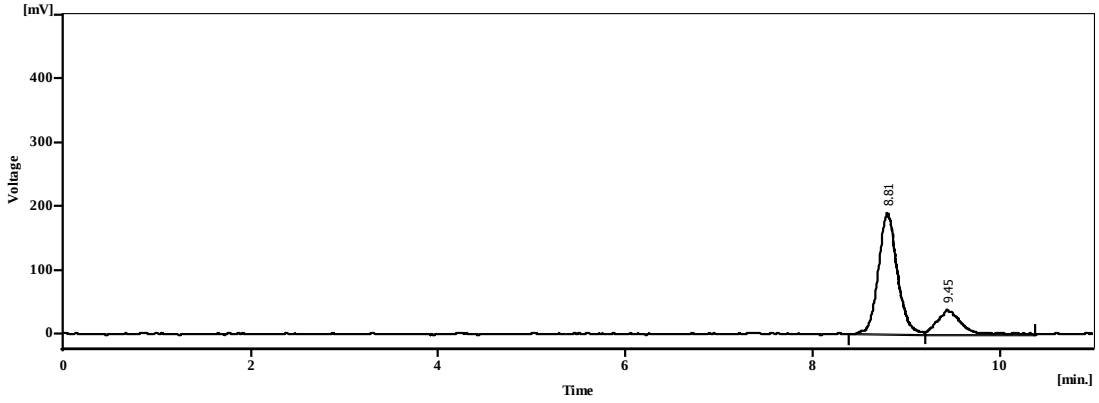
Standard of triacylglycerol, fatty acid methyl ester and free fatty acid

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	7.392	5930.062	475.499	30.2
2	8.56	6809.878	679.956	34.6
3	10.364	6919.899	431.756	35.2
	Total	19659.84	1587.212	100



Standard of diacylglycerol

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	8.808	2703.22	190.032	79
2	9.448	719.599	38.666	21
	Total	3422.818	228.698	100



Standard of monoacylglycerol

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	12.676	1676.472	105.058	75.6
2	13.248	541.467	27.634	24.4
	Total	2217.939	132.692	100

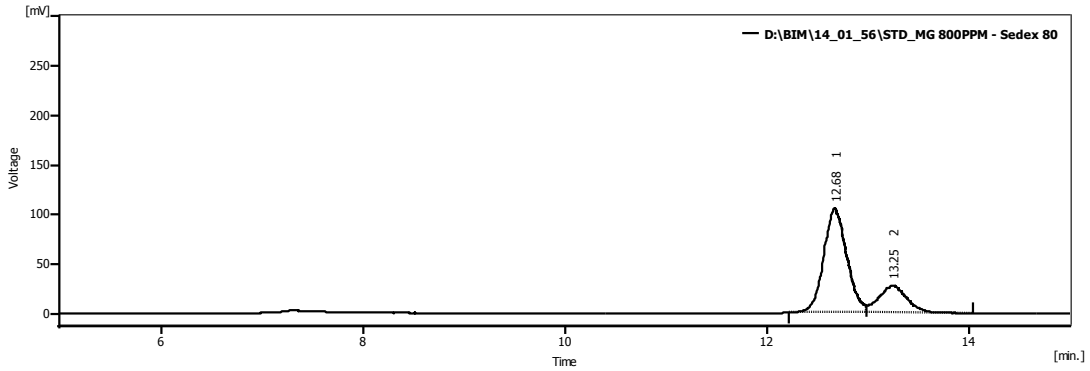
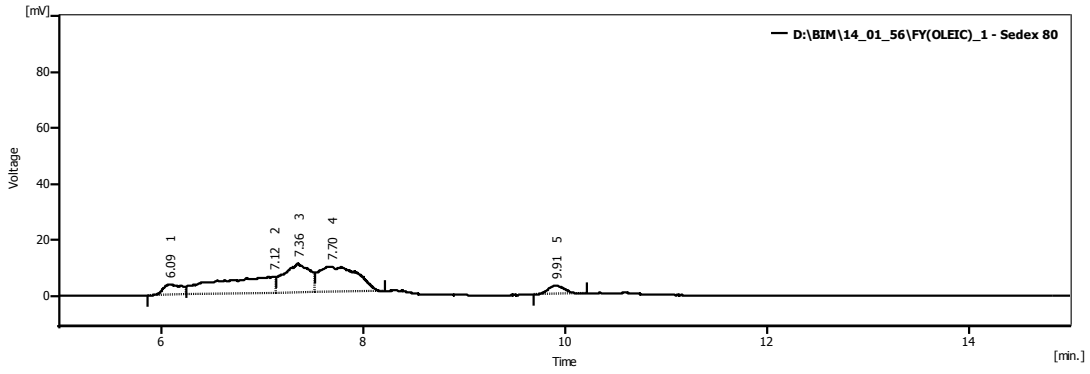


Table A.3 Retention time (min) and Area (%) obtained from chromatogram of HPLC analysis. HPLC analysis were used to analyzed lipid of the FY73 (wild-type) and zinc cluster deletion strains during growth in oleic acid during exponential growth phase.

Experiment 1

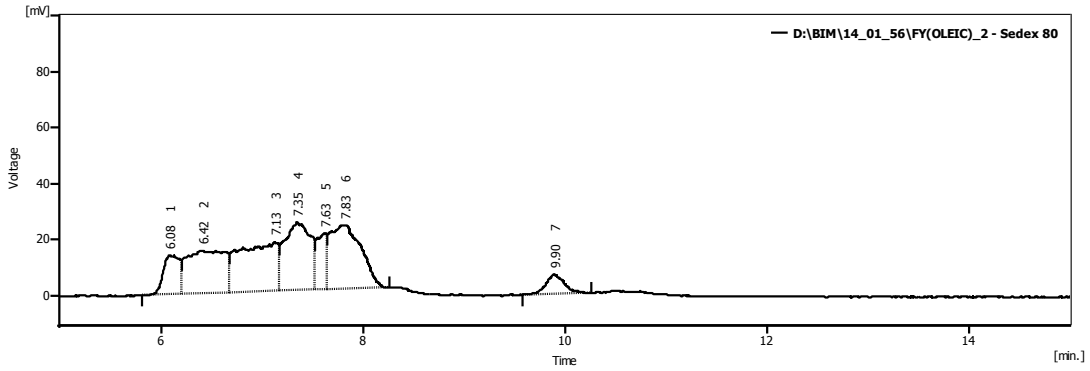
FY73 (replicate 1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.092	50.249	3.864	6.4
2	7.12	256.348	6.075	32.8
3	7.36	189.092	10.552	24.2
4	7.696	245.888	9.152	31.5
5	9.912	39.472	3.315	5.1
	Total	781.05	32.957	100



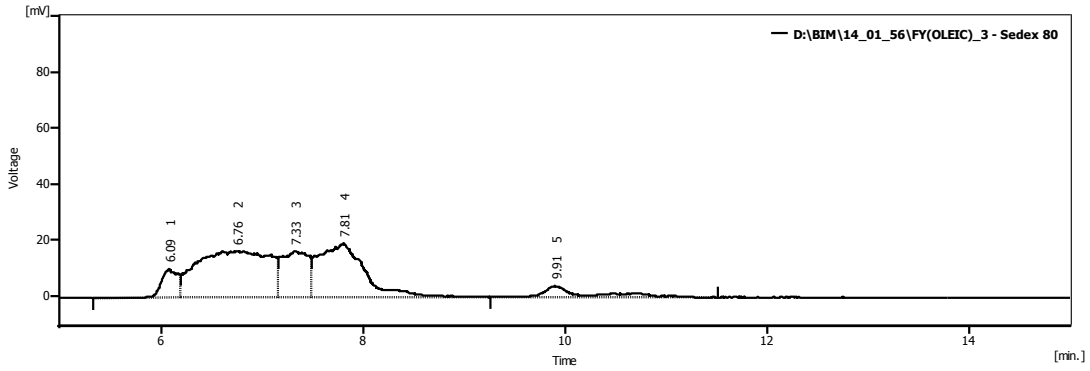
FY73 (replicate 2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.084	155.154	14.266	7.1
2	6.416	406.349	15.457	18.6
3	7.128	469.719	17.479	21.5
4	7.352	440.196	24.498	20.1
5	7.628	137.633	20.177	6.3
6	7.828	489.856	22.894	22.4
7	9.896	89.914	6.988	4.1
	Total	2188.822	121.759	100



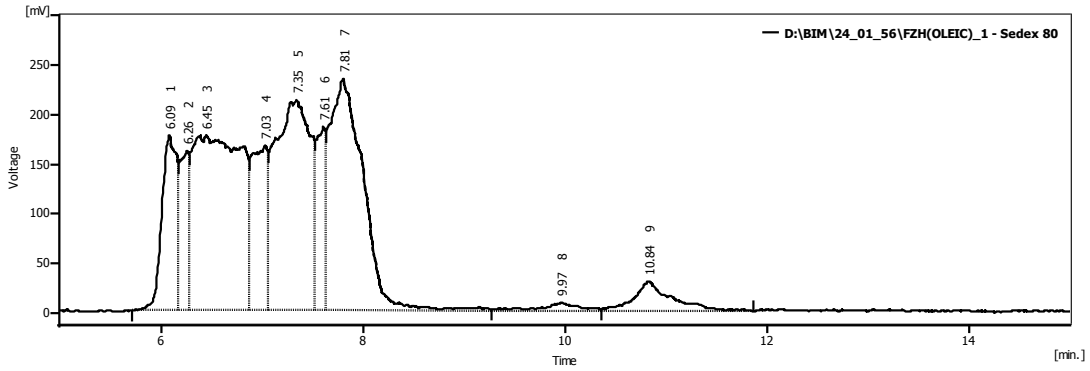
FY73 (replicate 3)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.088	122.268	10.371	5.9
2	6.76	845.505	16.955	40.9
3	7.328	315.085	16.872	15.3
4	7.812	638.163	19.4	30.9
5	9.908	144.591	4.232	7
	Total	2065.612	67.83	100



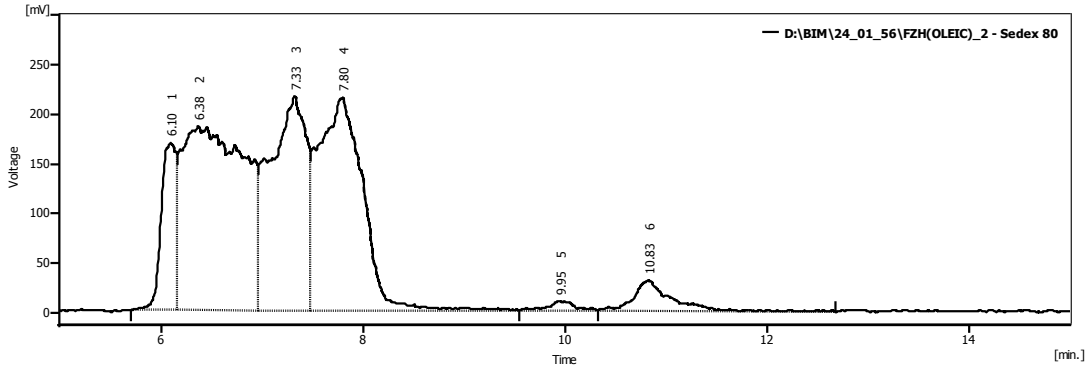
FZH (replicate 1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.088	1815.363	176.764	7.7
2	6.26	967.918	160.459	4.1
3	6.452	5934.743	175.819	25.3
4	7.032	1838.225	166.354	7.8
5	7.348	5153.976	212.125	22
6	7.612	1153.657	185.192	4.9
7	7.808	5488.787	233.727	23.4
8	9.972	245.496	8.724	1
9	10.84	828.832	30.506	3.5
	Total	23427	1349.671	100



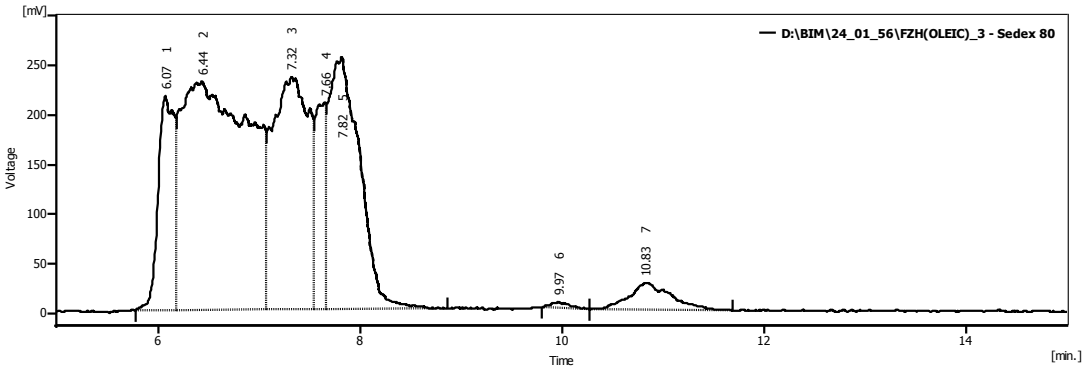
FZH in (replicate 2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.096	1749.043	168.387	7.6
2	6.376	7965.109	185.741	34.7
3	7.328	5490.362	215.886	23.9
4	7.804	6644.309	214.556	29
5	9.952	216.315	10.478	0.9
6	10.832	876.243	31.534	3.8
	Total	22941.38	826.582	100



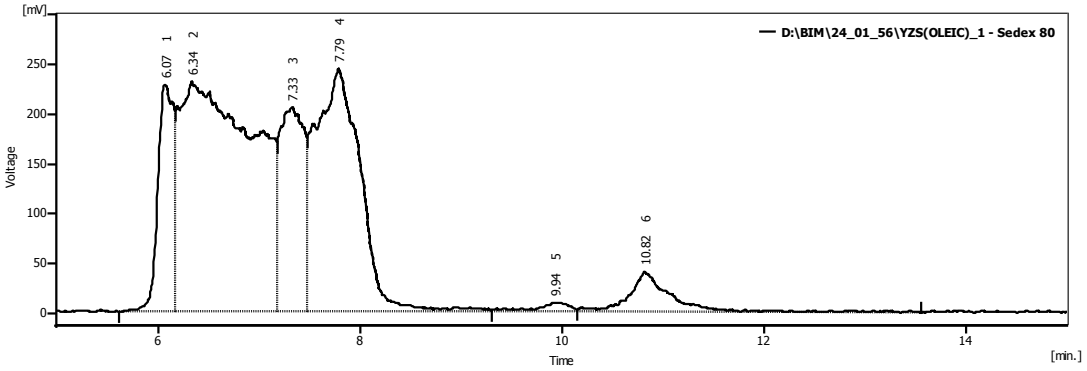
FZH (replicate 3)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.072	2361.862	215.952	8.8
2	6.44	10890.02	229.967	40.5
3	7.324	5824.601	234.123	21.7
4	7.656	1509.248	208.289	5.6
5	7.824	5415.006	254.061	20.1
6	9.968	87.039	6.409	0.3
7	10.828	805.723	27.513	3
	Total	26893.5	1176.313	100



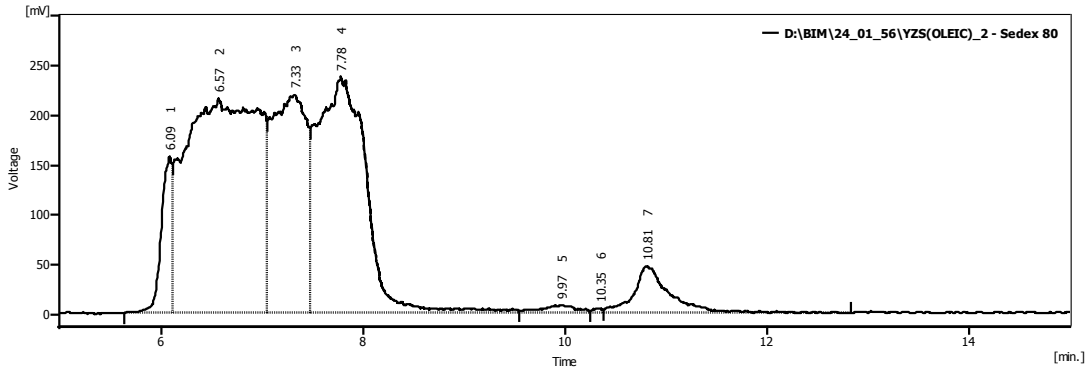
YZS (replicate 1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.072	2395.713	227.764	8.9
2	6.336	11929.18	230.642	44.4
3	7.332	3408.046	205.387	12.7
4	7.792	7640.146	243.809	28.4
5	9.936	241.226	9.581	0.9
6	10.82	1262.488	40.911	4.7
	Total	26876.8	958.092	100



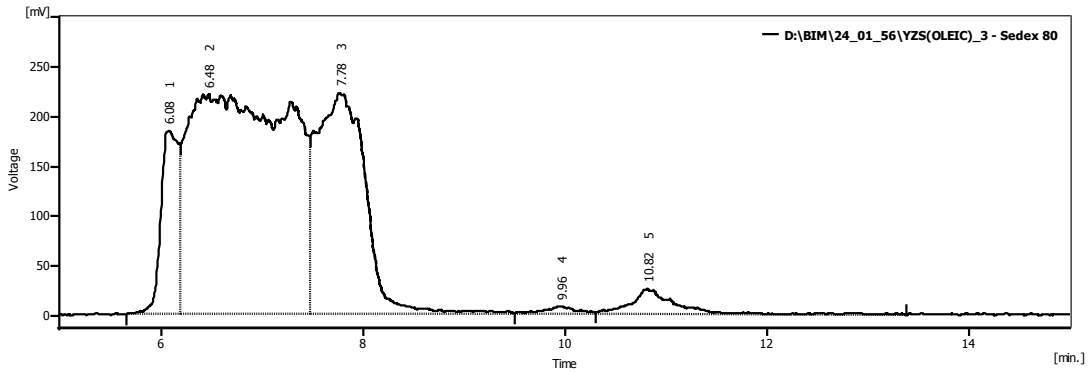
YZS (replicate 2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.088	1115.235	156.809	4.2
2	6.572	10917.32	215.654	40.8
3	7.328	5187.957	218.727	19.4
4	7.784	8026.041	237.097	30
5	9.968	210.298	8.347	0.8
6	10.352	33.192	5.266	0.1
7	10.808	1260.693	46.82	4.7
	Total	26750.73	888.72	100



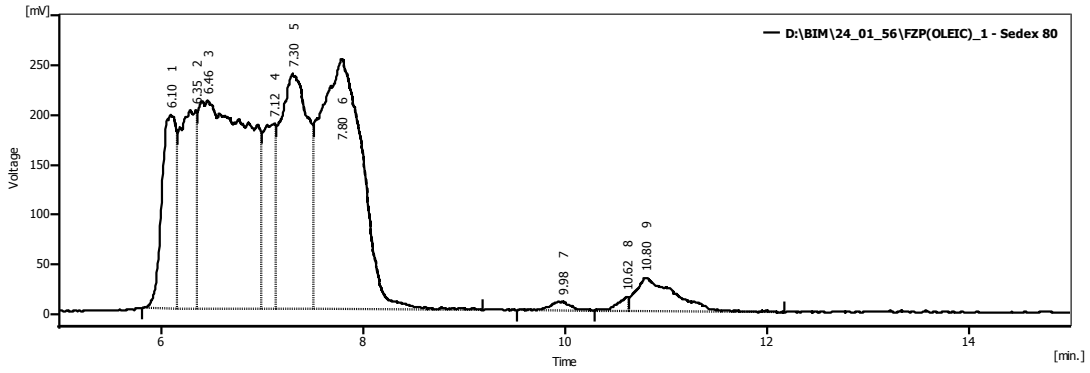
YZS (replicate 3)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.076	2230.751	184.08	8.4
2	6.476	15610.49	220.815	58.7
3	7.776	7625.091	222.239	28.7
4	9.964	214.133	8.806	0.8
5	10.82	909.306	25.818	3.4
	Total	26589.77	661.758	100



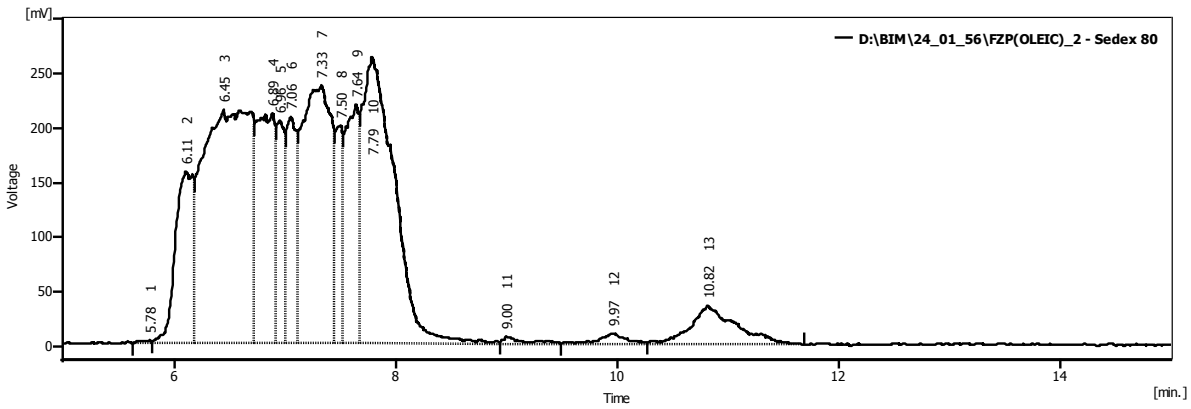
FZP (replicate 1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.1	1887.115	195.165	7.2
2	6.348	2330.263	199.121	8.9
3	6.464	7331.742	208.785	27.9
4	7.124	1580.877	185.928	6
5	7.304	4555.658	235.954	17.3
6	7.796	7381.742	251.77	28.1
7	9.98	150.978	9.178	0.6
8	10.62	120.29	14.79	0.5
9	10.8	951.437	33.801	3.6
	Total	26290.1	1334.491	100



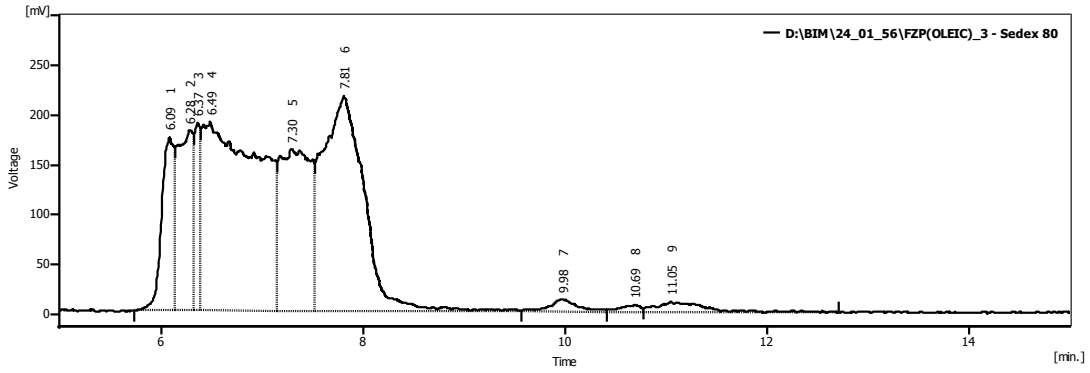
FZP (replicate 2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	5.784	21.118	2.867	0.1
2	6.112	1753.789	157.445	6.5
3	6.448	6395.911	213.695	23.9
4	6.892	2412.156	210.582	9
5	6.96	1007.286	204.726	3.8
6	7.056	1293.291	207.304	4.8
7	7.332	4424.854	236.261	16.5
8	7.504	898.939	199.585	3.4
9	7.644	1782.817	219.574	6.7
10	7.792	5433.604	262.538	20.3
11	9	107.456	7.325	0.4
12	9.972	203.509	10.511	0.8
13	10.82	1067.196	36.229	4
	Total	26801.93	1968.641	100



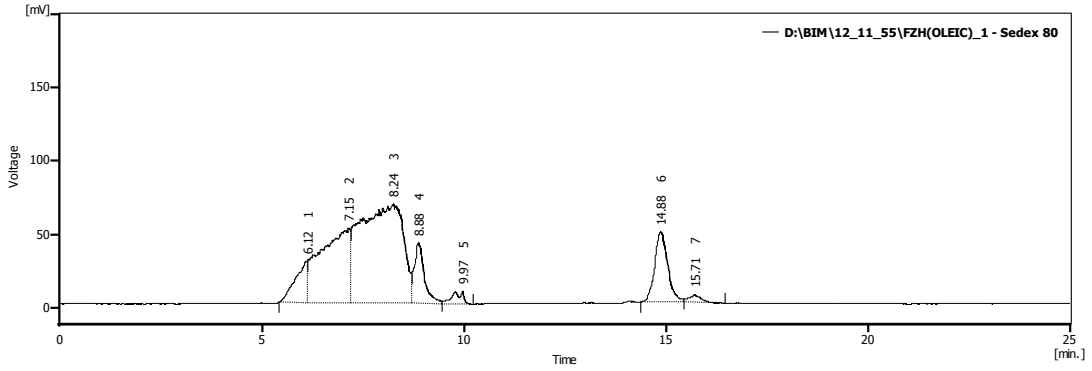
FZP (replicate 3)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.088	1448.391	173.847	6.5
2	6.284	1929.37	180.704	8.7
3	6.368	793.929	188.819	3.6
4	6.492	7406.674	189.7	33.4
5	7.296	3501.709	162.651	15.8
6	7.812	6268.801	215.659	28.3
7	9.98	275.115	12.834	1.2
8	10.692	109.652	7.223	0.5
9	11.052	423.952	10.854	1.9
	Total	22157.59	1142.29	100



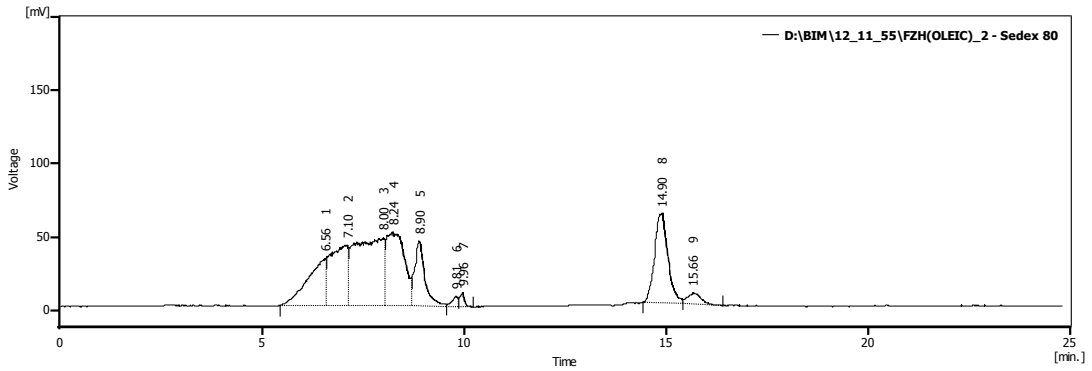
Experiment 2
FZH (replicate 1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.116	567.816	28.153	5.5
2	7.152	2590.39	51.344	25.1
3	8.236	5021.345	68.391	48.6
4	8.876	750.017	41.511	7.3
5	9.968	180.052	9.291	1.7
6	14.876	1094.258	47.841	10.6
7	15.712	129.491	5.563	1.3
	Total	10333.37	252.094	100



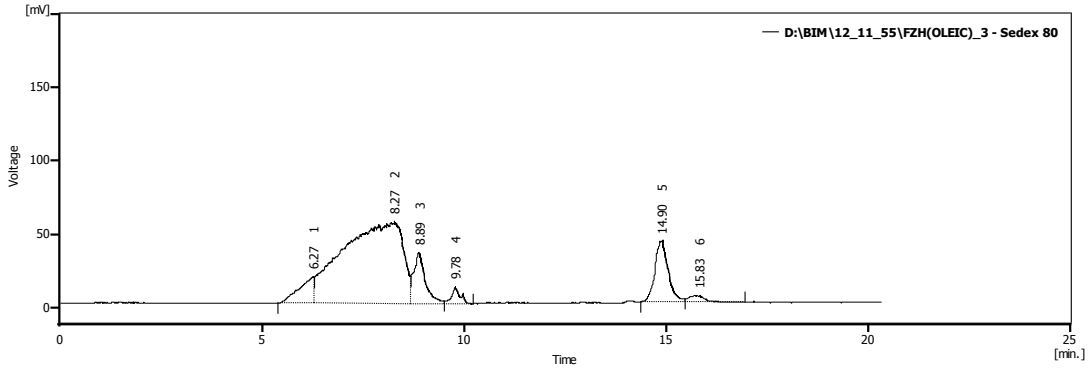
FZH (replicate 2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.564	1003.573	32.334	11.4
2	7.104	1249.386	41.518	14.2
3	8.004	2330.937	46.815	26.5
4	8.236	1609.307	50.729	18.3
5	8.896	817.876	44.512	9.3
6	9.812	78.123	7.335	0.9
7	9.96	81.005	9.832	0.9
8	14.9	1439.913	61.366	16.3
9	15.664	201.731	8.036	2.3
	Total	8811.851	302.477	100



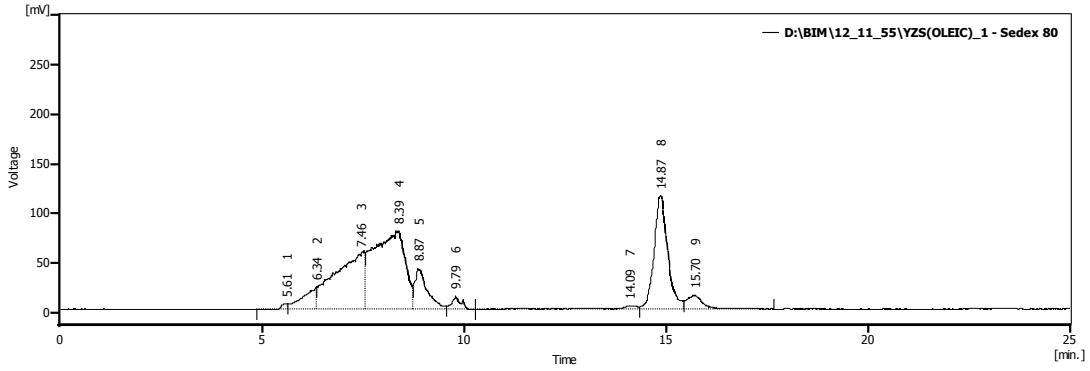
FZH (replicate 3)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.268	472.137	18.789	5.7
2	8.272	5800.255	56.162	70.1
3	8.888	711.803	34.856	8.6
4	9.78	179.502	11.504	2.2
5	14.9	970.693	42.036	11.7
6	15.832	143.386	4.875	1.7
	Total	8277.775	168.222	100



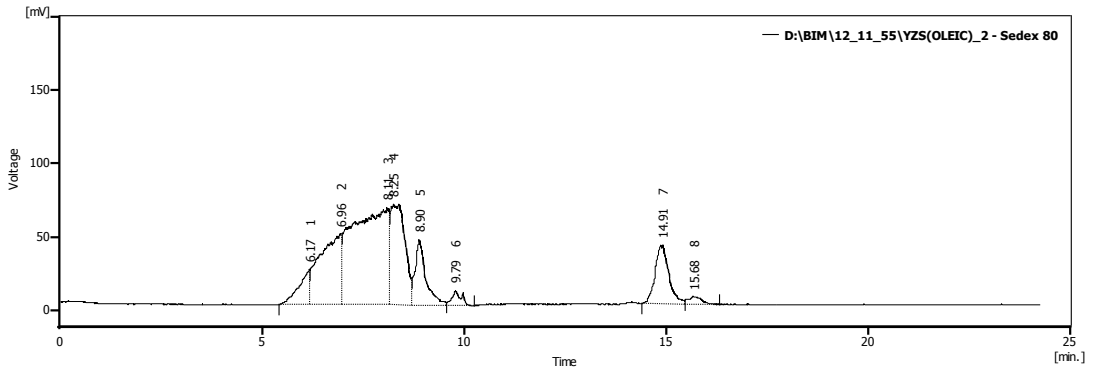
YZS (replicate 1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	5.608	69.226	5.869	0.6
2	6.336	521.64	22.634	4.2
3	7.456	2983.802	56.451	24
4	8.388	4302.822	79.259	34.7
5	8.872	920.064	41.166	7.4
6	9.792	228.732	13.424	1.8
7	14.088	247.923	3.676	2
8	14.872	2704.367	113.848	21.8
9	15.704	434.572	13.896	3.5
	Total	12413.15	350.224	100



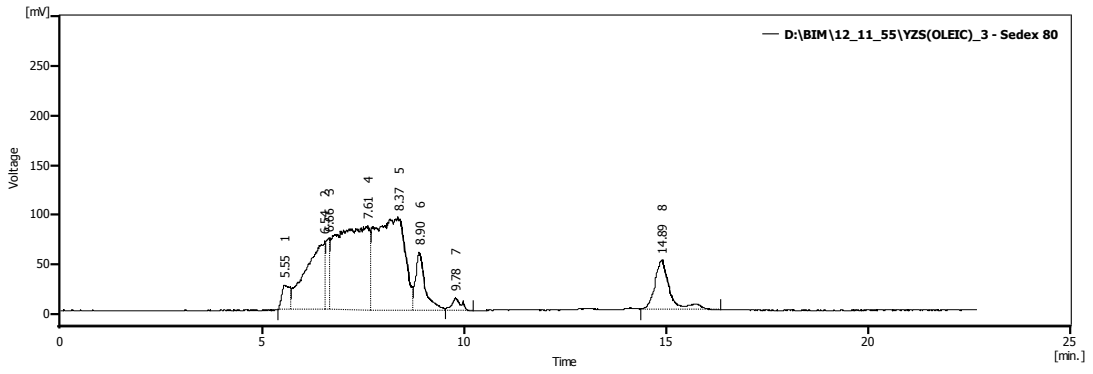
YZS (replicate 2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.168	453.46	24.491	4.4
2	6.964	1777.092	48.768	17.4
3	8.108	4139.524	66.703	40.6
4	8.248	1703.738	68.996	16.7
5	8.9	856.439	44.893	8.4
6	9.792	175.665	10.262	1.7
7	14.912	949.448	40.498	9.3
8	15.684	136.651	5.442	1.3
	Total	10192.02	310.053	100



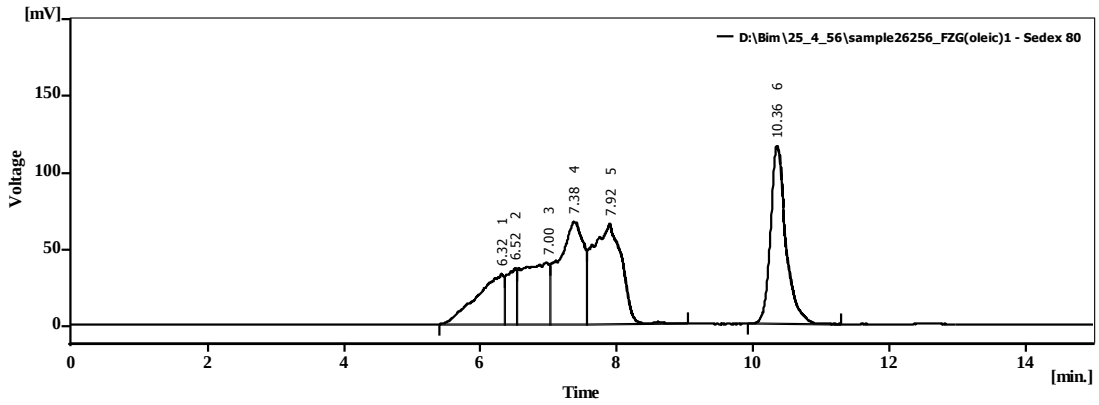
YZS (replicate 3)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	5.548	333.634	24.803	2.2
2	6.544	2265.249	69.144	15
3	6.656	508.957	72.194	3.4
4	7.612	4758.495	84.287	31.4
5	8.368	4783.566	93.392	31.6
6	8.9	970.444	58.467	6.4
7	9.78	220.191	12.95	1.5
8	14.892	1297.527	49.127	8.6
	Total	15138.06	464.364	100



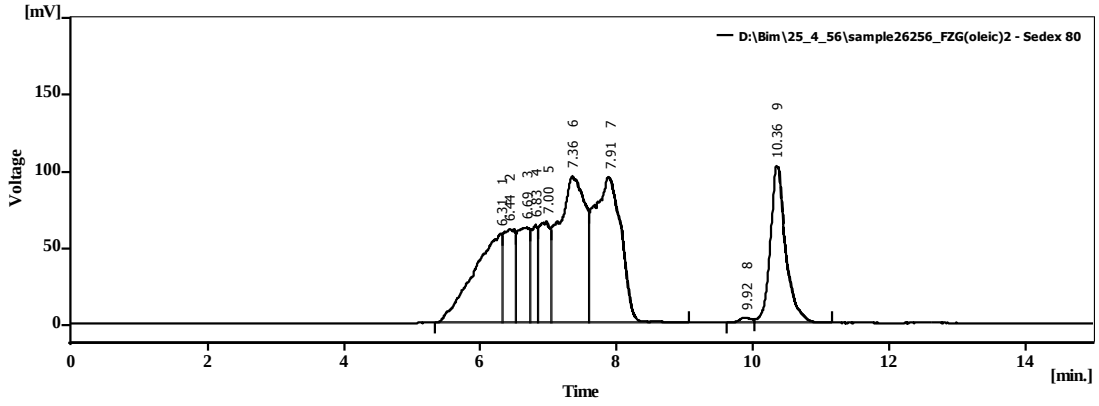
FZG (replicate 1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.324	894.488	32.888	11.5
2	6.52	345.451	36.452	4.4
3	6.996	1092.338	39.91	14
4	7.376	1691.901	66.628	21.7
5	7.916	1936.06	65.516	24.8
6	10.364	1849.172	115.623	23.7
	Total	7809.41	357.017	100



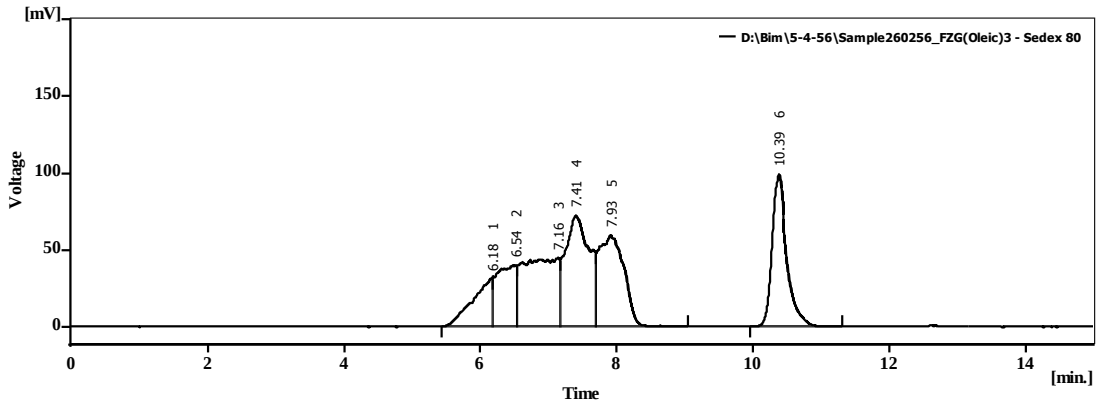
FZG (replicate 2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.312	1620.985	57.746	14.5
2	6.44	727.121	60.833	6.5
3	6.692	771.796	62.092	6.9
4	6.828	430.306	63.501	3.8
5	6.996	687.422	65.954	6.1
6	7.364	2620.445	95.094	23.4
7	7.908	2661.892	94.895	23.8
8	9.924	38.323	3.25	0.3
9	10.356	1631.372	102.154	14.6
	Total	11189.66	605.517	100



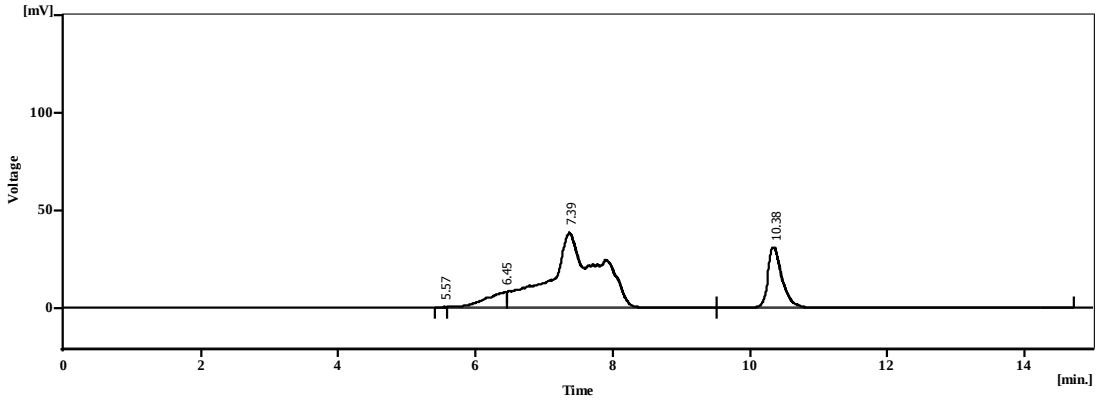
FZG (replicate 3)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.184	611.718	31.123	7.8
2	6.536	803.435	40.156	10.2
3	7.156	1594.302	44.636	20.3
4	7.408	1772.825	72.06	22.6
5	7.928	1552.573	59.616	19.8
6	10.392	1507.999	98.775	19.2
	Total	7842.853	346.366	100



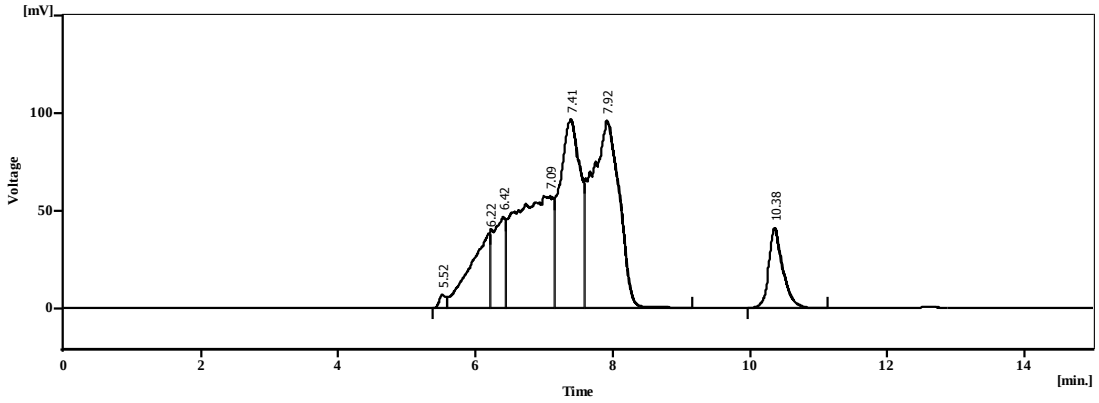
FZT (replicate 2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	5.568	3.809	0.55	0.1
2	6.452	184.162	8.161	7.1
3	7.388	1903.872	38.667	73.8
4	10.376	486.921	31.211	18.9
	Total	2578.764	78.59	100



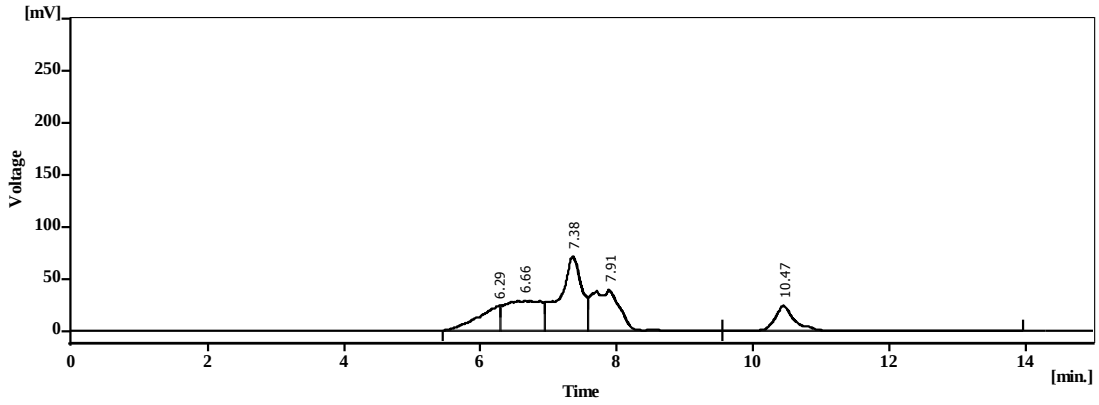
FZT (replicate 3)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	5.524	45.045	6.907	0.5
2	6.216	750.385	38.201	8.4
3	6.42	589.394	46.513	6.6
4	7.092	2228.857	57.237	25
5	7.408	1973.812	96.588	22.1
6	7.924	2749.877	96.289	30.8
7	10.376	594.504	40.979	6.7
	Total	8931.874	382.713	100



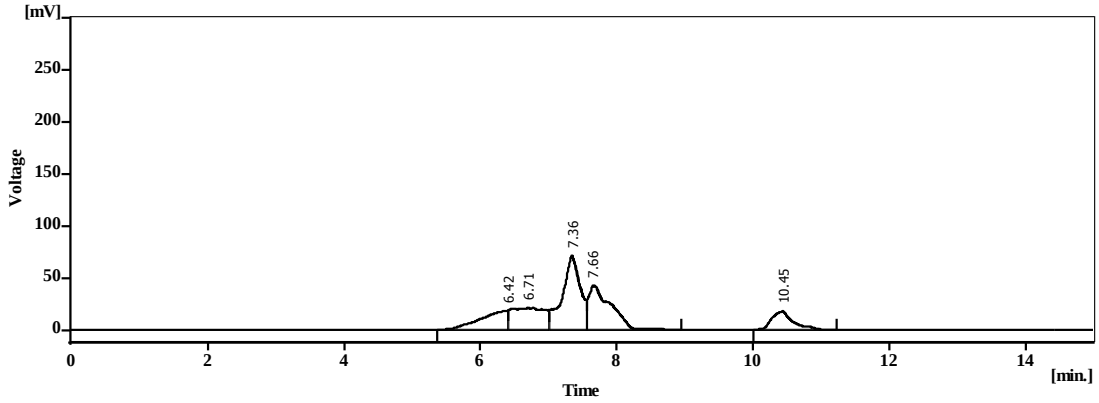
Experiment 3
FY (replicates 1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.292	507.563	23.44	10.7
2	6.66	1054.731	28.92	22.3
3	7.376	1595.345	71.235	33.7
4	7.908	1095.772	39.236	23.2
5	10.468	479.488	24.132	10.1
	Total	4732.899	186.962	100



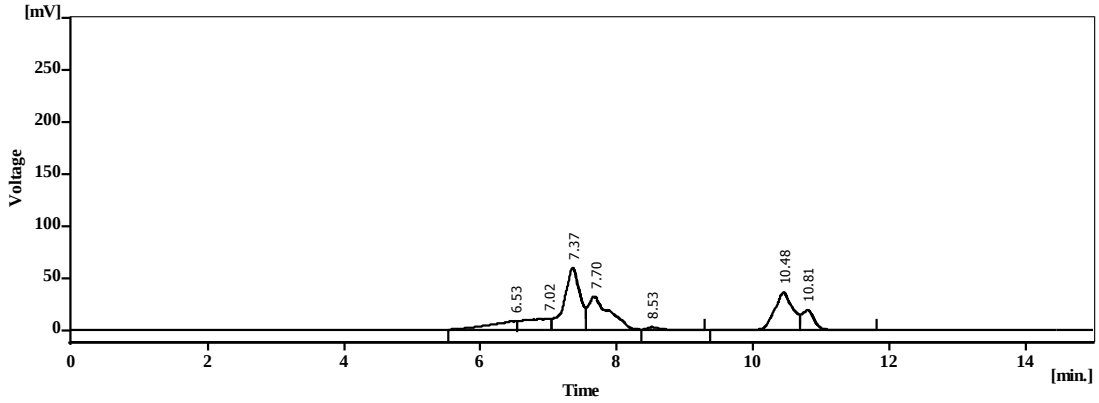
FY (replicates 2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.416	497.095	18.296	12.9
2	6.712	702.063	21.12	18.2
3	7.36	1282.935	70.656	33.3
4	7.66	978.753	42.065	25.4
5	10.448	386.815	17.841	10.1
	Total	3847.66	169.978	100



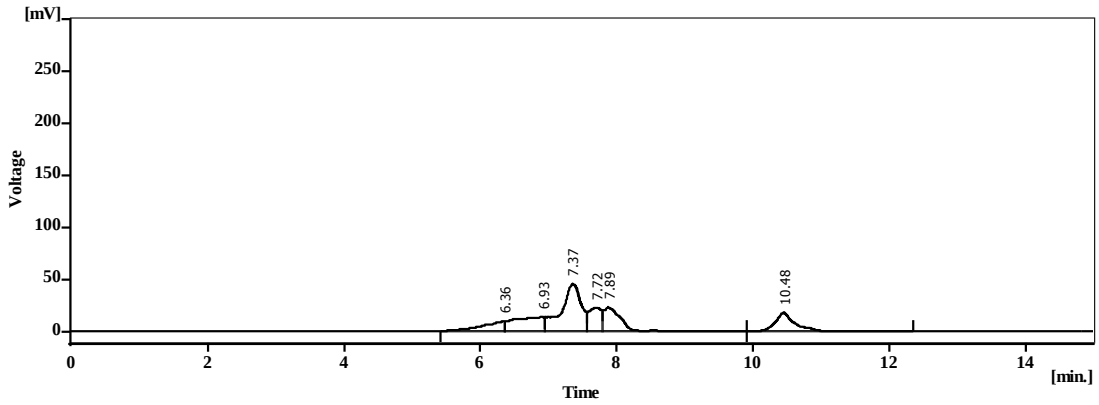
FY (replicates 3)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.532	219.756	8.182	7.1
2	7.02	284.932	10.478	9.2
3	7.372	941.293	59.404	30.3
4	7.696	708.896	31.26	22.8
5	8.532	31.558	2.444	1
6	10.48	680.501	36.358	21.9
7	10.812	237.783	19.471	7.7
	Total	3104.72	167.598	100



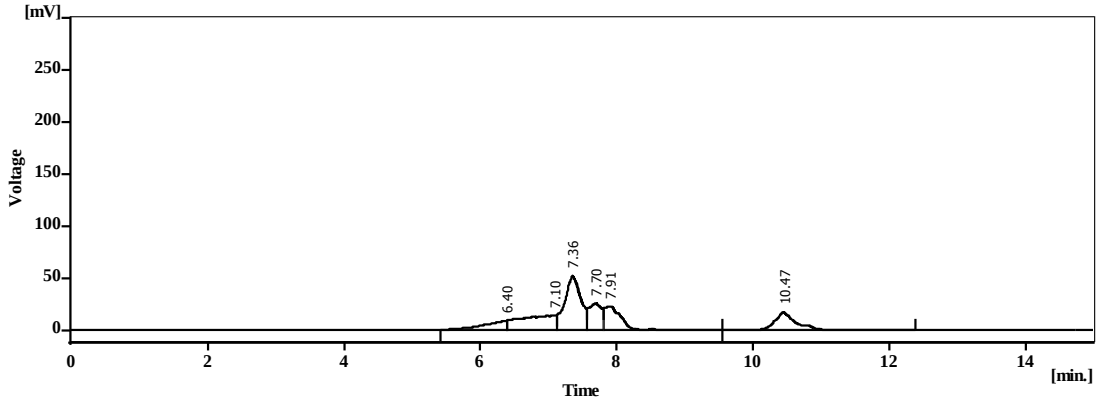
FZG (replicates 1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.364	201.208	9.114	7.9
2	6.932	416.665	13.837	16.4
3	7.368	889.395	45.166	35
4	7.72	296.412	22.033	11.7
5	7.892	383.825	22.794	15.1
6	10.48	350.07	17.734	13.8
	Total	2537.576	130.677	100



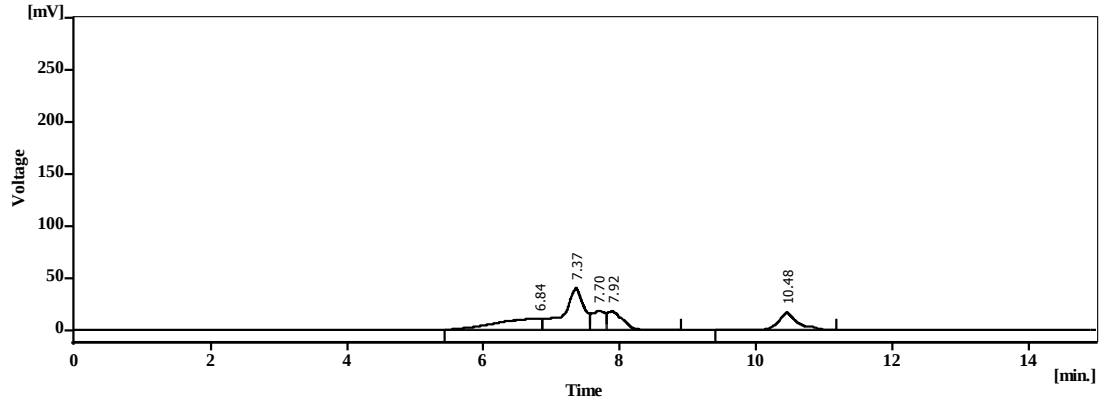
FZG (replicates 2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.4	195.277	8.731	7.5
2	7.104	509.806	14.102	19.6
3	7.364	831.721	51.616	32
4	7.704	333.643	25.53	12.8
5	7.912	378.422	22.527	14.5
6	10.472	353.224	17.009	13.6
	Total	2602.092	139.514	100



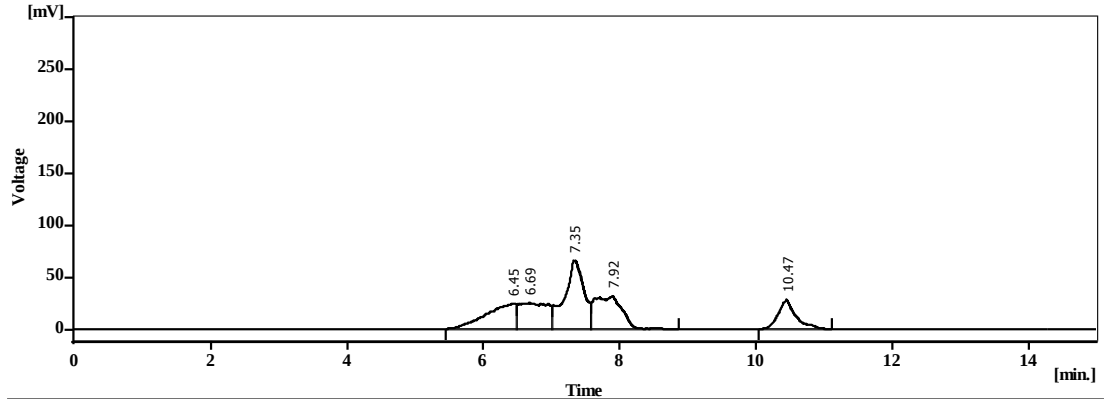
FZG (replicates 3)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.84	469.98	10.782	22.1
2	7.372	816.76	39.759	38.4
3	7.696	232.481	18.273	10.9
4	7.916	286.181	18.135	13.5
5	10.476	320.913	17.083	15.1
	Total	2126.316	104.033	100



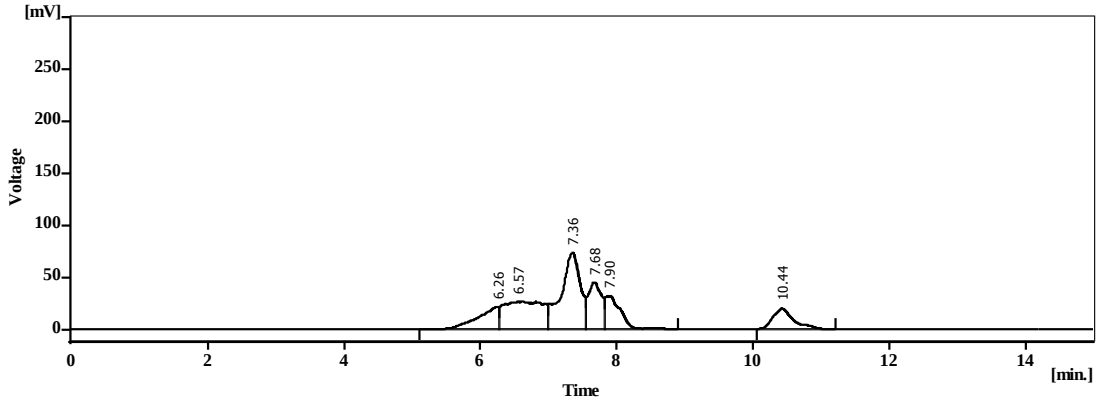
FZP (replicates 1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.452	728.75	24.433	17.6
2	6.688	736.351	24.906	17.7
3	7.352	1279.822	65.304	30.8
4	7.916	890.258	31.376	21.5
5	10.468	514.032	28.055	12.4
	Total	4149.213	174.075	100



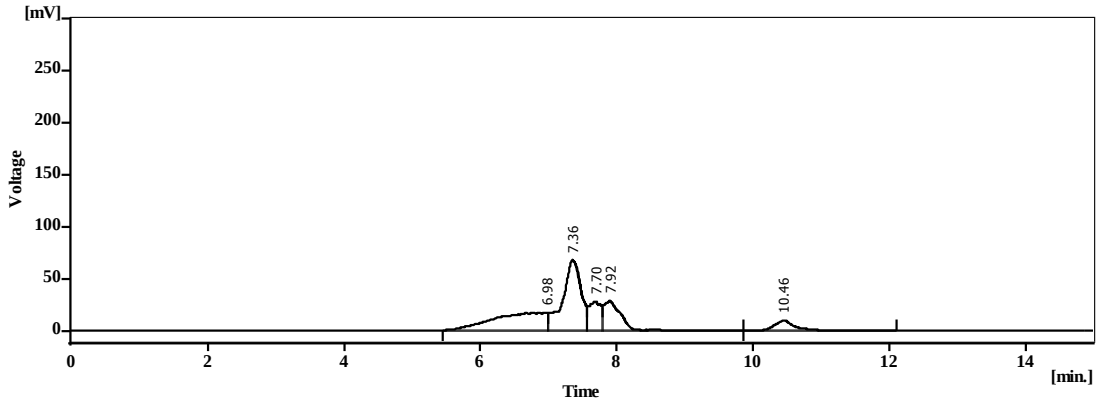
FZP (replicates 2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.264	430.031	21.127	9.7
2	6.568	1071.122	26.59	24.1
3	7.36	1414.924	73.409	31.8
4	7.684	612.992	44.917	13.8
5	7.904	502.895	31.318	11.3
6	10.436	413.564	19.872	9.3
	Total	4445.528	217.234	100



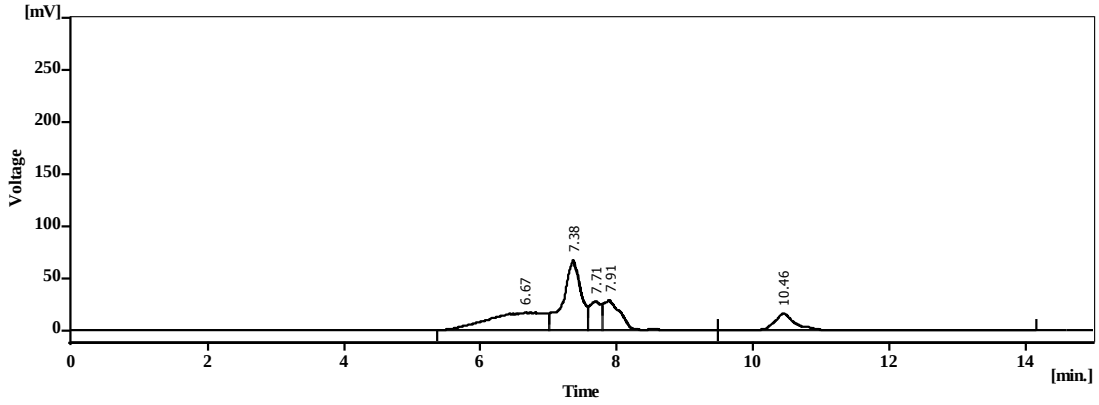
FZT (replicates 1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.98	912.172	17.179	28.8
2	7.36	1241.439	67.357	39.2
3	7.696	345.97	27.534	10.9
4	7.92	475.447	28.266	15
5	10.46	192.471	9.84	6.1
	Total	3167.499	150.175	100



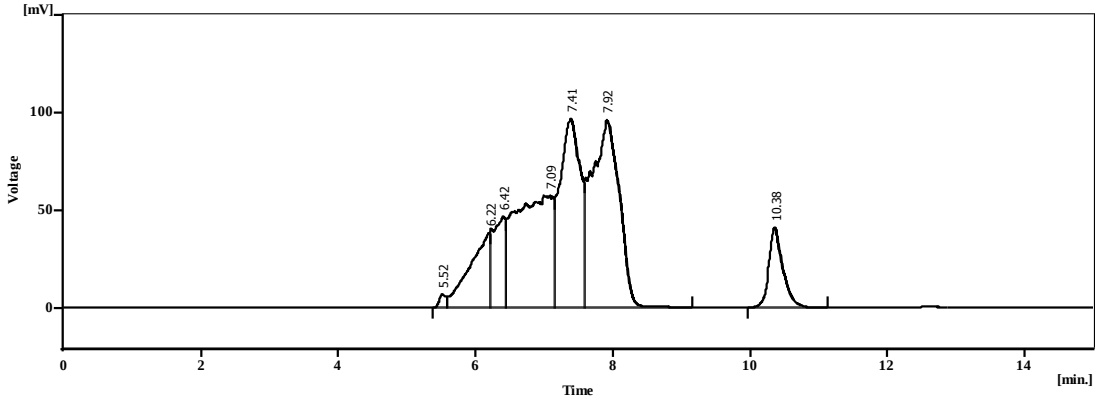
FZT (replicates 2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.668	955.271	16.842	28.9
2	7.376	1192.802	66.84	36.1
3	7.708	312.511	27.841	9.5
4	7.912	503.88	28.398	15.3
5	10.456	336.558	15.766	10.2
	Total	3301.022	155.686	100



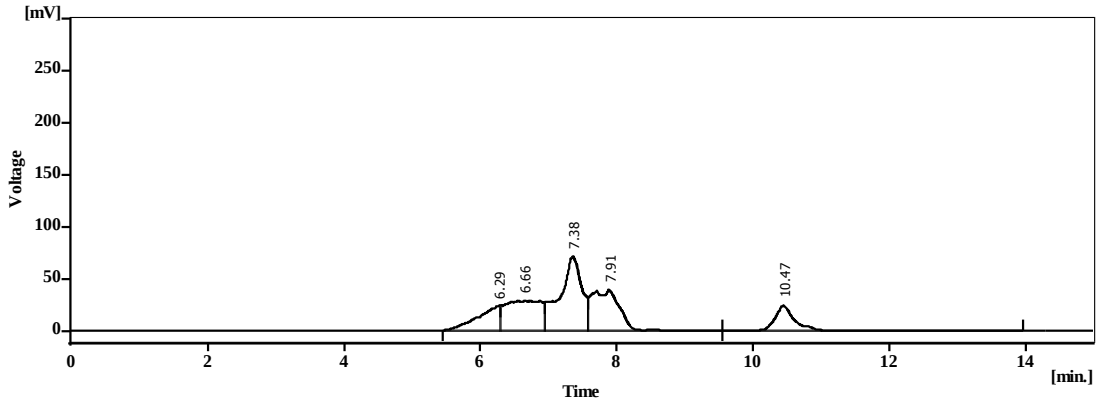
FZT (replicates 3)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	5.524	45.045	6.907	0.5
2	6.216	750.385	38.201	8.4
3	6.42	589.394	46.513	6.6
4	7.092	2228.857	57.237	25
5	7.408	1973.812	96.588	22.1
6	7.924	2749.877	96.289	30.8
7	10.376	594.504	40.979	6.7
	Total	8931.874	382.713	100



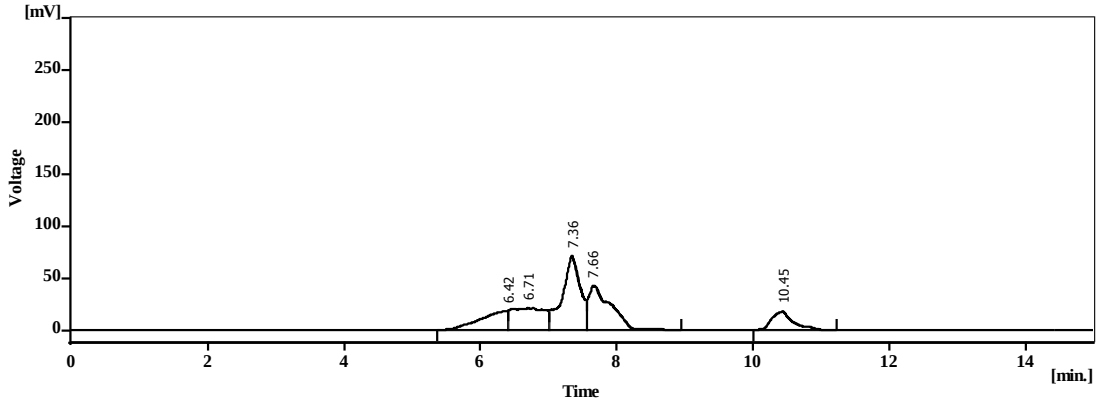
Experiment3
FY (replicate1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.292	507.563	23.44	10.7
2	6.66	1054.731	28.92	22.3
3	7.376	1595.345	71.235	33.7
4	7.908	1095.772	39.236	23.2
5	10.468	479.488	24.132	10.1
	Total	4732.899	186.962	100



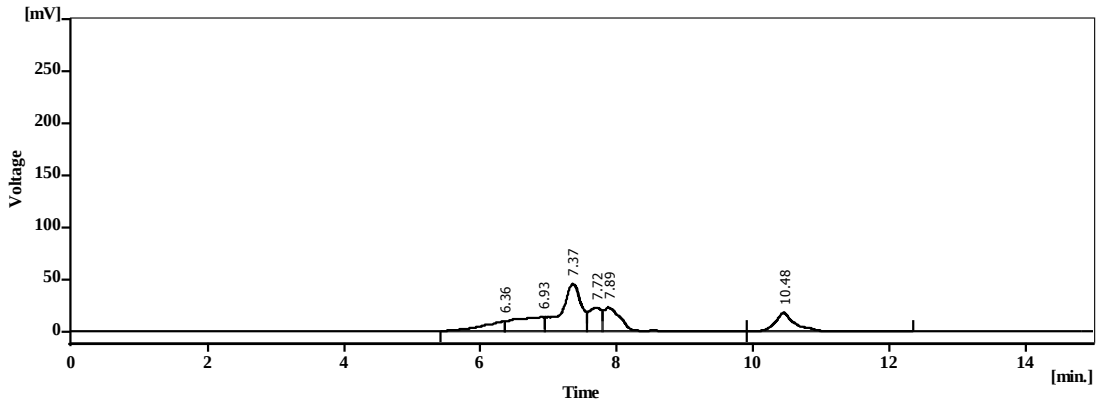
FY (replicate2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.416	497.095	18.296	12.9
2	6.712	702.063	21.12	18.2
3	7.36	1282.935	70.656	33.3
4	7.66	978.753	42.065	25.4
5	10.448	386.815	17.841	10.1
	Total	3847.66	169.978	100



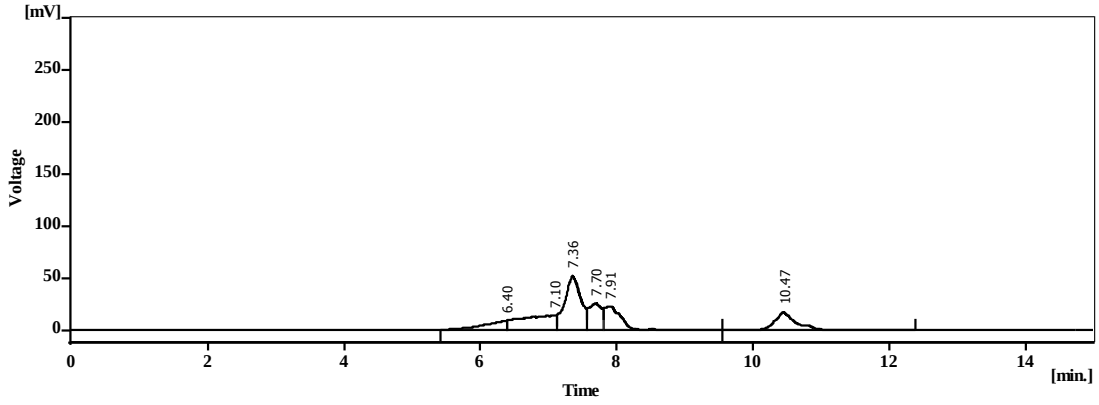
FZG (replicate1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.364	201.208	9.114	7.9
2	6.932	416.665	13.837	16.4
3	7.368	889.395	45.166	35
4	7.72	296.412	22.033	11.7
5	7.892	383.825	22.794	15.1
6	10.48	350.07	17.734	13.8
	Total	2537.576	130.677	100



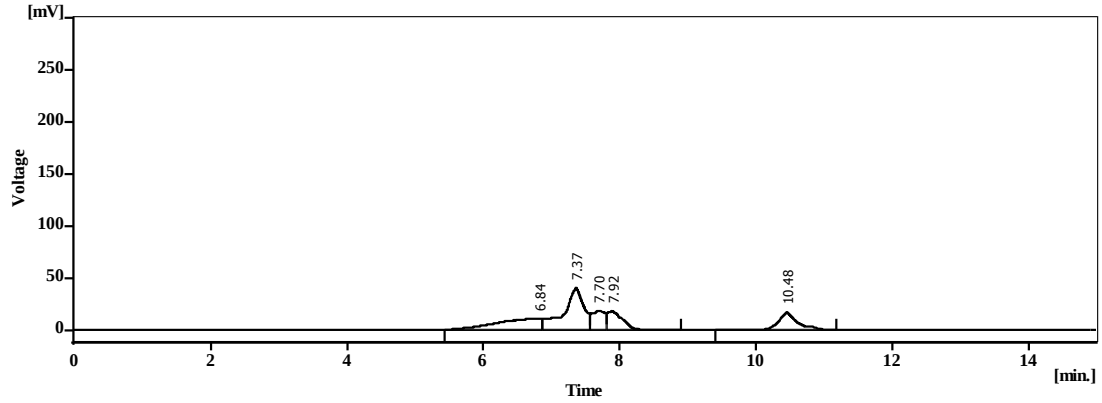
FZG (replicate2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.4	195.277	8.731	7.5
2	7.104	509.806	14.102	19.6
3	7.364	831.721	51.616	32
4	7.704	333.643	25.53	12.8
5	7.912	378.422	22.527	14.5
6	10.472	353.224	17.009	13.6
	Total	2602.092	139.514	100



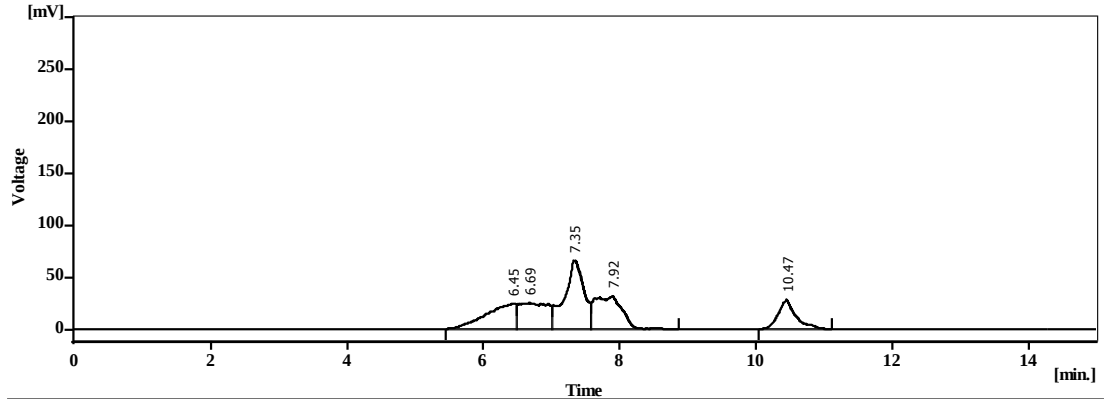
FZG (replicate3)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.84	469.98	10.782	22.1
2	7.372	816.76	39.759	38.4
3	7.696	232.481	18.273	10.9
4	7.916	286.181	18.135	13.5
5	10.476	320.913	17.083	15.1
	Total	2126.316	104.033	100



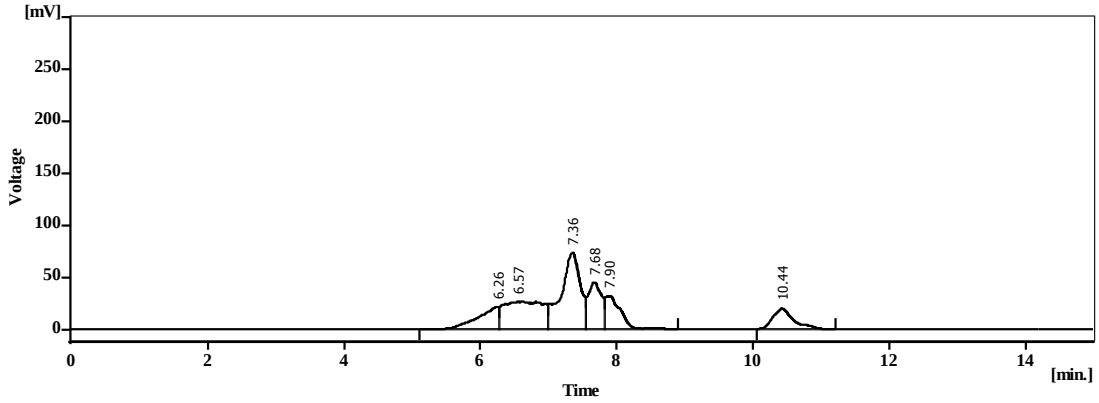
FZP (replicate1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.452	728.75	24.433	17.6
2	6.688	736.351	24.906	17.7
3	7.352	1279.822	65.304	30.8
4	7.916	890.258	31.376	21.5
5	10.468	514.032	28.055	12.4
	Total	4149.213	174.075	100



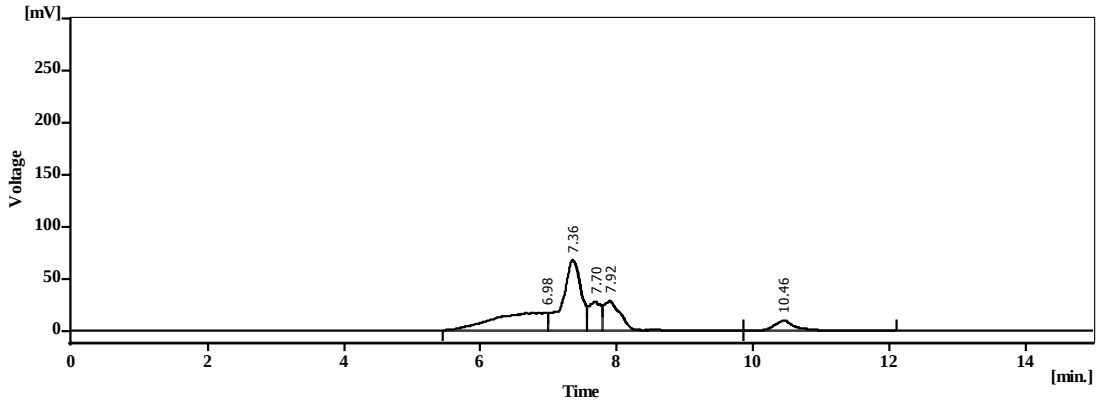
FZP (replicate2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.264	430.031	21.127	9.7
2	6.568	1071.122	26.59	24.1
3	7.36	1414.924	73.409	31.8
4	7.684	612.992	44.917	13.8
5	7.904	502.895	31.318	11.3
6	10.436	413.564	19.872	9.3
	Total	4445.528	217.234	100



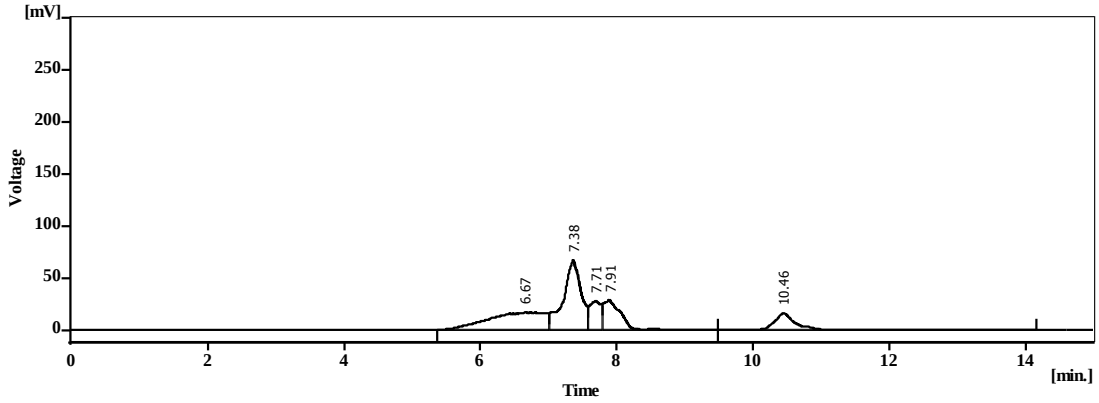
FZT (replicate1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.98	912.172	17.179	28.8
2	7.36	1241.439	67.357	39.2
3	7.696	345.97	27.534	10.9
4	7.92	475.447	28.266	15
5	10.46	192.471	9.84	6.1
	Total	3167.499	150.175	100



FZT (replicate2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.668	955.271	16.842	28.9
2	7.376	1192.802	66.84	36.1
3	7.708	312.511	27.841	9.5
4	7.912	503.88	28.398	15.3
5	10.456	336.558	15.766	10.2
	Total	3301.022	155.686	100



FZT (replicate3)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.216	301.613	15.573	8.6
2	6.932	1014.103	21.492	28.9
3	7.368	1144.433	64.153	32.6
4	7.684	376.382	27.446	10.7
5	7.9	447.563	27.986	12.8
6	10.456	225.692	11.371	6.4
	Total	3509.784	168.022	100

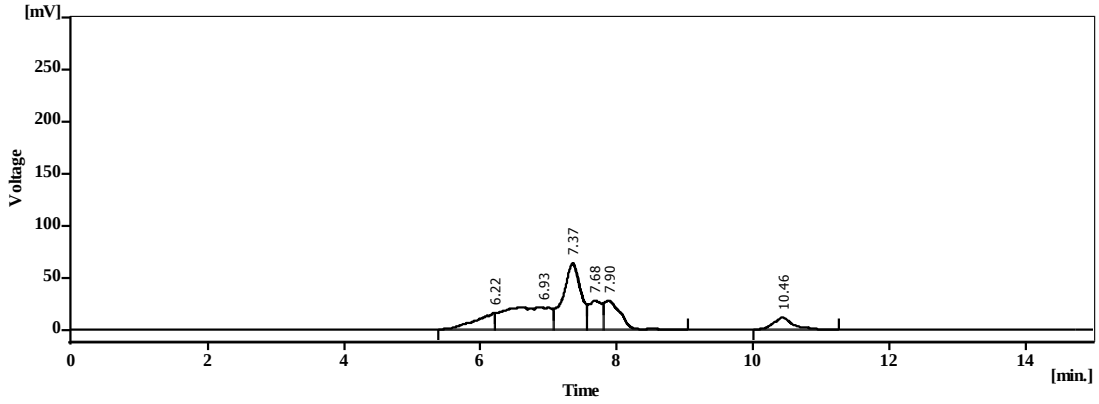
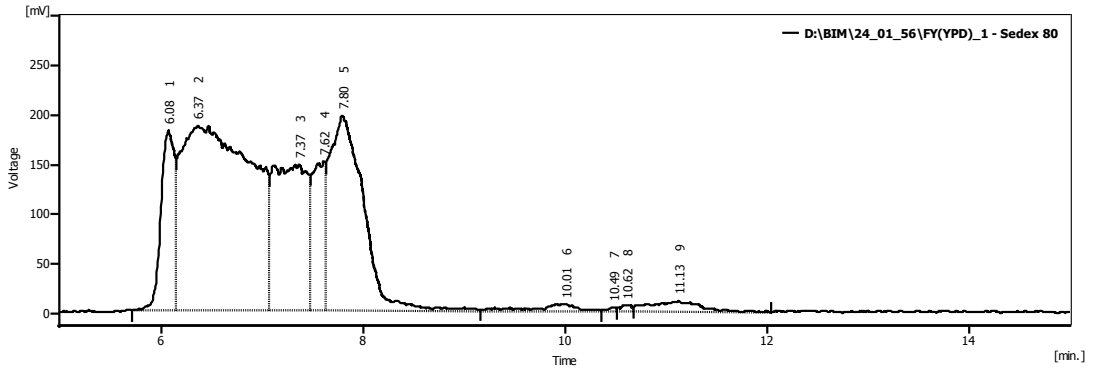


Table A.4 Retention time (min) and Area (%) obtained from chromatogram of HPLC analysis. HPLC analysis were used to analyzed lipid of the FY73 (wild-type) and zinc cluster deletion strains during growth in glucose during exponential growth phase.

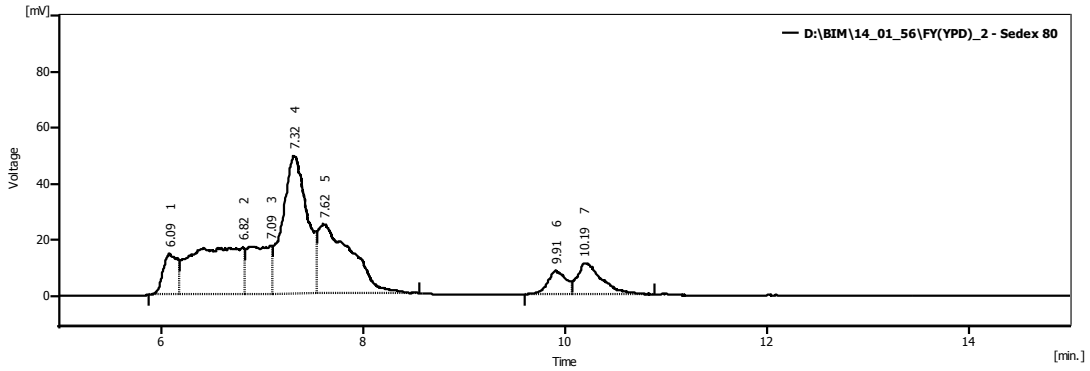
Experiment 1
FY (replicate1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.076	1704.916	181.003	8.1
2	6.368	9051.98	186.31	43.2
3	7.372	3503.997	147.477	16.7
4	7.616	1286.381	150.93	6.1
5	7.8	4656.086	196.934	22.2
6	10.012	260.747	8.192	1.2
7	10.488	28.442	4.55	0.1
8	10.616	55.255	7.307	0.3
9	11.132	414.967	11.199	2
	Total	20962.77	893.903	100



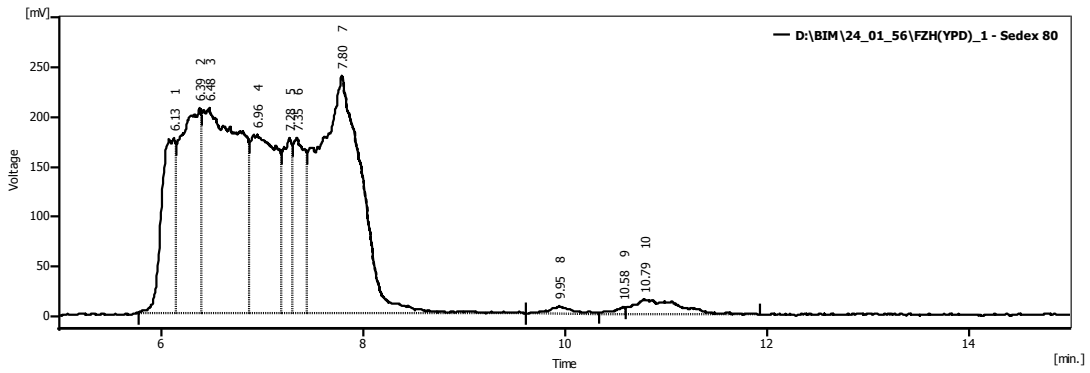
FY (replicate2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.088	146.462	14.617	5.3
2	6.816	600.619	16.833	21.9
3	7.092	272.61	17.447	9.9
4	7.316	835.091	49.153	30.4
5	7.616	590.146	24.844	21.5
6	9.912	106.747	8.462	3.9
7	10.192	190.832	11.054	7
	Total	2742.505	142.41	100



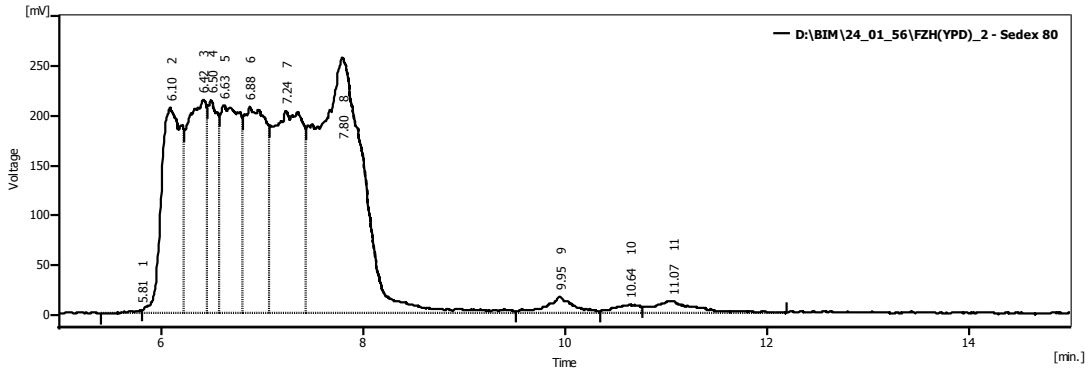
FZH (replicate1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.132	1686.526	175.434	7.1
2	6.388	2821.757	206.137	11.9
3	6.48	5373.048	205.875	22.7
4	6.956	3248.071	179.364	13.7
5	7.276	1132.529	176.448	4.8
6	7.352	1458.432	176.272	6.2
7	7.796	7194.642	238.481	30.4
8	9.948	142.154	7.769	0.6
9	10.58	62.331	7.169	0.3
10	10.788	517.853	15.031	2.2
	Total	23637.34	1387.979	100



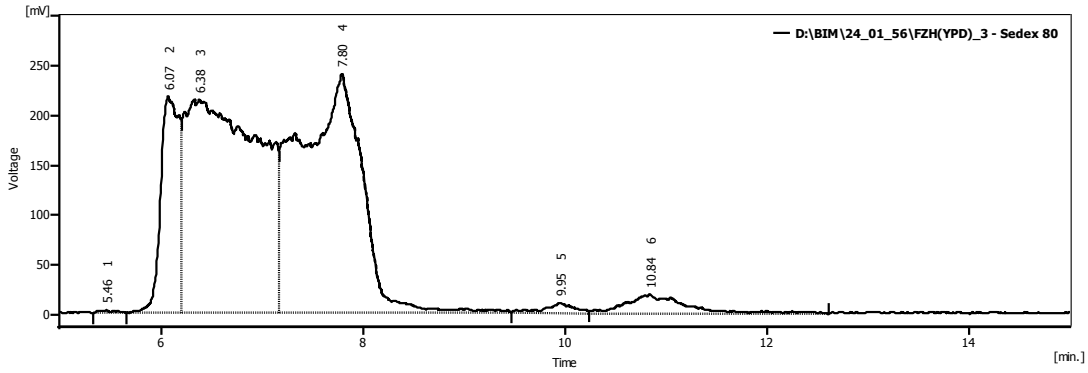
FZH (replicate2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	5.808	29.718	3.297	0.1
2	6.096	2885.194	206.594	10.9
3	6.42	2717.338	214.524	10.3
4	6.5	1532.989	213.718	5.8
5	6.628	2733.438	209.044	10.3
6	6.884	3250.438	206.909	12.3
7	7.24	4143.32	203.097	15.6
8	7.8	8382.158	256.753	31.6
9	9.952	316.975	16.887	1.2
10	10.644	146.035	8.482	0.6
11	11.072	364.317	12.366	1.4
	Total	26505.03	1552.968	100



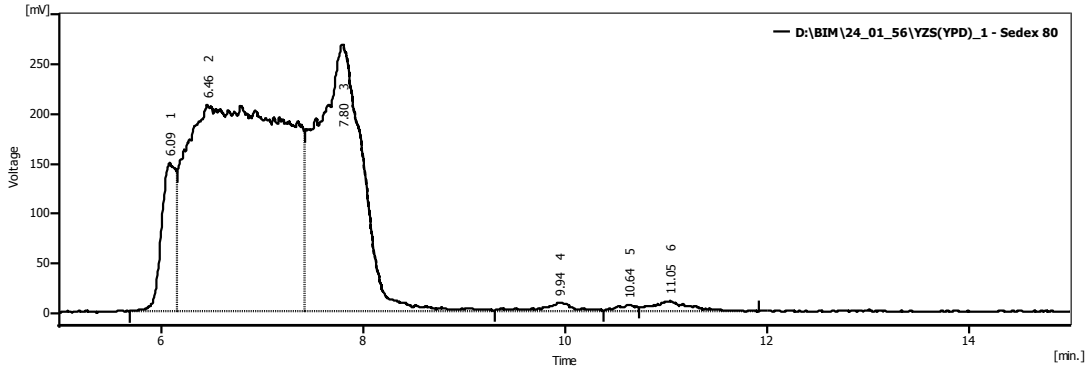
FZH (replicate3)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	5.464	36.128	3.066	0.1
2	6.072	2727.172	217.811	10.7
3	6.384	11003.69	214.625	43.4
4	7.796	10530.59	240.458	41.5
5	9.952	239.104	10.798	0.9
6	10.844	833.575	19.441	3.3
	Total	25370.26	706.198	100



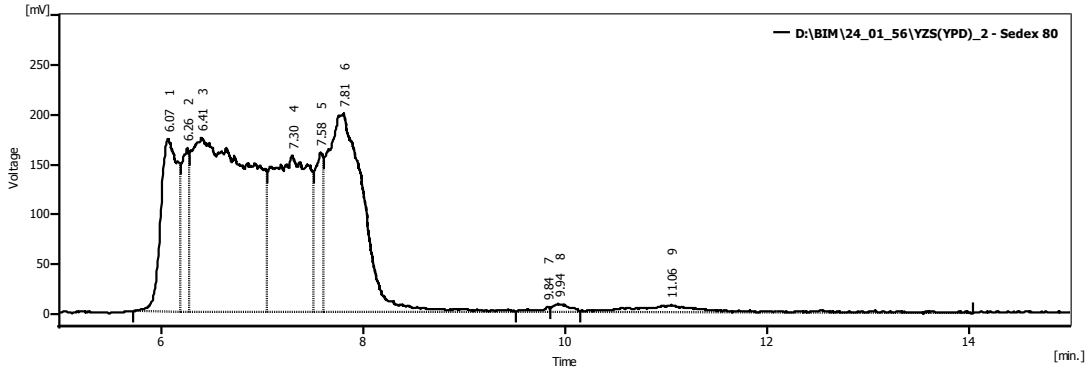
YZS (replicate1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.092	1432.414	148.783	5.7
2	6.464	14419.15	206.539	57.7
3	7.804	8525.807	267.741	34.1
4	9.94	219.247	8.938	0.9
5	10.636	90.723	6.984	0.4
6	11.048	286.762	10.479	1.1
	Total	24974.1	649.464	100



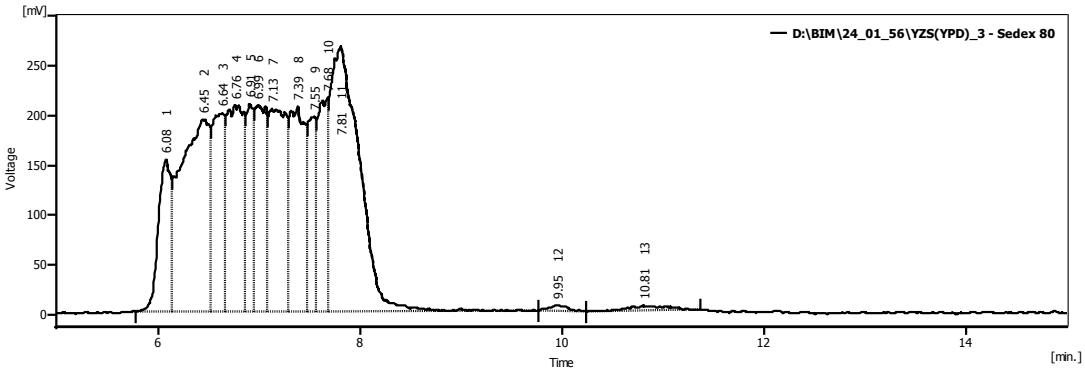
YZS (replicate2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.072	2071.864	173.412	10
2	6.264	789.523	163.986	3.8
3	6.408	7257.59	174.539	35
4	7.304	3999.579	157.005	19.3
5	7.584	946.782	159.787	4.6
6	7.812	4968.22	199.486	23.9
7	9.836	57.824	5.783	0.3
8	9.94	104.386	8.691	0.5
9	11.06	568.181	7.759	2.7
	Total	20763.95	1050.447	100



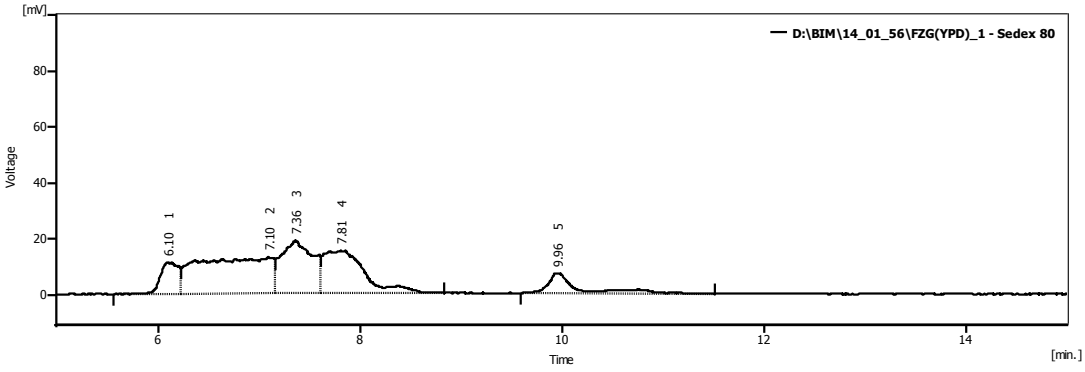
YZS (replicate3)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.08	1276.984	152.964	5.2
2	6.448	3824.352	193.03	15.5
3	6.64	1731.131	199.467	7
4	6.764	2413.364	207.094	9.8
5	6.912	1026.287	207.874	4.2
6	6.992	1661.797	207.355	6.7
7	7.128	2444.005	203.435	9.9
8	7.388	2155.117	205.494	8.7
9	7.552	1111.943	195.925	4.5
10	7.684	1485.465	214.8	6
11	7.812	5313.6	265.797	21.5
12	9.952	86.603	5.73	0.4
13	10.808	152.65	4.973	0.6
	Total	24683.3	2263.937	100



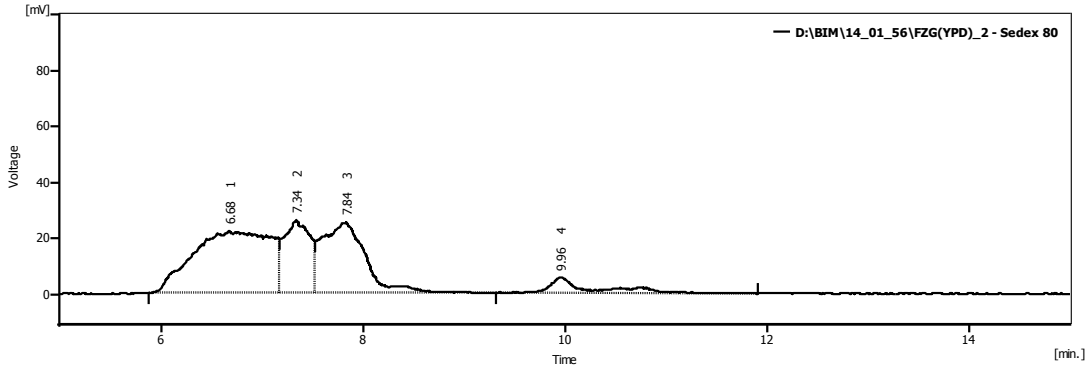
FZG (replicate1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.1	147.963	11.485	8.2
2	7.096	658.451	13.016	36.3
3	7.364	416.986	18.937	23
4	7.812	434.444	15.239	23.9
5	9.96	157.513	7.216	8.7
	Total	1815.356	65.892	100



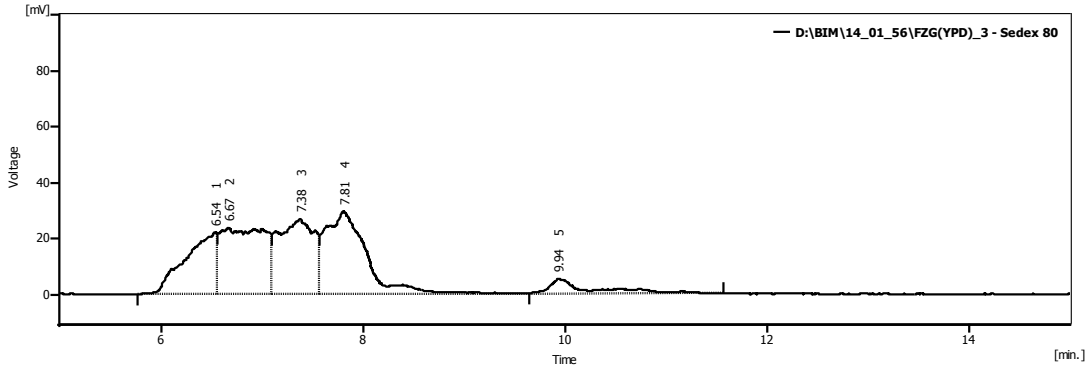
FZG (replicate2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.68	1181.373	22.099	45.5
2	7.344	473.169	26.223	18.2
3	7.836	766.538	25.243	29.6
4	9.956	172.7	5.551	6.7
	Total	2593.781	79.116	100



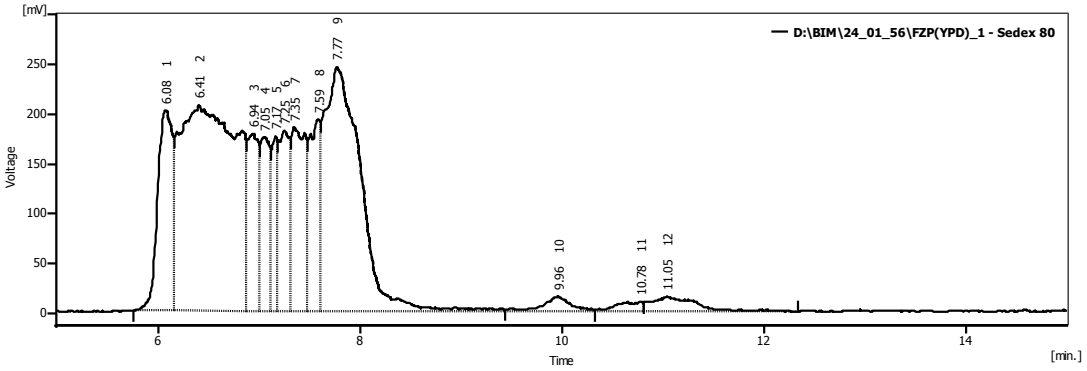
FZG (replicate3)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.536	472.277	22.068	16.5
2	6.672	728.648	23.824	25.4
3	7.38	655.468	26.874	22.9
4	7.812	843.998	29.474	29.5
5	9.936	163.663	5.43	5.7
	Total	2864.054	107.67	100



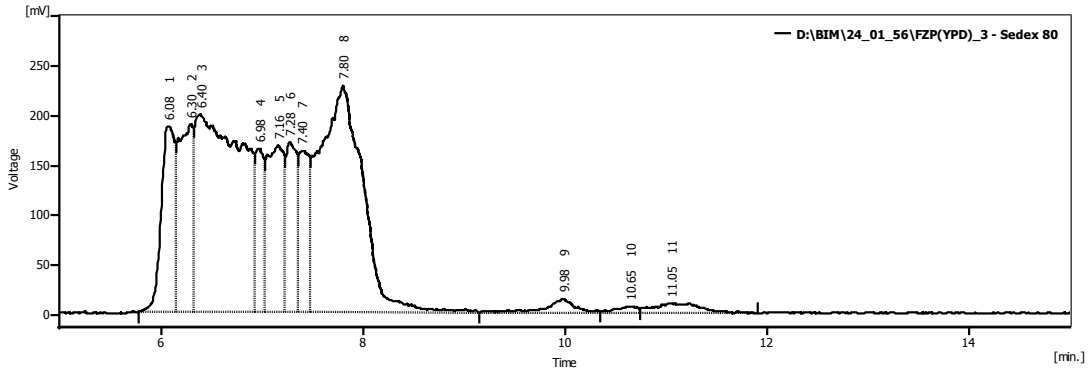
FZP (replicate1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.076	2016.045	201.68	8
2	6.412	8155.507	207.068	32.5
3	6.944	1373.212	178.279	5.5
4	7.052	1105.613	174.899	4.4
5	7.168	694.329	175.588	2.8
6	7.252	1305.206	181.504	5.2
7	7.348	1791.431	184.978	7.1
8	7.592	1392.028	192.359	5.6
9	7.772	6215.438	245.483	24.8
10	9.964	313.434	15.372	1.3
11	10.784	183.442	10.606	0.7
12	11.048	520.215	15.747	2.1
	Total	25065.9	1783.563	100



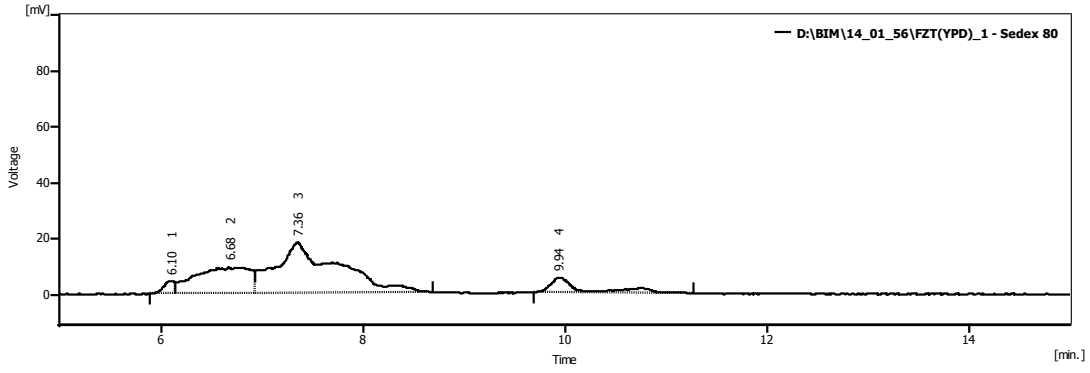
FZP (replicate3)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.08	1763.528	186.199	7.7
2	6.296	1844.042	188.349	8
3	6.396	6407.252	198.777	28
4	6.976	964.093	164.381	4.2
5	7.16	1964.34	167.57	8.6
6	7.28	1262.825	171.295	5.5
7	7.4	1150.649	162.372	5
8	7.804	6816.572	227.469	29.7
9	9.984	303.515	14.209	1.3
10	10.648	105.068	6.859	0.5
11	11.052	341.575	10.114	1.5
	Total	22923.46	1497.595	100



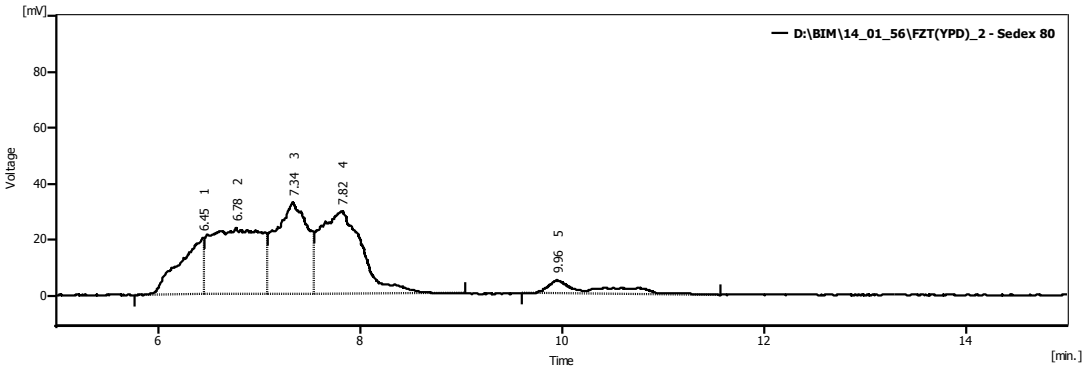
FZT (replicate1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.104	34.478	4.514	2.6
2	6.676	355.841	9.364	27.3
3	7.356	789.269	18.097	60.6
4	9.936	123.898	5.447	9.5
	Total	1303.486	37.423	100



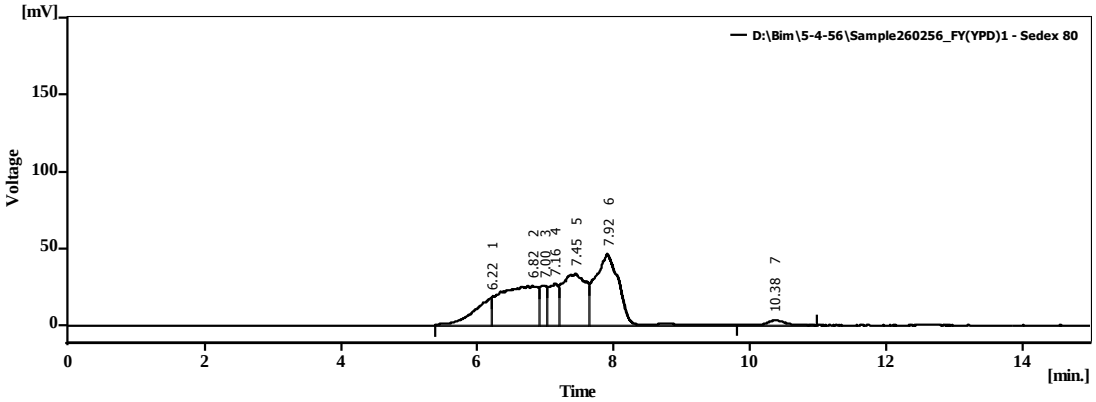
FZT (replicate2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.452	343.377	20.466	11.7
2	6.776	832.275	23.744	28.3
3	7.34	730.876	32.97	24.8
4	7.824	870.884	29.788	29.6
5	9.956	165.381	4.84	5.6
	Total	2942.792	111.807	100



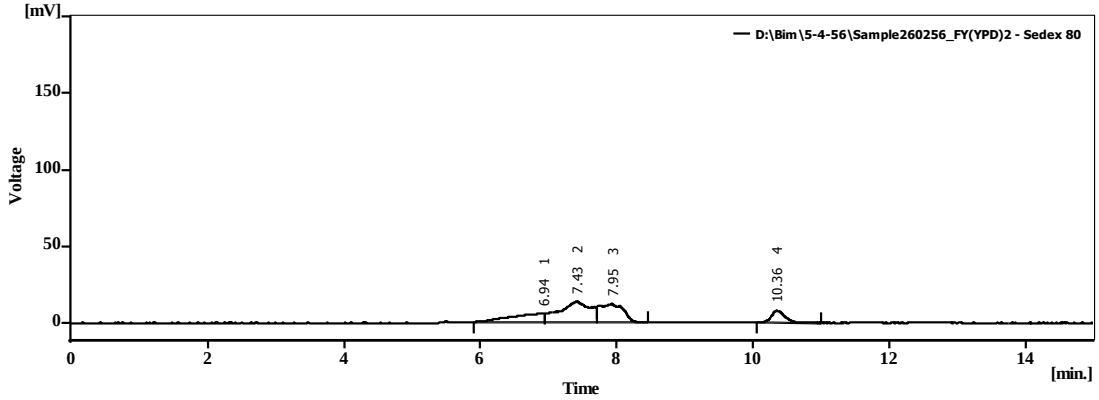
Experiment 2
FY73 (replicate 1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.216	312.72	17.665	8.4
2	6.82	976.226	25.822	26.2
3	7.004	169.778	25.867	4.6
4	7.156	259.214	27.085	7
5	7.452	797.146	33.396	21.4
6	7.924	1147.226	46.267	30.8
7	10.376	61.367	3.196	1.6
	Total	3723.676	179.298	100



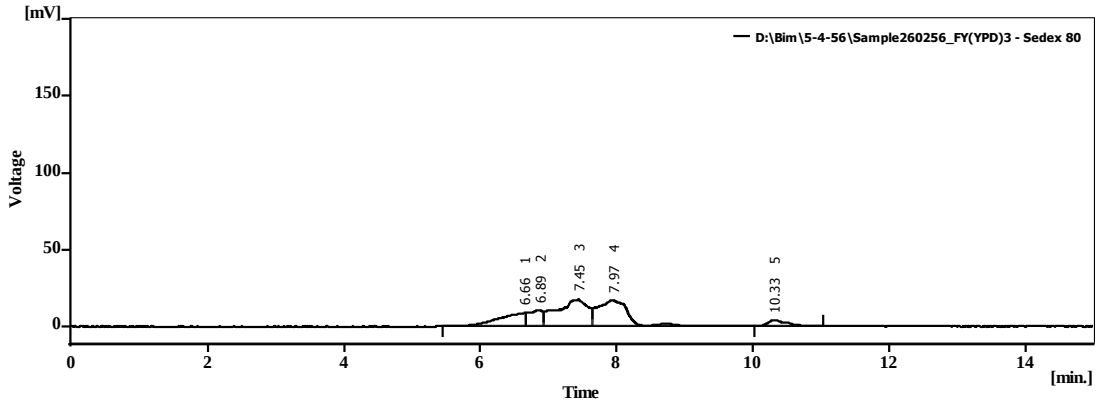
FY73 (replicate 2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.944	194.855	5.761	18.8
2	7.432	423.383	13.693	40.9
3	7.952	298.617	12.022	28.9
4	10.36	117.416	8.016	11.4
	Total	1034.271	39.493	100



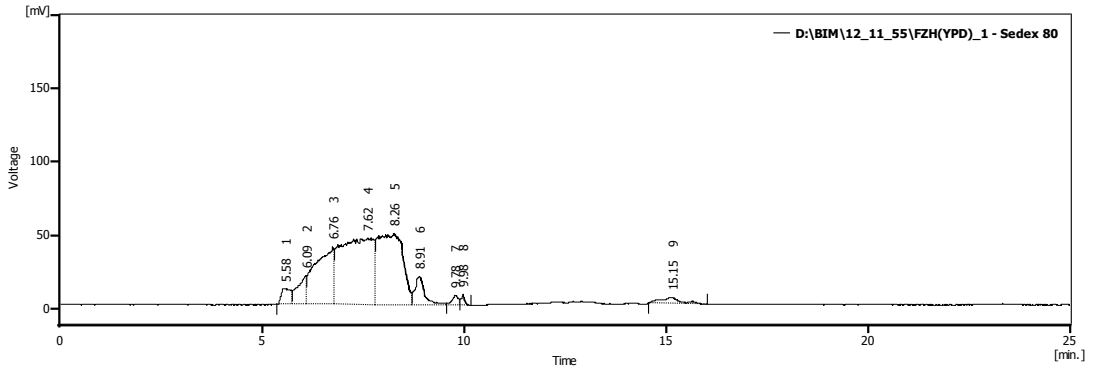
FY73 (replicate 3)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.66	238.47	8.695	15.1
2	6.892	147.502	10.421	9.3
3	7.452	561.193	17.515	35.6
4	7.968	545.587	17.272	34.6
5	10.332	85.194	4.064	5.4
	Total	1577.946	57.966	100



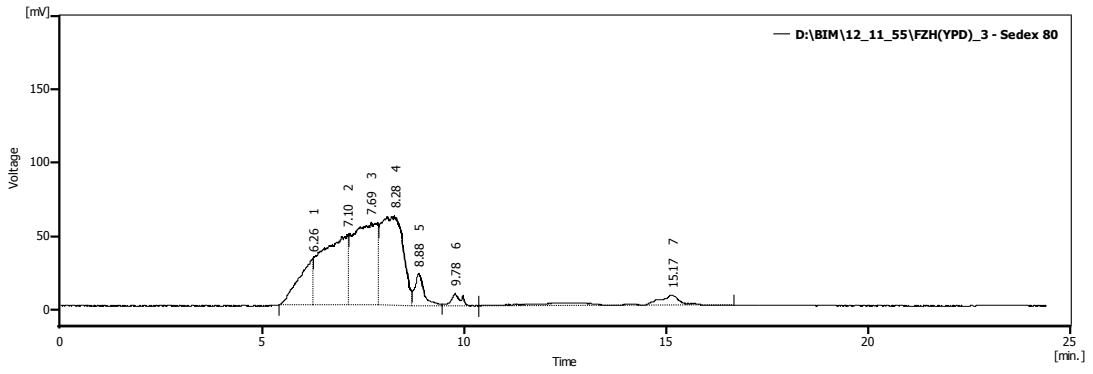
FZH (replicate 1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	5.576	167.536	11.015	2.4
2	6.088	309.119	20.109	4.4
3	6.756	1235.085	39.26	17.5
4	7.62	2583.146	45.472	36.6
5	8.256	2132.205	48.832	30.2
6	8.908	340.933	19.664	4.8
7	9.78	78.275	7.033	1.1
8	9.976	51.434	7.624	0.7
9	15.152	159.299	4.305	2.3
	Total	7057.033	203.314	100



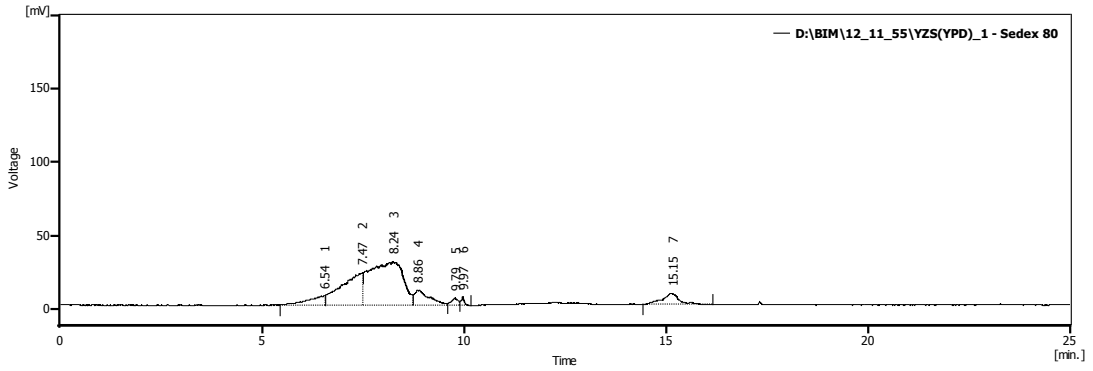
FZH (replicate 3)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.256	779.752	31.894	9
2	7.104	2163.004	48.427	24.9
3	7.692	2341.633	56.902	27
4	8.284	2357.156	61.667	27.1
5	8.88	360.483	21.877	4.2
6	9.78	150.899	8.624	1.7
7	15.172	529.353	7.168	6.1
	Total	8682.279	236.559	100



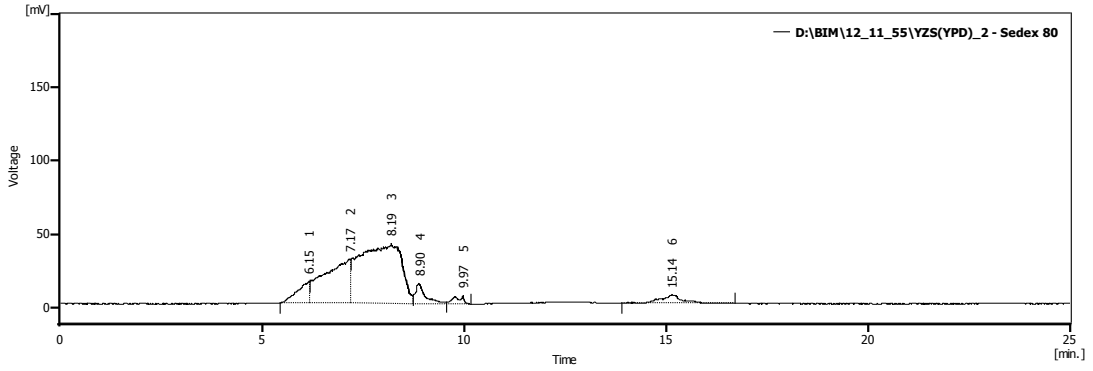
YZS (replicate 1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.544	190.84	6.89	5.7
2	7.468	815.045	22.08	24.3
3	8.236	1716.29	30.409	51.2
4	8.856	282.112	10.552	8.4
5	9.788	60.13	4.92	1.8
6	9.972	38.102	6.138	1.1
7	15.152	248.516	7.992	7.4
	Total	3351.036	88.982	100



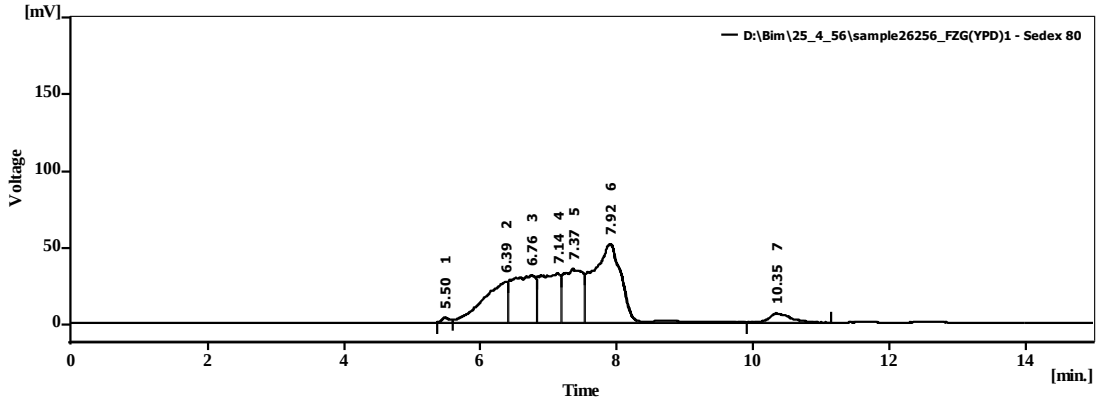
YZS (replicate 2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.152	305.111	15.055	5.9
2	7.172	1349.539	30.34	26
3	8.192	2948.108	40.984	56.9
4	8.896	246.34	13.752	4.8
5	9.972	97.026	5.566	1.9
6	15.14	235.271	5.806	4.5
	Total	5181.395	111.502	100



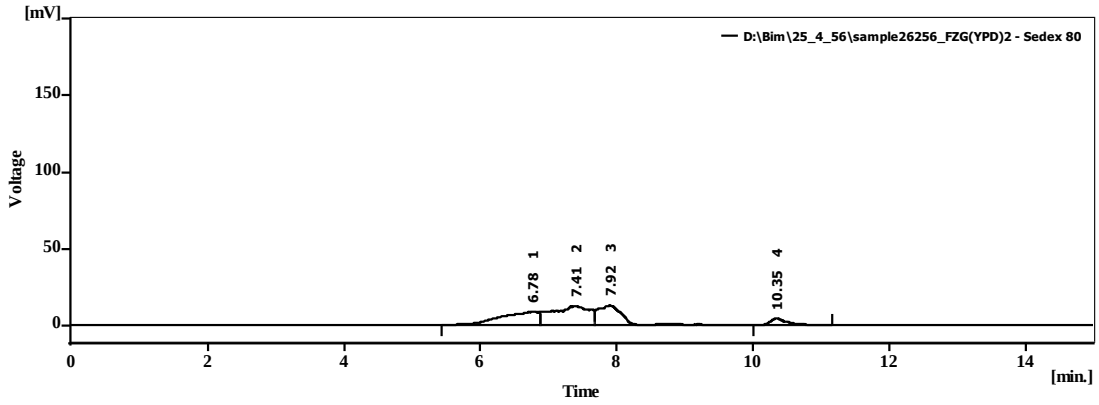
FZG (replicate 1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	5.496	24.18	3.197	0.6
2	6.392	649.16	26.769	14.9
3	6.756	729.482	30.78	16.7
4	7.144	670.22	32.09	15.3
5	7.372	675.061	35.16	15.4
6	7.916	1491.168	50.873	34.1
7	10.352	130.082	5.926	3
	Total	4369.353	184.795	100



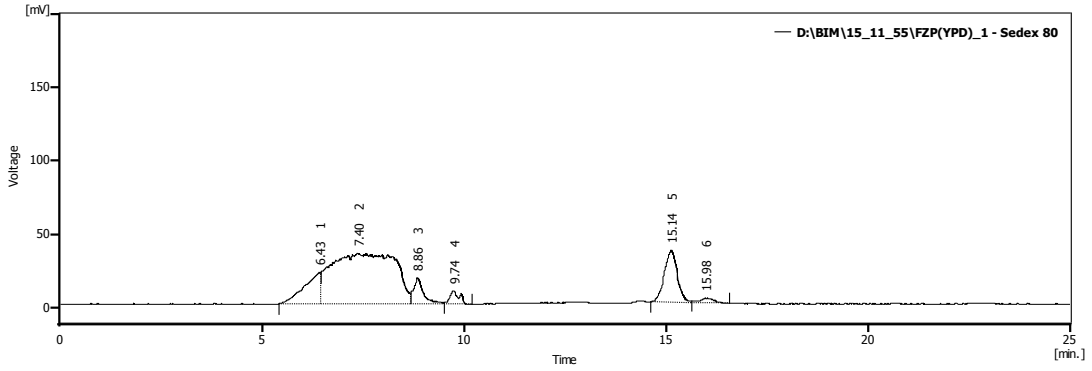
FZG (replicate 2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.776	344.024	8.762	27.4
2	7.408	481.066	12.432	38.3
3	7.92	349.625	13.344	27.8
4	10.352	82.231	4.6	6.5
	Total	1256.946	39.139	100



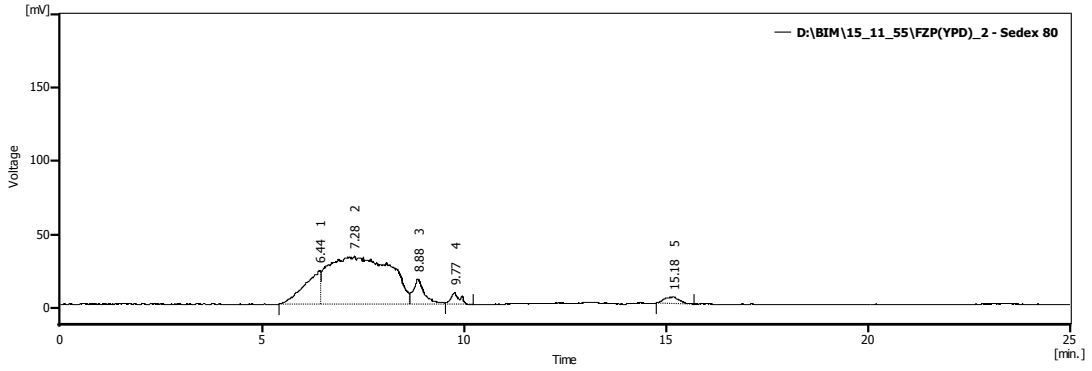
FZP (replicate 1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.428	604.994	21.775	10.3
2	7.396	3872.091	35.03	66.2
3	8.856	326.373	18.169	5.6
4	9.744	159.826	9.518	2.7
5	15.136	801.443	35.906	13.7
6	15.976	87.567	3.384	1.5
	Total	5852.294	123.782	100



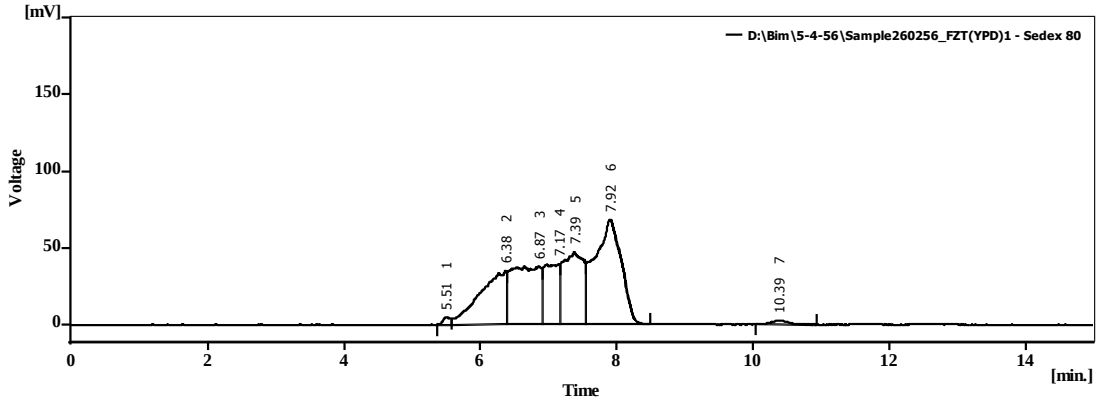
FZP (replicate 2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.436	660.753	23.515	13.7
2	7.284	3516.064	33.294	73
3	8.876	369.307	17.733	7.7
4	9.772	142.866	8.806	3
5	15.18	127.982	4.994	2.7
	Total	4816.972	88.341	100



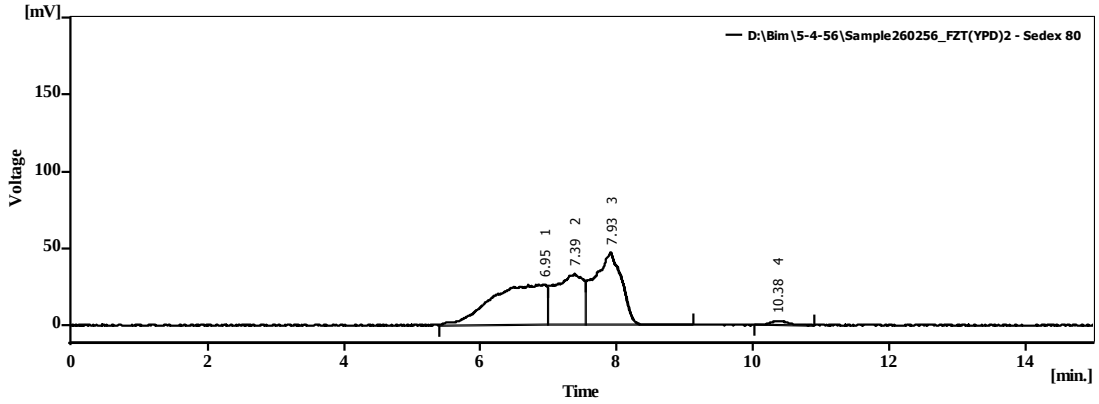
FZT (replicate 1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	5.512	34.256	4.992	0.6
2	6.384	940.403	34.888	16.9
3	6.872	1117.458	37.637	20.1
4	7.172	604.605	39.249	10.9
5	7.392	955.013	47.211	17.2
6	7.92	1853.927	67.736	33.4
7	10.392	48.75	2.73	0.9
	Total	5554.412	234.442	100



FZT (replicate 2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.952	1444.176	26.313	37.9
2	7.392	960.488	33.192	25.2
3	7.932	1352.56	47.48	35.5
4	10.38	49.523	2.684	1.3
	Total	3806.746	109.669	100



FZT (replicate 3)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	5.544	9.93	1.161	0.6
2	6.872	397.95	11.121	22.9
3	7.464	712.065	17.951	40.9
4	7.98	566.93	21.947	32.6
5	10.368	53.268	2.645	3.1
	Total	1740.143	54.825	100

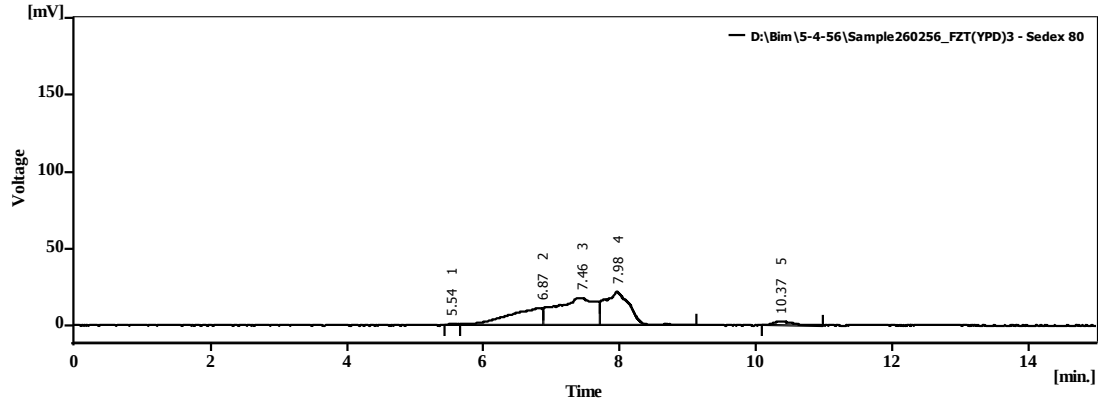
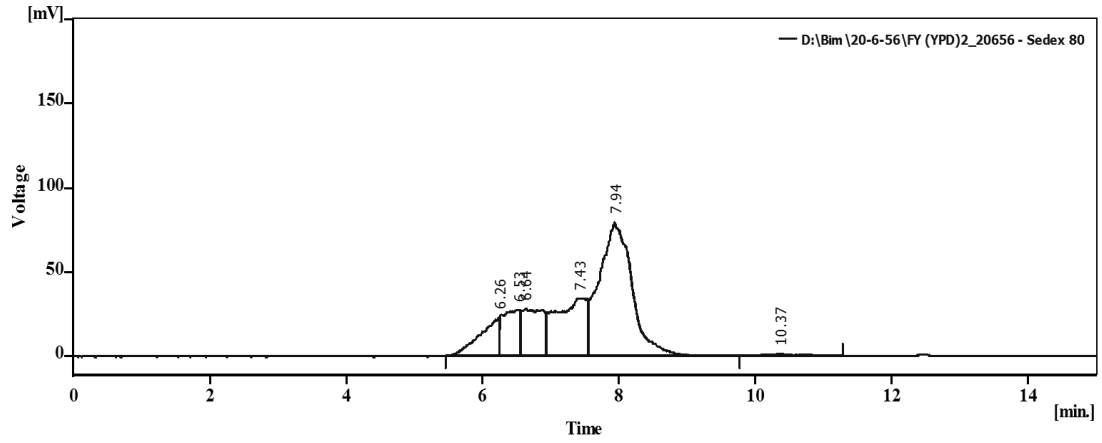


Table A.5 Retention time (min) and Area (%) obtained from chromatogram of HPLC analysis. HPLC analysis were used to analyzed lipid of the FY73 (wild-type) and FZG strains during growth in glucose during stationary growth phase.

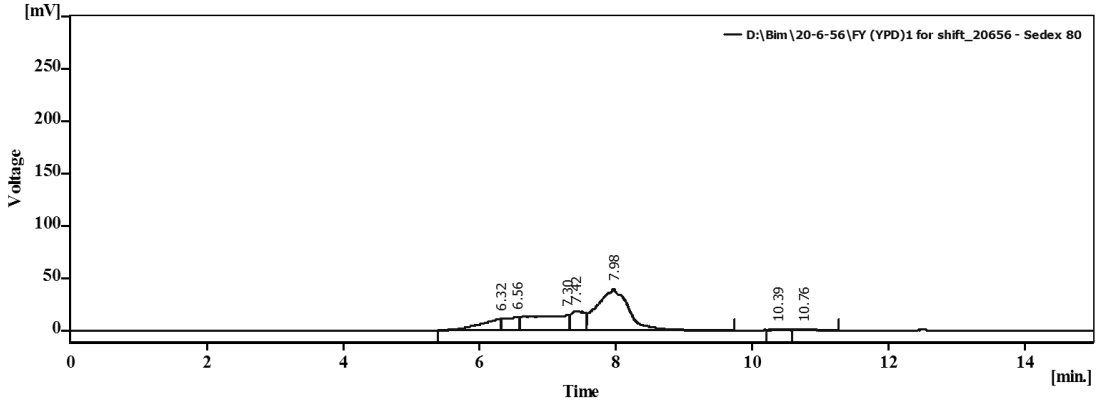
Experiment 1
FY73 (replicate 1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.256	466.07	23.211	8.7
2	6.532	480.097	27.655	9
3	6.636	605.498	28.202	11.3
4	7.432	1075.413	34.36	20.1
5	7.944	2679.611	79.507	50
6	10.372	52.901	1.45	1
	Total	5359.589	194.386	100



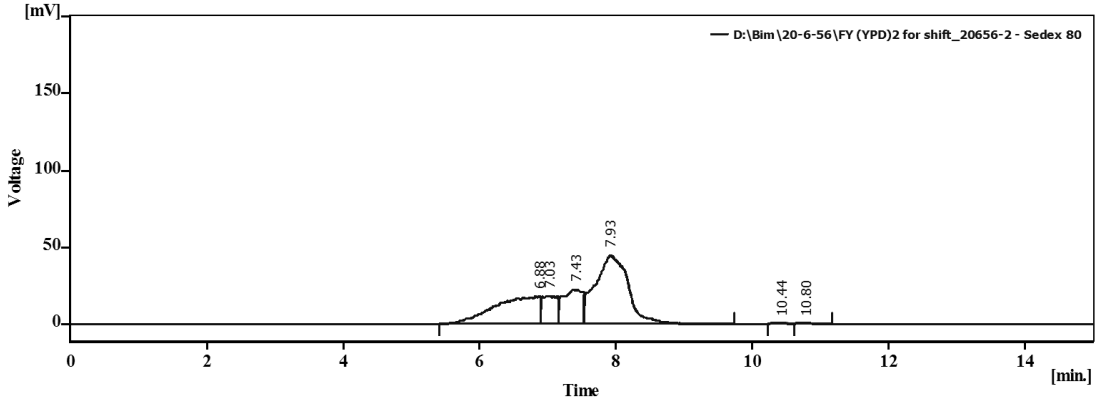
FY73 (replicate 2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.316	228.819	11.027	9
2	6.556	193.689	12.91	7.6
3	7.3	601.225	15.111	23.6
4	7.424	253.644	18.294	9.9
5	7.976	1260.058	39.501	49.4
6	10.392	5.189	0.374	0.2
7	10.764	10	0.553	0.4
	Total	2552.622	97.771	100



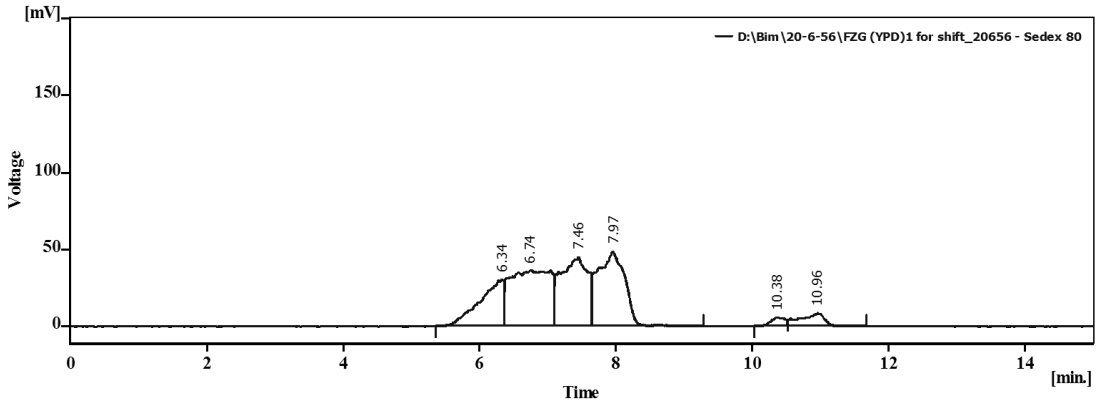
FY73 (replicate 3)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.884	850.393	18.021	27.3
2	7.032	282.614	18.498	9.1
3	7.428	456.863	22.746	14.6
4	7.932	1509.857	44.793	48.4
5	10.436	7.732	0.572	0.2
6	10.796	11.072	0.776	0.4
	Total	3118.532	105.406	100



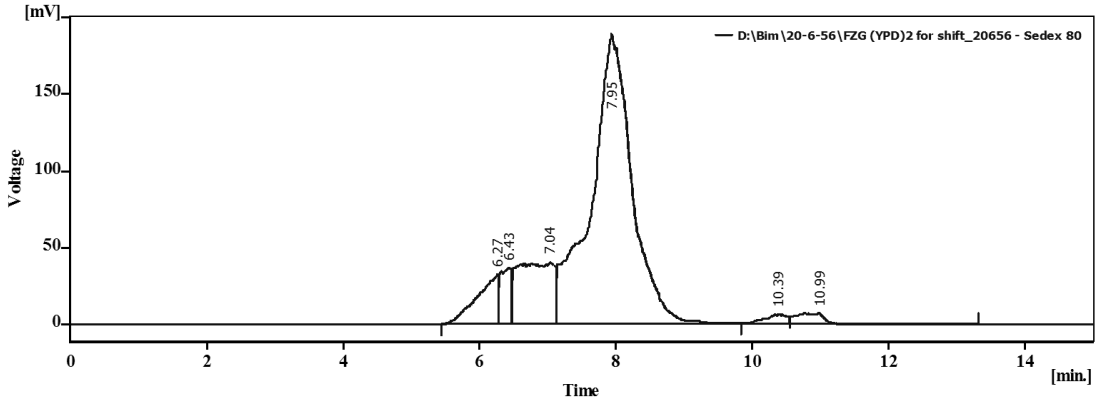
FZG (replicate 1)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.336	735.931	30.491	14.3
2	6.744	1525.281	36.33	29.6
3	7.46	1252.045	44.859	24.3
4	7.968	1345.167	48.315	26.1
5	10.38	88.85	5.866	1.7
6	10.964	214.027	8.629	4.1
	Total	5161.301	174.491	100



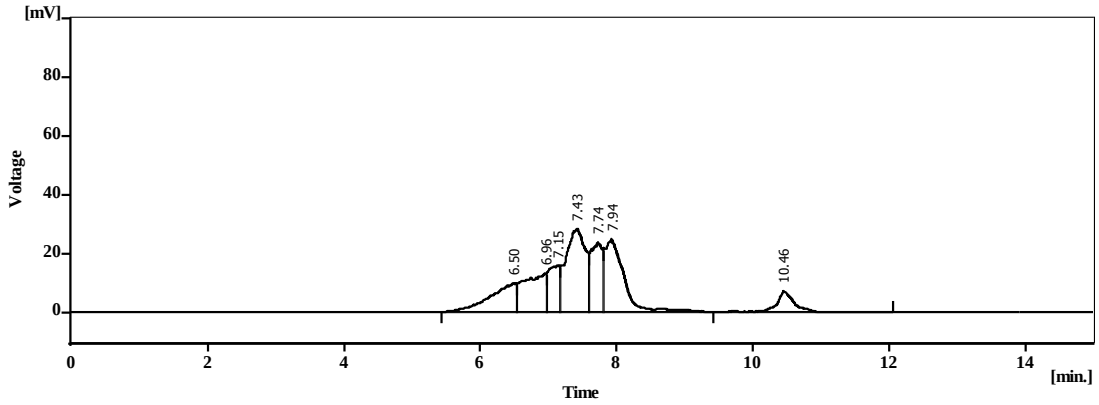
FZG (replicate 2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.272	705.747	32.586	6.5
2	6.428	411.165	36.866	3.8
3	7.04	1500.164	40.238	13.7
4	7.948	7908.286	189.014	72.4
5	10.388	161.155	6.601	1.5
6	10.992	232.817	7.392	2.1
	Total	10919.33	312.698	100



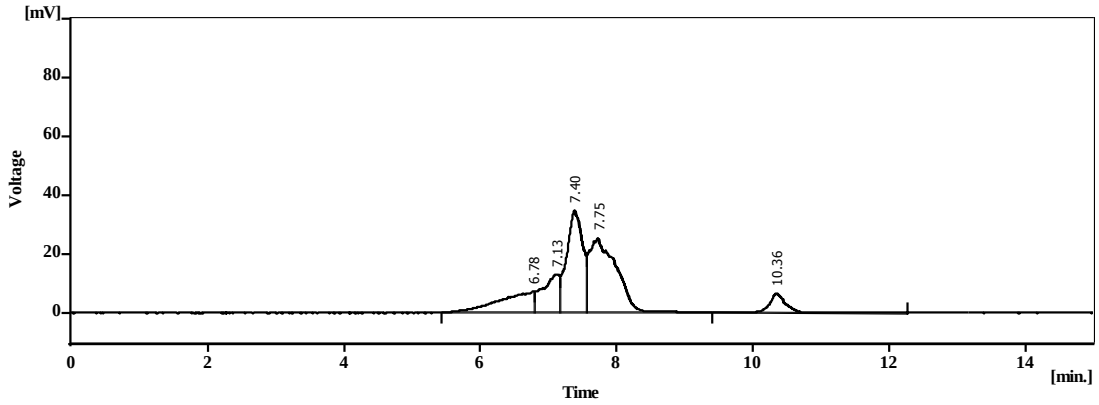
Experiment 2
FY73 (replicate 1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.5	257.268	9.853	11.4
2	6.964	296.2	13.145	13.2
3	7.152	186.492	15.973	8.3
4	7.432	571.012	28.452	25.4
5	7.744	265.235	23.748	11.8
6	7.944	517.66	24.878	23
7	10.464	149.564	7.249	6.9
	Total	2247.333	123.633	100



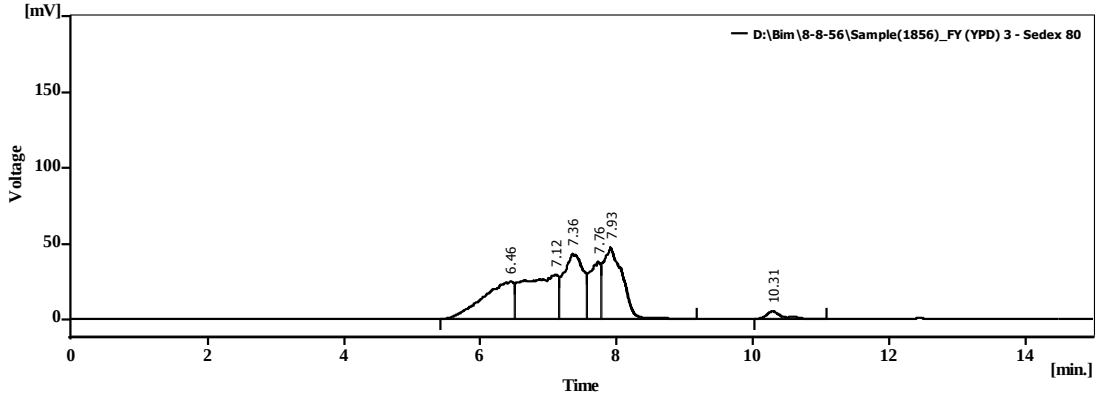
FY73 (replicate 2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.784	256.645	7.27	13.4
2	7.128	228.299	13.109	12
3	7.4	561.628	34.935	29.4
4	7.748	735.473	25.619	38.5
5	10.36	126.264	6.551	6.6
	Total	1908.309	87.484	100



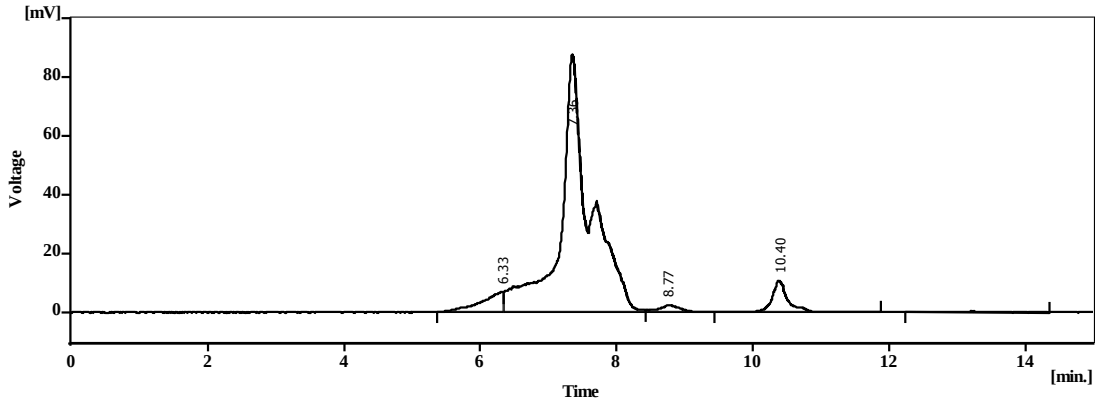
FY73 (replicate 3)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.456	775.969	24.899	18.9
2	7.12	1019.212	29.398	24.8
3	7.364	857.315	42.839	20.9
4	7.756	415.309	37.756	10.1
5	7.928	956.84	47.248	23.3
6	10.308	83.573	5.07	2
	Total	4108.218	187.211	100



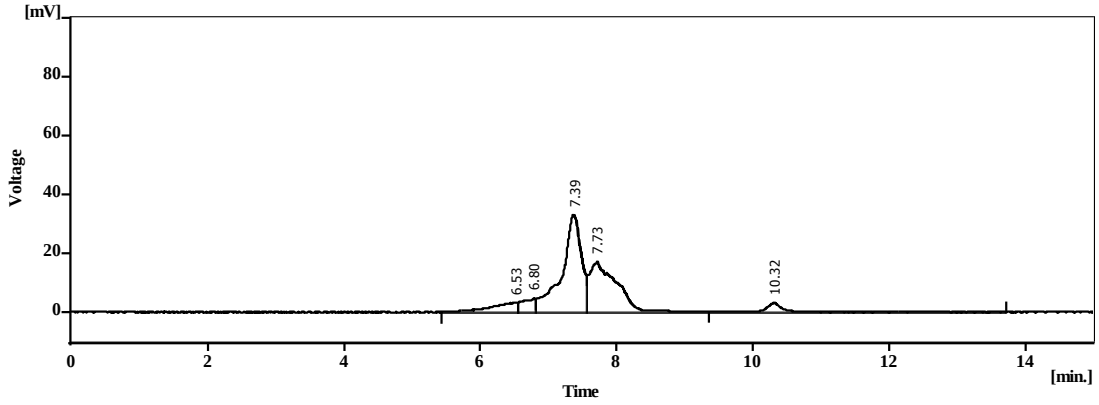
FZG (replicate 1)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.332	158.272	7.122	5.1
2	7.364	2688.349	87.62	86.4
3	8.768	57.165	2.346	1.8
4	10.4	187.566	10.921	6.6
	Total	3110.259	108.388	100



FZG (replicate 2)

Peak	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	6.528	90.28	3.342	6.6
2	6.796	64.047	4.587	4.7
3	7.388	650.009	33.14	47.3
4	7.728	496.119	17.219	36.1
5	10.324	74.002	3.132	5.4
	Total	1374.457	61.42	100



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	Jansuriyakul, S. and Soontorngun, N., 2013 "Identification of zinc cluster regulator involved in the oxidative stress response in the yeast *Saccharomyces cerevisiae*" **Burapha University International**

Conference 2013, July 4-6, 2013. Burapha University, Thailand. (Proceeding)

Jansuriyakul, S. and Soontorngun, N., 2012 "Phenotypic analysis of zinc cluster deletion strains on lipid utilization in the yeast *Saccharomyces cerevisiae*" **The 24th Annual Meeting of the Thai Society of Biotechnology (TSB)**, November 29-30, 2012. Ubon Ratchatani, Thailand. (Proceeding)

King Mongkut's University of Technology Thonburi

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