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Engineering *Saccharomyces cerevisiae* for the Secretion of an Extracellular Lipase

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Engineering *Saccharomyces cerevisiae* for the Secretion of an
Extracellular Lipase

A Thesis

Submitted to the Graduate Faculty of the
University of New Orleans
in partial fulfillment of the
requirements for the degree of

Master of Science
in
Biological Sciences

by

Gaynelle L. Stewart

B.A. Northwestern State University, 2001
B.S. Northwestern State University, 2001

August 2007

Dedication

This work is dedicated to my grandparents, Gustave and Willie Mae Stewart and Lawrence and Eugenia Blunt, for always believing in me.

Acknowledgements

First and foremost I want to thank God for making this possible. With God, all things are possible, even when situations seem impossible. I would like to gratefully acknowledge the enthusiastic supervision of Dr. John Dyer during this work. It is difficult to overstate my gratitude to my dedicated committee members, Dr. Mary Clancy and Dr. Candace Timpfe. Throughout my academic career and especially during my thesis-writing period, they provided encouragement, sound advice, good teaching, good company, and lots of new ideas. I would have been lost without them. I have been lucky enough to have the support of many good friends. A special gratitude is due to Dorselyn Chapital and Jay Shockey at the USDA Southern Regional Research Center for being knowledgeable and friendly people who helped me daily. I also want to thank my fellow lab-mates, Jami Bryan and Linda Bourassa, for their warm friendship, listening ear and continual support. I would like to acknowledge Dr. Kathleen Burt Utley for graciously coming to my rescue. From the staff of the Biology Department, Nereida Burns, Teresa Howell, and Yvette Stilley are especially thanked for their wealth of information and patience. I would also like to thank Dr. Bernard Rees and Dr. Kathleen Burt-Utley for their time and academic support as Graduate Coordinators for the Biology Department. I am grateful to all my friends who have acted interested in what I do regardless of their interest or understanding. I cannot end without thanking my family, on whose encouragement and love I have relied upon. I am forever indebted to my parents, Gustave and Betty Stewart, my sisters Collette Briley and Tiffany Stewart, and my husband, Patrick Davis, for their understanding, fervent prayers, endless patience and encouragement when I needed them the most.

Table of Contents

List of Figures	v
List of Tables	vii
Abstract	viii
Introduction	1
Materials and Methods	19
Results	32
Discussion	58
References	66
Appendix	72
Vita.....	85

List of Figures

<u>Figure number</u>	<u>Figure Title</u>	<u>Page</u>
Figure 1-1	Metabolic Engineering	2
Figure 1-2	Composition of Triacylglycerols	4
Figure 1-3	Fatty Acids native to <i>Saccharomyces cerevisiae</i>	4
Figure 1-4	The LIP2 gene isolated from <i>Yarrowia lipolytica</i>	8
Figure 1-5	Schematic representation of fatty acid metabolism	11
Figure 1-6	The Yeast Secretory Pathway	17
Figure 3-1	The pYes 2.1 galactose inducible yeast shuttle vector	32
Figure 3-2	LIP2, LIP2 NS	33
Figure 3-3	LIP2-Ser, LIP2-Ser NS	34
Figure 3-4	LIP2-CPY, LIP2-CPY NS	36
Figure 3-5	Properties and genetic modifications to the LIP2 gene	37
Figure 3-6	Growth and lipase activity of <i>S. cerevisiae</i> cells on agar containing tributyrin (pYes2.1 LIP2, LIP2 Ser and LIP2 CPY).	38
Figure 3-7	Growth and lipase activity of <i>S. cerevisiae</i> cells on agar containing tributyrin over a period of 72 hours (pYes2.1 LIP2, LIP2 Ser and LIP2 CPY).	39-40
Figure 3-8	Growth and lipase activity of <i>S. cerevisiae</i> cells on agar containing tributyrin (pYes2.1 LIP2, LIP2 opt and LIP2 Ser).	41
Figure 3-9	The Annulus	42
Figure 3-10	Lipase secretion as measured by annulus size at 15 hours	43
Figure 3-11	Lipase secretion as measured by annulus size at 24 hours	44
Figure 3-12	Lipase secretion as measured by annulus size at 72 hours.	45
Figure 3-13	Lipase secretion as measured by annulus size at 24 hours in serial diluted cultures	46
Figure 3-14	Lipase secretion as measured by annulus size at 48 hours in serial diluted cultures	47
Figure 3-15	Annulus size vs Concentration	48
Figure 3-16	The pEX-11 oil inducible yeast shuttle vector	50

Figure 3-17	Fatty acid composition of wild type yeast cells containing the empty plasmid pEX11-424.	52
Figure 3-18	Fatty acid composition of wild type yeast cells (pEX11-424) grown in the presence of the free fatty acid linoleic acid.	53
Figure 3-19	Fatty acid composition of pEX11-424 LIP2Ser cells grown in the presence of the free fatty acid linoleic acid.	54
Figure 3-20.	Fatty acid composition of Hain Soybean Oil.	55
Figure 3-21.	Fatty acid composition of wild type yeast containing the empty vector pEX11-424, grown in Soybean oil.	56
Figure 3-22.	Fatty acid composition of pEX11-424 LIP2Ser, grown in the presence of soybean oil.	57

List of Tables

<u>Table</u>	<u>Table Title</u>	<u>Page</u>
Table 1-1	Overview of Plasmid Constructs	25

Abstract

Developing microbial systems capable of converting low cost lipids into value added products depends on the ability to acquire substrates from the growth media. *Saccharomyces cerevisiae* can acquire free fatty acids from the growth media and a portion of these lipids can be converted into new lipid products. However, they cannot acquire complex lipids from the growth media unless a nonspecific lipase is included. To circumvent lipase addition, we are genetically engineering *S. cerevisiae* to secrete a lipase into the growth media. We selected the *LIP2* gene from *Yarrowia lipolytica*, which encodes a nonspecific lipase. Several modifications were made to the *LIP2* gene to improve processing. Results identified strains secreting the most lipase. From these results, high producing strains were inserted into an oil inducible vector. Halo assays confirmed lipase secretion, while measuring the fatty acid composition confirmed triacylglycerol breakdown, and yeast uptake of the free fatty acids released.

Keywords; Metabolic Engineering, *Saccharomyces cerevisiae*, lipase, yeast, carboxypeptidase Y, KEX2 protease, codon optimization, fatty acid methyl esters

Introduction

Metabolic Engineering

Metabolic engineering is the directed improvement of cellular properties through modification of specific biochemical reactions, introduction of new reactions and the application of genetic engineering (Stephanopoulos 1998; Ostergaard 2000). The science of metabolic engineering builds on a combination of traditional strain improvement and modern molecular biological tools. The specificity of the biochemical reactions targeted for modification is key. Once reactions and pathways are identified, molecular genetic techniques can be applied for amplification, deletion, inhibition, regulation, secretion of proteins or transfer of the gene for the enzyme. Metabolic engineering emphasizes the creation and introduction of integrated metabolic pathways as opposed to focusing on individual reactions. As a field, metabolic engineering is concerned with complete biochemical reactions, pathway synthesis, feasibility and control. This discipline has enormous potential for enhancing the production of desired metabolites from inexpensive sources.

A metabolic engineering strategy generally consists of two parts: analysis and synthesis. Analysis is the identification of potential targets and a thorough working knowledge of the genes, metabolites and features inherent to the cell in question. Potential targets are usually host organisms that allow for easy evaluation of responses to the new metabolic pathway. Synthesis is the genetic modifications of the organism and construction of recombinant strains with improved properties. Cellular targets for metabolic engineering include substrate and product range extension, productivity and yield improvement, by-product elimination, performance improvement, property enhancement or creation and heterologous protein production (Ostergaard *et al.* 2000; Cameron and Tong 1993).

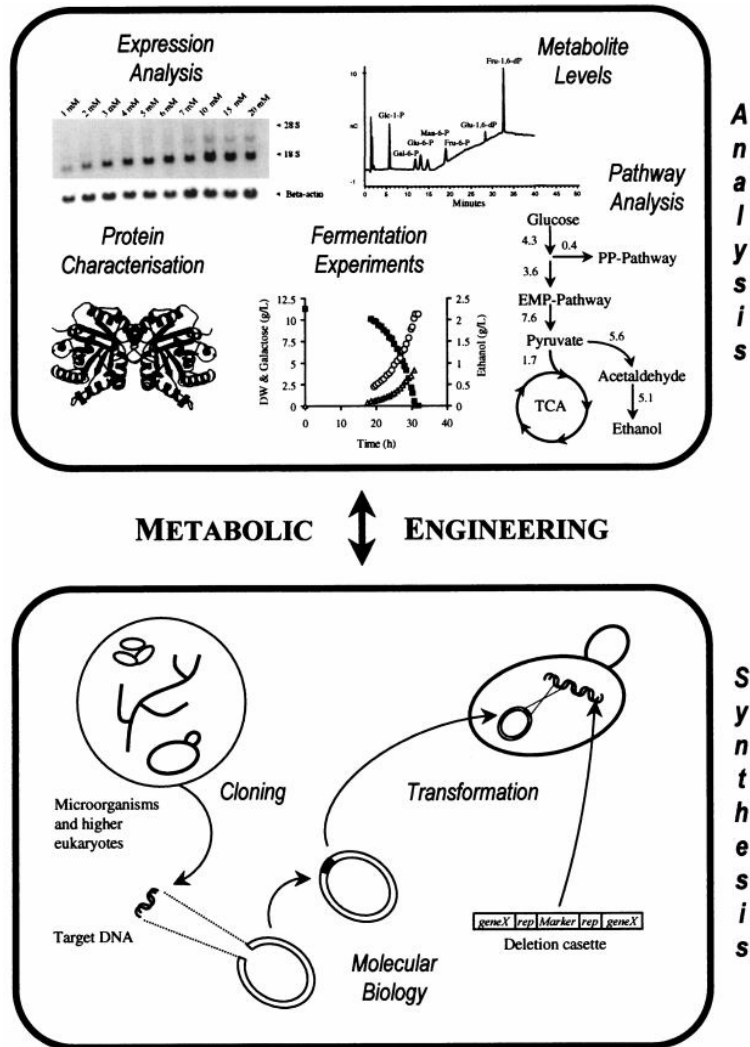


Figure 1-1: Various aspects of metabolic engineering, divided between analysis and synthesis (figure from Ostergaard et al 2000).

For metabolic engineering to be successful, there are some requirements. First, a robust host organism must be chosen. Host selection is based upon the availability of cloning vectors and the ease of cloning, pathogenicity, substrate range, hardiness, availability of physiological and genetic information, availability of regulatory mutants and the ability to use inexpensive nutritional sources. Furthermore, the use of organisms that are generally regarded as safe (GRAS) is desirable. Gene selection is also a vital part of a successful engineering strategy.

Selection of a gene is governed by availability of the gene, pathway requirements and construction, enzyme kinetics, thermal stability of the protein, gene complexity, cofactor requirements, regulatory properties and enzyme-enzyme interactions (Cameron and Tong 1993). Identification of the optimal genetic changes requires meticulous examination of gene expression and cellular metabolism at different conditions and the appropriate application of metabolic engineering to optimize metabolism and expression. The tools used in metabolic engineering include molecular biological tools (transformation systems, cloning vectors, promoters and genetic markers), analytical chemistry tools (mass spectrometry, isotope labeling, NMR and flow cytometry) and mathematical and computational tools (theoretical yield formulas, pathway synthesis algorithms, and kinetic calculations) (Cameron and Tong 1993).

While yeast is eukaryotic and more genetically complex than *E.coli*, yeast has some of the same useful qualities that make it a suitable research organism. Qualities such as rapid growth, easy replication and mutant isolation, fully characterized genome and versatile DNA transformation systems have made yeast a popular and successful model system for modern biology. *Saccharomyces cerevisiae*, commonly known as baker's yeast or budding yeast, is one of the most thoroughly investigated eukaryotic organisms. It is a desirable laboratory organism because it is non-pathogenic, generally regarded as safe, inexpensive to grow and maintain, viable with numerous auxotrophic selectable markers, susceptible and amenable to genetic modifications by recombinant DNA technology, and the total sequence of chromosomal DNA is available.

Lipids and Fatty Acids

Lipids are a large and diverse group of biological compounds, related by their solubility in nonpolar organic solvents and general insolubility in water. Lipids perform three major

functions in the cell. They are major components of biological membranes, and also serve as energy storage and intra- and intercellular signaling molecules (Voet *et al.* 1999). Cellular lipids are divided into five distinct categories: fatty acids, triacylglycerols (TAG), glycerophospholipids, sphingolipids and steroids. Fatty acids are the basic building blocks of more complex lipids. TAGs function as storage lipids, alternate energy storage, and a source of fatty acids for membrane phospholipid formation. Glycerophospholipids, cholesterol and sphingolipids are structural components of cellular membranes.

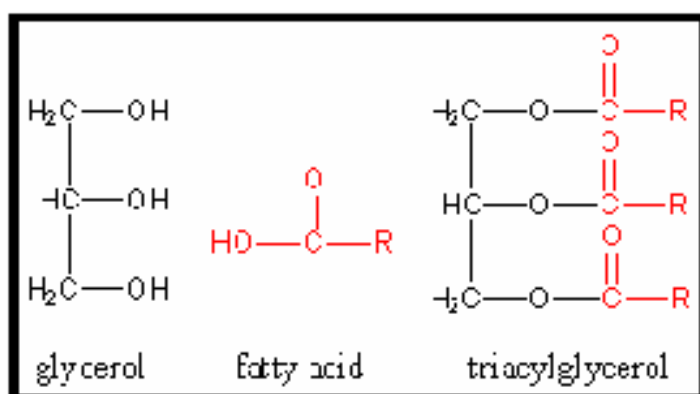


Figure 1-2: Composition of a triacylglycerol (TAG). TAGs are composed of glycerol and three fatty acid molecules linked by ester bonds. Lipases hydrolyze the reverse reaction.

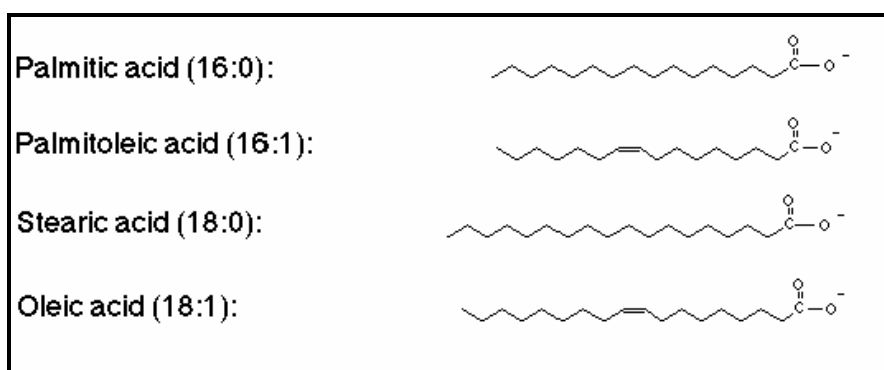


Figure 1-3: Fatty Acids native to *Saccharomyces cerevisiae*

Lipases

Lipases (triacylglycerols-acylhydrolases, EC 3.1.1.3) are water-soluble enzymes that catalyze the hydrolysis of triacylglycerols in a heterogeneous reaction. The reaction can be summarized by the following equation: Triglyceride \leftrightarrow Glycerol + Fatty acids. Another name for lipases is lipolytic enzymes, which are capable of hydrolyzing ester bonds of lipid substrates (Svendsen 2000). The lipase reaction occurs at an oil/water interface. The interface is usually provided by emulsion globules or lipoprotein particles (Jensen *et al.* 1983). Lipases have applications in several fields of industry including manufacture of foods, leather, cosmetics and pharmaceuticals.

Lipases occur naturally in plants, animals and microorganisms (Vakhlu and Koor 2006). Lipases function to catalyze hydrolysis of triglycerides to glycerol and fatty acid. The majority of yeast lipases are extracellular, monomeric glyceroproteins with molecular weights ranging from ~33 to ~65 kD (Vakhlu and Koor 2006). Lipases catalyze a wide range of reactions, including hydrolysis, inter-esterification, alcoholysis, acidolysis, esterification and aminolysis. They also catalyze the hydrolysis of fatty acid ester bond in the TAG and release free fatty acids (FFA).

Lipases are localized to different portions of the cell (or tissue) according to their physiological functions. Generally there are two classes of lipases: soluble and membrane bound. Membrane bound lipases are found in the cell wall and at the outer surface of the cytoplasmic membrane (Ota *et al.* 1982). Their primary function is the mobilization of lipid storage reserves. Soluble lipases are intra and extracellular. Intracellular soluble lipases are associated with cellular growth functions at specific phases (Pereira-Meirelles *et al.* 2000).

Extracellular soluble lipases are secreted into the growth media. Cells secrete lipases in order to break down TAGs in their environment for uptake and use in cellular processes.

Lipases from various sources have wide variations in their reaction specificities. Specificity of lipases is controlled by the molecular properties of the enzyme, structure of the substrate and factors affecting binding of the enzyme to the substrate. Types of specificity include substrate, positional, fatty acid, stereospecificity or combinations thereof. Substrate specificity describes the differing rates of hydrolysis for triglyceride, diglycerides and monoglycerides. Positional lipase refers to the position of the fatty acid cleaved off of glycerol; some lipases can cleave the primary, secondary or all three ester bonds, exhibiting nonspecific hydrolysis (Jensen *et al.* 1983). Lipases most often cleave the fatty acids at the 1 or 3 position of glycerol or both, but not the 2 position. Some lipases prefer cleaving the short-chain fatty acids (C_{2,4,6,8,10}), unsaturated fatty acids (oleic, linoleic, linolenic, etc) or randomly cleaving the fatty acids from the triglyceride in a non-specific fashion (Ghosh *et al.* 1996). Lipase stereospecificity describes faster hydrolysis of one primary sn ester as compared to the other. If the lipase is stereospecific, the sn-1,2- or 2,3-enantiomer will be the predominant substrate (Jensen *et al.* 1983). Triacylglycerol lipases catalyze the hydrolysis of TAGs at their 1 and 3 positions to form sequentially 1,2-diacylglycerols and 2-acylglycerols (Voet *et al.* 1999).

The LIP2 gene (GenBank Accession No. AJ012632) was isolated from the oleaginous yeast *Yarrowia lipolytica*. *Y. lipolytica* is an obligate aerobic dimorphic ascomycete yeast that naturally secretes large amounts of various metabolites. This yeast grows readily on hydrophobic substrates such as alkanes, fatty acids and oils (Barth and Gaillardin 1997). *Y. lipolytica* can utilize triglycerides as carbon sources and produce many lipases, including extracellular, cell bound and intracellular lipases (Yu et al 2007). Lipase production depends on

the media composition and environmental conditions (Guerzoni *et al.* 2001). The LIP2 gene encodes a 334 amino acid preproprotein that is responsible for most of the extracellular lipase activity in *Y. lipolytica*. Lip2 is able to catalyze the hydrolysis of long, medium and short chain triglycerides at high rates (Aloulou *et al.* 2007). The lipase has high activity in hydrolysis, esterification and transesterification reactions (Yu *et al.* 2007). Although LIP2 seems involved in the use of TAG as a carbon source for the yeast, the finding that a knockout strain is still able to use this source suggests the presence of other lipases in *Y. lipolytica*. Lip2 is secreted into the culture medium while Lip1 and Lip3 are thought to be intracellular lipases and Lip7 and Lip8 are mainly associated with the cell wall (Aloulou *et al.* 2007; Fickers *et al.* 2005). The LIP2 gene has a codon adaptation index of 0.246, indicating that it is a moderately expressed gene. The mature protein is a 301 amino acid glycosylated peptide classified as a triacylglycerol hydrolase (EC 3.1.1.3). It is optimally active at 37°C and pH7 and degrades vegetable oils well. Lipase expression is induced by olive oil and oleic acid and repressed by glucose (Pignède *et al.* 2000). The LIP2 gene contains several processing motifs. The first 33 amino acids consist of an endoplasmic reticulum (ER) signal sequence, followed by four dipeptides (X-Ala or X-Pro), a short 12-aa proregion, a Lys-Arg dipeptide, and the mature 301-aa lipase. There are also two potential signals for asparagine-linked glycosylation (Asn-X-Thr/Ser) (Yu *et al.* 2007). Protein processing involves cleavage of the signal sequence and endoprotease processing. The precursor protein is processed by a KEX2-like endoprotease. The signal sequence cleavage site has been predicted to be after amino acid 17 by SignalP prediction. The Lys-Arg dipeptide is the putative substrate of the KEX2 endopeptidase. Lip2p may be secreted via the signal recognition dependant pathway and its maturation is dependent upon KEX2 like processing (Pignède *et al.* 2000).

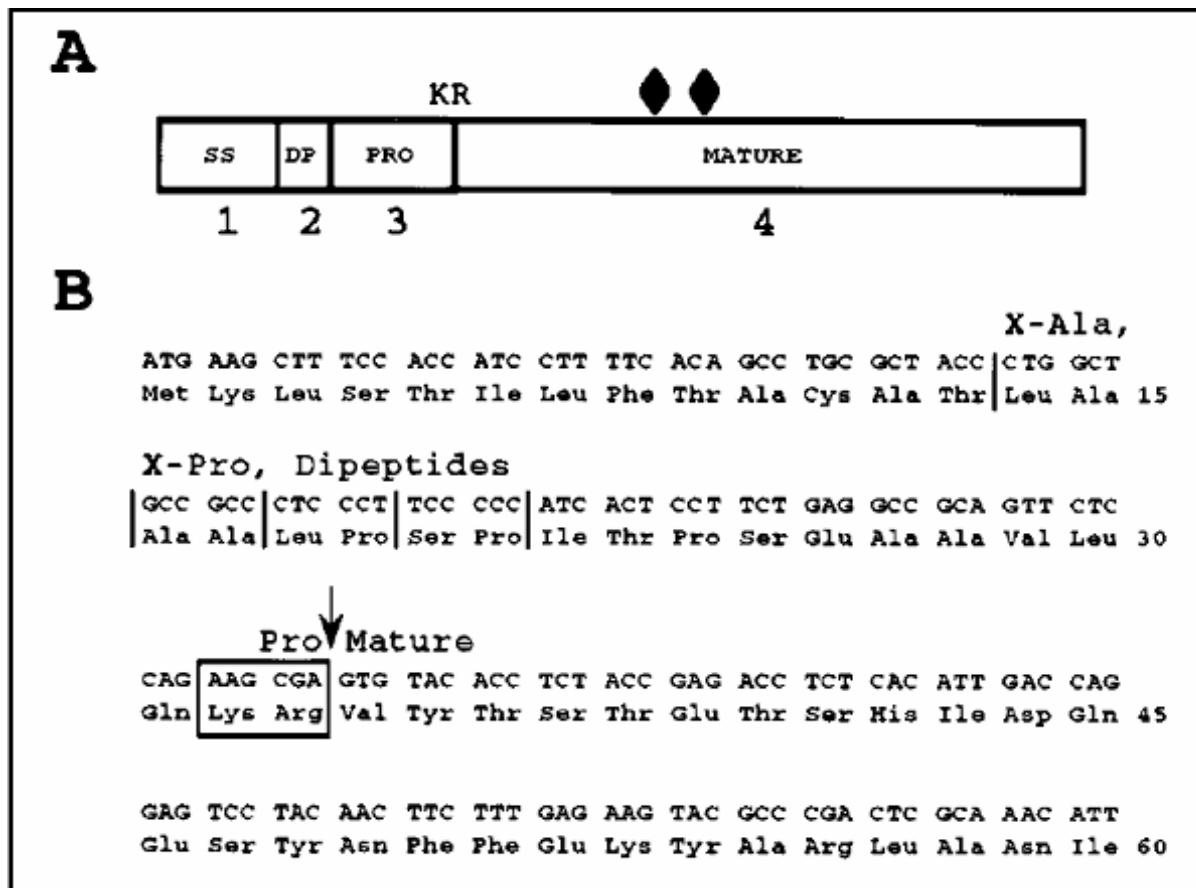


Figure1-4: The LIP2 gene isolated from *Yarrowia lipolytica* (A) Processing of *Y. lipolytica* Lip2p. Shown are the putative 13-aa signal sequence (SS), followed by a stretch of four dipeptides (DP), a short 12-aa pro region (PRO) including the Lys-Arg (KR) cleavage site of the KEX2-like XPR6 endoprotease, and the mature 301-aa lipase. The diamonds indicate the positions of the potential signals for asparagine-linked glycosylation (Asn-X-Thr/Ser). (B) Sequence of the preproregion and the first 27 N-terminal aa of the mature lipase encoded by the LIP2 gene from *Y. lipolytica*. The DNA sequence is shown together with the predicted amino acid sequence (three-letter code) and the N-terminal amino acid sequences (one-letter code) of the lipases secreted. The vertical arrow indicates the confirmed pro-mature lipase processing site, and the vertical lines represent the four X-Ala and X-Pro dipeptides which act as possible substrates for dipeptidyl aminopeptidase activity (Pignede *et al.* 2000).

Yeast Lipid Biotechnology

Yeast was the first microorganism recognized over a century ago to accumulate lipids (Leman 1997). Yeast has been widely and effectively used to produce high value pharmaceutical polypeptides, industrial enzymes and vitamins (Veen and Lang 2004). Yeast can

be genetically modified to produce selected substances in relatively high amounts. Additionally, *S. cerevisiae* has emerged as a viable and versatile host for the synthesis of lipid compounds of high commercial interest (Daum *et al.* 1998). *S. cerevisiae* has received attention as a source of lipid compounds and a model organism for multicellular eukaryotes, due to its similarity in subcellular structure of several of its lipid biosynthetic pathways (Veen and Lang 2004). Yeast has many advantages for lipid production because this organism (1) can produce lipids similar to vegetable oils and fats (2) can be grown on cheap agronomical and industrial wastes (3) can produce lipids at high rates in bulk using large capacity reactors and (4) produce products that are relatively non-toxic (Jacob 1993). Microbial lipids and plant oils have similar characteristics with regard to fatty acid distribution, triacylglycerol types and secondary metabolites.

Microorganisms are attractive alternate sources of lipids because of their enormous growth rates on a variety of substrates, their ability to synthesize a variety of products and ease of genetic manipulation (Ratledge 2002). Another advantage for choosing yeast as a target of lipid engineering is the fact that lipid pathways have been well characterized and thoroughly described (Veen and Lang 2004).

There are several strategies by which lipid modifications can be made. Rerouting metabolic pathways under optimized growth conditions in the presence of higher amounts of substrates or precursors, cloning and expression of specific, desired genes in yeast, conversion of post production lipids by enzymatic conversion and chemical conversion, are all feasible methods of yeast lipid modification (Jacob 1993). With any or all of the modification strategies utilized, the total lipid can be harvested by extraction with solvent systems or secreted by the yeast and purified from the growth media.

Fatty Acid Metabolism in Yeast

Cellular fatty acids are derived from three sources: external supply, endogenous lipid turnover and *de novo* synthesis and elongation. When *de novo* fatty acid metabolism is blocked or cells must bypass the energy expensive synthesis reactions, yeast can utilize fatty acids derived from the growth media. Formation of C-C bonds is a highly energy demanding process (Tehlivets *et al.* 2007). Cell types with high levels of fatty acid metabolism (either degradation or storage) transport exogenous fatty acids at higher rates than do to those with low levels of lipid metabolism. The processes governing the transport of fatty acids across the membrane are distinct from the transport of hydrophilic substrates such as sugars and amino acids.

Investigations into fatty acid transport address three central issues: (i) the low solubility of fatty acids under aqueous conditions; (ii) the physical and chemical parameters of fatty acids, which allows them to readily partition into a lipid bilayer; and (iii) the identification of membrane-bound and membrane-associated proteins (Black and DiRusso 2003). The mechanisms underlying fatty acid transport across the plasma membrane are poorly understood. Two possible mechanisms have been identified. In one mechanism, fatty acid transport is independent of membrane-bound fatty acid transporters and occurs by simple diffusion. This model suggests that transport is primarily diffusional and is independent of membrane-bound fatty acid transporters. Fatty acids bind to and diffuse through the lipid bilayer. Transport is driven by concentration gradients generated by either intracellular utilization (import) or extracellular fatty acid-binding proteins (export) (Zou *et al.* 2002). In the second, membrane-bound and membrane-associated proteins mediate fatty acid import and/or export. The second model suggests that membrane-bound and membrane-associated proteins mediate transport. Several lines of evidence favor the involvement of proteins in the transport mechanism, including

studies showing that this is a regulated process. In addition, cell types with high levels of fatty acid metabolism (either degradation or storage) transport exogenous fatty acids at higher rates when compared with those with low levels of lipid metabolism (Fargeman *et al.* 2001 and Zou *et al.* 2002).

In *S. cerevisiae*, fatty acid import is saturable and dependent on Fat1p, a homologue of the murine fatty acid transport protein, FATP. Four fatty acyl-CoA synthetases encoded by separate genes have been identified in *S. cerevisiae*: Faa1p, Faa2p, Faa3p, and Faa4p. Fat1p plays a role in very-long chain (C22-C26) acyl-CoA synthetase activity, a pivotal role in long chain fatty acid import. Faa1p and Faa4p are the primary enzymes involved in activation of imported long-chain fatty acids (C12–C18) while Faa2p appears to be involved in activation of medium-chain fatty acids directed toward peroxisomal β -oxidation. The physiological role of Faa3p, which is most active toward fatty acids larger than C18, has not yet been defined (Fargeman *et al.* 2001).

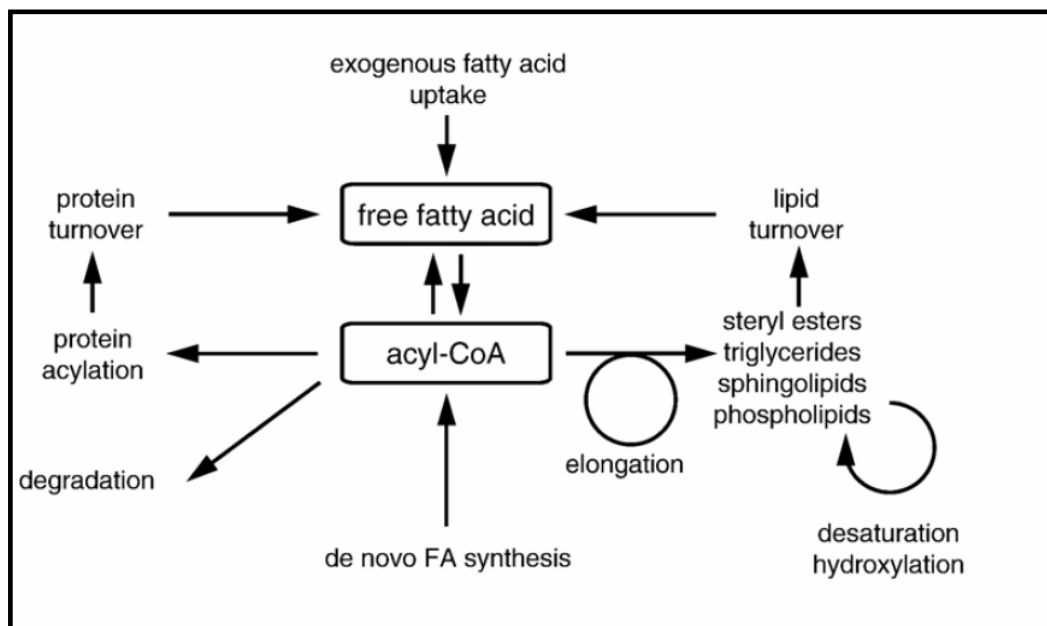


Figure 1-5: Schematic representation of fatty acid metabolism (Tehlivets *et al* 2007).

Glycerolipid Biosynthesis (TAG Formation)

Glycerophospholipids, which include phospholipids and triacylglycerols, are essential biomolecules. Phospholipids are the major component of membranes and determine membrane permeability and modulate the activity of membrane proteins. TAG by contrast, is the major storage form of energy. In yeast TAGs are synthesized through two pathways controlled by the acyl-CoA diacylglycerol acyltransferase Dga1p and the phospholipid diacylglycerol acyltransferase Lro1p. Formation of TAG requires the synthesis of phosphatidic acid (PA) and diacylglycerol (DAG), two key intermediates of lipid metabolism. The endoplasmic reticulum and lipid particles (LP) are TAG storage compartments in yeast and are the major sites of TAG synthesis (Sorger and Daum 2003).

The major de novo biosynthetic pathways that yield PA utilize glycerol-3-phosphate (G-3-P) or dihydroxyacetone phosphate (DHAP) as precursors. G-3-P is acylated by G-3-P acyltransferase (GAT) at the sn-1 position to form 1-acyl-G-3-P (lyso-phosphatidic acid, LPA), and then by 1-acyl-G-3-P acyltransferase (AGAT) in the sn-2 position, yielding PA. DHAP is acylated at the sn-1 position by DHAP acyltransferase (DHAPAT), and the product 1-acyl-DHAP formed is reduced by 1-acyl-DHAP reductase (ADR) to yield LPA, which is further acylated to PA by AGAT. PA can also be formed from phospholipids through the action of a phospholipase D, or by phosphorylation of DAG through DAG kinase. Activation of PA with CTP by a CDP-DAG synthase leads to the formation of CDP-DAG, the precursor for phosphatidylinositol, phosphatidylglycerol, cardiolipin, phosphatidyl-serine, phosphatidylethanolamine (PE), and phosphatidylcholine (PC). Dephosphorylation of PA by a phosphatidate phosphatase (PAP) yields DAG, which is also formed from TAG by TAG lipases or from phospholipids through the action of a phospholipase C. Acylation of DAG to yield TAG

is catalyzed by the two yeast proteins Dga1p and Lro1p, which utilize acyl-CoA or phosphatidylcholine as acyl donors. DAG is a precursor for aminoglycerophospholipids via the Kennedy pathway and therefore a key intermediate in membrane lipid biosynthesis and substrate to diacylglycerol acyltransferases (DAGATs), which convert DAG to TAG using different acyl donors (Wagner and Daum 2005; Dircks and Sul 1999; Sorger and Daum 2003).

Fatty Acid Storage

Fatty acids taken up by yeast are not stored as free fatty acids. FA esterified and stored as triacylglycerols. TAG are the most important storage form for energy and fatty acids required for the synthesis of complex membrane lipids in eukaryotic cells. In *S. cerevisiae*, formation of TAG can occur through various pathways with the contribution of different enzyme. Since TAG and steryl esters (STE) are unable to integrate into phospholipid membrane bilayers, they cluster and form the hydrophobic core of lipid particles. The hydrophobic core of yeast lipid particle is formed from TAG and STE and sequestered from the cytosolic environment by a phospholipid monolayer with a small amount of characteristic proteins embedded (Daum *et al.* 2007).

The current model of lipid particle biogenesis is based on a budding hypothesis. According to this model, proteins involved in neutral lipid metabolism including enzymes of TAG and STE formation accumulate in certain regions of the ER. Neutral lipids newly formed in these domains are unable to integrate into bilayer membranes and cluster in the hydrophobic region between the two leaflets of the ER membrane. During ongoing synthesis of TAG and STE the neutral lipid droplet grows and forms a bud. After reaching a certain size, the mature lipid particle buds off the ER and is released into the cytosol. Mechanisms and metabolic pathways appear to exist which allow the cell to grow lipid particles. With the exception of a few enzymes, lipid particles in *S. cerevisiae* harbor the whole set of proteins required for *de novo*

TAG formation including enzymes of fatty acid activation, PA synthesis and DAG acylation. lipid particle may, however, not only serve as a storage compartment for neutral lipids. It has been shown that neutral lipids of lipid particles may also play a role in stabilization of proteins. Once inside a lipid particle, TAG and STE can be rapidly mobilized to fulfill the cell's requirement for sterols and fatty acids. Hydrolysis of TAG by yeast lipases provides fatty acids and DAG for the biosynthesis of complex membrane lipids (Daum *et al.* 2007).

Fatty acid desaturases modify fatty acids by endogenous or transgenically expressed enzymes. Fatty acid desaturases are enzymes that convert a single bond between two carbon atoms to a double bond in fatty acyl chains. Unsaturated fatty acids in glycerolipids are important for the maintenance, structure and function of biological membranes. Each fatty acid desaturase introduces an unsaturated bond at a specific location in a fatty acyl chain (Los and Murata 1998).

While not an oleaginous yeast, *S. cerevisiae* has the potential to be oleaginous. An oleaginous species can accumulate between 30 – 70% of its total biomass in triacylglycerols as cellular storage lipids. Dyer *et al.* (2002) found that cultivation of yeast cells in media containing fatty acids as a sole carbon source increased the uptake of free fatty acids and increased total cellular lipid content sevenfold. Yeast cells grow very poorly on fatty acids, as evidenced by the slow metabolism of fatty acids and increase in doubling time from 1-4 hours to 20 hours. To help improve cellular capacity to degrade fatty acids to carbon metabolites (e.g., acetyl-CoA), the yeast cells respond by proliferating the number of peroxisomes in the cell (Trotter 2001). In addition, cells transcriptionally up-regulate the genes encoding enzymes of peroxisomal β -oxidation, the main pathway for degrading fatty acids (Hiltunen *et al.* 2003).

Fatty Acid Induction of Gene Expression

When glucose is depleted, a number of genes are expressed through *glucose* derepression (Trotter 2001). However, other genes obey additional cues relating to nutritional availability. For example, β -oxidation genes are repressed by glucose, derepressed on non-fermentable carbon sources, and are induced several fold in the presence of oleic acid. This *oleate induction* is accompanied by a drastic expansion of the peroxisomal compartment. (Gurvitz and Rottensteiner 2006). Peroxisome biogenesis and function is induced by exposure to fatty acids. In yeast, expression of such peroxisomal proteins is subject to several types of regulation, primarily at the level of transcription. When cells are cultured in medium containing 2% glucose, the enzymatic activity is nearly undetectable, but growth in a nonfermentable carbon source causes these activities to rise by 20 times. Exposure to oleic acid increases the activities of these enzymes further 20-fold (Trotter 2001; Gurvitz and Rottensteiner 2006).

Peroxisomes are cellular organelles that house enzymes that are involved in fatty acid β -oxidation. Yeast degrades fatty acids only in the peroxisome. Peroxisomes are characterized by the presence of oxidative enzymes such as catalase and peroxidase. Transfer of *S. cerevisiae* from a medium containing glucose to a medium containing fatty acids as the sole carbon source is accompanied by marked physiological changes. When grown in the presence of fatty acids yeast cells upregulate genes involved in fatty acid metabolism, increase the size and number of peroxisomes, and change cell structure (Smith *et al.* 2006). Two genes are known to regulate peroxisome multiplication, PEX10 and PEX11; both encode peroxisomal membrane proteins. When overexpressed, these genes cause the rapid increase in the number of peroxisomes.

In β -oxidation, fatty acids are broken down to generate acetyl-CoA. Overall the process involves four main steps: dehydrogenation, hydration, oxidation and thiolysis. The process

repeats until the fatty acid has been completely degraded to acetyl-CoA. Each round of β -oxidation yields 1 molecule of NADH, 1 molecule of FADH₂, acetyl CoA and 2 carbon fatty acid.

Yeast Secretory Pathway

Protein transport and secretion in eukaryotic cells involves a complex series of events associated with membrane bound cellular components. Protein secretion in *S. cerevisiae* follows a pathway that is similar to that of mammalian cells (Zsebo *et al.* 1986). *S. cerevisiae* has a multicomponent secretory pathway making posttranslational modifications of heterologous proteins possible (Ostergaard *et al.* 2000). Proteins are generally believed to move between the compartments of the secretory pathway by budding and fusion of transport vesicles between successive compartments. This method of transport maintains the distinct internal environments of the cellular compartments (Atkinson 2006). The *S. cerevisiae* α factor prepro-leader sequence is the most commonly used secretory expression system for heterologous proteins in yeast. Fusion of the prepro leader sequence and a heterologus gene directs *S. cerevisiae* to secrete heterologous proteins, since the leader sequence mediates cotranslational translocation of the fusion protein into the endoplasmic reticulum. Once the DNA is transcribed into mRNA and the mRNA is translated into protein, the secreted protein enters the endoplasmic reticulum (ER). Once in the ER, proteins are folded and undergo modifications such as glycosylation, disulfide bridge formation, phosphorylation and subunit assembly (Conesa *et al* 2001). The LIP2 gene products undergo N-linked glycosylation. N-linked glycosylation involves the attachment of a precursor oligosaccharide to the asparagines residue (Atkinson 2006). The proteins leave the ER packaged in transport vesicles and travel to the Golgi compartment where additional modifications can take place (Conesa *et al* 2001). The pre-region of the leader sequence is

cleaved by a signal peptidase and the Kex2 endoprotease cleaves the pro-region in the Golgi apparatus. Finally, the mature protein is packaged into secretory vesicles and directed to the plasma membrane. The secretory vesicles then fuse to the plasma membrane, releasing the fully processed protein into the extracellular space (Ostergaard *et al.* 2000). Some yeast proteins accumulate in the periplasmic space rather than passing through the cell wall and into the culture medium (Zsebo *et al.* 1986; Conesa *et al.* 2001).

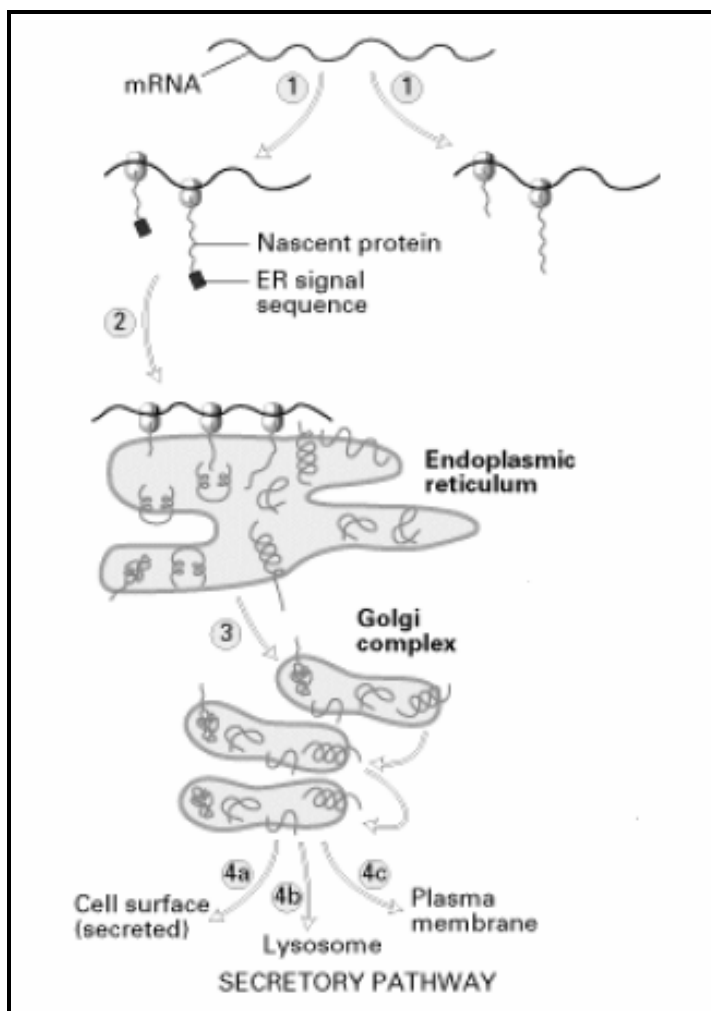


Figure 1-6: The Yeast Secretory Pathway. Proteins carry codes in their sequences that are read by targeting machinery at every stage. Proteins may be targeted to the cytosol, mitochondria, peroxisomes or chloroplasts. Proteins destined for secretion are synthesized on the membrane bound ribosomes of the rough ER. They are then targeted to the appropriate cellular compartment (Atkinson 2006).

The Current Study

Dyer *et al.* 2002 provided the analysis portion of the metabolic engineering strategy and set forth the elements for a lipid metabolic engineering strategy for *S. cerevisiae*. The strategy is to culture cells in media containing fatty acids as the sole carbon source, thus causing peroxisomal proliferation and fatty acid β oxidation. As a result of this growth strategy it was proposed that *S. cerevisiae* could acquire and retain fatty acids, in the form of lipid droplets, from the media and increase cellular lipid content from 2% to 15% dry weight. *S. cerevisiae* cannot take up triacylglycerols (the main component of oils) from the growth medium, but can do so if a nonspecific lipase is added to the growth medium to first break down the TAG into fatty acid components. Dyer tested this hypothesis and showed that yeast cells grown in the presence of TAGs and an exogenously added lipase results in TAG hydrolysis and uptake of the fatty acid components of the TAGs. From these promising results, Dyer hypothesized that *S. cerevisiae* could be engineered to secrete a nonspecific lipase for the hydrolysis of TAGs for the subsequent yeast uptake of component fatty acids. Furthermore, he introduced a long range goal of developing a microbial expression system for bioconversion of low cost lipids into value added products (Dyer *et al.* 2002).

Hypothesis

We hypothesize that insertion of the LIP2 gene from *Yarrowia lipolytica* into a yeast shuttle vector in *Saccharomyces cerevisiae* will cause the secretion of a nonspecific lipase into the growth medium. Once secreted, the nonspecific lipase will breakdown triacylglycerols in the growth media. Furthermore, modifications to the LIP2 gene will increase secretion in yeast cells.

Materials and Methods

Materials

DIFCO brand yeast media, yeast nitrogen base without ammonium sulfate and without amino acids, tributyrin, linoleic acid, trilinolein, and Tween 20 were purchased from Fisher Scientific (Hampton, NH). Synthetic complete amino acid supplement drop-out mix (SCM-URA, -TRP) was purchased from Bufferad (Lake Bluff, IL). Restriction enzymes, enzyme buffers, ligases, calf intestinal alkaline phosphatase (CIP), and DNA ladders were purchased from New England BioLabs (Ipswich, MA). RNA molecular weight marker was purchased from Roche Applied Science (Indianapolis, IN). TOPO cloning kits and chemically competent cells were purchased from Invitrogen (Carlsbad, CA). All other chemicals, unless otherwise noted, were purchased from Sigma-Aldrich (St. Louis, MO).

Yeast strains and growth conditions

Saccharomyces cerevisiae strain MMYO11 α (*MAT α ade 2-1 his3-11, 15 leu2-3, 112 tyr1-1 ura3-1 can1-100 Ole+*) and its subsequent derivatives were used for these studies. Untransformed yeast cells were maintained on YPD media (1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose) solidified with 2% w/v agar. Transformed cells containing plasmids were maintained on SD plates (2% w/v dextrose, 0.67% w/v yeast nitrogen base without amino acids, 2% w/v agar) with appropriate amino acid supplements. Single colonies were inoculated into SD liquid media and grown overnight at 30°C and 300 rpm. Cell growth was measured by optical density (OD) of the cultures at 600 nm on a Beckman spectrophotometer.

Yarrowia lipolytica E122 strain was also used for this study. Untransformed yeast cells were maintained on YPD media agar plates. Single colonies were inoculated in the same media and grown in the same manner as detailed above.

Recombinant DNA Protocols

E. coli transformations were performed using chemically competent cells (Invitrogen) or the colony method bacterial transformation protocol (Dyer and Murai, 1991). Yeast transformations were performed using the Quick and Easy Gietz method (1995). Plasmid DNA was isolated using the Wizard Miniprep DNA purification system (Promega). DNA bands were excised from 0.7% agarose gels and purified according to the GeneClean Gel Isolation and Reaction Cleanup protocol (II) from Q-BIO gene. Site directed mutagenesis was performed according to the Stratagene Quick Change Site Directed Mutagenesis protocol. DNA sequences were verified using and a Beckman CEQ8000 capillary sequencer. Prior to sequencing, salt and protein were removed from the sequencing reactions using cleanup cartridges from the Zymo Research DNA Clean & Concentrator Kit. All other cloning procedures were performed as described by Sambrook *et al.* (1989), with minimal modifications where necessary.

Plasmid Constructions

pYes2.1. pYES2.1/V5-His-TOPO is a 5.9 kb vector with the following features: 2 micron origin of replication for high-copy plasmid maintenance, GAL1 promoter for galactose-inducible expression of cloned genes, URA3 gene for prototrophic selection in host strains, C-terminal V5 epitope and polyhistidine (6xHis) tags for simplified detection and purification of the fusion protein, CYC1 terminator sequence for efficient transcription termination and mRNA stabilization, and an ampicillin resistance gene for selection of *E.coli* transformants. Transcription from the GAL1 promoter is repressed in the presence of glucose and induced by removing glucose and adding galactose as a carbon source (Invitrogen).

pYes2.1-LIP2 and pYes2.1-LIP2-V5/His (NS). A PCR reaction of *Yarrowia lipolytica* E122 genomic DNA using LIP2Forward (5'GCCACCATGAAGCTTTCCAC CATCCTTTTC

3') and LIP2Reverse (5'CCTTAGATACCACAGACACCCTCGGTGA C 3') primers was used to isolate the LIP2 gene. The PCR reactions (L2) were prepared in standard form and thermal cycled using *Pfu* polymerase and a 1 minute extension time corresponding to the 1kb fragment size. The resulting reaction was run on a 0.7% agarose gel against a 1kb marker. PCR products showing the highest concentration (as determined by band intensity) were isolated, cleaned and cloned. 2 µl of L2 were cloned directly into pYes2.1 TOPO TA vector using 10 minute incubation at room temperature (22°C). The cloning reaction was as follows: 2 µl L2, 1 µl salt solution, 1 µl sterile water and 1 µl vector. The reaction showing the highest DNA concentration was used for bacterial transformation. The remaining portion of the reaction was stored at 4°C. pYes2.1-LIP2NS (reaction L2NS) was constructed in the same fashion as pYes2.1-LIP2, but LIP2RevNS (5'GATACCACAGACACCCTCGGTGACGAAGT 3') was the reverse primer used to amplify the LIP2 gene from *Y. lipolytica* and pYes2.1/V5-HIS-TOPO vector for subcloning. All open reading frames of LIP2 were built with and without the stop codon. Removal of stop codon created C-terminal, V5-peptide fusion, which can be recognized by anti-V5 antibody in western blotting.

pYes2.1-LIP2-opt: Since the LIP2 gene was isolated from *Y. lipolytica*, a codon optimized version of the LIP2 gene was purchased from Genscript. The codons of the gene were optimized for expression in *S. cerevisiae*. PCR was employed to remove the codon optimized gene from the company vector (pUC57), and TOPO cloning was used to insert the gene into pYes2.1 vector. The PCR reactions were prepared in standard form and thermal cycled using a 1 minute extension time corresponding to the 1kb fragment size. The resulting reaction was isolated, cleaned and cloned. 2 µl of the reaction were cloned directly into pYes2.1 TOPO TA vector.

pYes2.1-LIP2SER and pYes2.1-LIP2-SERNS: A modified LIP2 gene was produced to optimize the propeptide cleavage site for the *S. cerevisiae* Kex2 protease. To accomplish this, a serine codon was inserted into the LIP2 gene at position adjacent to the KEX2 cleavage site. This insertion was created using site directed mutagenesis with pYes2.1-LIP2 and pYes2.1-LIP2NS plasmids as templates. For insertions 18 reaction cycles and 12 minutes (2 minutes/kb plasmid) extension was used for this reaction. The insertion was introduced using the following primers: SERTop (5'GCCGCAGTTCTCCAGAAGCGA TCTGTGTACACCTCTACCGAGACC 3') and SERBottom (5'GGTCTCGGTAGAGG TGTACACAGATCGCTTCTGGAGAACTGCGGC 3'). Following PCR-mediated mutagenesis, template plasmid DNA was digested using *DpnI* and the resulting PCR reaction was transformed using chemically competent DH5 α cells and incubated overnight at 37°C on LB-Amp agar plates. Single colonies were inoculated into liquid LB-Amp, cultured overnight and plasmid DNA was extracted using the Promega Wizard miniprep kit. The plasmids were sequenced using the GAL1 forward primer (5'AATATACCTCTATACTTTAACGTC 3').

To replace the LIP2 gene with LIP2-SER gene in pYes2.1-LIP2 and pYes2.1-LIP2NS, three restriction digests were performed. pYes2.1-LIP2, pYes2.1-LIP2NS and pYes2.1-LIP2SER were digested with *PvuII* and *SacI* at 37°C for 2 hours. The vector portion of pYes2.1-LIP2 and pYes2.1-LIP2NS as well as the LIP2-SER insert were isolated from the agarose gel and gene-cleaned. The plasmids were screened by restriction analysis for the correct digestion pattern. The vector and insert portions were then ligated and transformed using the colony method bacterial transformation protocol. Plasmids showing the highest concentrations were selected for yeast transformation.

pYes2.1-LIP2-CPY (LIP2-CPY-V5/His): The LIP2 gene was modified by the replacement of the prepro sequence of Lip2 with the carboxypeptidase Y (CPY) signal sequence from *S. cerevisiae*. This construct was created using gradient PCR, pYes 2.1-LIP2, CPY-Lip2Fusion primer (5'CCATGAAAGCATTACACCAGTTTACTATGTGGACTAGGCCTGTCC ACTACACTCGCTAAGGTGTACACCTCTACCGAGACCTCTC A 3') and Lip2Reverse primer. After PCR amplification, the fragment was gene-cleaned and transformed into bacteria. The resulting plasmids (pYes2.1-LIP2CPY and pYes2.1-CPYNS) were sequenced. The orientation of LIP2-CPY gene within pYes2.1 was confirmed using a *PvuII/SacI* double restriction digest. A *SacI* site is unique to the CPY sequence.

To replace the LIP2 gene with LIP2-CPY gene in pYes2.1-LIP2 and pYes2.1-LIP2NS, three restriction digests were performed. pYes2.1-LIP2, pYes2.1-LIP2NS and pYes2.1-LIP2CPY (from gradient PCR) were digested with *PvuII* and *SacI* at 37°C for 2 hours. The vector portion of pYes2.1-LIP2 and pYes2.1-LIP2NS as well as the LIP2-CPY insert were isolated from the agarose gel and gene-cleaned. The vector and insert portions were then ligated and transformed in bacteria, then yeast.

pEX11-424-LIP2-SER. pEX11-424 vector contains the TRP1 selectable marker. pEX11-424-SER was created using restriction digests to open the vector and remove the LIP2-SER gene from pYes2.1-LIP2-SER.

pEX11-426-LIP2, LIP2NS, LIP2-SER and LIP2-SERNS. pEX11-426 vector contains the URA3 selectable marker. pEX11-426-LIP2-SER and pEX11-426-LIP2-SERNS were created using restriction digests and custom PCR primers. To extract the LIP2 sequences from individual pYes 2.1 vectors, PCR primers PEXLip2Forward (5'CAGAGAATTCGCCACCATG AAGC TTTCC ACCATCCTTTTC 3') and PEXLip2Reverse (5'ACTTCCCGGGCTCTTAGAT

ACCACAGAC ACCCTCGGTGAC 3') were created. These primers add restriction sites *EcoRI* (forward primer) and *XmaI* (reverse) to the LIP2 genes as they are amplified out of the pYes2.1 vectors. These PCR reactions were run on a 1% agarose gel. The bands containing the amplified LIP2 genes were isolated and gene-cleaned. Once the genes are amplified with new restriction sites at both ends, the pEX11-426 plasmid was restriction cut with *EcoRI* and *XmaI*. Following that the vector and insert fragments were ligated together. The resulting plasmids were transformed into bacteria and yeast.

pEX11-426 LIP2-opt. In purchasing the codon optimized LIP2 gene, restriction sites *EcoRI* and *BglII* were added to the 5' and 3' ends, respectively, of the gene. pEX11-426 and pUC57 underwent restriction digest by *EcoRI* and *BglII*. The vector portion of pEX11-426 and the LIP2 gene were extracted from agarose gel and ligated together. The resulting plasmids were bacterial transformed and plasmids showing the highest concentrations (determined from agarose gel band intensity) were selected for yeast transformation.

pEX11-426 Δ PstI. pYes2.1 was digested with *PstI* to liberate a 488 bp fragment that included part of the URA promoter and 2 micron ori. Removal of this fragment from pYes2-based vectors was previously shown to significantly increase the copy number of plasmids in yeast cells. The digested pYes2.1 vector portion (pYes2.1 Δ) was then isolated from agarose and self-ligated. pYes2.1 Δ was then cut with *SpeI* and *XbaI* to remove the GAL1 promoter and CYC terminator region, and treated with T4 DNA polymerase to generate blunt ends. The reaction was run on an agarose gel and the vector portion was isolated by gene-cleaning. pEX11-426 was restriction cut with *KpnI* and *SacI*, which released a DNA fragment that included the pEX11 promoter, polylinker and terminator. Following restriction digest the fragments were treated with the blunt end protocol. The smaller fragment (pEX11 promoter, polylinker and terminator)

was isolated from agarose gel and gene-cleaned. The pEX11-426 promoter/terminator fragment was ligated to the blunt ended pYes2.1 Δ plasmid. The resulting plasmid was composed of pEX11-426 promoter/terminator fragment and pYes2.1 plasmid, without the *Pst*I fragment. All of the plasmid constructs created for this study are summarized in Table 1.

<u>Name</u>	<u>Functional Characteristics</u>
pYes2.1	High copy (2 micron), URA, GAL promoter, CYC terminator
pYES2.1 LIP2 pYES2.1 LIP2NS	Native LIP2 lipase from <i>Y. lipolytica</i>
pYES2.1 LIP2 opt	Native LIP2 lipase, codon optimized
pYES2.1 LIP2-Ser pYES2.1 LIP2-SerNS	LIP2 with improved Kex2 site
pYES2.1 LIP2-CPY pYES2.1 LIP2-CPYNS	LIP2 with carboxypeptidase Y signal sequence
pEX11	Oil inducible vector, high copy (2 micron), PEX11 promoter and terminator
pEX11-424, pEX11-426	TRP1 selectable marker, URA3 selectable marker
pEX 11-426 LIP2	Native LIP2 lipase from <i>Y. lipolytica</i> in oil inducible vector
PEX 11-426 LIP2-Ser	LIP2 with improved Kex2 site in oil inducible vector
PEX 11-426 LIP2-opt	Native LIP2 lipase, codon optimized
PEX11-426 Δ PstI	Ultra high copy constructed plasmid (Okkels 1996)
NS = No Stop, indicates V5/His tag at the C terminal end	

Table 1: Overview of plasmid constructs.

Cell culturing and lipid extraction

Preparation of individual synthetic galactose cultures for pYes-based plasmids.

Culturing of yeast cells containing pYes-based (galactose inducible) vectors was conducted over the course of two days. On the first day, 50 ml synthetic dextrose – 10 X URA media was inoculated with a single colony of yeast in a 250 ml flask. Cultures were grown 20 – 24 hrs at 30°C and 300 rpm. On the second day, the optical density of the culture at 600 nm (OD₆₀₀) was measured and the volume of culture representing 25 OD units was calculated and transferred to a centrifuge tube. The cells were then spun in a swinging bucket rotor (SH-3000) for 10 minutes and 4000 rpm at 25 - 28°C. The supernatant was discarded. The cells were resuspended in a small volume of synthetic galactose media and transferred to 100 ml synthetic galactose – 10 X URA media in a 500 ml flask (cell density was 25OD/100ml = 0.25 OD/ml). The appropriate lipids (0.1% v/v), trilinolein or linoleic acid, were added to their respective cultures. The cultures were then grown 20 – 24 hrs at 30°C and 300 rpm. On the following day the cultures were harvested, spheroplasted and lipids were extracted as described below.

Preparation of individual Holland Minimal Yeast Media (HMY) cultures for pEX11-based plasmids. Culturing of yeast cells containing pEX11-based (fatty acid inducible) plasmids took place over the course of two days. The first day of culturing proceeded in the same manner as those for pYes-based plasmids except the auxotrophic supplements were URA (pEX11-426) and TRP (pEX11-424). The second day of culturing proceeded in the same manner as previously described for pYes based vectors except the transferred cells were placed in 100 ml synthetic glycerol dextrose –10X amino acid in a 500 ml flask. Cell density was 25OD/100ml = 0.25 OD/ml.. The cells were grown 20 – 24 hrs at 30°C and 300 rpm. On the third day, the cultures were “boosted” by adding 10 ml of 10X yeast peptone (see Appendix B,

Solutions). The cultures were returned to the same growth conditions for 4 hours. During that time, HMY media was prepared for each culture in a 500 ml flask. The media consisted of 90 ml HMY – 10X amino acids, 10 ml 10X amino acids, and 100 μ l 1000X vitamins. After four hours the OD600 of each culture was determined and the volume of culture for 100 OD of cells to transfer was calculated. The cells were transferred to a centrifuge tube and spun in SH-3000 for 10 minutes and 4000 rpm at 25 - 28°C. The supernatant was discarded. The cells were resuspended in a small volume of HMY and transferred to 100 ml of HMY-aa. Cells were transferred to culture flasks (culture density is 100 OD/100 ml = 1 OD/ml). The appropriate lipids (0.1% v/v), trilinolein or linoleic acid, were added to their respective cultures. The cultures were then grown 20 – 24 hrs at 30°C and 300 rpm. On the following day the cultures were harvested, spheroplasted and lipids were extracted as described below.

Cell harvesting. Cells were harvested during late-log or early stationary phase. Starter cultures were grown overnight in synthetic dextrose media, then resuspended in synthetic galactose media. After 18-22 hours at 30°C incubation, cells were harvested in late-log phase. 100 ml of yeast culture was harvested and centrifuged at 4000 rpm, 30°C for 10 minutes. The supernatant was discarded and the pellet was resuspended by inversion in 30 ml sterile double distilled water or 1% v/v filter sterilized Tween-40 and centrifuged again at 4000 rpm, 30°C for 10 minutes. The supernatant was then removed and discarded. The pellet was then resuspended and washed with 30 ml sdd H₂O and centrifuged at 4000 rpm, 30°C for 10 minutes. The supernatant was aspirated and the pellet again resuspended in 20 – 30 ml water. 100 μ l cells were transferred to a labeled microfuge tube and OD600 was measured to determine the total OD of the culture. The remainder of the culture was centrifuged as above and aspirated carefully.

Preparation of spheroplasts. The aspirated cell pellet was resuspended in 10 ml 0.1 M Tris-SO₄ pH 9.2, 10 mM dithiothreitol (DTT), by inversion. The screw topped lids of the culture tubes were loosened and taped into place. The culture tubes were incubated in a shaker for 15 minutes at 30°C and 300 rpm. The culture was then centrifuged at 4000 rpm, 30°C for 10 minutes. Once aspirated the cells were washed with 10 ml of 1 M sorbitol, centrifuged at 4000 rpm, 30°C for 10 minutes, aspirated and the supernatant removed. Cells were resuspended in 5 ml of 1 M sorbitol, 20 mM potassium phosphate, pH 7.5, and a calculated amount of Zymolyase-20T solution (2 µg zymolyase/OD cells) was added to the cell suspension. The caps on the culture tubes were loosened and held in place using tape as above and incubated 1 hour at 30°C and 300 rpm. After 50 minutes, the progress of spheroplast formation was checked by osmotic lysis. A 5 µl aliquot of cells was removed and viewed under a microscope. Next, 5 µl of water was added near the cover slip and the cells observed for lysis. When approximately 70% of the cells under the microscope were disrupted, the culture tubes were removed from the incubator and transported on ice to the centrifuge. The spheroplasts were harvested by centrifugation as above and the supernatant removed. Lipids were harvested immediately.

Lipid extraction (Bligh and Dyer 1959, plus washes, Revision of Zanolari *et al.*, 2000). The spheroplast pellet was resuspended in 300 µl of 1 M sorbitol. 400 µl of dd H₂O was added and the suspension was vortexed briefly. 3 ml of chloroform/ methanol/ butylated hydroxytoluene (BHT) (1:2 v/v, 0.01% BHT) was added. The container was flushed with nitrogen, capped tightly and vortexed vigorously for 30 seconds. Each suspension was vortexed three times for 30 second intervals for a total of 2 minutes/sample. The denatured protein and cell debris were then pelleted by centrifugation at 4000 rpm, 30°C for 10 minutes. The supernatant containing lipids was then collected and transferred to a labeled glass centrifuge tube

(15 ml Corex II, #8441 or Kimax) with Nalgene push on caps or 15 ml red capped plastic centrifuge tubes, flushed with nitrogen and capped tightly. The remaining pellet was resuspended in 1ml chloroform/0.01% BHT, vortexed vigorously for 1 minute, and centrifuged as described above. The supernatant was carefully aspirated from the solution and combined with the first supernatant in the glass centrifuge tube or 15 ml red capped centrifuge tube. 1 ml of 0.88% KCl was added to the combined supernate fractions to promote phase separation, vortexed briefly and centrifuged for 5 minutes at 25°C and 2000 rpm. The upper aqueous/methanol layer as well as any interfacial material that may be associated with this layer was aspirated and discarded. The lower chloroform phase containing lipids was removed by aspiration. The chloroform layer was filtered through glass wool and into labeled 4 ml amber teflon capped vial and layered with nitrogen. Lipids were stored at –20°C.

Determination of lipid concentration. Labeled 1.5 ml serum vials were weighed and recorded. 150 µl of the chloroform extract was transferred to the weighed vial and recorded. The sample was evaporated under a stream of nitrogen. The weights of the vials were recorded at intervals of 60 and 90 minutes or until constant. The weight of lipids in the vials was determined and used to calculate the concentration of the original sample by dividing the weight of dried lipid residue (mg) by 1.5 ml.

Gas Chromatography

Preparation of fatty acid methyl esters (FAME). An aliquot of lipid extract representing 1 mg of lipid was transferred to glass screw capped tubes and the solvent was evaporated to dryness under a gentle stream of nitrogen. Dried lipid extract was reconstituted in 1 ml hexane and vortexed 1 minute. 0.2 ml 0.5 M sodium methoxide in methanol (NaOCH₃-CH₃OH) was added and the solution was vortex 1-1.5 minutes. The sample was incubated at

room temperature for 10 minutes to transmethyrate fatty acids. 3 ml saturated NaCl was added to terminate the reaction and the mixture was vortexed 1 minute. 40 μ l of 0.5 mg/ml methyl heptadecanoate (C17:0) was added as an internal standard, along with 3 ml hexane. The solution was vortexed 2 minutes and incubated at room temperature for 20 minutes or until clear phase separation occurred. The hexane (upper) layer was removed and filtered through 1 g Na₂SO₄ and into a clean 1 dram vial. Immediately before gas chromatography, the sample was concentrated under nitrogen to a volume of 1 ml. The concentrated sample was transferred to auto sampler vials.

Analysis of FAME. FAME were analyzed on an Agilent 6890N Gas Chromatograph. A temperature program of 110–160°C at 15°C/minute, to 170°C at 5°C, then to 200°C at 22.5°C/minute with a final hold for 3.3 minutes was employed. FAME were identified by comparison of retention times to FAME standards using methyl heptadecanoate as an internal standard, and percent FAME was calculated based on peak area counts.

Halo Assay for Lipase Activity

Cell Culturing. Yeast cells were prepared as follows: Single colonies were inoculated into SD liquid media and grown overnight at 30°C and 300 rpm. Cell growth was measured by optical density (OD) of the cultures at 600 nm on a spectrophotometer. The volume of culture representing 25 OD units was calculated and transferred to a centrifuge tube. The cells were then centrifuged for 10 minutes and 4000 rpm at 25 - 28°C. The supernatant was discarded. The cells were transferred to 100 ml synthetic galactose – 10 X URA media in a 500 ml flask.

Halo Assay. To qualitatively detect lipase activity, an agar plate assay was employed (Pignede *et al.* 2000). The media used (per liter) was 1.7 g yeast nitrogen base without ammonium sulfate and without amino acids, 4 g of ammonium chloride, 10 g tributyrin stock

solution, and 100 ml 10X-amino acid. A stock solution of the triacylglycerol tributyrin was composed of 20% tributyrin v/v, 1% Tween 20 v/v and subjected to sonication three times for 1 minute each on ice. Agar (2% w/v) was added for solid media formation (YNBT). Each halo assay plate contained about 100 ml YNBT agar. The halo assay plates were poured 5-12 hours before needed to prevent tributyrin pooling. 10 μ l yeast cell culture was placed onto the agar. A halo or clearing zone was visible after 36 - 48 hours of incubation at 30°C.

The LAS-3000 imaging system from Fuji Film Life Sciences (Tokyo, Japan), was used to create images of the agar plates. The LAS-3000 imager combines a CCD camera with a user interface. The Multi Gauge (version 3.0) software allowed for editing the saved images and drawing lines onto the image through the center of the cell and halo (radii). The software provides the length of the radii. The cell and the halo surrounding it can be defined as two concentric circles. Once measured, the annulus calculation (Figure 3-9), provides a numerical value for the region between the two circles (Weisstein, 2002).

Results

Cloning LIP2 into *Saccharomyces cerevisiae*

The LIP2 gene from *Y. lipolytica* was isolated and amplified from genomic DNA and cloned into the high copy yeast shuttle vector pYes2.1 (Figure 3-1), forming the plasmid pYes2.1-LIP2 (Figure 3-2). This molecule was expected to secrete the lipase protein into the growth medium. A second version of this molecule, pYes2.1-LIP2-V5/His (NS) was also constructed. In this molecule, the LIP2 open reading frame is extended to include amino acids encoding the V5 epitope to facilitate detection of the protein in yeast by western blotting. These constructs are diagrammed in Figure 3-3.

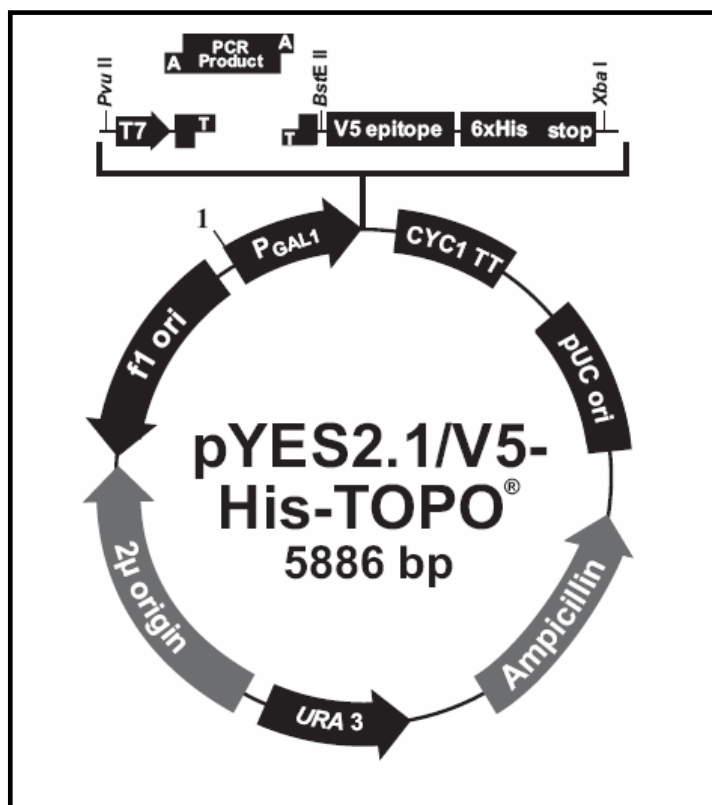


Figure 3-1: pYes2.1 yeast shuttle vector

It has been observed that expression of heterologous genes in yeast may be limited by features that are subtly different between organisms, factors such as codon usage or recognition by processing enzymes. Knowing this, plasmids were constructed with modifications to compensate for these subtle differences. To optimize lipase secretion, several modifications were made to the LIP2 gene. The modifications were designed to enhance the features of the gene that were specific to the *Y. lipolytica* expression system and tailor them for *S. cerevisiae* expression. The modifications involved the KEX2 cleavage site, the signal sequences and the codon preferences.

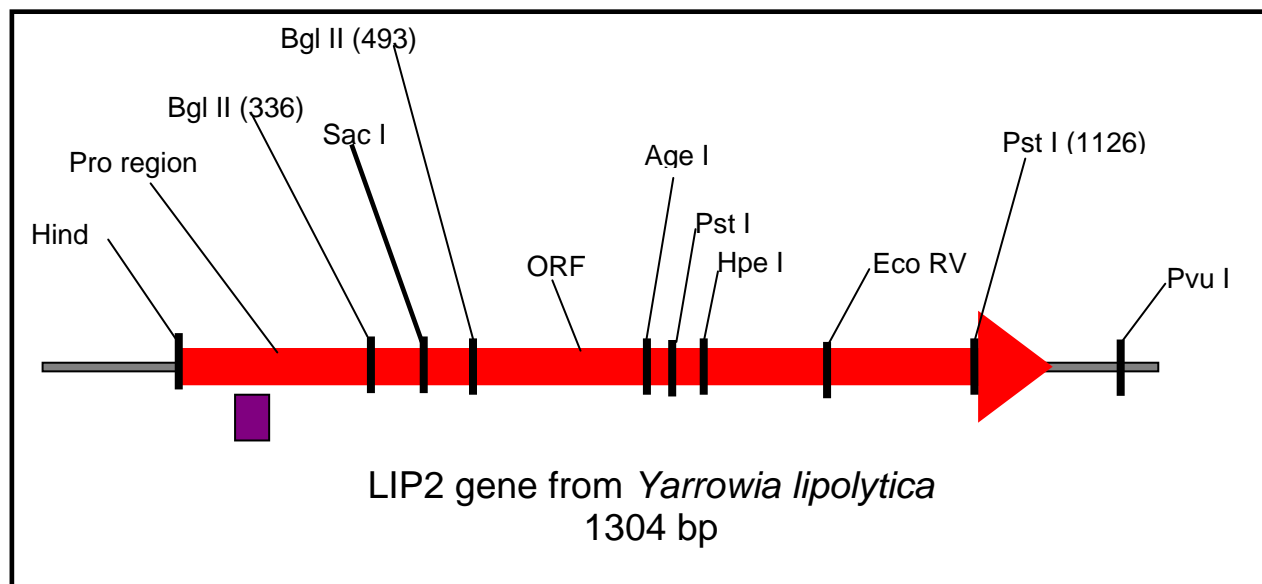


Figure 3-2: LIP2 / LIP2NS gene

KEX2 endoprotease *Yarrowia lipolytica* uses the XPR6 gene product to cleave the proregion and release the mature form of the protein. Pignede *et al.* found that the removal of the proregion is not required for release of the mature protein. The presence of the proregion does not inhibit lipase secretion and activity. The mature protein, however, is less stable. Unlike *Y. lipolytica*, *S. cerevisiae* utilizes the Kex2 endoprotease for prosequence cleavage. To ensure efficient cleavage in the *S. cerevisiae* system, the amino acid sequence of the cleavage site of the

LIP2 gene was optimized to include a serine codon in the P'₁ site. The serine codon was added based upon experiments examining the kinetic and enzymatic preferences of the KEX2 protease (Rholam *et al.* 1995). Site directed mutagenesis was used to add serine at position P'₁ in the LIP2 gene. pYes2.1-LIP2 and pYes2.1-LIP2NS were used as templates for extension using mutagenic primers as described in Materials and Methods. The resulting molecules were sequenced to ensure that the serine addition was the only mutation introduced. These plasmids are diagrammed in Figure 3-3.

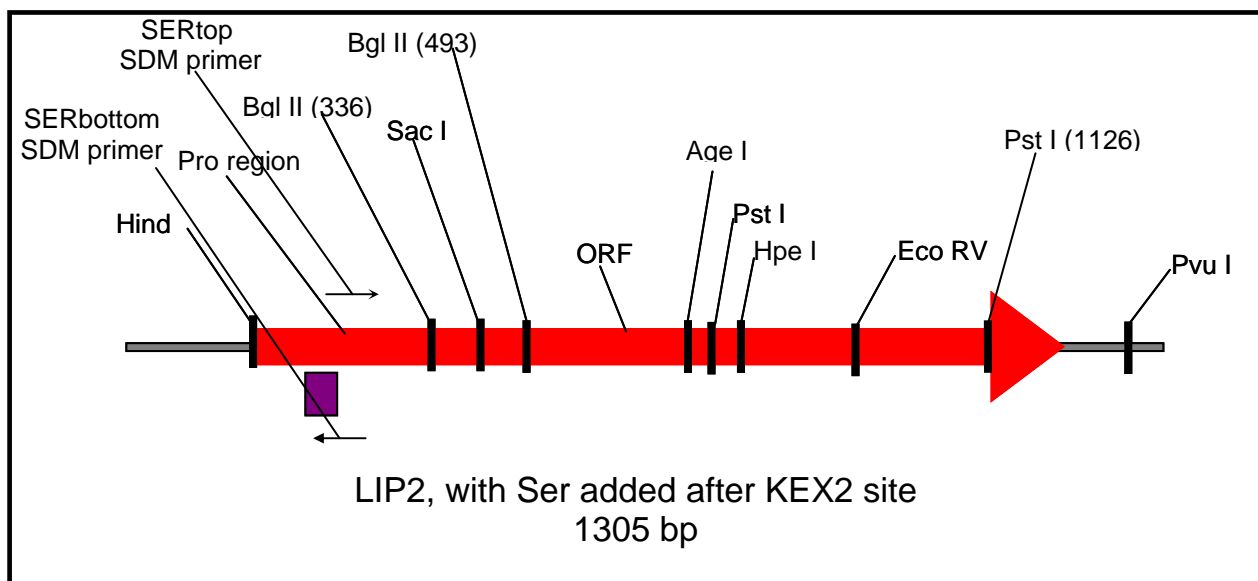


Figure 3-3: LIP2Ser / LIP2SerNS gene

Codon optimization. Codon usage has been correlated with expressivity (as measured by the codon adaptation index) in *S. cerevisiae*. This link makes codon optimization necessary in the case of foreign gene placement. The composition of genes is vital to translational efficiency. Presumably translational efficiency and codon usage are linked because some tRNAs are more abundant than others (Fuglsang 2004). More highly expressed genes tend to use the abundant tRNAs thus minimizing the risk of tRNA depletion during translation. Thus, to

enhance the expressivity of LIP2, the gene was codon optimized by the Genscript Corporation. In analyzing the final optimized DNA sequence, the LIP2opt sequence and LIP2Ser sequence have 72.03% sequence identity (757/1051 nucleotides) (Appendix D). The nucleotide changes are not in one particular area, but are spread evenly throughout the sequence. The nucleotide sequence has changed, while the amino acid sequence remained the same. The codon optimized gene was cloned into pYes2.1, forming pYes2.1-LIP2opt.

CPY signal sequence. Signal peptides govern the entry of almost all proteins to the secretory pathway. Signal sequences form the N-terminal portion of the amino acid chain and are cleaved off as the protein is translocated through the membrane (Nielsen 1990). Carboxypeptidase Y (CPY) contains the native signal sequence to the yeast vacuole in *S. cerevisiae*. CPY is a member of the serine carboxypeptidase family and a 61 kD vacuolar enzyme. It is synthesized on ER bound ribosomes as an active glycosylated precursor (preproCPY). The CPY signal sequence is removed by signal peptidase, forming proCPY. The presence of the propeptide is important for the correct folding of CPY and the role that the large carbohydrate moiety plays on the stability and function of CPY (Takahashi *et al.* 2001 and Kato 2003). Then, proCPY is transported from the endoplasmic reticulum to the Golgi apparatus, where it undergoes core glycosylation (unproCPY, if not glycosylated), then to the vacuole. At or immediately prior to its arrival at the vacuole, proCPY is proteolytically cleaved to its mature active form (Blachly-Dyson and Stevens 1987). The amino terminal signal sequence (pre region) is important for efficient translocation, but not absolutely required (Blachly-Dyson and Stevens 1987). In contrast, the pro region is essential for in vivo and in vitro protein folding and unfolding and ensuring cooperative structural transitions (Kato *et al.* 2003).

In choosing further modifications to the LIP2 gene for potential improvement, changing the signal sequence from the XPR6 sequence to a native *Saccharomyces* signal sequence seemed an obvious choice. Accordingly, the native pre-pro sequences have been replaced by ER targeting signal derived from *S. cerevisiae* carboxypeptidase Y (Lip2-CPY). Replacing the signal sequence was done to increase the likelihood of successful translocation, cleavage, and secretion of a true lipase. Only the proregion of CPY was cloned into *S. cerevisiae* because it has been established that the pre region was not necessary for correct transport of the mature protein (Pignede *et al.* 2000). The resulting plasmid is named pYes2.1-LIP2-CPY (Figure 3-4). All of the modified LIP2 strains are compared in Figure 3-5.

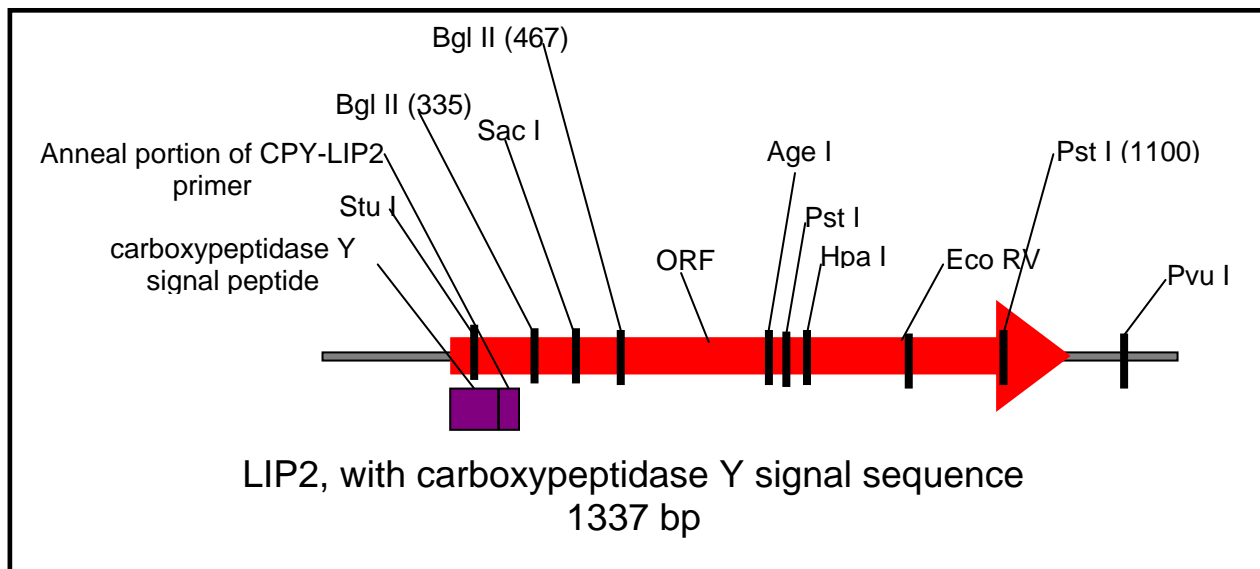


Figure 3-4: Schematic diagram of LIP2 CPY/CPYNS gene construct

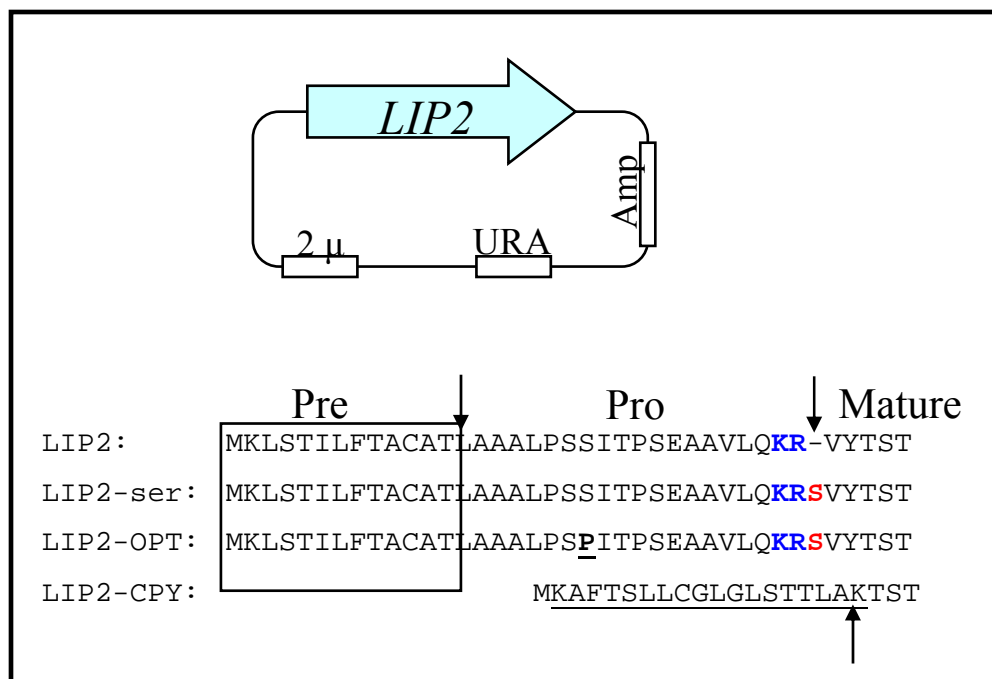


Figure 3-5: Properties and genetic modifications to the *LIP2* gene. Wild type *LIP2* sequence consists of three regions: pre-sequence (boxed), pro sequence, and mature protein. Cleavage sites are indicated by arrows, Lysine-Arginine dibasic cleavage site is in blue type. *LIP2*, wild-type amino acid sequence; *LIP2-ser*, *LIP2* sequence that has been modified to include an additional serine residue for optimal cleavage by the *S. cerevisiae* Kex2 protease (red); *LIP2-opt*, synthetic *LIP2* sequence optimized for *S. cerevisiae* codon preferences (bold, underlined); *LIP2-CPY*, *LIP2* sequence in which the native pre-pro sequences have been replaced by a *bona fide* ER targeting signal derived from *S. cerevisiae* carboxypeptidase Y (underlined).

Lipase Secretion

Halo Assay for Lipase Activity. *S. cerevisiae* cells containing the pYes 2.1 vector and respective *LIP2* gene variants were grown on agar plates containing the triacylglycerol tributyrin as the sole carbon source. The function of the agar plate assay was to produce a qualitative sign of lipolytic activity by the yeast cells. If the yeast cells were secreting a lipase, the tributyrin would be hydrolyzed, causing the region around the cells to become clear, forming a halo. The cells were plated in three groups: 1. pYes 2.1-*LIP2*, *LIP2-Ser*, *LIP2-CPY* (Figures 3-6 and 3-7); 2. *LIP2*, *LIP2-Ser*, *LIP2-opt*; 3. *LIP2NS*, *LIP2-SerNS*, *LIP2-CPYNS* (not shown). All plates included pYes2.1 (empty plasmid vector) as a negative control. Positive controls using

commercial lipase purchased from Sigma-Aldrich at concentrations of 1 mg/ml, 0.5 mg/ml and 0.1 mg/ml were performed on separate agar plates to prevent interference.

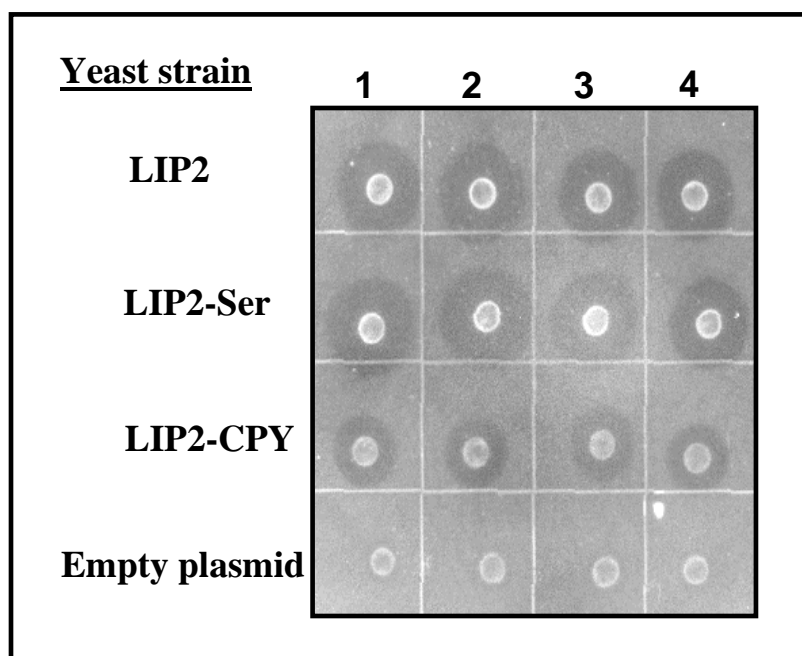
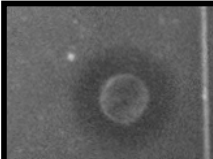
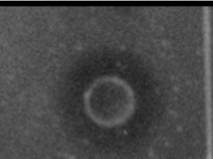
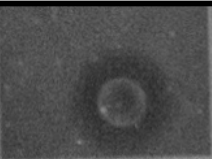
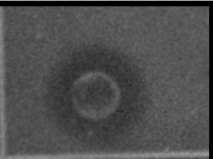
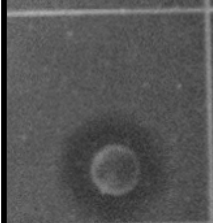
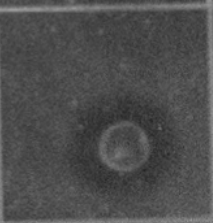

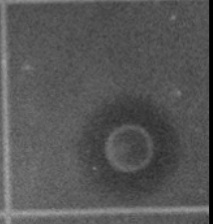
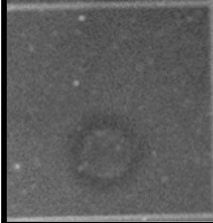
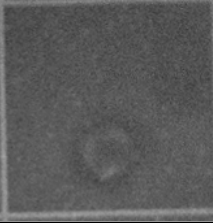

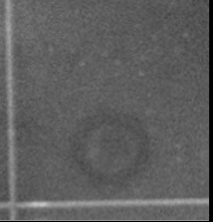
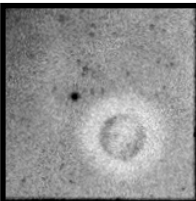
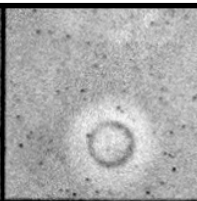
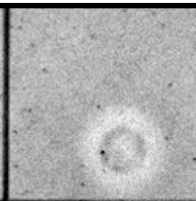
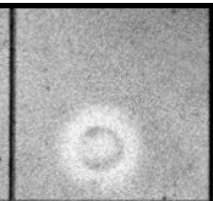
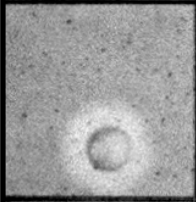
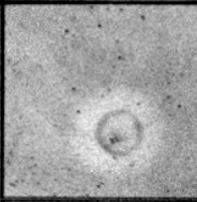
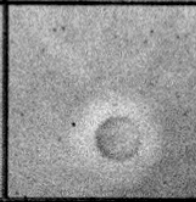
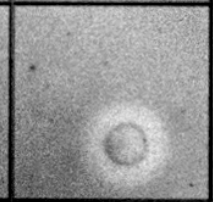
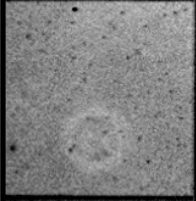
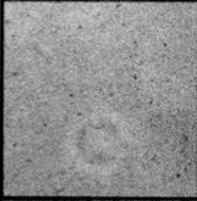
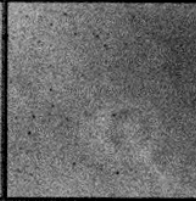
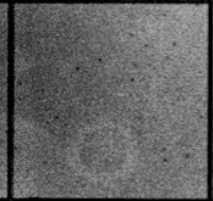


Figure 3-6. Growth and lipase activity of *S. cerevisiae* cells on agar containing tributyrin. Cells growing exponentially in SDextrose at 30° were diluted to 0.25 OD/ml and 10µl of cells were used to inoculate YNBT plates. The plate assay contains either wild-type yeast cells (empty plasmid), or yeast cells that have been engineered to secrete lipase enzymes into the growth media LIP2, LIP2-Ser and LIP2-CPY). The plates contain tributyrin, and the presence of lipase activity was detected by a “clearing zone” or halo around the colony. Lipase secretion was detected as a halo after 3 days incubation.

A

<u>Yeast strain</u>	1	2	3	4
LIP2				
LIP2-SER				
LIP2-CPY				

B

<u>Yeast strain</u>	1	2	3	4
LIP2				
LIP2-SER				
LIP2-CPY				

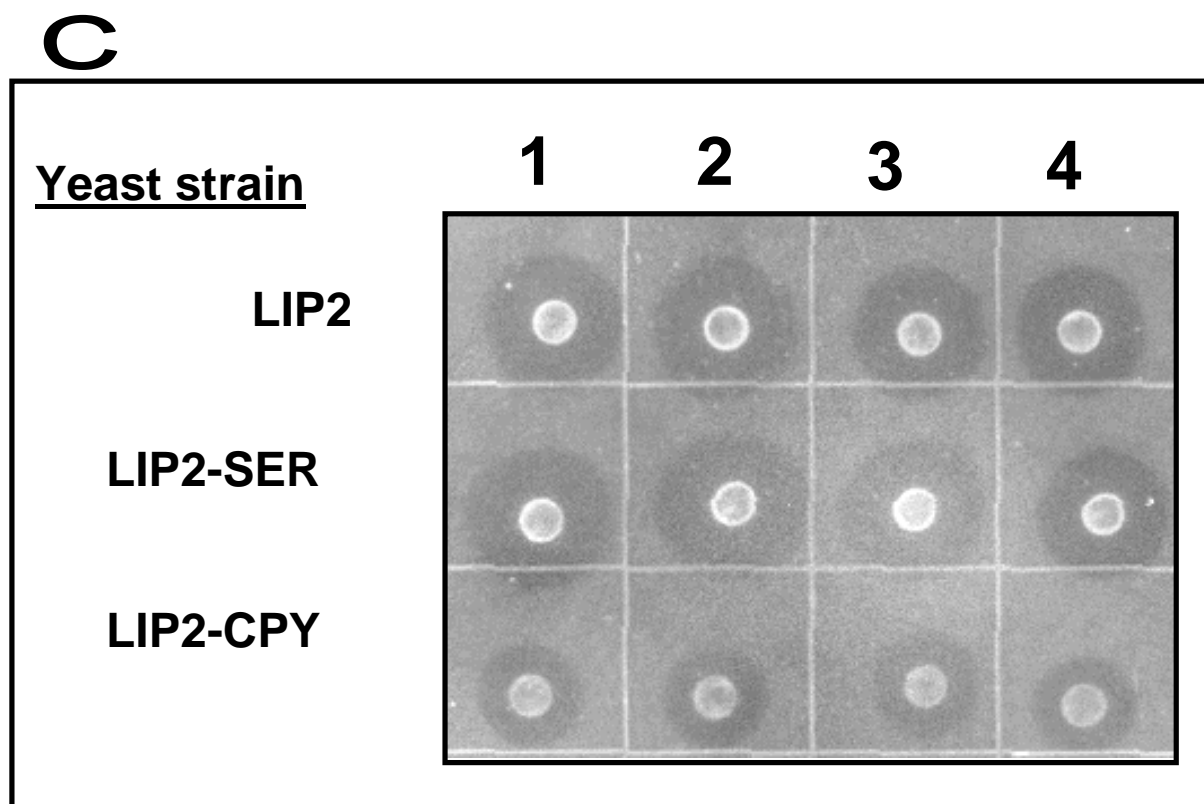


Figure 3-7. Growth and lipase activity of *S. cerevisiae* cells on agar containing tributyrin over a period of 72 hours (pYes2.1 LIP2, LIP2 Ser and LIP2 CPY). The growth conditions are as described in Figure 3-6. The progression of cell growth and lipase secretion on the agar plate lipase assay. Top (**A**): 15 hours incubation, middle (**B**): 24 hours, bottom (**C**) 48 hours.

The second group of plate assays consisted of cells containing the LIP2 gene, LIP2-Ser and LIP-opt genes (Figure 3-8). It is significant to note that this plate assay was done using cells salvaged from the basement. The cells were diluted to lower the possibility of contamination, plasmid isolation was repeated and the cells were re-transformed into yeast. Rather than spotting 10 μ l of culture the cells were serial diluted to concentrations of 100, 50, 10, 5, and 1 OD/ml then 10 μ l those cultures were spotted onto the agar plates. We serial diluted the cultures to determine the lowest concentration at which lipase production could be detected. The final group of plate assays contained the NS constructs (LIP2NS, LIP2-SerNS, LIP2-CPYNS). There is no figure because none of the NS constructs produced a halo.

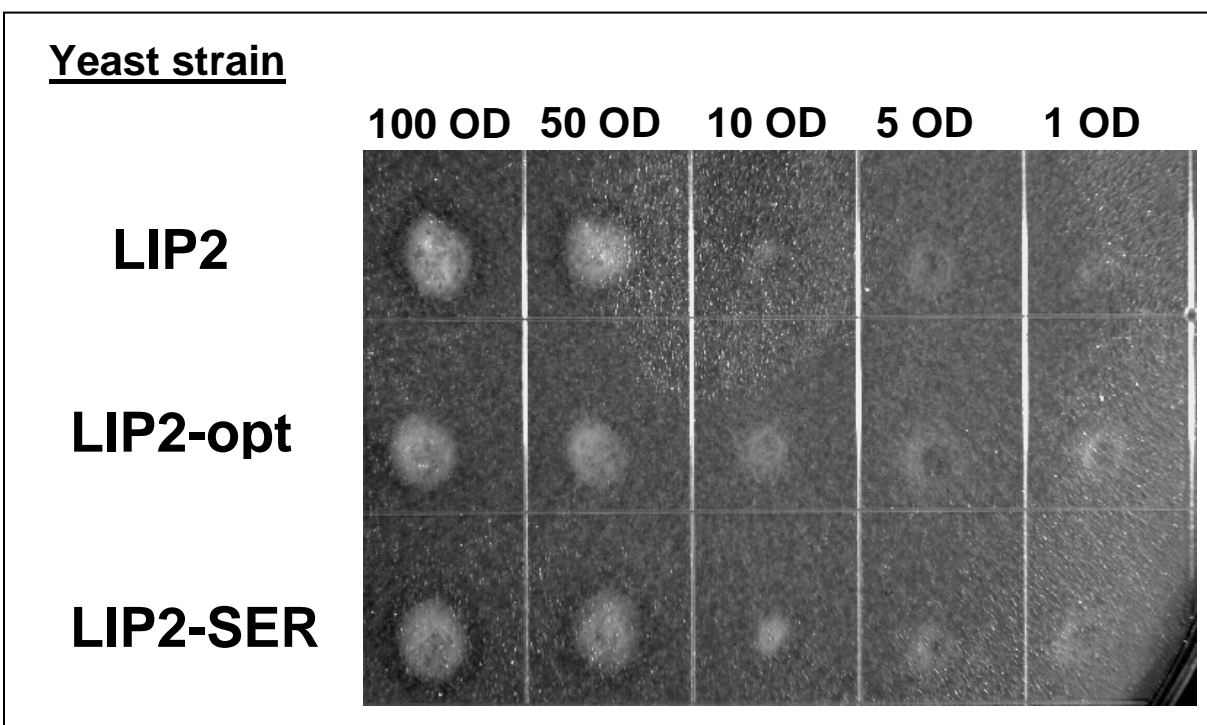


Figure 3-8. Growth and lipase activity of *S. cerevisiae* cells on agar containing tributyrin. The yeast strains growing on the plate are LIP2 (native gene), LIP2-opt (codon optimized) and LIP2-Ser (serine added after cleavage site) (empty plasmid not shown). The cells were diluted to 100 OD/ml, 50 OD/ml, 10 OD/ml, 5OD/ml and 1 OD/ml to determine the lowest concentration of cell culture that can produce and secrete a visible amount of lipase. The OD or OD/ml refers to the optical density of the culture which is a measure of the number of cells/ml present in a yeast culture.

The lipase activity of the yeast cells were qualitatively determined daily by visual assessment and quantitatively by using measurements of the cells and halo regions and annulus calculations. Using the Multi Gauge software from Fuji Film Life Sciences (Tokyo, Japan), the diameter of the cells and the halo were defined by lines drawn through the center of the cell and halo (radii). The software provides the length of the radii. The cell and the halo surrounding it can be defined as two concentric circles. Once measured, the annulus calculation (Figure 3-9), provides a numerical value for the region between the two circles. The data gives details on the amount of lipase produced by observing the size of the hydrolyzed region. Furthermore, information regarding the rate of lipase production can be gleaned from annulus data collected

over several days. Generally plate assays were discarded after 4 days because cell death and the subsequent release of cellular contents may hydrolyze tributyrin and artificially inflate the data.

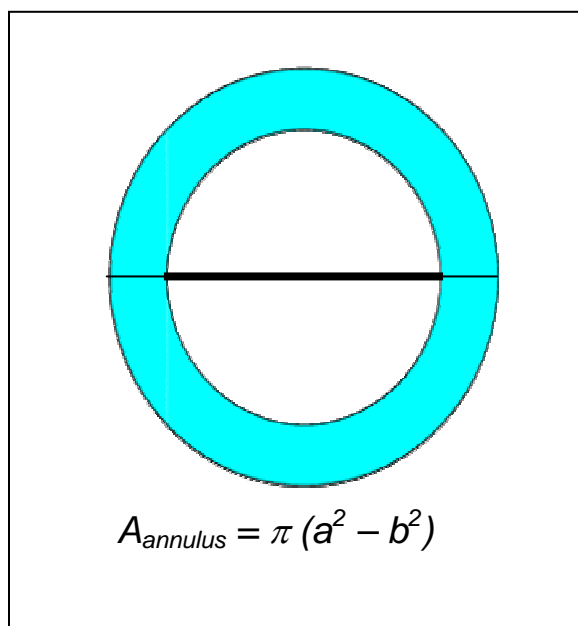


Figure 3-9. Annulus. An annulus is defined as the region lying between two concentric circles. The area of the annulus formed by two circles of radii a and b , with $a > b$. (Weisstein 2002).

In the first set of plates (LIP2, LIP2-Ser, LIP2-CPY), colonies containing the LIP2 and LIP2-Ser gene had the largest halos on visual inspection. Using the Student t-test we determined that there was no significant difference between the halos produced by the LIP2 and LIP2-Ser colonies ($P = 0.126$ at 24 hours, 0.14 at 48h and .05 at 72h). Surprisingly, cells containing the LIP2-CPY gene produced halos that were consistently smaller than the halos from LIP2 and LIP2-Ser colonies. According to the t-test there is a significant difference between the halos from LIP2 and LIP2-CPY ($P = 2.6 \times 10^{-8}$ at 24 hours, 1.6×10^{-5} at 48h and 8.9×10^{-8} at 72h) and LIP2-Ser and LIP2-CPY ($P = 3.6 \times 10^{-6}$ at 24 hours, 8.8×10^{-6} at 48h and 2.6×10^{-8} at 72h). From these results we concluded that the carboxypeptidase Y signal sequence may not function well without its pre-region despite reports to the contrary. We also speculated that incomplete

cleavage of the proregion from the mature protein may be a factor in poor or decreased action of the lipase gene. A simpler explanation is that the prepro signal sequence is not compatible with the LIP2 gene. From this experiment, we chose the yeast cells containing the LIP2-Ser gene for insertion into the oil inducible vector and further experiments because it promising levels of lipase secretion (Figure 3-10).

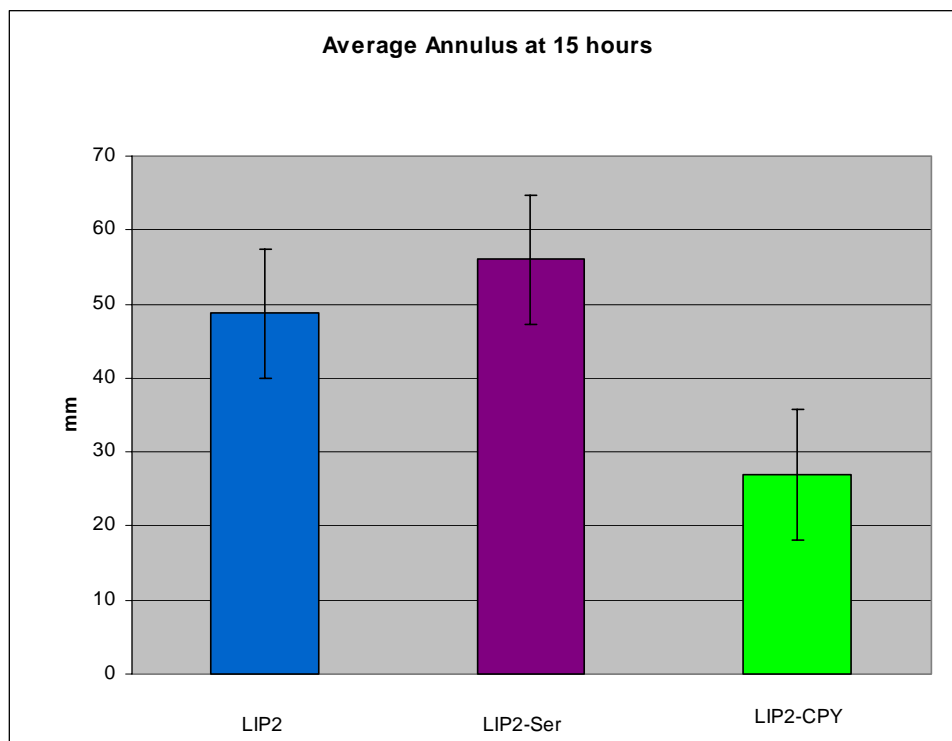


Figure 3-10. Lipase secretion as measured by annulus size at 15 hours. The annulus was produced by cell colonies spotted onto agar plate and grown at 30°C. Cells producing a lipase hydrolyzed the tributyrin in the agar, producing a halo that was measured using the annulus calculation. Bars denote standard error.

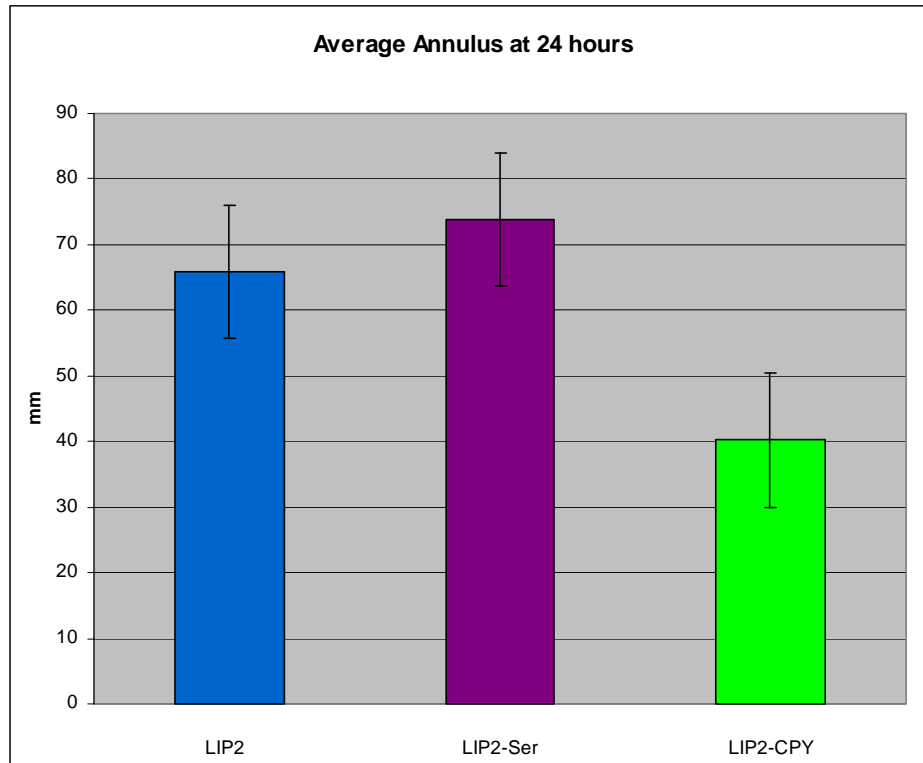


Figure 3-11 Lipase secretion as measured by annulus size at 24 hours. Cells were grown under the same conditions as described in Figure 3-10

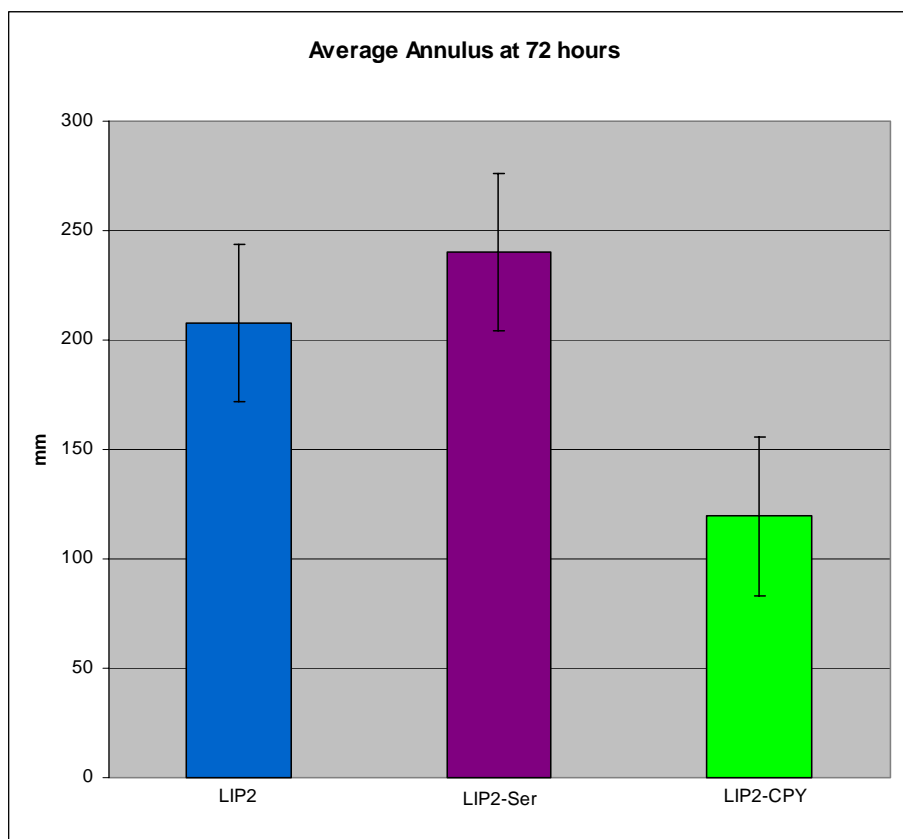


Figure 3-12 Lipase secretion as measured by annulus size at 72 hours.

In the second set of plates (pYes2.1- LIP2, LIP2-Ser, LIP2-opt) there was no significant difference between the size of the halos produced at 24 hours in 100 OD/ml colonies. At 50 OD/ml there was a significant difference between the LIP2 and LIP2-opt halo size. At 48 hours there was no significant difference between the sizes of any of the halos at any concentration. The average annulus size for the second group of plate assays at the 24 hours is shown in Figure 3-11 while average annulus size at 48 hours is shown in Figure 3-12.

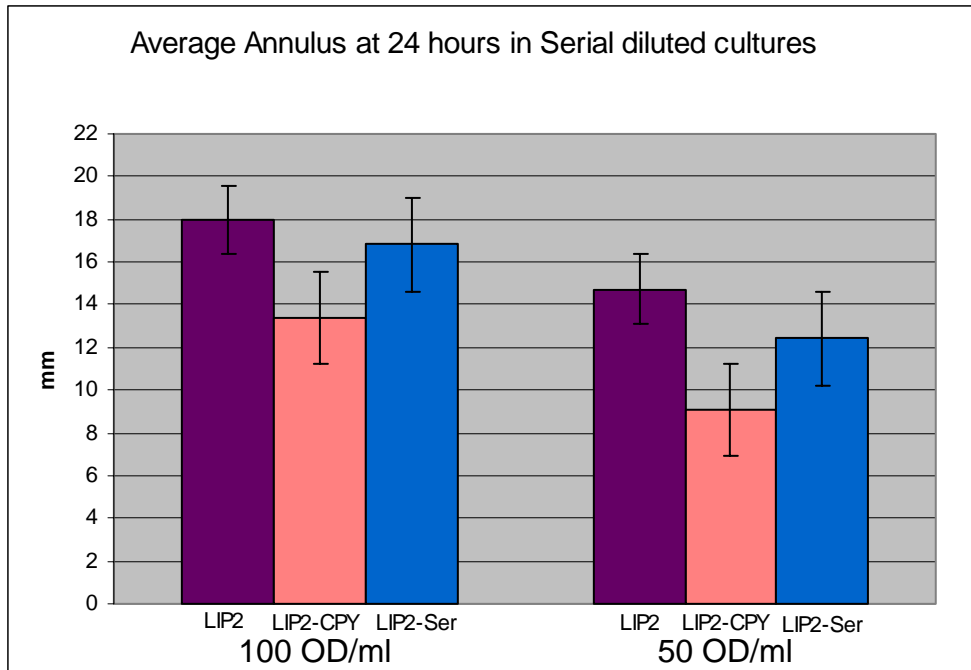


Figure 3-13: Average annulus size at 24 hours in serially diluted cultures. The annulus was produced by cell colonies spotted onto agar plate and grown at 30°C. Cells producing a lipase hydrolyzed the tributyrin in the agar, producing a halo that was measured using the annulus calculation. These cells were diluted to higher optical densities to determine at which concentration cells would cease to produce measurable amounts of lipase. Bars denote standard error.

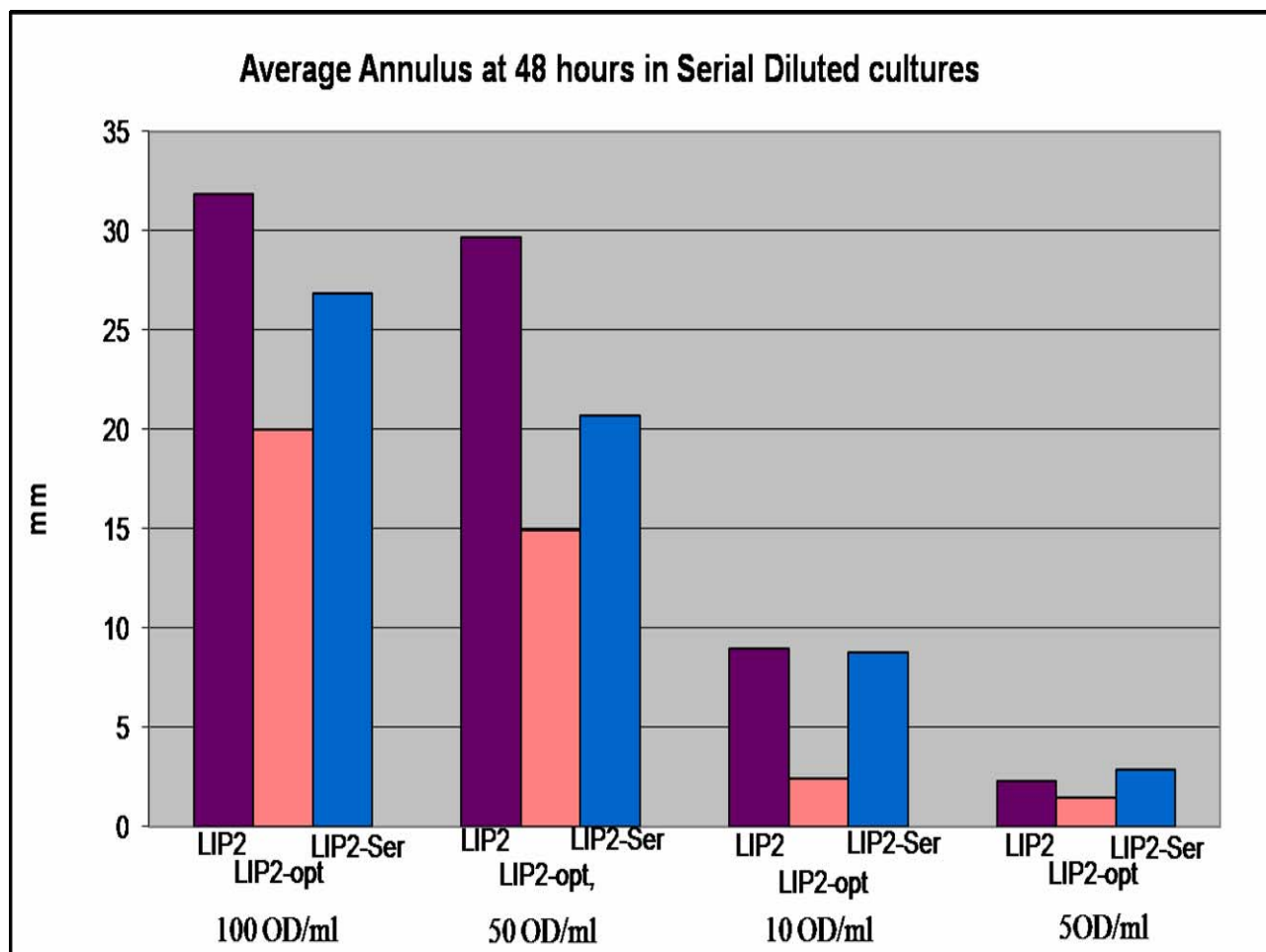


Figure 3-14: Average annulus size at 48 hours in serially diluted cultures. Cultures at 100 OD/ml, 50 OD/ml, 10 OD/ml, and 5 OD/ml are shown.

In the final set of plates that contained colonies of pYes2.1-LIP2NS, LIP2-SerNS, LIP2-CPYNS grown on agar containing tributyrin. All open reading frames were built with and without the stop codon which creates C-terminal V5-peptide fusion, which can be recognized by anti-V5 antibody. These cells were not thought to be very different from their stop codon counterparts. The C-terminal fusion was only looked upon as a convenience for western blotting. Surprisingly, the plate assay for cells constructed without the stop codon produced no halo (plate not shown).

To determine the quantitative value of measuring the annulus of lipase producing colonies we plotted annulus size versus cell number (Figure 3-15). We have speculated that the size of the annulus directly correlates to the amount of lipase secreted. From the data, annulus size is proportional to cell number to a point. At 100 OD/ml, the annulus size seems to reach saturation. The data does lend credence to using the annulus calculation as a quantitative measure of lipase secretion because lipase secretion is greater at higher cell numbers due to more active constructs.

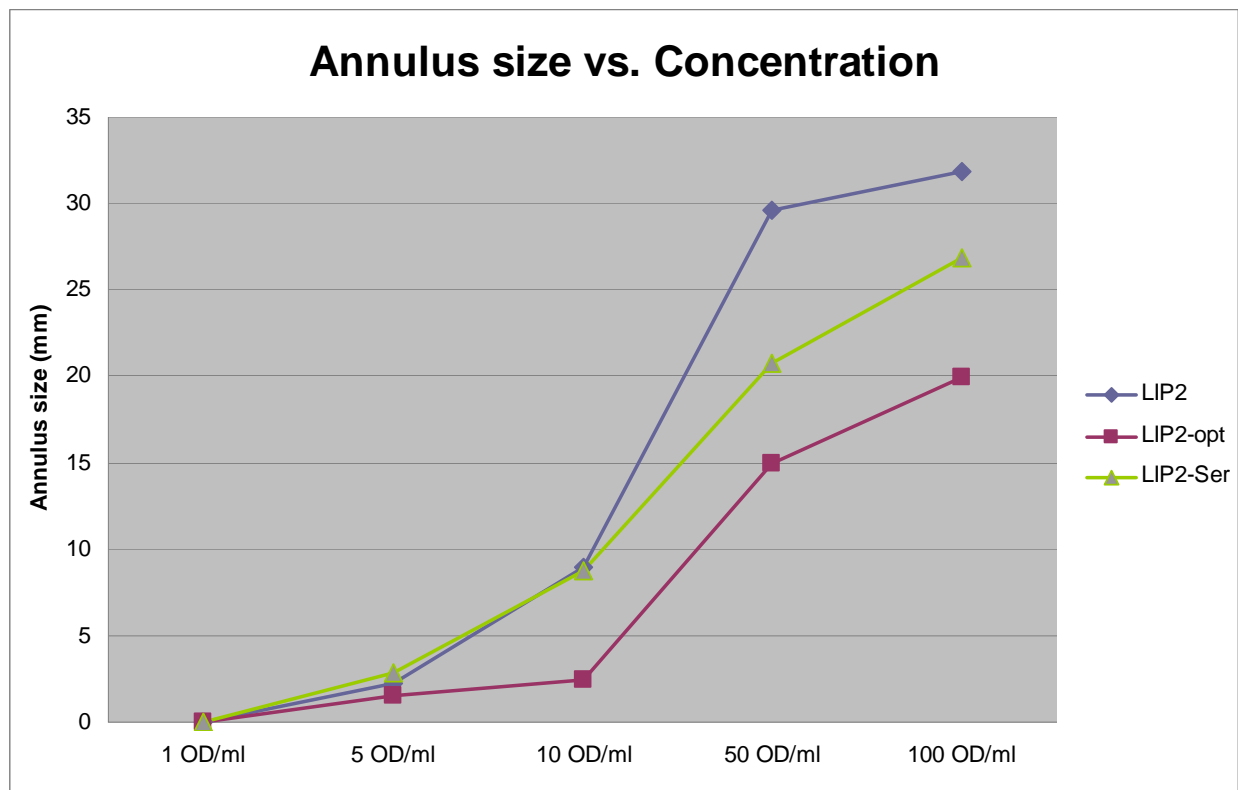


Figure 3-15: Annulus size vs. cell number.

Spectrophotometric Lipase Assay. To quantify the lipase secreted by our yeast cells we tried a quantitative spectrophotometric lipase assay. Despite repeated attempts and multiple modifications, no activity was detected. In general the spectrophotometric method is the most common procedure used for lipase assays due to its 10^{-4} U threshold of detection of lipase activity (U is the amount of enzyme required to yield 1 μ mol product per minute). The least amount of materials and enzyme are required for the spectrophotometric method (Pinsirodom and Parkin 2001). Modifications to the assay system included pH adjustments, length of culture incubation (growth), and reagent and culture volumes (Appendix E).

Expression of a lipase in yeast using a fatty-acid inducible gene promoter

pEX11 vector. To couple the processes of fatty acid uptake into *S. cerevisiae* cells and lipase production, open reading frames of lipase constructs showing robust activity were expressed in yeast cells using the fatty acid-inducible yeast *PEX11* gene promoter. The PEX11 gene encodes a protein (Pex11p) that is involved in proliferation of peroxisomes in response to certain environmental cues (Erdmann and Blobel 1995; Marshall *et al.* 1995). The PEX11 gene is expressed at low basal levels in glucose and induced approximately 100-fold when cells are shifted to media containing fatty acids as a sole carbon source (Marshall *et al* 1995). Thus, we chose to construct plasmids using this promoter creating plasmids pEX11-424-LIP2 Ser, pEX11-426-LIP2, LIP2 Ser, LIP2 opt and Δ PstI

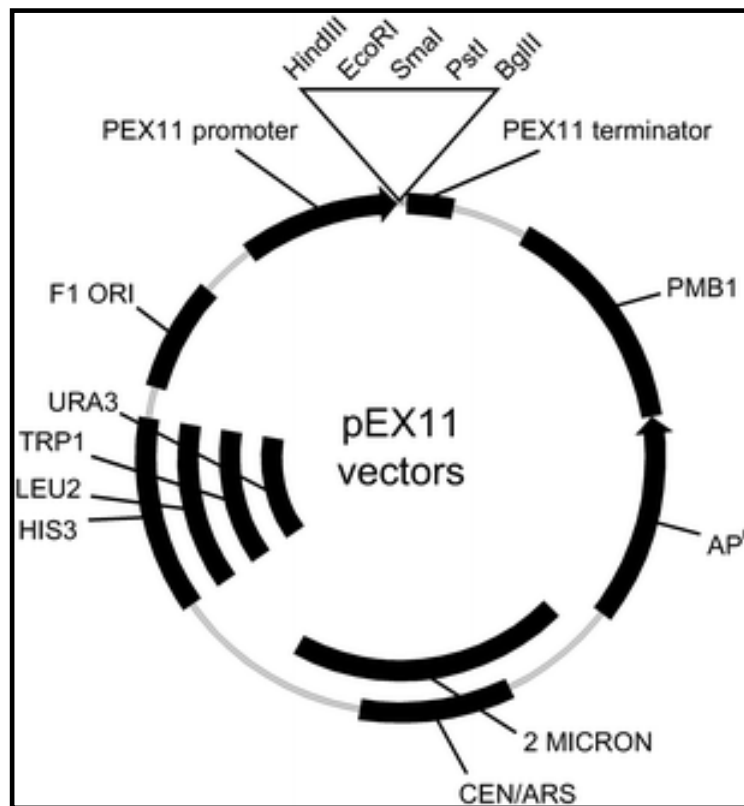


Figure 3-16. Single copy vectors for fatty acid-inducible gene expression in yeast (Dyer *et al.* 2002).

Analysis of FAME. Dyer *et al.* found that yeast cells could take up the free fatty acids of triglycerides from the growth media if a lipase was exogenously added to first breakdown the triacylglyceride (Dyer et al 2000). Under these conditions, the fatty acid composition of the yeast mimicked that of the sesame seed oil in the growth media and included linoleic and linolenic acids which are not normally present in yeast. The fatty acid composition of native *S. cerevisiae* cells consists of palmitic, palmitoleic, stearic and oleic acids (Figure 3-18). Yeast does not possess the enzyme to make linoleic acid and thus lacks this fatty acid. To circumvent the need to add a lipase to hydrolyze TAG in the growth media, the LIP2 gene and derivatives were transformed into the *S. cerevisiae* cells. Once it was confirmed by agar plate assay, that the

yeast cells containing the LIP2 gene and its derivatives were actively secreting a true lipase, we repeated the previous experiments. The yeast cells were cultured in linoleic acid or soybean oil as the sole carbon source. The cellular lipids were harvested, converted to their methyl esters by transmethylation and analyzed by gas chromatography.

Gas chromatography revealed several trends in the lipid profiles of cells. Both wild type and pEX11-LIP2Ser cells cultivated in the presence of free linoleic acid contained over 80% of their fatty acids as linoleic acid (Figure 3-17 and 18), implying that the cells can take up and store this molecule. Wild type cells cultivated in soybean oil (Figure 3-20), by contrast, contained 45% of their fatty acids as oleic acid and, only 13% as linoleic acid. The high percentage of oleic acid is more consistent with the profile of wild type cells (Figure 3-16), because yeast is unable to take up soybean oil as a triglyceride and cannot hydrolyze it extracellularly into component free fatty acids. Incubation of yeast cells containing the LIP2Ser gene in the presence of soybean oil (Figure 3-21) resulted in an uptake of linoleic acid, which is comparable to the amounts found in soybean oil (54%) (Figure 3-19). Most notably, cells containing the LIP2Ser gene had 50% of their fatty acids as linoleic acid and other fatty acids in similar percentages to soybean oil. From this data we conclude that the modified LIP2 strain, LIP2Ser in an oil inducible vector, is able to secrete a lipase into the growth media that hydrolyzes triacylglycerols. Once hydrolyzed, the free fatty acid component parts of the triacylglycerol are taken up by the yeast cell and stored.

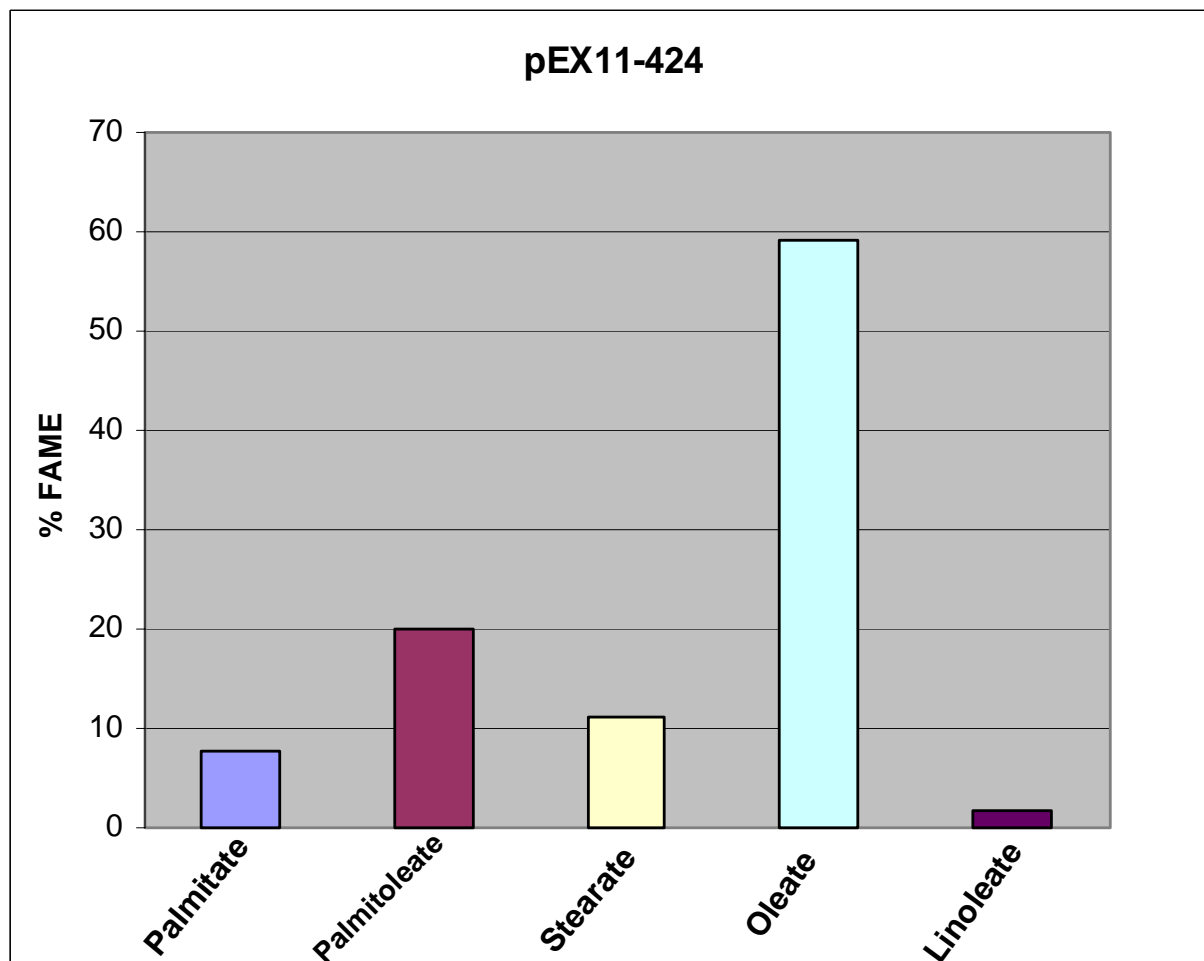


Figure 3-17 Fatty acid composition of wild type yeast cells (pEX11-424). Cells were grown under minimal media starvation then fatty acids were harvested.

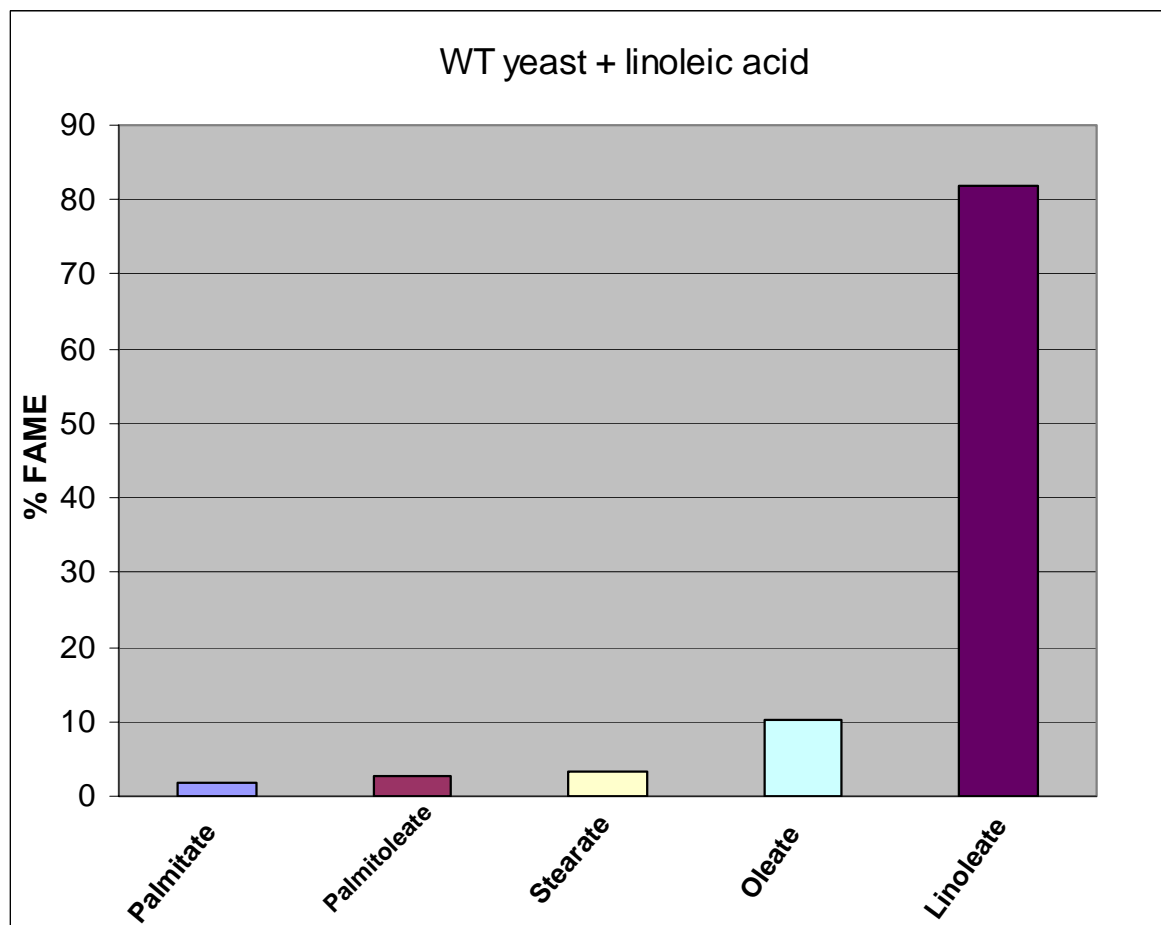


Figure 3-18 Fatty acid composition of wild type yeast cells (pEX11-424) grown in the presence of the free fatty acid linoleic acid. Cells were grown under minimal media starvation and in the presence of linoleic acid for 24 hours. After that period the cells were boosted with yeast peptone. The cells were then harvested and fatty acids were extracted.

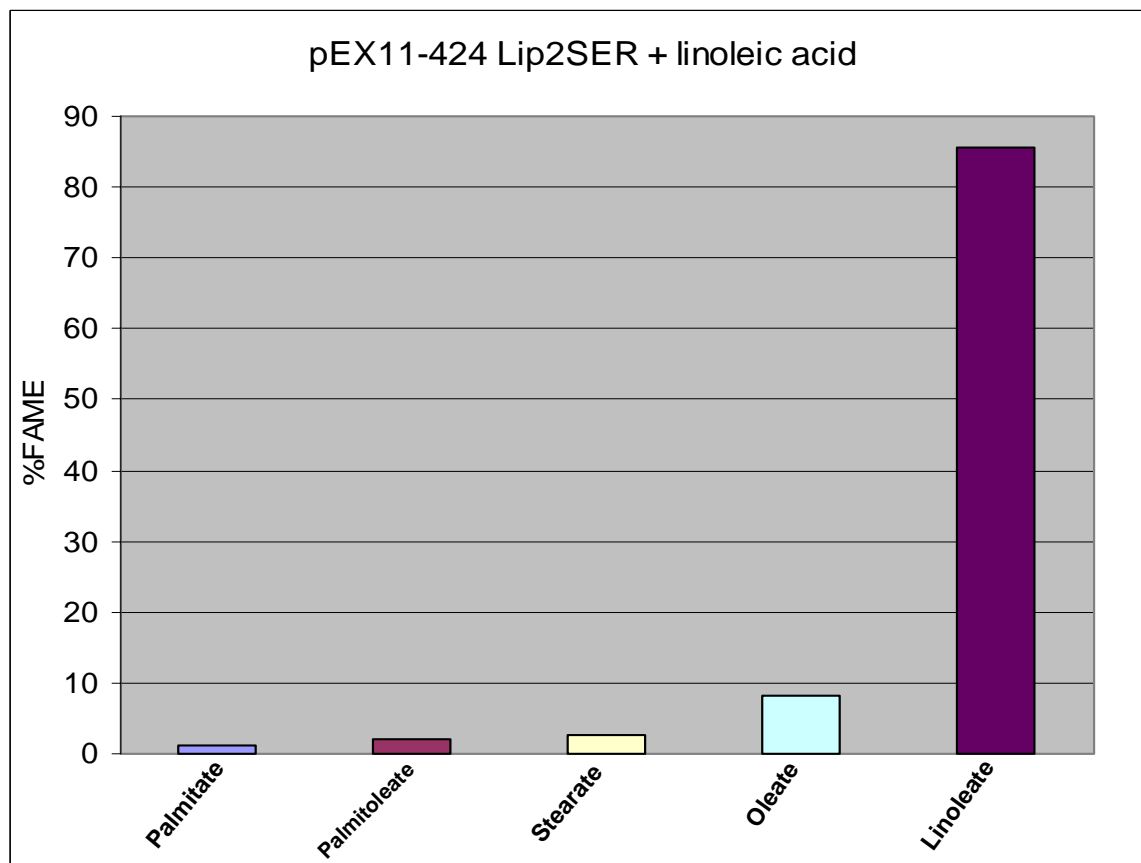


Figure 3-19 Fatty acid composition of pEX11-424 LIP2Ser cells grown in the presence of the free fatty acid linoleic acid.

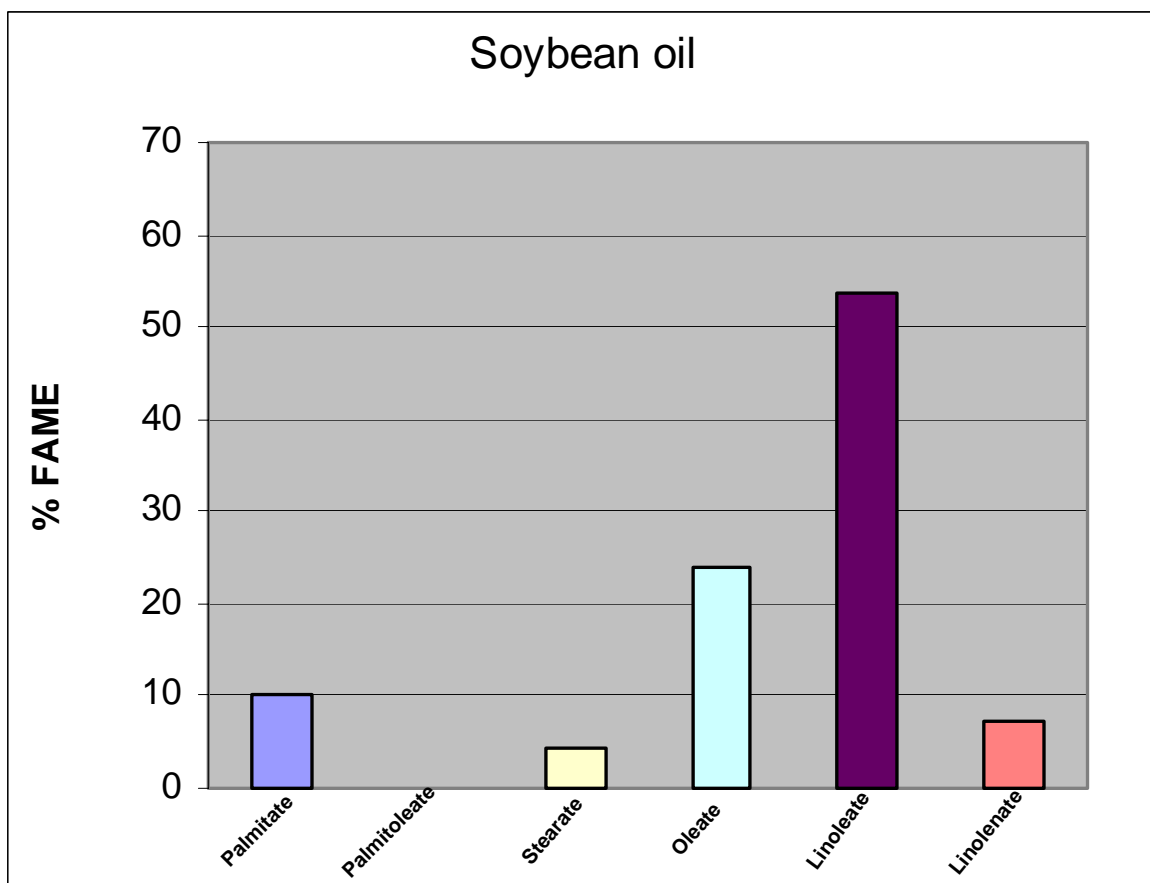


Figure 3-20. Fatty acid composition of Hain Soybean Oil.

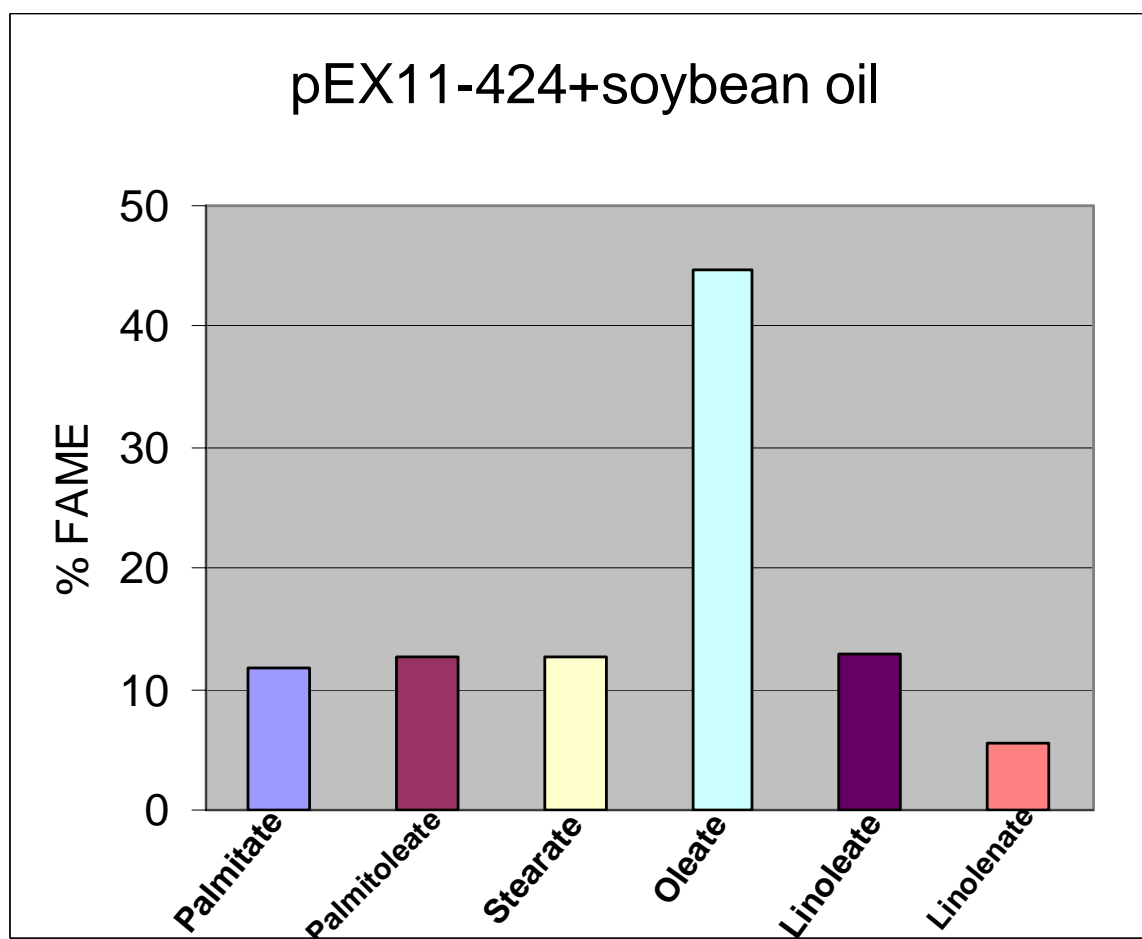


Figure 3-21. Fatty acid composition of wild type yeast containing the empty vector pEX11-424, grown in Soybean oil.

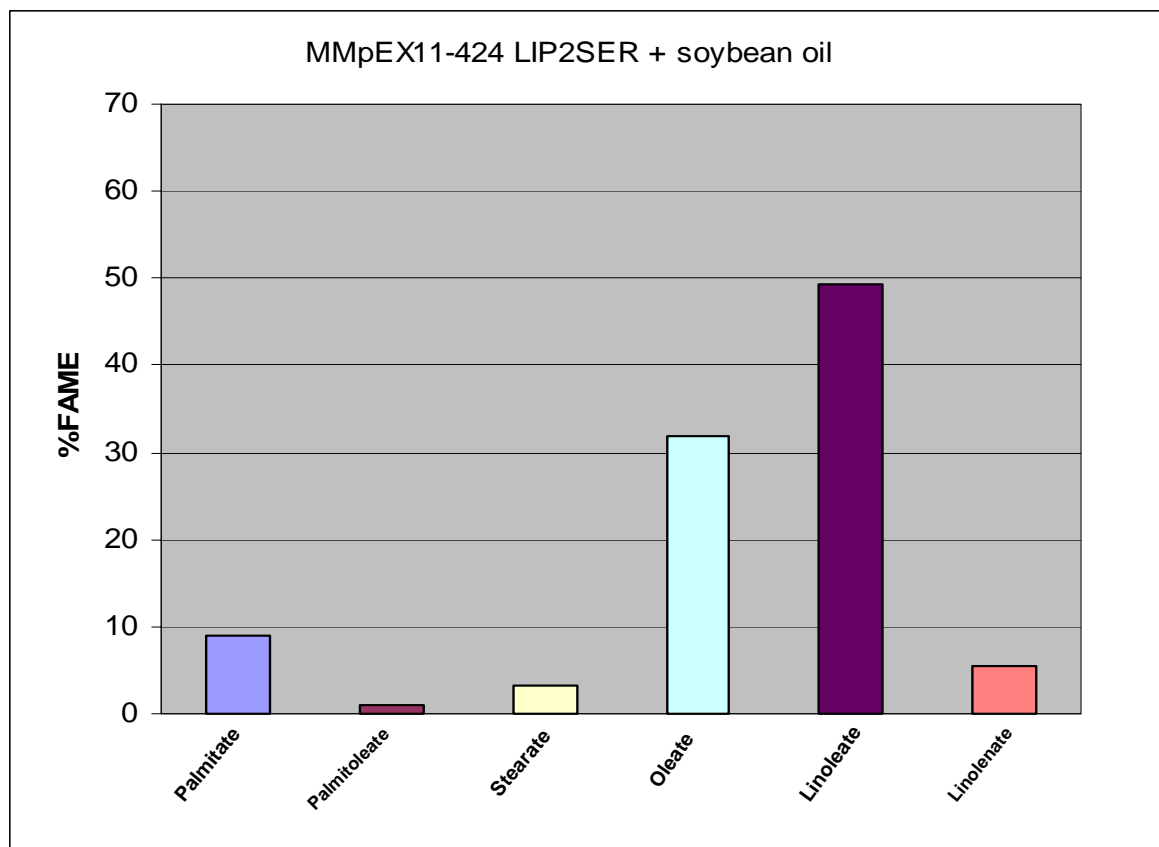


Figure 3-22. Fatty acid composition of pEX11-424 LIP2Ser, grown in the presence of soybean oil.

Discussion

Our laboratory is interested in development of microbial systems that are capable of converting low cost lipids into value added products. It has been demonstrated that *Saccharomyces cerevisiae* (bread yeast) is able to acquire free fatty acids from the growth media and that a portion of these lipids can be converted into new lipid products by transgenically expressed enzymes (Dyer *et al.* 2001). Yeast cells cannot, however, acquire complex lipids such as triacylglycerols from the growth media unless a nonspecific lipase is included to first release the fatty acid components. Economically, it is far more efficient to engineer the yeast cells to secrete this enzyme as needed to complete all of the necessary metabolic steps. In the work described above, we have engineered *S. cerevisiae* for secretion of lipase enzymes. In doing this we hope to use yeast as a platform for lipid metabolic engineering. The power and flexibility of yeast genetics/molecular biology can be used to develop methods for biotechnological conversion of low-cost oils into value-added products. The experimental strategy is to fuse a fatty acid inducible gene promoter to a secreted lipase enzyme sequence. The yeast PEX11 promoter is expressed at low, basal levels in all media and is strongly upregulated in the presence of free fatty acids. Expression of the *Yarrowia lipolytica* LIP2 gene behind the PEX11 promoter will allow basal levels of LIP2 expression in dextrose media, resulting in secretion of a small amount of lipase enzyme into the growth medium. Addition of TAGs will result in breakdown by the LIP2 enzyme, and fatty acid components will be taken up into yeast cells, which will upregulate expression of the LIP2 gene by the PEX11 promoter. Once the TAGs are broken down, fatty acid content of the media will decrease, and LIP2 expression will be downregulated.

Following initial observations and taking into account the features of the yeast secretory pathway and other inherent features of the cell, modifications were made to the signal sequence,

cleavage sites, promoter regions and codon usage of the LIP2 gene. Each modification was made individually and tailored to different phases of the secretory pathway. Through experimentation we found that inserting the lipase gene into *S. cerevisiae* secretes a nonspecific lipase into the growth medium. Early examination of the halo assay led us to believe that modifying the LIP2 gene by inserting a serine codon after the KEX2 protease cleavage site appears to have increased the amount of lipase secreted.

The P'₁ position, which is the amino acid residue immediately following the cleavage site, of the KEX2 gene has been found to exhibit preference for particular features of amino acids rather than strict specificity for a given amino acid residue. Several frequency calculations on the occurrence of particular residues at each position, P₆ – P'₄, illustrated several features that may increase the likelihood of cleavage at dibasic sites (Rholam *et al.* 1995). In analyzing the results for the P'₁ position, resulting in cleavage, it was found that a cleavage favorable P'₁ residue would be hydrophilic, a β -turn former, polar and tiny. From the classification provided, only one amino acid – serine – fit all of the criteria. Further analysis of ratios of amino acids at processing sites (cleaved: uncleaved) revealed another short list of amino acids (Phe, Tyr, His, Ala, Ser, Asn, Asp, Glu) producing a high frequency of cleavage at the P'₁ site. The last test for varying amino acids at the P'₁ position involved an examination of the kinetic parameters of Kex2 endoprotease cleavage. The K_m and V_{max} of several peptide substrates were collected from velocity measurements and plotted versus various substrate concentrations. A ratio of V_{max} to K_m provides data on the efficiency of the enzyme. Only 5 amino acids produced significant frequencies of cleavage, Ala, Asp, His, Ser, and Gly. Based on these results serine proved to be the best substrates for hydrolysis by the Kex2 endoprotease at the P'₁ position (Rholam *et al.* 1995). In choosing an amino acid residue to improve the XPR6 endoprotease signal on the LIP2

gene, serine was the best choice because it provides the properties most likely to cause cleavage at the P'₁ site. It is pertinent to note that in *Y. lipolytica* glutamine occupies the P'₁ position while the position is held by glutamic acid in *S. cerevisiae* (Fuller *et al.* 1999, Enderlin and Ogrydziak 1994). Glutamic acid, glutamine and serine have some features in common which provided further assurance regarding use of serine in the P'₁ position. All three amino acids have polar side chains. Glutamine and serine are both have neutral side chains (Voet *et al.* 1999).

Modifying the LIP2 gene by codon optimization and insertion of the carboxypeptidase Y signal sequence has proved less successful. Halo assays show that these two constructs secrete less lipase than cells containing the LIP2 and LIP2-Ser constructs. CPY is a vacuolar protein and is not normally secreted from the cell unless it is overexpressed (Westphal *et al.* 1996). Lipase may be produced as a result of the LIP2-CPY gene, but it may have been targeted to the vacuole, or the protein may remain in the endoplasmic reticulum.

Codon usage has been linked to gene expressivity (measured using the codon adaptation index). The composition of genes is critical for translational efficiency. Expressivity and codon usage are linked because some tRNAs are more abundant and the use of 'rare' tRNAs can limit translation rate and efficiency. Several studies have revealed that highly expressed genes use the more abundant tRNAs, which avoids the possibility of tRNA depletion during translation. Placement of foreign genes into cells and the resulting expressivity was originally studied in *E.coli*. Foreign genes introduced in *E.coli* having genes corresponding to rare codons, produced low yield despite high amounts of transcripts. When these codons of the introduced gene were point mutated to more commonly used codons, protein yield was improved in some cases. Similar results have been found in *S.cerevisiae*. These findings lend credence to the approach of

using codon optimization of foreign genes to ensure expressivity (Fuglsang 2004). In this study, the LIP2 gene was optimized by GenScript (<http://www.genscript.com>).

The LIP2-opt gene performed well below expectations. The decrease in lipase secretion could be due to several factors: inadvertent creation of an unstable RNA, protein aggregation caused by over expression, or protein folding problems. Another reason could be the loss of translational pausing. Non-optimal codons are defined by low usage and low abundance of corresponding tRNA and have an established role in translational pausing to allow the correct folding of proteins (Zalucki et al 2007). While this is a bacterial example, it is valid to note that the LIP2 lipase may not be produced because it is proceeding through translation too quickly either producing incorrectly folded lipases that are not secreted, or secreted lipase that has no enzymatic activity. Monitoring protein expression by northern blotting or RT-PCR would elucidate this situation.

The NS constructs, constructs with their stop codon removed and ORF fused to a V5/His reporter, were initially viewed as the same as their counterparts containing stop codons. These constructs were created mainly for use with Western blotting for ease of detection. The halo assay revealed that there was no lipase secreted or an inactive lipase was secreted from all of the NS constructs. Yu *et al.* (2007) in working with LIP2 and Fickers *et al.* (2005) working with LIP7 and LIP8 report loss of enzymatic activity and instability in proteins containing C-terminal His tags. The problem with our no stop constructs may lie in the addition of the C-terminal tags. It may be that there is a conserved region in the C-terminal region or that the protein is too rapidly degraded to be detected by the agar plate assay. Furthermore, there is a cysteine residue 3 amino acids from the end of the LIP2 gene. In RAS proteins the cysteine residues are 4 from the end and act as sites for post-translational modification. In our case, the NS gene constructs

may lack post translational modification due to the presence of the His tag. Rather than tag the C-terminal end of the protein, an N-terminal tag attached to the mature protein by site directed mutagenesis may prove successful. Song *et al.* (2006) reported that a 6-His tag at the N-terminus had little effect on the activity or stability of recombinant Lip7 and Lip8 lipases.

The annulus calculation has been helpful in this case, but the results are sharply limited by the human eye and the resolution of the monitor or printer displaying the plate image. Distortions in the plate image can easily be confused for halos. Furthermore cell death and lysis could be contributing to the resultant halo more than previously thought. While the assay is limited, the graph of annulus size in relation to cell number showed that lipase secretion is proportional to cell number, leading us to believe that using the annulus calculation is a good indicator of the quantity of lipase secreted. A sensitive and quantitative lipase assay would provide more definitive data regarding lipase secretion by cells containing each gene construct. We speculate that the spectrophotometric lipase assay did not work because there was not enough enzyme for detection or our lipase did not cleave the substrate used in the assay. While the assay was tested using a commercial lipase, the yeast enzyme may be more selective in cleaving substrates.

The results from gas chromatography are reliable. The results confirm that the lipase secreted from cells containing the LIP2-Ser gene confers uptake of triacylglycerols into yeast cells. All of the cells and experiments were in good working order at the time of culturing, harvesting and transmethylation. More gas chromatography would confirm the current results. While the halo assay confirms the secretion of a lipase and gas chromatography confirms that triacylglycerols are broken down by the lipase and taken up by the cells, there are further question and experiments needed.

Northern blotting would provide data regarding the activity of the LIP2 gene when a lipid is added to the growth media. Specifically, Northern blotting would let us know if the LIP2 gene is temporarily induced when lipid is added to the growth medium and is this induction dependent upon the presence of the LIP2 gene. The experimental setup would use the endogenous PEX11 gene as a control. The probe should be to the PEX11 open reading frame (endogenous gene) rather than the LIP2 gene to garner information on the behavior of the PEX11 promoter in the presence of TAG, but absence of lipase. Actin could be used as a control for the Northern blot. In comparison the wild type + pEX11-LIP2-SER, blotting for PEX11 and LIP2-SER would provide data on the induction of the LIP2 gene in the presence of TAG and the secreted lipase.

Western blotting would provide information on the lipase protein levels detectable in the growth medium. For western blotting, the cells are cultivated under normal conditions; supernatant is collected at specific time points, concentrated and analyzed on protein gel (Coomassie stain). Some of these experiments were performed as a part of this study, but did not produce useable results. In addition to confirming the current results, efforts to engineer *S. cerevisiae* to secrete large amounts of lipase for breakdown of triglycerides and incorporation of fatty acid components should be revived. Such strains of *S. cerevisiae* have previously been described (Okkels 1996), as well as other methods to produce an overproduction of a lipase. These efforts may permit the industrial- scale utilization of *S. cerevisiae* for bioconversion of low-cost starting materials into value-added lipid products.

We attempted to overproduce the LIP2 lipase by creating a system, based on the Leu2 system, but met with little success. Once constructed, mmPEX 11-426ΔPstI was transformed into yeast with very low transformation efficiency. J.S. Okkels (1996) found that deleting the

URA3 promoter region from the pYes2.0 plasmid increases the expression level of a fungal lipase several fold in *S. cerevisiae*. More specifically, the deletion originates 17 bp from the URA3 gene initiation sequence. The deletion includes the RNA polymerase initiation sequences, the TATA box, and the 3' overhang of the *Pst*I site. Overall lipase expression for URA3 deletion plasmids is higher than expression in plasmids containing the promoter region. After removing the *Pst*I sites in pYes2 to accommodate the insertion of the lipase gene from *Humicola lanuginosa*, it was found that plasmids giving the highest number of colonies were lacking the URA3 promoter. In removing the 484 bp *Pst*I fragment, the URA3 promoter region and part of the 2 μ m fragment was removed as well (Okkels et al 1996).

In a practical setting, the URA3 promoter is easily removed from pYes vectors by removing the two *Pst*I sites that flank the entire promoter. Okkels 1996 contends that the increase in expression is a result of poor expression of the URA3 selection marker resulting from the deletion. As a result of this poor expression there is a higher copy number per cell of the plasmid. Higher copy number can increase the transcript level per cell of the plasmid and subsequently the cellular expression level of the gene.

The primary goal of this project is to convert *S. cerevisiae* into a facultative oleaginous microbe through the use of yeast as a platform for lipid metabolic engineering. Complete conversion of *S. cerevisiae* requires four novel functions to occur within the yeast cell: (1) sense TAGs in the growth media, (2) upregulate expression of a lipase gene, (3) secrete a nonspecific lipase, and (4) down regulate gene expression once the TAGs have been utilized. These four functions establish a self-regulating positive feedback loop of gene regulation. The synthesis portion of metabolically engineering yeast involves the introduction of a strong promoter, for gene

regulation, selection of a non-specific lipase for TAG breakdown, and production of a TAG enriched growth media, to activate the promoter.

Establishing this closed system requires several genetic modifications. First we fused a fatty acid inducible gene promoter to a secreted lipase enzyme sequence. The yeast PEX11 promoter is expressed at low, basal levels in all media and is strongly up regulated in the presence of free fatty acids. The PEX11 promoter demonstrated 100-fold induction when cells were grown in oleic acid as the sole carbon source (Marshall *et al.* 1995, Kal *et al.* 1999). Expression of the *Yarrowia lipolytica* LIP2 gene behind the PEX11 promoter will allow basal levels of LIP2 expression in dextrose media, resulting in secretion of a small amount of lipase enzyme into the growth medium. The lipase produced as a result of the LIP2 gene will cleave TAGs in the growth media, and the fatty acid components will be taken up into yeast cells, which will upregulate expression of the LIP2 gene by the pEX11 promoter. Once the TAGs are cleaved, fatty acid content will decrease, and LIP2 expression will be downregulated.

Once these modifications are completed, these objectives can merge with other areas of this project. Another phase in the project is to co-express multiple lipid modifying enzymes to convert the exogenously acquired oils tung like drying oils. Currently there are plasmid-borne expression systems that will allow co-expression of up to 4 different genes in yeast cells (Dyer *et al.* 2002). There are also additional options such as yeast artificial chromosomes available for co-expressing many more genes if necessary. While the primary goal is to use this oleaginous yeast expression system for analysis and reconstitution of the tung oil biosynthetic pathway, the *S. cerevisiae* system allows any lipid-modifying enzyme to be used in our expression system. Therefore, successful development of an efficient bioconversion system could serve as a platform for conversion of low cost lipids into a variety of value added products.

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Appendix

A. Sequence of Oligonucleotide Primers

B. Plasmid Constructs

C. Solutions

D. GenScript Report

E. Additional Methods

Appendix A: Sequence of Oligonucleotide Primers

<u>Name</u>	<u>Sequence</u>	<u>Purpose</u>
LIP2 Forward	5'GCCACCATGAAGCTTTCCACCAT CCTTTTC 3'	To extract the LIP2 gene from <i>Yarrowia lipolytica</i>
LIP2 Reverse	5'CCTTAGATACCACAGACACCCTCG GTGAC3'	
LIP2RevNoStop	5'GATACCACAGACACCCTCGGTGAC GAAGT3'	
GAL1Forward (Invitrogen)	5' AATATACCTCTATACTTTAACGTC3'	To sequence pYes2.1 based plasmids
V5Reverse (Invitrogen)	5'ACCGAGGAGAGGGTTAGGGAT3'	
LIP2SeqForward	5'TCCCGGCACTAAGATCTTCAAGCCC TTC3'	To sequence the LIP2 regions of plasmid constructs
LIP2SeqReverse	5'ACTGCTGGTAACCGTCCCAGAGCAC3'	
SERTop	5'GCCGCAGTTCTCCAGAAGCGATC TGTGTACACCTCTACCGAGACC 3'	To improve the Kex2 cleavage site a serine codon is being inserted into the LIP2 gene
SERBottom	5'GGTCTCGGTAGAGGTGTACACAG ATCGCTTCTGGAGAACTGCGGC 3'	

CPY-Lip2Fusion	5'CCATGAAAGCATTACACCAGTTTAC TATGTGGACTAGGCCTGTCCACTAC ACTCGCTAAGGTGTACACCTCTACC GAGACCTCTCA 3'	To replace the prepro sequence of Lip2 with the carboxypeptidase Y signal sequence
PEXLip2Forward	5'CAGAGAATTTCGCCACCATGAAGCT TTCC ACCATCCTTTTC3'	To add an <i>EcoRI</i> restriction site to the beginning of the LIP2 gene for insertion into the pEX11 vector
PEXLip2Reverse	5'ACTTCCCGGGCTCTTAGATACCACA GAC ACCCTCGGTGAC3'	To add an <i>SmaI</i> restriction site to the end of the LIP2 gene for insertion into the pEX11 vector

Appendix B: Plasmid Constructs

<u>Name</u>	<u>Functional Characteristics</u>
pYes2.1	High copy (2 micron), URA, GAL promoter, CYC terminator
pYES2.1 LIP2 pYES2.1 LIP2NS	Native LIP2 lipase from <i>Y. lipolytica</i>
pYES2.1 LIP2 opt	Native LIP2 lipase, codon optimized
pYES2.1 LIP2-Ser pYES2.1 LIP2-SerNS	LIP2 with improved Kex2 site
pYES2.1 LIP2-CPY pYES2.1 LIP2-CPY (no stop)	LIP2 with a new signal peptide
pEX11	Oil inducible vector, high copy (2 micron), PEX11 promoter and terminator
pEX11-424, pEX11-426	TRP1 selectable marker, URA3 selectable marker
pEX 11-426 LIP2	Native LIP2 lipase from <i>Y. lipolytica</i>
PEX 11-426 LIP2-Ser	LIP2 with improved Kex2 site in oil inducible vector
PEX 11-426 LIP2-opt	Native LIP2 lipase, codon optimized
PEX11-426 Δ PstI	Ultra high copy (Okkels 1996)

NS = No Stop. Indicates V5/His (see Materials and Methods)

Appendix C: Solutions

10x-Ura

For 250 ml (final volume), dissolve 5 g SCM-Ura supplement to sterile water. Cover beaker with foil to protect from light. Stir for at least 1 hour. Filter sterilize. Wrap bottle in foil to protect from light. Store at 4°C.

10x Yeast Peptone (YP)

10% yeast extract, 20% Bacto peptone

For 100 ml, combine 10 g yeast extract and 20 g Bacto peptone in 30 ml sterile water. Stir until dissolved. Add sterile water to 100 ml final volume. Autoclave and store with lid closed tightly until used. Solution contaminates easily.

50X TAE

For 1L, combine 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml 0.5 EDTA (pH 8.0) in 500 ml sterile water. Add water to a final volume of 1L. Store at room temperature and dilute to 1X for use. 50X TAE may also be purchased commercially and diluted for use.

1000X Vitamins

For 1L, add the following: 2 mg biotin, 400 mg calcium pantothenate, 2 mg folic acid, 2000 mg inositol, 400 mg niacin, 200 mg p-amino benzoic acid, 400 mg pyridoxine-HCl, 200 mg riboflavin (does not dissolve completely), and 400 mg thiamine. Stir and store in 50 ml aliquots at 4°C.

1000X Trace Minerals

For 1 L combine the following in 800 ml sterile water: 50 mg boric acid (H_3BO_4), 4 mg copper sulfate (CuSO_4) (or 6.25 mg copper sulfate pentahydrate, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), 10 mg potassium iodide (KI), 20 mg iron (III) sulfate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), 40 mg manganese sulfate tetrahydrate ($\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$) (or 30 mg manganese sulfate, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$), 40 mg zinc sulfate heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) and ammonium molybdate tetra hydrate($(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$). Stir until dissolved, autoclave and store at 4°C.

Ampicillian Stock Solution (100 mg/ml)

For 5ml, dissolve 0.5 g ampicillin sodium salt in 5 ml sterile water. Vortex to dissolve and filter sterilize. Store in 1ml aliquots at -20°C.

Bacterial transformation Buffer (colony method)

For 50 ml, combine 5 ml 1M KCl (final concentration 100mM), 2.25 ml 1M MgCl_2 (45mM), 0.5 ml 1M CaCl_2 (10mM), 0.0401g HACoCl_3 (3mM) and 0.0980g MES (potassium methylethane sulfonate) (10mM) in 50 ml sterile water. Adjust to pH 6.2. Store at 4°C. Shake before use to resuspend the salts.

DEPC treated water (Ambion)

For 1 L, add 1 ml diethylpyrocarbonate (DEPC) to double-distilled, deionized H₂O (final concentration 0.1%). Stir well. Incubate 12 - 24 at 37°C. Autoclave for at least 45 minutes. The scent of DEPC should be either not detectable or only very slightly detectable.

Holland Minimal Yeast Media (HMY)

For 1 L add salts in this order to 600 ml sterile water:

(NH ₄) ₂ SO ₄	2.5g
MgSO ₄ · 7 H ₂ O	0.2g
NaH ₂ PO ₄ · H ₂ O	3.0g
K ₂ HPO ₄ H ₂ O	0.7g

Stir until all salts are dissolved. Adjust the pH to 6.0 with H₂SO₄ or 1M NaOH. Add 10 ml trace minerals. Mix trace minerals well prior to use. Add 0.5g yeast extract. Add sterile water to final volume of 900ml. Filter sterilize or transfer to media bottle, autoclave and cool. Add sterile carbon source and store at 4°C until ready to use.

Lipase stock solution (Sigma) (1 mg/ml)

Add 0.001 g lipase powder to 1 ml deionized, distilled water. Dilute stock solution to 0.5 mg/ml and 0.1 mg/ml.

Luria Bertani (LB) liquid media

For 300 ml, dissolve 7.5 g LB in 200 ml sterile water. Stir until dissolved. Add sterile water to final volume of 300ml. Autoclave and store at room temperature.

LB-Ampicillin (LB-Amp) plates

For agar plates, dissolve 7.5 g LB in 200 ml sterile water. Stir until dissolved. Add sterile water to final volume of 300ml. Add 4.5 g bactoagar and stir until dissolved. Autoclave. Cool solution to 55°C and add 30 µl ampicillin stock solution. Pour 25 ml per petri plate. Cool until agar hardens, store plates at 4°C.

p-Nitrophenyl laurate substrate solution, 420 µM

Place 0.0135 g p-nitrophenyl laurate (molecular weight 321.4), 0.017 g sodium dodecyl sulfate (SDS), and 1.00 g Triton X-100 into a 100 ml volumetric flask and bring to volume with water. Heat the mixture in a water bath at 65° C for 15 minutes, mix well, and let the solution cool to ambient temperature prior to use. Store up to 3 days at 4°C. Reheat if the solution becomes turbid.

p-Nitrophenol standard solution, 0.5 mM

Place 0.0869 g *p*-nitrophenol (molecular weight 139.1) in a 25 ml volumetric flask and bring to volume with 0.1 M Tris·Cl, pH 8.2. Store up to one month in a tightly sealed vessel at room temperature. Dilute 1 volume with 49 volumes of 0.1 M Tris·Cl buffer for a final 0.5 mM *p*-nitrophenol standard solution

SOC liquid media

For 1000 ml (final volume), combine 2g tryptone, 0.5 g yeast extract, 250 µl 1M KCl, 1 ml 1M MgCl₂, 1 ml 1M MgSO₄, and 0.36 g glucose in 600 ml sterile water. Adjust to pH 7.5 with NaOH and bring solution to final volume. Filter sterilize.

SOB agar plates

For 250 ml, combine 5g tryptone, 1.25 g yeast extract, 625 µl 1M KCl, 2.5 ml 1M MgCl₂ and 2.5 ml MgSO₄. Adjust solution to pH 7.5 with NaOH and add sterile water to final volume. Add 3.75 g agar to solution. Autoclave. Cool media to 55°C and pour approximately 25 ml per Petri plate. Allow plates to cool to room temperature. Store at 4°C.

1M Sorbitol

For 50 ml, combine 25 ml 2M Sorbitol and 25 ml sterile water in a graduated cylinder. Cover graduated cylinder with parafilm and mix by inversion.

1M Sorbitol, 20 mM KPi pH 7.5

For 50 ml, combine 25 ml 2M Sorbitol and 2 ml 0.5M KPi, pH 7.5. Add sterile water to reach final volume. Cover graduated cylinder with parafilm and mix by inversion.

Synthetic dextrose (SD) – 10X

2% dextrose, 0.67% YNB,

For 500 ml, dissolve 10g dextrose and 3.35 g yeast nitrogen base (YNB) in 450 ml sterile water (final volume). Filter sterilize and store at 4°C.

Agar plates: For 300 ml, dissolve 6 g dextrose and 2.01 g YNB in 270 ml sterile water (final volume). Add 6 g agar and dissolve. Autoclave. Cool to 55°C and add 30 ml of 10-Ura. Pour approximately 25 ml per plate.

Synthetic galactose (SGal) – 10X

2% galactose, 0.67% YNB

For 500 ml (final volume), dissolve 10g galactose and 3.35 g yeast nitrogen base (YNB) in 450 ml sterile water.

Synthetic glycerol dextrose (SGd)

3% glycerol, 0.1% dextrose, 0.67% YNB

For 500 ml, combine 15 ml glycerol, 0.5 g dextrose, and 3.36 g YNB to 450 ml (final volume) sterile water. Filter sterilize

TES solution

10 mM Tris-Cl, pH 7.5

10 mM EDTA (0.5 M)

0.5 % SDS (stock is 10%)

Can be stored indefinitely at room temperature

TES solution

50 x sample buffer

980 μ L sample buffer

20 μ L β -mercaptoethanol

0.1 M Tris·Cl pH 8.2

For 500 ml dissolve, 6.055 g Tris base in 300 ml deionized, distilled water. Bring solution to desired pH with concentrated hydrochloric acid (HCl_{aq}). Add water to 500 ml and store at room temperature.

Variations:

0.5 M Tris·Cl, pH 6.8, (Okkels 1996)

0.1 M Tris·Cl, pH 7.2 (V. Newman, personal communication 2003)

0.1 M Tris-SO₄ pH 9.2, 10 mM DTT

For 50 ml, combine 5 mL 1 M Tris SO₄, pH 9.2 and 0.08g DTT in 30 ml sterile water. Add sterile water to a final volume of 50 ml. Cover graduated cylinder with parafilm and mix by inversion.

Yeast Peptone Dextrose (YPD)

(1% yeast extract, 2% peptone, 2% dextrose) 2% agar for plates

For 300 ml, dissolve 15g YPD Broth sterile water. Stir until dissolved. Add 6 g agar for plates. Autoclave.

Zymolyase 20T

2 ug/OD, and volume of 10ug/uL Zymolyase stock needed to give desired amount of enzyme/sample.) Total OD (2ug/OD)=ug Zym.(1ul/10ug)=ul Zym. Soln.

Appendix D: GenScript Report



Codon Optimization Result

(confidential)

Organism: *Saccharomyces cerevisiae* ;

Gene Name: Gene_opt

Sequence Type: aa

Optimization Region: 13 - 1020

GC Range: 30 - 70

Addition 5' Sequence: GAATTCGCCACC

Addition 3' Sequence: AGATCT

Cut Offs:

Secondary Structure Stack Cutoff: 35

Repeat Cutoff: 21

Relative Frequency Cutoff: 50

5' Splice Score Cutoff: 85

Genetic Code: 1

RE Sites and CIS Pattern:

EcoRI (GAATTC), **BglII** (AGATCT), splice(GGTAAG), splice(GGTGAT), polyA(AATAAA), polyA(AAAAAA), destabilizing(ATTTA), polyt(TTTTTT), polyA(AAAAAA)

RE Check Sites: **SmaI** (CCCGGG), **EcoRV** (GATATC)

RE Keep Sites:

Protein Sequence:

MKLSTILFTACATLAAALPSPITPSEAAVLQKRSVYTSTETSHIDQESYNFFEKYARLAN
IGYCVGPGTKIFKPFNCGLQCAHFPNVELIEEFHDPRLIFDVSGYLAVDHASKQIYLVIR
GTHSLEDVITDIRIMQAPLTNFDLAANISSTATCDDCLVHNGFIQSYNNNTYNQIGPKLDS
VIEQYPDYQIAVTGHS LGGAAALLFGINLKVNGHDPLVVTLGQPIVGNAGFANWVDKLFF
GQENPDVSKVSKDRKLYRITHRGDIVQVPFWDGYQHCSGEVFIDWPLIHPPLSNVVMCQ
GQSNKQCSAGNTLLQQVNVIGNHLQYFVTEGVCGI*

Protein Alignment (Optimized Region):

Optimized	1	MKLSTILFTACATLAAALPSPITPSEAAVLQKRSVYTSTETSHIDQESYNFFEKYARLAN
Original	1	MKLSTILFTACATLAAALPSPITPSEAAVLQKRSVYTSTETSHIDQESYNFFEKYARLAN
Optimized	61	IGYCVGPGTKIFKPFNCGLQCAHFPNVELIEEFHDPRLIFDVSGYLAVDHASKQIYLVIR
Original	61	IGYCVGPGTKIFKPFNCGLQCAHFPNVELIEEFHDPRLIFDVSGYLAVDHASKQIYLVIR
Optimized	121	GTHSLEDVITDIRIMQAPLTNFDLAANISSTATCDDCLVHNGFIQSYNNNTYNQIGPKLDS
Original	121	GTHSLEDVITDIRIMQAPLTNFDLAANISSTATCDDCLVHNGFIQSYNNNTYNQIGPKLDS
Optimized	181	VIEQYPDYQIAVTGHS LGGAAALLFGINLKVNGHDPLVVTLGQPIVGNAGFANWVDKLFF
Original	181	VIEQYPDYQIAVTGHS LGGAAALLFGINLKVNGHDPLVVTLGQPIVGNAGFANWVDKLFF
Optimized	241	GQENPDVSKVSKDRKLYRITHRGDIVQVPFWDGYQHCSGEVFIDWPLIHPPLSNVVMCQ
Original	241	GQENPDVSKVSKDRKLYRITHRGDIVQVPFWDGYQHCSGEVFIDWPLIHPPLSNVVMCQ
Optimized	301	GQSNKQCSAGNTLLQQVNVIGNHLQYFVTEGVCGI*
Original	301	GQSNKQCSAGNTLLQQVNVIGNHLQYFVTEGVCGI*

Optimized Sequence: Length: 1026, GC%:37.33, Minimum Free Energy:-247.30

GAATTCGCCACCATGAAATTGTCTACTATTTTATTCACAGCTTGCGCTACCTTGGCTGCT
GCATTACCATCCCCAATCACTCCTTCTGAAGCAGCCGTTTTCAGAAAAGAGAAGTGTCTAC
ACTTCTACTGAAACTTCACATATTGACCAAGAATCTTATAATTTCTTGAAAAATACGCC
AGATTAGCAAATATCGGTTATTGTGTCGGTCCTGGTACCAAGATTTTAAACCATTCAAC
TGTGGTTTACAATGTGCTCATTTTCCAAATGTAGAATTAATAGAAGAATTTTCATGATCCT
AGATTGATTTTCGATGTATCAGGTTACTTAGCAGTTGACCATGCTTCTAAACAAATATAC

TTAGTCATCAGAGGTACACACTCCTTAGAAGATGTTATAACAGACATCAGAATTATGCAA
 GCCCCTTTAACCAATTTTGACTTAGCTGCTAACATTCCTCTACAGCTACATGTGACGAT
 TGTTTGGTCCACAACGGTTTTATACAAAGTTACAACAACACTTATAACCAAATTGGTCCT
 AAATTGGATTCAAGTTATCGAACAATATCCAGATTACCAAATTGCTGTCACTGGTCATTCA
 TTAGGTGGTGCTGCAGCCTTATTGTTCCGGTATAAAATTGAAAGTAAACGGTCATGATCCT
 TTAGTTGTTACATTAGGTCAACCAATTGTTGGTAATGCAGGTTTCGCCAACTGGGTCGAT
 AAGTTATTTTTTCGGTCAAGAAAACCTGATGTATCCAAGGTATCCAAAGACAGAAAGTTG
 TATAGAATTACTCATAGAGGTGACATAGTTCCACAAGTTCATTTTGGGATGGTTACCAA
 CATTGTAGTGGTGAAGTTTTTATCGACTGGCCATTGATTCATCCACCATTGTCTAATGTT
 GTCATGTGTCAAGGTCAATCCAATAAGCAATGCTCTGCAGGTAACACTTTATTGCAACAA
 GTAAATGTAATCGGTAACCACTTGCAATATTTTGTAAGTGAAGGTGTTTGCAGGTATCTAA
 AGATCT

Restriction Enzymes:

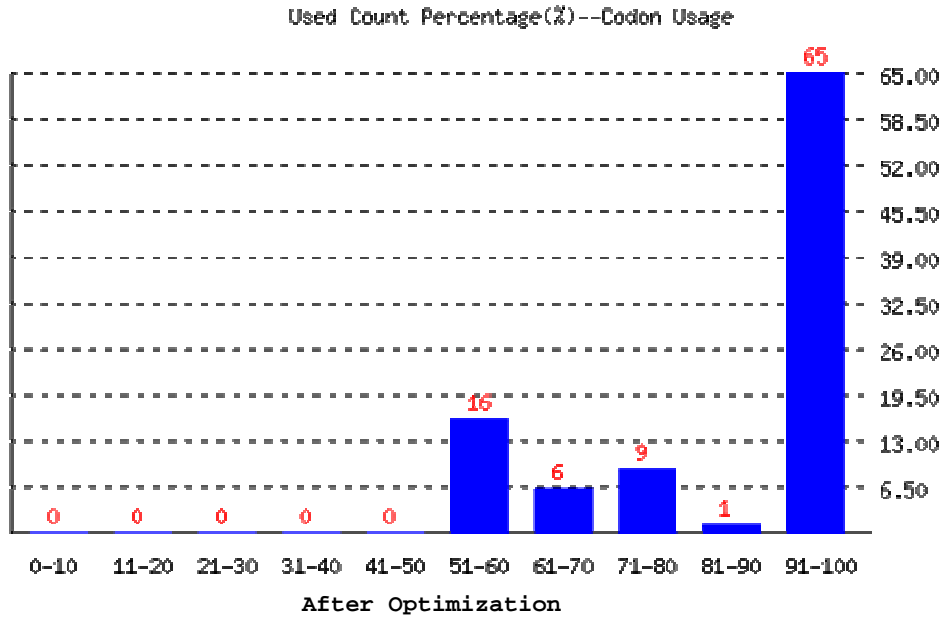
Fitering Enzymes and CIS Pattern:

EcoRI(GAATTC): 1 (1 - GAATTC)
 BglII(AGATCT): 1 (1021 - AGATCT)
 splice(GGTAAG): 0
 splice(GGTGAT): 0
 polyA(AATAAA): 0
 polyA(AAAAAA): 0
 destabilizing(ATTTA): 0
 polyt(TTTTTT): 0
 polyA(AAAAAA): 0

Checking Enzymes:

SmaI(CCCGGG): 0
 EcoRV(GATATC): 0

Keeping Enzymes:



Codon Frequency Table Used:

TTT 26.1(165543)	TCT 23.4(148304)	TAT 18.8(118913)	TGT 8.0(50767)
TTC 18.4(116276)	TCC 14.2(90037)	TAC 14.8(93431)	TGC 4.8(30093)
TTA 26.2(165644)	TCA 18.7(118123)	TAA 1.0(6581)	TGA 0.7(4222)
TTG 27.1(171648)	TCG 8.6(54354)	TAG 0.5(3226)	TGG 10.4(65681)
CTT 12.2(77527)	CCT 13.5(85758)	CAT 13.7(86674)	CGT 6.5(40930)
CTC 5.5(34552)	CCC 6.8(43129)	CAC 7.8(49136)	CGC 2.6(16463)
CTA 13.4(84648)	CCA 18.2(115045)	CAA 27.3(173012)	CGA 3.0(19067)

CTG 10.5(66471)	CCG 5.3(33393)	CAG 12.2(77168)	CGG 1.8(11103)
ATT 30.1(190652)	ACT 20.2(127988)	AAT 35.8(226818)	AGT 14.2(89887)
ATC 17.1(108276)	ACC 12.6(79840)	AAC 24.9(157427)	AGC 9.7(61558)
ATA 17.8(112751)	ACA 17.7(112317)	AAA 42.0(265942)	AGA 21.3(134653)
ATG 20.9(132601)	ACG 8.0(50519)	AAG 30.9(195431)	AGG 9.3(58663)
GTT 22.0(139064)	GCT 21.1(133660)	GAT 37.7(238643)	GGT 23.9(151282)
GTC 11.7(73837)	GCC 12.6(79826)	GAC 20.3(128466)	GGC 9.8(62157)
GTA 11.8(74832)	GCA 16.2(102601)	GAA 45.7(289526)	GGA 10.9(69181)
GTG 10.8(68181)	GCG 6.2(39179)	GAG 19.2(121785)	GGG 6.0(38293)

Appendix E: Additonal Methods

Spectrophotometric Determination of Lipase Activity using *p*-Nitrophenyl Laurate as Substrate (Pinsirodom and Parkin 2006)

This method quantifies the level of *p*-nitrophenol (λ_{max} 400 to 410 nm) released after the hydrolysis of *p*-nitrophenyl laurate substrate by lipase. Lipase activity can be calculated by comparing sample A_{410} values to those of a standard curve prepared with *p*-nitrophenol. While *p*-nitrophenyl laurate is considered a model, synthetic substrate, it provides only a presumptive test for lipase activity. The use of *p*-nitrophenyl acyl esters as chromogenic substrate analogs provides for a continuous, spectrophotometric assay. This assay is an effective method for sample screening or to provide an initial assessment of suitable assay conditions.

Cell Culturing. Yeast cells were prepared as follows: Single colonies were inoculated into SD liquid media and grown overnight at 30°C and 300 rpm. Cell growth was measured by optical density (OD) of the cultures at 600 nm on a spectrophotometer. The volume of culture representing 25 OD units was calculated and transferred to a centrifuge tube. The cells were then centrifuged for 10 minutes and 4000 rpm at 25 - 28°C. The supernatant was discarded. The cells were resuspended in a small volume of synthetic galactose and transferred to 100 ml synthetic galactose – 10 X URA media in a 500 ml flask (Cell density was $.25\text{OD}/3 \text{ ml} = 0.75 \text{ OD/ml}$).

Positive control assay. The *p*-nitrophenol standard curve was omitted because no *p*-nitrophenol was available. Beer's law and the extraction coefficient coupled with known information about *p*-nitrophenol were used to produce a standard curve.

Spectrophotometric Lipase Assay. For each lipase activity, 2.5 ml of 0.1 M Tris·Cl buffer, pH 8.2 and 2.5 ml of 420 μM *p*-nitrophenyl laurate substrate solution were combined in a 15 ml test

tube. An extra test tube containing all reagents and 1 ml of water was prepared as a reagent blank. The spectrophotometer was set to the Lip2_1 program, a Kinetics/Time program that recorded A_{410} every 5 seconds for 5 minutes. 1 ml of the reagent blank was transferred to a cuvette, then to the spectrophotometer. The instrument was set to blank. 1 ml of the lipase solution was added to the next reagent containing solution to initiate the reaction. The mixture was vortexed briefly and 1 ml of the reaction mixture was immediately transferred to a cuvette then to the spectrophotometer. A_{410} was recorded every 5 seconds for 5 minutes. The reactions using cell culture was performed in the same manner as the lipase solution. The graph and resulting raw data were printed and saved. The p-nitrophenol standard curve was used to convert absorbances to mM substrate hydrolyzed using the following formula; $\mu\text{mol } p\text{-nitrophenol/ml reaction mixture} = (A_{410} - y \text{ intercept}) / (\text{slope} \times 6 \text{ ml reaction mixture})$. Lipase activity was determined by constructing a reaction progress curve. A reaction progress curve was generated using the concentration of p-nitrophenyl (mM) released versus reaction time

To optimize this reaction, several variations were employed. Instead of 0.1 M Tris·Cl buffer, 0.5 M Tris·Cl, pH 6.8, (Okkels 1996), 0.1 M or 0.5M Tris·Cl, pH 7.2 (V. Newman, personal communication 2003), were employed as part of the substrate mixture. In other trials the cell cultures were centrifuged for 10 minutes at 14,000 rpm and the supernatant removed. The supernatant fraction was used instead of cell culture.

Vita

Gaynelle Lucretia Stewart Davis was born on May 22, 1979 to Gustave and Betty Stewart. She received her Bachelor of Arts in Liberal Arts and Sciences with a Scientific Inquiry concentration and Bachelor of Science in Biological Science from the Louisiana Scholars College at Northwestern State University in Natchitoches, LA in 2001. She received her Masters of Science in Biological Science from the University of New Orleans in 2007.