

Exploring the effects of evolution of a hybrid Fatty Acid Synthase type II in *Saccharomyces cerevisiae*

João Pedro Gomes Cruz

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Trabalho efetuado sob a orientação do(a)
Professor Doutor Björn Fredrik Johansson
Doutora Tatiana Andréevna Pozdniakova

Janeiro 2023

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STATEMENT OF INTEGRITY

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Explorando os efeitos da evolução da via híbrida de síntese de ácidos gordos do tipo II em *Saccharomyces cerevisiae*

RESUMO

A necessidade na substituição dos combustíveis fósseis inflamou o crescente interesse por biomoléculas renováveis, como os ácidos gordos (FA) de cadeia média. A sua produção atual em sistemas biológicos não atinge um rendimento suficientemente alto para que seja economicamente viável. A levedura *Saccharomyces cerevisiae* é um modelo genético e bioquímico amplamente utilizado, assim como um organismo de produção industrial. A sua produção de FA está associada a um complexo proteico multifuncional, codificado pelos genes *FAS1* e *FAS2*. Em outros organismos, como *Escherichia coli* e *Arabidopsis thaliana*, a mesma via é catalisada por enzimas monofuncionais expressas por genes únicos. Uma via monofuncional híbrida de *E. coli* e *A. thaliana* expressando doze genes individuais fora previamente desenvolvida, sendo o objetivo deste trabalho a investigação das consequências fisiológicas da substituição dos genes nativos *FAS1* ou *FAS2* pela mesma. As leveduras recombinantes foram sujeitas a um processo de adaptação em vários meios sem FA adicionados, assim como cada unidade genética fora explorada pela introdução de cópias adicionais de genes da via. Os perfis lipídicos foram determinados por fluorometria, gravimetria e cromatografia gasosa. As estirpes dependentes da via híbrida para a síntese de FA cresceram a um ritmo lento, com uma fase de latência extensa em comparação com a estirpe controlo. Em termos lipídicos, houve uma redução de 7 vezes no ácido Oleico em comparação com o sistema nativo. A adaptação das estirpes proporcionou um crescimento consideravelmente mais rápido e uma fase de latência reduzida. Cópias adicionais dos genes *fabH* de *E.coli* e *mod1* de *A. thaliana* resultaram numa maior produção de C14 e a diminuição da saturação face aos determinados pela estirpe controlo.

Palavras chave: Ácido gordo sintase tipo I; Ácido gordo sintase tipo II; Ácido gordo; Adaptação metabólica; Engenharia metabólica.

Exploring the effects of evolution of a hybrid Fatty Acid Synthase type II in *Saccharomyces cerevisiae*

ABSTRACT

The urgency in substituting fossil fuels has triggered a growing interest in renewable biomolecules, such as medium-chain fatty acids (FA). Current fatty acid production in biological systems does not reach high enough titers for this to be economically viable. The yeast *Saccharomyces cerevisiae* is a widely used genetic and biochemical model and an industrial production organism. Yeast FA pathway is associated with a multifunctional protein complex, encoded by the *FAS1* and *FAS2* genes. In other organisms such as *Escherichia coli* and *Arabidopsis thaliana*, the same pathway is catalyzed by monofunctional enzymes expressed by single genes. A hybrid *E. coli* and *A. thaliana* monofunctional pathway expressing twelve individual genes had been previously established and this work aimed at investigating the physiological consequences of replacing the function of native *FAS1* and *FAS2* genes with this pathway. Recombinant yeast strains were subjected to adaptation on media without exogenous FA and gene dosage was explored by adding additional copies of individual pathway genes. Lipid profiles were determined by fluorometry, gravimetry and gas chromatography. Strains relying on the hybrid pathway for fatty acid synthesis grew at a low rate and with a prolonged lag phase compared to the control strain. In terms of lipids, a 7-fold reduction in Oleic acid compared to those with the native system. Strain adaptation resulted in strains that grew considerably faster and with a reduced lag phase. Additional copies of either *E. coli fabH* or *A. thaliana mod1* genes resulted in a greater production of C14 FA, and a decrease in saturation compared to the results determined by the control strain.

Keywords: Fatty acid synthase type I; Fatty acid synthase type II; Fatty acid; Metabolic adaptation; Metabolic engineering.

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ABBREVIATIONS

ACP – Acyl carrier protein;

ATP – Adenosine triphosphate;

bp – base pairs;

DMSO – Dimethyl sulfoxide;

EtOH – Ethanol;

FA – Fatty acid;

FAME – Fatty acid methyl ester;

FAS – Fatty acid synthase;

FAS I – FAS type I;

FAS II – FAS type II;

FID – Flame ionization detector;

G418 – Geneticin;

GC – Gas chromatography;

GRAS – Generally recognized as safe;

Hmy– Myristic acid;

HPLC – High-performance liquid chromatography;

LCFA – Long-chain fatty acid;

MCFA – Medium chain fatty acid;

MM – Master mix;

MS – Mass spectrometry;

MW – Weight marker;

NADPH – Nicotinamide adenine dinucleotide phosphate;

OD_{600} – Optical density at 600 nm;

PBS – Phosphate-buffered saline;

PCR – Polymerase chain reaction;

PPT – Phosphopantetheinyl transferase;

PTFE – Polytetrafluoroethylene;

RCF – Relative centrifugal force;

RI – Refractive index;

RFU – Relative fluorescence unit;

RNA - Ribonucleic acid;

RPM - Rotations per minute;

SCFA – Short chain fatty acid;

SC – Synthetic Complete;

SD – Standard deviation;

SOC – Super Optimal Broth with glucose;

TCA – Citrate cycle;

TEM - Transmission electronic microscopy;

UV - Ultraviolet;

VLCFA – Very long chain fatty acid;

YNB – Yeast nitrogen base;

YPD – Yeast extract peptone dextrose;

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1. Introduction

1. Introduction

The use of various microorganisms is centered on a practice that has been adopted by humans for the most basic fermentation processes, such as the production of wine, bread, beer and in others procedures that require the capabilities of microorganisms (Legras et al., 2007). Despite a practice rooted in the beginnings of human knowledge, it remains essential for more recent procedures, such as the synthesis of insulin (Baeshen et al., 2014), vitamins (Sun et al., 2019) and biopharmaceutical compounds (Kulagina et al., 2021).

1.1 Yeast, the biological friend of the human factory

One of these fermentative microorganisms corresponds to *Saccharomyces cerevisiae*, a species of single-cell eukaryotic fungi with a globular shape (Tofalo et al., 2013), considered a key model for eukaryotic organisms as it has characteristics such as short duplication time, ability to grow under very varied conditions, ease of genetic transformation, low cultivation costs and a fully sequenced genome (Kovačević, 2015; Sherman, 2002). Considered a generally recognized as safe organism (GRAS; Sewalt et al., 2016), it is present in several of the processes, playing an important role in the fermentation of wine and bread (Birch et al., 2013; K. Fugelsang & Edwards, 2007).

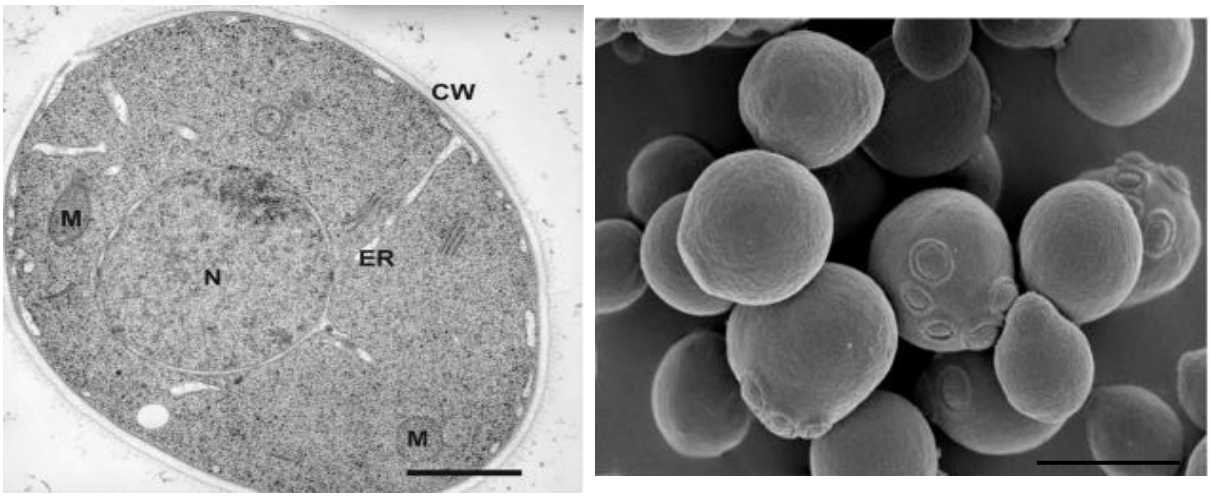


FIGURE 1.1: Morphology of yeast cells. Left: Cells were high-pressure frozen, freeze-substituted in acetone and embedded in a mixture of Epon-Spurr's resin. CW- cell wall; ER- endoplasmic reticulum; M- mitochondria; N- nucleus. Scale Bar- 1.0 µm. Transmission electronic microscopy (TEM) image (Frank et al, 2015). Right: Cell morphology detected by field emission scanning electron microscopy. Scale Bar- 5.0 µm (Liu et al., 2022).

1.1.1 Metabolism network of yeast

In metabolic terms, the adenosine triphosphate (ATP) production process is based on the oxidation of compounds, like alcohols, organic acids, FAs and amino acids that depend on the conditions of the cell and the environment (Walker & Stewart, 2016). However, there is a preference to metabolize sugars, which can be hexoses, disaccharides or even two-carbon compounds (Rodrigues et al., 2006). The oxidation of the carbon source makes ATP via substrate-level phosphorylation (in the case of fermentation) or oxidative phosphorylation (in the case of oxidative catabolism) (Somsen et al., 2000). In both cases, the sugars are metabolized to pyruvate, a central molecule for yeast metabolism - **Figure 1.2**.

S. cerevisiae ferments sugars to ethanol even in the presence of oxygen, a phenomenon called the Crabtree effect, resulting in the repression of respiratory enzymes. The partial oxidation of the carbon source by the fermentation process results in a lower generation of ATP (2 molecules instead of the 18 obtained by one molecule of glucose through the aerobic route). From the cell point of view, fermentation can be the rational choice since fewer and fewer enzymes are involved and the ATP generation is faster. This pathway also produces less toxic reactive oxygen species associated with oxidative catabolism (Goddard, 2008; Pfeiffer & Morley, 2014).

For aerobic respiration, a two-carbon unit in form of a pyruvate molecule is directed to the citrate cycle (TCA), in the form of acetyl-CoA and undergoes a series of reactions, where the main goal is to provide reducing equivalents for the respiratory chain. However, this cycle also provides a biosynthetic function in the generation of metabolites that can be used in other pathways (Rodrigues et al., 2006).

The electron respiratory chain uses the reducing power of all these processes in which the chemical energy stored in these molecules will be converted into potential energy in the form of a proton gradient (Schäfer, 2018). Traditionally, the chain is composed of five complexes, but in the case of *S. cerevisiae*, only three are present: the mitochondrial dehydrogenase supercomplex (Matus-Ortega et al., 2015), the super complex bc1-COX and the F_1F_0- ATP synthase complex (Stuart, 2008).

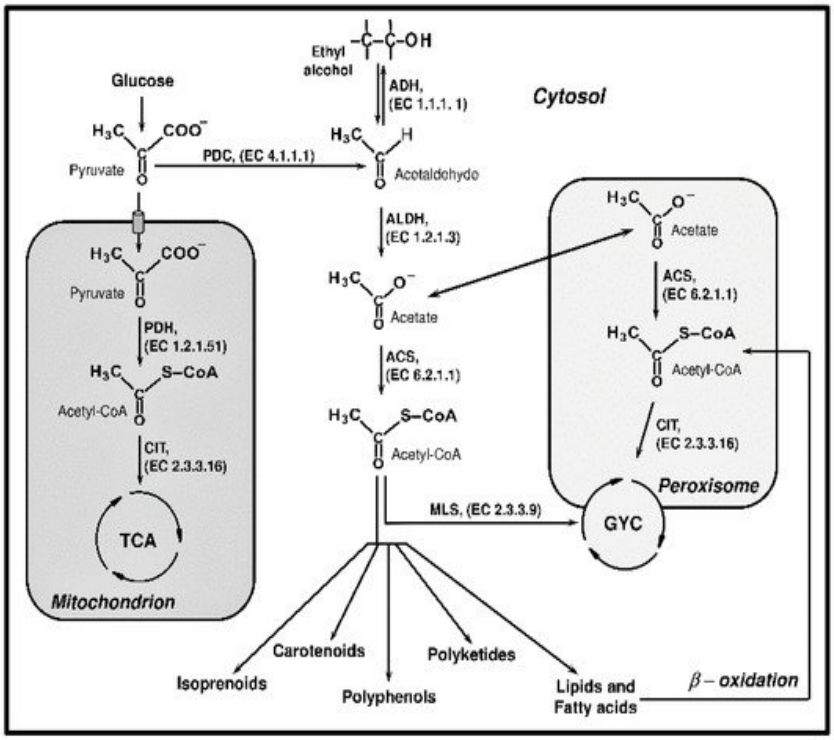


FIGURE 1.2: Schematic representation of the principal pyruvate "fate" in *S. cerevisiae*. Depending on the conditions of the biological system, pyruvate may travel to the citrate cycle (TCA) corresponding to aerobic respiration. If this is not possible, the yeast opts for the fermentation process with the production of ethanol. In both cases, energy is produced (Chrzanowski, 2020).

When aerobic respiration is not possible, either by the absence of oxygen or by high concentrations of glucose, the metabolism is directed towards fermentation, where the accumulation of pyruvate in the cell forces its decarboxylation to the acetaldehyde, and through the action of the alcohol dehydrogenase, to ethanol with NAD^+ regeneration - **Figure 1.2**. This keeps the redox potential balanced (Otterstedt et al., 2004) allowing the continuation of the glycolytic process and consequently the extension of energy production (Walker & Stewart, 2016). Yeast is also capable of producing glycerol, an important metabolite that allows the formation of NAD^+ under anaerobic conditions or hyperosmotic stress (Modig et al., 2007), as well as acetic and propionic acids (Ahmadi et al., 2017; Giannattasio et al., 2013). These products can be used as carbon sources after hexose consumption.

In addition to the metabolic products generated, the fundamental role of metabolism is centered on ATP synthesis. This molecule conserves energy for several cellular processes, either

catabolic, such as the initiation of the glycolytic pathway (Hynne et al., 2001) or anabolic, such as the production of proteins, nucleic acids and lipids (Pontes et al., 2015). The molecule is also involved in cell homeostasis, required for the maintenance of intracellular pH (Pampulha & Loureiro-Dias, 2000) or in division processes (Caplow, 2020).

S. cerevisiae still requires a source of nitrogen, as it cannot fix atmospheric form. Therefore, it depends on its assimilation, either in the form of organic nitrogen (e.g. amino acids) or inorganic nitrogen (e.g. ammonium salts). The role of nitrogen is important in anabolism to produce functional enzymes (Walker & Stewart, 2016). Nitrogen also plays a regulatory role in TCA, through the α-ketoglutarate, influencing the central metabolic pathway of the yeast (Aon & Cortassa, 2001).

1.1.2 Yeast growth

In terms of growth, the yeast follows the microbial growth model - **Figure 1.3**. Its optimal growth conditions are 32.3 °C (Salvadó et al., 2011) at pH 4 (Salari & Salari, 2017), with an average doubling time of 90 minutes (Sherman, 2002). After incubation, cell growth enters a latency phase called the lag phase, which allows the adaptation to the new conditions of the culture medium (Rolfe et al., 2012). An exponential phase of cell growth follows, reaching the maximum growth rate. The presence of an excess of nutrients leads to the channeling of carbon and nitrogen to the production of biomolecules (carbohydrates, lipids, proteins and nucleic acids) essential for cell proliferation. While the beginning of this stage corresponds to the fermentation of sugars, the later stages of this are characterized by the respiration of certain metabolites, such as ethanol. This metabolism switch is called the diauxic shift (Gibb, 2018). With the scarcity of nutrients and the accumulation of toxic metabolites for growth, the stationary phase is reached in which growth is residual, but cells remain metabolically active (Jaishankar & Srivastava, 2017).

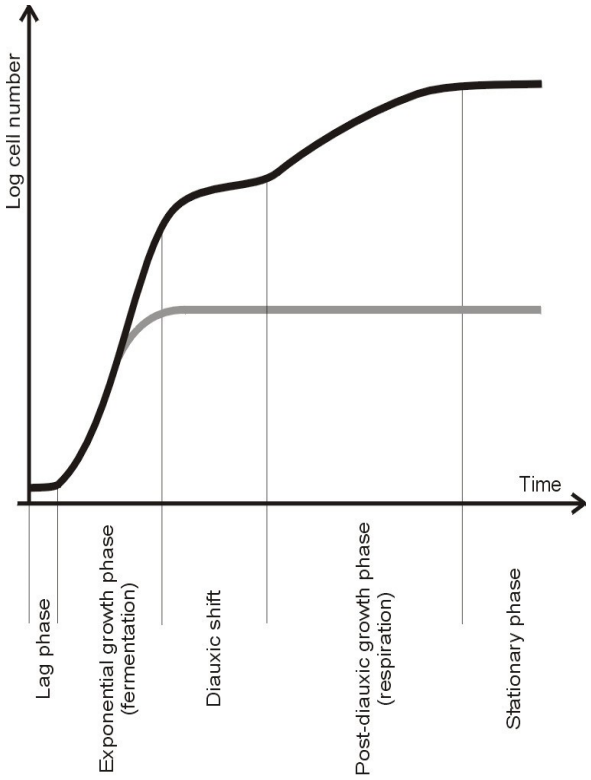


FIGURE 1.3: Characteristic growth model of *S. cerevisiae*. Black line- Under optimal growth conditions, yeast enters an adaptation phase (lag phase) after inoculation in the new medium. The fermentation of sugars leads to exponential growth, which due to the limitation of the carbon source, enters in the diauxic shift. Thus, the metabolism is reprogrammed for the respiration of the compounds generated in the fermentation, allowing their growth at a lower rate than that recorded. In an environment adverse to cell growth, cells enter in the stationary phase of growth to ensure their survival in that conditions. Grey line- The transfer of cells in the exponential phase to a culture medium that does not have the minimum growth conditions implies that the cells enter a stationary phase enhanced by nutritional deficiency (Swinnen et al., 2006) .

1.2 Lipids – the good, the bad and the powerful molecule

Lipids are a family of organic molecules present in all living organisms (Mbuyane et al., 2021) and characterized by their hydrophobic nature. These molecules participate in various cellular functions, such as biological scaffolding in cell membranes (Ferreri et al., 2017), energy reserves (Turner et al., 2014) and precursors of cofactor molecules such as biotin (H. Wu et al., 2005). They are also potent signaling molecules, regulating various cellular processes such as gene expression and direct enzyme activities (Papackova & Cahova, 2015). This family of biomolecules includes triacylglycerides (Sorger & Daum, 2003), sterol (Piva et al., 2022), vitamins (Klassert et al., 2014) and FAs.

Oleaginous microorganisms stand out from other microorganisms due to the amount of lipids they have, about 20% of cell mass (Meng et al., 2009). Within this group, microorganisms like bacteria, filamentous algae and yeasts have a prominent role (Caporusso et al., 2021). However, due to its duplication capabilities and its ease of cultivation, yeast assumes a role of excellence among these (Adrio, 2017). Species such as *Yarrowia lipolytica* (J. Wang et al., 2011), *Rhodotorula glutinis* (Pi et al., 2018) and *Rhodosporidium toruloides* (Singh et al., 2018) constitute some of the yeasts included in this group. *S. cerevisiae* is considered non-oleaginous, having no more than 10-15% of cell mass (Knoshaug et al., 2018).

1.2.1 Fatty acids – small but “huge” molecules

FAs are organic molecules that have a carboxyl group and carbon chain that varies in length. They are rarely found in their free form, organised into more complex structures to prevent effects related to lipotoxicity (Eisenberg & Büttner, 2014). FAs are categorised by chain length: up to 6 carbons long, they are classified as short chain fatty acid (SCFA); between 6 to 12 carbons are designated as medium chain fatty acid (MCFA); between 12 and 22 carbons are identified as long chain fatty acid (LCFA) and after 22 carbons referred to as very long chain fatty acid (VLCFA). They are also classified according to the number of unsaturations, which correspond to the number of double bonds, predominantly in the *cis* configuration, present in the carbon chain. Thus, three classes of FAs appear: saturated, which corresponds to those that do not present any double bond in the carbon chain; unsaturated, a denomination for FAs that has a double bond; polyunsaturated, when two or more double bonds are present in the carbon chain. Some of the most common FAs

in biological systems correspond to those with an even number of carbon atoms, with LCFA being the most prevalent FA class - **Table 1.1**.

TABLE 1.1: Main FAs in biological systems.

Common name	*Systematic name*	*Systematic nomenclature*	*Structure*
Capric acid	Decanoic acid	10:0	$CH_3(CH_2)_8COOH$
Lauric acid	Dodecanoic acid	12:0	$CH_3(CH_2)_{10}COOH$
Myristic acid	Tetradecanoic acid	14:0	$CH_3(CH_2)_{12}COOH$
Palmitic acid	Hexadecanoic acid	16:0	$CH_3(CH_2)_{14}COOH$
Stearic acid	Octadecanoic acid	18:0	$CH_3(CH_2)_{16}COOH$
Arachidic acid	Eicosanoic acid	20:0	$CH_3(CH_2)_{18}COOH$
Unsaturated fatty acids			
Palmitoleic acid	9-Hexadecanoic acid	16:1n7	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$
Oleic acid	9-Octadecanoic acid	18:1n9	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$
Linoleic acid	9,12-Octadecanoic acid	18:2n6	$CH_3(CH_2)_4(CH=CHCH_2)_2(CH_2)_6COOH$
α-Linolenic acid	9,12,15-Octadecatrienoic acid	18:3n3	$CH_3CH_2(CH=CHCH_2)_3(CH_2)_6COOH$
γ-Linolenic acid	6,9,12-Octadecatrienoic acid	18:3n6	$CH_3(CH_2)_4(CH=CHCH_2)_3(CH_2)_3COOH$

1.2.2 Microbial fatty acids synthesis system

FAs synthesis consists of several enzymatic reactions that vary according to the type of FA produced – **Figure 1.4**. Starting with acetyl-CoA, the crossroad molecule between anabolism and catabolism (L. Shi & Tu, 2015), the process can be characterized by 3 phases: initiation, elongation, and release. The synthesis begins with the transfer of a carboxylic group to acetyl-CoA, forming a malonyl-CoA molecule, requiring a biotin molecule as an enzyme cofactor as well as energy in the form of an ATP molecule. This reaction is the limiting step in the FAs production process (Sheng & Feng, 2015). The following reactions occur with the binding of the product to the acyl carrier protein (ACP), in which the introduction of a post-translational modification (4′-phosphopantetheine) allows the enzyme residue binding to ACP through a phosphodiester bond (Majerus, 1968). After activation of the acetyl and malonyl groups by the respective transferases,

there is the condensation of malonyl-[acp] with acetyl-[acp], leading to the production of acetoacetyl-[acp] and CO_2 from the malonyl group (H. Zhang et al., 2010).

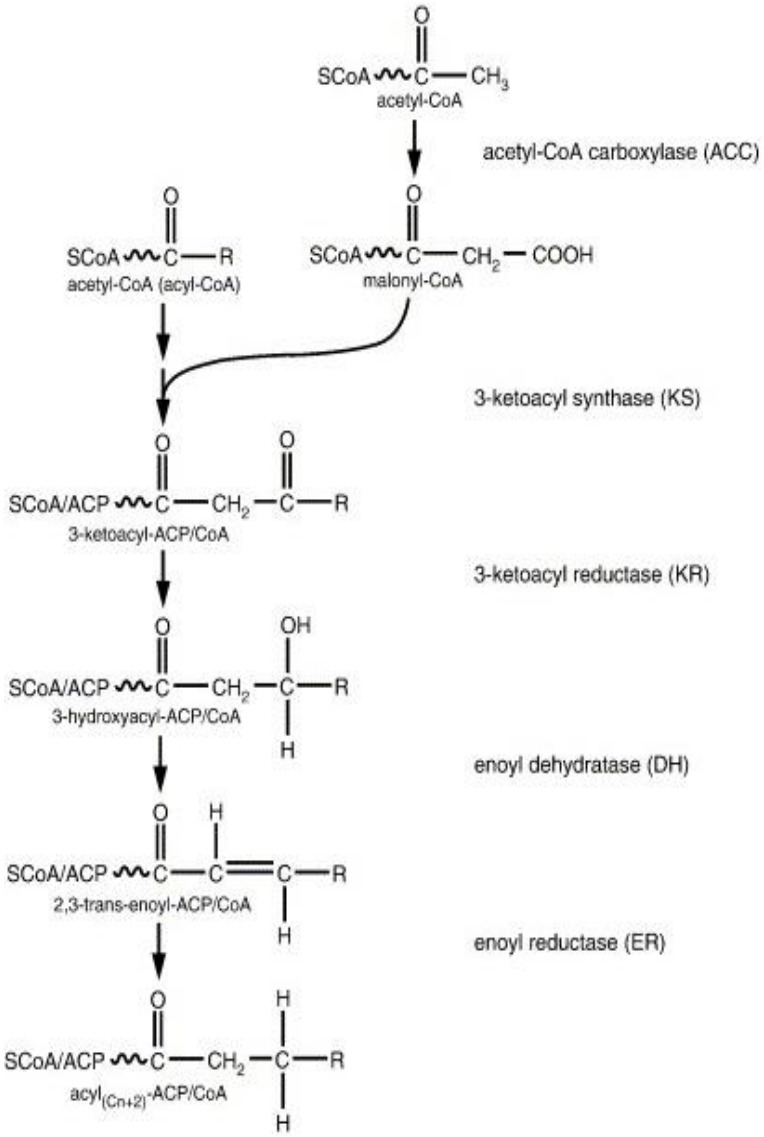


FIGURE 1.4: Representation of the initiation phase of fatty acid synthesis. The synthesis of FA starts with the acetyl-CoA molecule generating the malonyl-CoA molecule. Malonyl group will be inserted for each cycle of extension of the FA synthesis. The process entails a series of condensation, reduction and dehydration reactions being catalyzed by enzymatic units (Tehlivets et al., 2007).

Using the reducing power conferred by nicotinamide adenine dinucleotide phosphate (NADPH), the reduction of the C-3 of the newly formed molecule occurs, resulting in 3-hydroxybutanoyl-[acp] (Prescott et al., 1972). The water elements are now removed, in a dehydration reaction, obtaining the 2,3-trans-enoyl-ACP (Knobling et al., 1975) through the

formation of a double bond in the molecule. A second reduction for the synthesis of butanoyl–[acp] again expends a molecule of NADPH (Berglert et al., 1994). After all these initiation steps, the next acetyl group binds to butanoyl-[acp], restarting the reactions. For each cycle, the FA molecule increases by two carbons which continues to the required FA length. The release of the molecule occurs by thioesterase action. This enzymatic unit has different affinities for FAs of different sizes, breaking the ester bond with the enzyme and consuming an H_2O molecule (Lu et al., 2004).

Despite sharing the same type of reactions, the production of FAs differs between prokaryotes and eukaryotes, as the enzymatic units that catalyze the series of reactions present themselves differently, either as independent units or as large enzymatic complexes.

1.2.2.1 Fatty acid synthase type I

Fatty acid synthase type I (FAS I) comprises a multi-enzymatic dimer set that integrates several enzymes, which catalyze the most diverse reactions related to the FA synthesis – **Figure 1.5**. This type of system is found in the cytoplasm of eukaryotic cells (Zhou et al., 2014) such as plants, animals, fungi and in some bacteria (Shuib et al., 2022). FAS I in *S. cerevisiae* consists of a complex that contains six copies of independent domains in the form of an α6β6 molecular complex of 2.6 MDa (Gipson et al., 2010). Each subunit contains four functional domains (Fichtlscherer et al., 2000) encoded by two genes: *FAS1* (β subunit) and *FAS2* (α subunit), being present on chromosomes XI and XVI, respectively (Schuller et al., 1992).

These two chains embody different catalytic domains, where the β chain carries the acetyl-transferase, enoyl reductase, dehydratase, and most of the malonyl/palmitoyl transferase. The ACP, ketoacyl reductase, ketoacyl synthase and phosphopantetheine transferase are bound to the α chain (Jenni et al., 2006). The structure is complex in the form of a barrel, and non-coding parts for the enzymes are necessary as elements of building scaffolding elements (Jenni et al., 2007).

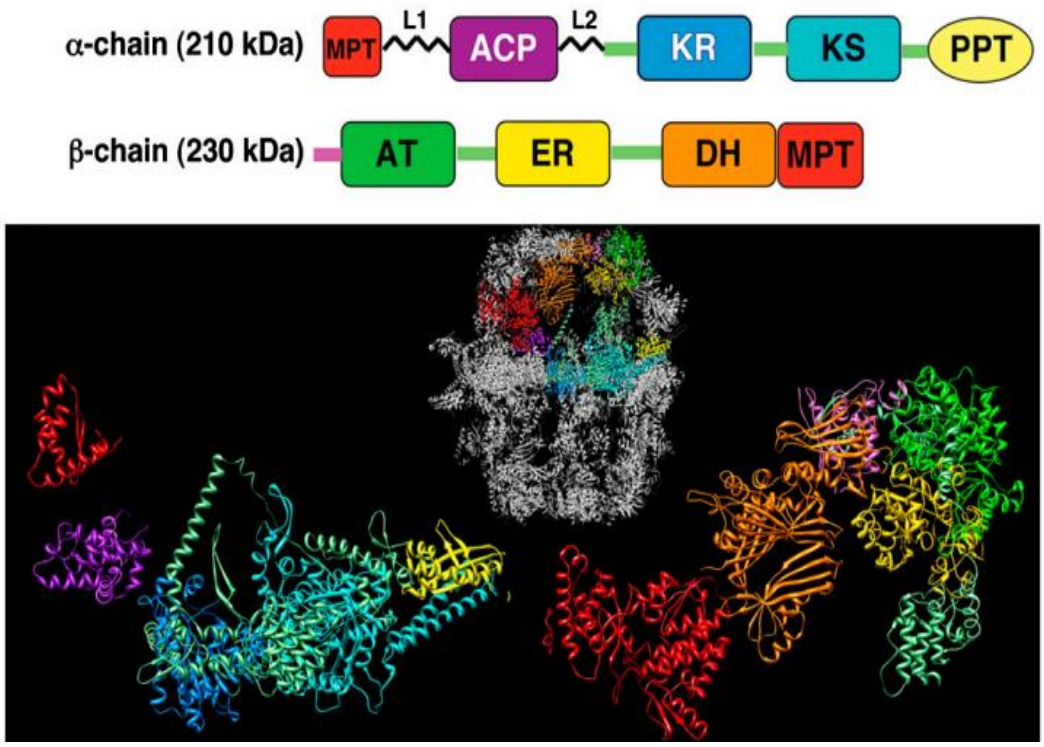


FIGURE 1.5: Model of the yeast structure fatty acid synthase. Organization of the two FAS I domains α and β, as well as their three-dimensional models. The colors are in accordance with the diagram shown above. MPT- malonyl-palmitoyl transferase; ACP- acyl carrier protein; KR- ketoacyl reductase; KS- ketoacyl synthase; PPT- phosphopantetheinyl transferase; AT- acetyl transferase; ER- enoyl reductase; DH- dehydratase; L1/L2- Peptide linkers (Gipson et al., 2010).

This system requires a specific ACP activation mechanism, likewise requiring a specific transferase (PPT) for the phosphopantetheine group of coenzyme A to be covalently linked to ACP (Fichtlscherer et al., 2000). Since the PPT domain is spatially separate, it is not able to catalyze the reaction in the ACP domain. It is thus believed that there is self-activation on the part of the FAS before the reactions that culminate in the formation of the structure, and consequently in the impediment of activation by the PPT (Johansson et al., 2009). Another feature of this system lies in the MPT domain, which has a dual function, either in the transfer of malonyl groups for chain elongation, as well as a catalyst for the release of FA from ACP (Engeser et al., 1979). This versatility of functions is due to the large hydrophobic pocket of the domain, which allows it to host extensive FAs stabilizing through hydrophobic bonds and an active site of high affinity for malonyl-CoA for FA chain extension (Engeser et al., 1979). This enzyme thus plays a regulatory role, as it

prevents the start of a new extension cycle while the process is ended for another FA (Lynen et al., 1980).

The β subunit is shown to be essential for the assembly of the FAS I multienzyme complex and thus influencing the FA output (Furukawa et al., 2003; Wenz et al., 2001). Deletion of one of the two showed that the *fas2* deletion gene has little influence, but there is a decrease in the amount of messenger RNA from this gene with the deletion of the *fas1* gene (Egner et al., 1993).

1.2.2.2 Fatty acid synthase type II

Unlike the FAS I system, fatty acid synthase type II (FAS II) is commonly found in the cytoplasm of bacteria and is still compartmentalized in eukaryotic cell organelles, such as mitochondria and chloroplasts (Hiltunen et al., 2009). Its function in that organelles is based on the production of certain FA, such as lipoic acid in mitochondria (Nowinski et al., 2020).

This system is expressed in several genes whose transcripts originate catalytic units that act independently, without the formation of complexes (Marrakchi et al., 2002) – **Table 1.2**. Despite other enzymes and their different arrangement, the synthesis follows the same generalized reaction cycle. However, they express different affinities for the synthesized compounds. In the condensation process, FabH only has specificity for the initial condensation, since its specificity focuses on the acetyl-CoA molecule (Musayev et al., 2005), while FabB is used for the condensation of unsaturated fatty acids (Heath & Rock, 1996b).

TABLE 1.2: Key enzymes involved in the FAS II system.

Enzyme group	***Enzyme***	***Cofactor***	***Activity***	***Reference***
ACP	*AcpP*	–	ACP	Rawlings, 1992
Condensing enzymes	*FabH*	–	β-Ketoacyl-ACP synthase III	Lai & Cronan, 2003
	FabB	–	β-Ketoacyl-ACP synthase I	Gerusz, 2010
	FabF	–	β-Ketoacyl-ACP synthase II	Huang et al., 1998
Enoly-ACP reductases	*FabG*	NADPH	3-ketoacyl-ACP reductase	Hu et al., 2001
	FabI	NADH	Enoyl-ACP reductase I	Berglert et al., 1994
	FabK	NADH	Enoyl-ACP reductase II	Lu et al., 2004
	FabL	NADPH	Enoyl-ACP reductase III	Lu et al., 2004
Dehydratases/ Isomerases	*FabA*	–	β-Hydroxyacyl-ACP dehydratase/isomerase	Feng, 2009
	FabZ	–	β-Hydroxyacyl-ACP dehydratase	Heath & Rock, 1996b

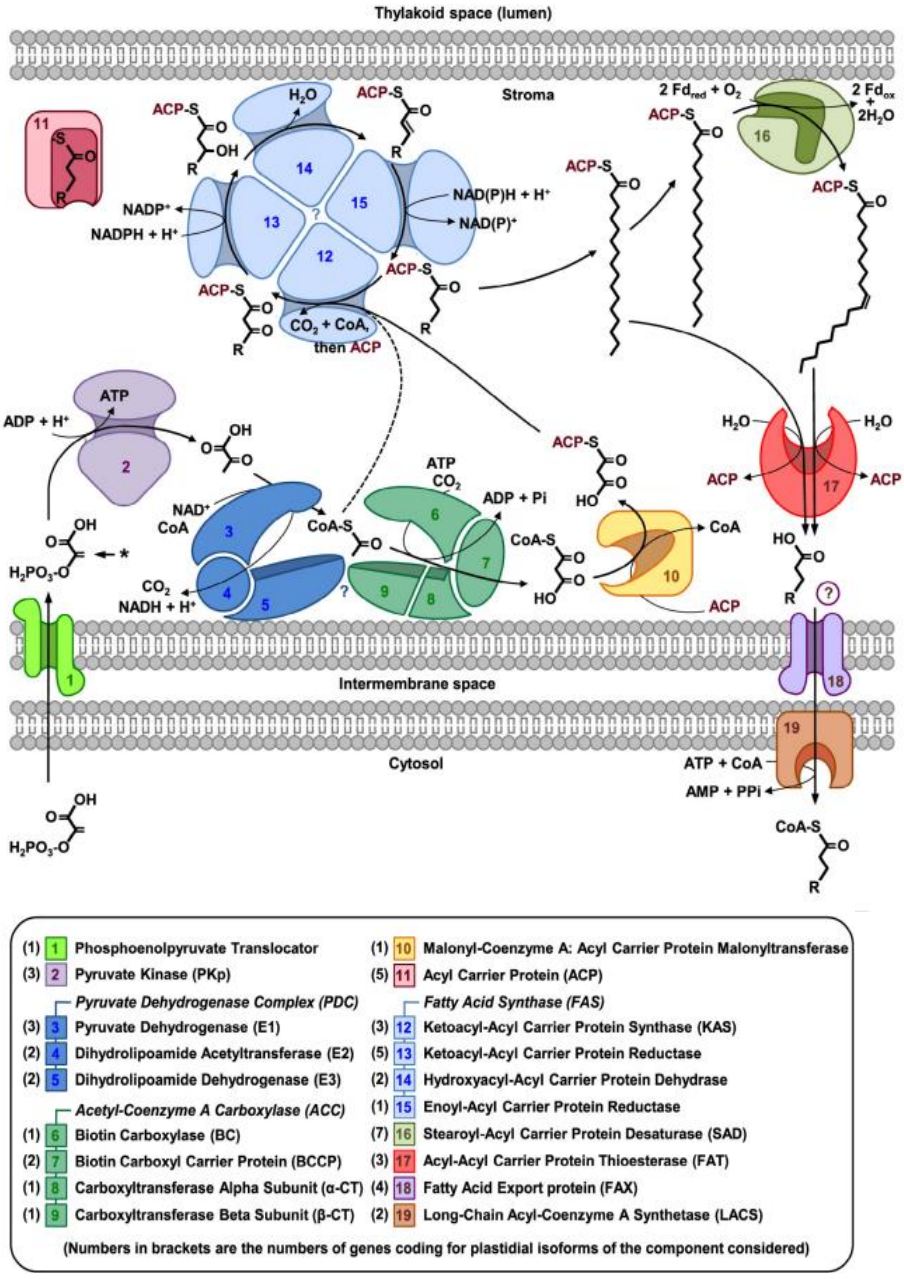


FIGURE 1.6: Schematic organization of FAs synthesis in plastids. There is the importation of phosphoenolpyruvate into the plastid (1), where it's converted to pyruvate (2). Through the action of the pyruvate dehydrogenase complex (3-5) the formation of acetyl-CoA occurs. Synthesis depends only on this molecule produced in the plastid. The following steps correspond to the general steps of FAS II: formation of malonyl-CoA (6-9); activation of the malonyl-CoA group (10-11) and the cyclic elongation process in FAS II (12-15). The recent FA can be unsaturated by the action of stearoyl-ACP desaturase and then it is released by the action of acyl-ACP thioesterases (17), being exported by the plastids (18). FAs can also be modified into CoA esters by the action of acyl-CoA synthetase (19) (Troncoso-Ponce et al., 2016).

In terms of regulation, the synthesis focuses on enzymes with a key role in the process: AcpP, FabH and FabI. The first focuses on the supply of malonyl groups to the extended chain and their inhibition prevents the advance of synthesis, avoiding the expenditure of unnecessary FA to the cell (Davis & Cronan, 2001). With the same aim of making a process metabolically efficient, the regulation also takes place in the FabH enzyme, which prevents a new extension cycle (Heath & Rock, 1996a), as well as in FabI, which catalyzes the reduction during elongation, preventing complete elongation of the FA (Heath & Rock, 1996).

FAS II system in plants is a special case because the FA production does not occur in the cytosol but in plastids (Nishida, 2004) – **Figure 1.6**. Its system is associated with the production of long chains, with 16 and 18 carbon, representing 30% and 70% of all FAs in the cell (Tjellström et al., 2012). Its synthesis consists of the repetition of several cycles, being of extreme importance an effective regulation of the process, either to avoid unnecessary expenses or to dissuade premature cell death. (Y. Zhao et al., 2018). These long FAs play a key role in lipid membrane dynamics, like the formation and maintenance of its integrity (Meï et al., 2015).

1.2.3 Importance of fatty acids

1.2.3.1 In society

In addition to the most varied functions that they present in biological systems, the FAs obtain their huge importance in the daily life of society. These molecules are present in the most varied industries, from the simplest ones, such as in the food industry to produce supplements and components rich in unsaturated fatty acids (Gammone et al., 2019), to the most complex stages of energy, chemical and pharmaceutical industries.

The use of lipid is a practice that comes from the dawn of civilization. The ancient Egyptians used scented oil for hygiene processes, cosmetics or mummification, being considered a luxury resource (Denavarre, 1978). Its use in the Middle Ages is also associated with healing properties as well as used in alchemy (L.-C. Wu et al., 1932). The advent of the industrial revolution and advances in the field of organic chemistry allowed the use of lipids on a large scale, through saponification, substantially allowing the increase in soap production. Its use in candles, soaps and cosmetics led to a search for new sources, such as sperm whales to replace animal fat (Irwin & Washington, 2012). Nowadays, its use still focuses on cosmetics and soap production, as well as components in detergents, gaining an important role in the diet for eye development (A. C. Zhang

et al., 2020), in the prevention of cardiovascular disease (Jain et al., 2015), strengthening joints (Kostoglou-Athanassiou et al., 2020) and preventing cancer (Bischoff-Ferrari et al., 2022).

Each industry requires FAs to a specific chain length and given the needs of each one, we can consider that all FAs are important raw materials in all sectors. In the case of soap, the combination of Palmitic and Stearic acid contributes to the detergent properties and together with Lauric and Myristic acid, the soap has its lathering and washing properties. MCFA is required in capsaicin synthesis (Keum et al., 2012) and LCFA for biofuel production (Pinzi et al., 2013).

1.2.3.2 Microbial fatty acids vs petrol fatty acids

At an urgent time to address global warming and the reduction of the ecological footprint, it becomes necessary to find alternative sources to the so-called fossil fuels (Zhou et al., 2014). Green technologies, such as hydroelectric and nuclear energy require large initial investments not easily accessible. One of the most effective options for green energy is biodiesel, a fuel obtained by transesterifying oils with short-chain alcohols or by esterifying FAs from vegetable oils or animal fats (Vasudevan & Briggs, 2008). In addition to generating less pollution, certain of these green fuels provide about 93% more energy when compared to energy generated by fossil fuels.

The property of biodiesel depends on certain characteristics such as density, viscosity, cloud point and ignition quality (Ramos et al., 2009) that are associated with the FAs that compose it. An excess of polyunsaturated will affect oxidative stability, emissions of nitrogen oxides and thermal efficiency (Puhan et al., 2010). But, an overload of saturates will show poor fuel properties at low temperatures (Cao et al., 2010). Feedstocks rich in monounsaturated FAs correspond to the best-performing fuel (Tyson, 2006) as they combine oxidative stability with adequate performance capacity even at non-ideal temperatures.

The production of green fuel can be based on several compounds, highlighting edible oil, palm oil, soybean oil and waste oil (Elgharbawy et al., 2021). However, spending on the use of pesticides, irrigation of fields and the presence of irregular weather are some of the obstacles raised in the production of this type of energy. In addition, the increase in the energy demand triggers unsustainable cultivation techniques and deforestation for new fields of culture, ending up camouflaging the benefits for the sustainability of the planet. One of the most plausible solutions is the use of FAs produced by microorganisms, which due to their ease of maintenance and

pollution reduction could be used as substitutes for petroleum fuels, in the production of biodiesel and other compounds of interest such as lubricants, rubbers and plastics.

1.2.3.3 Current status of microbial fatty acid synthesis

Despite the use of FAs from microorganisms being a good bet, its low prevalence becomes a limiting factor in its applicability for energy feedstock. *S. cerevisiae*, due to its robust and tolerant response to environmental stress, makes it the first viable option for applicability in the industrial context. However, its production leaves much to be desired, not meeting industrial demands. The possibility of other oleaginous organisms is an excellent response to produce FAs, as in the case of *Y. lipolytica*. Although they are amply used for citric acid and protein production, the modulation of synthesis of FAs in this yeast is still a little explored issue (Tai & Stephanopoulos, 2013).

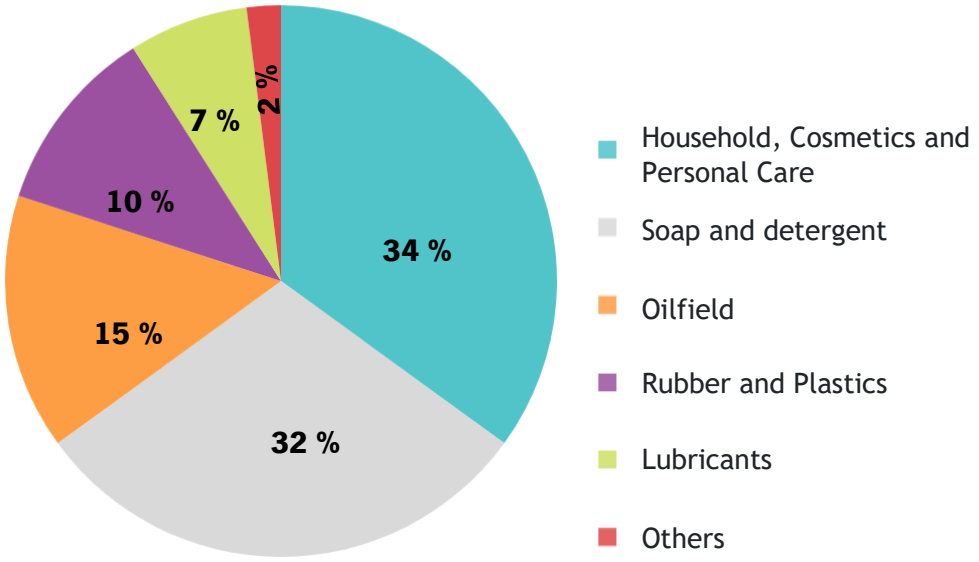


FIGURE 1.7: Naturally sourced FAs market in 2018. (*Natural Fatty Acids Market (2022 - 2027)*, 2020).

Furthermore, the chemical synthesis of FAs entails specific chemical means, long times, and large expenses. The great variability in the length of the FAs chain that is required by the industry requires the divergence of investments and developments for different solutions. Even so, with all these difficulties, the market for FAs has been increasing, estimating that the US market associated with FAs of natural origin could reach up to $4.99 billion in 2025 (J. Feng et al., 2021).

1.2.3.4 Improving fatty acids synthesis

The need to improve the natural production of FAs and the modulation of its hydrophobic tail has become a "hot topic" in the world of science communication. Understanding its accumulation process, through the perception of the effect of key enzymes in the synthesis pathway, increasingly becomes the key to achieving production that satisfies industrial demands (Fortman et al., 2008; Knoshaug et al., 2018; Zhou et al., 2018).

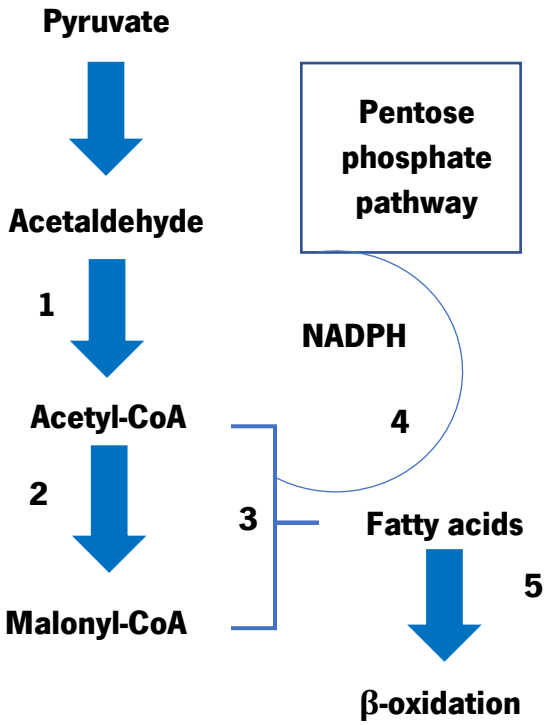


FIGURE 1.8: Metabolic strategies to increase FAs production in *S. cerevisiae*. Increasing the transcription of key enzymes in the initial pathway like acetyl-CoA synthase (**1**) and acetyl-CoA carboxylase (**2**) or overexpressing the *FAS1* and *FAS2* genes of FAS II complex (**3**) leads to a higher FA pool. Improving the production of NADPH, by expression of isocitrate dehydrogenase (**4**), increases the redox potential equivalents for FAS II and reveals the same effect. The disruption of the functioning of the FAs degradation pathway causes them to remain in the biological system (**5**).

The increase in FAs can be achieved through the overexpression of several genes that encode various enzymes, such as acetyl-CoA synthase (**Figure 1.8-1**; F. Chen et al., 2010) or acetyl-CoA carboxylase (**Figure 1.8-2**; Runguphan & Keasling, 2014). Some studies report an increase in FAs through the overexpression of genes encoding the multienzyme complex FAS II (**Figure 1.8-3**; Runguphan & Keasling, 2014), or even the expression of isocitrate dehydrogenase

to generate redox potential in the form of NADPH (**Figure 1.8-4**; Partow 2017). An increase can also be generated by the silencing of FAs consumption pathways, through the deletion of the *pox1* gene that is associated with the β-oxidation pathway (**Figure 1.8-5**). However, the reduction in pathway activity causes a decrease in precursors such as acetyl-CoA, culminating in less pronounced growth (L. Chen et al., 2014). Resorting to the heterologous expression of enzymes is also addressed to influence the amount of FAs, using those associated with microorganisms with high lipid production (S. Shi et al., 2016), as well as the study of the effect of each enzyme unit belonging to FAS II in *S. cerevisiae* (Fernandez-Moya et al., 2015).

Fernandez-Moya et al., (2015) describes the replacement of the function of the native *S. cerevisiae* pathway by the expression of a FAS II system to increase the production of SCFA and MCFA. As it is a simpler system compared to FAS I, they also report the expression of each FAS II enzyme and the addition of heterologous thioesterases to study their effect on the structure of FAs. The results were promising with an increase in the production of Myristic acid (C14:0) and a decrease in the production of FAs from 18 carbons of length.

1.2.3.5 "Project FATVAL – Systematic metabolic engineering of *Saccharomyces cerevisiae* for high value fatty acids and derived products"

This research project consists of improving the metabolic system of *S. cerevisiae* to produce FAs to be used for the synthesis reaction of certain compounds with therapeutic properties, such as Capsaicin and Cannabinol. One of the steps of the synthesis requires a specific FA, 8-methyl-*trans*-6-Nonanoic acid, a mono-unsaturated medium chain length, which the microorganism lipidome does not include (Muratovska et al., 2022). The modulation of lipid synthesis becomes essential, requiring exhaustive knowledge of the lipid synthesis system to determine the key points needed to modulate production to the desired FA. Due to the complexity of the FAS I system, the project promoted the expression of simpler and more independent production systems, such as the FAS II system. The perception of the effect of each gene selects the essential ones for the prevalence of MCFA.

The increase of these FAs is not sufficient to produce Capsaicin, so the project foresees the expression of desaturases capable of catalyzing the formation of the necessary mono-unsaturation since the yeast only has one desaturase that is directed for the formation of long poly-unsaturated FA instead of MCFA (Muratovska et al., 2022). It is still necessary to proceed with the activation to generate the free FA, thus being available for the condensation reaction with the vanillyl amine for capsaicin production (Muratovska et al., 2022) – **Figure 1.9**.

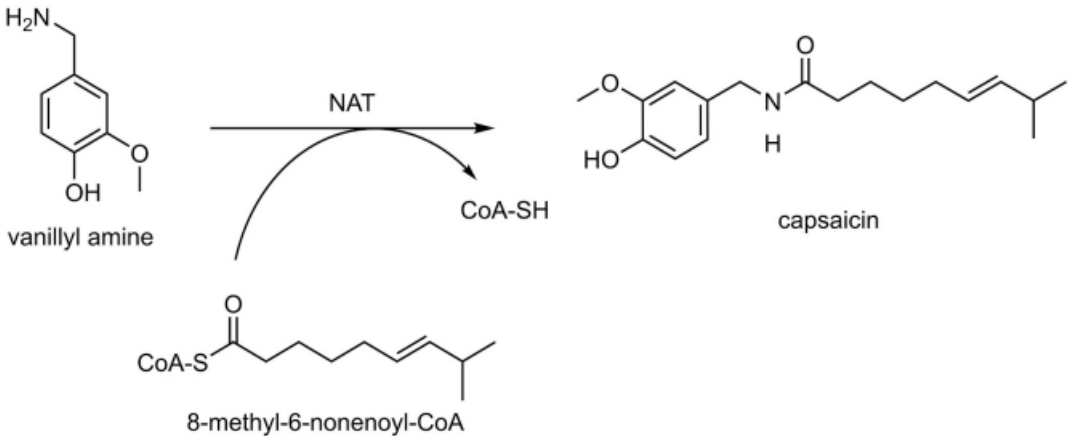


FIGURE 1.9: Mechanism of production of Capsaicin. The capsaicin synthase catalyzes the reaction between vanillyl amine and 8-methyl-6-nonenoyl-CoA, generating the capsaicin molecule, a powerful therapeutic agent (Muratovska et al., 2022).

1.2.4 Methods for lipid examination

1.2.4.1 Industrial field

All industries need quality controls to analyze and monitor industrial activity, allowing the detection of problems and preventing their recurrence. Industries within the scope of FAs require specific methods for their analysis, developing protocols that allow ascertaining the quality of the product and the continuation of the production chain. Depending on the FAs and the final objective, the most frequent lipid analyses carried out in the industrial context are:

- **Fatty acid profiling –** determines the composition of FAs in relative percentage values or absolute values (mg/g). Esterification is carried out into fatty acids methyl esters (FAMEs) using alkylation derivatization reagent, and then analyzed by gas-chromatography (GC) with flame ionization detector (FID; AOCS Official Method Ce 1i-07, 2022);

- **Oxidation testing –** tests the degree of oxidation of lipids, considering three stages of oxidation: Peroxide value, which consists of the determination by titration of hydroperoxides, the primary oxidation form (AOCS Official Method Cd 8b-90, 2022); Anisidine value, which determines the amount of aldehyde from the decomposition of hydroperoxides using a spectrophotometer (AOCS Official Method Cd 18-90, 2022); Acid value, specific to determine the hydrolysis of triglycerides and other lipids based on titration (AOCS Official Method Cd 3d-63, 2022);
- **Sterols and stanols -** evaluates the amount of sterols/stanols, using the GC-FID after a basic sialylation process to derivatize the lipid molecules (AOCS Official Method Ce 12-16, 2022);
- **Phospholipids –** lipid extraction is applied to thin-layer plates coated with silica gel. The bands are then identified using UV and subjected to transmethylation to be analyzed by GC-FID (Phospholipids Analysis, 2022);
- **Neutral lipid –** after separating the extracted sample content by solid-phase extraction on silica gel, the layers of interest are identified using radio frequency compared to standards analyzed by UV light. After identification and transmethylation, the sample is analyzed by GC-FID (Phospholipids Analysis, 2022).

1.2.4.2 Laboratory domain

The analysis of lipid content in a laboratory environment consists of the characterization of the most diverse types of lipids. In general, the analysis begins with the growth of cells and generating the necessary biomass for lipid examination. With the sample, the use of fluorescence is used to survey the lipid content. This initial perception by fluorescence becomes indispensable for the quantification process to adjust the lipid extraction protocols with the necessary volumes. The amount of the lipid content of the extracted sample can be estimated and through chromatographic analysis, a full characterization of the lipids profile.

A. Staining approach – using Nile red dye

The use of this technique is based on the use of fluorescence to estimate intracellular lipids, without lipid extraction, and can be approached with various equipment such as the fluorescence microscope, fluorimetry and flow cytometry. There are several stains used for the

detection of lipids, including Sudan III, Bodipy 493/503 and Nile red, the most used for intracellular lipid determination (Fam et al., 2018).

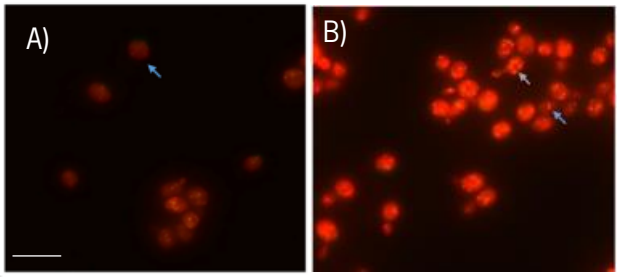


FIGURE 1.10: Fluorescence microscope images of dye Nile red reporting the lipid droplets accumulation in yeasts. Cells correspond in the mind-exponential phase of growth. Filter set Red (emission wavelength: 515-560 nm; excitation wavelength: 572-590 nm). Images obtained by 100x magnification; Bar scale of 5 µm. The arrows indicates lipids lower number of lipid droplets (Blue arrow) or higher (Gray arrow). A- Control; B- Strain SK22-E58 (Paramasivan et al., 2021).

Nile red (9-diethylamino-5H-benzo[α]phenoxazine-5-one) has an emission and excitation wavelength of 530 nm and 590 nm, respectively (Hicks et al., 2019). Nile red can be applied to live cells in the aqueous medium (Greenspan et al., 1995), and strongly fluoresces when in a hydrophobic environment of non-polar lipids. It is widely used due to the high number of samples that can be analyzed. Dimethylsulfoxide (DMSO) is used to increase its permeability in the cell by the formation of water pores allowing the penetration of the dye through lipid membranes (Notman et al., 2006), increasing its contact with the hydrophobic environment, as well as applying phosphate-buffered saline (PBS) wash solution to eliminate any background fluorescence from the sample (Ramírez-Castrillón et al., 2021). It is also reported that microplates affect the fluorescence readings, so black microplates are used to avoid emission from adjacent wells (Sitepu et al., 2012).

B. Lipids extraction

Lipid extraction can start from wet material, but extractions from dry samples are more effective (Athenaki et al., 2018). The use of freeze-drying in biological samples is more efficient (Ferrari et al., 2020), without the process affecting the structural conformation of lipids (Murphy et al., 2003). The method further requires the use of an organic solvent capable of dissolving non-polar lipids. The choice of solvent depends on the type of lipid that is to be extracted (González-Fernández et al., 2020). For the extraction of FA, the most used are n-hexane (Shin et al., 2018)

as well as chloroform: methanol mixtures (Axelsson & Gentili, 2014) or chloroform:methanol: water (Breil et al., 2017), in different proportions. An important step during the extraction protocol is the use of glass since the use of polymers such as plastic is likely to react with the organic extraction solution (Z. Zhao & Xu, 2010).

For intracellular lipids quantification, methods of disrupting cell integrity are needed. Several have been developed being categorized as mechanical, which comes from the application of physical forces to disintegrate the cell structure (Patel et al., 2018). Examples of these are the use of bead beating (Halim et al., 2012), ultrasonic frequencies (Günerken et al., 2015) or microwave-assisted extraction (Mwaurah et al., 2020). While the former increases the diffusion between the matrix and the extraction solvent (Xie et al., 2013), the latter takes advantage of the heat generated by the process and increases the efficiency of the derivatization protocols for further analysis (Söderholm et al., 2010). The other category focuses on the use of chemical properties for the destabilization of cell permeability, being categorized as non-mechanical methods. Enzymatic, acid-base and osmotic shock are some treatments that fall into this group. Unlike mechanical, these methods consume more time as well as more energy, not being economically viable (Yenkie et al., 2017).

A correct extraction means transferring the maximum lipid content to the extraction solvent, repeating the process as many times as necessary to recover the greatest number of lipids. The appearance of a bi-phase system after centrifugation allows the recovery of lipids in the organic phase, promoting the retention of polar compounds (like proteins or carbohydrates) in the polar phase. The addition of 10 % NaCl increases lipid extraction (Halim et al., 2012) by further increasing the polarity of the polar phase for more efficient phase separation.

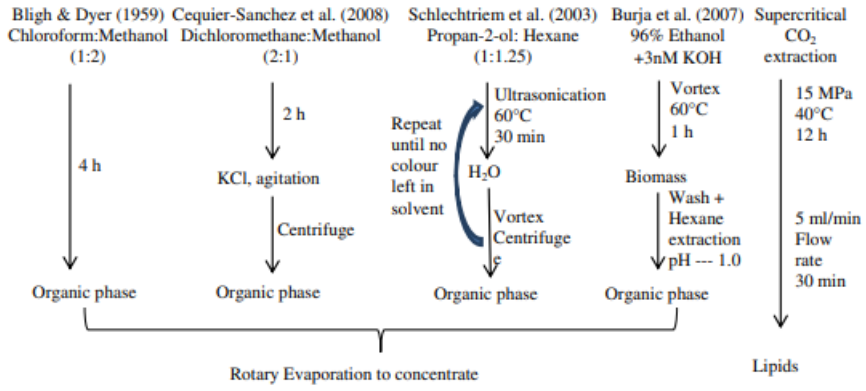


FIGURE 1.11: Overview of some methods used for lipid extraction. (Y. Li et al., 2014).

C. Gravimetric method

This method allows for lipid quantification, but it is associated with a protocol that comprises lipid extraction to determine the lipid content (Halim et al., 2012). The procedure considers the mass difference between an empty container and the container with lipids after the evaporation of the solvent. Results are expressed in relative terms (% lipids) or absolute terms (grams of lipids/ grams of dry cell mass). This method can be classified as macro-gravimetric, if the mass of the samples used is between 0.5 – 5 g (Inouye & Lotufo, 2006), and micro-gravimetric if the samples are of a lower mass (approximately 100 mg) (Van Handel, 1985).

The evaporation of the organic compound from the phase is required which can be catalyzed by N_2 current (Q. Wang et al., 2021); rotary evaporator (Jeevan Kumar & Banerjee, 2019) or by heating (Bengtsson et al., 2021). The first two evaporation systems are the most suitable for carrying out evaporation since the great advantage resides in preventing contact with oxygen from the surrounding air and preventing oxidation. However, both require specialized equipment. The last evaporation system stands out for its applicability in any laboratory. However, the probability of lipid oxidation increases significantly and high-temperature contact may lead to variations in the structure level of lipid samples (Szabo et al., 2022). The choice of this method depends on whether the analysis of the same sample will proceed to other studies that have the sensitivity to describe the lipid profile. These structural changes do not affect the determination by gravimetry, since this method only quantifies, ignoring the lipid class. As it is easy to apply, a less detailed study of lipid classes allows the use of the gravimetric method for lipid quantification.

D. Apply the chromatographic features to strain analysis

The chromatographic technique is a method of separating components from a mixture. This process uses two immiscible phases called the mobile phase, the solvent containing the sample and the stationary phase, where the mobile phase moves. In contact with these two phases, the different components of the mixture have different affinities, ending up behaving differently – **Figure 1.12**. This behavior is expressed by the distribution coefficient, the concentration ratio in each of the phases. According to the physical state of the mobile phase, it is possible to divide two large groups: liquid chromatography and gas chromatography (Coskun, 2016). The two most used techniques correspond to high-performance liquid chromatography (Protzko et al., 2018) and gas chromatography (Tejero Rioseras et al., 2017). These methods are the most used in biological

analyses, and their stationary phase corresponds to a column, in which the mobile phase passes inside the column. The time it takes for each constituent of the mixture to be detected from its injection is referred to as the retention time (RT) being characteristic of the same for the chromatographic conditions subjected. The longer the RT of a compound, the greater its affinity for the stationary phase.

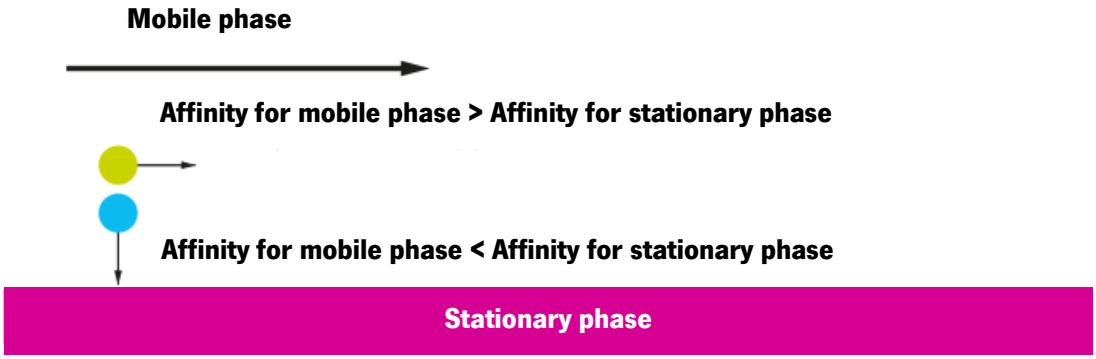


FIGURE 1.12: Basic principles of separation by chromatographic methods.

D.1. High-performance liquid chromatography

As a chromatographic method with a versatile application window, high-performance liquid chromatography (HPLC) is one of the most used in the food industry (J. Lee et al., 2008), in the determination of the active substance of drugs (M. Li et al., 2011) and even in anti-doping controls (Thevis & Schanzer, 2005). The process is characterized by the passage of a solvent into a column (stationary phase) through a pump. When injecting the sample, the solvent that constitutes the mobile phase passes through the stationary phase, separating the constituents of the mixture according to their distribution coefficients for the two phases. After the column, the existence of a detector can register and save a certain component, and through a data interpretation system, it is possible to identify the sample (Dong, 2006). Each HPLC analysis needs to be sensitive to the type of sample being analysed, so a compatible column, solvent and detector are required for the compounds to be analysed. Normally the concentration of the target compound is low, so it is necessary to improve the sensitivity of the analysis. For this, it is necessary to optimize the method to be used for the type of compounds to be analysed through standard curves to determine both the quantification limit of the method as well as the RT of the compound.

One of the most important parameters of HPLC analysis is the choice of the appropriate detector since it must respond even at low analyte concentrations. In addition, it should respond

with increasing analyte concentration linearly, always responding in the same way with a fast response. There are several detectors available, which can be consulted in **Table 1.3**. The most used is the Refractive Index (RI) detector, which allows its application to any type of analyte in the mobile phase (da Silva Medeiros et al., 2021), being used for the analysis of any biomolecule (Chávez-Servín et al., 2004; K. W. Y. Lee et al., 2013; Wen et al., 1996). HPLC-RI system is the most sophisticated for the phenotypic analysis and characterization of the most varied biological systems.

HPLC analysis is an ideal tool for characterizing yeast metabolism since it reports the presence and concentration of compounds such as glucose, ethanol, glycerol and others important for the development of cell growth. The equipment can also analyse the lipid profile using the derivatization process to increase the detection sensitivity (Moldoveanu & David, 2015). However, although HPLC operates at lower temperatures than those practised by GC that reduce the risk of isomerizations, co-elution of different compounds makes it hard to determine the type of lipid that corresponds, making it a fruitless analysis for complex FAs samples (Sanches-Silva et al., 2004).

TABLE 1.3: Characteristics of some HPLC detectors. RI- Refractive index; UV- Ultraviolet; VIS- Visible light; PDA- Photo diode array; FL- Fluorescence; EC- Electrochemical (Swartz, 2010).

Detector	***Range of applications***	***Minimal detectable quantity***	***Linear range***
RI	Universal	µg	10^3
Conductivity	Selective	High ng	10^4
Light Scattering	Universal	High ng	10^3
Corona Discharge	Universal	Low ng	10^4
UV/VIS/PDA	Selective;Universal at low λ	ng	10^5
FL	Very selective	pg	10^3-10^4
Radioactivity	Selective	pg	10^3-10^4
EC	Very Selective	fg-pg	10^5

D.2. Gas chromatography

Despite following the same principle as HPLC, gas chromatography (GC) has certain essential components for the analysis of its mobile phase in the gaseous state. After sample injection, the mobile phase transports it to the chromatographic column, coupled to an oven. The increase in temperature in the column causes the constituents of the sample to pass into the gas phase, allowing them to be reached and noticed by the detector. Throughout the analysis, there is a gradual increase in temperature, allowing the volatilization of more compounds. The signal is then amplified, stored, and interpreted by the system. Normally, the mobile phase is composed of different gases, among them: He_2, called carrier gas which, being an inert gas, has the function of transporting the sample (Hajšlová & Ajka, 2007); N_2 termed auxiliary gas which has the same function as carrier gas (Ashour & Kattan, 2010); H_2, termed as fuel gas, is used in some analyses, depending on the detector functioning as an oxidizer of the flame (Henriksen et al., 2017).

GC analysis becomes ideal for compounds with a boiling point suited to the capabilities of the equipment, which does not happen with the boiling point of lipids. Since FAs have very high points, it is necessary to reduce them, by derivatization (Chiu & Kuo, 2020). The transformation into esters through a reaction of the lipids with alcohol with an acidic catalyst allows the boiling point to be accessible at temperatures suitable for GC.

Several detectors are available for GC analysis, highlighting the flame ionization detector (FID) and mass spectrometer detectors (MS) as the most popularly used in the analysis. Being the most used, due to its fast response to any compound (Sevcik, 2011), the MS advantage is concentrated in identifying unknown peaks and doubly confirming the compounds through their RT and according to their mass as a function of charge (Lovestead & Urness, 2019).

2. Aims

2. Aims

The objective of this work is centered on the hybrid FASIIb pathway in two distinct parts:

I. Validation/Expression of a *de novo FASIIb* pathway for fatty acids production, in *S. cerevisiae* with silent native FAS: Analysis of the growth, metabolome and lipid profile, proving the effectiveness of the pathway as a substitute for the native pathway and on cellular viability. Besides the use of HPLC-RI for product monitoring in a culture medium, the fluorometric assay was performed to monitor lipids accumulation within the cell over time, the gravimetric method to quantify lipids and the GC analysis to characterize the lipidic profile.

II. Influence of adaptation and additional copies of every single FASIIb genes on lipidome of the yeast cell: Evaluation of the growth of strains and selection of those with the most promising phenotypes. Test the select strains in different cultures to improve the growth phenotype. As before, lipids were evaluated by fluorometric assay, gravimetric method and GC analysis to determine the influence of each gene on the lipid output of the cell.

The impact of this work is the identification of a FASII system in yeast that facilitates future metabolic engineering, aiming at the future production of distinct lipids with high productivity.

3. Material and methods

3. Material and methods

3.1. Culture media

The media used throughout this work for growing *S. cerevisiae* are detailed in **Table 3.1**. Yeast peptone dextrose (YPD) medium consists of 10 g/L yeast extract (PanReac AppliChem®), 20 g/L peptone (Grisp®) and 20 g/L glucose (Scharlau®). YPD + Hmy consists of YPD supplemented with 0.46 g/L Myristic acid (Hmy; Sigma®) and 10 g/L Tween 40 (Polysorbate 40; Sigma®) and YPD + EtOH consists of sterile YPD supplemented with 10 g/L ethanol (EtOH). Synthetic Complete (SC) medium contains 5 g/L ammonium sulfate (PanReac AppliChem®), 20 g/L glucose, 1.70 g/L yeast nitrogen base (YNB; Grisp®), 1.40 g/L of a mixture of amino acids without HULT (histidine, uracil, leucine and tryptophan) called "Drop-out" ((Kaiser et al., 1994), and more information in **Appendix 8.1**), 80 mg/L histidine (H; Formedium®), 80 mg/L uracil (U; Formedium®), 80 mg/L tryptophan (T; Formedium®) and 400 mg/L leucine (L; Formedium®). Ammonium sulfate and glucose were sterilised by autoclaving (121 °C, 20 min; Uniclave, AJC) and a solution with the remaining ones was added in aseptic conditions after sterilised by syringe filter (0.22 µm cellulose acetate (Whatman®)). L (SC-L), U (SC-U), LU (SC-L-U) and HULT (SC-HULT) were omitted as required for the selection of auxotrophic markers in the SC medium.

For bacteria, we used sterile Lysogeny-Broth-Miller (LB) medium which is composed of 10 g/L peptone, 5 g/L yeast extract and 10 g/L NaCl (PanReac AppliChem®). For transformations, we used the super optimal broth medium with glucose (SOC) prepared with 20 g/L peptone, 5 g/L yeast extract, 0.5 g/L NaCl and 0.19 g/L KCl (PanReac AppliChem®) proceeding to the autoclave process. To the sterilized mixture was added, through filtration 0.22 µm cellulose acetate filters, $MgCl_2$, $MgSO_4$ and glucose with final concentrations of 0.95 g/L, 1.20 g/L and 4 g/L, respectively.

All medium components were dissolved in deionized H_2O (Elix® system; Merck). For solid media, we added 20 g/L agar (LabChem®). We used the antibiotics for the selection of strains, 0.2 g/L Geneticin (YPD + G418; Gibco®) on YPD and 0.1 g/L Ampicillin on LB (LB + Amp; Formedium®).

TABLE 3.1: Composition of different culture media.

Compounds (g/L)	*YPD*	*YPD + Hmy*	*YPD + EtOH*	*SC*	*SC-L*	*SC-U*	*SC-L-U*	*SC-HULT*
Yeast extract	10	10	10	—	—	—	—	—
Peptone	20	20	20	—	—	—	—	—
Glucose	20	20	20	20	20	20	20	20
Myristic acid	—	0.46	—	—	—	—	—	—
Tween 40	—	10	—	—	—	—	—	—
Ethanol	—	—	10	—	—	—	—	—
Agar (If required)	20	20	20	20	20	20	20	20
G418 (If required)	0.20	0.20	0.20	—	—	—	—	—
Ammonium sulfate	—	—	—	5	5	5	5	5
YNB	—	—	—	1.70	1.70	1.70	1.70	1.70
"Drop out"	—	—	—	1.40	1.40	1.40	1.40	1.40
(mg/L)								
H	—	—	—	80	80	80	80	—
U	—	—	—	80	80	—	—	—
L	—	—	—	400	—	400	—	—
T	—	—	—	80	80	80	80	—

3.2 Identification of biological material

Strains of *S. cerevisiae* developed previously were the basis of the current project. These are based on laboratory strain CEN.PK2-1C (*MATa ura3-52 trp1-289 leu2-3,112 his3Δ1 MAL2-8C SUC2*) obtained from Dr. Peter Kötter (Frankfurt am Main, Germany). A unique identifier (ID) was defined for each strain listed in **Table 3.2**. Ω183 is the ID for this CEN.PK2-1C strain.

Some of the strains have either a *fas1* or *fas2* gene deletion at their native FAS genomic loci. In both cases, the gene was replaced by a KanMX4 cassette, providing the strain with resistance to G418 (Guldener, 1996). With the loss of functionality of the native lipid production, the pathway constructed in previous works (Pozdniakova et al., 2023 submitted) is composed of twelve genes: nine of which come from *E. coli* (*fabA*, *fabB*, *fabD*, *fabF*, *fabG*, *fabH*, *fabZ*, *acpH* and *acpP*) and three from *A. thaliana* (*mod1, fatA1* and *fatB*). The pathway was built using the Yeast

Pathway Kit (Pereira et al., 2016). The plasmid pTA1 (Pozdniakova et al. 2023 submitted; see **Appendix 8.4**) was used as a backbone providing sequences for maintenance in yeast and *E. coli*, assuming the name pTA1_FASIIb when loading the developed pathway (see **Appendix 8.4.1**). In addition, twelve strains were created based on CEN.PK2-1C *fas2* :: KanMX4 pTA1_FASIIb, with the incorporation of a second plasmid. Named pYPK0 (Pereira et al. 2015), it contains each unit that constitutes pTA1_FASIIb. All of these strains carry both pTA1_FASIIb and a pYPK0_Promoter_Gene_Terminator (see **Appendix 8.5.1 – 8.5.13**).

TABLE 3.2: Identification of biological material. Strains are based on CEN.PK2-1C (Ω183) with mutation on *fas1* (only µ1255) or *fas2* gene by introducing the KanMX4 cassette except for µ1128, µ1206 and µ1239. µ1128 is a strain only with pTA1 and pYPK0 vectors. µ1206 and µ1239 transformed with pTA1 (empty vector) and with pTA1_FASIIb, respectively. The µ1202 is a mutant strain with pTA1 and the µ1255 and µ1257 are mutants only with pTA1_FASIIb. Twelve mutant strains have pTA1_FASIIb and a second plasmid (pYPK0) with individual genes of FASIIb pathway.

Strain ID Original	***Strain ID Evolved***	***Genome***	***Plasmid pTA1***	***Plasmid pYPK0***
µ 1128	–	–	pTA1	pYPK0
µ 1202	–	*fas 2::KanMX4*	pTA1	–
µ 1206	–	–	pTA1	–
µ 1239	–	–	pTA1_FASIIb	–
µ 1255	µ 1420	*fas 1::KanMX4*	pTA1_FASIIb	–
µ 1257	µ 1421	*fas 2::KanMX4*	pTA1_FASIIb	–
µ 1290	µ 1412	*fas 2::KanMX4*	pTA1_FASIIb	pYPK0_PDC1_*fabH*_TEF1
µ 1292	µ 1405	*fas 2::KanMX4*	pTA1_FASIIb	pYPK0_TEF1_*fabD*_FBA1
µ 1294	µ 1404	*fas 2::KanMX4*	pTA1_FASIIb	pYPK0_FBA1_*fabG*_RPL22A
µ 1296	µ 1411	*fas 2::KanMX4*	pTA1_FASIIb	pYPK0_RPL22A_*acpP*_TDH3
µ 1298	µ 1408	*fas 2::KanMX4*	pTA1_FASIIb	pYPK0_TDH3_*fabF*_UTR2
µ 1301	µ 1410	*fas 2::KanMX4*	pTA1_FASIIb	pYPK0_UTR2_*fabB*_TPI1
µ 1303	µ 1409	*fas 2::KanMX4*	pTA1_FASIIb	pYPK0_TPI1_*fabA*_PMP3
µ 1306	µ 1407	*fas 2::KanMX4*	pTA1_FASIIb	pYPK0_PMP3_*fabZ*_ENO2
µ 1309	µ 1418	*fas 2::KanMX4*	pTA1_FASIIb	pYPK0_ENO2_*mod1*_RPL5
µ 1312	µ 1406	*fas 2::KanMX4*	pTA1_FASIIb	pYPK0_RPL5_*fatA1*_RPL16A
µ 1315	µ 1419	*fas 2::KanMX4*	pTA1_FASIIb	pYPK0_RPL16A_*fatB*_RPL17A
µ 1318	µ 1417	*fas 2::KanMX4*	pTA1_FASIIb	pYPK0_RPL17A_*acpH*_RPL16B

3.3 Standard colony polymerase chain reaction conditions

The colony polymerase chain reaction (PCR) consists of the amplification of a certain segment of DNA from a colony of cells, which requires a reaction mixture with the following composition: 50% master mix (MM), 5% Primer_Forward, 5% Primer_Reverse, and 40% ultrapure H_2O (Milli-Q®). The MM is made up of 20% Taq Buffer (made in house), 4% Taq DNA polymerase (made in house), 8% $MgCl_2$ (50 mM; Bioline®), 4% Nucleoside triphosphate (10 mM; NZYTech®) topped up with ultrapure H_2O. Each PCR tube contains a small portion of cells, adding about 10 µL of the reaction mixture. A PCR tube only with the reaction mixture was the negative control. After spin down, the PCR tubes were placed in the thermocycler (T100; Bio-Rad®) with the temperature program obtained according to the specifications of the annealing and extension steps – **Table 3.3**. PCR products were analyzed by agarose gel (1% tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer 1x (Bio-Rad®), 10 g/L agarose (Grispr®), 0.5 µg/ml MidoriGreen® (NipponGenetics) under electrophoresis process (5-7 V/cm). To each PCR product, 1x loading buffer (2 % FICOLL® 400, 0.0025 % Xylene Cyanol FF®, 0.3 % VAHINE® Yellow, 0.024% Orange G®) was added for DNA staining before loading the samples in the agarose gel. The weight marker (Mw) used was in the range of 250 base pairs (bp) to 10,000 bp (Gene ruler 1 kb Thermo Scientific). The electrophoresis was run at 200 V during 20-25 minutes in a horizontal electrophoresis system and DNA bands were visualized and photographed under UV light on the GenoSmart® UV transilluminator (VWR).

TABLE 3.3: Time set on thermocycler used in this work.

Target	Denaturation	*Annealing*	*Extension*
pTA1_FASIIb	95 °C, 30 s (initial denaturation: 3 min)	53.3 °C, 30 s	72 °C, 45 s/kb 1:06 min (final extension: 5 min)
pYPK0_TPI1_*fabA*_PMP3		58.9 °C, 30 s	72 °C, 45 s/kb 1:49 min (final extension: 5 min)
pYPK0_PDC1_*fabH*_TEF1		58.8°C, 30 s	72 °C, 45 s/kb 2:17 min (final extension: 5 min)
pYPK0_ENO2_*mod1*_RPL5		58.0 °C, 30 s	72 °C, 45 s/kb 1:54 min (final extension: 5 min)

3.4 Verification of FASIIb function

The capacity of the pathway was tested by growing strains on SC-L medium (µ1206, µ1239, µ1255 and µ1257) under ideal growth conditions (30 °C, 200 rpm, orbital shaker,

Certomat® HK). The growth was recorded by reading a small volume of the sample on the Genesys 20 spectrophotometer (Thermo Scientific) at 600 nm (OD_{600}). The reading was performed in 1.5 mL plastic cuvettes (Sigma-Aldrich®) and the blank was performed with the culture medium. The strains were grown in triplicate, in 100 mL Erlenmenyers, containing 20 mL of the respective medium after inoculating to an OD_{600} of 0.5. Each strain was cultivated in twenty-four identical Erlenmeyer flasks, reserving three for continuous OD_{600} measurement. For every 24h, three flasks were removed for OD_{600} reading, pH determination, HPLC metabolites characterization and lipid estimation by fluorimetry. Intracellular lipids were analyzed at the last time point using gravimetry and GC-FID analysis.

3.5 Adaptation process

A small portion of every cell with pTA1_FASIIb and pYPK0, µ1255 and µ1257 from freezer stock (-80°C with previous 50% glycerol) was spread out in solid YPD. They were placed in an incubator at 30°C (INCU-line® VWR) for 1 to 3 days, depending on the growth rate of the strain. From the biomass that emerged, part of it was transferred to a 50 mL centrifuge tube, containing 5 mL of YPD + G418 and incubated at 30 °C, 200 rpm for six days. About 300 µL of cells were transferred to a culture tube containing 5 mL of the same fresh medium. They proceeded to incubate under the same conditions (30°C, 200 rpm), recording initial OD_{600}. The cyclic process of adaptation consisted of recording the OD_{600} of the culture medium over time. When the culture reached the fourth generation, a new cycle was started: 100 µL of the culture was transferred to a new tube with fresh 5 mL of YPD + G418, repeating the absorbance readings and restarting the cycle. A total of seven cycles was considered for a strain to be said to have adapted.

At the end of the adaptation process, plasmids presence was confirmed by plating the strains on SC-HULT (negative control), YPD (positive control) and selective medium (SC-L for µ1255 and µ1257; SC-L-U for strains with two plasmids) at 30 °C. The confirmed strains were then stored at -80°C in cryotubes (AHN® myTube) with previous 50% glycerol (Fischer Chemical®). To be able to differentiate the ancestral strains from the newly adapted strains, they obtained a new ID, found in **Table 3.2**.

3.5.1 Strains selection

To understand which of the adapted strains had a distinct phenotype, a pre-inoculum was prepared in 5 mL YPD + G418 and incubated (30 °C, 200 rpm). After two days of growth, the

volume necessary for an initial OD_{600} of 0.05 was transferred to a new culture tube with 5 mL YPD + G418 and incubated at the same conditions for three days. The OD_{600} was recorded at the start and after three days of incubation. The number of generations was then determined for that interval of growth.

The strains with a higher number of generations were then grown again in 100 mL Erlenmeyer flasks with 20 mL of YPD + G418 and an initial OD_{600} of 0.05, in triplicate. The growth was recorded as OD_{600} and the model (**Section 3.9**) was adjusted to the data. When the growth reached the stationary phase, an aliquot of culture was plated in SC-L-U to confirm the presence of the plasmids (µ1239 used as control). Additionally, confirmation by polymerase chain reaction (PCR) and sequencing was also performed.

3.5.2 Confirmation of strains by colony PCR and plasmid sequencing

To guarantee the presence of the plasmid, and the correct selection in **Section 3.5.1**, we performed the extraction of the plasmids from yeast strains using the commercial kit Miniprep (NZYTech®) according to its specifications. With this extraction material, we increased the number of copies of plasmids by the transformation of *E.coli* competent cells (Inoue et al., 1990). The transformation of *E. coli* (XL1-Blue strain) was carried out using 20 µL of competent cells and 5 µL of the extraction material from samples. The two process controls contained the same volume of competent cells used, being added to one of them 0.2 µL of previously extracted pTA1 (Positive Control) and to the other ultrapure H_2O (Negative Control). After remaining on ice for 30 minutes, the samples are subjected to a temperature of 42 °C for 45 seconds, returning to the ice for 3 minutes. Approximately 4 volumes of SOC were added to the samples and later plated in LB+Amp. After incubation of the plates at 37 °C (Medcenter 55; Incucell), colonies were selected and transferred to a new plate for incubation at the same temperature. Through the isolated cells, we started a new plasmid extraction using the same method (commercial kit NZYMiniprep®), and the purified vectors were used by PCR and sequencing.

For colony PCR, to detect both plasmids (pTA1_FASIIb and pYPK0), two reaction mixtures were prepared, each one with different pair of primers: p577 and p578 to detect pYPK0; p856 and p623 to identify pTA1_FASIIb (according to **Figure 3.1**; see **Appendix 8.2** for more information). The amplification occurred following the parameters in **Table 3.3.**

To know effectively the individual genes of FASIIb in the pYPK0 vector, we send for sequencing (LightRun® tube - Eurofins Genomics) a microtube with a final concentration of 50 ng/µL purified plasmids and 5 pmol/µL of one of the primes: p468; p467; p444; p577 and p578 (see **Appendix 8.2**). The sequence generated in FASTA format is aligned with the template, using the *Benchling* software.

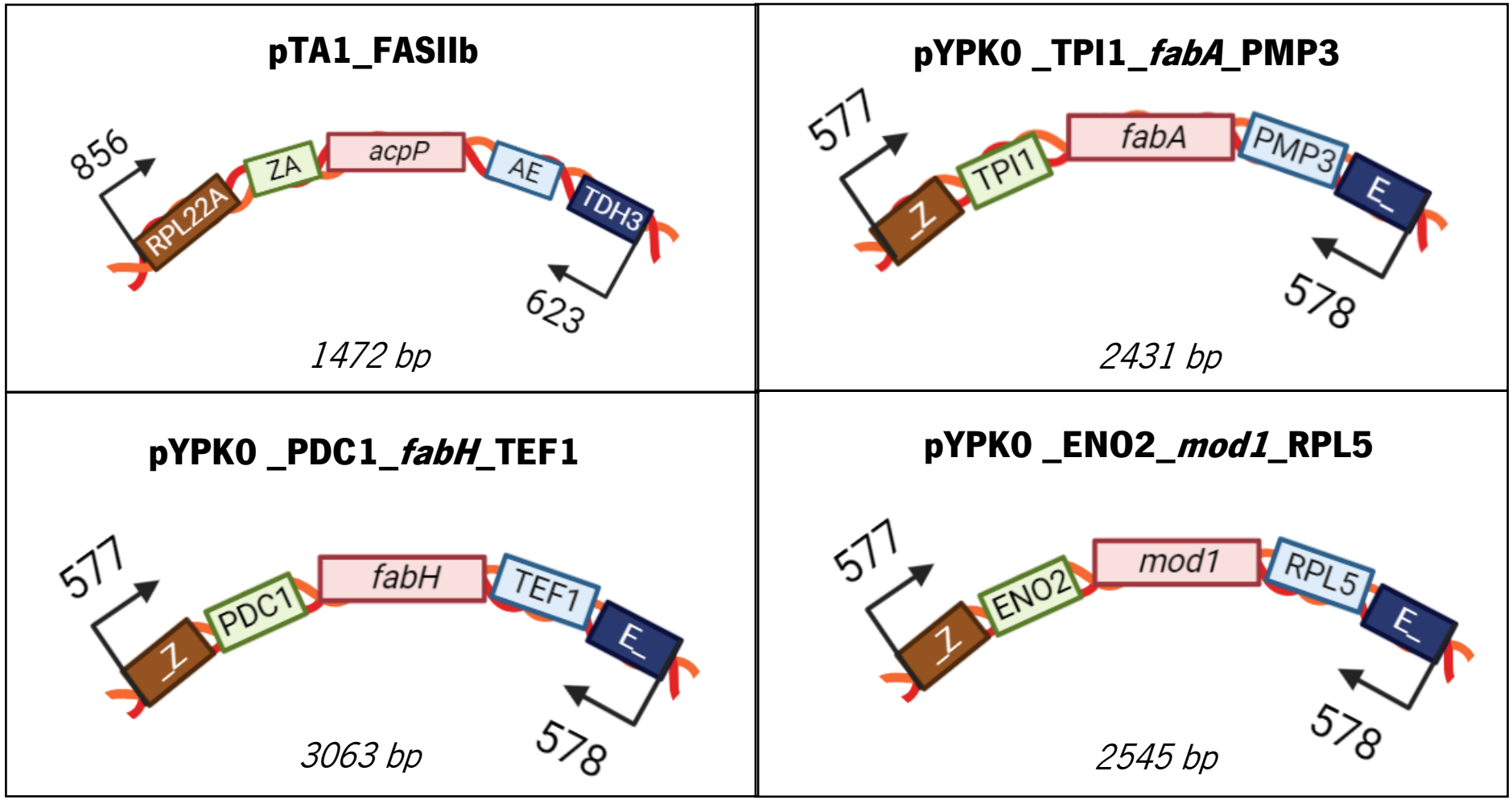
3.6 Effect of carbon source in adapted strains growth

After confirmation of the presence of plasmids from adapted strains, to understand the influence of the adaptation process and the overexpression of individual genes (pYPK0 plasmid), we proceeded with the phenotypic assays of the strains using two different media (YPD + EtOH and YPD + Hmy). A pre-inoculum was prepared in 5 mL YPD and incubated at 30 °C, 200 rpm for 24h. For inoculum, one first in YPD + EtOH with G418 for the two adapted strains positively confirmed (**Section 3.5.2)** and the respective originals in triplicate and another in YPD + Hmy with G418 for the same strains plus the control strain adapted (µ1421) and ancestral one (µ1257). The OD_{600} was 0.005 and 0.2, respectively, and its difference is due to previous experiments in order to match the experimental growth of the strains with the schedule of the laboratory.

3.7 Effect of individual FASIIb gene on lipid profile

With the µ1128 strain, we can investigate the effect of each gene of pYPK0 in the adapted strains (**Section 3.5.2**) in lipid production. To access this information, a pre-inoculum was prepared in 5 mL YPD and incubated at 30 °C, 200 rpm for 48 h to reach the stationary phase. From this point, we inoculated (OD_{600} of 0.05) to a new 20 mL of YPD + G418 and incubated at 30 °C, 200 rpm until 192 h. In duplicate, each strain had one Erlenmeyer flask for continuous measurement at OD_{600} and seven Erlenmeyer flasks for each 24 h (24,48,72,120,168 and 192 h) measured of OD_{600} at the end of each time point and for lipid analysis. For time point 0 h, the inoculum was carried out in 50 mL of YPD, to increase the biomass quantity. The samples were stored in 50 mL tubes, transferring about 1.6 mL to a 2 mL microcentrifuge tube with the remaining volume being stored at -20 °C until use. The microcentrifuge tube was centrifuged (1 min at 16110 RCF Eppendorf 5415D) and kept pellet for fluorometry analysis.

FIGURE 3.1: Schematic representation of the annealing zone for the different primers and the size expected according to the plasmid.



3.8 Analytical methods

3.8.1 Determination of the pH of the medium

The cultures flasks (**Section 3.4**) were centrifuged (3 min at 4400 RCF, Eppendorf 5804R) and the supernatant was used for pH measurement and the pellet was stored at -20 °C for gravimetric analysis. The pH was measured in 5 mL of supernatant using the pH meter micro pH 2002 (Crison®) coupled with the electrode 52 10 (Crison®). The pH values were recorded and expressed as the mean of triplicates affected by standard deviation.

3.8.2 Analysis of medium composition by HPLC

From the culture tubes (**Section 3.4**), 1.6 mL was transferred to a 2 mL microcentrifuge tube and centrifuged (1 min at 16110 RCF Eppendorf 5415D), with the supernatant transferred to a clean microcentrifuge tube while the pellet was reserved for fluorometric analysis. Since for HPLC analysis, the samples should be free of proteins, a volume corresponding to 10% of the total sample volume of 100% (w/v) trichloroacetic acid ($C_2HCl_3O_2$; PanReac AppliChem®) was added and led to react overnight at room temperature. The samples were then centrifuged (5 min at 4500 RCF, 4°C Eppendorf 5804R) and the supernatant was filtered through a 0.22 µm hydrophilic polytetrafluoroethylene (PTFE) syringe filter (VWR) into a 1.5 mL HPLC vial. A calibration curve was also prepared with the components we intend to analyze in order to determine the retention time plus detection and quantification limits - **Table 3.4**.

The HPLC analysis was performed using column chromatography HyperRE® XP Carbohydrate H^+ 8 µm (Thermo Electron Corporation) on a LaChrom Elite® (VWR Hitachi) chromatography system with a LaChrom Elite® L-2490 RI detector (VWR Hitachi). Sample injection was performed by an autosampler system (LaChrom Elite® L-2200, VWR Hitachi) with a volume of injection of 10 µL. The flow rate was 0.5 mL/min and both the detector and the column were at 45 °C. The analysis was run for 30 min. For the mobile phase, a solution of 2.5 mM sulfuric acid (95-97% Merck®) in ultrapure H_2O was prepared and vacuum filtered through 0.22 µm acetate cellulose filter.

The results of the different components detected are expressed as mean ± SD.

TABLE 3.4: Detection and quantification limits of compounds under HPLC analysis.

Compound	*Detection limit*	*Quantification limit*
	g/L	
Acetic acid	0.082	0.275
Glucose	0.181	0.603
Glycerol	0.057	0.019
Ethanol	0.057	0.190
Propionic acid	0.079	0.262

3.8.3 Detection of lipids environment by fluorometry

This method was performed as described by Miranda, (2018) and consists of the resuspension of the pellet (**Sections 3.7** and **3.8.2**) with 1 mL of 1x PBS (VWR), centrifuge (1 min at 16110 RCF Eppendorf 5415D) and resuspension with PBS. All samples were adjusted to OD_{600} of 1 with 1x PBS in a 2 mL dark microtube. This was followed by the addition of 83 µL PBS: DMSO (1:1 v/v; Sigma-Aldrich®) and 100 µL Nile red fluorophore (50 ug/mL 98% Sigma-Aldrich®) in acetone (99.5 % PanReac AppliChem®). The volume added in this step is removed previously before the incorporation of these compounds. In a twilight environment, 200 µL of homogenized suspension of cells was transferred to a 96-well fluorometer black microplate (Polystyrene Costar®), in triplicate and immediately analysed by the fluorometer (Fluoroskan Ascent FL, Thermo Scientific). The analysis parameters were: kinetic readings at 30 °C, during 20 minutes with 60 seconds break and 30 seconds of shaking previously to each reading; fluorescence excitation at 530 nm and emission at 590 nm. As a blank, a solution of PBS: DMSO (1:1 v/v) and Nile red without cells, were used. As a control, a cell suspension with known RFU value was used allowing us to compare the values between different microplate readings.

3.8.4 Determination of lipidic quantity by gravimetric process

For lipid quantification, cells stored at -20°C in tubes (**Section 3.4**) were defrosted and centrifuged two times (3 min at 4400 RCF, Eppendorf 5804R) with the pellet resuspended between centrifugation in 10 mL deionized H_2O. The final pellet was resuspended in 700 µL of deionized H_2O and from cell suspension, 1 mL was pipetted to 2 mL microtubes. This one was centrifuged

(1 min at 16110 RCF Eppendorf 5415D) for supernatant removal and the pellet was stored at -80 °C until freeze-dried (24 h, -50 °C, 0.140 mBar, Labconco Freezone 2.5).

The extraction and gravimetric analysis were performed according to Bettencourt, (2019), and consisted in weight of 20 mg of lyophilized cells (register the exact value) to 2 mL microtubes with a screw lid (VWR) with 400 mg of glass beads (diameter 0.4-0.6 mm Sartorius). After the addition of 1 mL of n-hexane (99 % PanReac AppliChem®), cell lysis was performed in Gene Disruptor (5 min at 2850 rpm; Scientific Industries) twice with 1 min on the ice at the end. Solvent and lipids were recovered after centrifugation (2 min at 14100 RCF miniSpin plus Eppendorf) to a glass centrifuge tube. The glass beads were washed three times and the solvent was added to the glass centrifuge tube. Each washing time consisted of the addition of 0.5 mL n-hexane and homogenized in the Gene Disruptor (1 min at 2850 rpm) and cold 1 min on ice before supernatant recovery to minimize solvent volatilization when opening the microtubes. After adding 1/5 of the total volume of NaCl (0.9 % w/v Fluka) to the solvent with lipids (glass centrifuge tube), and vortexing for 40 seconds (to remove any component soluble in water), the samples were centrifuged (5 min at 1000 RCF 4 °C Eppendorf 5804R) and the supernatant was filtered by 0.22 µm PTFE (13 mm diameter filter VWR) using glass syringe into a previously weighed and labeled 4 mL amber vial. The solvent was then evaporated on an evaporation system with a heated stream of N_2 for about 40 minutes in a fume hood. The vial with lipids was then placed in a desiccator to remove any humidity before weighing.

For batch assay using YPD medium (**Section 3.7**), the protocol was modified to increase the lyophilized biomass, so the pellet from 50 mL culture was resuspended in 5 mL of deionized H_2O (instead of 700 µL), transferred to a 15 mL tube and stored at -80 °C until freeze-dried (24 h, -50 °C, 0.140 mBar, Labconco Freezone 2.5). Furthermore, we increased the lyophilized sample to 50 mg and the glass beads to 800 mg. The initial cell lysis and homogenization during glass beads were also extended from 5 min to 15 min, as well as 1 min to 5 min, respectively. Centrifugation was also incremented by an extra 1 min . Solvent evaporation was also different since the previous evaporation system was no longer available. For these samples, the evaporation of the organic phase took place using a stirring hot plate (Velp Scientific) at 70 °C, for approximately 2 h in a fume hood. The vials were then placed in the desiccator until weighed.

For both protocols, a control sample containing olive oil (*Oliveira da Serra Extra Virgem*) was also added to determine the extraction efficiency. The lipid content was then calculated by the

weight difference between the vial with lipids and the empty vial weight and normalized by the dry cell mass used in the extraction. Samples were analyzed in triplicate, and the data were expressed as mean ± SD. All material used after the lyophilization process was previously washed with n-hexane, preferring the use of glass material.

3.8.5 Evaluation of lipidic profile by gas chromatography with mass spectrometry detector or flame ionization detector

After the lipids extraction with n-hexane described in **Section 3.8.4**, lipids were transesterified to reduce the boiling point of the FAs and thus, the lipidic profile could be measured using gas chromatography with mass spectrometry (GC-MS) or flame ionization detector (GC-FID). The transesterification step was performed according to Bettencourt, (2019) and consisted of: lipids suspended in 1 mL n-hexane (99% PanReac AppliChem®) were transferred to a glass-capped tube with the screw. After the addition of 1 ml of H_2SO_4: MeOH (1:10 (v/v)) and 100 µl of heptadecanoic acid (98% Sigma®) as an internal control, the well-closed tubes were incubated at 100°C for 2 h, in a water bath. After reaching room temperature, 1 mL n-hexane was added to the recent FAMEs, transferring the upper phase to a conical glass tube. To this, a micro spatula of Na_2SO_4 (Merck®) was added, and if a resistant aggregate formed, one more was added. Transfer the upper phase to a 2 ml amber vial and analyze it by GC. In addition to these samples, two more were prepared, containing Oleic (Sigma-Aldrich®) and Stearic acid (Sigma-Aldrich®) alone, following the same procedure as the remaining samples. These were made to identify the retention time of these very similar compounds at a structural level that can lead to the appearance of a single peak during chromatographic analysis.

GC-MS analysis was performed by the Agilent Varian 450 gas chromatograph coupled to an ion trap mass detector Varian 4000 performance. The column used was low-bleed ZB-5MSi (5% phenyl; 95% dimethylpolysiloxane phase) with the dimensions: 30m x 0.25mm x 0.25 µm (Zebron). The carrier gas was He_2 at constant pressure with a flow rate of 1.0 mL/min. The injection was manual, and the volume was 0.4 µL with a split ratio of 1:20, changing to 1:100 after 2 minutes of the run. The injector was at 250 °C and the column temperature was increased from 80 °C up to 250 °C at 8 °C/min and held for 5 minutes at this last temperature. The mass spectrometry was performed in EI mode in full scan, with a mass range of mass/charge 35 - 1000 and an

ionization energy of 70 eV. The solvent n-hexane was injected between samples for column washing.

GC-FID analysis was performed in a gas chromatograph (CP-3800 Varian) with a FID detector and auto-sampler module. The chromatography column had 30m x 0.25mm x 0.2 µm (TRWAX, Teknokroma). The carrier gas was He_2 with a flow of 1 mL/min at a constant pressure of 16 to 17 psi. The column, in the first 2 minutes was at 50 ^{o}C, followed by a heating ramp of 10 ^{o}C/min to 225 ^{o}C, keeping this same temperature for 10 minutes. The injector and the FID detector were up to 250 °C. The split ratio was 1:20 injection volume, with the sample volume being 1 µL.

The retention time of the peaks in the samples was compared to the retention times of the standard mixture of 37 FAMEs (SupelcoTM 37 Component FAME mix, Sigma-Aldrich®), aided by the mass of the ions generated, in the case of GC-MS. Data were analyzed using Varian PFFD Analytical Software (GC Workstation Multi Instrument Version 6.3). The results are expressed as percentage of the detected FAMEs peaks, showing their mean of the triplicates ± SD.

3.9 Growth curves and adjustment model

The growth assays performed throughout this work consist of the periodic measurement of OD_{600}. From each replica, its average was displayed, with a standard deviation (SD) associated with each point. This is represented by an error bar.

$$OD_t = OD_0 \times \exp^{ut}$$

Equation 1: Adjustment model used in the exponential growth phase. Equation used to fit the experimental points in the exponential phase of the growth curve outlined by the OD_{600} vs Time (h) graph.**t –** time; **OD –** Optical density at 600 nm; **OD_0 –** OD at t=0; **µ -** growth rate.

Data points for the exponential phase of each strain were selected, and choosing at least three points from this growth phase are incorporated into a new graph OD_{600} vs Time (h). These points were then fitted to the model described in **Equation 1**. This exponential equation estimates the growth rate (µ) allowing us to compare the growth between different strains and experiments. The quality of the fit was given by the coefficient of determination (R^2) between 0 and 1, where 1 is a perfect fit.

4. Results

4. Results

4.1 Effect of the FASIIb pathway on strain phenotype

S. cerevisiae strain CEN.PK2-1C carrying pTA1 (empty plasmid, µ1206) or pTA1_FASIIb (containing the FASIIb pathway, µ1239) with deletions in genes *fas1* (µ1255) or *fas2* (µ1257) were cultivated for 168h at 30 °C, 200 rpm in SC-L medium (**Section 3.4**). Samples were periodically withdrawn for OD_{600}, lipids quantification and FAs composition, substrate and fermentation products analysis.

The OD_{600} was plotted against time in **Figure 4.1**. The data shows that strains retaining the wild-type FAS (µ1206 and µ1239) had a relatively short lag phase of 4h. These two strains reached an OD_{600} of 1.2 to 1.8 after 6h. The *fas1* or *fas2* mutant strains (µ1255 and µ1257, respectively) reached about half of the OD_{600} after this time. A lower final OD_{600} for strains with gene deletions was observed (4 compared to around 7). Growth rates calculated (**Equation 1**) from the data for the mutant strains were 0.06 - 0.07 h^{-1} ($R^2 \approx 0.99$) compared to the growth rate of µ1206 and µ1239 (0.48 h^{-1} and 0.35 h^{-1}, respectively; $R^2 \approx 0.96$).

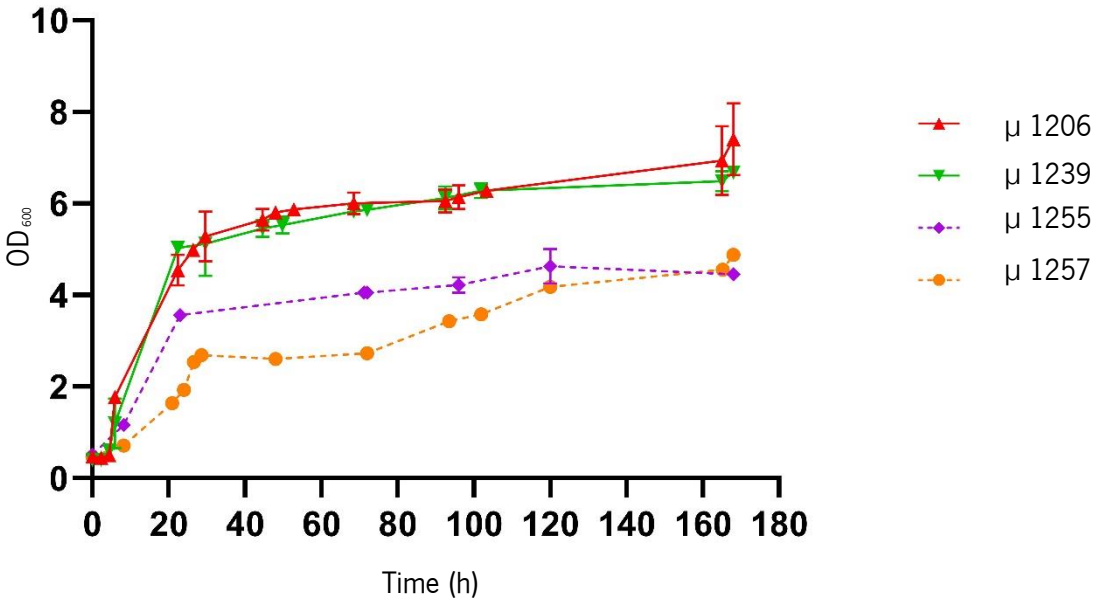


FIGURE 4.1: Representation of the growth curve on SC-L. Graph of the growth in SC-L, incubated at 30 °C, 200 rpm for 168h of control strains µ 1206 (pTA1) , µ 1239 (pTA1_FASIIb) and the mutant strains with pTA1_FASIIb with the deletion of *fas1* (µ 1255) or *fas2* (µ 1257) native genes. Each point corresponds to the average of at least two replicates, with an error bar proportional to the SD.

Substrate and fermentation products (acetic acid, ethanol, glucose, glycerol and propionic acid) in the medium were analysed by HPLC (**Figure 4.2**) at several intervals between 0 and 168h. Glucose was rapidly consumed within 24h, and after this time, the concentration was lower than the detection limit (0.181 g/L). Glycerol and propionic acid were detectable at 24h at a concentration that remained stable over time. Acetic acid peaked at 168h for all strains except for μ1257, where it is below the detection limit (0.082 g/L) after 96h. Ethanol reached the maximum peak after 24h of cultivation except for strain μ1257 where ethanol reached its maximum for 48h after incubation. After the maximum peak, the concentration of ethanol decreases for all strains.

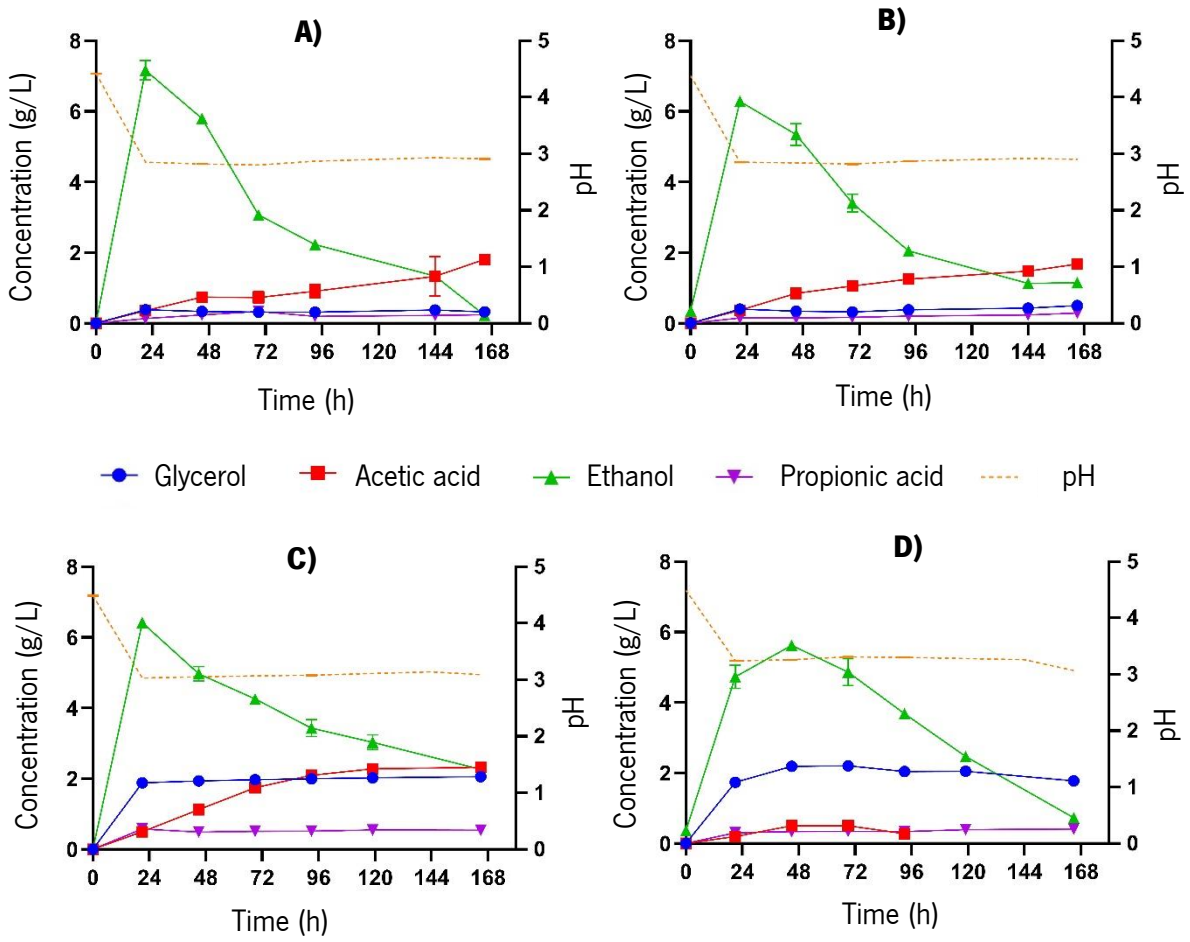


FIGURE 4. 2: HPLC and pH results over time. Concentrations are expressed in g/L. Each graph corresponds to the analysis of a single strain. A) μ 1206 (pTA1); B) μ 1239 (pTA1_FASIIb); C) μ 1255 (*fas 1* :: KanMX4 pTA1_FASIIb); D) μ 1257 (*fas 2 ::* KanMX4 pTA1_FASIIb). The results are expressed as mean ± SD.

Relative fluorescence units (RFUs) were plotted over time for the four strains in **Figure 4.3**. The strains with native FAS (µ1206 and µ1239) display a jump in relative fluorescence units between 72h and 96h after which there is only a slow increase (left panel – **Figure 4.3**). For strains depending on the FASIIb pathway (µ1255 and µ1257), the RFUs remain low (right panel – **Figure 4.3**) until 96h, when RFUs suffer a big jump. The final value is around 9 for all strains except for µ1257 which reached only 6 at 168h.

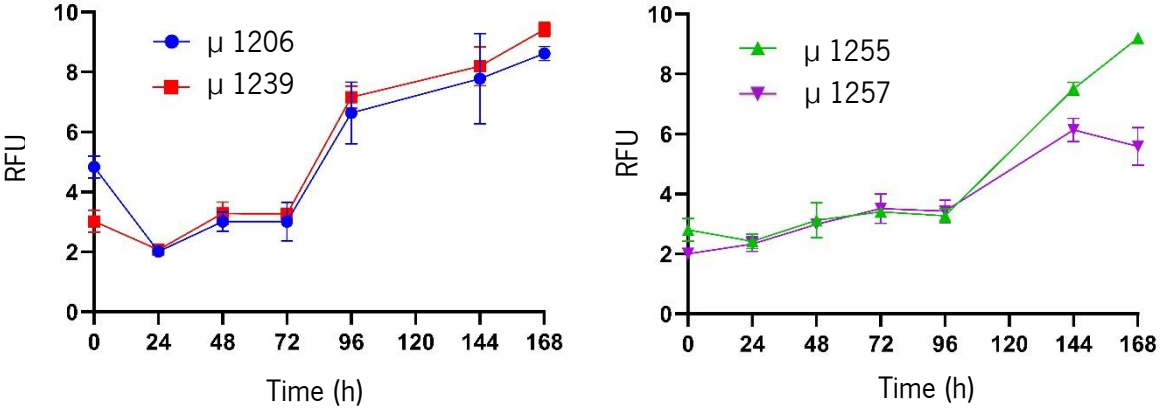


FIGURE 4.3: Variation of relative fluorescence units over time. Graphic representation of fluorometry results for strains with FAS native (µ 1206 and µ 1239) and for dependent FASIIb strains (µ 1255 and µ 1257) over the incubation time. The stain used was Nile red, and the values were calculated by the difference between the sample value and the blank. Data are expressed as mean ± SD of triplicates.

The relative fluorescence was plotted against OD_{600} for the four cultures in **Figure 4.4**. There is a decrease in detected RFU between the two first measurements a trend that is counteracted at the beginning of the stationary phase, in which the rapid increase in OD_{600} is replaced by a rapid increase in RFU. All strains represented display this general phenomenon, except for the *fas2* strain (µ1257) which shows a steady increase in RFU/OD_{600} over time. It can be noted that the maximum peak of RFU occurs during the stationary phase around OD_{600} 6 - 7 for native FAS strains and 4 - 4.5 for mutant strains. This indicates that even with the cessation of growth, cells continue to accumulate lipids.

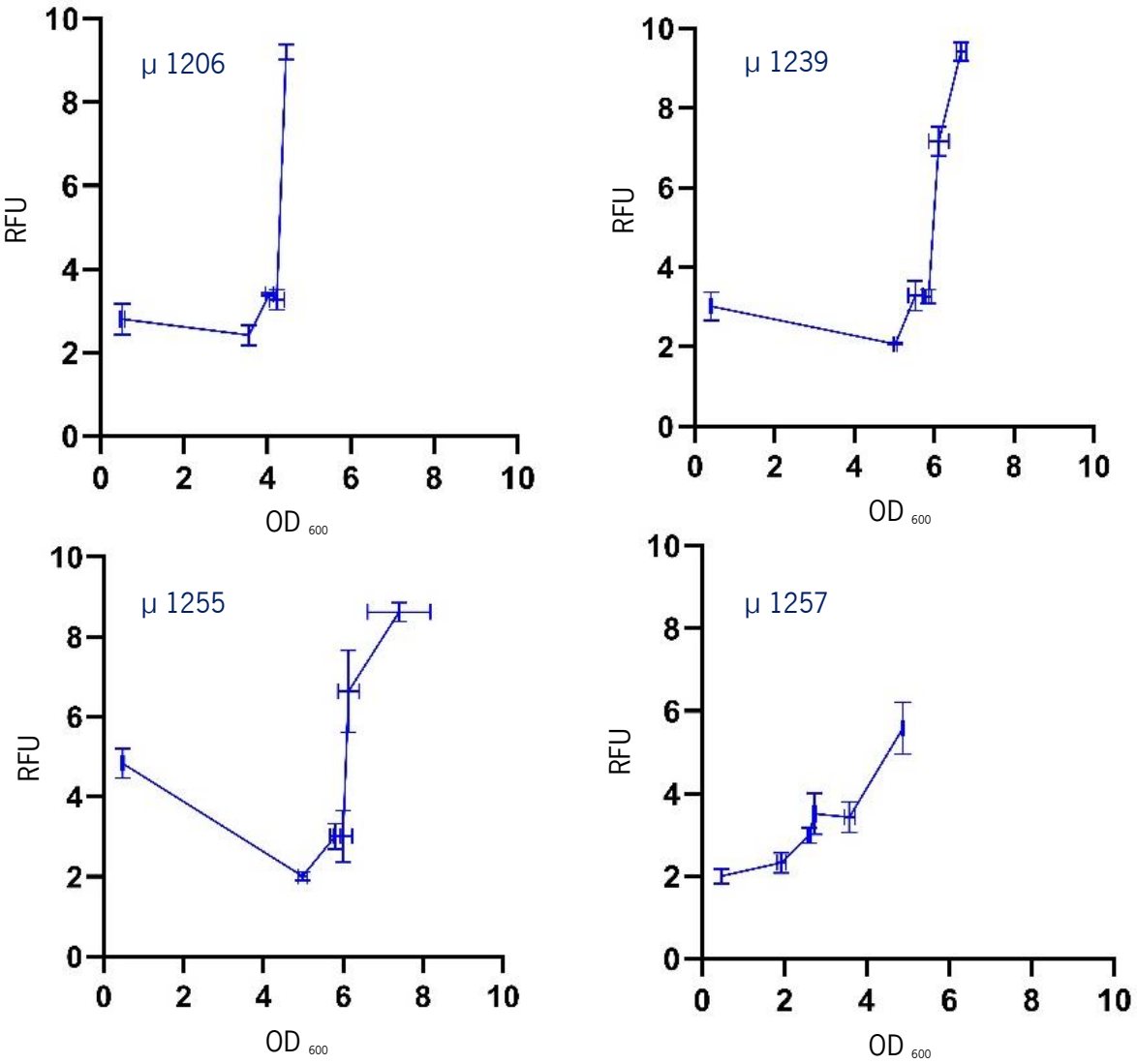


FIGURE 4.4: Correlation of RFU over OD_{600} . Representation of RFU values according to the OD_{600} from the growth curve results. The continuous time-points (0,24,48,72,96,144 and 168h) are selected, and a graph drawn with the RFU values at these time-points. Two error bars are associated with each point, a horizontal one that codes for the error in the determination of the OD_{600} and a vertical one, which concerns the calculation of the RFU. µ1206 (pTA1); µ1239 (pTA1_FASIIb); µ1255 (*fas 1* :: KanMX4 pTA1_FASIIb); µ1257 (*fas 2* :: KanMX4 pTA1_FASIIb).

Quantitative analysis of cellular lipid content after 168h of incubation was determined by gravimetry after extraction with n-hexane – **Table 4.1**. Intracellular lipids reached 4.5% of dry cell mass for *fas1* and *fas2* strains (µ1255 and µ1257, respectively), while the native FAS strains reached 6%. Protocol efficiency was calculated to 91%, based on the assumption of 100% lipid content of a commercial olive oil control sample.

The lipids profile as FAMEs was determined by GC-FID and compared between strains to see if there were any differences due to the pTA1_FASIIb pathway. Since the RFU values were higher for 168h of incubation, these samples were used for lipids extraction and GC-FID analysis. The results are expressed as mean ± SD - **Table 4.1**.

In all strains, the predominant FAs correspond to C16:1 (Palmitoleic acid), about 70% in FASIIb dependent strains (µ1255 and µ1257). On the other hand, these strains had much less C18:1n9c (around 4%) compared with 27 - 29% in strains with native FAS (µ1206 and µ1239). The two FAs mentioned correspond to about 80% of all FAs detected for native FAS strains. The remaining 20% are distributed between C16:0 (Palmitic acid), C18:0 (Stearic acid) and C18:1n9t (Elaidic acid) and just for µ1255 and µ1257, shorter chain FAs such as C14:0 (Myristic acid) and C15:0 (Pentadecanoic acid). Myristoleic acid (C14:1) is detected only in µ1257 with a percentage of 1.14%. Lipids detected with less than 1% were not considered. All strains produce five times more saturated than unsaturated fatty acids.

TABLE 4.1: Percentage of lipids and FAs determined by GC-FID. Lipid content is expressed as % lipids by dry cell mass and lipidic profile determined by GC-FID after 168h of incubation, 30 °C, 200 rpm. Results are expressed as mean ± SD of triplicates. In addition to the samples with the strains, a sample containing olive oil is used as a protocol efficiency control. µ1206 (pTA1); µ1239 (pTA1_FASIIb); µ1255 (*fas1* :: KanMX4 pTA1_FASIIb); µ1257 (*fas2* :: KanMX4 pTA1_FASIIb).

ID	Lipids (% w/w)	% FAs									
		C14:0 Myristic acid	C14:1 Myristoleic acid	C15:0 Pentadecanoic acid	C16:0 Palmitic acid	C16:1 Palmitoleic acid	C18:0 Stearic acid	C18:1n9c Oleic acid	C18:1n9t Elaidic acid	C18:2n6c + C18:2n6t Linoleic + Linolelaidic acid	Sum (%)
µ 1206	5.97 ± 0.22				9.70 ± 0.60	52.00 ± 3.00	4.40 ± 0.40	27.00 ± 2.00	5.00 ± 0.20		98
µ 1239	5.99 ± 0.18				10.30 ± 0.20	49.50 ± 0.10	4.82 ± 0.05	29.00 ± 0.40	5.02 ± 0.07		99
µ 1255	4.56 ± 0.07	1.20 ± 0.05		1.07 ± 0.70	12.70 ± 0.30	74.10 ± 0.60	1.40 ± 0.30	4.10 ± 0.10	4.09 ± 0.20		99
µ 1257	4.43 ± 0.25	2.08 ± 0.05	1.14 ± 0.03	1.02 ± 0.04	11.00 ± 1.00	72.00 ± 2.00	3.00 ± 1.00	4.00 ± 0.10	5.00 ± 0.30		99
Olive oil	91.33 ± 0.21				16.90 ± 0.10	1.23 ± 0.06	2.50 ± 0.01	69.10 ± 0.30	2.10 ± 0.40	7.05 ± 0.03	99

4.2 Optimization of the FASIIb pathway

Single gene expression vectors for each gene in the FASIIb pathway are available as a by-product of the assembly of the pathway. Strains were generated carrying the pTA1_FASIIb vector (LEU2 auxotrophic marker) and one additional expression vector (URA3 auxotrophic marker) for each of the genes (**Section 3.2**). An adaptation to a medium without FAs was carried out (**Section 3.5**). Cultures were serially diluted seven times into fresh YPD + G418 medium at 30 °C, 200 rpm. The resulting strains were collected and frozen (**Table 3.2**).

4.2.1 Confirmation of both plasmids after the adaptation process

Three strains originally transformed with a single expression vector for *fabD*, *fabZ* and *acpH* (µ1405, µ1407 and µ1417) failed to grow in medium selective for both plasmids while all other strains grew, indicating the presence of both pTA1_FASIIb and the single expression vector - **Table 4.2**. Strains containing only pTA1_FASIIb (µ1420 and µ1421) did not grow on SC-L-U medium as expected but grew on SC-L.

TABLE 4.2: Growth of strains in different media. µ1420 and µ1421 were negative growth for SC-L-U and positive for SC-L. **+** : Positive growth; **-** : Negative growth.

Strains	***SC-L-U***	***SC-HULT***	***YPD***
µ 1404	+	-	+
µ 1405	-	-	+
µ 1406	+	-	+
µ 1407	-	-	+
µ 1408	+	-	+
µ 1409	+	-	+
µ 1410	+	-	+
µ 1411	+	-	+
µ 1412	+	-	+
µ 1417	-	-	+
µ 1418	+	-	+
µ 1419	+	-	+
µ 1420	- (+ for SC-L)	-	+
µ 1421	- (+ for SC-L)	-	+

4.2.2 Adapted vs original strains

Strains were grown in YPD + G418, 30°C, 200 rpm for 72h. Knowing the initial OD_{600} and the OD_{600} after incubation, the number of generations was calculated. The adapted *fas2* strain with FASIIb grew 6.43 generations in 72h. Three strains obtained a higher value, µ1409 (7.30), µ1412 (6.86) and µ1418 (7.17). These strains had been transformed with extra copies of the *fabA*, *fabH* and *mod1* genes. This three best-performing strains as well as the control strains µ1420 and µ1421 were cultured a second time in YPD + G418 medium (**Section 3.5.1**). Using the model described in **Section 3.9**, the exponential phase and respective growth rates were described - **Figure 4.5**. The µ1421 strain grew faster at 0.24 h^{-1} followed by µ1412 carrying extra copies of *fabH.* All other strains grew at 0.19 - 0.21 h^{-1}. There were no large differences in the length of the lag phase.

The strains were collected and cultivated on solid SC-L-U to check for plasmid loss (**Section 3.5.1**). The µ1409, µ1412 and µ1418 strains grew, indicating that both plasmids had been retained during growth in a rich medium (YPD + G418) - **Figure 4.6**.

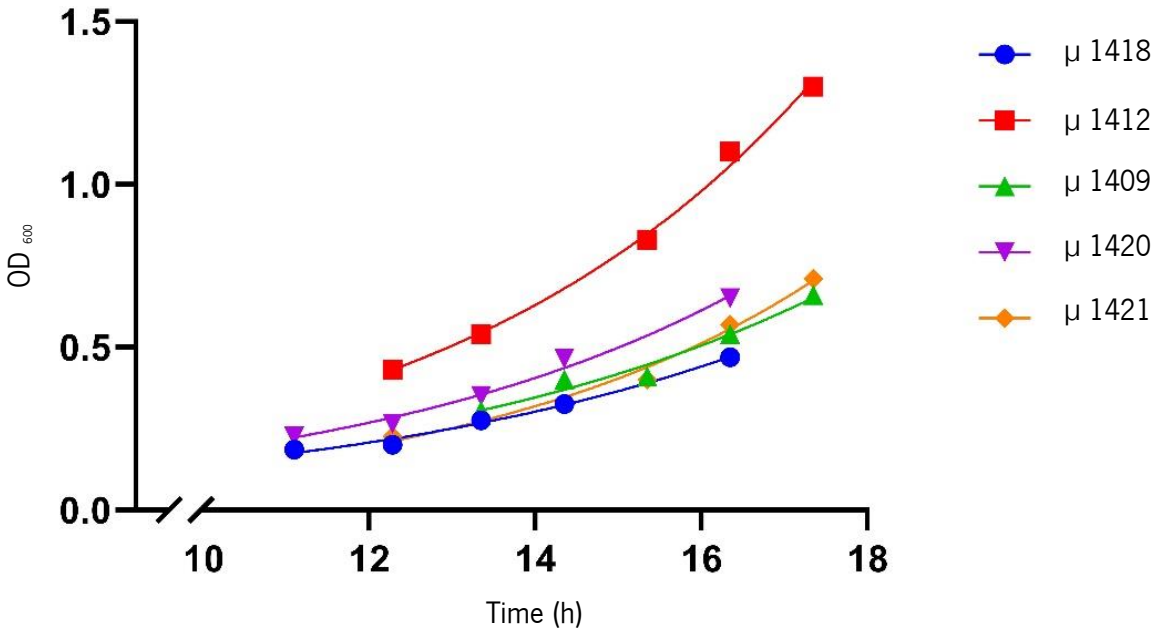


FIGURE 4.5: Growth curve of selected adapted strains in YPD + G418. Representation of adjusted OD_{600} values of exponential phase with the model to determine the growth rate of the strains. Minimal R^2 of 0.98. All strains have pTA1_FASIIb and *fas2 ::* KanMX4, except µ1420 (*fas1* ::KanMX4). µ1409, µ1412 and µ1418 have, in addition, the pYPK0 with extra copies of *fabA*, *fabH* and *mod1* genes, respectively.



FIGURE 4.6: Strain confirmation through solid SC-L-U selective medium. Representation of a section of the photographic record, of the growth obtained on each plate, after incubation at 30 °C. **+** : Positive growth; **-** : Negative growth. All strains have pTA1_FASIIb and *fas2 ::* KanMX4, except µ1420 (*fas1* ::KanMX4) and µ1239 (CEN.PK2-1C). µ1409, µ1412 and µ1418 have, in addition, the pYPK0 with extra copies of *fabA*, *fabH* and *mod1* genes, respectively.

Amplification of extracted DNA from transformed strains µ1409, µ1412 and µ1418 (**Section 3.5.2**) revealed that most colonies show a PCR product indicative of the single gene expression vector for each strain – **Figure 4.7**. Regarding strain µ1409, three colonies showed a band in the expected region between 2500 - 3000 bp, consistent with the *fabA* gene. Two of the colonies showed the expected band around 3000 bp for µ1412, indicating the presence of the *fabH* gene. All colonies of strain µ1418 showed a band approximately the size of the expected 2545 bp. The results for the amplification of pTA1_FASIIb (**Figure 4.7B**), using primers p856 and p623, showed that for µ1409 and µ1412, at least two colonies of each showed a band with the correct size (approximately 1500 bp). One colony from µ1418 showed the appearance of a band. All controls performed for the amplification process had the expected result.

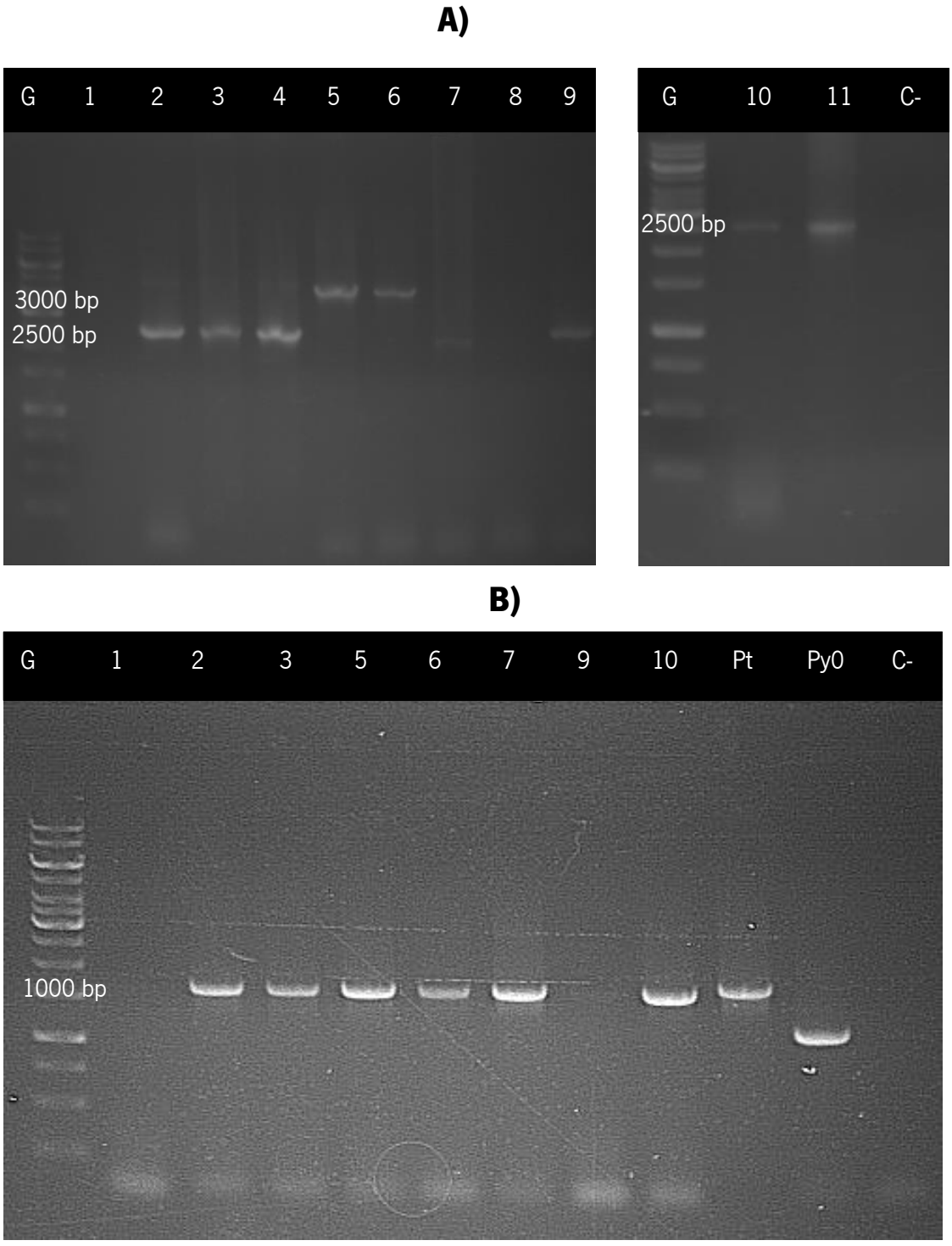


FIGURE 4.7: Agarose gel electrophoresis for amplified pYPK0 and pTA1_FASIIb. **A)** DNA fragments amplified by PCR through primers p577 and p578, for detection of pYPK0. **B)** PCR amplification, using primers p856 and p623, for detection of pTA1_FASIIb. **1-11**: Numbering of the respective colony (1-4: µ 1409; 5-8: µ 1412; 9-11: µ 1418); **G**: GeneRuler 1 kb DNA Ladder (Thermo Fischer); **Pt**: pTA1_FASIIb; **Py0**: empty pYPK0; **C-**: Negative control (without DNA template).

TABLE 4.3: Sequencing results for strains µ1409, µ1412 and µ1418 according to the primer used. X: no alignment; **ND**: not performed.

Strain ID	*p444*	*p467*	*p468*	*p577*	*p578*
µ 1409	ND	*mod1*	*mod1*	*mod1*	X
µ 1412	*fabH*	X	X	*fabH*	E-2_E-_Tef1_*fabH*
µ 1418	ND	*mod1*	*mod1*	*mod1*	X

The plasmids in the adapted strains were rescued to *E. coli* and pure DNA sequenced (**Section 3.5.2**.). Sequencing with primer p578 confirmed the presence of the *fabH* gene together with the expected promoter (PDC1 from *S. cerevisiae*) in strain µ1412 as well as the *mod1* gene in µ1418 as expected. Sequence information derived from strain µ1409 indicated the presence of *mod1* instead of the expected *fabA*. The µ1409 strain frozen right after adaptation also showed the presence of *mod1* - **Table 4.3**.

4.2.3 Effect of each FASIIb gene in growth on different media

Strains with a *fas1* or *fas2* background can grow on a medium supplemented with Myristic acid (Hmy). FAS mutants with the pTA1_FASIIb pathway partially complement this phenotype. Adaptation improved the degree of this complementation, but the nature of the adaptation is not known. The partial adaptation could have improved FASIIb capacity in which case the adaptation could be mimicked by the addition of FAs in the medium. The adapted strains (µ1412, µ1418 and µ1421) and the original strains from which they were derived (µ1290, µ1309 and µ1257) were cultivated in YPD + Hmy - **Figure 4.8**.

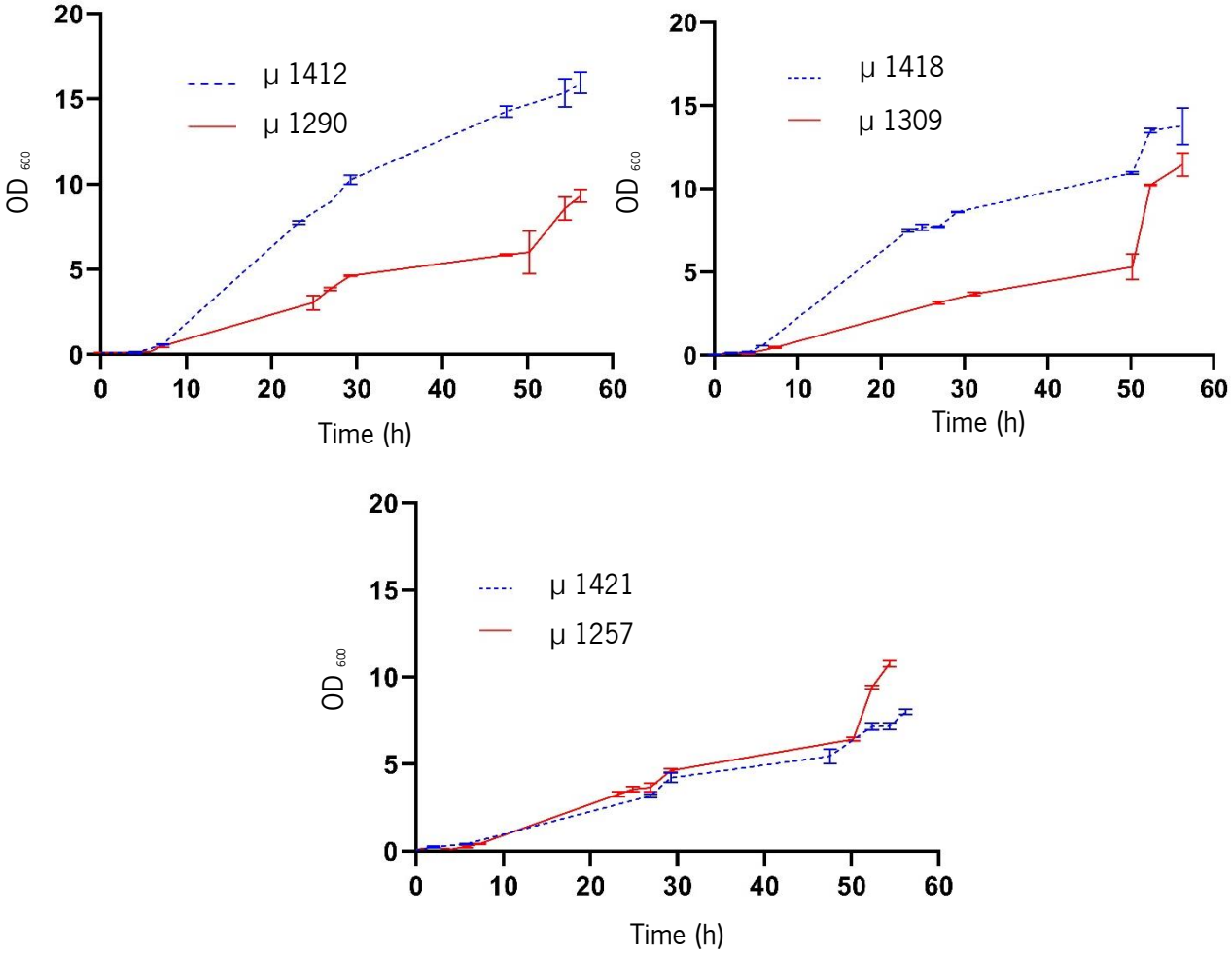


FIGURE 4.8: Comparison of the effect of Hmy on growth curves between adapted and original strains. Graphical representation of the mean values of OD_{600} over time of the adapted strain and respective original strain. All strains have pTA1_FASIIb and *fas2 ::* KanMX4. μ1412 and μ1418 have, in addition, the pYPK0 with extra copies of *fabH* and *mod1* genes, respectively. Strains μ1412, μ1418 and μ1421 are the adapted strains of μ1290, μ1309 and μ1257, in that order.

The growth of the two strains only carrying the pTA1_FASIIb (μ1421 and μ1257) is rather similar between the adapted and unadapted strains in the presence of exogenous FA. For the strains carrying extra copies of *fabH* and *mod1* (μ1412/μ1290 and μ1418/μ1309), growth was considerably quicker for the adapted strains even with fatty acids supplementation.

As discussed in **Section 1.2.3.4**, one of the approaches used to improve the amount of FAs involves the production of acetyl-CoA. One way to achieve this effect is by increasing the

availability of compounds upstream of the acetyl-CoA synthesis pathway, like ethanol (**Section 1.1.1**). We observed in the results of **Section 4.1** that peak ethanol concentration is reached before the stationary phase, coinciding with an increase in FAs. Adapted strains (µ1412 and µ1418) and the respective originals strains (µ1290 and µ1309) were cultivated in YPD supplemented with 10 g/L EtOH (**Section 3.1**). OD_{600} was plotted against time in **Figure 4.9**.

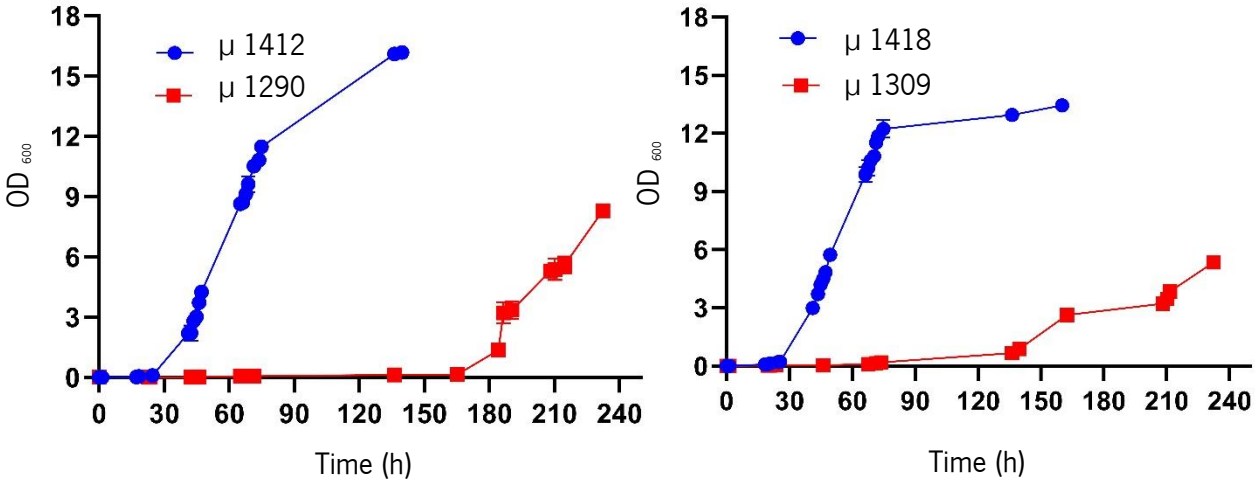


FIGURE 4.9: Impact of EtOH on growth curves. Average values of OD_{600} over time, with the error associated with each point. µ1412/ µ1290 (pYPK0 with *fabH* gene) and µ1418/ µ1309 (pYPK0 with *mod1* gene).

Even with ethanol supplementation, adapted and unadapted strains differ greatly mainly in the length of the lag phase (less than 30h for adapted; greater than 60h for µ1309 and greater than 150h for µ1290). For adapted strains, it is possible to define two distinct growth rates: the first up to 60 - 70 h of incubation and the second after that point (**Figure 4.9**).

4.2.4 Influence of Tween 40 on strain phenotype

Tween 40 (polyoxyethylene sorbitan monopalmitate) is a commercial emulsifier used in the pharmaceutical, cosmetic and food industries as a vehicle for fat-soluble compounds (O'Sullivan et al., 2004). This emulsifier has a Palmitic acid (C16:0) FA moiety that helps dissolve Myristic acid when supplemented to the medium (**Section 3.1**). Some components of high structural similarity, such as Tween 80 (polyoxyethylene sorbitan monooleate), were reported as a source of FAs (Dekker et al., 2019). To rule out this possibility of Tween 40 interference in the results, strains Ω183 (CEN.PK2-1C), µ1202 (*fas 2* :: KanMX4 pTA1) and µ1257 (*fas 2* :: KanMX4

pTA1_FASIIb) - **Section 3.2** - were cultured in three different media: YPD, YPD +Tween 40 and YPD + Tween 40 + Hmy. All media was supplemented with G418, except for Ω183 culture.

OD_{600} for the cultures were plotted against time in **Figure 4.10**. Growth of the Ω183 (wild-type) was not affected much by supplements in the media. For the remaining two strains, Tween 40 + Hmy affected growth positively and Tween 40 only had a small positive effect. These results do not rule out a small effect by Tween 40.

After the growth curve, a sample from the cultures was grown in solid media, whose results are shown in **Table 4.4.** This reveals a generalized growth in all solid media, as expected. Strain µ1202 does not show any growth on solid YPD without Hmy.

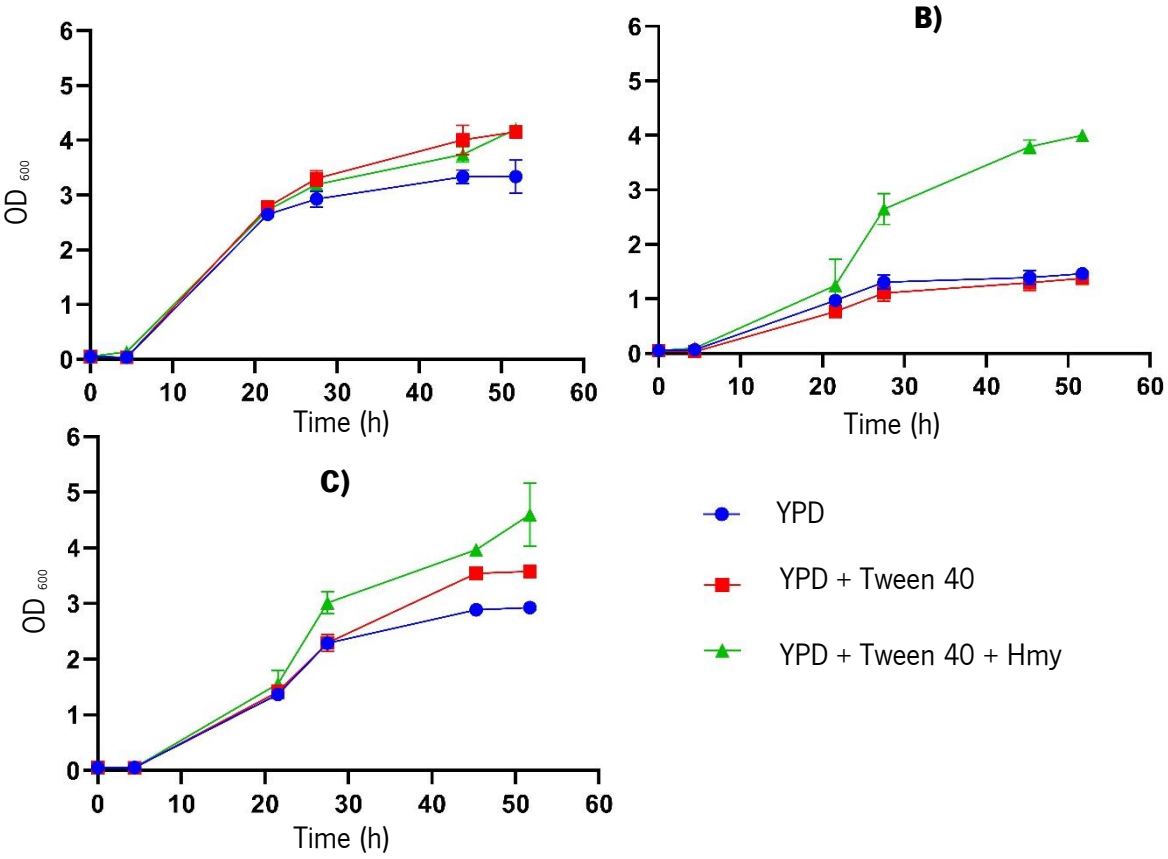


FIGURE 4.10: Growth curves in YPD, YPD + Tween 40 and YPD + Tween 40 + Hmy. Representation of the variation of the OD_{600} over time according to the medium used. All media with G418 (except for Ω183). **A)** Ω183 (CEN.PK2-1C); **B)** µ1202 (*fas 2* :: KanMX4 pTA1); **C)** µ1257 (*fas 2* :: KanMX4 pTA1_FASIIb).

TABLE 4.4: Verification of strains growth on solid medium. The result of each sample's growth from the aqueous culture,according to different solid media. The results were made in duplicate, consistent with each other. **+** - Significant growth; **-** - No pronounced growth. Ω183 (CEN.PK2-1C); µ1202 (*fas 2* :: KanMX4 pTA1); µ1257 (*fas 2* :: KanMX4 pTA1_FASIIb).

From growth curve	***Growth on solid medium***		
	YPD	***YPD + Tween 40***	***YPD + Tween 40 + Hmy***
Ω 183 - YPD	+	+	+
Ω 183 – YPD + Tween 40	+	+	+
Ω 183 – YPD + Tween 40 + Hmy	+	+	+
µ 1202 - YPD	-	-	+
µ 1202 – YPD + Tween 40	-	-	+
µ 1202 – YPD + Tween 40 + Hmy	-	-	+
µ 1257 - YPD	+	+	+
µ 1257 – YPD + Tween 40	+	+	+
µ 1257 – YPD + Tween 40 + Hmy	+	+	+

4.2.5 Effect of each FASIIb gene on lipid profile

Strains µ1128 (CEN.PK2-1C, pTA1, pYPK0), µ1412 (*fas2*, pTA1_FASIIb, pYPK0_PDC1_*fabH*_TEF1) and µ1418 (*fas2*, pTA1_FASIIb, pYPK0_ENO2_*mod1*_RPL5) were cultivated on YPD + G418 or YPD for µ1128. Incubation was carried out at 30 °C from an initial OD_{600} of 0.05 until 192h (**Section 3.7**). The reading of the OD_{600} was carried out continuously over time, and at specific time points and plotted in **Figure 4.11**.

Adapted strains µ1412 and µ1418 started growing noticeably after 24h while the strain with the native FAS had reached an OD_{600} of about 7 at this time. Additionally, this strain reached a maximal OD_{600} of around 15, much superior to the same parameter for adapted strains (around 10).

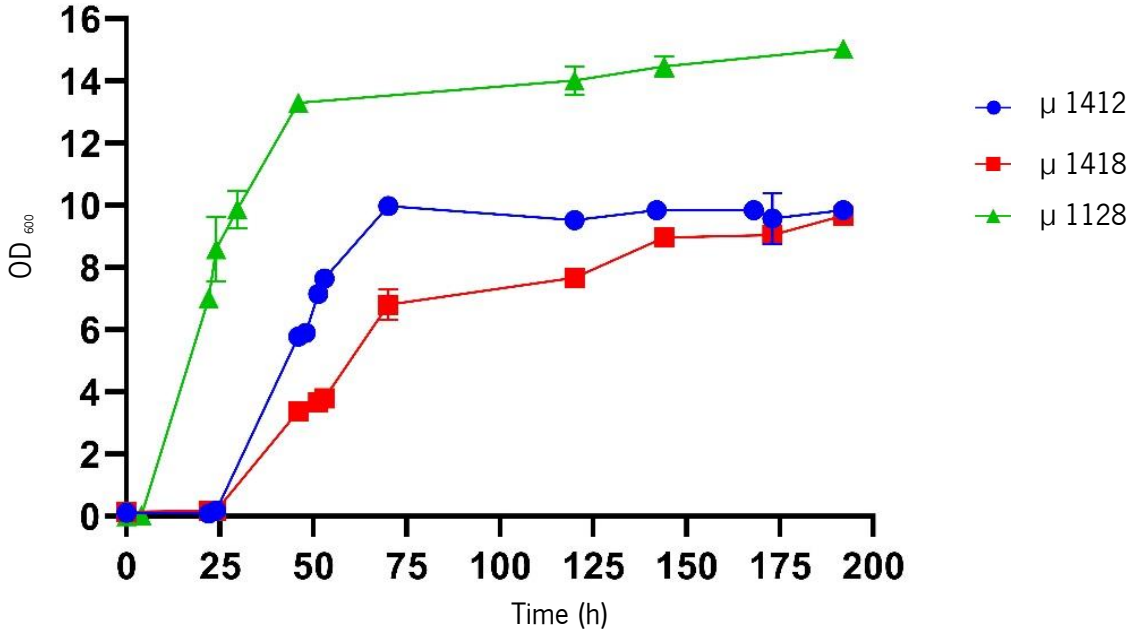


FIGURE 4.11: Graphical arrangement of OD $_{600}$ values of YPD growth curve over time. Each OD_{600} point displayed on the graph is associated with SD. For plotting the graph, all OD $_{600}$ points were included. µ1412 (*fas2 ::* KanMX4 pTA1_FASIIb, pYPK0_PDC1_*fabH*_TEF1); µ1418 (*fas2 ::* KanMX4 pTA1_FASIIb, pYPK0_ENO2_*mod1*_RPL5); µ1128 (CEN.PK2-1C pTA1, pYPK0).

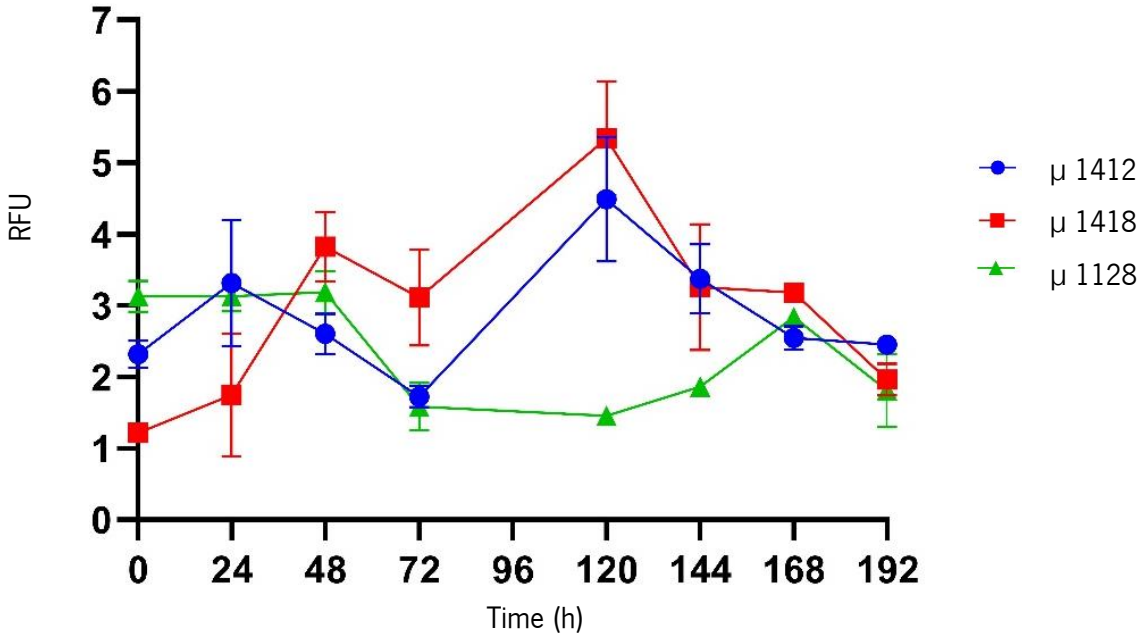


FIGURE 4.12: Graphic representation of the registered values of RFU. Mean values of RFU detected for each strain over time, with an error bar corresponding to the SD associated with each point. µ1412 (*fas2 ::* KanMX4 pTA1_FASIIb, pYPK0_PDC1_*fabH*_TEF1); µ1418 (*fas2 ::* KanMX4 pTA1_FASIIb, pYPK0_ENO2_*mod1*_RPL5); µ1128 (CEN.PK2-1C pTA1, pYPK0).

The amount of lipids in each strain was estimated by fluorimetry over various time points (**Section 3.8.3**). For each time point, the sample was performed in duplicate, and for the fluorimeter analysis, each replicate was performed in triplicate, with six values for the same time point. The results are expressed in RFU and obtained as the difference between the value recorded in each sample and the value recorded by the blank. The results obtained are shown in **Figure 4.12**. The strain µ1128 shows maximum fluorescence at 48h while adapted strains with FASIIb reach maximum only after 120 h. Strains µ1412 and µ1418 show a relative fluorescence of 4.5 - 5.3, while strain µ1128 only reaches 3.2.

Relative fluorescence was plotted against OD_{600} in **Figure 4.13**. The plot reveals that the productivity of two FASIIb strains has a rather narrow maximum compared to the strain with native

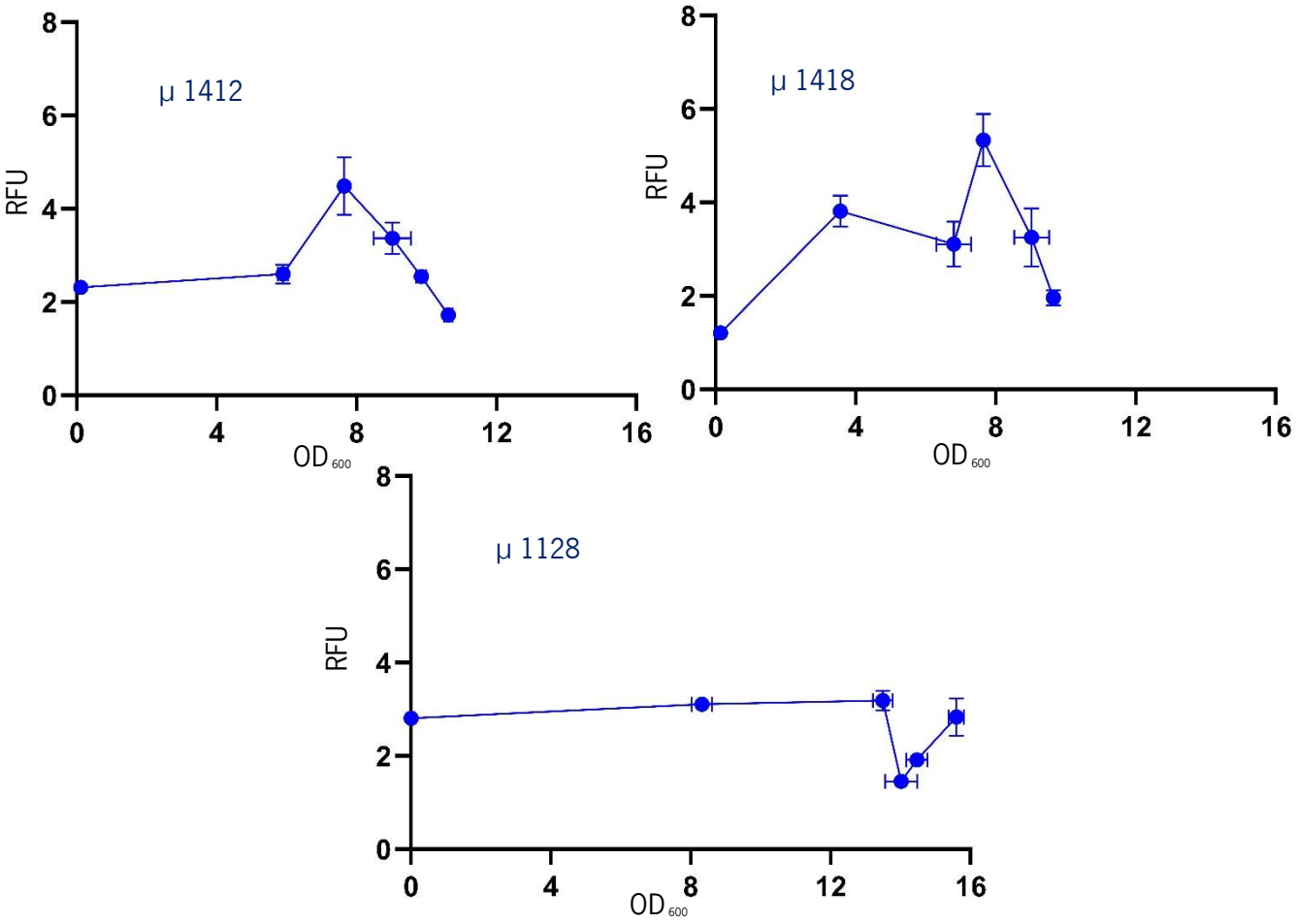


FIGURE 4.13:Variation of RFU's obtained for adapted strains in function of OD $_{600}$. Graphic layout between the two experimental variables: OD_{600} vs RFU. Each point is associated with horizontal and vertical error bars, which indicate the SD.

FAS (μ1128). After approximately OD_{600} of 7, there is a sharp drop in RFU. This indicates that FAs are consumed or secreted. This narrow optimum means that a potential process based on *S. cerevisiae* with a FASIIb pathway in its present form has to be monitored closely so as not to miss the point of maximum productivity.

Cells were collected after 48 h (during exponential growth) and lipids were analyzed by gravimetry and GC-MS (**Section 3.8.5**; **Table 4.5**). The strains μ1412 and μ1418 both had about 8% lipid dry mass while the control strain (μ1128) only had 2.5%. The effectiveness of the process can be proven with the standard sample (olive oil) that was subjected to the same protocol conditions and obtained 95% lipids by dry cell mass. This agrees with the fluorometry results indicating a higher lipid portion in the adapted strains. The GC-MS analysis (**Table 4.5**) suggests differences between the mutant strains with the μ1128 strain. C16:1 (Palmitoleic acid) was the major FAs detected for μ1412 and μ1418 (51.47 % and 40.11 %, respectively) instead of C18:0 (Stearic acid) for μ1128 (35.55 %). Exclusively in strains μ1412 and μ1418, Myristic acid was detected with a higher percentage in strain μ1418. Oleic acid was found only in μ1128 (18.39 %) and the mixture of γ-Linolenic and α-Linolenic acids only in μ1418 (6.63 %). About the sample with olive oil, the results focus on the presence of 74.35% of Oleic acid, being present in smaller percentages, the Palmitic acid (10.83 %), Linoleic acid (8.62 %), Stearic acid (3.53 %) and Palmitoleic acid (1.70%). Less than 1 %, cis-10-Heptadecanoic acid, Arachidic acid and cis-11-Eicosenoic acid were detected for olive oil. Adapted strains had a ratio close to even between unsaturated and saturated acids. The μ1128 strain tends to favour saturated acids, a ratio of 1.97 times higher.

TABLE 4.5: Percentage of lipids and FAs determined by GC-MS. Lipid content is expressed % lipids by dry cell mass and lipidic profile determined by GC-MS. Results are expressed as mean ± SD. In addition to the samples with the strains, a sample containing olive oil is used as a protocol efficiency control. µ1412 (*fas2 ::* KanMX4 pTA1_FASIIb, pYPK0_PDC1_*fabH*_TEF1); µ1418 (*fas2 ::* KanMX4 pTA1_FASIIb, pYPK0_ENO2_*mod1*_RPL5); µ1128 (CEN.PK2-1C pTA1, pYPK0). ***** Less than 1% - **C17:1** (*cis*-10-Heptadecenoic acid): 0.30 ± 0.09; **C20:0** (Arachidic acid): 0.40 ± 0.10; **C20:1n9** (*cis*-11-Eicosenoic acid): 0.40 ± 0.10.

		% FAs							
ID	**Lipids (% w/w)**	**C14:0** Myristic acid	**C16:0** Palmitic acid	**C16:1** Palmitoleic acid	**C18:0** Stearic acid	**C18:1n9c** Oleic acid	**C18:2n6c** Linoleic acid	**C18:3n6 + C18:3n3** γ-Linolenic + α-Linolenic acids	**Sum (%)**
µ 1412	8.63 ± 0.61	5.30 ± 0.81	30.87 ± 2.57	51.47 ± 1.95	12.36 ± 0.72				100
µ 1418	8.14 ± 0.23	6.81 ± 1.19	31.00 ± 2.13	40.11 ± 5.08	15.44 ± 1.26			6.63 ± 0.49	100
µ 1128	2.48 ± 1.03		30.73 ± 2.30	15.33 ± 0.47	35.55 ± 2.15	18.39 ± 2.92			100
Olive oil	95.02 ± 2.10		10.83 ± 2.53	1.70 ± 0.14	3.53 ± 0.11	74.35 ± 0.64	8.62 ± 0.20		100*****

5. Discussion

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5.1 Verification of the FASIIb pathway

The possibility of shifting the native lipid profile of *Saccharomyces cerevisiae* leads to the opening of new approaches using different FAs of high industrial value for the most varied processes. The higher complexity of FAS I in yeast blocks the modulation of FAs profile, a fact that does not happen in FAS II. The development of a FAS pathway with independent catalytic units (FASIIb) opens doors to an approach for redirecting FA production of greater industrial interest.

Only with the FASIIb pathway for the production of FA, strains µ1255 (*fas1* :: KanMX4 pTA1_FASIIb) and µ1257 (*fas2* :: KanMX4 pTA1_FASIIb) demonstrated lower initial RFU and lower growth rate in comparison than strains with native FAS fully functional (µ1206 and µ1239). However, in the exponential phase, the RFU of these strains decays for values similar to each other, indicative of the consumption of stored lipids. The same doesn't happen with the mutant strains, in which it is detected that their RFU levels remain similar after 24h of incubation. This distinct phenotype of the mutant strains can be possibly explained by the lipid output induced by the FASIIb pathway. Even in a short-term lag phase, the cells are in a period of adaptation to the new medium and, therefore, metabolize their reserves for energy production (Weber et al., 2020). It's known that the presence of Oleic acid proves to be an effective inducer in the formation of peroxisomes, organelles with a higher role in the β-oxidation of FAs (Stanway et al., 1995). A possible reason may be due to the storage of a different type of FAs (due to FASIIb pathway) less effective in inducing the formation of peroxisomes and therefore a lower impact in the catabolism of molecules of lipid character, justifying the minimal RFUs variation in mutant strains.

From 96h of incubation, the RFUs from mutant strains are increased and in the last time point, the RFUs for all strains are 9 (6 for µ1257). Although they have the same RFUs, the final OD_{600} differs between wild-type FAS (µ1206 and µ1239) and mutant strains (µ1255 and µ1257). The lack of optimization of the FASIIb pathway in comparison with native FAS blocks strains from reaching values close to wild-type ones. The presence of other proportions of FAs than usual for the biological system may prove to be less effective (as in the case of Oleic acid) for metabolism, also being a possible reason for the results described.

The HPLC analysis of the media reveals the exhaustion or accumulation of certain products, like ethanol and acetic acid. The highest concentration of ethanol is detected after 24h

of incubation, except for µ1257 (*fas2* :: KanMX4 pTA1_FASIIb) which is achieved in the next time point (48h) of incubation. For this same strain, acetic acid is not detected after 96h. The partial disruption of native FAS can disturb the homeostasis of yeast metabolism, in terms of FAs production. An imbalance in acetyl-CoA and NADPH molecules can affect directly the production of acetate and ethanol. Curiously, in an identical genomic situation, strain µ1255 (*fas1* :: KanMX4 pTA1_FASIIb) does not share the same disturbance that affects the concentrations of this both molecules. A possible hypothesis can be illustrated based on the highly regulated mechanisms of native FAS. Although both have the FASIIb pathway, there is no transcription of the two genes involved, *fas1* gene in case of µ1255 and *fas2* gene in the µ1257 strain. These two genes affect, in different ways, the regulation of FAs synthesis and therefore, different steps of yeast metabolism (Wenz et al., 2001).

We can say that the presence of exogenous DNA from plasmid and hybrid pathway does not alter the patterns of native FAs production, as the µ1206 and µ1239 strains have the same % lipids/ dry cell mass. A lower amount of lipids in the mutant strains is expected compared to the control strains since the native pathways of FAs synthesis are partially replaced by the FASIIb hybrid pathway. The µ1239 strain (pTA1_FASIIb) uses both the native and FASIIb pathway systems and does not have a large % of lipid/dry cell mass since the FAs synthesis is highly regulated by the generated products. Long-chain FAs act as an inhibitor of the FAs biosynthesis process, acting on key enzymes for the synthesis pathway, such as the FabH enzyme (López-Lara & Soto, 2019). This enzyme is also inhibited when the FAs concentration in the cell is significantly greater than 1 µM (Yu et al., 2011).

Given the lipid profile, the results in the literature indicate that the most abundant FAs in *S. cerevisiae* are Palmitic (15%; C16:0), Palmitoleic (57%; C16:1), Stearic (2%; C18:0) and Oleic (26%; C18:1n9c) acids (Besada-Lombana et al., 2017). According to these values, the control strains (µ1206 and µ1239) assume these FAs, discarding any interference either from the empty plasmid (in the case of µ1206) or from the plasmid with the FASIIb pathway (in the case of µ1239) in the native elongation pattern and FAs unsaturation.

Regarding the mutant strains (µ1255 and µ1257), the increase in the percentage of Palmitoleic acid (C16:1) agrees with the action of the enzyme encoded by the *fabZ* gene, which catalyzes the dehydration with a preference for the substrate between four and six carbons, as well as chains with more than fourteen carbons in length (Finzel et al., 2015). The prevalence of shorter-

chain FAs than those found in control strains is suggestive of the action of palmitoyl-ACP thioesterase, encoded by the *fatB* gene from *A. Thaliana* (Jones et al., 1995). This enzyme prefers releasing chains of size C16:0 (Dö et al., 2000). The action of these two elements prevents chain elongation after C16:0, as well as the unsaturation of chains smaller than this carbon extension, which agrees with the results presented. These results reinforce the hypothesis of the influence of the distinct FAs in the growth parameters of the mutant strains.

The replacement of native FAS by the FASIIb pathway allowed the growth of strains and the production of different FAs. Although with lower growth rates, the effectiveness of the hybrid pathway in replacing the native system is shown to be a correct approach to obtain a dynamic system in terms of lipid modulation.

5.2 Optimization of the FASIIb pathway

The inability of strains with only the FASIIb pathway for FA synthesis to achieve higher growth rates directs them towards the adaptation process to suppress this fragility at the phenotypic level. Additionally, the incorporation of each genetic unit of the FASIIb pathway into a new vector (pYPK0) reveals a direct effect on the profile of FAs produced.

From the adaptation process, it is observed that the strains that were subjected to this procedure had higher growth rates than the respective strains that did not undergo the procedure (**Section 4.2.2**). This process possibly allows the accumulation of adaptive changes in metabolic mechanisms under specific growth conditions, like YPD medium (Dragosits & Mattanovich, 2013).

In the YPD + EtOH medium (**Section 4.2.3**), the time duration of the lag phase is different between unadapted and respective adapted strains. These had a short lag phase with a diauxic shift occurring approximately up to 60 - 70h after incubation. This prolongation in time compared to the results obtained from growth on YPD or YPD + Hmy is due to the low OD_{600} of the inoculum at the initial time point of incubation.

Comparing the different growth curves performed for adapted strains µ1412 (pYPK0 with *fabH*) and µ1418 (pYPK0 with *mod1*) in different media, the YPD + Hmy medium constitutes the richest growth medium with the better growth performance. The lipid supplement allows its use in cellular catabolism to increase the ATP yield.

Between adapted strains, µ1418 achieves a different phenotype than µ1412 possibly due to the additional gene held by the pYPK0 plasmid. While µ1418 has an extra *mod1* gene from *A. thaliana*, µ1412 has *fabH* from *E. coli*, both involved in the synthesis of FAs. However, they influence different stages of the synthesis process, with the enzyme transcribed from *mod1* gene acting in more advanced stages of synthesis such as elongation, while the enzyme transcribed from the *fabH* gene is involved in the catalysis of the first reaction, and therefore an important regulator for production of FAs (Choi et al., 2000). The Hmy supplementation will encourage strains to β-oxidation, which culminates in the generation of large amounts of acetyl-CoA. Since the enzyme encoded by the *fabH* gene has a strong preference for acetyl-CoA, there will be an increase in the FAs production (S. Lee et al., 2011). The improved metabolism of the strain to produce this type of compound shifts the focus from growth to the production of FAs. The extra gene of µ1418 strain (*mod1*) has a later influence on the FA synthesis process, not being directly influenced by the increase in acetyl -CoA, and therefore with a less pronounced effect. Through the visualization by optical microscopy (**Appendix 8.3**), it is possible to observe that cells of the µ1412 strain have larger dimensions than those from µ1418. In other media with a lower lipid content (YPD and YPD + EtOH), this effect is not observed.

The adapted strains have better growth parameters than the respective unadapted ones. However, the unadapted strain µ1128 achieves higher OD_{600} values and a shorter lag phase compared to the adapted strains (**Section 4.2.5**). Although µ1128 is an unadapted strain, this has the native FAS fully operational with intrinsic pathways and FA molecules completely optimized for the biological system.

The fluorometric analysis reveals that in the first 24h of incubation, the RFU values recorded for the adapted strains undergo a slight increase, which in turn corresponds to an increase in the amount of FAs. For µ1128, these values are shown to be approximately unchanged until 50h of incubation. In the early stages of growth, the results suggest that the adapted strains direct their metabolism to FAs production instead of typical ethanol production. It knows that glucose exhaustion triggers yeast metabolism for the diauxic shift, which comprises the use of ethanol produced in the early stages (Yang et al., 2022). With the lower concentrations of this compound and due to the higher quantity of the FAs produced previously, the cell chooses to metabolize these molecules. Strain µ1128, after the diauxic shift, uses ethanol as an energy source and can subsequently starts FAs production. This situation can be corroborated by looking for the

last points of incubation. There is a decrease in the RFU values for adapted strains indicating the consumption of the FAs, and an increase in RFU values for µ1128.

By gravimetric quantification, it is assumed that µ1412 and µ1418 show a higher % of lipids/dry cell mass than that calculated for the µ1128 strain. This proves the effectiveness of the FASIIb pathway, the action of the additional gene incorporated into the pYPK0 plasmid and the consequences of the adaptation process, as well as reiterating the hypothesis that there is a greater production of FA in the exponential phase in the mutant strains than in the µ1128. Comparing with the results obtained for the strain µ1257 in SC-L, we obtain a higher percentage of lipids in the YPD medium, a difference that also comes from the adaptation process, the presence of the pYPK0 plasmid, the sampling time point and the culture media.

Observing the lipid profile quantified by the GC-MS, it is noted that mutant strains have a higher percentage of Palmitoleic acid (C16:1). With the µ1412, about 82% are FAs with 16 carbons long, in agreement with the effect of an additional copy of the *fabH* gene that causes a greater abundance of FA with 14 and 16 carbons long (Janßen & Steinbüchel, 2014). The two mutant strains have the same FAs, with similar percentages. The exception is for the mixture of γ-linolenic and α-Linolenic acids only present in µ1418 (6.63 %). An extra copy of *mod1* gene increases the production of FAs with 16 – 18 carbons long with unsaturations, the typical profile of FAs found in plants (He et al., 2020; Mou et al., 2000).

Compared with the results obtained for the strain µ1257 in SC-L, only 17% of FAs are saturated, while the effect of the adaptation process and the pYPK0 plasmid with the extra gene increase this percentage to 48%, in the case of µ1412 and 53% for the strain µ1418. However, the hypothesis of these results is due to exclusively from the effects mentioned above does not make sense, due to the sample resorted to different stages of growth, different culture media and different GC analyses (Olson & Ingram, 1975). In addition, the extraction process is modified, using different evaporation mechanisms, which can lead to changes in the structure of the FA. Between these two analyses, the control sample (olive oil) was taken from the same stock, and between analyses, it presents the same FAs, which corroborates a good analysis.

Looking at the control strain µ1128, the literature indicates that Palmitoleic, Stearic, Palmitic and Oleic acids are the predominant FAs in *S. cerevisiae* (Yamada & Sgarbieri, 2005). All of these are found in the strain, with different percentages described earlier, with prevalence for saturated acids. The use of a different medium or the evaporation mechanism can both explain

this quantitative variation since, there is a different nutritional content and the high temperatures used promoted the transformation of unsaturated acids into saturated ones (Zhuang et al., 2022).

6. Final remarks and future perspectives

6. Final remarks and future perspectives

Given all the proposed results, the hybrid pathway developed - FASIIb - allows the growth of the strains, despite a lipid profile different from what is expected by the native yeast system. This proves the functioning of the pathway, even if the growth of these strains is more limited compared to the same strains with the native pathway in full operation. The advantage of this pathway is centered on the possibility of studying the effect of each enzyme involved in the pathway, concentrating the positive effects on each enzymatic unit of the synthesis pathway for modulation of FAs production to a certain type of extension.

The optimization of the same, either by the adaptation process or by expression of each gene constituting the FASIIb pathway, results in a shorter lag phase duration giving an interest in use in industrial and biotechnological areas with a lipid focus. The production of FA is directed to C16 to smaller extensions opening a door to a profitable and sustainable synthesis for these smaller chains.

However, it is believed that the large production of FA in the exponential phase is used as a source of energy for the maintenance of the strains in the most advanced stages of the stationary phase. Possible considerations for further studies are centered on this growth phase, through the analysis of the lipid profile. Other future approaches are based on the use of an inductive promoter to grow cells and limitate the β-oxidation with the increase of the permanence of FAs in the cell. Another strategy is aligned in thioesterases with a higher affinity for fatty acyl moiety from the fatty acyl-ACPs of shorter length. These studies could be translated into a type of FAs with a distinct structure and amount, making available the modulation of FA production as required.

7. References

7. References

Adrio, J. L. (2017). Oleaginous Yeasts: Promising Platforms for the Production of Oleochemicals and Biofuels. *Biotechnol. Bioeng*, *114*, 1915–1920. https://doi.org/10.1002/bit.26337/abstract

Ahmadi, N., Khosravi-Darani, K., & Mortazavian, A. M. (2017). An overview of biotechnological production of propionic acid: From upstream to downstream processes. *Electronic Journal of Biotechnology*, *28*, 67–75. https://doi.org/10.1016/j.ejbt.2017.04.004

AOCS Official Method Cd 3d-63. Retrieved 14 November 2022, from https://myaccount.aocs.org/PersonifyEbusiness/Store/Product-Details?productId=111545

AOCS Official Method Cd 8b-90. Retrieved 14 November 2022, from https://myaccount.aocs.org/PersonifyEbusiness/Store/Product-Details?productId=111547

AOCS Official Method Cd 18-90. Retrieved 14 November 2022, from https://myaccount.aocs.org/PersonifyEbusiness/Store/Product-Details?productId=111529

AOCS Official Method Ce 1i-07. Retrieved 14 November 2022, from https://myaccount.aocs.org/PersonifyEbusiness/Store/Product-Details?productId=111781

AOCS Official Method Ce 12-16. Retrieved 14 November 2022, from https://myaccount.aocs.org/PersonifyEbusiness/Store/Product-Details?productId=87372599

Aon, J. C., & Cortassa, S. (2001). Involvement of Nitrogen Metabolism in the Triggering of Ethanol Fermentation in Aerobic Chemostat Cultures of *Saccharomyces cerevisiae*. *Metabolic Engineering*, *3*(3), 250–264. https://doi.org/10.1006/mben.2001.0181

Ashour, S., & Kattan, N. (2010). Simultaneous Determination of Miconazole Nitrate and Metronidazole in Different Pharmaceutical Dosage Forms by Gas Chromatography and Flame Ionization Detector (GC-FID). In *International Journal of Biomedical Science* (Vol. 6, Issue 1). www.ijbs.org

Athenaki, M., Gardeli, C., Diamantopoulou, P., Tchakouteu, S. S., Sarris, D., Philippoussis, A., & Papanikolaou, S. (2018). Lipids from yeasts and fungi: physiology, production and analytical considerations. In *Journal of Applied Microbiology* (Vol. 124, Issue 2, pp. 336–367). Blackwell Publishing Ltd. https://doi.org/10.1111/jam.13633

Axelsson, M., & Gentili, F. (2014). A single-step method for rapid extraction of total lipids from green microalgae. *PLoS ONE*, *9* (2). https://doi.org/10.1371/journal.pone.0089643

Baeshen, N. A., Baeshen, M. N., Sheikh, A., Bora, R. S., Morsi, M., Ahmed, M., Ramadan, H. A. I., Saini, K. S., & Redwan, E. M. (2014). Cell factories for insulin production. https://doi.org/10.1186/s12934-014-0141-0

Bengtsson, J. D., Wallis, J. G., & Browse, J. (2021). Lipid isolation from plants. In *Methods in Molecular Biology* (Vol. 2295, pp. 3–13). Humana Press Inc. https://doi.org/10.1007/978-1-0716-1362-7_1

Berglert, H., Wallnert, P., Ebelingt, A., Leitingert, B., FuchsbichlerS, S., Aschaued, H., KollenzII, G., Hogenauert, G., & Turnowskyt, F. (1994). Protein EnvM Is the NADH-dependent Enoyl-ACP Reductase (FabI) of *Escherichia coli*. In *THE JOURNAL OF BIOLOGICAL CHEMISTRY* (Vol. 269, Issue 8).

Besada-Lombana, P. B., Fernandez-Moya, R., Fenster, J., & da Silva, N. A. (2017). Engineering *Saccharomyces cerevisiae* fatty acid composition for increased tolerance to octanoic acid. *Biotechnology and Bioengineering*, *114* (7), 1531–1538. https://doi.org/10.1002/bit.26288

Bettencourt, S. (2018). Oleaginous yeast selection for the production of lipidic compounds: development of an optimized fermentation protocol regarding nutrient requirements and growth conditions. University of Minho.

Birch, A. N., Petersen, M. A., Arneborg, N., & Hansen, Å. S. (2013). Influence of commercial baker's yeasts on bread aroma profiles. *Food Research International*, *52*(1), 160–166. https://doi.org/10.1016/j.foodres.2013.03.011

Bischoff-Ferrari, H. A., Willett, W. C., Manson, J. E., Dawson-Hughes, B., Manz, M. G., Theiler, R., Braendle, K., Vellas, B., Rizzoli, R., Kressig, R. W., Staehelin, H. B., da Silva, J. A. P., Armbrecht, G., Egli, A., Kanis, J. A., Orav, E. J., & Gaengler, S. (2022). Combined Vitamin

D, Omega-3 Fatty Acids, and a Simple Home Exercise Program May Reduce Cancer Risk Among Active Adults Aged 70 and Older: A Randomized Clinical Trial. *Frontiers in Aging*, *3*. https://doi.org/10.3389/fragi.2022.852643

Breil, C., Abert Vian, M., Zemb, T., Kunz, W., & Chemat, F. (2017). "Bligh and Dyer" and Folch methods for solid–liquid-liquid extraction of lipids from microorganisms. Comprehension of solvatation mechanisms and towards substitution with alternative solvents. *International Journal of Molecular Sciences*, *18*(4). https://doi.org/10.3390/ijms18040708

Cao, Y., Yang, J., Xian, M., Xu, X., & Liu, W. (2010). Increasing unsaturated fatty acid contents in *Escherichia coli* by coexpression of three different genes. *Applied Microbiology and Biotechnology*, *87*(1), 271–280. https://doi.org/10.1007/s00253-009-2377-x

Caplow, M. (2020). ATP Mediates Yeast Cell-Cell Communication.

Caporusso, A., Capece, A., & de Bari, I. (2021). Oleaginous Yeasts as Cell Factories for the Sustainable Production of Microbial Lipids by the Valorization of Agri-Food Wastes. *Fermentation*, *7* (2), 50. https://doi.org/10.3390/fermentation7020050

Chávez-Servín, J. L., Castellote, A. I., & López-Sabater, M. C. (2004). Analysis of mono- and disaccharides in milk-based formulae by high-performance liquid chromatography with refractive index detection. *Journal of Chromatography A*, *1043*(2), 211–215. https://doi.org/10.1016/j.chroma.2004.06.002

Chen, F., Zhou, J., Shi, Z., Liu, L., Du, G., & Chen, J. (2010). Effect of acetyl-CoA synthase gene overexpression on physiological function of *Saccharomyces cerevisiae*. *Wei Sheng Wu Xue Bao = Acta Microbiologica Sinica*, *50* (9), 1172–1179.

Chen, L., Zhang, J., & Chen, W. N. (2014). Engineering the *Saccharomyces cerevisiae* β-oxidation pathway to increase medium chain fatty acid production as potential biofuel. *PLoS ONE*, *9* (1). https://doi.org/10.1371/journal.pone.0084853

Chiu, H. H., & Kuo, C. H. (2020). Gas chromatography-mass spectrometry-based analytical strategies for fatty acid analysis in biological samples. In *Journal of Food and Drug Analysis* (Vol. 28, Issue 1, pp. 60–73). Elsevier Taiwan LLC. https://doi.org/10.1016/j.jfda.2019.10.003

Choi, K.-H., Heath, R. J., & Rock, C. O. (2000). Ketoacyl-Acyl Carrier Protein Synthase III (FabH) Is a Determining Factor in Branched-Chain Fatty Acid Biosynthesis. In *JOURNAL OF BACTERIOLOGY* (Vol. 182, Issue 2).

Chrzanowski, G. (2020). *Saccharomyces cerevisiae* — An Interesting Producer of Bioactive Plant Polyphenolic Metabolites. *International Journal of Molecular Sciences*, *21*(19), 7343. https://doi.org/10.3390/ijms21197343

Coskun, O. (2016). Separation Techniques: Chromatography. *Northern Clinics of Istanbul*. https://doi.org/10.14744/nci.2016.32757

da Silva Medeiros, T., Pinto, E. C., Cabral, L. M., & de Sousa, V. P. (2021). Tobramycin: A review of detectors used in analytical approaches for drug substance, its impurities and in pharmaceutical formulation. In *Microchemical Journal* (Vol. 160). Elsevier Inc. https://doi.org/10.1016/j.microc.2020.105658

Davis, M. S., & Cronan, J. (2001). Inhibition of *Escherichia coli* acetyl coenzyme A carboxylase by acyl-acyl carrier protein. *Journal of Bacteriology*, *183* (4), 1499–1503. https://doi.org/10.1128/JB.183.4.1499-1503.2001

Dekker, W. J. C., Wiersma, S. J., Bouwknegt, J., Mooiman, C., & Pronk, J. T. (2019). Anaerobic growth of *Saccharomyces cerevisiae* CEN.PK113-7D does not depend on synthesis or supplementation of unsaturated fatty acids. *FEMS Yeast Research*, *19*(6). https://doi.org/10.1093/femsyr/foz060

Denavarre, M. G. (1978). *SD&C Technical Oils and Fats, The Historical Cosmetics 1*.

Dö, P., Voelker, T. A., & Ohlrogge, J. B. (2000). Accumulation of Palmitate in Arabidopsis Mediated by the Acyl-Acyl Carrier Protein Thioesterase FATB1 1. www.plantphysiol.org

Dong, M. (2006). Modern HPLC practising scientists. John Wiley & sons.

Dragosits, M., & Mattanovich, D. (2013). Adaptive laboratory evolution - principles and applications for biotechnology. In *Microbial Cell Factories* (Vol. 12, Issue 1). https://doi.org/10.1186/1475-2859-12-64

Egner, R., Thumm, M., Straub, M., Simeon, A., Schuller, H. J., & Wolf, D. H. (1993). Tracing intracellular proteolytic pathways. Proteolysis of fatty acid synthase and other cytoplasmic

proteins in the yeast *Saccharomyces cerevisiae*. *Journal of Biological Chemistry*, *268*(36), 27269–27276. https://doi.org/10.1016/s0021-9258(19)74245-8

Eisenberg, T., & Büttner, S. (2014). Lipids and cell death in yeast. *FEMS Yeast Research*, *14*(1), 179–197. https://doi.org/10.1111/1567-1364.12105

Elgharbawy, A. S., Sadik, W. A., Sadek, O. M., & Kasaby, M. A. (2021). A review on biodiesel feedstocks and production technologies. In *J. Chil. Chem. Soc* (Vol. 66).

Engeser, H., Hübner, K., Straub, J., & Lynen, F. (1979). Identity of Malonyl and Palmitoyl Transferase of Fatty Acid Synthetase from Yeast: 2. A Comparison of Active-Site Peptides. *European Journal of Biochemistry*, *101*(2), 413–422. https://doi.org/10.1111/j.1432-1033.1979.tb19734.x

Fam, T. K., Klymchenko, A. S., & Collot, M. (2018). Recent advances in fluorescent probes for lipid droplets. In *Materials* (Vol. 11, Issue 9). MDPI AG. https://doi.org/10.3390/ma11091768

Feng, J., Zhang, J., Ma, Y., Feng, Y., Wang, S., Guo, N., Wang, H., Wang, P., Jiménez-Bonilla, P., Gu, Y., Zhou, J., Zhang, Z. T., Cao, M., Jiang, D., Wang, S., Liu, X. W., Shao, Z., Borovok, I., Huang, H., & Wang, Y. (2021). Renewable fatty acid ester production in Clostridium. *Nature Communications*, *12*(1). https://doi.org/10.1038/s41467-021-24038-3

Fernandez-Moya, R., Leber, C., Cardenas, J., & da Silva, N. A. (2015). Functional Replacement of the *Saccharomyces cerevisiae* Fatty Acid Synthase With a Bacterial Type II System Allows Flexible Product Profiles. *Biotechnol. Bioeng*, *112*, 2618–2623. https://doi.org/10.1002/bit.25679/abstract

Ferrari, F. B., Fortunato De Oliveira, R., Alves De Souza, R., Lolli, J., de Mello, M., Santos Fávero, M., Pereira, M. R., Giampietro-Ganeco, A., Borba, H., Alves, P., & Souza, D. (2020). Freeze-Dried Cattle Meat Improves the Measurement of Total Cholesterol. https://doi.org/10.19080/ARTOAJ.2020.24.556285

Ferreri, C., Masi, A., Sansone, A., Giacometti, G., Larocca, A. V., Menounou, G., Scanferlato, R., Tortorella, S., Rota, D., Conti, M., Deplano, S., Louka, M., Maranini, A. R., Salati, A., Sunda, V., & Chatgilialoglu, C. (2017). Fatty acids in membranes as homeostatic, metabolic and

nutritional biomarkers: Recent advancements in analytics and diagnostics. In *Diagnostics* (Vol. 7, Issue 1). MDPI. https://doi.org/10.3390/diagnostics7010001

Fichtlscherer, F., Wellein, C., Mittag, M., & Schweizer, E. (2000). A novel function of yeast fatty acid synthase. Subunit α is capable of self-pantetheinylation. *European Journal of Biochemistry*, *267*(9), 2666–2671. https://doi.org/10.1046/j.1432-1327.2000.01282.x

Finzel, K., Nguyen, C., Jackson, D. R., Gupta, A., Tsai, S. C., & Burkart, M. D. (2015). Probing the Substrate Specificity and Protein-Protein Interactions of the *E. coli* Fatty Acid Dehydratase, FabA. *Chemistry and Biology*, *22*(11), 1453–1460. https://doi.org/10.1016/j.chembiol.2015.09.009

Fortman, J. L., Chhabra, S., Mukhopadhyay, A., Chou, H., Lee, T. S., Steen, E., & Keasling, J. D. (2008). Biofuel alternatives to ethanol: pumping the microbial well. In *Trends in Biotechnology* (Vol. 26, Issue 7, pp. 375–381). Elsevier Ltd. https://doi.org/10.1016/j.tibtech.2008.03.008

Fugelsang, K., & Edwards, C. (2007). *Wine Microbiology* (K. C. Fugelsang & C. G. Edwards, Eds.). Springer US. https://doi.org/10.1007/978-0-387-33349-6

Furukawa, K., Yamada, T., Mizoguchi, H., & Hara, S. (2003). Increased Ethyl Caproate Production by Inositol Limitation in *Saccharomyces cerevisiae*. In *JOURNALOFBIOSCENCE AND BIOENGINEWNG* (Vol. 95, Issue 5).

Gammone, M. A., Riccioni, G., Parrinello, G., & D'orazio, N. (2019). Omega-3 polyunsaturated fatty acids: Benefits and endpoints in sport. In *Nutrients* (Vol. 11, Issue 1). MDPI AG. https://doi.org/10.3390/nu11010046

Gerusz, V. (2010). Recent Advances in the Inhibition of Bacterial Fatty Acid Biosynthesis. In *Annual Reports in Medicinal Chemistry* (Vol. 45, Issue C). https://doi.org/10.1016/S0065-7743(10)45018-6

Giannattasio, S., Guaragnella, N., Ždralević, M., & Marra, E. (2013). Molecular mechanisms of *Saccharomyces cerevisiae* stress adaptation and programmed cell death in response to acetic acid. *Frontiers in Microbiology*, *4*. https://doi.org/10.3389/fmicb.2013.00033

Gibb, E. (2018). Understanding the diauxic shift in *Saccharomyces cerevisiae* and its evolutionarily conserved mechanisms of regulation [University of California]. https://escholarship.org/uc/item/5z61r1tf

Gipson, P., Mills, D. J., Wouts, R., Grininger, M., Vonck, J., & Kühlbrandt, W. (2010). Direct structural insight into the substrate-shuttling mechanism of yeast fatty acid synthase by electron cryomicroscopy. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(20), 9164–9169. https://doi.org/10.1073/pnas.0913547107

Goddard, M. R. (2008). Quantifying the complexities of *Saccharomyces cerevisiae*'s ecosystem engineering via fermentation. *Ecology*, *89*(8), 2077–2082. https://doi.org/10.1890/07-2060.1

González-Fernández, M. J., Manzano-Agugliaro, F., Zapata-Sierra, A., Belarbi, E. H., & Guil-Guerrero, J. L. (2020). Green argan oil extraction from roasted and unroasted seeds by using various polarity solvents allowed by the EU legislation. *Journal of Cleaner Production*, *276*. https://doi.org/10.1016/j.jclepro.2020.123081

Greenspan, P., Mayer, E. P., & Fowler, S. D. (1995). *Nile red'' A Selective Fluorescent Stain for Intracellular Lipid Droplets*.

Guldener, U. (1996). A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Research*, *24* (13), 2519–2524. https://doi.org/10.1093/nar/24.13.2519

Günerken, E., D'Hondt, E., Eppink, M. H. M., Garcia-Gonzalez, L., Elst, K., & Wijffels, R. H. (2015). Cell disruption for microalgae biorefineries. In *Biotechnology Advances* (Vol. 33, Issue 2, pp. 243–260). Elsevier Inc. https://doi.org/10.1016/j.biotechadv.2015.01.008

Hajšlová, J., & ajka, T. (2007). *Gas chromatography-mass spectrometry (GC-MS)*.

Halim, R., Danquah, M. K., & Webley, P. A. (2012). Extraction of oil from microalgae for biodiesel production: A review. In *Biotechnology Advances* (Vol. 30, Issue 3, pp. 709–732). https://doi.org/10.1016/j.biotechadv.2012.01.001

He, M., Qin, C.-X., Wang, X., & Ding, N.-Z. (2020). Plant Unsaturated Fatty Acids: Biosynthesis and Regulation. *Frontiers in Plant Science*, *11*. https://doi.org/10.3389/fpls.2020.00390

Heath, R. J., & Rock, C. O. (1996a). Inhibition of β-ketoacyl-acyl carrier protein synthase III (FabH) by acyl-acyl carrier protein in *Escherichia coli*. *Journal of Biological Chemistry*, *271*(18), 10996–11000. https://doi.org/10.1074/jbc.271.18.10996

Heath, R. J., & Rock, C. O. (1996b). Roles of the FabA and FabZ β-hydroxyacyl-acyl carrier protein dehydratases in *Escherichia coli* fatty acid biosynthesis. *Journal of Biological Chemistry*, *271*(44), 27795–27801. https://doi.org/10.1074/jbc.271.44.27795

Heath, R. J., & Rock, C. O. (1996c). Regulation of fatty acid elongation and initiation by acyl-acyl carrier protein in *Escherichia coli*. *Journal of Biological Chemistry*, *271*(4), 1833–1836. https://doi.org/10.1074/jbc.271.4.1833

Henriksen, M., Bjerketvedt, D., Vaagsaether, K., Gaathaug, A. v., Skjold, T., & Middha, P. (2017). Accidental hydrogen release in a gas chromatograph laboratory: A case study. *International Journal of Hydrogen Energy*, *42*(11), 7651–7656. https://doi.org/10.1016/j.ijhydene.2016.05.299

Hicks, R. H., Chuck, C. J., Scott, R. J., Leak, D. J., & Henk, D. A. (2019). Comparison of Nile red and Cell Size Analysis for High-Throughput Lipid Estimation Within Oleaginous Yeast. *European Journal of Lipid Science and Technology*, *121*(11). https://doi.org/10.1002/ejlt.201800355

Hiltunen, J. K., Schonauer, M. S., Autio, K. J., Mittelmeier, T. M., Kastaniotis, A. J., & Dieckmann, C. L. (2009). Mitochondrial fatty acid synthesis type II: More than just fatty acids. In *Journal of Biological Chemistry* (Vol. 284, Issue 14, pp. 9011–9015). https://doi.org/10.1074/jbc.R800068200

Hu, Z., Ma, J., Chen, Y., Tong, W., Zhu, L., Wang, H., & Cronan, J. E. (2021). *Escherichia coli* FabG 3-ketoacyl-ACP reductase proteins lacking the assigned catalytic triad residues are active enzymes. *Journal of Biological Chemistry*, *296*. https://doi.org/10.1016/j.jbc.2021.100365

Huang, W., Jia, J., Edwards, P., Dehesh, K., Schneider, G., & Lindqvist, Y. (1998). Crystal structure of β-ketoacyl-acyl carrier protein synthase II from *E.coli* reveals the molecular architecture of condensing enzymes. In *The EMBO Journal* (Vol. 17, Issue 5).

Hynne, F., Danø, S., & Sørensen, P. G. (2001). Full-scale model of glycolysis in *Saccharomyces cerevisiae*. In *Biophysical Chemistry* (Vol. 94).

Inoue, H., Nojima, H., & Okayama, H. (1990). High efficiency transformation of *Escherichia coli* with plasmids. *Gene*, *96*(1), 23–28. https://doi.org/10.1016/0378-1119(90)90336-P

Inouye, L. S., & Lotufo, G. R. (2006). Comparison of macro-gravimetric and micro-colorimetric lipid determination methods. *Talanta*, *70*(3), 584–587. https://doi.org/10.1016/j.talanta.2006.01.024

Irwin, E., & Washington, G. (2012). *The Spermaceti Candle and the American Whaling Industry*. http://www.nha.org/history/hn/HNWhalingmus.htm.

Jain, A. P., Aggarwal, K. K., & Zhang, P.-Y. (2015). Omega-3 fatty acids and cardiovascular disease. *European Review for Medical and Pharmacological Sciences*, *19*(3), 441–445.

Jaishankar, J., & Srivastava, P. (2017). Molecular basis of stationary phase survival and applications. In *Frontiers in Microbiology* (Vol. 8, Issue OCT). Frontiers Media S.A. https://doi.org/10.3389/fmicb.2017.02000

Janßen, H. J., & Steinbüchel, A. (2014). Fatty acid synthesis in *Escherichia coli* and its applications towards the production of fatty acid based biofuels. http://www.biotechnologyforbiofuels.com/content/7/1/7

Jeevan Kumar, S. P., & Banerjee, R. (2019). Enhanced lipid extraction from oleaginous yeast biomass using ultrasound assisted extraction: A greener and scalable process. *Ultrasonics Sonochemistry*, *52*, 25–32. https://doi.org/10.1016/j.ultsonch.2018.08.003

Jenni, S., Leibundgut, M., Boehringer, D., Frick, C., Mikolásek, B., & Ban, N. (2007). Structure of fungal fatty acid synthase and implications for iterative substrate shuttling. *Science*, *316*(5822), 254–261. https://doi.org/10.1126/science.1138248

Jenni, S., Leibundgut, M., Maier, T., & Ban, N. (2006). Architecture of a fungal fatty acid synthase at 5 Å resolution. *Science*, *311*(5765), 1263–1267. https://doi.org/10.1126/science.1123251

Johansson, P., Mulinacci, B., Koestler, C., Vollrath, R., Oesterhelt, D., & Grininger, M. (2009). Multimeric Options for the Auto-Activation of the *Saccharomyces cerevisiae* FAS Type I Megasynthase. *Structure*, *17*(8), 1063–1074. https://doi.org/10.1016/j.str.2009.06.014

Jones, A., Davies, H. M., & Voelker', T. A. (1995). Palmitoyl-Acyl Carrier Protein (ACP) Thioesterase and the Evolutionary-Origin of Plant ACyl-ACP Thioesterases. In *The Plant Cell* (Vol. 7).

Kaiser, A., Michaelis, S., & Mitchell, A. (1994). *Methods in Yeast Genetics: A cold Spring Harbor Laboratory Course Manual*.

Keum, Y. S., Park, H. W., Song, H.-H., Kim, B.-D., Kang, B.-C., & Kim, J.-H. (2012). Metabolite analysis of long chain branched fatty acids and capsaicin biosynthesis in Capsicum annuum placenta. *Journal of the Korean Society for Applied Biological Chemistry*, *55*(2), 189–195. https://doi.org/10.1007/s13765-012-1163-0

Klassert, T. E., Hanisch, A., Bräuer, J., Klaile, E., Heyl, K. A., Mansour, M. M., Tam, J. M., Vyas, J. M., & Slevogt, H. (2014). Modulatory role of vitamin A on the *Candida albicans*-induced immune response in human monocytes. *Medical Microbiology and Immunology*, *203*(6), 415–424. https://doi.org/10.1007/s00430-014-0351-4

Knobling, A., Schiffmann, D., Sickinger, H.-D., & Schweizer, E. (1975). Malonyl and Palmityl Transferase-less Mutants of the Yeast Fatty-Acid-Synthetase Complex. In *Eur. J. Biochem* (Vol. 56).

Knoshaug, E. P., van Wychen, S., Singh, A., & Zhang, M. (2018). Lipid accumulation from glucose and xylose in an engineered, naturally oleaginous strain of *Saccharomyces cerevisiae*. *Biofuel Research Journal*, *5*(2), 800–805. https://doi.org/10.18331/BRJ2018.5.2.3

Kostoglou-Athanassiou, I., Athanassiou, L., & Athanassiou, P. (2020). The effect of omega-3 fatty acids on rheumatoid arthritis. *Mediterranean Journal of Rheumatology*, *31*(2), 190–194. https://doi.org/10.31138/mjr.31.2.190

Kovačević, M. (2015). Morphological and physiological characteristics of the yeast *Saccharomyces cerevisiae* cells differing in the life span. University of Zagreb - Faculty of Pharmacy and Biochemistry.

Kulagina, N., Besseau, S., Godon, C., Goldman, G. H., Papon, N., & Courdavault, V. (2021). Yeasts as Biopharmaceutical Production Platforms. *Frontiers in Fungal Biology*, *2*. https://doi.org/10.3389/ffunb.2021.733492

Lai, C. Y., & Cronan, J. E. (2003). β-Ketoacyl-Acyl Carrier Protein Synthase III (FabH) Is Essential for Bacterial Fatty Acid Synthesis. *Journal of Biological Chemistry*, *278*(51), 51494–51503. https://doi.org/10.1074/jbc.M308638200

Lee, J., Rennaker, C., & Wrolstad, R. E. (2008). Correlation of two anthocyanin quantification methods: HPLC and spectrophotometric methods. *Food Chemistry*, *110*(3), 782–786. https://doi.org/10.1016/j.foodchem.2008.03.010

Lee, K. W. Y., Porter, C. J. H., & Boyd, B. J. (2013). A simple quantitative approach for the determination of long and medium chain lipids in bio-relevant matrices by high performance liquid chromatography with refractive index detection. *AAPS PharmSciTech*, *14*(3), 927–934. https://doi.org/10.1208/s12249-013-9976-7

Lee, S., Jeon, E., Yun, H. S., & Lee, J. (2011). Improvement of fatty acid biosynthesis by engineered recombinant *Escherichia coli*. *Biotechnology and Bioprocess Engineering*, *16*(4), 706–713. https://doi.org/10.1007/s12257-011-0034-6

Legras, J. L., Merdinoglu, D., Cornuet, J. M., & Karst, F. (2007). Bread, beer and wine: *Saccharomyces cerevisiae* diversity reflects human history. *Molecular Ecology*, *16*(10), 2091–2102. https://doi.org/10.1111/j.1365-294X.2007.03266.x

Li, M., Holi, X.-F., Zhang, J., Wang, S.-C., Fu, Q., & He, L.-C. (2011). Applications of HPLC/MS in the analysis of traditional Chinese medicines. In *J Pharm Anal* (Vol. 1, Issue 2). http://www.j-phann-anal.com

Li, X., Guo, D., Cheng, Y., Zhu, F., Deng, Z., & Liu, T. (2014). Overproduction of fatty acids in engineered *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering*, *111*(9), 1841–1852. https://doi.org/10.1002/bit.25239

Li, Y., Ghasemi Naghdi, F., Garg, S., Adarme-Vega, T. C., Thurecht, K. J., Ghafor, W. A., Tannock, S., & Schenk, P. M. (2014). A comparative study: The impact of different lipid extraction methods on current microalgal lipid research. *Microbial Cell Factories*, *13*(1). https://doi.org/10.1186/1475-2859-13-14

Liu, H., Zhou, P., Qi, M., Guo, L., Gao, C., Hu, G., Song, W., Wu, J., Chen, X., Chen, J., Chen, W., & Liu, L. (2022). Enhancing biofuels production by engineering the actin cytoskeleton in

Saccharomyces cerevisiae. *Nature Communications*, *13*(1). https://doi.org/10.1038/s41467-022-29560-6

López-Lara, I. M., & Soto, M. J. (2019). Fatty Acid Synthesis and Regulation. In *Biogenesis of Fatty Acids, Lipids and Membranes* (pp. 391–407). Springer International Publishing. https://doi.org/10.1007/978-3-319-50430-8_26

Lovestead, T. M., & Urness, K. N. (2019). *Gas Chromatography-Mass Spectrometry (GC–MS) **.

Lu, Y.-J., Zhang, Y.-M., & Rock, C. O. (2004). Product diversity and regulation of type II fatty acid synthases. *Biochemistry and Cell Biology*, *82*(1), 145–155. https://doi.org/10.1139/o03-076

Lynen, F., Engeser, H., Foerster, E.-C., Fox, J. L., Hess, S., Kresze, G.-B., Schmitt, T., Schreckenbach, T., Siess, E., Wieland, F., & Winnewisser, W. (1980). On the Structure of Fatty Acid Synthetase of Yeast. In *Eur. J. Biochem* (Vol. 112).

Majerus, P. W. (1968). Acyl Carrier Protein: Effects of Acetylation and Tryptic Hydrolysis on Function in Fatty Acid Synthesis. *Science*, *159*(3813), 428–430. https://doi.org/10.1126/science.159.3813.428

Marrakchi, H., Zhang, Y.-M., & Rock, C. O. (2002). Mechanistic diversity and regulation of Type II fatty acid synthesis. *Biochemical Society Transactions*, *30*(6), 1050–1055. https://doi.org/10.1042/bst0301050

Matus-Ortega, M. G., Cárdenas-Monroy, C. A., Flores-Herrera, O., Mendoza-Hernández, G., Miranda, M., González-Pedrajo, B., Vázquez-Meza, H., & Pardo, J. P. (2015). New complexes containing the internal alternative NADH dehydrogenase (Ndi1) in mitochondria of *Saccharomyces cerevisiae*. *Yeast*, *32*(10), 629–641. https://doi.org/10.1002/yea.3086

Mbuyane, L. L., Bauer, F. F., & Divol, B. (2021). The metabolism of lipids in yeasts and applications in oenology. In *Food Research International* (Vol. 141). Elsevier Ltd. https://doi.org/10.1016/j.foodres.2021.110142

Meï, C., Michaud, M., Cussac, M., Albrieux, C., Gros, V., Maréchal, E., Block, M. A., Jouhet, J., & Rébeillé, F. (2015). Levels of polyunsaturated fatty acids correlate with growth rate in plant cell cultures. *Scientific Reports*, *5*. https://doi.org/10.1038/srep15207

Meng, X., Yang, J., Xu, X., Zhang, L., Nie, Q., & Xian, M. (2009). Biodiesel production from oleaginous microorganisms. In *Renewable Energy* (Vol. 34, Issue 1, pp. 1–5). Elsevier BV. https://doi.org/10.1016/j.renene.2008.04.014

Miranda, C. (2018). Screening of single cell oils producing yeasts using volatile fatty acids as carbon sources: optimisation of Fourier transform infrared spectroscopy as a method to study lipid accumulation. University of Minho.

Modig, T., Granath, K., Adler, L., & Lidén, G. (2007). Anaerobic glycerol production by *Saccharomyces cerevisiae* strains under hyperosmotic stress. *Applied Microbiology and Biotechnology*, *75*(2), 289–296. https://doi.org/10.1007/s00253-006-0821-8

Moldoveanu, S., & David, V. (2015). The Role of Derivatization in Chromatography. In *Modern Sample Preparation for Chromatography* (pp. 307–331). Elsevier. https://doi.org/10.1016/b978-0-444-54319-6.00009-8

Mou, Z., He, Y., Dai, Y., Liu, X., & Li, J. (2000). Deficiency in Fatty Acid Synthase Leads to Premature Cell Death and Dramatic Alterations in Plant Morphology. In *The Plant Cell* (Vol. 12). www.plantcell.org

Muratovska, N., Silva, P., Pozdniakova, T., Pereira, H., Grey, C., Johansson, B., & Carlquist, M. (2022). Towards engineered yeast as production platform for capsaicinoids. In *Biotechnology Advances* (Vol. 59). Elsevier Inc. https://doi.org/10.1016/j.biotechadv.2022.107989

Murphy, K. J., Mann, N. J., & Sinclair, A. J. (2003). Fatty acid and sterol composition of frozen and freeze-dried New Zealand Green Lipped Mussel (*Perna canaliculus*) from three sites in New Zealand. In *Asia Pacific J Clin Nutr* (Vol. 12, Issue 1).

Musayev, F., Sachdeva, S., Scarsdale, J. N., Reynolds, K. A., & Wright, H. T. (2005). Crystal structure of a substrate complex of Mycobacterium tuberculosis β-ketoacyl-acyl carrier protein synthase III (FabH) with lauroyl-coenzyme A. *Journal of Molecular Biology*, *346*(5), 1313–1321. https://doi.org/10.1016/j.jmb.2004.12.044

Mwaurah, P. W., Kumar, S., Kumar, N., Attkan, A. K., Panghal, A., Singh, V. K., & Garg, M. K. (2020). Novel oil extraction technologies: Process conditions, quality parameters, and

optimization. *Comprehensive Reviews in Food Science and Food Safety*, *19*(1), 3–20. https://doi.org/10.1111/1541-4337.12507

Natural fatty acids market (2022 - 2027). Mordor Intelligence(2020).

Nishida, I. (2004). *Molecular Biology and Biotechnology of Plant Organelles 543-564*. Springer.

Notman, R., Noro, M., O'Malley, B., & Anwar, J. (2006). Molecular basis for dimethylsulfoxide (DMSO) action on lipid membranes. *Journal of the American Chemical Society*, *128*(43), 13982–13983. https://doi.org/10.1021/ja063363t

Nowinski, S. M., Solmonson, A., Rusin, S. F., Maschek, J. A., Bensard, C. L., Fogarty, S., Jeong, M. Y., Lettlova, S., Berg, J. A., Morgan, J. T., Ouyang, Y., Naylor, B. C., Paulo, J. A., Funai, K., Cox, J. E., Gygi, S. P., Winge, D. R., Deberardinis, R. J., & Rutter, J. (2020). Mitochondrial fatty acid synthesis coordinates oxidative metabolism in mammalian mitochondria. *ELife*, *9*, 1–35. https://doi.org/10.7554/ELIFE.58041

Olson, G. J., & Ingram, L. O. (1975). Effects of temperature and nutritional changes on the fatty acids of *Agmenellum quadruplicatum*. *Journal of Bacteriology*, *124*(1), 373–379. https://doi.org/10.1128/jb.124.1.373-379.1975

O'Sullivan, S. M., Woods, J. A., & O'Brien, N. M. (2004). Use of Tween 40 and Tween 80 to deliver a mixture of phytochemicals to human colonic adenocarcinoma cell (CaCo-2) monolayers. *British Journal of Nutrition*, *91*(5), 757–764. https://doi.org/10.1079/bjn20041094

Otterstedt, K., Larsson, C., Bill, R. M., Ståhlberg, A., Boles, E., Hohmann, S., & Gustafsson, L. (2004). Switching the mode of metabolism in the yeast *Saccharomyces cerevisiae*. *EMBO Reports*, *5*(5), 532–537. https://doi.org/10.1038/sj.embor.7400132

Pampulha, M. E., & Loureiro-Dias, M. C. (2000). Energetics of the effect of acetic acid on growth of *Saccharomyces cerevisiae*. *FEMS Microbiology Letters*, *184*(1), 69–72. https://doi.org/10.1111/j.1574-6968.2000.tb08992.x

Papackova, Z., & Cahova, M. (2015). Fatty acid signalling: The new function of intracellular lipases. In *International Journal of Molecular Sciences* (Vol. 16, Issue 2, pp. 3831–3855). MDPI AG. https://doi.org/10.3390/ijms16023831

Paramasivan, K., Aneesha, A., Gupta, N., & Mutturi, S. (2021). Adaptive evolution of engineered yeast for squalene production improvement and its genome-wide analysis. *Yeast*, *38*(7), 424–437. https://doi.org/10.1002/yea.3559

Patel, A., Mikes, F., & Matsakas, L. (2018). An overview of current pretreatment methods used to improve lipid extraction from oleaginous microorganisms. In *Molecules* (Vol. 23, Issue 7). MDPI AG. https://doi.org/10.3390/molecules23071562

Pereira, F., Azevedo, F., Parachin, N. S., Hahn-Hägerdal, B., Gorwa-Grauslund, M. F., & Johansson, B. (2016). Yeast Pathway Kit: A Method for Metabolic Pathway Assembly with Automatically Simulated Executable Documentation. *ACS Synthetic Biology*, *5*(5), 386–394. https://doi.org/10.1021/acssynbio.5b00250

Pfeiffer, T., & Morley, A. (2014). An evolutionary perspective on the Crabtree effect. *Frontiers in Molecular Biosciences*, *1*. https://doi.org/10.3389/fmolb.2014.00017

Phospholipids analysis. Retrieved 14 November 2022, from https://myaccount.aocs.org/PersonifyEbusiness/Store/Product-Details?productId=112590

Pi, H. W., Anandharaj, M., Kao, Y. Y., Lin, Y. J., Chang, J. J., & Li, W. H. (2018). Engineering the oleaginous red yeast *Rhodotorula glutinis* for simultaneous β-carotene and cellulase production. *Scientific Reports*, *8*(1). https://doi.org/10.1038/s41598-018-29194-z

Pinzi, S., Rounce, P., Herreros, J. M., Tsolakis, A., & Pilar Dorado, M. (2013). The effect of biodiesel fatty acid composition on combustion and diesel engine exhaust emissions. *Fuel*, *104*, 170–182. https://doi.org/10.1016/j.fuel.2012.08.056

Piva, G. G., Casalta, E., Legras, J. L., Tesnière, C., Sablayrolles, J. M., Ferreira, D., Ortiz-Julien, A., Galeote, V., & Mouret, J. R. (2022). Characterization and Role of Sterols in *Saccharomyces cerevisiae* during White Wine Alcoholic Fermentation. In *Fermentation* (Vol. 8, Issue 2). MDPI. https://doi.org/10.3390/fermentation8020090

Pontes, M. H., Sevostyanova, A., & Groisman, E. A. (2015). When Too Much ATP Is Bad for Protein Synthesis. In *Journal of Molecular Biology* (Vol. 427, Issue 16, pp. 2586–2594). Academic Press. https://doi.org/10.1016/j.jmb.2015.06.021

Pozdniakova, T., Cruz, J. P., Silva, P. C., Azevedo, F., Parpot, P., Domingues, M. R., Carlquist, M., & Johansson, B. (2023). *Optimization of a hybrid bacterial/Arabidopsis thaliana fatty acid synthase system II in Saccharomyces cerevisiae* – submitted.

Prescott, D. J., J7agelos, P. R., & Louis, S. T. (1972). *ACYL CARRIER PROTEIN*.

Protzko, R. J., Latimer, L. N., Martinho, Z., de Reus, E., Seibert, T., Benz, J. P., & Dueber, J. E. (2018). Engineering *Saccharomyces cerevisiae* for co-utilization of d-galacturonic acid and d-glucose from citrus peel waste. *Nature Communications*, *9*(1). https://doi.org/10.1038/s41467-018-07589-w

Puhan, S., Saravanan, N., Nagarajan, G., & Vedaraman, N. (2010). Effect of biodiesel unsaturated fatty acid on combustion characteristics of a DI compression ignition engine. *Biomass and Bioenergy*, *34*(8), 1079–1088. https://doi.org/10.1016/j.biombioe.2010.02.017

Ramírez-Castrillón, M., Jaramillo-Garcia, V. P., Lopes Barros, H., Pegas Henriques, J. A., Stefani, V., & Valente, P. (2021). Nile red Incubation Time Before Reading Fluorescence Greatly Influences the Yeast Neutral Lipids Quantification. *Frontiers in Microbiology*, *12*. https://doi.org/10.3389/fmicb.2021.619313

Ramos, M. J., Fernández, C. M., Casas, A., Rodríguez, L., & Pérez, Á. (2009). Influence of fatty acid composition of raw materials on biodiesel properties. *Bioresource Technology*, *100*(1), 261–268. https://doi.org/10.1016/j.biortech.2008.06.039

Rawlings, M., & Cronan, J. E. (1992). The gene encoding *Escherichia coli* acyl carrier protein lies within a cluster of fatty acid biosynthetic genes. *Journal of Biological Chemistry*, *267*(9), 5751–5754. https://doi.org/10.1016/s0021-9258(18)42616-6

Rodrigues, F., Ludovico, P., & Leão, C. (2006). Sugar Metabolism in Yeasts: an Overview of Aerobic and Anaerobic Glucose Catabolism. In *Biodiversity and Ecophysiology of Yeasts*. Springer-Verlag. https://doi.org/10.1007/3-540-30985-3_6

Rolfe, M. D., Rice, C. J., Lucchini, S., Pin, C., Thompson, A., Cameron, A. D. S., Alston, M., Stringer, M. F., Betts, R. P., Baranyi, J., Peck, M. W., & Hinton, J. C. D. (2012). Lag phase is a distinct growth phase that prepares bacteria for exponential growth and involves

transient metal accumulation. *Journal of Bacteriology*, *194*(3), 686–701. https://doi.org/10.1128/JB.06112-11

Runguphan, W., & Keasling, J. D. (2014). Metabolic engineering of *Saccharomyces cerevisiae* for production of fatty acid-derived biofuels and chemicals. *Metabolic Engineering*, *21*, 103–113. https://doi.org/10.1016/j.ymben.2013.07.003

Salari, R., & Salari, R. (2017). Investigation of the Best *Saccharomyces cerevisiae* Growth Condition. *Electronic Physician*, *9*(1), 3592–3597. https://doi.org/10.19082/3592

Salvadó, Z., Arroyo-López, F. N., Guillamón, J. M., Salazar, G., Querol, A., & Barrio, E. (2011). Temperature adaptation Markedly Determines evolution within the genus *Saccharomyces*. *Applied and Environmental Microbiology*, *77*(7), 2292–2302. https://doi.org/10.1128/AEM.01861-10

Sanches-Silva, A., Rodríguez-Bernaldo De Quirós, A., López-Hernández, J., & Paseiro-Losada, P. (2004). Comparison between high-performance liquid chromatography and gas chromatography methods for fatty acid identification and quantification in potato crisps. *Journal of Chromatography A*, *1032*(1–2), 7–15. https://doi.org/10.1016/j.chroma.2003.11.012

Schäfer, J. (2018). *Wiring components of the respiratory chain*. University of Stockholm.

Schuller, H.-J., Hahn, A., Troster, F., Schutz, A., & Schweizer, E. (1992). Coordinate genetic control of yeast fatty acid synthase genes FAS1 and FAS2 by an upstream activation site common to genes involved in membrane lipid biosynthesis. In *The EMBO Journal* (Vol. 1, Issue 1).

Sevcik, J. (2011). *Detectors in gas chromatography*.

Sewalt, V., Shanahan, D., Gregg, L., la Marta, J., & Carillo, R. (2016). The Generally Recognized as Safe (GRAS) process for industrial microbial enzymes. In *Industrial Biotechnology* (Vol. 12, Issue 5, pp. 295–302). Mary Ann Liebert Inc. https://doi.org/10.1089/ind.2016.0011

Sheng, J., & Feng, X. (2015). Metabolic engineering of yeast to produce fatty acid-derived biofuels: Bottlenecks and solutions. In *Frontiers in Microbiology* (Vol. 6, Issue JUN). Frontiers Media S.A. https://doi.org/10.3389/fmicb.2015.00554

Sherman, F. (2002). *Getting Started with Yeast*. http://dbb.urmc.rochester.edu/labs/sherman_f/yeast/Index.html

Shi, L., & Tu, B. P. (2015). Acetyl-CoA and the regulation of metabolism: Mechanisms and consequences. In *Current Opinion in Cell Biology* (Vol. 33, pp. 125–131). Elsevier Ltd. https://doi.org/10.1016/j.ceb.2015.02.003

Shi, S., Ji, H., Siewers, V., & Nielsen, J. (2016). Improved production of fatty acids by *Saccharomyces cerevisiae* through screening a cDNA library from the oleaginous yeast *Yarrowia lipolytica*. *FEMS Yeast Research*, *16*(1). https://doi.org/10.1093/femsyr/fov108

Shin, H. Y., Shim, S. H., Ryu, Y. J., Yang, J. H., Lim, S. M., & Lee, C. G. (2018). Lipid Extraction from Tetraselmis sp. Microalgae for Biodiesel Production Using Hexane-based Solvent Mixtures. *Biotechnology and Bioprocess Engineering*, *23*(1), 16–22. https://doi.org/10.1007/s12257-017-0392-9

Shuib, S., Nazir, M. Y. M., Ibrahim, I., Song, Y., Ratledge, C., & Hamid, A. A. (2022). Co-existence of type I fatty acid synthase and polyketide synthase metabolons in Aurantiochytrium SW1 and their implications for lipid biosynthesis. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, *1867*(12). https://doi.org/10.1016/j.bbalip.2022.159224

Singh, G., Sinha, S., Bandyopadhyay, K. K., Lawrence, M., & Paul, D. (2018). Triauxic growth of an oleaginous red yeast *Rhodosporidium toruloides* on waste 'extract' for enhanced and concomitant lipid and β-carotene production. *Microbial Cell Factories*, *17*(1). https://doi.org/10.1186/s12934-018-1026-4

Sitepu, I. R., Ignatia, L., Franz, A. K., Wong, D. M., Faulina, S. A., Tsui, M., Kanti, A., & Boundy-Mills, K. (2012). An improved high-throughput Nile red fluorescence assay for estimating intracellular lipids in a variety of yeast species. *Journal of Microbiological Methods*, *91*(2), 321–328. https://doi.org/10.1016/j.mimet.2012.09.001

Söderholm, S. L., Damm, M., & Kappe, C. O. (2010). Microwave-assisted derivatization procedures for gas chromatography/mass spectrometry analysis. *Molecular Diversity*, *14*(4), 869–888. https://doi.org/10.1007/s11030-010-9242-9

Somsen, O. J. G., Hoeben, M. A., Esgalhado, E., Snoep, J. L., Visser, D., Heijden, R. T. J. M., Heijnen, J. J., & Westerhoff, H. v. (2000). Glucose and the ATP paradox in yeast. *Biochemical Journal*, *352*(2), 593–599. https://doi.org/10.1042/bj3520593

Sorger, D., & Daum, G. (2003). Triacylglycerol biosynthesis in yeast. In *Applied Microbiology and Biotechnology* (Vol. 61, Issue 4, pp. 289–299). Springer Verlag. https://doi.org/10.1007/s00253-002-1212-4

Stanway, C. A., Gibbs, J. M., Berardi, E., A Stanway, P. C., Gibbs, J. M., & Berardi, E. I. (1995). Expression of the FOX1 gene of *Saccharomyces cerevisiae* is regulated by carbon source, but not by the known glucose repression genes. In *Curr Genet* (Vol. 27). Springer-Verlag.

Stuart, R. A. (2008). Supercomplex organization of the oxidative phosphorylation enzymes in yeast mitochondria. *Journal of Bioenergetics and Biomembranes*, *40*(5), 411. https://doi.org/10.1007/s10863-008-9168-4

Sun, L., Kwak, S., & Jin, Y. S. (2019). Vitamin A Production by Engineered *Saccharomyces cerevisiae* from Xylose via Two-Phase in Situ Extraction. *ACS Synthetic Biology*, *8*(9), 2131–2140. https://doi.org/10.1021/acssynbio.9b00217

Swartz, M. (2010). HPLC detectors: A brief review. In *Journal of Liquid Chromatography and Related Technologies* (Vol. 33, Issues 9–12, pp. 1130–1150). https://doi.org/10.1080/10826076.2010.484356

Swinnen, E., Wanke, V., Roosen, J., Smets, B., Dubouloz, F., Pedruzzi, I., Cameroni, E., de Virgilio, C., & Winderickx, J. (2006). Rim15 and the crossroads of nutrient signalling pathways in *Saccharomyces cerevisiae*. In *Cell Division* (Vol. 1). https://doi.org/10.1186/1747-1028-1-3

Szabo, Z., Marosvölgyi, T., Szabo, E., Koczka, V., Verzar, Z., Figler, M., & Decsi, T. (2022). Effects of Repeated Heating on Fatty Acid Composition of Plant-Based Cooking Oils. *Foods*, *11*(2). https://doi.org/10.3390/foods11020192

Tai, M., & Stephanopoulos, G. (2013). Engineering the push and pull of lipid biosynthesis in oleaginous yeast *Yarrowia lipolytica* for biofuel production. *Metabolic Engineering*, *15*(1), 1–9. https://doi.org/10.1016/j.ymben.2012.08.007

Tehlivets, O., Scheuringer, K., & Kohlwein, S. D. (2007). Fatty acid synthesis and elongation in yeast. In *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* (Vol. 1771, Issue 3, pp. 255–270). https://doi.org/10.1016/j.bbalip.2006.07.004

Tejero Rioseras, A., Garcia Gomez, D., Ebert, B. E., Blank, L. M., Ibáñez, A. J., & Sinues, P. M. L. (2017). Comprehensive Real-Time Analysis of the Yeast Volatilome. *Scientific Reports*, *7*(1). https://doi.org/10.1038/s41598-017-14554-y

Thevis, M., & Schanzer, W. (2005). Examples of Doping Control Analysis by Liquid Chromatography-Tandem Mass Spectrometry: Ephedrines, -Receptor Blocking Agents, Diuretics, Sympathomimetics, and Cross-Linked Hemoglobins. *Journal of Chromatographic Science*, *43*(1), 22–31. https://doi.org/10.1093/chromsci/43.1.22

Tjellström, H., Yang, Z., Allen, D. K., & Ohlrogge, J. B. (2012). Rapid kinetic labelling of *Arabidopsis* cell suspension cultures: Implications for models of lipid export from plastids. *Plant Physiology*, *158*(2), 601–611. https://doi.org/10.1104/pp.111.186122

Tofalo, R., Perpetuini, G., Schirone, M., Fasoli, G., Aguzzi, I., Corsetti, A., & Suzzi, G. (2013). Biogeographical characterization of *Saccharomyces cerevisiae* wine yeast by molecular methods. *Frontiers in Microbiology*, *4*. https://doi.org/10.3389/fmicb.2013.00166

Troncoso-Ponce, M. A., Nikovics, K., Marchive, C., Lepiniec, L., & Baud, S. (2016). New insights on the organization and regulation of the fatty acid biosynthetic network in the model higher plant *Arabidopsis thaliana*. In *Biochimie* (Vol. 120, pp. 3–8). Elsevier B.V. https://doi.org/10.1016/j.biochi.2015.05.013

Turner, N., Cooney, G. J., Kraegen, E. W., & Bruce, C. R. (2014). Fatty acid metabolism, energy expenditure and insulin resistance in muscle. In *Journal of Endocrinology* (Vol. 220, Issue 2). https://doi.org/10.1530/JOE-13-0397

Tyson, S. (2006). Biodiesel Handling and Use Guidelines. U.S. Department of Energy, Energy Efficiency and Renewable Energy. http://www.osti.gov/bridge

van Handel, E. (1985). Rapid determination of total lipids in mosquitoes. *Journal of the American Mosquito Control Association*, *1*(3), 302–304.

Vasudevan, P. T., & Briggs, M. (2008). Biodiesel production - Current state of the art and challenges. In *Journal of Industrial Microbiology and Biotechnology* (Vol. 35, Issue 5, pp. 421–430). https://doi.org/10.1007/s10295-008-0312-2

Vermeersch, L., Perez-Samper, G., Cerulus, B., Jariani, A., Gallone, B., Voordeckers, K., Steensels, J., & Verstrepen, K. J. (2019). On the duration of the microbial lag phase. *Current Genetics*, *65*(3), 721–727. https://doi.org/10.1007/s00294-019-00938-2

Walker, G., & Stewart, G. (2016). *Saccharomyces cerevisiae* in the Production of Fermented Beverages. *Beverages*, *2*(4), 30. https://doi.org/10.3390/beverages2040030

Wang, J., Zhang, B., & Chen, S. (2011). Oleaginous yeast *Yarrowia lipolytica* mutants with a disrupted fatty acyl-CoA synthetase gene accumulate saturated fatty acid. *Process Biochemistry*, *46*(7), 1436–1441. https://doi.org/10.1016/j.procbio.2011.03.011

Wang, Q., Oshita, K., & Takaoka, M. (2021). Effective lipid extraction from underwater microalgae liquid using subcritical dimethyl ether. *Biotechnology for Biofuels*, *14*(1). https://doi.org/10.1186/s13068-020-01871-0

Weber, C. A., Sekar, K., Tang, J. H., Warmer, P., Sauer, U., & Weis, K. (2020). β-Oxidation and autophagy are critical energy providers during acute glucose depletion in *Saccharomyces cerevisiae*. https://doi.org/10.1073/pnas.1913370117/-/DCSupplemental

Wen, J., Arakawa, T., & Philo, J. S. (1996). REVIEW Size-Exclusion Chromatography with On-Line Light-Scattering, Absorbance, and Refractive Index Detectors for Studying Proteins and Their Interactions. In *Analytical Biochemistry* (Vol. 240).

Wenz, P., Schwank, S., Hoja, U., & Schüller, H.-J. (2001). A downstream regulatory element located within the coding sequence mediates autoregulated expression of the yeast fatty acid synthase gene *FAS2* by the *FAS1* gene product. In *Nucleic Acids Research* (Vol. 29).

Wu, H., Ito, K., & Shimoi, H. (2005). Identification and characterization of a novel biotin biosynthesis gene in *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology*, *71*(11), 6845–6855. https://doi.org/10.1128/AEM.71.11.6845-6855.2005

Wu, L.-C., David, T., & Po-Wang, W. (1932). *An Ancient Chinese Treatise on Alchemy Entitled Ts'an T'ung Ch'i*. www.journals.uchicago.edu

Xie, J., Lin, Y. S., Shi, X. J., Zhu, X. Y., Su, W. K., & Wang, P. (2013). Mechanochemical-assisted extraction of flavonoids from bamboo (*Phyllostachys edulis*) leaves. *Industrial Crops and Products*, *43*(1), 276–282. https://doi.org/10.1016/j.indcrop.2012.07.041

Yamada, E. A., & Sgarbieri, V. C. (2005). Yeast (*Saccharomyces cerevisiae*) protein concentrate: Preparation, chemical composition, and nutritional and functional properties. *Journal of Agricultural and Food Chemistry*, *53*(10), 3931–3936. https://doi.org/10.1021/jf0400821

Yang, P., Jiang, S., Lu, S., Jiang, S., Jiang, S., Deng, Y., Lu, J., Wang, H., & Zhou, Y. (2022). Ethanol yield improvement in Saccharomyces cerevisiae GPD2 Delta FPS1 Delta ADH2 Delta DLD3 Delta mutant and molecular mechanism exploration based on the metabolic flux and transcriptomics approaches. *Microbial Cell Factories*, *21*(1), 160. https://doi.org/10.1186/s12934-022-01885-3

Yenkie, K. M., Wu, W., & Maravelias, C. T. (2017). Synthesis and analysis of separation networks for the recovery of intracellular chemicals generated from microbial-based conversions. *Biotechnology for Biofuels*, *10*(1). https://doi.org/10.1186/s13068-017-0804-2

Yu, X., Liu, T., Zhu, F., & Khosla, C. (2011). In vitro reconstitution and steady-state analysis of the fatty acid synthase from *Escherichia coli*. *Proceedings of the National Academy of Sciences*, *108* (46), 18643–18648. https://doi.org/10.1073/pnas.1110852108

Zhang, A. C., Singh, S., Craig, J. P., & Downie, L. E. (2020). Omega-3 Fatty Acids and Eye Health: Opinions and Self-Reported Practice Behaviors of Optometrists in Australia and New Zealand. *Nutrients*, *12*(4). https://doi.org/10.3390/nu12041179

Zhang, H., Machutta, C., & Tonge, P. (2010). *Fatty Acid Biosynthesis and Oxidation*. Elsevier.

Zhang, X., Nijland, J. G., & Driessen, A. J. M. (2022). Combined roles of exporters in acetic acid tolerance in *Saccharomyces cerevisiae*. *Biotechnology for Biofuels and Bioproducts*, *15*(1). https://doi.org/10.1186/s13068-022-02164-4

Zhao, Y., Luo, L., Xu, J., Xin, P., Guo, H., Wu, J., Bai, L., Wang, G., Chu, J., Zuo, J., Yu, H., Huang, X., & Li, J. (2018). Malate transported from chloroplast to mitochondrion triggers production of ROS and PCD in *Arabidopsis thaliana*. *Cell Research*, *28*(4), 448–461. https://doi.org/10.1038/s41422-018-0024-8

Zhao, Z., & Xu, Y. (2010). An extremely simple method for extraction of lysophospholipids and phospholipids from blood samples. *Journal of Lipid Research*, *51*(3), 652–659. https://doi.org/10.1194/jlr.D001503

Zhou, Y. J., Buijs, N. A., Siewers, V., & Nielsen, J. (2014). Fatty acid-derived biofuels and chemicals production in *Saccharomyces cerevisiae*. In *Frontiers in Bioengineering and Biotechnology* (Vol. 2, Issue SEP). Frontiers Media S.A. https://doi.org/10.3389/fbioe.2014.00032

Zhou, Y. J., Kerkhoven, E. J., & Nielsen, J. (2018). Barriers and opportunities in bio-based production of hydrocarbons. In *Nature Energy* (Vol. 3, Issue 11, pp. 925–935). Nature Publishing Group. https://doi.org/10.1038/s41560-018-0197-x

Zhuang, Y., Dong, J., He, X., Wang, J., Li, C., Dong, L., Zhang, Y., Zhou, X., Wang, H., Yi, Y., & Wang, S. (2022). Impact of Heating Temperature and Fatty Acid Type on the Formation of Lipid Oxidation Products During Thermal Processing. *Frontiers in Nutrition*, *9*. https://doi.org/10.3389/fnut.2022.913297

8. Appendix

8. Appendix

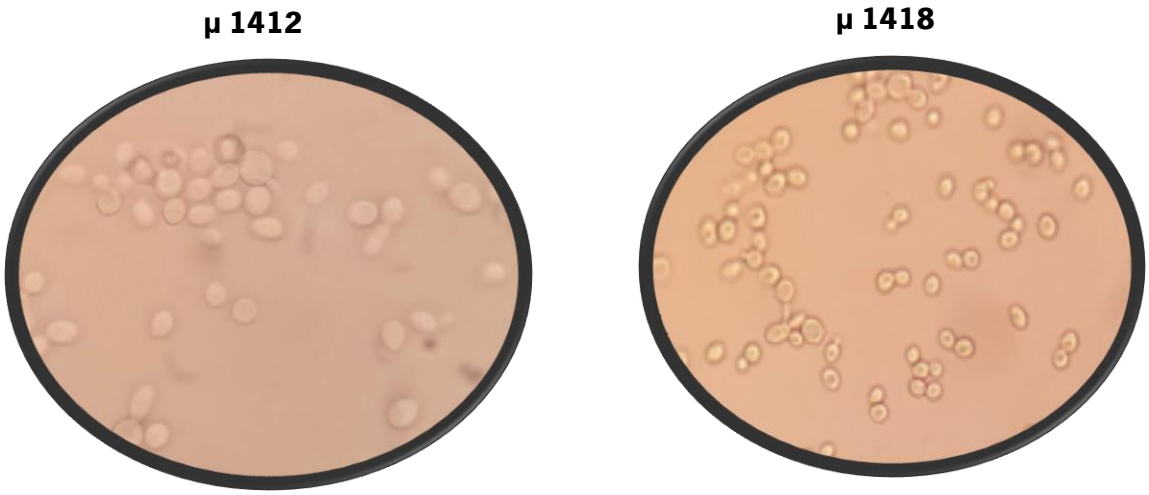
8.1 Drop-out – a mixture of amino acids

Compound	Code	Concentration mg/L
Alanine	A	19.74
Arginine	R	78.96
Asparagine	N	78.96
Aspartic Acid	D	78.96
Cysteine	C	78.96
Glutamic Acid	E	78.96
Glutamine	Q	78.96
Glycine	G	78.96
Isoleucine	I	78.96
Lysine	L	78.96
Methionine	M	78.96
Phenylalanine	F	78.96
Proline	P	78.96
Serine	S	78.96
Threonine	T	78.96
Tyrosine	Y	78.96
Valine	V	78.96
Myo-inositol	–	78.96
para-Aminobenzidine	–	7.896
Total	g/L	1.37

8.2 Primers used in this work

Primer	*Sequence (5'→3')*
p467	ATTTAAATCCTGATGCGTTTGTCTGCACAGA
p468	GTCGAGGAACGCCAGGTTGCCCACT
p577	GTTCTGATCCTCGAGCATCTTAAGAATTC
p578	GTTCTTGTCTCATTGCCACATTCATAAGT
p623	TAATTAATTTGTTTGTTTATGTGTGTTTATTCG
p856	TTAAATTGTAATGTTCTAACAAAAATTT
p1216	TAATAACTGATATAATTAAATTGAAGC

8.3 Microscope visualization of cells on YPD + Hmy medium



8.4 pTA1 plasmid

[.....**Variable zone**.....]

ATCCGGATTTACCTGAATCAATTGGCGAAATTTTTTGTACGAAATTTCAGCCACTTCACAGGCGGTTTTC
GCACGTACCCATGCGCTACGTTCCTGGCCCTCTTCAAACAGGCCCAGTTCGCCAATAAAATCACCCTG
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8.4.1 pTA1_FASIIb plasmid

Variable zone:

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8.5 pYPK0 plasmid

[.....**Variable zone**.....]

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8.5.1 pYPK0 plasmid – PDC1_*fabH*_TEF1

Variable zone:

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TTTCTTGTTCTATTACAACTTTTTTTACTTCTTGCTCATTAGAAAGAAAGCATAGCAATCTAATCTAAGTTT
TAATTACAAATTAATTA

8.5.2 pYPK0 plasmid – TEF1_*fabD*_FBA1

Variable zone:

TTAAATAACAATGCATACTTTGTACGTTCAAAATACAATGCAGTAGATATATTTATGCATATTACATATAAT
ACATATCACATAGGAAGCAACAGGCGCGTTGGACTTTTAATTTTCGAGGACCGCGAATCCTTACATCAC
ACCCAATCCCCCACAAGTGATCCCCCACACACCATAGCTTCAAAATGTTTCTACTCCTTTTTTACTCTTC
CAGATTTTCTCGGACTCCGCGCATCGCCGTACCACTTCAAAACACCCAAGCACAGCATACTAAATTTCC
CCTCTTTCTTCCTCTAGGGTGTCGTTAATTACCCGTACTAAAGGTTTGGAAAAGAAAAAAGAGACCGCCT
CGTTTCTTTTTCTTCGTCGAAAAAGGCAATAAAAATTTTTATCACGTTTCTTTTTCTTGAAAATTTTTTTTTT
TGATTTTTTTCTCTTTCGATGACCTCCCATTGATATTTAAGTTAATAAACGGTCTTCAATTTCTCAAGTTTC
AGTTTCATTTTTCTTGTTCTATTACAACTTTTTTTACTTCTTGCTCATTAGAAAGAAAGCATAGCAATCTAA
TCTAAGTTTTAATTACAAATTAATTAGTCGAGGAACGCCAGGTTGCCCACTTTCTCACTAGTGACCTGCA
GCCGACAAATGACGCAATTTGCATTTGTGTTCCCTGGACAGGGTTCTCAAACCGTTGGAATGCTGGCTG
ATATGGCGGCGAGCTATCCAATTGTCGAAGAAACGTTTGCTGAAGCTTCTGCGGCGCTGGGCTACGAC
CTGTGGGCGCTGACCCAGCAGGGGCCAGCTGAAGAACTGAATAAAACCTGGCAAACTCAGCCTGCGCT
GTTGACTGCATCTGTTGCGCTGTATCGCGTATGGCAGCAGCAGGGCGGTAAAGCACCGGCAATGATGG
CCGGTCACAGCCTGGGGGAATACTCCGCGCTGGTTTGCGCTGGTGTGATTGATTTCGCTGATGCGGTG
CGTCTGGTTGAGATGCGCGGCAAGTTCATGCAAGAAGCCGTACCGGAAGGCACGGGCGCTATGGCGG
CAATCATCGGTCTGGATGATGCGTCTATTGCGAAAGCGTGTGAAGAAGCTGCAGAAGGTCAGGTCGTTT
CTCCGGTAAACTTTAACTCTCCGGGACAGGTGGTTATTGCCGGTCATAAAGAAGCGGTTGAGCGTGCTG
GCGCTGCCTGTAAAGCGGCGGGCGCAAAACGCGCGCTGCCGTTACCAGTGAGCGTACCGTCTCACTG
TGCGCTGATGAAACCAGCAGCCGACAAACTGGCAGTAGAATTAGCGAAAATCACCTTTAACGCACCAAC
AGTTCCTGTTGTGAATAACGTTGATGTGAAATGCGAAACCAATGGTGATGCCATCCGTGACGCACTGGT
ACGTCAGTTGTATAACCCGGTTCAGTGGACGAAGTCTGTTGAGTACATGGCAGCGCAAGGCGTAGAACA
TCTCTATGAAGTCGGCCCGGGCAAAGTGCTTACTGGCCTGACGAAACGCATTGTCGACACCCTGACCG
CCTCGGCGCTGAACGAACCTTCAGCGATGGCAGCGGCGCTCGAGCTTTAAGTGCCATCTGTGCAGACA
AACGCATCAGGATTTAAATAATAACAATACTGACAGTACTAAATAATTGCCTACTTGGCTTCACATACGTT
GCATACGTCGATATAGATAATAATGATAATGACAGCAGGATTATCGTAATACGTAATAGTTGAAAATCTCA
AAAATGTGTGGGTCATTACGTAAATAATGATAGGAATGGGATTCTTCTATTTTTCCTTTTTCCATTCTAGC
AGCCGTCGGGAAAACGTGGCATCCTCTCTTTCGGGCTCAATTGGAGTCACGCTGCCGTGAGCATCCTC
TCTTTCCATATCTAACAACTGAGCACGTAACCAATGGAAAAGCATGAGCTTAGCGTTGCTCCAAAAAAGT
ATTGGATGGTTAATACCATTTGTCTGTTCTCTTCTGACTTTGACTCCTCAAAAAAAAAAAATCTACAATCA
ACAGATCGCTTCAATTACGCCCTCACAAAAACTTTTTTCCTTCTTCTTCGCCCACGTTAAATTTTATCCCT
CATGTTGTCTAACGGATTTCTGCACTTGATTTATTATAAAAAGACAAAGACATAATACTTCTCTATCAATTT

CAGTTATTGTTCTTCCTTGCGTTATTCTTCTGTTCTTCTTTTTCTTTTGTCATATATAACCATAACCAAGTA
ATACATATTCAAATTAATTA

8.5.3 pYPK0 plasmid – FBA1_*fabG*_RPL22A

Variable zone:

TTAAATAATAACAATACTGACAGTACTAAATAATTGCCTACTTGGCTTCACATACGTTGCATACGTCGATA
TAGATAATAATGATAATGACAGCAGGATTATCGTAATACGTAATAGTTGAAAATCTCAAAAATGTGTGGGT
CATTACGTAAATAATGATAGGAATGGGATTCTTCTATTTTTCCTTTTTCCATTCTAGCAGCCGTCGGGAAA
ACGTGGCATCCTCTCTTTCGGGCTCAATTGGAGTCACGCTGCCGTGAGCATCCTCTCTTTCCATATCTA
ACAACTGAGCACGTAACCAATGGAAAAGCATGAGCTTAGCGTTGCTCCAAAAAAGTATTGGATGGTTAAT
ACCATTTGTCTGTTCTCTTCTGACTTTGACTCCTCAAAAAAAAAAAATCTACAATCAACAGATCGCTTCAA
TTACGCCCTCACAAAAACTTTTTTCCTTCTTCTTCGCCCACGTTAAATTTTATCCCTCATGTTGTCTAACG
GATTTCTGCACTTGATTTATTATAAAAAGACAAAGACATAATACTTCTCTATCAATTTCAGTTATTGTTCTT
CCTTGCGTTATTCTTCTGTTCTTCTTTTTCTTTTGTCATATATAACCATAACCAAGTAATACATATTCAAAT
TAATTAGTCGAGGAACGCCAGGTTGCCCACTTTCTCACTAGTGACCTGCAGCCGACAAATGAATTTTGA
AGGAAAAATCGCACTGGTAACCGGTGCAAGCCGCGGAATTGGCCGCGCAATTGCTGAAACGCTCGCAG
CCCGTGGCGCGAAAGTTATTGGCACTGCGACCAGTGAAAATGGCGCTCAGGCGATCAGTGATTATTTAG
GTGCCAACGGCAAAGGTCTGATGTTGAATGTGACCGACCCGGCATCTATCGAATCTGTTCTGGAAAAAA
TTCGCGCAGAATTTGGTGAAGTGGATATCCTGGTCAATAATGCCGGTATCACTCGTGATAACCTGTTAAT
GCGAATGAAAGATGAAGAGTGGAACGATATTATCGAAACCAACCTTTCATCTGTTTTCCGTCTGTCAAAA
GCGGTAATGCGCGCTATGATGAAAAAGCGTCATGGTCGTATTATCACTATCGGTTCTGTGGTTGGTACC
ATGGGAAATGGCGGTCAGGCCAACTACGCTGCGGCGAAAGCGGGCTTGATCGGCTTCAGTAAATCACT
GGCGCGCGAAGTTGCGTCACGCGGTATTACTGTAAACGTTGTTGCTCCGGGCTTTATTGAAACGGACAT
GACACGTGCGCTGAGCGATGACCAGCGTGCGGGTATCCTGGCGCAGGTTCCTGCGGGTCGCCTCGGC
GGCGCACAGGAAATCGCCAACGCGGTTGCATTCCTGGCATCCGACGAAGCAGCTTACATCACGGGTGA
AACTTTGCATGTGAACGGCGGGATGTACATGGTCTGAGTGCCATCTGTGCAGACAAACGCATCAGGATT
TAAATTGTAATGTTCTAACAAAAATTTTTACTGATTTATAAAACTTATATAGATAGATAGACACATATATATA
TCTATATATAGAAACACAACTAAAGTTTACCATGTTTTATATAATATTACTACCGCTACTGAGTAAATGTTT
AGCTTGTACTTAACTTTCAAAGACAGTTTTTTTGATAAACATCAGTACATTTAATAATACATCCATACATTT
ATGTTAATTTGTTTTTCCTTTCCACCTCAGTGCGCTTCTCTTTTGAAAAAATCCTCAACAGGCCACGCGA
ACAAATCTTACCGAGAATTTCCTAAAATGTTTCAAACTATCATTTAGCTGCATACAATATGTACATTATTAA

TTGAGATTAACAACTTTACCTGTACTTTACCGTTATCAAATTATAAACCACCAAAACAACAGATTAAGAAA
CATTAATTA

8.5.4 pYPK0 plasmid – RPL22A_*acpP*_TDH3

Variable zone:

TTAAATTGTAATGTTCTAACAAAAATTTTTACTGATTTATAAAACTTATATAGATAGATAGACACATATATAT
ATCTATATATAGAAACACAACTAAAGTTTACCATGTTTTATATAATATTACTACCGCTACTGAGTAAATGTT
TAGCTTGTACTTAACTTTCAAAGACAGTTTTTTTGATAAACATCAGTACATTTAATAATACATCCATACATT
TATGTTAATTTGTTTTTCCTTTCCACCTCAGTGCGCTTCTCTTTTGAAAAAATCCTCAACAGGCCACGCG
AACAAATCTTACCGAGAATTTCCTAAAATGTTTCAAACTATCATTTAGCTGCATACAATATGTACATTATTA
ATTGAGATTAACAACTTTACCTGTACTTTACCGTTATCAAATTATAAACCACCAAAACAACAGATTAAGAA
ACATTAATTAGTCGAGGAACGCCAGGTTGCCCACTTTCTCACTAGTGACCTGCAGCCGACAAATGAGCA
CTATCGAAGAACGCGTTAAGAAAATTATCGGCGAACAGCTGGGCGTTAAGCAGGAAGAAGTTACCAACA
ATGCTTCTTTCGTTGAAGACCTGGGCGCGGATTCTCTTGACACCGTTGAGCTGGTAATGGCTCTGGAAG
AAGAGTTTGATACTGAGATTCCGGACGAAGAAGCTGAGAAAATCACCACCGTTCAGGCTGCCATTGATT
ACATCAACGGCCACCAGGCGTAAGTGCCATCTGTGCAGACAAACGCATCAGGATTTAAATAATAAAAAA
CACGCTTTTTCAGTTCGAGTTTATCATTATCAATACTGCCATTTCAAAGAATACGTAAATAATTAATAGTA
GTGATTTTCCTAACTTTATTTAGTCAAAAAATTAGCCTTTTAATTCTGCTGTAACCCGTACATGCCCAAAA
TAGGGGGCGGGTTACACAGAATATATAACATCGTAGGTGTCTGGGTGAACAGTTTATTCCTGGCATCCA
CTAAATATAATGGAGCCCGCTTTTTAAGCTGGCATCCAGAAAAAAAAAAGAATCCCAGCACCAAAATATTG
TTTTCTTCACCAACCATCAGTTCATAGGTCCATTCTCTTAGCGCAACTACAGAGAACAGGGGCACAAAC
AGGCAAAAAACGGGCACAACCTCAATGGAGTGATGCAACCTGCCTGGAGTAAATGATGACACAAGGCAA
TTGACCCACGCATGTATCTATCTCATTTTCTTACACCTTCTATTACCTTCTGCTCTCTCTGATTTGGAAAA
AGCTGAAAAAAAAGGTTGAAACCAGTTCCCTGAAATTATTCCCCTACTTGACTAATAAGTATATAAAGAC
GGTAGGTATTGATTGTAATTCTGTAAATCTATTTCTTAAACTTCTTAAATTCTACTTTTATAGTTAGTCTTTT
TTTTAGTTTTAAAACACCAAGAACTTAGTTTCGAATAAACACACATAAACAAACAAA

8.5.5 pYPK0 plasmid – TDH3_*fabF*_UTR2

Variable zone:

TTAAATAATAAAAAACACGCTTTTTCAGTTCGAGTTTATCATTATCAATACTGCCATTTCAAAGAATACGTA
AATAATTAATAGTAGTGATTTTCCTAACTTTATTTAGTCAAAAAATTAGCCTTTTAATTCTGCTGTAACCCG

TACATGCCCAAAATAGGGGGCGGGTTACACAGAATATATAACATCGTAGGTGTCTGGGTGAACAGTTTAT
TCCTGGCATCCACTAAATATAATGGAGCCCGCTTTTTAAGCTGGCATCCAGAAAAAAAAAGAATCCCAG
CACCAAAATATTGTTTTCTTCACCAACCATCAGTTCATAGGTCCATTCTCTTAGCGCAACTACAGAGAAC
AGGGGCACAAACAGGCAAAAAACGGGCACAACCTCAATGGAGTGATGCAACCTGCCTGGAGTAAATGA
TGACACAAGGCAATTGACCCACGCATGTATCTATCTCATTTTCTTACACCTTCTATTACCTTCTGCTCTC
TCTGATTTGGAAAAAGCTGAAAAAAAAGGTTGAAACCAGTTCCCTGAAATTATTCCCCTACTTGACTAATA
AGTATATAAAGACGGTAGGTATTGATTGTAATTCTGTAAATCTATTTCTTAAACTTCTTAAATTCTACTTTT
ATAGTTAGTCTTTTTTTTAGTTTTAAAACACCAAGAACTTAGTTTCGAATAAACACACATAAACAAACAAAT
TAATTAGTCGAGGAACGCCAGGTTGCCCACTTTCTCACTAGTGACCTGCAGCCGACAAATGTCTAAGCG
TCGTGTAGTTGTGACCGGACTGGGCATGTTGTCTCCTGTCGGCAATACCGTAGAGTCTACCTGGAAAGC
TCTGCTTGCCGGTCAGAGTGGCATCAGCCTAATCGACCATTTCGATACTAGCGCCTATGCAACGAAATT
TGCTGGCTTAGTAAAGGATTTTAACTGTGAGGACATTATCTCGCGCAAAGAACAGCGCAAGATGGATGC
CTTCATTCAATATGGAATTGTCGCTGGCGTTCAGGCCATGCAGGATTCTGGCCTTGAAATAACGGAAGA
GAACGCAACCCGCATTGGTGCCGCAATTGGCTCCGGGATTGGCGGCCTCGGACTGATCGAAGAAAACC
ACACATCTCTGATGAACGGTGGTCCACGTAAGATCAGCCCATTCTTCGTTCCGTCAACGATTGTGAACA
TGGTGGCAGGTCATCTGACTATCATGTATGGCCTGCGTGGCCCGAGCATCTCTATCGCGACTGCCTGTA
CTTCCGGCGTGCACAACATTGGCCATGCTGCGCGTATTATCGCGTATGGCGATGCTGACGTGATGGTT
GCAGGTGGCGCAGAGAAAGCCAGTACGCCGCTGGGCGTTGGTGGTTTTGGCGCGGCACGTGCATTATC
TACCCGCAATGATAACCCGCAAGCGGCGAGCCGCCCGTGGGATAAAGAGCGTGATGGTTTCGTACTGG
GCGATGGTGCCGGTATGCTGGTACTTGAAGAGTACGAACACGCGAAAAAACGCGGTGCGAAAATTTACG
CTGAACTCGTCGGCTTTGGTATGAGCAGCGATGCTTATCATATGACGTCACCGCCAGAAAATGGCGCAG
GCGCAGCTCTGGCGATGGCAAATGCTCTGCGTGATGCAGGCATTGAAGCGAGTCAGATTGGCTACGTT
AACGCGCACGGTACTTCTACGCCGGCTGGCGATAAAGCTGAAGCGCAGGCGGTGAAAACCATCTTCGG
TGAAGCTGCAAGCCGTGTGTTGGTAAGCTCCACGAAATCTATGACCGGTCACCTGTTAGGTGCGGCGG
GTGCAGTAGAATCTATCTACTCCATCCTGGCGCTGCGCGATCAGGCTGTTCCGCCAACCATCAACCTG
GATAACCCGGATGAAGGTTGCGATCTGGATTTCGTACCGCACGAAGCGCGTCAGGTTAGCGGAATGGA
ATACACTCTGTGTAACTCCTTCGGCTTCGGTGGCACTAATGGTTCTTTGATCTTTAAAAAGATCTAAGTG
CCATCTGTGCAGACAAACGCATCAGGATTTAAATCGATTTCCCACATCCTGCGTTTCCGTAGTCAAGGG
TTCATAATGGATGATGAGCCTGTAAACAGCTTTGTATTTACCTCTTTTACTTCAAGAATGCTCTTGCGTCG
TTTGCAGTGTCGAAGAAGATCCAGTAGAGCTATACAAAATGGAGAAATGAAACAGTTGAACCAGAGCGC
CGCATTTGGCAGGTAGTTTCGCTAAATTTCCTCATGCGCGTTTGTAATTATGCTAGTTAACCATATGATAA
GGTTGCCTAACTTGCAGTCACCGGAAGGTTATTTCCGGAATGGTGCAGAATAATTTTTAAAATAGTAGCA

TATATTTCGTTCCAATTCCCAAATAGGGAATTATAATTATGACAGATAAATTCCCGATTAGGAAGAAGCGC
GAAAATTCTTGAAAAACTTTTTTCAACCTCGAAGAAAATCGTCGTCCTTCGAAGATTTAGTGGGAATCTTC
AAGGTGGATAGTTTCCAAATTAAAACTGCAGAAGAGGCTTTTAAAACATGCCTGAGGTATAAAAAGAACA
ACATTCCTGGTGATTATGGTTTCTGTTGGACCCTGTTCCCTTTGTAAAACTTCTATTTATTGTCCTGCAAA
TAAGCTAATTATATAATTAATATTAATTA

8.5.6 pYPK0 plasmid – UTR2_*fabB*_TPI1

Variable zone:

TTAAATCGATTTCCCACATCCTGCGTTTCCGTAGTCAAGGGTTCATAATGGATGATGAGCCTGTAAACAG
CTTTGTATTTACCTCTTTTACTTCAAGAATGCTCTTGCGTCGTTTGCAGTGTCGAAGAAGATCCAGTAGA
GCTATACAAAATGGAGAAATGAAACAGTTGAACCAGAGCGCCGCATTTGGCAGGTAGTTTCGCTAAATTT
CCTCATGCGCGTTTGTAATTATGCTAGTTAACCATATGATAAGGTTGCCTAACTTGCAGTCACCGGAAGG
TTATTTCCGGAATGGTGCAGAATAATTTTTAAAATAGTAGCATATATTTCGTTCCAATTCCCAAATAGGGA
ATTATAATTATGACAGATAAATTCCCGATTAGGAAGAAGCGCGAAAATTCTTGAAAAACTTTTTTCAACCT
CGAAGAAAATCGTCGTCCTTCGAAGATTTAGTGGGAATCTTCAAGGTGGATAGTTTCCAAATTAAAACTG
CAGAAGAGGCTTTTAAAACATGCCTGAGGTATAAAAAGAACAACATTCCTGGTGATTATGGTTTCTGTTG
GACCCTGTTCCCTTTGTAAAACTTCTATTTATTGTCCTGCAAATAAGCTAATTATATAATTAATATTAATTA
GTCGAGGAACGCCAGGTTGCCCACTTTCTCACTAGTGACCTGCAGCCGACAAATGAAACGTGCAGTGA
TTACTGGCCTGGGCATTGTTTCCAGCATCGGTAATAACCAGCAGGAAGTCCTGGCATCTCTGCGTGAAG
GACGTTCAGGGATCACTTTCTCTCAGGAGCTGAAGGATTCCGGCATGCGTAGCCACGTCTGGGGCAAC
GTAAAACTGGATACCACTGGCCTCATTGACCGCAAAGTTGTGCGCTTTATGAGCGACGCATCCATTTAT
GCATTCCTTTCTATGGAGCAGGCAATCGCTGATGCGGGCCTCTCTCCGGAAGCTTACCAGAATAACCC
GCGCGTTGGCCTGATTGCAGGTTCCGGCGGCGGCTCCCCGCGTTTCCAGGTGTTCGGCGCTGACGCA
ATGCGCGGCCCGCGCGGCCTGAAAGCGGTTGGCCCGTATGTGGTCACCAAAGCGATGGCATCCGGCG
TTTCTGCCTGCCTCGCCACCCCGTTTAAAATTCATGGCGTTAACTACTCCATCAGCTCCGCGTGTGCGA
CTTCCGCACACTGTATCGGTAACGCAGTAGAGCAGATCCAACTGGGCAAACAGGACATCGTGTTTGCTG
GCGGCGGCGAAGAGCTGTGCTGGGAAATGGCTTGCGAATTCGACGCAATGGGTGCGCTGTCTACTAAA
TACAACGACACCCCGGAAAAAGCCTCCCGTACTTACGACGCTCACCGTGACGGTTTCGTTATCGCTGG
CGGCGGCGGTATGGTAGTGGTTGAAGAGCTGGAACACGCGCTGGCGCGTGGTGCTCACATCTATGCTG
AAATCGTTGGCTACGGCGCAACCTCTGATGGTGCAGACATGGTTGCTCCGTCTGGCGAAGGCGCAGTA
CGCTGCATGAAGATGGCGATGCATGGCGTTGATACCCCAATCGATTACCTGAACTCCCACGGTACTTCG
ACTCCGGTTGGCGACGTGAAAGAGCTGGCAGCTATCCGTGAAGTGTTCGGCGATAAGAGCCCGGCGAT

TTCTGCAACCAAAGCCATGACCGGTCACTCTCTGGGCGCTGCTGGCGTACAGGAAGCTATCTACTCTCT
GCTGATGCTGGAACACGGCTTTATCGCCCCGAGCATCAACATTGAAGAGCTGGACGAGCAGGCTGCGG
GTCTGAACATCGTGACCGAAACGACCGATCGCGAACTGACCACCGTTATGTCTAACAGCTTCGGCTTCG
GCGGCACCAACGCCACGCTGGTAATGCGCAAGCTGAAAGATTAAGTGCCATCTGTGCAGACAAACGCA
TCAGGATTTAAATTGTTTAAAGATTACGGATATTTAACTTACTTAGAATAATGCCATTTTTTTGAGTTATAA
TAATCCTACGTTAGTGTGAGCGGGATTTAAACTGTGAGGACCTTAATACATTCAGACACTTCTGCGGTAT
CACCCTACTTATTCCCTTCGAGATTATATCTAGGAACCCATCAGGTTGGTGGAAGATTACCCGTTCTAAG
ACTTTTCAGCTTCCTCTATTGATGTTACACCTGGACACCCCTTTTCTGGCATCCAGTTTTTAATCTTCAGT
GGCATGTGAGATTCTCCGAAATTAATTAAAGCAATCACACAATTCTCTCGGATACCACCTCGGTTGAAAC
TGACAGGTGGTTTGTTACGCATGCTAATGCAAAGGAGCCTATATACCTTTGGCTCGGCTGCTGTAACAG
GGAATATAAAGGGCAGCATAATTTAGGAGTTTAGTGAACTTGCAACATTTACTATTTTCCCTTCTTACGTA
AATATTTTTCTTTTTAATTCTAAATCAATCTTTTTCAATTTTTTGTTTGTATTCTTTTCTTGCTTAAATCTATA
ACTACAAAAAACACATACATAAACTAAAATTAATTA

8.5.7 pYPK0 plasmid – TPI1_*fabA*_PMP3

Variable zone:

TTAAATTGTTTAAAGATTACGGATATTTAACTTACTTAGAATAATGCCATTTTTTTGAGTTATAATAATCCTA
CGTTAGTGTGAGCGGGATTTAAACTGTGAGGACCTTAATACATTCAGACACTTCTGCGGTATCACCCTA
CTTATTCCCTTCGAGATTATATCTAGGAACCCATCAGGTTGGTGGAAGATTACCCGTTCTAAGACTTTTC
AGCTTCCTCTATTGATGTTACACCTGGACACCCCTTTTCTGGCATCCAGTTTTTAATCTTCAGTGGCATG
TGAGATTCTCCGAAATTAATTAAAGCAATCACACAATTCTCTCGGATACCACCTCGGTTGAAACTGACAG
GTGGTTTGTTACGCATGCTAATGCAAAGGAGCCTATATACCTTTGGCTCGGCTGCTGTAACAGGGAATAT
AAAGGGCAGCATAATTTAGGAGTTTAGTGAACTTGCAACATTTACTATTTTCCCTTCTTACGTAAATATTT
TTCTTTTTAATTCTAAATCAATCTTTTTCAATTTTTTGTTTGTATTCTTTTCTTGCTTAAATCTATAACTACA
AAAAACACATACATAAACTAAAATTAATTAGTCGAGGAACGCCAGGTTGCCCACTTTCTCACTAGTGACC
TGCAGCCGACAAATGGTAGATAAACGCGAATCCTATACAAAAGAAGACCTTCTTGCCTCTGGTCGCGGT
GAACTGTTTGGCGCTAAAGGCCCGCAATTGCCAGCACCGAACATGCTGATGATGGACCGTGTGGTCAA
AATGACCGAAACGGGTGGTAACTTCGACAAAGGGTATGTTGAAGCAGAACTGGATATCAATCCGGATCT
GTGGTTCTTCGGATGCCACTTTATTGGCGATCCGGTTATGCCGGGATGCCTGGGCCTGGACGCAATGT
GGCAGCTGGTAGGGTTCTACCTCGGCTGGCTGGGCGGCGAAGGTAAAGGCCGCGCGCTGGGCGTTGG
CGAAGTGAAATTCACTGGTCAGGTACTGCCGACAGCGAAAAAAGTGACCTACCGTATTCACTTTAAACG
CATTGTTAACCGTCGTCTGATTATGGGCCTGGCGGATGGCGAAGTGCTGGTTGATGGTCGTCTGATCTA

TACCGCCAGCGACCTGAAAGTCGGTCTGTTCCAGGATACGTCTGCCTTCTGAGTGCCATCTGTGCAGA
CAAACGCATCAGGATTTAAATAGATACTAAAAAGAAACCTTCCCGATGGTTTTTTTGGAGACTCTTCGATT
GTATCAATACTATCCCATGGACATCAGTTTTAATAGAGGAGCTCCAAATTCCCAAGGAATTGAGGAAGAA
GCGCCAATGATTTATATATACAAACTAGCTGTTGCAGCTTTATAGAGATTTAGTAATAAGAATTTTATGGG
AACGTGGTACCGTTATCGCAGTAAAGAGTCAAGGGTAGCGAAGATCAAACCATACGATGACGAAATACC
TTTAGTTCTTAGCATCACCAATCGCAGACATCCAACGTATCCGTGCGCGTAATCCTTCTCTTGGTAGTTG
AGCACAGCATACAGAAGAAGCCGCGCGCAAGCGGTAAATGTCTTTCCTCCGGCCTTCTAACCACCAAA
ACCGATCTCGGAACATGGGGGGGGGAAGGTCCTCTGAATCGAAAAACCCGAGACAGCGAGAGGGATTT
TGCAGAAAATTACAAAGATCACTATTTACTGCTCCCCTCACTTCCGCAGTCCCCTAATAGCGGAAGATG
CAATGGGTGTGGGCTCTGGGTGCCCTTTAACCACGCCCTCAAAAGGGGGTCCTGGTTATTTTGCGATG
GGCGCCTCTATAAATACAAAAGAGGAAGTGAGTGTTTTTGTTTTGGAAGAGGGAAAGGAAAAAAAGAAGA
AAATTTACTATCGGTTGTTGTTTTTCGCCAGTATAATACAATTGATTATACATTTTGAACTAAACAGCACA
GCACAATACAACATTAATTA

8.5.8 pYPK0 plasmid – PMP3_*fabZ*_ENO2

Variable zone:

TTAAATAGATACTAAAAAGAAACCTTCCCGATGGTTTTTTTGGAGACTCTTCGATTGTATCAATACTATCC
CATGGACATCAGTTTTAATAGAGGAGCTCCAAATTCCCAAGGAATTGAGGAAGAAGCGCCAATGATTTAT
ATATACAAACTAGCTGTTGCAGCTTTATAGAGATTTAGTAATAAGAATTTTATGGGAACGTGGTACCGTTA
TCGCAGTAAAGAGTCAAGGGTAGCGAAGATCAAACCATACGATGACGAAATACCTTTAGTTCTTAGCATC
ACCAATCGCAGACATCCAACGTATCCGTGCGCGTAATCCTTCTCTTGGTAGTTGAGCACAGCATACAGA
AGAAGCCGCGCGCAAGCGGTAAATGTCTTTCCTCCGGCCTTCTAACCACCAAAACCGATCTCGGAACA
TGGGGGGGGGAAGGTCCTCTGAATCGAAAAACCCGAGACAGCGAGAGGGATTTTGCAGAAAATTACAAA
GATCACTATTTACTGCTCCCCTCACTTCCGCAGTCCCCTAATAGCGGAAGATGCAATGGGTGTGGGCTC
TGGGTGCCCTTTAACCACGCCCTCAAAAGGGGGTCCTGGTTATTTTGCGATGGGCGCCTCTATAAATAC
AAAAGAGGAAGTGAGTGTTTTTGTTTTGGAAGAGGGAAAGGAAAAAAAGAAGAAAATTTACTATCGGTTG
TTGTTTTTCGCCAGTATAATACAATTGATTATACATTTTGAACTAAACAGCACAGCACAATACAACATTAA
TTAGTCGAGGAACGCCAGGTTGCCCACTTTCTCACTAGTGACCTGCAGCCGACAAATGACTACTAACAC
TCATACTCTGCAGATTGAAGAGATTTTAGAACTTCTGCCGCACCGTTTCCCGTTCTTACTGGTGGATCGC
GTGCTGGATTTTGAAGAAGGTCGTTTTCTGCGCGCAGTAAAAAATGTCTCTGTCAATGAGCCATTCTTCC
AGGGCCATTTCCCTGGAAAACCGATTTTCCCGGGTGTGCTGATTCTGGAAGCAATGGCACAGGCAACA
GGTATTCTGGCGTTTAAAAGCGTAGGAAAACTGGAACCGGGTGAGCTGTACTACTTCGCTGGTATTGAC

GAAGCGCGCTTCAAGCGCCCGGTCGTGCCTGGCGATCAAATGATCATGGAAGTCACTTTCGAAAAAAC
GCGCCGCGGCCTGACCCGTTTTAAAGGGGTTGCTCTGGTCGATGGTAAAGTAGTTTGCGAAGCAACGA
TGATGTGTGCTCGTAGCCGGGAGGCCTGAGTGCCATCTGTGCAGACAAACGCATCAGGATTTAAATACG
CGGCGTTATGTCACTAACGACGTGCACCAACTTGCGGAAAGTGGAATCCCGTTCCAAAACTGGCATCCA
CTAATTGATACATCTACACACCGCACGCCTTTTTTCTGAAGCCCACTTTCGTGGACTTTGCCATATGCAA
AATTCATGAAGTGTGATACCAAGTCAGCATACACCTCACTAGGGTAGTTTCTTTGGTTGTATTGATCATTT
GGTTCATCGTGGTTCATTAATTTTTTTTCTCCATTGCTTTCTGGCTTTGATCTTACTATCATTTGGATTTTT
GTCGAAGGTTGTAGAATTGTATGTGACAAGTGGCACCAAGCATATATAAAAAAAAAAAAGCATTATCTTCC
TACCAGAGTTGATTGTTAAAAACGTATTTATAGCAAACGCAATTGTAATTAATTCTTATTTTGTATCTTTTC
TTCCCTTGTCTCAATCTTTTATTTTTATTTTATTTTTCTTTTCTTAGTTTCTTTCATAACACCAAGCAACTAA
TACTATAACATACAATAATAATTAATTA

8.5.9 pYPK0 plasmid – ENO2_*mod1*_RPL5

Variable zone:

TTAAATACGCGGCGTTATGTCACTAACGACGTGCACCAACTTGCGGAAAGTGGAATCCCGTTCCAAAAC
TGGCATCCACTAATTGATACATCTACACACCGCACGCCTTTTTTCTGAAGCCCACTTTCGTGGACTTTGC
CATATGCAAAATTCATGAAGTGTGATACCAAGTCAGCATACACCTCACTAGGGTAGTTTCTTTGGTTGTA
TTGATCATTTGGTTCATCGTGGTTCATTAATTTTTTTTCTCCATTGCTTTCTGGCTTTGATCTTACTATCAT
TTGGATTTTTGTCGAAGGTTGTAGAATTGTATGTGACAAGTGGCACCAAGCATATATAAAAAAAAAAAGCA
TTATCTTCCTACCAGAGTTGATTGTTAAAAACGTATTTATAGCAAACGCAATTGTAATTAATTCTTATTTTG
TATCTTTTCTTCCCTTGTCTCAATCTTTTATTTTTATTTTATTTTTCTTTTCTTAGTTTCTTTCATAACACCA
AGCAACTAATACTATAACATACAATAATAATTAATTAGTCGAGGAACGCCAGGTTGCCCACTTTCTCACT
AGTGACCTGCAGCCGACAATGATGGCGGCTACAGCAGCTTCAAGCTTACAAATTGCTACACGAAGGCC
GAGCATGTCTTCGCCTAGCAAAATTCTTAAAGCAGGAACCTACATTGTTGGTGCCAATCCTGGAAACGC
TTCATGGGATAAACTTTCATGCACTCGCCAATTATCAAACCTTGGATGTTTGAGAAACCACAGTGCTGTT
CCAACTTGTAAGAGACCCTTTTCTTTTTCCACAAGGGCAATGTCTGAATCCAGTGAAAACAAGGCTCCTT
CAGGGCTTCCAATTGATTTGAGAGGGAAAAGAGCTTTCATTGCTGGTATAGCTGATGATAATGGCTATGG
TTGGGCCATAGCAAAATCTCTTGCTGCTGCTGGAGCTGAAATATTGGTTGGGACTTGGGTTCCTGCACT
TAATATATTTGAGACAAGCTTGAGACGTGGAAAATTCGACCAGTCACGAGTGTTGCCTGACGGGTCATTG
ATGGAGATTAAGAAGGTTTATGCTTTGGATGCTGTGTTTGACAATCCTGAAGATGTGCCTGAAGATGTGA
AAACGAATAAGCGATATGCAGGATCGTCAAACTGGACCGTACAGGAAGCTGCTGAATGTGTGAAAAAAG
ATTTTGGAAGCATTGACATTCTTGTCCATTCCCTTGCAAATGGCCCAGAGGTTAGCAAACCTCTTCTGGA

GACATCAAGGAAAGGCTATCTCGCTGCTATCTCTGCTTCGAGTTACTCCTTTGTTTCCTTGCTGAGGCAT
TTTCTGCCAATTATGAACCCAGGAGGTGCTTCAATATCTCTTACTTACATTGCCTCTGAAAGAATCATTC
CTGGGTATGGTGGAGGTATGAGTTCTGCCAAAGCTGCACTAGAGAGTGACACACGGGTGCTTGCATATG
AAGCTGGAAGGAAATCAAACATTAGGGTCAACACCATCTCTGCGGGTCCTTTGGGAAGCCGAGCAGCAA
AAGCCATTGGGTTCATAGACACCATGATTGAGTATTCCTACAATAATGGGCCTATTCAGAAAACACTGAC
CGCAGATGAAGTTGGGAATGCAGCAGCCTTCTTGGCATCTCCATTGGCCTCTGCCATAACCGGTGCAA
CCATATATGTGGACAATGGTTTGAATGCAATGGGCGTTGCACTGGACAGCCCGGTGTTCAAAGACCTTA
ACAGCAAGAATTAGGTGCCATCTGTGCAGACAAACGCATCAGGATTTAAATTTGTAAATACAAAGTCATT
CAATGTACATAAATACTGTTATGCTTTTGAATTAAGTAAAACACCATGACTTCATATTGAGATGTTTTAAG
CATATAATCACGGCACCGAACTTCTTGTAATCATCAAATCTTTGTTTTCTAGCCGTCATAGAAATGAAGAA
ATGAAGATTCTTGTTCATGTGATACAGCTTTTCTAGACAGCTAACTTGTACATCCAGACATCCTAAAACA
CCCATAAAGCAAAAAAAATTATTGAAATATTCTCTTTTTTTTCTTTTTTTTTTTTCTGGTGGCTTTCAATGC
GGAATCGCTCACATCCGAAAATTTCAGAGGCTTCACTTAGCGTCGCAGATCCCAGTAGTCGCCAGATGC
TTTGAAAAAAAAAAAAATAAATAGAAAAGTTTGTAGACATATCCGATGTAGTATATTTCAGAGGCCAACCT
TTACTTTTGTTTTCTCCTACACTTCAAAATAAAAGACACCGTCACAAATCTCAGCAACATTAATTA

8.5.10 pYPK0 plasmid – RPL5_*fatA1*_RPL16A

Variable zone:

TTAAATTTGTAAATACAAAGTCATTCAATGTACATAAATACTGTTATGCTTTTGAATTAAGTAAAACACCAT
GACTTCATATTGAGATGTTTTAAGCATATAATCACGGCACCGAACTTCTTGTAATCATCAAATCTTTGTTT
TCTAGCCGTCATAGAAATGAAGAAATGAAGATTCTTGTTCATGTGATACAGCTTTTCTAGACAGCTAACTT
GTACATCCAGACATCCTAAAACACCCATAAAGCAAAAAAAATTATTGAAATATTCTCTTTTTTTTCTTTTTT
TTTTTTCTGGTGGCTTTCAATGCGGAATCGCTCACATCCGAAAATTTCAGAGGCTTCACTTAGCGTCGCA
GATCCCAGTAGTCGCCAGATGCTTTGAAAAAAAAAAAAATAAATAGAAAAGTTTGTAGACATATCCGATG
TAGTATATTTCAGAGGCCAACCTTTACTTTTGTTTTCTCCTACACTTCAAAATAAAAGACACCGTCACAAA
TCTCAGCAACATTAATTAGTCGAGGAACGCCAGGTTGCCCACTTTCTCACTAGTGACCTGCAGCCGGAA
ATGTGTTCTCAGACGAAGAAGACAGGTTTGGTTCCTTTGCGTGCTGTTGTATCTGCTGATCAAGGAAGTG
TGGTTCAAGGTTTGGCTACTCTCGCGGATCAGCTCCGATTAGGTAGTTTGACTGAAGATGGTTTATCTTA
TAAAGAGAAGTTTGTTGTTAGATCTTACGAAGTGGGTAGTAACAAAACCGCTACTGTTGAAACCATTGCT
AATCTTTTACAGGAGGTGGGATGTAATCATGCACAAAGTGTTGGTTTTTCGACTGATGGGTTTGCAACAA
CAACTACTATGAGGAAGTTGCATCTCATTTGGGTTACTGCGAGAATGCATATCGAGATCTATAAGTACCC
TGCTTGGGGTGATGTGGTTGAGATAGAGACTTGGTGTCAGAGTGAAGGAAGGATTGGGACAAGGCGTGA

TTGGATTCTTAAGGATTCTGTCACTGGTGAAGTCACTGGCCGTGCTACAAGCAAGTGGGTGATGATGAA
CCAAGACACGAGACGGCTTCAGAAAGTTTCTGATGATGTTCGGGACGAGTACTTGGTCTTCTGTCCTCA
AGAACCGAGGTTAGCATTTCCGGAAGAGAATAACAGAAGCTTGAAGAAAATCCCGAAACTCGAAGATCC
GGCTCAGTATTCAATGATTGGGCTTAAGCCTAGACGAGCTGATCTCGACATGAACCAGCATGTCAATAA
TGTCACCTATATTGGATGGGTTCTCGAGAGCATACCACAAGAAATTGTAGACACGCACGAGCTTCAGGT
CATAACTCTGGATTATAGAAGAGAATGTCAACAAGACGATGTGGTGGATTCACTCACCACCACCACCTC
TGAAATTGGTGGAACCAATGGCTCTGCCACGTCTGGCACACAGGGCCACAACGATAGCCAGTTCTTGC
ACCTCCTGAGGTTGTCTGGAGATGGTCAGGAGATCAACCGCGGGACAACTCTGTGGAGAAAGAAGCCT
TCAAGTTAACCATCTGTGCAGACAAACGCATCAGGATTTAAATCTACATAAGTGCAATTAAAGCATCGGT
ACACCATGTAACACCAGTACATTTATTCATGAAACATTAAATTTTTCTGCGTCGGGCCGAGAGATTATTTT
CATGATACTTCGGCCTAGCTATGCTCCTACGCCCGCCAATGCAGAACAAATCCTGCCCGGGAAAACTA
CCCCGATGCTAGACTTCTCCCTCTGTATCCATGCCCGGCAACAGAGACCCTTGGCTAAGCCCGATGCG
CAATAGTACCTGTAGTGCACTCCCCGAGCTTATGCCTGAGAATATGCTATTGCAATGGGTCAATGTAGG
TACTGCAGGGAAATTCAATCTTGGGCTGCTGTGTAATAATAATTAAATTAGCAATTTAGTAATCACACTGT
ATAATTGCACGCTATCGTTATACATATTATTTCTCTTTGAAAACGGAAGGTGAAGAACAAATCGAGAAATT
AATTA

8.5.11 pYPK0 plasmid – RPL16A _*fatB*_RPL17A

Variable zone:

TTAAATCTACATAAGTGCAATTAAAGCATCGGTACACCATGTAACACCAGTACATTTATTCATGAAACATT
AAATTTTTCTGCGTCGGGCCGAGAGATTATTTTCATGATACTTCGGCCTAGCTATGCTCCTACGCCCGC
CAATGCAGAACAAATCCTGCCCGGGAAAACTACCCCGATGCTAGACTTCTCCCTCTGTATCCATGCCCG
GCAACAGAGACCCTTGGCTAAGCCCGATGCGCAATAGTACCTGTAGTGCACTCCCCGAGCTTATGCCT
GAGAATATGCTATTGCAATGGGTCAATGTAGGTACTGCAGGGAAATTCAATCTTGGGCTGCTGTGTAATA
ATAATTAAATTAGCAATTTAGTAATCACACTGTATAATTGCACGCTATCGTTATACATATTATTTCTCTTTG
AAAACGGAAGGTGAAGAACAAATCGAGAAATTAATTAGTCGAGGAACGCCAGGTTGCCCACTTTCTCAC
TAGTGACCTGCAGCCGGAAATGGCTCAGGCTCCACCTAAGATTAATGGGAAAAAGGTTGGTTTGCCTGG
TTCTGTAGATATTGTAAGGACTGATACCGAGACCTCATCACACCCTGCGCCGAGAACTTTCATCAACCA
GTTACCTGACTGGAGCATGCTTCTTGCTGCTATAACTACGATTTTCTTAGCGGCTGAGAAACAGTGGATG
ATGCTTGATTGGAAACCTAGGCGTTCTGACATGCTGGTGGATCCTTTTGGTATAGGGAGAATTGTTCAGG
ATGGCCTTGTGTTCCGTCAGAATTTTTCTATTAGGTCATATGAAATAGGTGCTGATCGCTCTGCATCTAT
AGAAACCGTCATGAATCATCTGCAGGAAACGGCGCTTAATCATGTTAAGACTGCTGGATTGCTTGGAGA

TGGGTTTGGCTCTACACCTGAGATGTTTAAGAAGAACTTGATATGGGTTGTCACTCGTATGCAGGTTGTG
GTTGATAAATATCCTACTTGGGGAGATGTTGTTGAAGTAGACACCTGGGTCAGTCAGTCTGGAAAGAATG
GTATGCGTCGTGATTGGCTAGTTCGGGACTGTAATACTGGAGAAACCTTAACACGAGCATCAAGTGTGT
GGGTGATGATGAATAAACTGACAAGGAGATTGTCAAAGATTCCTGAAGAGGTTCGAGGGGAAATAGAGC
CTTATTTTGTGAATTCTGATCCTGTCCTTGCCGAGGACAGCAGAAAGTTAACAAAAATTGATGACAAGAC
TGCTGACTATGTTCGATCTGGTCTCACTCCTCGATGGAGTGACCTAGATGTTAACCAGCATGTGAATAAT
GTAAAGTACATTGGGTGGATCCTGGAGAGTGCTCCAGTGGGAATAATGGAGAGGCAGAAGCTGAAAAGC
ATGACTCTGGAGTATCGGAGGGAATGCGGGAGAGACAGTGTGCTTCAGTCCCTCACTGCAGTTACGGG
TTGCGATATCGGTAACCTGGCAACAGCGGGGGATGTGGAATGTCAGCATTTGCTCCGACTCCAGGATG
GAGCGGAAGTGGTGAGAGGAAGAACAGAGTGGAGTAGTAAAACACCAACAACAACTTGGGGAACTGCA
CCGTAACCATCTGTGCAGACAAACGCATCAGGATTTAAATACAGAAGAGAGTGTGCGCAATATTTTAGTT
CTTAAATATGACATAGATTATCAAAAAACTTTGTATGTTTGCCAGATTTCAGTGAATCTGCAAGAGTATAA
ATTATAGGAATTTGGGTGAATTAAACCATTTATTATGTTTTATGTATCATGGAAAAAACAGAACCTTTATCA
AATCATCTATGAAATTTTGACAGGTTAGCCATTTTCCTCATTTTATTGCTCTGCAATAGTAAATTATATAGA
TTTATGCAATGCTCTAGGTTCCGGCGGCTTTTCATTTAATGTGAACTCAAGACTATTATCATCCAAACAC
ATTACAACATCCGTACATTTAGTTGGCTCCATTTTTTGAAAAAATACTTCCGAGTAAGATGCTGAAAGTAA
TCAAATTCCGTGAAAAAGGAACCGATTTGGTAAGGGCGGACCTGCCGGAAGAAAATCGCTCCCGCGTG
TCTACTGGACTTTGCTTCTTTGCCAGGCGGGAGAATGAAGCCCACCGGCCTAGCTCTATGGGCGCAGA
CAGGGATGGTCTCGATTTGCTTTCTCACTGCAATGGAAATTTCTAACATTAGAGCTTCAACGGCTAGCAT
AAAACAACTACTAACGTTTTCAGTGCTATATTCGAAAGAGCAAACAAAGAGTCCTCAAGAACTACTAATA
GATTAAATTAATTA

8.5.12 pYPK0 plasmid – RPL17A _*acpH*_RPL16B

Variable zone:

TTAAATACAGAAGAGAGTGTGCGCAATATTTTAGTTCTTAAATATGACATAGATTATCAAAAAACTTTGTAT
GTTTGCCAGATTTCAGTGAATCTGCAAGAGTATAAATTATAGGAATTTGGGTGAATTAAACCATTTATTAT
GTTTTATGTATCATGGAAAAAACAGAACCTTTATCAAATCATCTATGAAATTTTGACAGGTTAGCCATTTT
CCTCATTTTATTGCTCTGCAATAGTAAATTATATAGATTTATGCAATGCTCTAGGTTCCGGCGGCTTTTCA
TTTAATGTGAACTCAAGACTATTATCATCCAAACACATTACAACATCCGTACATTTAGTTGGCTCCATTTT
TTGAAAAAATACTTCCGAGTAAGATGCTGAAAGTAATCAAATTCCGTGAAAAAGGAACCGATTTGGTAAG
GGCGGACCTGCCGGAAGAAAATCGCTCCCGCGTGTCTACTGGACTTTGCTTCTTTGCCAGGCGGGAGA
ATGAAGCCCACCGGCCTAGCTCTATGGGCGCAGACAGGGATGGTCTCGATTTGCTTTCTCACTGCAATG

GAAATTTCTAACATTAGAGCTTCAACGGCTAGCATAAAACAACTACTAACGTTTTCAGTGCTATATTCGAA
AGAGCAAACAAAGAGTCCTCAAGAACTACTAATAGATTAAATTAATTAGTCGAGGAACGCCAGGTTGCCC
ACTTTCTCACTAGTGACCTGCAGCCGACAAATGAATTTTTTAGCTCACCTGCATTTAGCCCATCTCGCG
GAAAGCTCGCTTTCCGGCAATTTACTGGCTGATTTCGTACGCGGAAATCCTGAAGAAAGTTTTCCGCCC
GACGTCGTGGCTGGCATTCATATGCATCGACGTATCGACGTATTGACTGACAATCTGCCGGAAGTCCGC
GAAGCACGGGAGTGGTTTCGTAGTGAAACGCGCCGCGTTGCGCCTATTACGCTGGATGTCATGTGGGA
TCACTTTCTTTCCCGCCACTGGTCGCAGCTGTCGCCGGACTTTCCGCTACAGGAATTTGTCTGTTATGC
CCGCGAGCAAGTGATGACGATTTTGCCGGACTCACCGCCACGTTTTATCAATCTGAACAATTACTTGTG
GTCAGAGCAGTGGCTGGTGCGCTATCGCGATATGGATTTCATCCAGAACGTGTTAAACGGCATGGCAAG
CCGCCGCCCACGTCTGGATGCCCTGCGTGACTCCTGGTACGATTTAGACGCTCATTATGACGCCCTCG
AAACCCGCTTCTGGCAGTTTTATCCGCGGATGATGGCGCAGGCGTCACGCAAGGCGTTATAAGTGCCA
TCTGTGCAGACAAACGCATCAGGATTTAAATGACAAGAAACAAGGTGAATCACTAAGACTAAAGAAATGA
ATGAGAAATGGCTAAGCTGCAGCCAGTCATATGAGCTGCTTTCCTTTCATTCTATTGTCGTCCGAATCTA
TGTCACGAAGTACATATATAACTGTCTTTCATATATAATATTTAATCACCGTGCATTCCCTATTTTACTCTT
CCTCATGCGTTTGATCATCTGACCTTTATGATTTTGAACACCCCGCAATGTTCACTCTAATTTATGCAAC
TGAACTTTATTTTCGCTGCTGTGTTTGCATCCTTGACATATTCAATCCGTACACTATACACACCCAAACC
TCCTTTAAAAATTTCCTATGAATCAGAAAATTTGGCTGTTTCTCAACGCTTCGGCCAACGGGTGAAAAAC
AGCGGCAGAAGGACTTCCTTCGGAGAACGCTCCTAGAACCTGAAACTGCCCGCTAAAGCGTCTCCTGG
GCTGTCCCCAAGTGGGTGATTTTCCATTTGATCCTTGTATCAGCTTGGGAGGGTGGTGATCTCTTCTTAG
GCAGACACTTGGTCCCTCCGGCTAAACATTCGCACTGCGGACCGCTGCAATTTCGAATTCTGGAAAATT
ATTCTGATATTGGTAAAACTGATTCTTAAAACTATAACACATGTAGATATTCCACTAAGTGCATTTTGTTAA
GGTATTAAACAACCAACCAACCATAAAAGTTAATTA

8.5.13 pYPK0 plasmid – RPL16B _*acpS*_TMA19

Variable zone:

TTAAATGACAAGAAACAAGGTGAATCACTAAGACTAAAGAAATGAATGAGAAATGGCTAAGCTGCAGCCA
GTCATATGAGCTGCTTTCCTTTCATTCTATTGTCGTCCGAATCTATGTCACGAAGTACATATATAACTGTC
TTTCATATATAATATTTAATCACCGTGCATTCCCTATTTTACTCTTCCTCATGCGTTTGATCATCTGACCT
TTATGATTTTGAACACCCCGCAATGTTCACTCTAATTTATGCAACTGAACTTTATTTTCGCTGCTGTGTTT
GCATCCTTGACATATTCAATCCGTACACTATACACACCCAAACCTCCTTTAAAAATTTCCTATGAATCAG
AAAATTTGGCTGTTTCTCAACGCTTCGGCCAACGGGTGAAAAACAGCGGCAGAAGGACTTCCTTCGGAG
AACGCTCCTAGAACCTGAAACTGCCCGCTAAAGCGTCTCCTGGGCTGTCCCCAAGTGGGTGATTTTCC

ATTTGATCCTTGTATCAGCTTGGGAGGGTGGTGATCTCTTCTTAGGCAGACACTTGGTCCCTCCGGCTA
AACATTCGCACTGCGGACCGCTGCAATTTCGAATTCTGGAAAATTATTCTGATATTGGTAAAACTGATTC
TTAAAACTATAACACATGTAGATATTCCACTAAGTGCATTTTGTTAAGGTATTAAACAACCAACCAACCAT
AAAAGTTAATTAGTCGAGGAACGCCAGGTTGCCCACTTTCTCACTAGTGACCTGCAGCCGACAAATGGC
AATATTAGGTTTAGGCACGGATATTGTGGAGATCGCTCGCATCGAAGCGGTGATCGCCCGATCCGGTGA
TCGCCTGGCACGCCGCGTATTAAGCGATAACGAATGGGCTATCTGGAAAACGCACCACCAGCCGGTGC
GTTTTCTGGCGAAGCGTTTTGCTGTGAAAGAAGCCGCAGCAAAAGCGTTTGGCACCGGGATCCGCAATG
GTCTGGCGTTTAATCAATTTGAAGTATTCAATGATGAGCTCGGCAAACCACGGCTACGGCTATGGGGCG
AGGCATTAAAACTGGCGGAAAAGCTGGGCGTTGCAAATATGCATGTAACGCTGGCAGATGAGCGGCACT
ATGCTTGTGCCACGGTAATTATTGAAAGTTAAGTGCCATCTGTGCAGACAAACGCATCAGGATTTAAATA
AGAAAATCGAAACAAATAATAGATTTGTGTCTGTTCAATGTGTATATCGATGAGCTCAGTGAACACATTTT
CACATCACGTTTCTTTTTTTTCTTTTTTCCGCTAAGAAAAATTTTCCAGAATTTTTCAGAGGCAAAACAAAA
CTCATCGATTTCGCACGCATTTCTCGTAGAATTGATATTTTTTTATTTTTTTATTGGTTTTTTTTTTTTTTT
CACTTTTTTTTCTTCCTTTCATGATATTTATAAGGAGAAGTATTTCAATTGGGACTTAAAAAAAAATTTTAT
TTTGGTTAATGTTTTCATTCAATGGTTTACTACTTTCATTTTAGCTTTTTAACAATTTTCTTTCTGGGGTCT
CAGTTGCGGTTAGCAGATTAACACAGAACATACTATAGACAAATTTAATTA