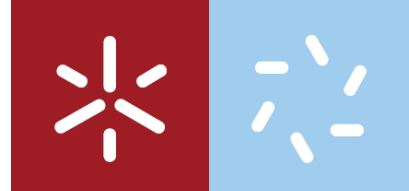




Júlio Filipe Antunes Freire

**Metabolic engineering for the
improvement of fatty acid synthesis in
industrial *Saccharomyces cerevisiae*
strains**

Universidade do Minho
Escola de Ciências





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of fatty acid synthesis in industrial
Saccharomyces cerevisiae strains**

Dissertação de Mestrado
Mestrado em Genética Molecular

Trabalho efetuado sob a orientação do:
Professor Doutor Björn Johansson

abril 2024

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AGRADECIMENTOS

Este trabalho não seria possível sem o apoio de um conjunto de pessoas que, de várias maneiras, contribuíram para a realização de mais uma etapa na minha vida. Deixo aqui os meus agradecimentos:

Ao professor Doutor Björn Johansson por me ter aceitado neste projeto de braços abertos, por toda a orientação que prestou ao longo do ano, por toda a profissionalidade e rigor científico que sempre demonstrou e a confiança depositada no meu trabalho.

Ao Paulo por ter desempenhado o papel de um orientador dentro e fora do laboratório. Toda a ajuda que disponibilizou durante a realização desta tese é inestimável.

Ao Humberto por toda a ajuda prestada dentro do laboratório e por todas as ideias e conselhos que foram providenciados de boa vontade.

Ao resto dos membros do LGM: Cláudia, Inês, Faezhe, Rosana, João e Vítor. Obrigado por contribuírem para o bom ambiente vivido dentro do laboratório e por toda a ajuda prestada ao longo do ano.

Aos membros do LBM por me aturarem sempre que ia fazer lá uma visita.

A todos os meus coleguinhas que sempre torceram por mim. Em especial aos meus colegas de casa Rui e Rúben que acompanharam de perto a realização desta tese. São todos igualmente especiais.

Ao resto dos meus amigos todos. Não apenas aqueles que conheci na universidade, mas também aos ansianenses que me viram chegar até à universidade e que eu não pude ver tantas vezes durante estes anos.

À minha namorada Ana Lope(s) por todo o apoio que me deu até chegar aqui. Não conseguiria ter acabado sem toda a motivação e ajuda. Estarei sempre grato.

Em último e principalmente aos meus pais e irmã que permitiram que tudo isto fosse possível. Tenho completa noção de todo o sacrifício feito para que eu pudesse ter chegado aqui. Não há palavras para descrever a minha gratidão.

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

RESUMO

A exploração da capacidade dos microrganismos na síntese industrial de uma ampla variedade de ácidos gordos emerge como uma estratégia promissora para impulsionar as indústrias biotecnológicas. Na natureza, existem dois mecanismos principais responsáveis pela produção de ácidos gordos: os sistemas de síntese de ácidos gordos do tipo I e do tipo II. *S. cerevisiae* é uma espécie um fungo unicelular com funções importantes em indústrias fermentativas e investigação em biologia. A robustez das suas estirpes industriais pode ser utilizada na produção de ácidos gordos. Contudo, o sistema nativo FAS de tipo I em *S. cerevisiae* representa um desafio para a produção de ácidos gordos não tipicamente produzidos em levedura. Por meio de recombinação homóloga, foi construído um vetor de expressão que contém um sistema FAS de tipo II e o marcador dominante *KanMX* em levedura. A natureza do marcador seletivo possibilitou a sua expressão em estirpes industriais de *S. cerevisiae*. No entanto, a expressão heteróloga desse plasmídeo em uma estirpe mutante $\Delta fas2$ de *S. cerevisiae* não complementou a produção de ácidos gordos e o seu crescimento em meio de cultura onde não foram adicionados ácidos gordos.

A Enoyl-ACP reductase é a enzima que catalisa a última etapa do ciclo de elongação no sistema de síntese de ácidos gordos. Embora esta reação enzimática seja conservada na natureza, a estrutura e a organização da enzima varia consideravelmente entre organismos. Este estudo avaliou o impacto da atividade de diferentes Enoyl-ACP redutases no crescimento de uma estirpe de levedura $\Delta fas2$ cuja produção de ácidos gordos estava dependente de um sistema heterólogo FAS tipo II. A preferência dessas enzimas pelo uso de NADP ou NADPH como cofatores não impactou a produção de ácidos gordos na levedura. A melhoria mais significativa no crescimento foi observada com a sobreexpressão do gene *MOD1* derivado da *Arabidopsis thaliana*. Ainda assim, a expressão adicional de uma enoyl-ACP redutase resultou em aumento do crescimento em todas as estirpes. Este passo na síntese de ácidos gordos em levedura é um potencial alvo para aumentar a produção desses compostos.

Palavras-chave: Ácido gordo; ácido gordo sintetase; estirpes industriais; enoyl-ACP redutase; recombinação homóloga

ABSTRACT

Exploiting the industrial-scale proficiency of microorganisms in the synthesis of a wide array of fatty acids stands as a promising strategy for advancing bio-based industries. There are two main mechanisms in which fatty acids are produced in nature: type I and type II fatty acid synthase systems. *S. cerevisiae* is a unicellular fungus that has important functions in fermentative industries and biological research. The robust nature of industrial *S. cerevisiae* strains is advantageous in the production of fatty acids. However, the nature of the native type I FAS system in *S. cerevisiae* is a major setback in the production of fatty acids that aren't normally produced in this organism. An expression vector containing a type II FAS system and the dominant *KanMX* marker was constructed in yeast by homologous recombination. The nature of the selective marker allowed for its expression in industrial *S. cerevisiae* strains. However, the heterologous expression of this plasmid in a $\Delta fas2$ mutant *S. cerevisiae* strain did not complement the production of fatty acids and its growth in media depleted of exogenous fatty acids.

The enoyl-ACP reductase is the enzyme that catalyses the last reaction of the elongation cycle in the fatty acid synthase system. This enzymatic reaction is conserved in nature, however, the structure and organization of the enzyme is unusually diverse among organisms. This study assessed the impact of the activity of different Enoyl-ACP reductases on the growth of a $\Delta fas2$ yeast strain that relied on a heterologous type II FAS system for the production of fatty acids. The preference of the Enoyl-ACP reductases for the use of either NADP or NADPH as the cofactor didn't seem to impact the production of fatty acids in yeast. The most substantial improvement in growth was observed with the overexpression of the *MOD1* gene derived from *Arabidopsis thaliana*. However, the expression of an additional Enoyl-ACP reductase resulted in increased growth across all strains. This step in the synthesis of fatty acids in yeast is a potential target for the upscaling of the production of fatty acids in yeast.

Keywords: Fatty acid; fatty acid synthase; industrial strains; Enoyl-ACP reductase; homologous recombination

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LIST OF ABBREVIATIONS

ACC-	acetyl-CoA carboxylase
ACP-	Acyl carrier protein
BLAST-	Basic local alignment sequence tool
DO-	Dropout mixture
ER-	Enoyl-ACP reductase
FA-	fatty acid
FAS-	Fatty acid synthase
G418-	geneticin
GRAS-	Generally regarded as safe
Hmy-	Myristic acid
LB-	Lisogeny Broth
LEU-	leucine
MCS-	Multi cloning site
OD-	Optical density
PCA-	Phenol-Chloroform-Isoamylalcohol
PCR-	Polymerase Chain Reaction
PEG-	Polyethylene glycol
PUFA-	Polyunsaturated fatty acids
SC-	Synthetic complete
SOC-	Super optimal broth with catabolite repression
TE-	Tris/EDTA
TP-	Terminator/Promoter
TU-	Transcription unit
URA-	Uracil
YNB-	Yeast Nitrogen Base
YPD-	Extract-peptone-dextrose

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1. INTRODUCTION

1.1. Microorganisms for a greener future, big problems sometimes require microscopical solutions

The increasing demand for basic human needs like food, clothes and medicine generates an immense amount of waste products and the increased employment of non-renewable sources of energy (Akinsemolu, 2018). Currently, anthropogenic activities are the leading cause of climate change (Abbass et al., 2022). As consequence, human health and food security are negatively impacted by the degradation and deterioration of the natural environment (Xin et al., 2022). However, climate change is a multidisciplinary problem that requires the combined efforts of several entities in order to effectively solve such a complex problem (Middleton, 2011). The mitigation of the environmental impacts caused by the industrial sector is a critical step in the prevention of climate change. In order to achieve optimal growth, industries consume natural resources and fossil fuels indiscriminately, making them the largest consumers of these raw materials worldwide (Xin et al., 2022). The uncontrolled use of natural resources, however, causes an increase in greenhouse gas emissions (Sovacool et al., 2021) and the loss of biodiversity (Seddon et al., 2016). Chemical industries are, particularly, responsible for the release of toxic chemicals and hazardous pollutants in the air and water, having a negative impact on the environment while causing adverse health problems to humans (Kätelhön et al., 2019). Chemical industries also consume large amounts of fossil fuels, mainly used for energy production and carbon feedstock (Lopez et al., 2023). Finally, these industries have the tendency to produce large amounts of waste products while utilising toxic reagents and highly reactive solvents. Still, a majority of chemical industries still focus most of their efforts in optimizing existent production processes, disregarding the change to greener alternative methods (Gunningham, 1995). In order to achieve environmental stability, there needs to be an increasing effort to gradually implement sustainable alternatives to common industrial practices that are harmful to the environment. However, a greener alternative has to accommodate the demand for a large variety of chemicals in a cost-effective manner (Walley & Whitehead, 1994). This makes the shift from traditional chemical processes to greener equivalents a time consuming and expensive

process. In recent times, one of the most popular alternatives is the use of microorganisms to replace traditional chemical processes and products.

Microorganisms have long impacted the lives of humans, plants and animals in one way or the other (N. Singh et al., 2018). Being present in the most diverse habitats, microorganisms have acquired extreme adaptations and capabilities that contribute to their success in survivability and also as potential tools for human applications (Niehaus et al., 1999). While the majority of microbes haven't been identified yet, there are some who have a long record of impacting human lives across history. While being essential in the production of fermentative drinks like wine and beer, microorganisms have also been responsible for all pandemics across human history, earning them a negative reputation (Rodríguez-Frías et al., 2021). This narrative started to change at the same time that more and more information was collected regarding their ecological role and their uses in everyday life (Cockrell, 2005).

Microorganisms such as fungi, microalgae and bacteria are currently being used as indispensable tools in industries ranging from biotechnology to agriculture. For example, microbial biofertilizers and biopesticides offer a solution to agricultural problems that used to heavily rely on chemical substances that are harmful to the environment (Seiber et al., 2014). In biotechnology and industrial production, microorganisms are employed in the synthesis of bio-based materials like biofuels, biopharmaceuticals and bioplastics – **Table1**. Actinomycetes, for example, are gram-positive bacteria that have long aided humanity in the discovery and production of all classes of antibiotics (De Simeis & Serra, 2021). Organic acids like citric acid or lactic acid are two economically important products which production mainly derives from microorganisms as well (Chen & Nielsen, 2016). Microbes are also employed in the production of industrial enzymes, food additives and numerous therapeutical agents. By utilizing renewable resources and general waste from other industries, the use of microbes contributes to a circular economy with low environmental impact.

Table 1 – Examples of products with high market value that are produced using engineered microorganisms.

Organism	Name	Applications	Reference
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<i>Saccharomyces cerevisiae</i>	Artemisinin	Anti-malarial drug	(Westfall et al., 2012)
<i>Taxomyces andreanae</i>	Taxadiene	Precursor of taxol – an anticancer agent	(Malcı et al., 2023)
<i>Aspergillus terreus</i>	Lovastatin	Cholesterol lowering agent	(Mulder et al., 2015)
<i>Streptomyces cinnamonensis</i>	Monensin	Antibiotic used in livestock farming	(Beckett et al., 1998)
<i>Propionibacterium shermanii</i>	Vitamin B12	Health supplements	(Piwowarek et al., 2018)
<i>Bacillus sp.</i>	Proteases	Detergents component	(Vojcic et al., 2015)
<i>Aspergillus niger</i>	Citric acid	Food additive and preservative	(Yang et al., 2017)
<i>Saccharomyces cerevisiae</i>	Bioethanol	Biofuel	(Buijs et al., 2013)
<i>Escherichia coli</i>	L-Threonine	Essential aminoacid	(Dong et al., 2011)

1.2. *Saccharomyces cerevisiae*, old but reliable

Saccharomyces cerevisiae, commonly known as baker's yeast, is a unicellular eukaryotic microorganism belonging to the fungi kingdom. Even though it was only identified in 1859, it has played an important role throughout human history, being present in the first forms of biotechnology, through the production of different beverages and in bread baking techniques (Sicard & Legras, 2011). In recent times, *Saccharomyces cerevisiae* has proven to be a multi-function tool as it still is widely used in fermentative industries but also as a model organism for biological research (Botstein et al., 1997). Its effectiveness in the fermentative industry is partially due to its unique cellular energy metabolism, mostly caused by the Crabtree effect (Nielsen, 2019). This strategy allows the yeast cells to grow under aerobic conditions while still producing ethanol and energy, through pyruvate. Since ethanol is toxic, yeast cells create an environment with very limited competition where they thrive due to its

remarkable robustness (De Deken, 1966). Additionally, *S. cerevisiae* has a high tolerance to sugar rich environments and can produce a high variety of aromatic compounds, making it a valuable asset in a wide range of fermentative industries (Suástegui & Shao, 2016). Besides the bread and alcoholic beverages industries, one of the most distinct applications of yeast fermentation is in the production of bioethanol from plant based raw materials (Rodionova et al., 2017). In this process, starchy raw materials like corn and sugar cane, serve as feedstock in the alcoholic fermentation that ultimately results in ethanol production. The produced bioethanol is mostly used as fuel, usually combined in a mixture with either gasoline or isobutylene (Bušić et al., 2018). As a result, bioethanol is by far the most used biofuel worldwide (Balat, 2011), providing a greener alternative to the use of fossil fuels. Apart from bioethanol, genetically modified yeast strains have the potential to also produce propanol and butanol, suitable for jets and rockets that require fuels with higher density. Moreover, *S. cerevisiae* has been employed in the production of other chemicals with high market potential. The production of organic acids like lactic acid (Lee et al., 2015), succinic acid (Xiberras et al., 2020) and 3-Hydroxypropionic acid (Ferreira et al., 2019) is already possible at an industrial scale by yeast. Yeast has also the potential to produce isoprenoids with high commercial value (Sun et al., 2014) at a larger scale. This group of secondary metabolites derive from isopentenyl diphosphate (IPP) and include carotenoids, sterols, terpenes, ubiquinone and polyprenyl alcohols. Their properties include their use as food colorant, aroma and flavour enhancers as well as anticarcinogenic agents (Chemler et al., 2006). With the growing understanding of yeast's cellular mechanisms and the advances in genetic engineering techniques, the range of products is limitless.

1.3. *Saccharomyces cerevisiae* as a model organism

Saccharomyces cerevisiae has had a remarkable role as a model organism in a wide variety of biological research fields. When it comes to maintenance, yeast cells are relatively easy to grow in cheap media, their duplication time is only about ninety minutes under optimal conditions and it is considered a “generally regarded as safe” (GRAS) organism. In addition, *S. cerevisiae* has been leading the way as a powerful genetic system (Hanson, 2018). In fact, it was the first eukaryotic organism to have its genome sequenced (Goffeau et al., 1996), leading

the way to the development of other important genetic tools. The successful transformation of yeast with a prokaryotic plasmid (Hinnen et al., 1978) paved the way for the creation of numerous vectors and selection markers (Vanderwaeren et al., 2022). One of the most advantageous attributes of yeast is its homologous recombination machinery. This self-repairing process allowed for the integration of DNA fragments in very specific locations of the yeast genome and lead the way to the manipulation of the yeast genome (Kevin R. et al., 1997). From then on, new techniques emerged and more data has been collected which made *S. cerevisiae* one of the best understood organisms in science. The ease of manipulation combined with the extensive array of technologies optimised in yeast further improved the use of yeast as a model organism in the study of human related diseases.

Because many key cellular processes are highly conserved between higher eukaryotes and yeast, the latter is often preferred as an alternative to the testing of novel therapeutic agents and the understanding of cellular mechanisms in animal cells (Lum et al., 2004). In addition, 30% of known genes involved in human diseases have orthologs in yeast (Foury, 1997). By using a collection of yeast deletion mutants, it has been possible to screen the effect of multiple drugs on a genome wide level. This method has helped with the identification of genes involved in specific cellular processes and also new targets for therapeutic treatments. The robustness and simplicity of *S. cerevisiae* has resulted in major advances in the understanding of complex human diseases like cancer, aging, neurological conditions, infectious diseases and many others (Matuo et al., 2012).

1.4. Industrial *S. cerevisiae* strains, well-oiled machines

Many fundamental studies prefer the use of tamed laboratory yeast strains, this is due to the fact that their growth has been optimised in standard conditions, they have been carefully sexually selected and are overall easier to handle (Steensels et al., 2014). These characteristics, however, don't hold up when faced with industrial bioreactor conditions, making these strains unsuitable for industrial use. Thus, the common practice is to genetically engineer industrial strains using applicable techniques and research conducted on laboratory strains. However, the applicability of such techniques is dependent on the strain genetic background. Most industrial strains possess a more complex genetic architecture when compared to laboratory

strains. For instance, laboratory strains are usually haploid while industrial strains can exist as polyploid, diploidy and aneuploid (Codón et al., 1998). Additionally, industrial strains are characterized by having poor sporulation efficiency, unstable mating types (Codón et al., 1995) and are prototrophic. Such characteristics limit the use of traditional auxotrophic markers which have an important role in genetic engineering. One way to counter this impediment is to use selection markers that are based around antibiotic resistance. The *KanMX* selective marker grants resistance to geneticin and has proven to work in industrial *S. cerevisiae* strains (M. E. Walker et al., 2003). This marker does not have a significant impact on the growth rate of industrial strains unlike some auxotrophic markers (Baganz et al., 1997), making it a suitable selection approach in industrial yeast strains.

1.5. Fatty acids

Fatty acids (FA), prevalent in all living beings, hold pivotal significance in numerous biological mechanisms. Chemically, they consist of a carboxylic acid linked to a hydrocarbon chain, varying in length and saturation – either saturated or unsaturated. Consequently, organisms harbour a diverse array of these acids, each performing distinct functions. These compounds act as primary energy stores and constitute a fundamental element within cell membranes. The composition of FA in membranes contributes to the compositional diversity in cell membranes by changing the physical properties of phospholipids that constitute most bilayer membranes (De Carvalho & Caramujo, 2018). Moreover, they undertake crucial roles as signalling molecules, integral to cellular membrane structure and function (He et al., 2020). FA are also economically important as they can be used as precursors of high value chemicals in oleochemical industries (Biermann et al., 2021). Thus, their synthesis constitutes an important process in which optimisation has high marker potential.

1.6. Fatty acid synthesis

Having an essential role in all organisms, the synthesis of fatty acids is, therefore, a highly regulated and complex mechanism in nature. Moreover, the pathway in which FAs are synthesised is highly conserved between all kingdoms of life (De Carvalho & Caramujo, 2018).

The synthesis requires a substantial amount of ATP, metabolites, acetyl-CoA and NADPH. The first step in the synthesis of fatty acids is the conversion of acetyl-CoA to malonyl-CoA by the acetyl-CoA carboxylase (ACC). Malonyl-CoA is converted to malonyl-ACP by transferring the malonate group to the prosthetic group of an acyl carrier protein (ACP). The elongation phase is characterized by the repeated condensation, reduction, dehydration and again reduction of malonyl-ACP that adds two carbons to the fatty acyl chain. The number of cycles in the elongation step determines the length of the resultant fatty acid. The final step involves a thioesterase that releases the newly synthesised FA from the FA synthase system.

In Nature, there are two main systems in which fatty acids are synthesised: type I and type II fatty acid synthases (FAS). Eukaryotes and advanced prokaryotes possess a type I synthase that is characterized by the activity of large heterodimeric enzyme complexes which carry out multiple enzymatic reactions. Meanwhile, Bacteria and mitochondria utilise a type II FAS system in which the necessary enzymatic reactions are carried out by a set of separated enzymes which, together, compose the metabolic pathway.

1.7. In *Saccharomyces cerevisiae*

Fatty acids are naturally produced in yeast as they carry out important functions as structural and signalling molecules (Roermund et al., 2003). Yeast mainly produces saturated and monounsaturated fatty acids with 16 or 18 carbons in length (Uemura, 2012). Though, being an extensively studied model organism, the utilisation of genetic engineering techniques has allowed the production of economically relevant fatty acids that are not naturally produced in yeast. For example, the production of polyunsaturated fatty acids (PUFAs) in yeast has been achieved by expressing exogenous genes that express the required desaturases (Beaudoin et al., 2000).

Just like in other eukaryotes, the synthesis of fatty acids in *S. cerevisiae* is carried out through a type I fatty acid synthase complex. This mechanism is made up of a hexamer with two non-identical multifunctional subunits, α and β (**Figure 1**), encoded by the *FAS2* and *FAS1* genes, respectively (Schweizer et al., 1978). Unless the medium is supplemented with an exogenous fatty acid, like myristic acid, *fas1* or *fas2* mutants are not viable. Additionally, when there are exogenous fatty acids in the medium, the *de novo* synthesis of fatty acids decreases

substantially in yeast. Like any other FAS system, the production of fatty acids in yeast is mainly dependent on the availability of the main carbon precursor, acetyl-CoA, the general activity of the responsible enzymes, energy in the form of ATP and NAD(P)H (Sheng and Feng, 2014). The first limiting step in the fatty acid pathway is the activity of Acc1, a carboxylase that converts acetyl-CoA to malonyl-CoA. Several studies have been done around the improvement of the activity of this enzyme (Chen et al., 2018) or its overexpression (Choi et al., 2014) which translated in effectively increasing fatty acid production in yeast. There are several other challenges that need to be surpassed in order to produce fatty acids at an industrial level. The availability of cytosolic acetyl-CoA, the main building block of fatty acids, is conditioned by the fact that this molecule is also the precursor of other major metabolic pathways. The suppression of key enzymes that enhance the carbon flux to the production of acetyl-CoA has shown an increase in the fatty acid accumulation in yeast (Schadeweg & Boles, 2016). One of the major setbacks of the *de novo* synthesis of FAs is the overall molecular complex in the cytosol. From a metabolic engineering standpoint, because the native type I FAS system is highly regulated and its reactions are catalysed in only two separate domains (Leibundgut et al., 2008), it is harder to optimise and diversify the production of FAs in *S. cerevisiae*.

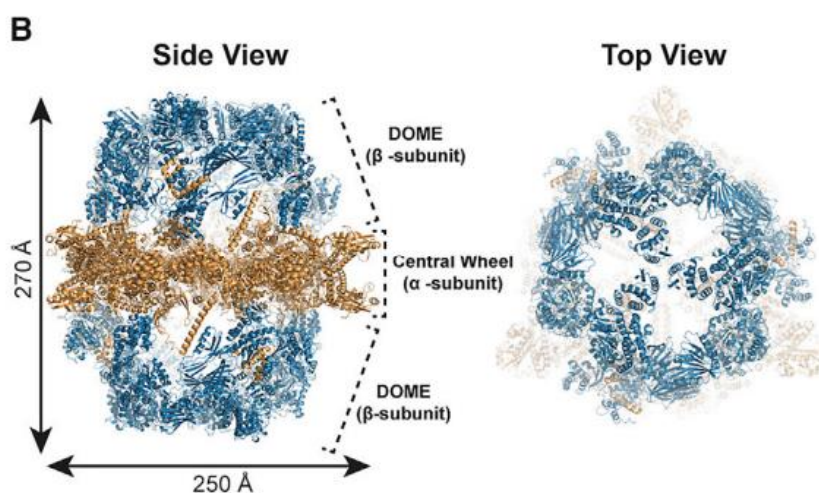


Figure 1 – Overall structure of the D-3 symmetric yeast FAS. Six α-subunits (ochre) form a central wheel, three β-subunits (blue) each form the two domes of the barrel-like structure. The 2-fold axis dissects the central wheel perpendicularly, whereas the 3-fold axis runs down the length of the molecule, where the β-subunits join. Adapted from K. Singh et al., 2020.

1.8. Heterologous FAS type II system in *S. cerevisiae*

One of the most promising strategies to overcome the setback caused by the structural nature of the native FAS system is the expression of a heterologous FAS type II system. The expression of a type II FAS system allows for a greater level of manipulation and the possible outcome of a larger variety of fatty acids that wouldn't normally be synthesised. The changes in chain length and level of unsaturation of fatty acids result in unique properties that can be economically relevant. Expressing a FAS type II system in yeast was first achieved by (Fernandez-Moya et al., 2015), through the insertion of eight *E. coli* genes in the genome of a yeast strain that lacked the *FAS2* gene. This was the first time a heterologous FAS II type system complemented the production of fatty acids in yeast. More recently, the same feat was accomplished, but this time, all the required genes were expressed in an heterologous expression plasmid containing *E. coli* and *Arabidopsis thaliana* genes (Pozdniakova et al., 2023). The use of an expression vector instead of the insertion in the yeast genome allows for an easier manipulation of each gene, which lays the foundation for the specialized production of desired fatty acids. This plasmid goes by the name of pTA1_FASIIb and is made up of nine genes from *E. coli* and three genes from *Arabidopsis thaliana* – **Table 2**. The assembly was done through the Yeast Pathway Kit protocol (Pereira et al., 2016) which uses intergenic elements that serve as both as a promoter and a terminator (TP). This allows for the homologous recombination of cassettes in which a terminator serves as the promoter of the consecutive gene.

Table 2 – All the genes that make up the pTA1_FASIIb plasmid, along with origin, function and respective promoters and terminators.

Source	Name	Promoter / Terminator	Function
<i>E. coli</i>	EcfabH	PDC1 / TF1	β -ketoacyl-ACP synthase III
<i>E. coli</i>	EcfabD	TF1 / FBA1	ACP S-malonyl transferase
<i>E. coli</i>	EcfabG	FBA1 / RPL22A	3-oxoacyl- ACP reductase

<i>E. coli</i>	EcacpP	RPL22A / pTDH3	Acyl carrier protein
<i>E. coli</i>	EcfabF	pTDH3 / UTR2	β -ketoacyl-ACP synthase II
<i>E. coli</i>	EcfabB	UTR2 / PTI1	3-oxoacyl- ACP synthase I
<i>E. coli</i>	EcfabA	PTI1 / PMP3	3-Hidroxyacyl ACP dehydrogenase
<i>E. coli</i>	EcfabZ	PMP3 / ENO2	3-Hidroxyacyl ACP dehydrogenase
<i>A. thaliana</i>	Athmod1	ENO2 / RPL5	Enoyl-ACP reductase
<i>A. thaliana</i>	Athfat1	RPL5 / RPL16A	Thioesterase
<i>A. thaliana</i>	Athfatb	RPL16A / RPL17A	Thioesterase
<i>E. coli</i>	EcacpS	RPL17A / TMA19	Holo-acp synthase

1.9. Enoyl-ACP reductases

The enoyl-acyl carrier protein reductase (ER) is the enzyme involved in the last step of the elongation cycle in the fatty acid synthesis. This enzyme catalyses the reduction of the *trans*-2-acyl-ACP to the fully saturated acyl-ACP species. Despite being conserved among all organisms, the enzyme required for this reaction varies in structure and action between species. Both NADH and NADPH can be used as the primary reductant in this reaction. However, some ERs have an higher affinity to one of these cofactors whereas other ERs don't have a preference for neither NADH or NADPH (Massengo-Tiassé & Cronan, 2009). The ER used in the pTA1_FASIIb plasmid, Athmod1, comes from the plant *Arabidopsis thaliana* and can use both NADPH and NADH as cofactors. Though, it has been observed that this reductase highly favours the NADH dependent activity (de Boer et al., 1998). Because *S. cerevisiae* has a higher availability of NADPH in the cytosol when compared to NADH (Zhang et al., 2015), the activity of this enzyme can be a limiting factor in the process of fatty acid synthesis. This aspect alone can have a bottleneck effect in the production of fatty acids by the pTA1_FASIIb plasmid. Thus, there's the possibility of improving the total fatty acid production by using an ER that is in line with the cofactor abundance. The ER responsible for this reaction in *Bacillus subtilis*, BsfabL, has a higher affinity for NADPH (Heath, Su, et al., 2000). The same happens with the ER in

Streptococcus aureus (Heath, Li, et al., 2000). The substitution of the *MOD1* gene with the *B. subtilis* *BsfabL* gene or the *S. aureus* *SafabI* gene can lead to an increase in FA production in yeast.

1.10. Objectives

The primary goal of this study was to develop a heterologous type II FAS system within a vector incorporating the dominant genetic marker *KanMX*, enabling its expression in industrial strains of *Saccharomyces cerevisiae*. The *KanMX* marker allows for the selection of yeast on media containing geneticin and the selection of *E. coli* in media supplemented with kanamycin. To achieve this, we switched the *LEU2* auxotrophic marker with the *KanMX* in a vector that expressed a previously described type II FAS system. This substitution was carried out by homologous recombination in a $\Delta fas2$ mutant *S. cerevisiae* strain. By using the Cre-loxP deletion system, we created a $\Delta fas1 \Delta fas2$ double mutant yeast strain. The *KanMX* gene was used as part of the deletion cassette and was later recycled from the yeast's genome. The main purpose was to have a *S. cerevisiae* strain that could be selected in media containing geneticin and by its growth in media depleted of exogenous fatty acids.

The other objective of this work was to evaluate the effect of the expression of different Enoyl-ACP reductases in the growth of a $\Delta fas2$ yeast strain containing a vector that expressed a heterologous type II FAS system. The *FABL* gene from *Bacillus subtilis*, the *FABI* gene from *Streptococcus aureus* and the *MOD1* gene from *Arabidopsis thaliana* were expressed in single gene expression vectors assembled using the Yeast Pathway Kit system. Several growth curves were performed and used to calculate the specific growth rate of the different yeast strains.

2. MATERIALS AND METHODS

2.1. Yeast strains and growth conditions

S. cerevisiae strains (**Table 4**) were grown on Yeast extract-peptone-dextrose (YPD) rich media containing 1 % (w/v) yeast extract (PanReac AppliChem®), 2 % (w/v) bacto-peptone (BD Bioscience®) and 2 % (w/v) glucose. Yeast strains that lacked the ability to synthesize fatty acids, were cultivated in YPD medium supplemented with 0.46 g/L of myristic acid (Hmy; Sigma®) and 0.1 % (v/v) of Tween 40 (Polysorbate 40; Sigma®). Depending on the auxotrophic selective marker, yeast strains were also grown in synthetic complete media (SC) made up of 0.67 % (w/v) yeast nitrogen base (YNB; Grisp®) without amino acids, 0.14 % (w/v) amino acid dropout mixture (DO; **Appendix -7.1**) and 2 % of glucose (w/v). The histidine, leucine, tryptophan and uracil amino acids were omitted from the mixture according to the respective auxotrophy – **Table 3**. If yeast strains contained the *KanMX* dominant marker, 0.2 g/L of geneticin (G418) was added to the respective media.

The *E.coli* XL1-Blue strain (Stratagene) was routinely used to prepare and store plasmids. This strain was grown in Lisogeny-Broth-Miller (LB) medium containing 0.5 % (w/v) yeast extract, 1 % (w/v) tryptone, 0.5 % (w/v) sodium chloride and also 100 mg/L of ampicillin.

For solid media, 2 % (w/v) agar was added. Yeast liquid cultures were incubated at 37 °C in a shaking incubator at the speed of 200 rpm.

Table 3 – Composition of the Synthetic complete (SC) media used in this work. The addition of Tryptophan, Uracil, Histidine and Leucine was according to the auxotrophy of the yeast strain to be

grown. Reagent	Concentration (g/L)
Glucose	20
Yeast Nitrogen Base(YNB) w/o amino acids	1.70
Ammonium sulphate	5
DO	1

Tryptophan; Uracil; Histidine	0.08
Leucine	0.4
Agar	20

Table 4 – *S. cerevisiae* strains used in this work.

No.	Strain	Features	Reference
-	CEN.PK-21C	<i>MATa ura3-52 his3-Δ1 leu2-3,112 trp1-289 MAL2-8c SUC2</i>	Entian and Kötter (2007)
u1421	CEN.PK-21C	<i>Δfas2:KanMX + pTA1_FASIIb</i>	Pozdniakova et al. (2023)
u1483	CEN.PK-21C	<i>Δfas2:KanMX</i>	Pozdniakova et al. (2023)
u1767	CEN.PK-21C	u1483 with no <i>KanMX</i>	This work

2.2. *S. cerevisiae* transformation using the lithium acetate method

A pre culture containing the intended *S. cerevisiae* strain was grown overnight in the respective media and was then transformed using the lithium acetate method (Gietz & Woods, 2002). The *S. cerevisiae* cells were then grown starting at an Optical Density (OD_{600nm}) of 0.17 until they reached an OD₆₀₀ of around 0.8. The remaining culture was centrifuged for 5min at 5000 rpm and then washed with the same volume of sterile water. The pellet was then transferred to a 1.5 mL tube and resuspended with 1 mL of sterile water. For each transformation, 100 µL of the previous suspension was then centrifuged for 30s at max speed and the supernatant was discarded. To the remaining pellet, it was added 300 µL of a transformation mix containing polyethylene glycol (PEG) 50 % w/v, lithium acetate 1 M and single stranded carrier DNA (ss-DNA) (previously boiled), the plasmid DNA to be transformed and sterile water to make up a total volume of 360 µL. The cell suspension was then vortexed and incubated at 42 °C for 40min. The cell suspension was then centrifuged for a short period of time and the transformation mix was removed. The cells were resuspended in sterile water and plated on selective media. When using antibiotics for selections, the cells were previously incubated at 30 °C for 4h and only then plated in their selective media.

2.3. *E. coli* transformation by the heat shock method

The *E. coli* transformation was performed using the heat shock method, using calcium frozen competent XL-1Blue *E. coli* cells. Each tube, containing the competent cells, was defrosted in ice for about 5 min and then aliquoted in different tubes containing 20 µL of cells. The DNA was then added to each tube and incubated in ice for 30 min. The cells were then heat shocked in a water bath at 42 °C for exactly 45 s and immediately after, incubated in ice for 2 min. Afterwards, it was added Super optimal broth with catabolite repression (SOC) media to 1 mL total volume and the cells recovered at 37 °C for about an hour. The cell suspension was then centrifuged for 30 s at max speed and the supernatant was removed so that roughly 200 µL remained on the tube. The cells were then plated in selective media and grown overnight at 37 °C.

2.4. Standard PCR conditions and Colony PCR

During our work, several Polymerase Chain Reactions (PCR) were performed in order to amplify specific DNA sequences which then served different purposes. These reactions were carried out using a standard reaction mix. The final reaction mix included a mastermix twice concentrated which contained homemade Taq polymerase, Taq buffer (1 %), MgCl₂ (50 mM), dNTPs (10 mM) and topped up with ultrapure water. The rest of the reaction included both primers (0,5 µM each), the DNA (10 % v/v, 1-30 ng) and topped up with ultrapure H₂O. The PCR conditions were predicted by software and, depending on the characteristics of our work, some conditions were slightly altered in order to optimise each reaction . When unspecific bands were detected, the annealing temperature was increased. In the absence of bands, the annealing temperature was often increased. The extension time was also adjusted to the length of the resulting DNA product.

The PCR products were finally analysed by agarose (1 %, TAE 1x) gel electrophoresis, performed at 5-7 V/cm. For DNA staining, a 1x loading buffer (2 % FICOLL® 400, 0.0025 % Xylene Cyanol FF®, 0.3 % VAHINE® Yellow, 0.024 % Orange G®) was added to each PCR product. In each gel, 2 µL of a molecular weight marker (Gene Ruler 1 kb Thermo Scientific) was added. The

electrophoresis was then run at 200 V for 25 minutes and the gels were finally visualized in the Geno Smart UV transilluminator.

After the transformation of a specific bacterial or yeast strain, a colony PCR was performed, which allowed us to do the screening of the resultant transformant colonies. Instead of using purified DNA in the PCR mix, we used a small portion of cells in the PCR tubes. The only difference of the colony PCR relatively to the conventional PCR was that the initial denaturation step was extended to 15 min instead of 5 min at 95 °C.

2.5. Plasmid DNA purification and rescue from *S. cerevisiae* transformant colonies

Plasmid DNA was purified from *E. coli* overnight cultures, using the NZYTech Miniprep protocol. To perform a plasmid rescue from yeast transformant colonies, we started by collecting the cells from an overnight culture. Then, in a 2 mL Eppendorf tube, we added 200 µL of glass beads (0,5 mm) along with the recommended amount of resuspension solution in the NZYTech Miniprep kit. The cells were then fast prepped (Vortex-Genie 2) for 2 min and collected through centrifugation. We then followed the rest of the plasmid purification protocol according to the manufacturer's manual. The purified DNA was then used in the transformation of competent *E. coli* cells. Finally, these cells were cultivated and the plasmid DNA was purified.

2.6. Genomic DNA extraction

The genomic DNA extractions performed used the phenol-chloroform-isoamylalcohol (25:24:1 (v/v)) (PCA) method. We started by growing the intended strain in a petri dish with the respective media for 24-28 h. Afterwards, we added about half of the biomass in the dish to a 2 mL tube. We then added 200 µL of PCA, 300 µL of 10x concentrated Tris-EDTA (TE) and 200 µL glass beads (0.5 mm). The tube was fast-prepped (Vortex-Genie 2) for 2-3 min and then centrifuged for 10 min at 14000 rpm. We then collected the supernatant from the tube and added it another tube. In that tube, we added 50 µL of potassium acetate and 200 µL of Isopropanol and let it incubate at -20 °C for 20 min. The tube was then centrifuged for 10 min

at max speed and the supernatant was carefully removed so that only the precipitated DNA remained. It was added 200 μ L of 70 % ethanol to the tubes and then vortexed for 5 s. The tubes were then centrifuged for 2 min at max speed, the supernatant was meticulously removed and the tubes were left to dry at room temperature for 2-6 h. We finally added 50 μ L of TE 1x.

2.7. Growth Assays

In order to save time and preserve the repeatability of the different growth curves, we first prepared ten pre inoculum tubes of each strain and kept them at -80 °C. The four strains were first cultivated in 5 mL of synthetic complete liquid media lacking uracil and leucine and containing myristic acid and Tween 40 (SC-LU + Hmy), until the cultures reached the stationary phase. From the pre inoculum tubes, the cells were diluted in a new erlenmeyer flask with 20 mL of SC-LU + HMY media, so that the initial OD₆₀₀ was 0.2. These cultures were then grown at 30 °C until they reached an OD₆₀₀ of 1.5. These cultures were then washed twice with 10 mL of ultrapure water. 500 μ L of each cell suspension was transferred to ten different 1,5 mL tubes containing glycerol to a final concentration of 25 % v/v. These pre-inoculum tubes had an optical density of 1.5, so that when they were poured in 25 mL of media, the initial optical density of this culture was 0.07. The growth curves started by pouring one of each pre-inoculum tubes into an Erlenmeyer flask with 25 mL of SC-URA + Hmy media. Immediately after, the optical density of each culture was measured at 600 nm. After 10 hours of growth, the optical density was once again measured in intervals of 1 hour across a span of 10 hours of growth. The two final measurements were done at the 22 h and 24 h timepoints. The aim was to measure the growth rate of all cultures during their exponential phase. The optical density of the growing cultures has a direct correlation with their density in the medium and was used to calculate the specific growth rate of each culture.

3. RESULTS

3.1. Construction of the pTA5 plasmid

This work required the construction of an expression vector with the *KanMX* dominant marker that could be used in industrial *S. cerevisiae* strains. This plasmid, named pTA5, was constructed through the assembly of five different DNA fragments, each serving a functional purpose. The required DNA fragments were obtained by PCR (**Table 5**). Each primer carried a 30 bp long tail that allowed for the homologous recombination between adjacent fragments (**Figure 2**). The pBR *E. coli* origin of replication was amplified from the pBR322 vector and retained the ROP gene which limits the number of copies in *E. coli*. The yeast 2 μ origin of replication was amplified from the YEplac181 vector. The bacterial selective marker, amp, was amplified from the pUG35 CYC1-GFP vector. A deletion allele of the *E. coli* CRP gene was amplified from the pTA1 vector. The dominant selection marker in yeast, *KanMX4*, was amplified from the pFA6a-GFPS65T-*KanMX6* vector.

Table 5 – DNA segments used in the construction of the pTA5 plasmid and the respective PCR conditions for their amplification by PCR.

Source	Target feature	Forward primer	Reverse primer	Annealing temperature	Extension time (min)	Size (bp)
YEplac181	2 μ	984	983	53°C	1:13	1644
pFA6a-GFPS65T- <i>KanMX6</i>	<i>KanMX</i>	1350	1349	56°C	1:03	1417
pBR322	pBR ori	1196	1195	57°C	1:07	1497
pUG35 CYC1-GFP	amp	1113	987	55°C	0:48	1072
pTA1	Δ CRP	978	977	65°C	0:30	445

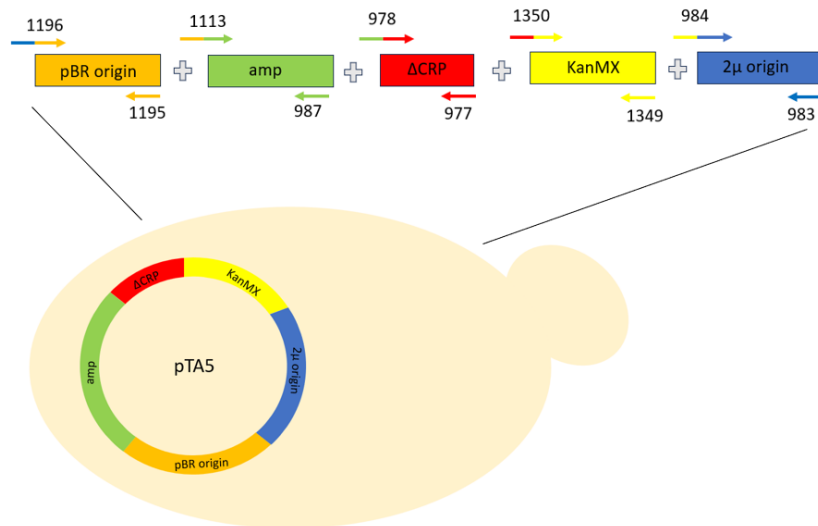


Figure 2 – Schematic representation of the construction of the pTA5 plasmid by homologous recombination. This plasmid is made up of five DNA fragments: pBR322 bacterial origin of replication; amp selective marker; Δ CRP multi cloning site; *KanMX* selective marker; 2 μ yeast origin of replication. All fragments are amplified by PCR, using primers that carry a tail that allows for the recombination between all PCR products (pictured in respective colours). The simultaneous transformation of all PCR products in yeast leads to the coordinated assembly of the final plasmid.

The PCR products were analysed by gel electrophoresis. All resulting PCR products appeared to have the predicted size in the gel (**Figure 3**). The comparison of the different bands in the gel helped confirm the amplification of the PCR products that shared a similar size. This confirmation was a powerful indicative of the success of the amplification.

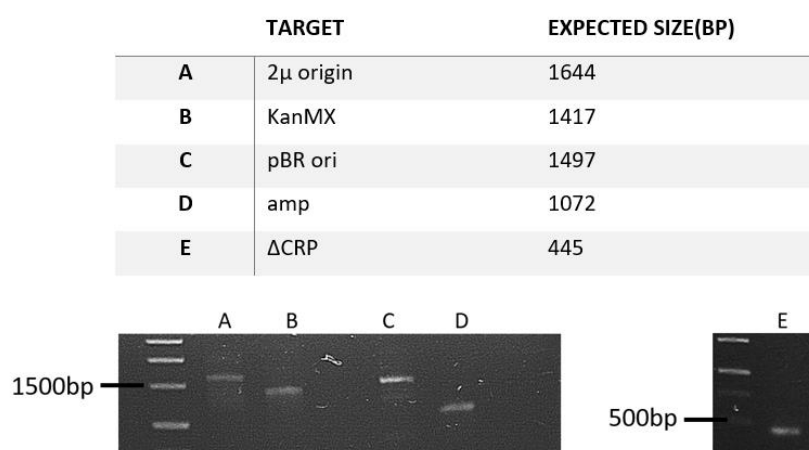


Figure 3– Agarose gel of the DNA fragments resulting from the five different PCR reactions used to obtain the necessary fragments for the construction of the pTA5 plasmid. The different fragments are represented from the letter A to E and their expected size is displayed in the table above. The molecular weight marker used was the GeneRuler 1kb DNA ladder (Thermo Scientific).

The plasmid was assembled by transforming the yeast strain CEN.PK2-1C with 10 μ L of each PCR product and plating the cells in YPD + G418 selective media. Simultaneously, the same cells were transformed in other conditions to serve as controls. These included the transformations in which the cells were transformed with only the *KanMX* PCR product and with all the PCR products but the Δ CRP fragment (**Figure 4**). The results showed that both the cells transformed with all PCR products and the cells transformed with four of the products were able to grow in media with geneticin. In theory, the transformation with four of the five fragments should not result in cell growth due to the lack of homologous regions between two of the fragments. However, the cells have found a way to reconstruct a truncated plasmid containing the KanMX selection marker, but with less efficiency than the transformation with the five fragments. This was observed more clearly when the remaining transformed cells stored at 4 $^{\circ}$ C were diluted 100x and plated again in selective media. The culture transformed with the five fragments resulted in substantially more transformants than the cultures transformed with only four.

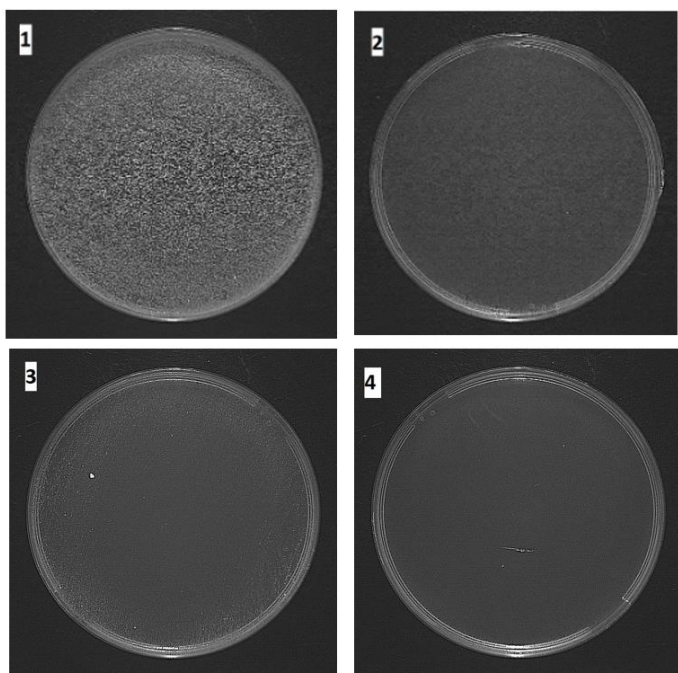


Figure 4 – Resulting colonies from the transformation of the CEN.PK2-1C yeast strain with the five PCR fragments that compose the pTA5 plasmid, in YPD + G418 media. (1) Transformation with five PCR products. (2) Transformation with all fragments but the Δ CRP PCR products. (3) Transformation with the *KanMX* PCR product. (4) Negative control (only water)

The construction of the plasmid was first confirmed through Colony PCR in which a set of primers was strategically used so that one primer annealed in the middle of the Δ CRP fragment, because of its importance as a Multi Cloning Site (MCS), and the other primer annealed in the middle of the adjacent *KanMX* gene (**Figure 5**). The screening by Colony PCR allowed us to identify the colonies that had the right plasmid orientation by using primers that hybridized in two adjacent DNA fragments.

DNA	Forward primer	Reverse primer	Annealing temperature(°C)	Extension time(min)	Product size(bp)
pTA5	977	1481	59	1:00	1345



Figure 5– Colony PCR conditions and representation to test the pTA5 construction. The pair of primers used were the 977 (forward) and 1481 (reverse) primers. The 977 primer annealed inside the Δ CRP fragment while the 1481 primer annealed in the adjacent *KanMX* gene. The PCR product was 1345bp long.

The results displayed in the gel showed that more than half of the colonies had the right plasmid orientation out of 21 tested colonies (**Figure 6**). The 13,17 and 18 colonies were picked based on the intensity of the respective band and had their plasmid rescued.

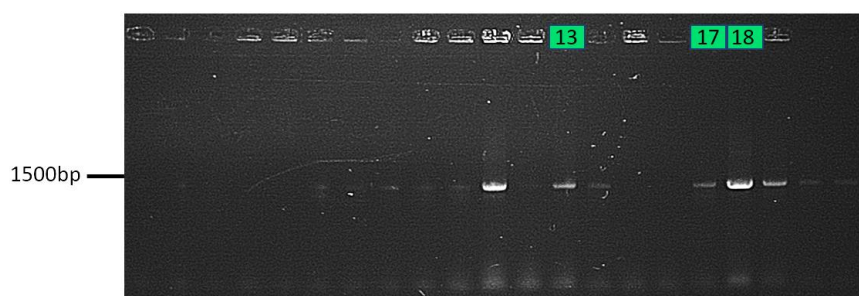


Figure 6 – Agarose gel displaying the DNA fragments that resulted from the colony PCR performed in the colonies transformed with all five fragments that form the pTA5 plasmid. The PCR products from the 13,17 and 18 colonies are marked on the gel.

The plasmid constructions were then tested by enzymatic digestion, PCR and finally sequenced (**Figure 7**). The plasmids were first digested with the *Bgl*I, *Eco*321 and *Scal* restriction enzymes. A series of digestions were carried out: only the *Bgl*I enzyme, only the *Eco*321 enzyme, both *Bgl*I and *Eco*321 enzymes and only the *Scal* enzyme. The digestions were analysed in gel electrophoresis and compared to an *in silico* prediction made in Benchling®.

Upon confirming the digestions, several PCR reactions were carried out to confirm the construction. Three pairs of primers were used so that each pair flanked a determined gene. Finally, the ΔCRP fragment was sequenced as it was crucial to know if there were any mutations in the cloning site of the plasmid. Mutations in the multi cloning site can lead to errors in the insertion of DNA sequences. Two primers flanking the ΔCRP fragment were used and the results were matched to the nucleotide sequence.

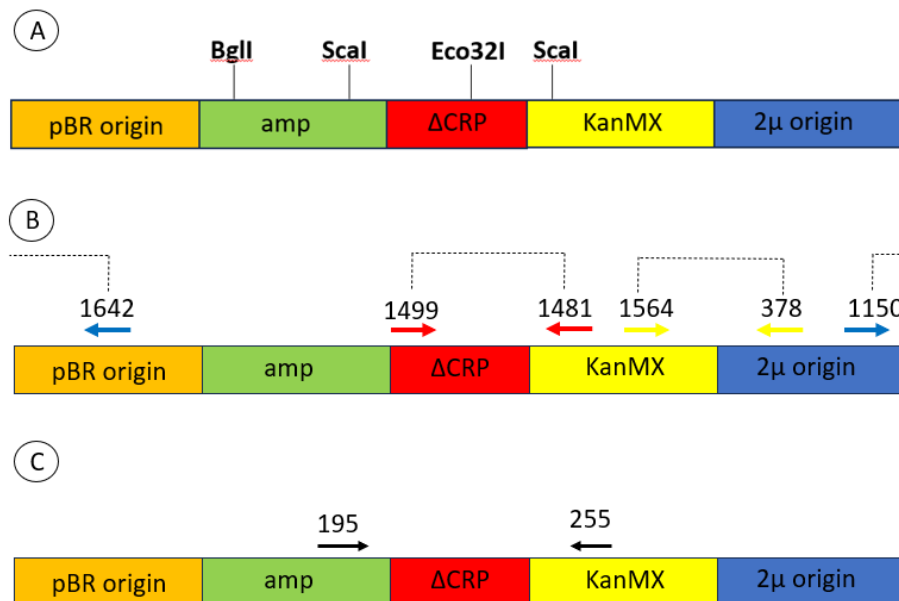


Figure 7 – Schematic representation of different methods used to test the construction of the pTA5 plasmid by enzymatic digestion, PCR and sequencing. A – Enzymatic digestion of the pTA5 plasmid using the *BglI*, *Eco32I* and *Scal* enzymes. The *BglI* enzyme cuts once, inside the amp gene. The *Eco32I* cuts in the multi cloning site, inside the ΔCRP fragment. The *Scal* enzyme cuts twice, inside the KanMX gene and inside the amp gene. B – The construction was tested by PCR. Three pairs of primers were used and divided by colours. The 1499 and 1481 primers (in red) amplified the region between the ΔCRP and KanMX genes. The 1564 and 378 primers (in yellow) amplified the region between the KanMX and the 2 μ origin of replication genes. The 1642 and 1150 primers (in blue) amplified the region between the 2 μ origin of replication and the pBR origin of replication. C – The ΔCRP fragment was sequenced using the 195 and the 255 primers.

The use of either the *Eco32I* or the *BglI* enzyme results in the linearization of the plasmid, as each enzyme has one cutting site inside the plasmid. Meanwhile, *ScaI* cuts the plasmid in two different sites. We performed four different digestions: *Eco32I*; *BglI*; *Eco32I* and *BglI*; *ScaI* (**Figure 8**). The results depicted in the gel match *in silico* prediction of the digestions. Both the *Eco32I* and the *BglI* enzymes linearized the plasmid. The simultaneous use of both enzymes resulted in two DNA fragments similar to those produced by the digestion of the plasmid with the *ScaI* enzyme.

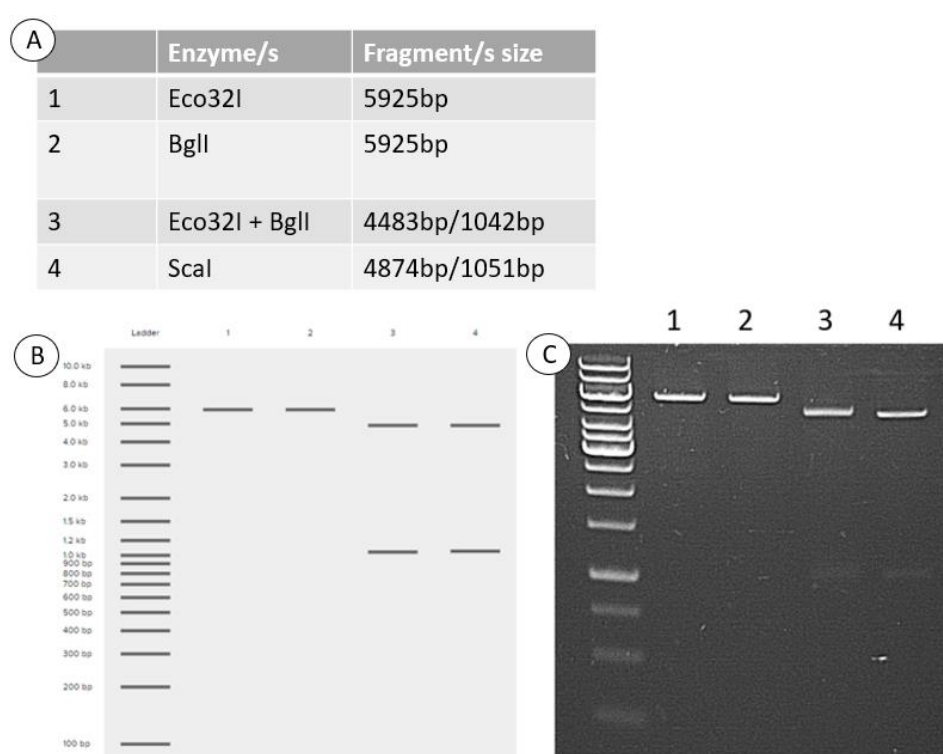


Figure 8 – Confirmation of the pTA5 construction by enzymatic digestion. A – Tabel representing the different enzymatic digestions, the enzymes involved and the size of the produced fragments. B – *In silico* prediction of the agarose gel containing the fragments produced by the enzymatic digestions. C – Agarose gel displaying the fragments produced by the enzymatic digestions. The molecular weight marker used was the GeneRuler 1kb DNA ladder (Thermo Scientific).

The construction of the plasmid was confirmed by amplifying regions between adjacent genes. The PCR products were analysed by gel electrophoresis. All the resulting bands appear as being between 1000bp and 1500bp long, which matches the expected size for all of the products (**Figure 9**). This confirms the order in which the DNA fragments were assembled.

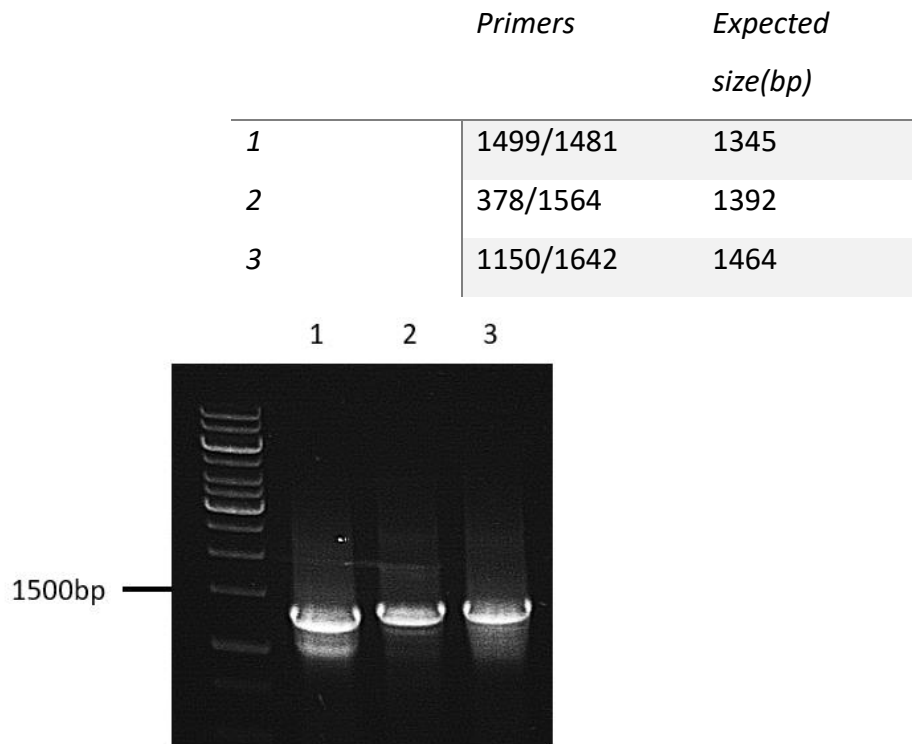


Figure 9 – Confirmation of the pTA5 construction by PCR. The table above shows the three different PCR reactions, with their respective primers and the expected size of the PCR product. The results are displayed in the agarose gel below, each reaction is represented by their respective numbers. The molecular weight marker used was the GeneRuler 1kb DNA ladder (Thermo Scientific).

The results given by PCR and enzymatic digestions suggested that the pTA5 plasmid was well constructed, however, it was still crucial to know if there were any mutations in the plasmid, especially in the MCS. The primers chosen for the sequencing analysis flanked the ΔCRP fragment which contains the MCS. The sequencing results matched the predicted nucleotide sequence of the plasmid except for two nucleotide mutations(a deletion and a substitution) inside the terminator region of the *KanMX* gene (**Figure 10**). Looking at the sequencing results, we could see that these mutations occurred in a repetitive stretch of the DNA chain. It has been documented that high throughput sequencing techniques are often accompanied by errors in repetitive areas of the DNA sequence(Treangen & Salzberg, 2012). Therefore, the errors in the sequencing data could be caused by the nature of the DNA sequence. The mutations don't belong to the coding sequence of the gene which means that the protein is not affected by them.

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                2689                                                    2800
pTA5      agatacctgattttacagttcagtatagcgaccagcattcacatagcattgacgcgatgattactttctgcgcaacttaacttcgcatctgggcagatgatgtcgaggcg-a
DVI683_25... AGATACCTGATTTACAGTTCAGTATAGCGACCAGCATTACATACGATTGACGCATGATATTACTTTCTGCGCACTTAACCTCGCATCTGGGCAGATGATGTCGAGGCGA
.....

                2801                                                    2912
pTA5      aaaaaataataatcacgctaacatttgattaaaaatagaacaactacaataataaaaaaactatacaaatgacaagttcttgaaaacaagaatctttttattgtcagtactga
DVI683_25... AAAAAAATATAAATCACGCTAACATTTGATTAAATAGAACAACTACAATATAAAAAAATAACAATGACAAGTCTTGAAAACAAGAATCTTTTATTGTCAGTACTGA
.....

                2913                                                    3024
pTA5      ttagaaaaactcatcgagcatcaaatgaaactgcaatttattcattatcaggattatcaataccatatttttgaaaaagccgtttctgtaataaggagaaaaactcaccgagg
DVI683_25... TTAGAAAACATCATCGAGCATCAATGAACTGCAATTTATTCATATCAGGATTATCAATACCATATTTTGAAAAGCCGTTTCTGTAATGAGGAATAAACTCACCAGG
.....

```

Figure 10 – Nucleotide sequence obtained from the sequencing of the pTA5 plasmid. The segment ranging from the 2689 to the 3024 nucleotides of the theoretical pTA5 sequence is aligned with the results from the sequencing of the constructed plasmid. The mutations are highlighted in orange.

3.2. Construction of the pYPKpw_ENO2_BsfabL_RPL5, pYPKpw_ENO2_SafabI_RPL5 and pYPKpw_ENO2_Athmod1_RPL5 Transcription Unit vectors

3.2.1 Construction of the pYPKa_BsfabL and pYPKa_SafabI vectors

To determine whether the expression of different Enoyl-ACP reductases had an impact on the growth of a $\Delta fas2$ *S. cerevisiae* strain, we simultaneously expressed the pTA1_FASIIb plasmid and a vector expressing two different Enoyl-ACP reductases. To serve as our controls, we also tested the expression of a copy of the *AthMOD1* gene and an empty vector that served as the backbone of the other vectors. This work required *S. cerevisiae* expression vectors with single gene transcription units (TUs). This was achieved through the Yeast Pathway Kit (Pereira et al., 2016) cloning strategy. We started by cloning the *fabL* (from *Bacillus subtilis*) and the *fabI* (from *S. aureus*) genes in the pYPKa vector (**Figure 11**). The pYPKa plasmid is a positive selection vector for cloning in *E. coli* that serves the function of providing building blocks for expression vectors in *S. cerevisiae*. Both the *BsfabL* and *SafabI* genes were amplified by PCR from purified genomic DNA and cloned in the pYPKa vector linearized by *AjlI*. The ligation mixture containing all PCR products along with the linear pYPKa plasmid was transformed in *E. coli* and left to grow in LB+ ampicillin media.

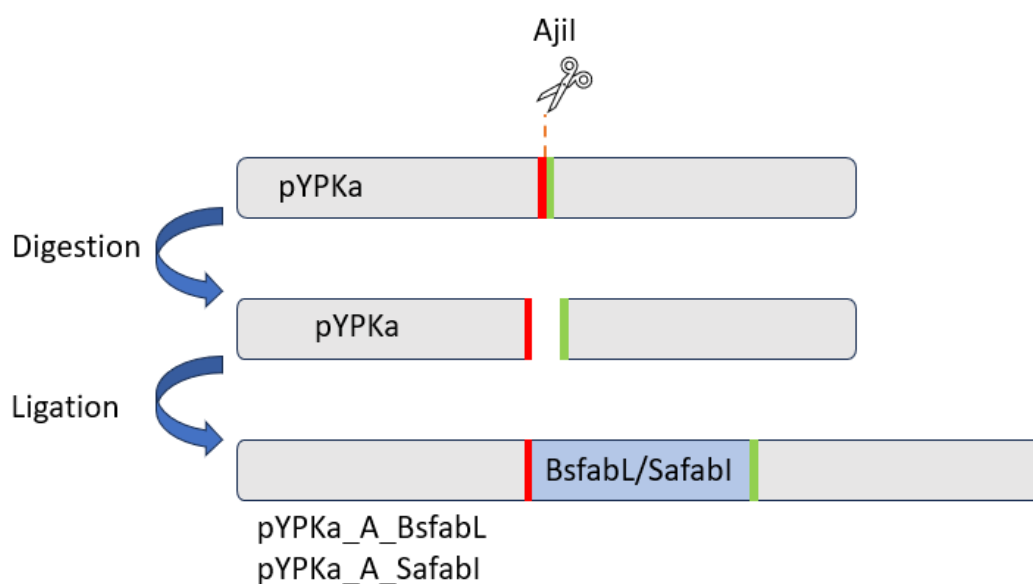


Figure 11 – Schematic representation of the cloning of the BsfabL and SafabI genes in the pYPKa plasmid. The pYPKa plasmid was first linearized using the AjiI enzyme that cuts a specific site that is flanked by two specific sequences pictures in red and green. The linearized plasmid is then ligated with either the BsfabL or the SafabI genes, producing the pYPKa_A_BsfabL/SafabI vector that will be used as a building block for single gene expression vectors.

Both transformations were successful so we proceeded to screen the cloning of both genes by colony PCR (**Figure 12**). The used primers amplified a region that included the insert and the adjacent regions belonging to the pYPKa vector. The linearized plasmid carried blunt ends which limits the maximum theoretical cloning efficiency to 50% given the equal probability of insertion in one of either direction. As expected, about half of the tested colonies gave PCR products with the expected length indicating the presence of the insert in the correct orientation within the vector. The confirmation of both constructions by PCR along with the lack of colonies in the negative control transformation highlighted the success of this cloning strategy. The resulting vectors, pYPKa_BsfabL and pYPKa_SafabI, were used as building blocks for the construction of expression vectors.

Template	Primers	Expected size(bp)
pYPKa_BsfabL	577/1737	977
pYPKa_Safabl	577/1739	996

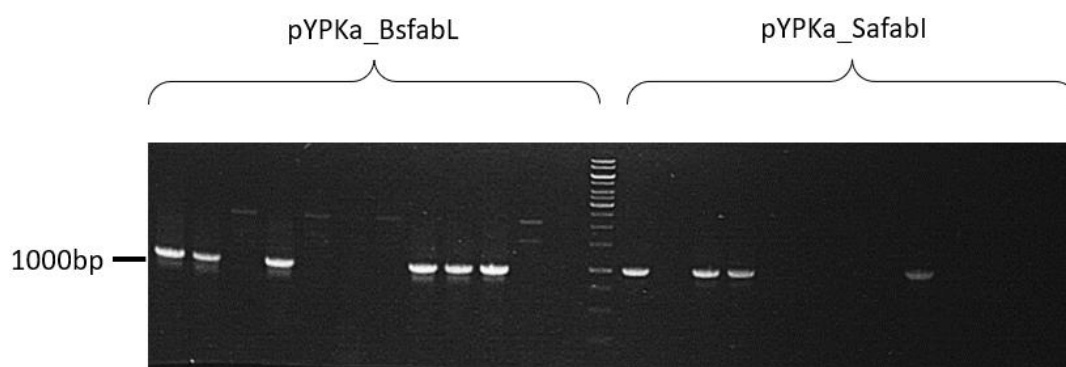
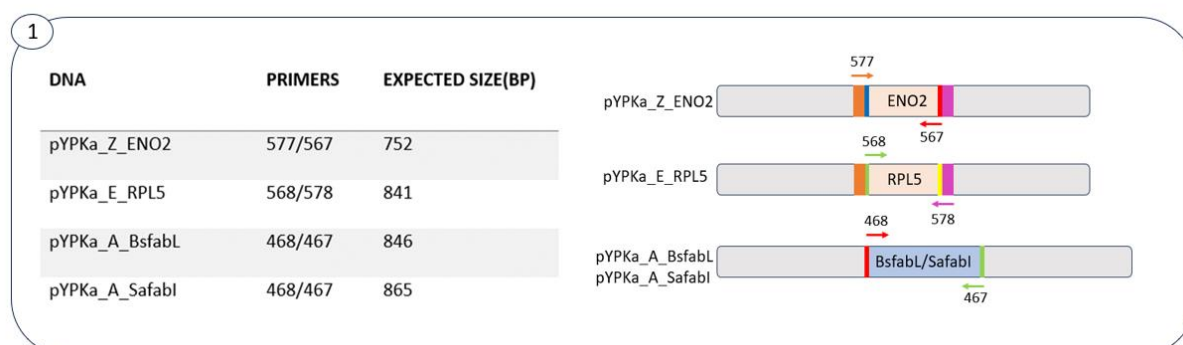


Figure 12 – Confirming the construction of the pYPKa_BsfabL and pYPKa_Safabl plasmids my Colony PCR. The PCR conditions are described in the table above. The agarose gel shows the results from both plasmids, separated by the molecular weight marker. The molecular weight marker used was the GeneRuler 1kb DNA ladder (Thermo Scientific).

3.2.2 Construction of the pYPKpw_ENO2_BsfabL_RPL5, pYPKpw_ENO2_Safabl_RPL5 and pYPKpw_ENO2_Athmod1_RPL5 Transcription Unit vectors

The *S. cerevisiae* expression vectors used in this work were assembled *in vivo* through homologous recombination in yeast (**Figure 13**). The expression vectors consisted of single gene TUs that were made up of the gene of interest flanked by two TPs : ENO2 and RPL5.



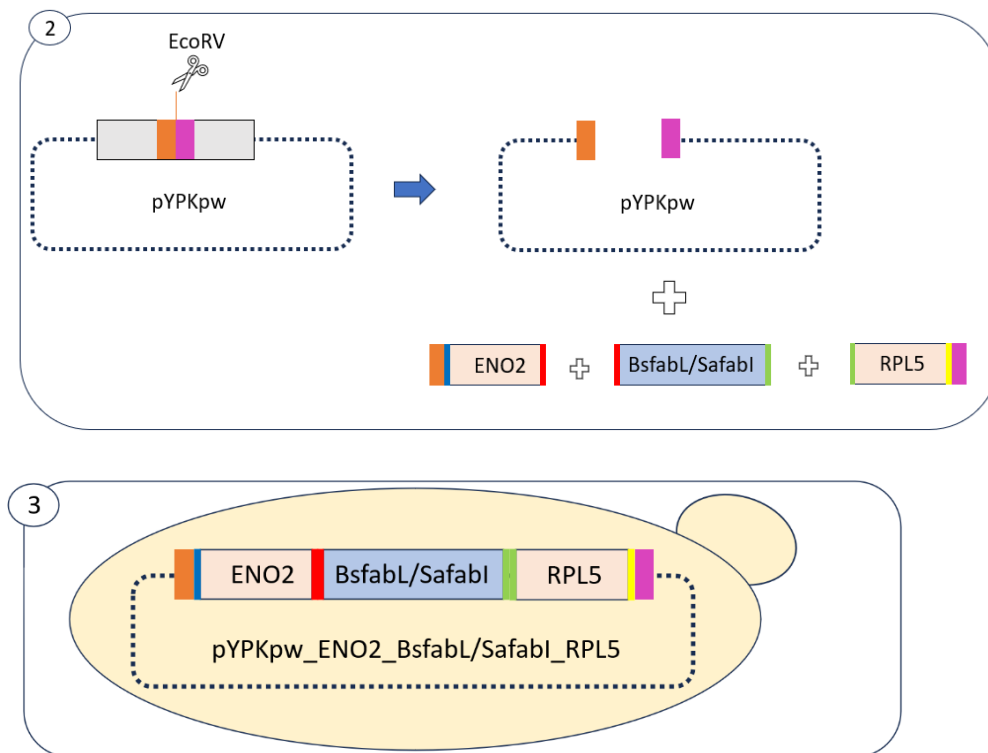


Figure 13 – Schematic representation of the construction of the pYPKpw_ENO2_BsfabL/SafabI_RPL5 expression vectors by homologous recombination. 1 – The required DNA fragments that compose the required vectors were amplified from the pYPKa. The primers used also amplified the flanking regions that have homology between themselves, allowing for homologous recombination (pictured in different colours). 2 – The pYPKpw plasmid was used as the backbone of the final expression vectors. This plasmid was first linearized using the EcoRV enzyme. The cutting site of this enzyme is flanked by regions homologous to the PCR products (pictured in orange and pink). All PCR products and the linearized pYPKpw plasmid were simultaneously transformed in yeast. 3 – The homology between PCR products and the linearized plasmid allowed for the homologous recombination and construction of the pYPKpw_ENO2_X_RPL5 expression vectors (X being either the BsfabL or the SafabI genes).

Using the pYPKa_BsfabL, pYPKa_SafabI, pYPKa_Z_ENO2 and pYPKa_E_RPL5 plasmids as templates, the required DNA fragments were obtained by PCR by using designated primers that allow for the further homologous recombination (**Figure 14**). The results displayed in the agarose gel indicate that all the products appear to have the predicted size.

NUMBER	TEMPLATE	PRIMERS	EXPECTED SIZE(BP)
1	pYPKa_ENO2	577/567	752
2	pYPKa_BsfabL	468/467	846
3	pYPKa_SafabI	468/467	865
4	pYPKa_RPL5	568/578	841

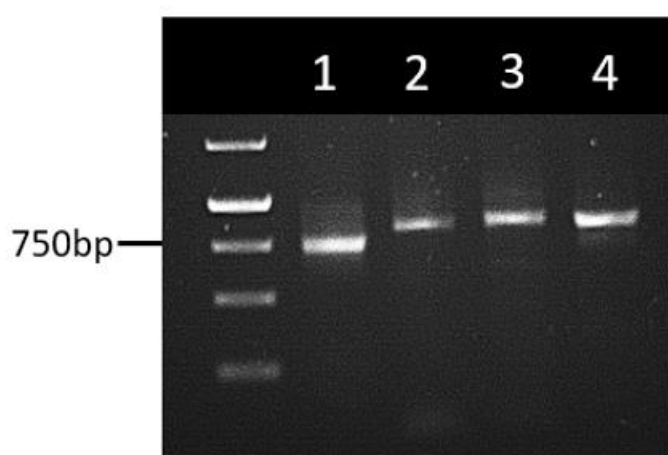


Figure 14 – Amplification of the DNA fragments used to construct the pYPKpw_ENO2_gene_RPL5 expression vectors by PCR. The different PCR reactions were named from 1 to 4 and described in the table above. The agarose gel displays the DNA fragments obtained from the PCR reactions. The molecular weight marker used was the GeneRuler 1kb DNA ladder (Thermo Scientific).

The plasmid backbone of the final construction was originated from the pYPKpw plasmid. This plasmid is a *S. cerevisiae*/*E. coli* shuttle vector that contains the *URA3* auxotrophic marker. The linearization of this plasmid was done through enzymatic digestion using the *EcoRV* enzyme . In this particular reaction, alkaline phosphatase was added in order to prevent the possible vector regeneration (Dale & Greenaway, 1984). The final plasmid constructions were assembled by transforming the CEN.PK2-1C *S. cerevisiae* strain with both TP, one of the *BsfabL*/*SafabI* genes and the linear pYPKpw plasmid. The transformants were selected in SC-URA media and their constructions were confirmed by colony PCR (**Figure 15**). The results showed in the agarose gel allowed for the identification of the colonies that had

the right insertion orientation. Based on the bands in the agarose gel, one colony of each construction has its plasmid rescued and sequenced.

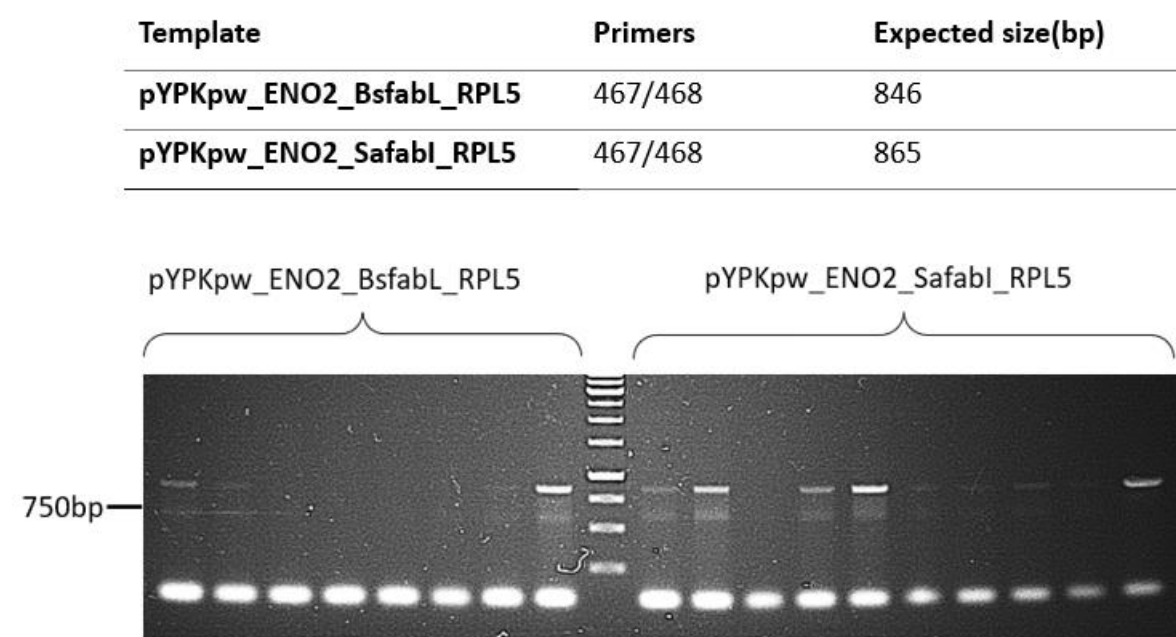
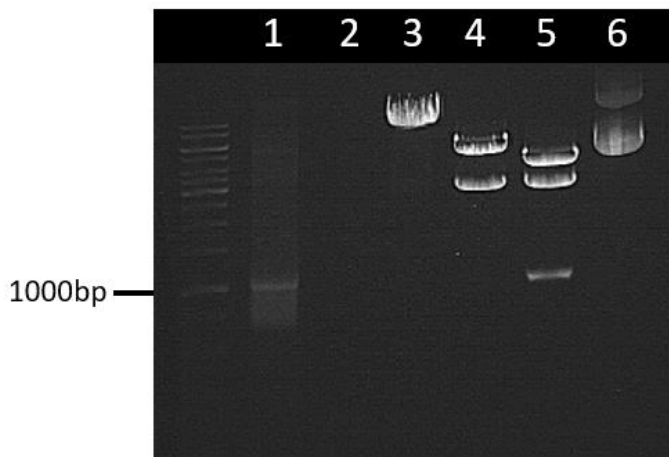


Figure 15 – Colony PCR reactions that were used to confirm the construction of the pYPKpw_ENO2_gene_RPL5 vectors. Both reactions are described in the table above. The agarose gel shows the resulting DNA fragments from all colonies tested. Both constructions are separated by the molecular weight marker. The molecular weight marker used was the GeneRuler 1kb DNA ladder (Thermo Scientific).

The previously constructed pYPKpw_ENO2_Athmod1_RPL5 plasmid was extracted from the *E. coli* u168 strain and was tested by PCR and enzymatic digestion (Figure 16). The screening through PCR was done using the 1304 and 1305 primers which produced a 1004bp product. The confirmation by enzymatic digestion was done using the *Bam*HI and *Bgl*II enzymes, separately and together. The PCR reaction successfully amplified the desired DNA fragment, while the negative control reaction (only missing the DNA template) didn't produce any fragment. All enzymatic digestion matched the predicted bands in the agarose gel. The digestion with *Bam*HI linearized the plasmid (7988bp) while the digestion with *Bgl*II produced two fragments, one 4953bp long and the other 3035bp long. The digestion with both enzymes

produced three fragments: 3980bp long, 3035bp long and 973bp long. The results showed in the agarose gel confirmed the constitution of the pYPKpw_ENO2_Athmod1_RPL5 vector.



- 1- PCR; 1304 and 1305 Primers; 1004bp product
- 2- C-
- 3- Digestion with BamH
- 4- Digestion with BglI
- 5- Digested with BamH+BglI
- 6- Undigested plasmid

Figure 16 – Confirmation of the composition of the pYPKpw_ENO2_Athmod1_RPL5 vector by PCR and enzymatic digestion. The agarose gel shows the resulting products of all the different reactions. The reactions are named from 1 to 6 and are described in the list below. The used molecular weight marker was the GeneRuler 1kb DNA ladder (Thermo Scientific).

3.3 Growth assays

All single gene expression vectors were transformed into the $\Delta fas2$ u1420 *S. cerevisiae* strain. The empty pYPKpw plasmid that served as the backbone of the other vectors was also transformed to serve as a control. The u1420 strain already contained the pTA1_FASIIb plasmid, that complemented the synthesis of fatty acids and growth of the strain in media without the addition of myristic acid. All expression vectors were successfully transformed and the transformant colonies were used to prepare the pre inoculums. The pre-inoculums were kept at -80°C for preservation so that all growth curves started with a similar number of

cells. The four pre inoculums containing all the strain to be tested were poured into pre heated erlenmeyers with SC-LU media and the initial OD₆₀₀ (optical density) was measured. All cultures had an initial OD that ranged between 0.5 to 0.6. The use of pre inoculums seemed to work perfectly as all cultures had a similar initial OD₆₀₀. All cultures entered the exponential phase around after 10 hours of growth (**Figure 17**). At that time, the OD₆₀₀ of the cultures was measured again and at that point in time, the cells had duplicated one and a half times. Two hours later, after 12 hours of growth, the OD₆₀₀ was measured again. From that point on, the OD₆₀₀ was measured at one hour intervals spanning for 8 hours. The two last measures were done 2 and four hours after that, totalling a 24 hour growth period. The growth experiments were performed in triplicate. The specific growth rate was determined by using the mean value of the OD₆₀₀ at the same timepoints between equal strains (**Figure 18**). All enoyl-ACP reductases expressed here conferred a higher growth rate than the empty plasmid indicating that low gene dosage may be a bottleneck of the FASII system. Nonetheless, differences among the different enzymes were observed. The results showed that the highest growth rate was observed in the culture expressing the *MOD1* gene. The second highest growth rate was observed in the culture expressing the *BsfabL* gene, followed by the culture expressing the *SafabL* gene. The strain containing the empty pYPKpw plasmid had the lowest growth rate among all cultures.

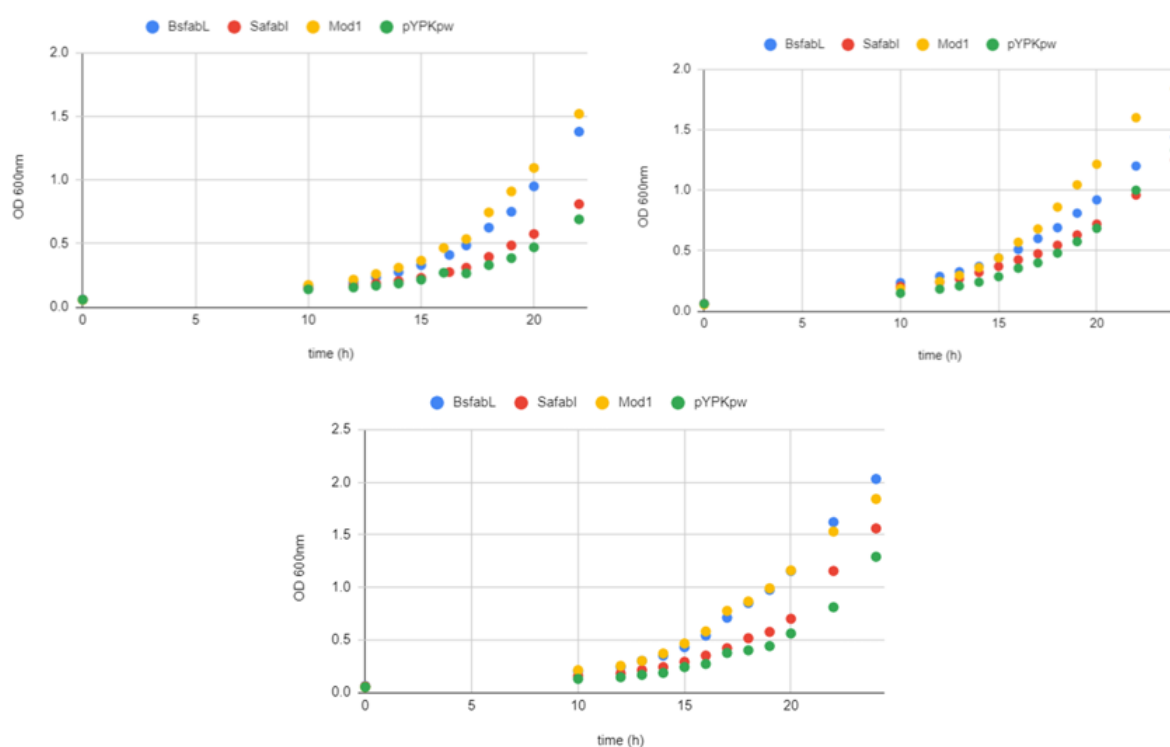


Figure 17 – Graphic display of the results obtained from the OD₆₀₀ measurements of the growth curves. Each graph accounts for one of the three growth curves done on the exact same condition. Each growth curve had a time span of 24 hours in a total of twelve OD₆₀₀ measurements.

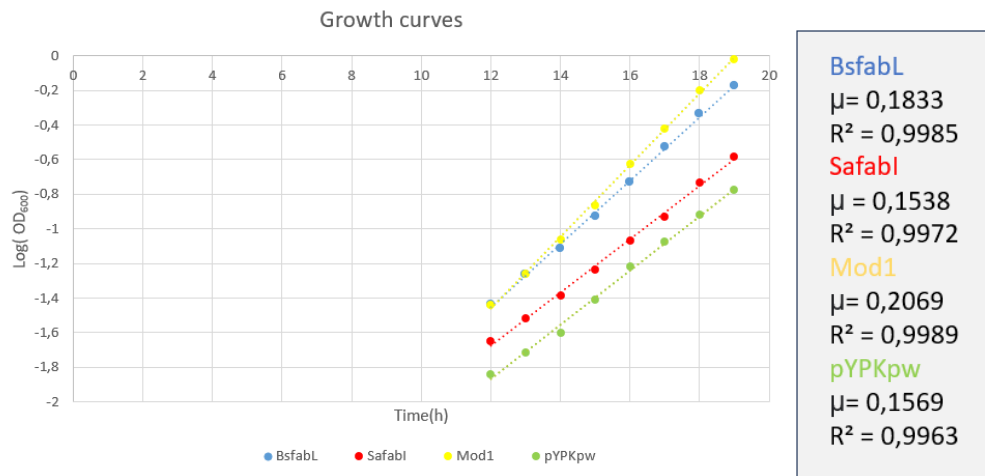


Figure 18 – Specific growth rate of the four different strains. Each point represents the mean of the three absorbances obtained in the same timepoints, between all growth curves.

3.4 Deletion of the *FAS1* gene using the Cre/loxP method and recycling of the *KanMX* marker

To prevent the interaction between the native FAS machinery and the heterologous type II FAS system in yeast, the deletion of both the *FAS1* and *FAS2* genes was required. The use of a double mutant strain prevents this possibility and guarantees that the heterologous FAS system complements the synthesis of fatty acids.

The u1483 yeast strain lacks the *FAS2* gene. This deletion was carried out by replacing the gene with the *KanMX* marker. In order to delete the *FAS1* gene with the same marker, *KanMX* was recycled using the Cre/loxP recombination system.

Traditionally, the deletion of yeast genes involves the cloning of the DNA fragments flanking the gene of interest, left and right of a yeast marker gene (Szostak et al., 1983). After transformation, this deletion cassette is inserted by homologous recombination in the yeast genome, resulting in the deletion of the target gene (Orr-Weaver et al., 1981). The required length of the flanking regions is usually small, allowing the construction of gene disruption cassettes by PCR (Baker Brachmann et al., 1998). The following recycling of the marker is also

of great importance due to the limited availability of markers and the attributes they grant to the mutant strain. The Cre-loxP recombination system is often a preferred option to the deletion of yeast genes because of its efficiency in rescuing the selective marker (Guldener, 1996). The Loxp/Cre recombinase deletion method involves two main components: the Cre protein, usually expressed in a vector, is a site-specific recombinase that mediates intramolecular recombination events between cis-linked loxP sites (Abremski & Hoess, 1984). The loxP sequence is 34bp in length and serves as the DNA binding site to the Cre recombinase. This sequence is made up of two 13bp palindromic inverted sequences and an 8bp spacer sequence that confers directionality of the lox site (Ghosh & Van Duyne, 2002). The gene disruption cassette is produced by PCR and consists of a selective marker flanked by two 34bp long loxP sequences. The transformation of the cassette induces the excision of the target gene and the hybridization of the designated selective marker in the genome.

3.4.1 Recycling of the *KanMX* gene

We started out by thawing the u839 *E. coli* strain that had the pSH47 plasmid. This plasmid is characterized by expressing the Cre protein, under the control of the GAL1 promoter. The plasmid DNA was purified from the *E. coli* culture and then tested by PCR. The primers used flanked the Cre gene. Upon confirming the presence of the Cre gene, we transformed 10 µL of the pSH47 plasmid in the u1483 yeast strain($\Delta fas2$, *KanMX4*) and selected the transformants in SC-URA + Hmy media since pSH47 has the URA3 auxotrophic marker. The transformant colonies were then grown in 5mL of YPD + Hmy liquid culture to induce Cre expression. A 10^{-4} dilution of the liquid culture was plated on YPD + Hmy media as a way to test single colonies. We then picked 25 colonies and grew them in YPD + Hmy + G418 solid media to test their resistance to geneticin. The colonies that didn't grow in YPD + Hmy + G418 media were believed to have lost the *KanMX* cassette. To further confirm the removal of the cassette, a PCR was conducted using three primers (**Figure 19**). Two of the primers annealed in the flanking regions of the *FAS2* gene while the other primer hybridized inside the *KanMX* cassette. The size of the produced fragment would indicate the presence or absence of the *KanMX* gene. As a control, the genomic DNA of a colony that grew in YPD + Hmy + G418 media was also tested.

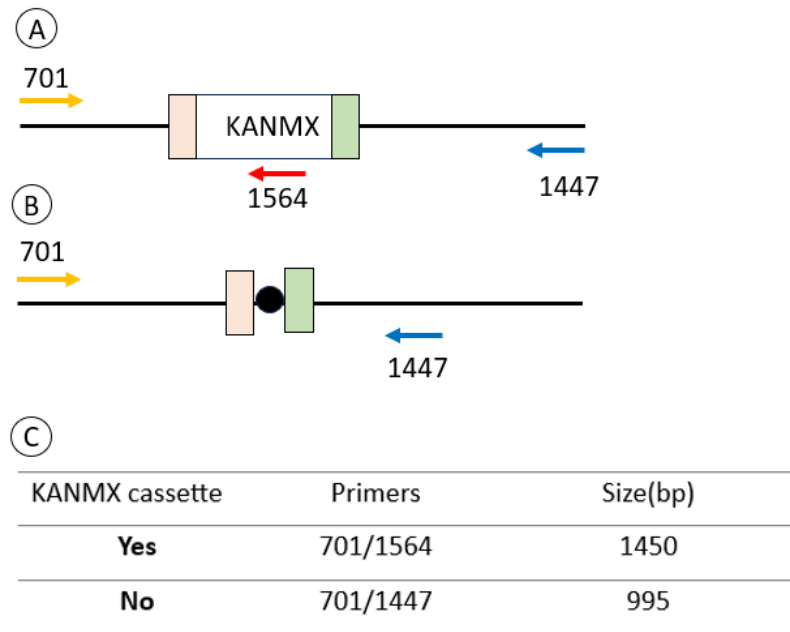


Figure 19 – Schematic representation of the PCR reaction to confirm the recycling of the *KanMX* gene. A – When the *KanMX* gene is present, the 1564 primer will hybridize inside this gene B – When the *KanMX* gene is not present, the 1564 primer cannot hybridize C – Depending on the presence of the *KanMX* gene, the final PCR product can be either 1450bp long or 995bp long.

The results showed that both colonies didn't effectively have the cassette inside the genome while the colony used as a control still had the *KanMX* cassette (**Figure 20**). In order to lose the pSH47 plasmid, both colonies were grown in non-selective media YPD + Hmy. After reaching the stationary phase, the cells were recovered and grown again in YPD + Hmy. The resulting cultures were diluted and plated in YPD + Hmy media. The presence of the plasmid was tested by plating the resulting colonies in SC-URA + Hmy. Most of the colonies didn't grow in this media, meaning they had lost the pSH47 plasmid. The final strain was named u1767.

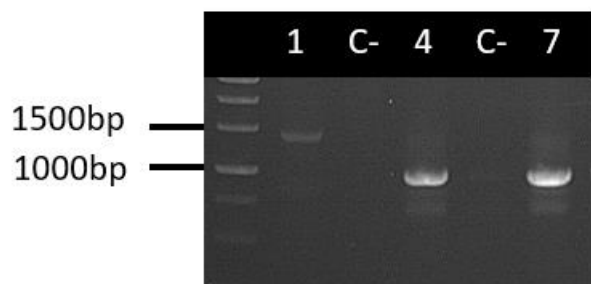


Figure 20 – Gel agarose showing the resulting DNA fragments from the PCR reactions used to confirm the presence of the *KanMX* gene. The 4 and 7 columns contain the PCR products from the genomic DNA of the two colonies that didn't grow in geneticin media. The column number 1 contains the PCR product from the genomic DNA of a colony before inducing the excision of the *KanMX* gene. The used molecular weight marker was the GeneRuler 1kb DNA ladder (Thermo Scientific).

3.4.2 Deletion of the *FAS1* gene and recycling of the *KanMX* marker

In order to obtain a $\Delta fas1 \Delta fas2$ yeast strain, the *FAS1* gene was deleted in the u1767 yeast strain. The deletion was done using the Cre/loxP deletion method. We started by amplifying the *KanMX* cassette that to replace the *FAS1* gene, by homologous recombination. The pUG6 plasmid was used as the template for the PCR reaction (**Figure 21**). In this reaction, a large quantity of insert was used maximise the number of transformants as genomic substitutions tend to be inefficient. Therefore, we did three identical 100uL PCR reactions. The three PCR products were pooled together followed by precipitation and resuspension in the maximum volume allowed for the transformation. All DNA was used to transform the u1767 yeast strain. The transformed cells were grown in YPD + Hmy + G418 media.

Template	Forward primer	Reverse primer	Expected size(bp)
pUG6	1394	1393	1703

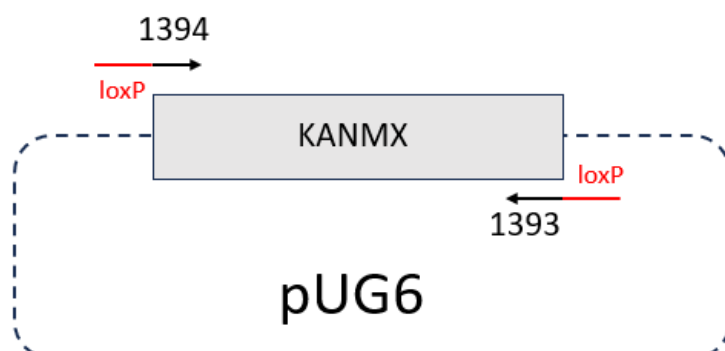


Figure 21 – Schematic representation of the PCR reaction used to obtain the *FAS1* deletion cassette. To delete the *FAS1* gene, the Cre/loxP method was used. This method uses deletion cassettes that have homology with the flanking regions of the target gene. In order to delete this gene, a deletion cassette containing the *KanMX* selective marker was used. This cassette was obtained by PCR, using the 1394 and 1393 primers and using the pUG plasmid as a template. The final PCR product was 1703bp long, was homologous to the flanking regions of the *FAS1* gene and had the loxP sequence.

The transformation was successful and 10 transformants were again plated in YPD + Hmy + G418(600mg/L) media. All colonies appeared to have the *KanMX* cassette inserted in their genome, as they were able to grow in media with geneticin. From this petri dish, 4 colonies were picked and had their genomic DNA extracted to confirm the deletion by PCR (**Figure 22**). Two PCR reactions were performed, one that confirmed the absence of the *FAS1* gene and the other to confirm the presence of the *KanMX* cassette in its place. The 1418 forward primer annealed in the flanking region of the *FAS1* gene and was used in both PCR reactions. The 1564 reverse primer annealed inside the *KanMX* gene and was used to test for the insertion of the cassette in the genome. When using the 1459 reverse primer that anneals inside the *FAS1* gene, no PCR products were produced in the $\Delta fas1$ mutant strains. The results displayed in the agarose gels indicate that all the colonies tested positive for the presence of the *KanMX* gene. Simultaneously, the PCR reaction used to test the presence of the *FAS1* gene produced no results in any of the colonies. The combined results of both gels indicate the successful deletion of the *FAS1* gene in all the tested colonies.

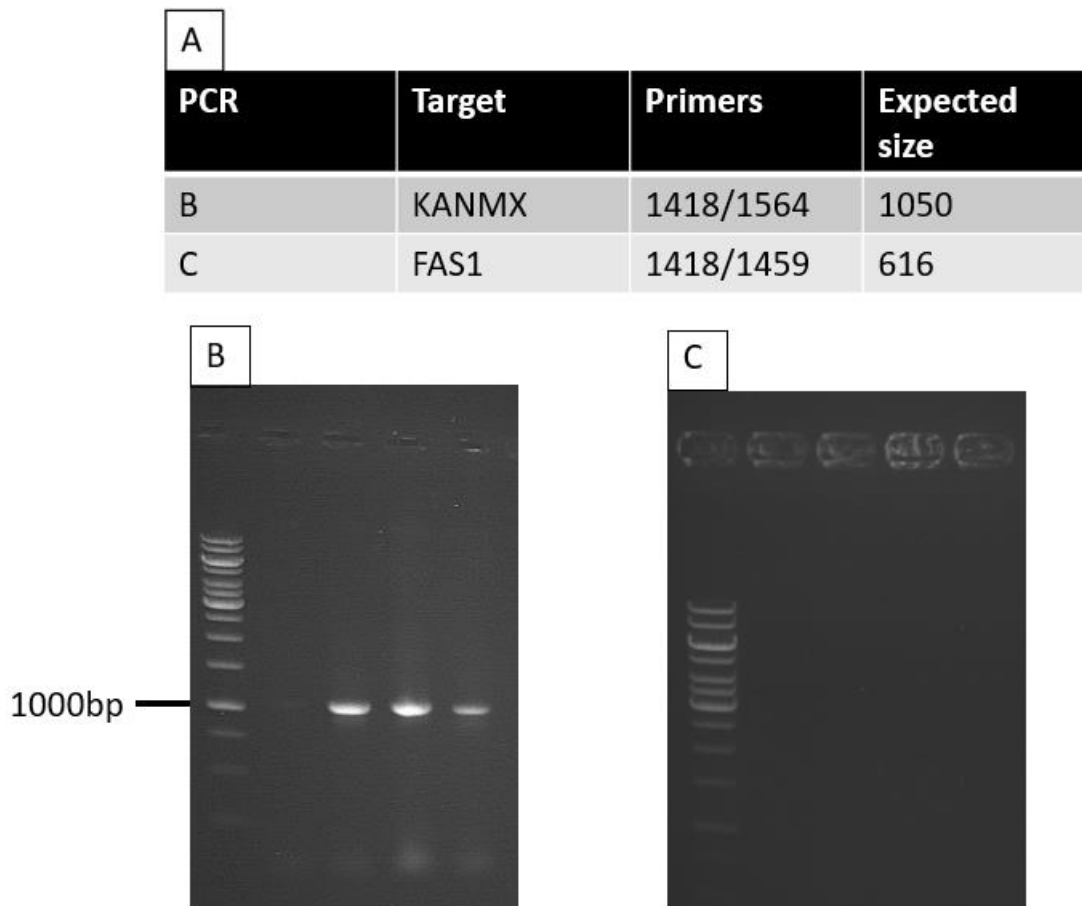


Figure 22 – Confirmation of the yeast *FAS1* deletion by PCR. A- Table representing both PCR reactions(B and C), represented in the agarose gels below; The used molecular weight marker was the GeneRuler 1kb DNA ladder (Thermo Scientific).

3.4.3 Recycling of the *KanMX* cassette

Having confirmed the deletion of the *FAS1* gene, *KanMX* gene remaining in the genome, was removed. The approach was identical to the one mentioned previously in this work. The strain was first transformed with the pSH47 plasmid and grown in SC-U + Hmy media. Two of the transformant colonies were then transferred to a YPD + Hmy liquid culture to induce the expression of the Cre protein. To select individual colonies, the liquid culture was diluted and the cells were plated in solid YPD + Hmy media. From that petri dish, we picked twenty colonies that were plated in YPD + Hmy + G418 media. The colonies that didn't grow

in this media were selected and their genomic DNA was extracted in order to test their genotype. By using three primers, we were able to confirm the *KanMX* gene by PCR (**Figure 23**).

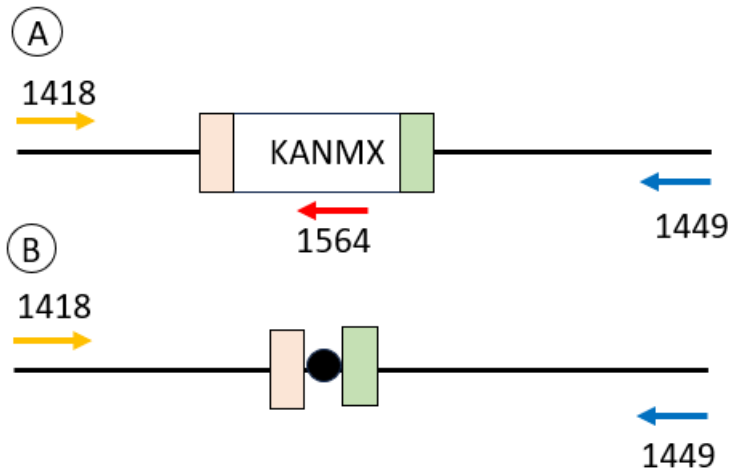


Figure 23 – Graphical representation of the PCR reactions used to confirm the recycling of the *KanMX* cassette.

3.5 Construction of the pTA5_FASIIb plasmid- first strategy

The pTA1 and pTA5 are identical with exception to the selective markers, which are *LEU2* and *KanMX*, respectively. We hypothesised that this homology between the two plasmids can theoretically allow for the transfer of the genes belonging to the FASII pathway from the pTA1_FASIIb plasmid into the pTA5 plasmid. By transforming a yeast strain with both the pTA1_FASIIb plasmid and the linearized pTA5 plasmid, we aimed to clone the FASIIb pathway inside the pTA5 plasmid. The pTA5 plasmid was linearized with the *EcoRV* restriction enzyme, which cuts the plasmid in the multi cloning site, inside the Δ CRP fragment (**Figure 24**). The multi cloning site is homologous to the flanking regions of the FAS genes in the pTA1_FASIIb plasmid.

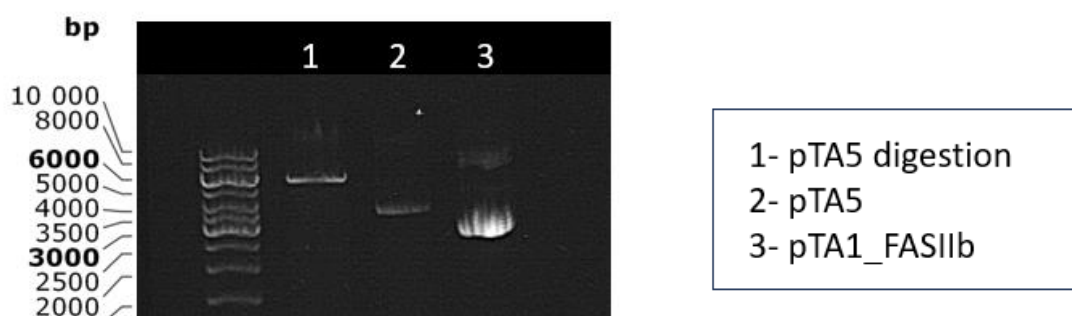


Figure 24 – Results from the linearization of the pTA5 plasmid by the EcoRV enzyme. All the different reactions are numbered from 1 to 3 and displayed in the agarose gel. The used molecular weight marker was the GeneRuler 1kb DNA ladder (Thermo Scientific).

Both the linearized pTA5 plasmid and the pTA1_FASIIb plasmid were simultaneously transformed in the yeast strain CEN.PK2-1C. The transformed cells were selected in YPD + G418 media. The petri dish with cells transformed with both plasmids had about 10 colonies while the petri dish with cells transformed with only the linear pTA5 didn't have any transformant colonies. The construction was first tested by Colony PCR (**Figure 25**). In this reaction, the 714 and 715 primers were used to amplify the *fabG* gene, part of the FASIIb pathway.

Template	Primers	Expected size(bp)
pTA5_FASIIb	714/715	749

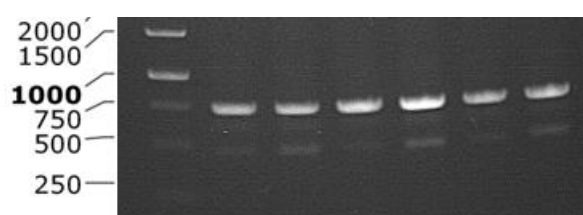


Figure 25 – Confirmation of the pTA5_FASIIb construction by Colony PCR. The agarose gel shows the resulting fragments from six different colonies. The used molecular weight marker was the GeneRuler 1kb DNA ladder (Thermo Scientific).

To further test the construction, we started by rescuing the plasmids from the transformant colonies (Figure 21). During the rescue process, LB + Kanamycin media was used in the competent *E. coli* transformation. The pTA5_FASIIb plasmids were first analysed by gel electrophoresis while using the pTA1_FASIIb plasmid as a control since their size and composition are very similar. The results in the agarose gel display a big disparity between the plasmids of all the transformed colonies. Additionally, there were no plasmids that appeared to have a similar composition to the pTA1_FASIIb plasmid (C+) .

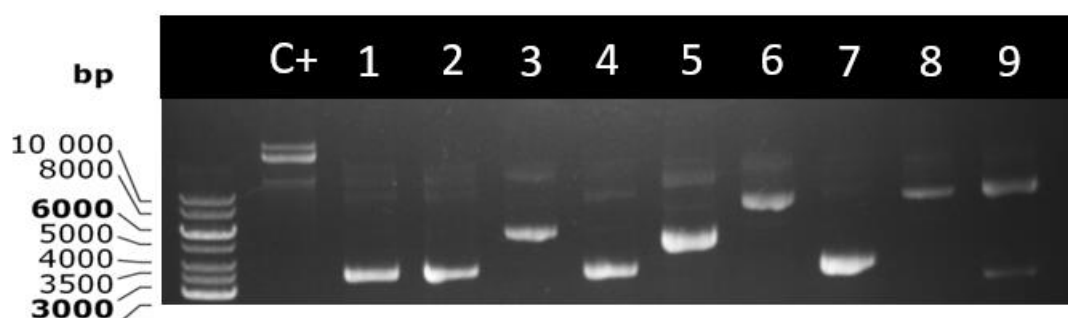


Figure 26 – Agarose gel displaying the comparison between the extracted plasmids from the pTA5_FASIIb construction and the pTA1_FASIIb plasmid. The different constructions are numbered from 1 to 9. The positive control (C+) is made up of the pTA1_FASIIb plasmid. The used molecular weight marker was the GeneRuler 1kb DNA ladder (Thermo Scientific).

3.6 Construction of the pTA5_FASIIb by homologous recombination – Second strategy

The second attempt at constructing the pTA5_FASIIb plasmid by homologous recombination was done by only replacing the selective marker in the pTA1_FASIIb plasmid instead of the whole vector backbone . In this approach, a Δfas yeast strain plasmid was transformed with the KANMX gene , obtained from the pTA5 plasmid. This strain was first transformed with the pTA1_FASIIb plasmid and plated in SC-LEU + Hmy media. The transformant colonies were then plated in YPD media, successfully complementing the synthesis of fatty acids by the native type I FAS system. To switch the LEU2 auxotrophic marker to a *KanMX* dominant selective marker, we transformed this strain with a PCR product from pTA5 (**Figure 27**). This PCR reaction involved the amplification of the *KanMX* gene along with its flanking regions that have homology with the adjacent regions of the *LEU2* marker in the

pTA1_FASIIb plasmid. The pTA5 template was diluted 10x, 100x, 1000x and 10000x in order to minimise the risk of having false positives transformants.

Template	Forward primer	Reverse primer	Expected size(bp)
pTA5	1742	1748	1417

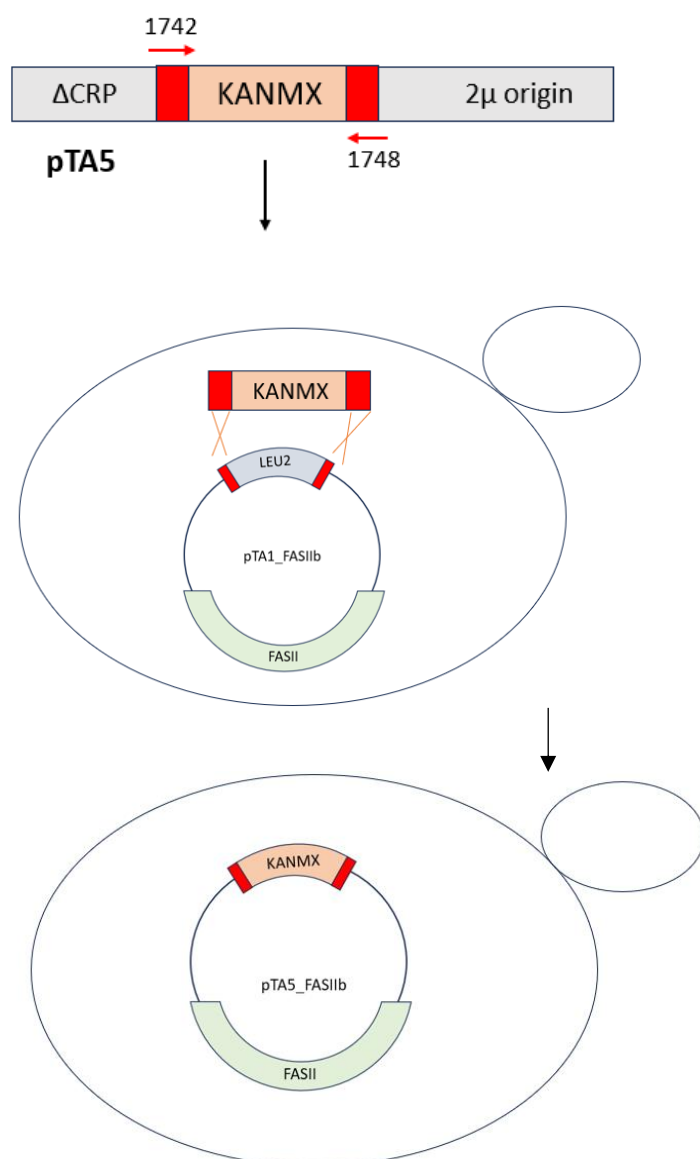


Figure 27 – Graphical representation of the construction of the pTA5_FASIIb plasmid by homologous recombination. In this process, the *KanMX* gene was amplified by PCR from the pTA5 plasmid. The transformation of this PCR product in a Δ *fas2* mutant yeast strain containing the pTA1_FASIIb plasmid results in the homologous recombination between the flanking regions of both

selective markers. By growing the transformed colonies in media with geneticin, it is possible to select the colonies with the pTA5_FASIIb construction.

All PCR reactions successfully amplified the desired DNA fragment. Therefore, this product was transformed into the u1767 yeast strain and the transformed cells were plated in YPD+Hmy+G418 media. The colonies that grew in media with geneticin were grown in new YPD + Hmy + G418 (3x concentrated) media. Having confirmed the growth in this media, the pTA5_FASIIb plasmid was rescued from two of the colonies.

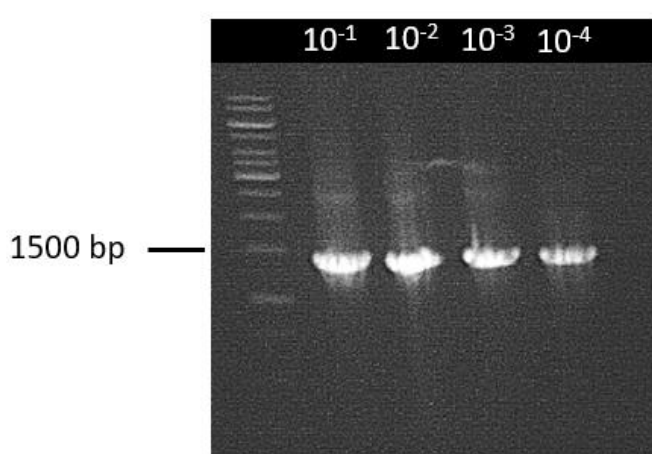


Figure 28 – Agarose gel with the PCR products used in the construction of the pTA5_FASIIb plasmid. The pTA5 template used for the reactions was diluted 10, 100, 1000 and 10000 times, represented in the different gel columns. The molecular weight marker was the GeneRuler 1kb DNA ladder (Thermo Scientific).

The construction was first confirmed by enzymatic digestion, using the *AflwNI* enzyme (**Figure 29**). This enzyme only cuts inside the FASIIb pathway so the digestion of the pTA1_FASIIb plasmid by the same enzyme served as the control. The results in the gel showed that both colonies appeared to have the right construction as all resulting fragments had the predicted size.

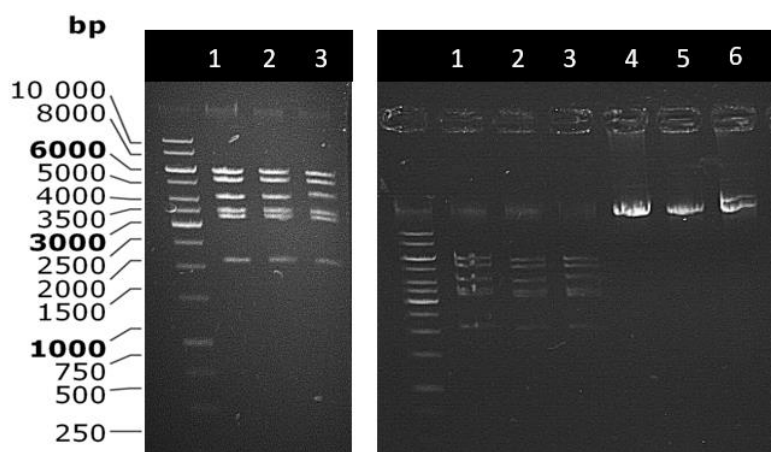


Figure 29 – Agarose gels showing the resulting fragments from the digestion of the pTA5_FASIIb plasmids with the AlwNI enzyme. The different reactions were numbered from 1 to 3 while the 4,5,6 reactions represent the respective undigested plasmids.

In order to confirm that the pTA5_FASIIb plasmids contained all the genes in the FASIIb pathway, the constructions were confirmed by PCR. Six different PCR reactions were performed, each pair of primers amplified two adjacent genes that comprised all the twelve genes in the pathway (**Figure 30**).

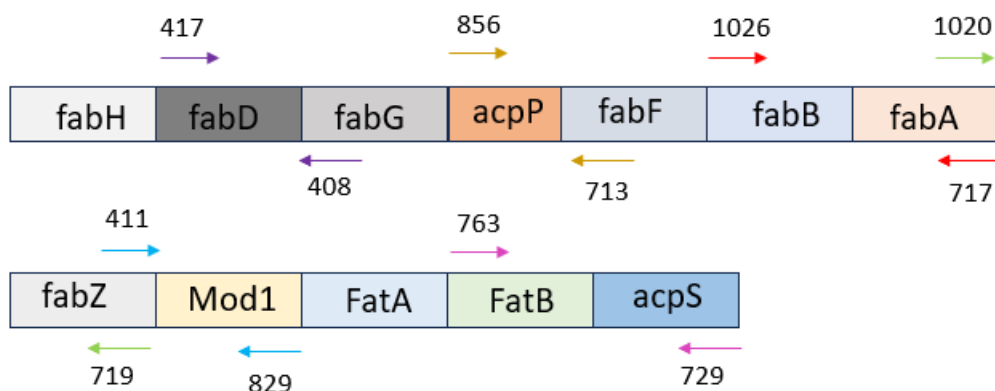


Figure 30 – Schematic representation of the primers used to confirm the presence of the genes belonging to the FASIIb pathway. Each pair of primers is represented in the same colour.

All PCR products appeared to have the right size in the agarose gel, confirming the constitution of the plasmids (**Figure 31**). The two pTA5_FASIIb plasmids were sequenced. The sequencing revealed that both plasmids had the predicted construction as the FASIIb sequence perfectly matched the pTA1_FASIIb plasmid. In order to test the functionality of the pTA5_FASIIb plasmid, the yeast strain transformed with the plasmid was grown in 50 mL of YPD media. However, it appeared that there was no growth. Despite its sequence being known and the FASIIb pathway being identical to the pathway in the pTA1_FASIIb plasmid, the pTA5_FASIIb plasmid did not complement the growth of a $\Delta fas2$ mutant strain in media without the addition of fatty acids. Considering the similarity between both plasmids, the KANMX selective marker might be considered the determining factor that prevents the growth in media depleted of fatty acids. Additionally, the lack of a native FAS system on top of a required resistance to an antibiotic may cause too much of a metabolic burden to the cells, hindering the growth.

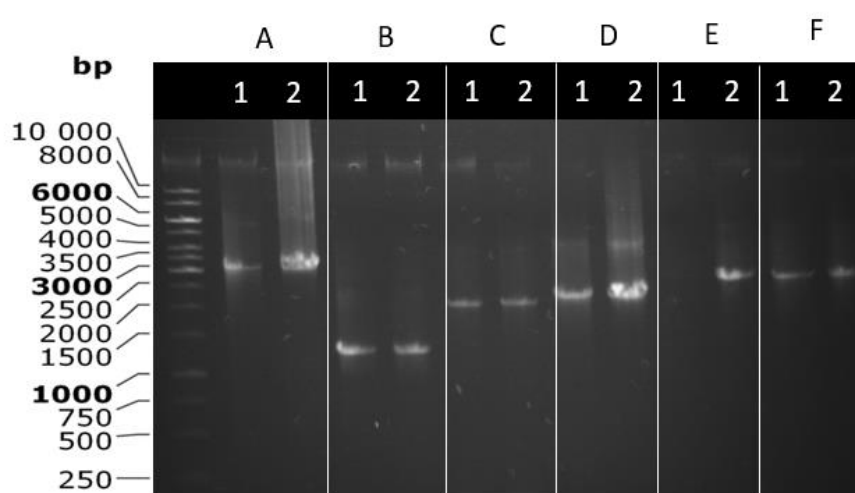


Figure 31 – Agarose gel showing the DNA products from the PCR reaction used to confirm the construction of the pTA5_FASIIb plasmids. The six different reactions are named from A to F. The two tested colonies are named 1 and 2. The used molecular weight marker was the GeneRuler 1kb DNA ladder (Thermo Scientific).

4. DISCUSSION

Saccharomyces cerevisiae has an important role across different industries. The production of high value chemicals is dependent on industrial *S. cerevisiae* strains that display metabolic adaptations that are best suited for those conditions. The production of economically important fatty acids and derivatives in yeast has the potential to be an alternative to traditional processes that are harmful to the environment. However, the production of fatty acids in *S. cerevisiae* is limited by the nature of the native type I fatty acid synthase system. The expression of a heterologous type II FAS system in yeast tackled this problem offering a range of possibilities regarding the production of different types of fatty acids in yeast.

The Enoyl-ACP reductase (ER) is the enzyme that catalyses the last reaction of the elongation phase in the production of fatty acids. Even though the general enzymatic reaction is conserved in nature, there is a wide variety of these enzymes (Massengo-Tiassé & Cronan, 2009). One of the major differences between different ERs is their preference in the use of NADH or NADPH as a cofactor. In this work, we tested the activity of different ERs in a *S. cerevisiae* strain which FA production depended on a heterologous type II FAS system (pTA1_FASIIb plasmid). The *AthMOD1* gene expresses the designated NADH-dependent ER enzyme in the pTA1_FASIIb plasmid. The results showed that the activity of an additional ER led to an increase in the growth rate of the designated yeast strain. This indicated that the enzymatic reaction catalysed by the ER enzyme can be a limiting factor in the production of fatty acids in yeast. The strains expressing the *BsfabL* and *SafabI* genes had a slightly lower growth rate compared to the strain expressing an extra *AthMOD1* gene. The *AthMOD1* appeared to be more efficient in yeast despite being dependent on NADH as a cofactor. A more effective interaction between the *Athfata* and *Athfatb* thioesterases with the *AthMOD1* enzyme could explain the increased efficiency in FA production.

In this work, we developed an expression vector that is controlled by a dominant selective marker and can be expressed in industrial *S. cerevisiae* strains. This was done by successfully assembling five DNA fragments by homologous recombination. By taking advantage of yeast's own DNA repair machinery, we were able to devise a strategy for the construction of several plasmids. The pTA5_FASIIb plasmid is controlled by the dominant *KanMX* marker and was successfully constructed by homologous recombination. This was achieved by transforming a

Δfas2 S. cerevisiae strain with a PCR product made up of the *KanMX* marker, flanked by two homologous regions to the pTA1_FASIIb plasmid that was being expressed.

The use of a high amount of PCR product increased the number of transformant colonies but not necessarily the efficiency of the homologous recombination. Additionally, the use of a highly diluted DNA template in the PCR reaction also reduced the risk of the occurrence of false positive colonies containing the pTA5 plasmid. However, the pTA5_FASIIb plasmid did not complement the growth of the *Δfas2* mutant yeast strain in media depleted of fatty acids. Meanwhile, the pTA1_FASIIb plasmid complemented the growth of the same strain under the same conditions. The *KanMX* marker gene, serving as the distinguishing factor between the two plasmids, could potentially exhibit toxicity under those circumstances. Additionally, the lack of the native FAS system, the required resistance to an antibiotic and the carrying of a large plasmid (nearly 25kbp long) may be too much of a metabolic burden to the cells.

5. CONCLUSION

The utilization of a heterologous type II FAS system in *S.cerevisiae* has the potential of increasing the production of a wider variety of FAs, by overcoming the robustness of the native type I FAS system. The pTA1_FASIIb plasmid is an expression vector that contains all the required genes for the heterologous production of fatty acids as a type II FAS system. Inside this plasmid, the *MOD1* gene from *Arabidopsis thaliana* encodes for the Enoyl-ACP reductase enzyme that catalyses the last step of the fatty acid production. Unlike the *MOD1* enzyme, the *FABL* and the *FABI* genes from *Bacillus subtilis* and *Streptococcus aureus*, respectively, encode for an ER enzyme that prefers NADH as a cofactor. In this work, we compared the growth of a $\Delta fas2$ yeast strain when expressing different Enoyl-ACP reductases. The results showed that the overexpression of the *MOD1* gene caused the highest growth rate of this yeast strain. Nevertheless, all ER enzymes improved the growth of this yeast strain which indicates that the gene dosage for this reaction could be a limiting factor in the production of fatty acids by the pTA1_FASIIb plasmid.

In the hopes of expressing the pTA1_FASIIb plasmid in industrial *S. cerevisiae* strains, we needed to change the selective marker of this plasmid. To do this, we took advantage of the fact that the backbone of the pTA5 plasmid was homologous to that of pTA1_FASIIb plasmid. By homologous recombination we set out to express the *KanMX* dominant marker in the pTA1_FASIIb plasmid, which we named pTA5_FASIIb. We required a mutant yeast strain that had no resistance to genetic. Therefore, we conceived a $\Delta fas1 \Delta fas2$ mutant strain by the Cre/loxP method and recycled the *KanMX* marker used in the deletion of these genes. By sequencing the pTA5_FASIIb plasmid, we were able to confirm the nucleotide sequence that compose this vector. Despite having an identical composition to the pTA1_FASIIb plasmid, the pTA5_FASIIb plasmid does not complement the growth of a $\Delta fas1 \Delta fas2$ mutant strain in media lacking fatty acids.

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7. APPENDIX

7.1 Dropout amino acid mixture

Compound	Concentration (mg/L)
Alanine	19.74
Arginine	78.96
Asparagine	78.96
Aspartic Acid	78.96
Cysteine	78.96
Glutamic Acid	78.96
Glutamine	78.96
Glycine	78.96
Isoleucine	78.96
Lysine	78.96
Methionine	78.96
Phenylalanine	78.96
Proline	78.96
Serine	78.96
Threonine	78.96
Tyrosine	78.96
Valine	78.96
Myo-inositol	78.96
para-Aminobenzidine	78.96
Total	1.37 g/L

7.2 pTA5 construction

7.2.1 Used primers

Primer name	Sequence (5'→3')
195	ATAATACCGCGCCACATAGC
255	TGATTTTGATGACGAGCGTAAT
977	TACAATAGAGTTCCGAGGTAAACGCTTTTCGTTCTTGTCTCATTGCC
978	AACTGTAAAATCAGGTATCTCGTAGTCCGTGTTCTGATCCTCGAGC
983	CAGAGCAGACAGTTCCTTTACGAGATTTTAGATCCAATATCAAAGGAA
984	ATCGTATCTGCTGCGTAAATAGTAGTCAACGATCGTACTTGTTACCCAT
987	AGAAAGTCTACACCTTACGCTGATTGGATTGAAGTTTTAAATCAATCTAAA
1113	GAAAAGCGTTTACCTCGGAACTCTATTGTAGAACCCCTATTTGTTTATTTTCT A
1195	GTTGACTACTATTTACGCAGCAGATACGATCTCGTTTCATCGGT
1196	AATCCAATCAGCGTAAGGTGTAGACTTTCTCTGTCAGACCAAGTTTACT
1349	ACGGACTACGAGATACCTGATTTTACAGTTCAGTATAGCGACCAGC
1350	TAAAATCTCGTAAAGGAACTGTCTGCTCTGGACATGGAGGCC
1481	CTATCGATTGTATGGGAAGCCCG

7.2.2 pTA5 Sequencing results

```

1987                                     2068
pTA5      cgaaactctcaaggatcttaccgctgttgagatccagttcgatgtaacccactcgtgcacccaactgatcttcagcatctt
Sequencin... CGAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTGATGTAACCCACTCGTGCACCCAACGATCTTCAGCATCTT
.....

2069                                     2150
pTA5      ttactttcaccagcgtttctgggtgagcaaaaacaggaaggcaaaatgccgcaaaaagggaataagggcgacacggaaatg
Sequencin... TTACTTTCAACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAGGGAATAAGGGCGACACGGAATG
.....

2151                                     2232
pTA5      ttgaatactcatactcttcctttttcaatattattgaagcatttatcagggttattgtctcatgagcggatacatatttgaa
Sequencin... TTGAATACTCATACTCTTCCTTTTCAATATTATTGAAGCATTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAA
.....

2233                                     2314
pTA5      tgtatttagaaaaataaacaatagggttctacaatagagttccgaggtaaacgcttttcgttcttctcattgccacat
Sequencin... TGTATTTAGAAAAATAACAAATAGGGGTTCTACAATAGAGTTCGAGGTAAACGCTTTTCGTTCTGTCTCATTGCCACAT
.....

2315                                     2396
pTA5      tcataagtacccatccaagagcacgcttattcaccaggtgaaaaagcggaaacgctgtactacatcgtaaaggctcctgtg
Sequencin... TCATAAGTACCCATCCAAGAGCACGCTTATTCACCAGGGTGAAAAAGCGGAAACGCTGTACTACATCGTTAAAGGCTCTGTG
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2397                                     2478
pTA5      gcagtgctgatcaaaagacgaagagggtaaagaaatgatcctctcctatctgaatcaggggtgattttattggcgaactgggcc
Sequencin... GCAGTGCTGATCAAGACGAAGAGGGTAAAGAAATGATCCTCTCCTATCTGAATCAGGGTGATTTTATTGGCGAAGTGGGCC
.....

2479                                     2560
pTA5      tgtttgaagagggccaggaaacgtagcgcaggtgacgtgcgaaaaacgcctgtgaagatatcatgcgcagtcagtcaccaga
Sequencin... TGTTTGAAGAGGGCCAGGAACGTAGCGCATGGGTACGTGCGAAAAACGCCTGTGAAGATATCATGCGCATGAOGTCAACGAGA
.....

2561                                     2642
pTA5      cgctatgactcaccggacggcagcgtgcaaatcaaaattaccogtcaggaaattggccagattgtcggtgctctagacaaacc
Sequencin... CGCTATGACTCACCCGGACGGCATGCAAAATCAAAATTACCGTCAGGAAATTGGCCAGATTGTGGGCTGCTCTAGACAAACC
.....

2643                                     2724
pTA5      gtgggaacgaattcttaagatgctcgaggatcagaacacggactacgagatacctgattttacagttcagtatagcgaccagc
Sequencin... GTGGGACGAATTCTTAAGATGCTCGAGGATCAGAACACGGACTACGAGATACCTGATTTTACAGTTCAGTATAGCGACCCAGC
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2725                                     2806
pTA5      attcacatacagattgacgcagatgattactttctgcgcacttaacttcgcatctgggcagatgatgtcgaggcg-aaaaaaa
Sequencin... ATTCACATACGATTGACGCATGATATTACTTTCTGCGCACTTAACCTTCGCATCTGGGCAGATGATGTGCGAGGGGAAAAAAA
.....

2807                                     2888
pTA5      atataaatcacgctaacatttgattaaaatagaacaactacaatataaaaaaactatacaaatgacaagttcttgaaaacaa
Sequencin... ATATAAATCACGCTAACATTTGATTAAAAATAGAACAACATACAATATAAAAAAATAACAATGACAAGTTCCTTGAAAACAA
.....

2889                                     2970
pTA5      gaatctttttattgtcagttactgattagaaaaactcatcgagcatcaaatgaaactgcaatttattcatatcaggattatca
Sequencin... GAATCTTTTTATTGTGCTAGTACTGATTAGAAAAACTCATCGAGCATCAAATGAAACTGCAATTTATTATATCAGGATTATCA
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2971                                     3052
pTA5      ataccatatttttgaaaaagccgtttctgtaataagggagaaaaactcaccgaggcagttccataggatggcaagatcctggg
Sequencin... ATACCATATTTTGA AAAAGCCGTTTCTGTAATGAAGGAAAAAACTCACCGAGGCAGTTCATAGGATGGCAAGATCCTGGT
.....

3053                                     3112
pTA5      atcggctctgcgattccgactcgtccaacatcaatacaacctattaatttccctcgtcaa
Sequencin... ATCGGCTCTGCGATTCCGACTCGTCCAACATCAATACAACCTATTAATTTCCCTCGTCAA
.....

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7.3 pYPKpw_ENO2_gene_RPL5 expression vectors

7.3.1 Used primers

Primer name	Sequence (5'→3')
467	ATTTAAATCCTGATGCGTTTGTCTGCACAGA
468	GTCGAGGAACGCCAGGTTGCCCACT
567	GTCGGCTGCAGGTCACTAGTGAG
568	GTGCCATCTGTGCAGACAAACG
577	GTTCTGATCCTCGAGCATCTTAAGAATTC
578	GTTCTTGTCTCATTGCCACATTCATAAGT
1737	ATTTTAAACGAGCAGTGAGCGTC
1739	ATATTATTTAATTGCGTGGAATCC

pYPKpw_ENO2_GENE_RPL5

Primer name	Sequence (5'→3')
1304	TTCTCACTAGTGACCTGCAGCCGAAATGGCAATGTCTGAATCCA
1305	TGATGCGTTTGTCTGCACAGATGGCTAATTCTTGCTGTAAAGGTC

7.3.2 Sequencing of the ENO2_BsfabL_RPL5 transcription unit

TTAAATACGCGCGTTATGTCACTAACGACGTGCACCAACTTGCGGAAAGTGGAATCCCGTTCCAAA
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TGCCATATGCAAAATTCATGAAGTGTGATACCAAGTCAGCATAACCTCACTAGGGTAGTTTCTTTGG
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TATCATTTGGATTTTTGTGCAAGGTTGTAGAATTGTATGTGACAAGTGGCACCAAGCATATATAAAAA
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TTTCATAACACCAAGCAACTAATACTATAACATACAATAATAAGTTTAAAGTCGAGGAACGCCAGGTT
GCCCACTTTCTCACTAGTGACCTGCAGCCGACAAATGGAACAAAATAAATGTGCACTCGTAACAGGA
AGCAGCCGCGGTGTCGGAAAAGCGGCCGCGATCAGACTTGCTGAGAACGGCTATAACATCGTCATT

AACTATGCACGCAGCAAAAAAGCAGCATTAGAAACAGCGGAAGAAATCGAAAAGCTTGGCGTTAAA
 GTGCTTGTCTGTAAGCAAAACGTAGGACAGCCTGCAAAAATCAAAGAAATGTTTCAGCAAATTGATG
 AAACGTTCCGGCAGACTTGATGTTTTGTCAATAATGCCGCTTCAGGAGTACTAAGACCTGTCATGGAA
 TTAGAAGAAACACACTGGGACTGGACGATGAACATTAATGCGAAAGCATTGCTTTTCTGCGCTCAGG
 AAGCTGCCAAGCTAATGGAGAAGAACGGTGGCGGGCATATTGTCAGCATTAGTTCATTAGGCTCTAT
 CCGCTATCTTGAAAACACACCACGGTCGGTGTATCAAAAGCAGCGTTAGAGGCTTTAACCCGTTATC
 TTGCCGTTGAGCTTTACCAAAACAAATTATCGTCAATGCTGTTTCAGGCGGAGCGATCGACACAGAT
 GCGCTGAAGCACTTCCCGAATAGAGAAGATCTGCTTGAGGATGCGCGCCAAAATACGCCGGCGGGA
 CGCATGGTCGAAATTAAGACATGGTTGATACTGTGGAGTTTCTAGTGTCTTCCAAGGCTGACATGA
 TCCGCGGACAGACAATTATCGTTGACGGCGGACGCTCACTGCTCGTTTAAAAATGTGCCATCTGTGC
 AGACAAACGCATCAGGATTTAAATTTGTAAATACAAAGTCATTCAATGTACATAAATACTGTTATGCT
 TTTGAATTAAGTAAACACCATGACTTCATATTGAGATGTTTTAAGCATATAATCACGGCACCGAACT
 TCTTGTAATCATCAAATCTTTGTTTTCTAGCCGTCATAGAAATGAAGAAATGAAGATTCTTGTTTCATGT
 GATACAGCTTTTCTAGACAGCTAACTTGTACATCCAGACATCCTAAAACACCCATAAAGCAAAAAAAA
 TTATTGAAATATTCTCTTTTTTTCTTTTTTTTTCTGGTGGCTTTCAATGCGGAATCGCTCACATCCGA
 AAATTTAGAGGCTTCACTTAGCGTCGCAGATCCCAGTAGTCGCCAGATGCTTTGAAAAAAAAAAAA
 TAAATAGAAAAGTTTGTAGACATATCCGATGTAGTATATTTAGAGGCCAACCTTTACTTTTGTCTTCT
 CCTACACTTCAAATAAAAAGACACCGTCACAAATCTCAGCAACA

7.3.3 Sequencing of the ENO2_Safabl_RPL5 Transcription unit

TTAAATACGCGCGTTATGTCACTAACGACGTGCACCAACTTGCGGAAAGTGGAATCCCGTTCCAAA
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 TGCCATATGCAAAATTCATGAAGTGTGATACCAAGTCAGCATACACCTCACTAGGGTAGTTTCTTTGG
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 TATCATTTGGATTTTTGTGGAAGGTTGTAGAATTGTATGTGACAAGTGGCACCAAGCATATATAAAAA
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 GCCCACTTTCTCACTAGTGACCTGCAGCCGACTACATGTTAAATCTTGAAAACAAAACATATGTCATC
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 TACGCGGACGCTTTTCTGAACTTCACGTGAAGGCTTCTTGTTAGCTCAAGACATTAGTTCTTACTCAT
 TAACAATTGTGGCTCATGAAGCTAAAAAATTAATGCCAGAAGGTGGTAGCATTGTTGCAACAACATA
 TTTAGGTGGCGAATTCGCAGTTCAAATTATAATGTGATGGGTGTTGCTAAAGCGAGCTTAGAAGCA
 AATGTTAAATATTTAGCATTAGACTTAGGTCCTGATAATATTCGCGTTAATGCAATTTAGCTGGTCCA
 ATCCGTACATTAAGTGCAAAGGTGTGGGTGGTTTCAATACAATTCTTAAAGAAATCGAAGAGCGTG
 CACCTTTAAAACGTAACGTTGATCAAGTAGAAGTAGGTAAAACAGCGGCTTACTTATTAAGTGACTTA
 TCAAGTGGCGTTACAGGTGAAAATATTCATGTAGATAGCGGATTCCACGCAATTAAATAATATGTGC
 CATCTGTGCAGACAAACGCATCAGGATTTAAATTTGTAAATACAAAGTCATTCAATGTACATAAATAC
 TGTTATGCTTTTGAATTAAGTAAAACACCATGACTTCATATTGAGATGTTTTAAGCATATAATCACGGC
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 GTTCATGTGATACAGCTTTTCTAGACAGCTAACTTGTACATCCAGACATCCTAAAACACCCATAAAGC
 AAAAAAATTATTGAAATATTCTCTTTTTTTCTTTTTTTTTCTGGTGGCTTTCAATGCGGAATCGCT
 CACATCCGAAAATTTAGAGGCTTCACTTAGCGTCGCAGATCCCAGTAGTCGCCAGATGCTTTGAAA
 AAAAAAATAAATAGAAAAGTTTGTAGACATATCCGATGTAGTATATTTAGAGGCCAACCTTTACT
 TTTGTTTTCTCCTACACTTCAAATAAAAGACACCGTCACAAATCTCAGCAACA

7.4 *FAS1* deletion and marker recycling

7.4.1 Used primers

Primer name	Sequence (5' → 3')
99	GTCAAGCTTCGTACGATCGCCATCTTCCAG
114	GTACTGTACAAGGGTGGAGGTGGAGGATCTATGTCCAATTTACTGAC
1418	CAAGAACATCATCCGGAAAATCAGATTATAGACTAGGATTCCGCT
1449	TTAAGACTATTAAACGGTAGAATCTGC
1564	CACTCGCATCAACCAAACC
1459	AATTCTTCAAAGTAGTCGTCGGTG
1742	ACGGACTACGAGATAC
1748	AATCCAATCAGCGTAAG

7.5 pTA5_FASIIb construction

7.5.1 Used primers

Primer name	Sequence (5'→3')
714	AAATGAATTTTGAAGGAAAAATC
715	TCAGACCATGTACATCCC
408	TTTAAACTTTTGAATATGTATTACTTGGTTATGGT
411	TTAAATACGCGGCGTTATGTCAC
417	TTAAATAACAATGCATACTTTGTACGTTCA
713	TTAGATCTTTTAAAGATCAAAGAAC
717	TCAGAAGGCAGACGTATC
719	TCAGGCCTCCCGGCTA
729	TTAACTTTCAATAATTACCGTGG
763	TTAAATCTACATAAGTGCAATTAAAGCAT
829	TAATTAATGTTGCTGAGATTTGTG
856	TTAAATTGTAATGTTCTAACAAAAATTT
1020	TTAAATAGATACTAAAAAGAAACCTTC
1026	TTAAATCGATTTCCCACATCCT

7.5.2 pTA5_FASIIb sequencing

Page 1 pTA1_FASIIb.plasmid_annotations

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3727                                     3808
pTA5_FASIIb1 atgtatacgaagattattggtactggcagctatctgccgaacaagtgcggacaaacgcgatttggaaaaaatggtggaca
pTA1_FASI... atgtatacgaagattattggtactggcagctatctgccgaacaagtgcggacaaacgcgatttggaaaaaatggtggaca
.....

3809                                     3890
pTA5_FASIIb1 cctctgacgagtggtattgtcactcgtacgggtatccgcgaacgccacattgccgcgcaaacgaaacggtttcaaccatggg
pTA1_FASI... cctctgacgagtggtattgtcactcgtacgggtatccgcgaacgccacattgccgcgcaaacgaaacggtttcaaccatggg
.....

3891                                     3972
pTA5_FASIIb1 ctttgaagcggcgacacgcgcaattgagatggcgggcattgagaaagaccagattggcctgatcgttgtggcaacgacttct
pTA1_FASI... ctttgaagcggcgacacgcgcaattgagatggcgggcattgagaaagaccagattggcctgatcgttgtggcaacgacttct
.....

3973                                     4054
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pTA1_FASI... gctacgcacgctttcccgagcgcagcttgtcagattcaaaagcatgctgggcattaaaggctgcccggcatttgacgttgcag
.....

4055                                     4136
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.....

4137                                     4218
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pTA1_FASI... cggttccgatgtactggcgcgcacctcgggtccaaccgatcgtgggactattattatttttggcgatggcgcgggcgtcgcg
.....

4219                                     4300
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pTA1_FASI... gtgctggcgccctctgaagagccgggaatcatctccaccatctgcatgcgcacggtagctatggtgagttgctgacgctgc
.....

4301                                     4382
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.....

4383                                     4464
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.....

4465                                     4546
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4547                                     4628
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.....

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4629                                     4710
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.....

4711                                     4792
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pTA1_FASI... aaataacaatgcatactttgtacgttcaaaatacaatgcagtagatatatttatgcataattacataataacacatatcacata
.....

4793                                     4874
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pTA1_FASI... ggaagcaacaggcgctggacttttaattttcgaggaccgcgaatccttacatcacaccaatccccacaagtgtatcccc
.....

4875                                     4956
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.....

4957                                     5038
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.....

5039                                     5120
pTA5_FASIIb1 aaagaaaaagagacgcctcgtttctttttctctcgtcgaaaaaggcaataaaaaatttttatcacgtttctttttcttgaaa
pTA1_FASI... aaagaaaaagagacgcctcgtttctttttctctcgtcgaaaaaggcaataaaaaatttttatcacgtttctttttcttgaaa
.....

5121                                     5202
pTA5_FASIIb1 a-tttttttttttgatttttttctctttcogatgacctccattgatatttaagttaataaacggtcttcaatttctcaagtt
pTA1_FASI... atttttttttttgatttttttctctttcogatgacctccattgatatttaagttaataaacggtcttcaatttctcaagtt
.....

5203                                     5284
pTA5_FASIIb1 tcagtttcatttttctgtttctattacaacttttttacttcttgcctcattagaagaaagcatagcaatcctaatcctaaagtt
pTA1_FASI... tcagtttcatttttctgtttctattacaacttttttacttcttgcctcattagaagaaagcatagcaatcctaatcctaaagtt
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5285                                     5366
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16847                                     16928
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16929                                     17010
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17011                                     17092
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17093                                     17174
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17175                                     17256
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17257                                     17338
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17339                                     17420
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17421                                     17502
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17503                                     17584
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17585                                     17666
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17995                                     18076
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18241                                     18322
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18323                                     18404
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18405                                     18486
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18487                                     18568
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18569                                     18650
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18651                                     18732
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18733                                     18814
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18815                                     18896
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18897                                     18978
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18979                                     19060
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19143                                     19224
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19225                                     19306
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19307                                     19388
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19389                                     19470
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19471                                     19552
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19553                                     19634
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19635                                     19716
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19717                                     19798
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19799                                     19880
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19881                                     19962
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20045                                     20126
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20127                                     20208
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20209                                     20290
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20291                                     20372
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20373                                     20454
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20455                                     20536
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20537                                     20618
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20619                                     20700
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20701                                     20782
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20783                                     20864
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