PONTIFICIA UNIVERSIDAD CATOLICA DE CHILE

SCHOOL OF ENGINEERING

CONSTRUCTION OF A YEAST PLATFORM FOR THE SYNTHESIS OF HIGH VALUE NATURAL FLAVOURS

JAVIERA C. LÓPEZ SALINAS

Thesis submitted to the Office of Graduate Studies in partial fulfillment of the requirements for the Degree of Master of Science in Engineering (or Doctor in Engineering Sciences)

Advisor:

EDUARDO AGOSIN TRUMPER

Santiago de Chile, (January, 2016)

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To my parents, Cecilia and Hector.

To my family, Francisco and Trinidad

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SCHOOL OF ENGINEERING

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ABSTRACT

Metabolic engineering has opened an opportunity for the production of a vast array of valuable compounds, especially for those naturally produced in small amounts from non-renewable resources, or whose chemical synthesis are inherently difficult and expensive. In the case of terpenes, numerous opportunities and strategies exist for biochemical synthesis using natural and engineered microorganisms.

Saccharomyces cerevisiae is one of the main microorganisms used for the expression of heterologous genes, due mostly to its fast growth on different carbon sources, the vast knowledge of its genome and tools for its manipulation, and the robustness in large-scale fermentations. Moreover, in the case of *S. cerevisiae,* its mevalonate pathway, which finally produces ergosterol, is also capable of producing precursors of high-value compounds. In the recent decades, advances in the fields of system biology, evolutionary engineering, and synthetic biology have accelerated the progresses for the use of this yeast as the microbial cell factory of choice.

We constructed a series of yeast platforms for the synthesis of two types of terpenes: the monoterpene carvone and the norisoprenoid β-ionona. Monoterpenes are mostly

produced by plants as a defense mechanism against depredators and can be used by the food industry as antiseptic compounds, fragrances and additives. Norisoprenoids are knows by their pleasant flavor and are used by the food industry as additives, but are also highly appreciated by the cosmetic industry. In this study, we used different gene expression promoters and plasmids, to control gene transcription and gene dosage.
For the norisoprenoid synthesis, we constructed several β-ionone producing strains. We first integrated three heterologous genes in the engineered SCGI22a strain: the carotenogenic genes *crtYB* and *crtI*, from *Xanthophyllomyces dendrorhous,* to produce β-carotene, and the carotenoid cleavage dioxygenese (*PhCCD1* gene), from *Petunia hybrida*, to produce β-ionone. In this strain we reached a maximal concentration of 0.63 ± 0.02 mg/g biomass in shake flask cultures. Further gene expression of these three genes in high copy number plasmids, improved the final titer 15-fold, reaching more than 5 mg/L in a batch culture (Chapter 2).

The accumulation of ß-carotene, in mostly all constructed strains, may indicate a poor efficiency in the CCD1 enzyme activity or a poor expression of the gene. Since in this study the constitutive over-expression of the *PhCCD1* resulted in low growth strains, we decided to swap the original constitutive promoter used for this gene (P_{TEF1}) for the *Gal1*promoter, inducible by light. In this system, the Gal1 transcription factor was split in its DNA-binding domain (BD) and its activation domain (AC) and these two components were fused to two *Neurospora crassa* cryptochromes, called White collar -1 (WC-1) and Vivid-1 (VVD1). These two flavoproteins responded to blue light, interacting with each other and reconstituting the Gal1 transcription factor. Even though this system worked with a reporter gene, we could not improve the β-ionone production, probably for the prolonged light induction which affected carotenes (Chapter 3). Nevertheless, this system has several advantages and further optimizations are promising.

Finally, utilizing the same design used for norisoprenoids synthesis, we constructed a yeast strain for the production of the monoterpenes limonene and carvone. We expressed five heterologous genes: The *GGPS* gene from *Abies grandis* which encodes a geranyl diphosphate synthase; the *Mentha piperita* genes *LS*, *LH* and *CD* for the production of the monoterpenes limonene, trans carveol and carvone respectively; and a mutated version of the yeast gene *ERG20*, which encodes the enzyme that produces geranyl diphosphate (GPP) and farnesyl diphosphate (FPP). No monoterpenes were detected in cultives of this strain, possibly explained by monoterpenes volatility/toxicity and by the absence of their direct precursor, GPP. Further optimizations are required (Chapter 4).

There still plenty of room for improvements in all the platforms created here. The knowledge generated, together with the extraordinary advances in synthetic biology, will allow us the possibility to produce industrially competitive compounds, through a sustainable and low-cost production process.

Keywords: Terpenes, *Saccharomyces cerevisiae*, ß-ionone, carvone, limonene, metabolic engineering.

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PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE
ESCUELA DE INGENIERÍA

CONSTRUCCIÓN DE UNA PLATAFORMA EN LEVADURA PARA LA SÍNTESIS DE COMPUESTOS AROMÁTICOS NATURALES DE ALTO VALOR

Tesis enviada a la Dirección de Investigación y Postgrado en cumplimiento parcial de los requisitos para el grado de Doctor en Ciencias de la Ingeniería.

JAVIERA C. LÓPEZ SALINAS

RESUMEN

La ingeniería metabólica ha abierto una oportunidad para la producción de una amplia variedad de compuestos de alto valor industrial, especialmente aquellos producidos en bajas concentraciones desde fuentes no-renovables, o cuya síntesis química es difícil y costosa. En el caso de los terpenos, existen numerosas oportunidades y estrategias para su síntesis bioquímica usando microorganismos naturales o "*ingenierizados"*.

Saccharomyces cerevisiae es uno de los principales microorganismos usados para la expresión heteróloga de genes, debido principalmente a su rápido crecimiento en diferentes fuentes de carbono, el amplio conocimiento de su genoma y las herramientas para su manipulación, y su robustez en fermentaciones a gran escala. Además, en el caso de *S. cerevisiae,* la vía del mevalonato que lleva finalmente a la producción de ergosterol, es también capaz de producir precursores de compuestos de alto valor. Avances en el campo de la biología de sistemas, ingeniería evolutiva, y biología sintética han acelerado el progreso para el uso de esta levadura como la factoría celular microbiana de elección.

En este trabajo se construyeron una serie de plataformas en levadura para la síntesis de dos tipos de terpenos: el monoterpeno carvona y el norisoprenoides β-ionona. Los monoterpenos son principalmente producidos por las plantas como mecanismo de defensa contra depredadores, y pueden ser usados por la industria de los alimentos como antisépticos, fragancias y aditivos. Los norisoprenoides son conocidos por su agradable aromas y también usados en la industria de alimentos como aditivos, pero además, altamente apreciados por la industria cosmética. En este estudio, se usaron diferentes promotores de expresión y plasmidios para controlar la transcripción y la dosis génica.
Para la síntesis de norisoprenoides, se construyeron varias cepas de levadura, capaces de producir β-ionona. Primero, se integraron tres genes heterólogos en la cepa *ingenierizada* SCGI22a: los genes carotenogénicos *crtYB* y *crtI* de *Xanthophyllomyces dendrorhous,* para producir β-caroteno, y el gen dioxigenasa de escisión de carotenos, (gen *PhCCD1*)*,* de *Petunia hybrida*, para producir β-ionona. En esta cepa se alcanzó una concentración final de 0,63 ± 0,02 mg/g biomasa en matraces. Una posterior expresión de estos tres genes en vectores de alta copia, mejoró la concentración final, alcanzando mas de 5 mg/L en cultivos *batch* (Capítulo 2).

La acumulación de ß-caroteno en todas las cepas construidas, puede indicar una baja eficiencia en la actividad de la enzima CCD1, o una baja expresión de su gen. Debido a que en este estudio, la sobre-expresión de este gen resultó en cepas que crecían muy lento, se decidió cambiar el promotor original usado para este gen (P_{TEF1}) por el promotor *Gal1* inducible por luz. En este sistema, el factor de transcripción Gal1 fue dividido en sus dominios de unión a ADN (BD) y de activación (AC), y estos dos componentes fusionados a dos criptocromos de *Neurospora crassa* llamados White collar -1 (WC-1) y Vivid-1 (VVD1). Estas dos flavoproteínas responden a luz azul interaccionando una con otra, reconstituyendo el factor de transcripción Gal1. A pesar de que este sistema funcionó con un gen reportero, no se logró optimizar la producción de β-ionona, probablemente por la inducción prolongada con luz, lo que afectaría a los

carotenos (Capítulo 3). Sin embargo, este sistema presenta varias ventajas y futuras optimizaciones son promisorias

Finalmente, utilizando el mismo diseño usado para la producción de norisoprenoides, se construyó una cepa de levadura para la producción de los monoterpenos limoneno y carvona (Chapter 4). Para ello, se expresaron cinco genes heterólogos: El gen *GPPS* de *Abies grandis* que codifica para la enzima geranil difosfato sintasa; los genes de *Mentha piperita*, *LS*, *LH* y *CD* para la producción de los monoterpenos limoneno, trans-carveol y carvona respectivamente; y una versión mutada del gen de levadura *ERG20*, que codifica para la enzima que produce geranil difosfato (GPP) y farnesil difosfato (FPP). Ningún monoterpeno fue detectado en cultivos de esta cepa, debido posiblemente a la volatilidad/toxicidad de los monoterpenos y la ausencia de su precursor directo GPP. Otras optimizaciones son necesarias.

Aún hay mucho espacio para la optimización de las cepas creadas en este estudio. El conocimiento generado, junto con los extraordinarios avances en biología sintética, nos permitirán la posibilidad de producir compuestos industrialmente competitivos, a través de procesos sustentables y de bajo costo.

Palabras claves: Terpenos, *Saccharomyces cerevisiae*, ß-ionone, carvona, limoneno, ingeniería metabólica.

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1. INTRODUCTION

1.1 METABOLIC ENGINEERING

During the last decades, biotechnology is redefining the industrial production of chemical compounds, providing the technology and knowledge to replace petrochemical production processes by more sustainable alternatives. The efforts to become a greener society, together with new consumer preferences and acceptance, have forced a change in the manufacturing and use of petrochemical feedstocks toward the use of biological systems for the synthesis of natural products [1, 2]. Since the 1980s, significant advances in genetic engineering and metabolic modeling have allowed to employ some well-known microorganisms as cell factories, for the production of compounds of both natural and heterologous origin.

Metabolic Engineering (ME) focuses on this task, allowing the construction of new platforms. Even though there are several definitions of ME, most of them agree in the design of new hybrid organisms with novel properties and capabilities for the biosynthesis of a wide range of products [3–5]. Advances in ME have allowed the construction of multi-step enzymatic pathways to synthesize complex molecules, such as isoprenoids [6] (Chapter2 of this thesis), alkaloids [7], and steroids [8, 9]; from renewable raw materials. An increasing number of engineered microorganisms have been developed, and now the next challenge is to reconstruct efficient pathways/platforms by redirecting carbon flux from the precursor metabolites toward the compound of interest [10]. In addition, the advances in the fields of systems biology, synthetic biology, and evolutionary engineering have allowed performing ME in a more systematically and holistic way in the industrially well-known microorganisms [5].

The yeast *Saccharomyces cerevisiae*, traditionally employed for beer, wine, and bread production is currently extensively used for bio-based applications [4, 11, 12]. *S. cerevisiae* is a well-characterized microorganism with a wide range of available tools for genetic manipulation, making it very attractive for biotechnological applications. Genes can be easily introduced, deleted, replaced, over- or under -expressed in this organism. It is also considered a GRAS (Generally Regarded As Safe) organism, with industrial robustness and product yields capacity in the *multi*gram range [13]. One of the most remarkable examples of heterologous compound production in this microorganism is the synthesis of artemisinic acid, precursor of the artemisinin. Artemisinin, a sesquiterpene lactone extracted from *Artemisia annua* leaves, is highly effective against multi-drug resistant *Plasmodium* spp. However, as this compound is present in very low quantities in the plant, it is unaffordable to most malaria sufferers [14]. Through the expression - and further optimization - of a multistep heterologous pathway in yeast, it is now possible to provide up to one-third of the global need for this antimalarial drug, with a more sustainable and ecological strategy [15].

With the increase of platforms developed, as well as optimization in fermentation and downstream processes, the range and variety of industrial compounds that can be synthesized by this microorganism is continuously growing. Synthetic biology and system biology tools are two key components that are accelerating this development [16]. Therefore, the first part of this introduction will focus on synthetic and systems biology tools and how these allow the development of different platforms in *S. cerevisiae*. In the second part, the isoprenoid biosynthetic pathways will be introduced, together with the main transformations required in *S. cerevisiae* for economically-feasible synthesis of bioproducts.

1.1.1 SYSTEMS BIOLOGY

ME was initially employed to manipulate microorganisms as chemical factories, based in intuitive or traditional approaches, i.e. screening the best strains with improved

production performance [17]. Nevertheless, the goal has always been to establish the design process in a rational way [18]. The understanding of how cells use energy and substrates for growth and product synthesis, was boosted by the advances in the –*Omics*[*], which allowed to accelerate the improvements of these cell factories during the last decade [19]. On the other hand, the availability of detailed mathematical models expanded the predictive capacity of *in silico* analysis of metabolic flux distribution. The combination of these two tools delimits the **systems biology** field. The latter allows obtaining new, integrative insights of the molecular mechanisms occurring inside the cell. These insights are reached through the combination of high-throughput experimental methods (top-down approaches) and mathematical modeling (bottom-up approaches) [4]. Systems biology tools can provide targeted predictions for genetic modifications that improve strain performances, avoiding unnecessary or random changes to the host [17].

With the arrival of whole-genome massive sequencing techniques, together with the exponential increase of computational performance, the first major advancements in the field of systems biology arose with the development of Genome Scale Metabolic models (GSM). GSM models describe cellular metabolism based on gene-protein-reaction associations, as well as physiological and transcriptional data under steady-state conditions [10, 20]. These advances, and the methods derived from GSM models, like Flux Balance Analysis (FBA), enabled the first studies in the field of systems ME, where the strains were built and improved through the combination of systems biology, synthetic biology and evolutionary engineering [17]. For example, *in silico* ME algorithms were used to identify target reactions in the metabolic network of the synthesis of vanillin by *S. cerevisiae*, which led to an improved production rate [21]. The current availability of high- throughput metabolomic and fluxomic data, along with thermodynamics information, opens the possibility to build genome-scale kinetics models, in an effort to develop more inclusive, robust, and realistic models [22]. Kinetic

[*] Fields of large-scale data-rich biology, like genomics, transcriptomics, proteomics and metabolomics.

models have been developed for specific pathways in few microorganisms, in addition to metabolic flux balances, to achieve a dynamic description of metabolic responses of the cell [20]. These efforts are limited by incomplete or missing information about kinetic properties of enzymes and their regulation, but the new methodologies will allow a more accurate representation of the metabolic networks.

1.1.2 SYNTHETIC BIOLOGY

Besides computational tools, **synthetic biology** is also boosting the development of cell factories. Synthetic biology encompasses the construction of non-natural biologically functional DNA parts, modules and systems by employing molecular biology and synthetic DNA tools [5]. The proper functioning of synthetic circuits relies on precise control and tuning of the expression levels and activities of the protein components [23]. The balance of enzyme levels avoids the metabolic burden due to over-expression of proteins [24, 25], accumulation of toxic intermediates [26], and permits redirecting metabolic fluxes without affecting cell growth and viability [27]. There are multiple levels inside the cell at which metabolic fluxes can be modified. The classical ones (and best understood) are gene copy number, transcription efficiency, mRNA stability, translation efficiency, protein stability and allosteric control. Some of the most common strategies are summarized below.

Promoters

Among the tools available to redesign the cellular metabolism within a cell factory, one of the most popular is the control of transcription efficiency. Promoters are DNA parts that naturally control the expression of a gene in a given physiology condition or period of time. Their structural diversity within a genome allows a complex gene- and condition- dependent transcription regulation [28].

In *S. cerevisiae*, several constitutive promoters are well characterized in terms of relative activity. Many of them originate from genes encoding enzymes of the yeast glycolytic

pathway, for instance the promoters of genes encoding for phosphoglycerate kinase (P_{PGK1}), alcohol dehydrogenase (P_{ADH1}), glyceraldehyde-3-phosphate dehydrogenase (P_{TDH3} also known as P_{GPD}) and pyruvate kinase (P_{PYK1}). Other frequently used promoters derive from genes encoding the translation elongation factor 1 (P_{TEF1}), a cytochrome c isoform (P_{CYC1}), actin (P_{ACT1}) or the hexose transporter 7 (P_{HXT7}). Partow et al. (2010) [29], compared the relative strength of seven different constitutive promoters (P_{TEF1}, P_{TPI1}, P_{TDH1}, P_{ADH1}, P_{PGK1}, P_{HXT7} and P_{PYK1}) against the inducible *GAL1/GAL10* promoter by using *LacZ* as a reporter gene. They observed that the promoter relative activity varied with the glucose concentration and whether the cells were growing on glucose or ethanol. This indicated that a better strategy to over-express a gene is the use of different promoters at different physiological conditions or metabolic states. Another study by Sun et al., (2012) [30], complemented this conclusion by evaluating 14 pairs of constitutive promoters and terminators in oxygen-limited and oxygen-sufficient conditions, using GFP as a reporter. Constitutive promoters were used for the production of the flavour ß-ionone and carvone, as discussed in Chapters 2 and 4 of this thesis.

When the final product of a pathway can be synthesized during the entire fermentation process, constitutive promoters represent a useful tool, and still are considered the simplest way to control protein levels inside the cell. However, fine-tuning is required when the central carbon and energy metabolism are the targets, in order to ensure the appropriate balancing of all the cell requirements for their basic metabolism and the compounds production. In those cases, a better strategy for heterologous compound production is to uncouple biomass synthesis from product formation, allowing the cell to grow without the burden caused by the former. Inducible or regulatory promoters have been usually chosen when control of gene expression is needed as an on-off switch [28]. Promoter regulation usually occurs at the *cis*- regulatory elements through recruitment of a transcriptional activator or by the release of a transcriptional repressor [31]. Specific nutrients can be used as chemical stimuli when added to the medium. Other stimuli can be exerted by the accumulation of intracellular metabolites or fermentation products during cell growth [32]. The galactose pathway system is a classic example of an

inducible system in *S. cerevisiae*, where the promoters of *GAL1*, *GAL7* and *GAL10* genes are glucose-repressed and galactose inducible [33]. This system has been employed for improving isoprenoid biosynthesis by engineering of the acetyl-CoA and mevalonic acid pathways in wild yeast with a push-pull-restrain strategy [34]. Other examples include the *CUP1* [35] and *MET25* [36] promoters, which are tightly regulated by copper and methionine, respectively, via endogenous pathways.

The re-use of multiple parts, like the same promoters in the same genome, can threaten the integrity of these synthetic designs. Modifications of the promoter sequence can optimize some features, like activity and regulation, and avoid instability problems. Libraries based on error-prone PCR are available with several constitutive promoters based on *TEF1* promoter [37, 38], while conserving key elements for gene expression, such as the TATA box [39], allows both up and down regulation of genes in *S. cerevisiae*. Combining sections from different natural or heterologous promoters to produce chimeric promoters can also regulate genes without affecting other natural networks. This property, referred as *orthogonality*, allows the regulation of the DNA parts of a synthetic circuit in an independent way. Nevertheless, a much attractive approach to accomplish this orthogonality is by fusing endogenous domains from different transcription factors to responsive proteins, inducing the formation of chimeric complexes. Shimizu-Sato et al., (2002) [40] demonstrated that the expression of two light responsive proteins, a phytochrome and a phytochrome interacting protein (PIF3), fused respectively to the DNA binding domain and activation domain of Gal4 transcription factor, allowed the induction of genes containing in their promoters Gal4 DNA binding sites. This induction is triggered by red light, which can be controlled precisely by titration of the number of photons delivered to the cell by a light pulse. This system is non-invasive, fast and could be a rapidly switchable control for expression of genes. Other light systems have been developed, with other features and advantages, as discussed in Chapter 3 of this thesis.

RNA-based control elements

RNA parts as gene expression control devices have generated increasing attention than other biomolecules present in the cell. RNA devices are capable of performing several biological functions, such as gene regulation, directed conformational change and ligand binding. Hence, RNA molecules can regulate gene expression by antisense binding, by conformational changes that disrupt transcription/translation and by ribozyme activity [41]. In general terms, RNA parts can be grouped into three categories based on function: sensors, actuators and transmitters. RNA sensors detect diverse signals, such as temperature and molecular ligands, mostly by conformation changes; RNA actuators control the activity of other biological molecules, either *in cis* or *in trans*; and RNA transmitters are RNA sequences that translate an informational event, such as signal detection by a sensor, from one RNA part to another through conformational changes. Some RNA devices are composed by more than one functionally distinct part. For example, riboswitches are complex folded RNA domains, present in the non-coding portions of various mRNAs that serve as receptors for specific metabolites, which finally result in the control of gene expression [42]. Although riboswitches have not been explored in yeast for isoprenoid production, they can be used as an approach to control proteins levels. For example, riboswitches could sense metabolites from the isoprenoid pathway to feedback control and maximize flux toward the heterologous pathway. The use of antisense RNA is another alternative successfully employed to down-regulate gene expression. The use of small regulatory RNAs is a rapid and easy method to knockdown simultaneously several transcripts. Recently, Liu et al., (2015) [43] employed the antisense RNA strategy to weaken three genes of the heterologous mevalonate pathway in *E. coli*, increasing final product 8-fold. Clearly, this strategy is a useful and effective method to diminish the concentration of some by-products, allowing the accumulation of precursors of interest.

Protein engineering

The synthesis of bioproducts at industrial scale by metabolically engineered pathways are usually suboptimal, due to the highly regulated nature of biological systems. This regulation mostly prevents over-production of metabolites through feedback inhibition of enzymes. Sometimes, heterologous enzymes or proteins show low activities in the new host or alter the balance of native metabolism, affecting mostly cell growth by the accumulation of cytotoxic intermediates and by products [44, 45]. Protein engineering has been evaluated to modify natural proteins to suit the needs or requirements of any particular synthetic metabolic pathway [46]. Low enzymatic activities can be compensated by increasing its expression level, mostly by increasing the gene copy numbers into the genome or by the use of stronger promoters. Nevertheless, in order to reduce energy and metabolites (cell resources) expenses, sometimes it is more beneficial to engineer the target enzyme for higher *in vivo* activity [46]. The approaches used to modify proteins rely on the available information of the target protein. Rational design allows the engineering of new enzyme properties or the improvement of the existing ones, based on the three-dimensional structure and the knowledge of protein structure and function. Thus, the alterations of selected residues of the protein will cause predicted changes in its function [47]. An alternative approach is directed evolution, which can dispense of all the detailed information needed for the rational design [48, 49]. This molecular technique mimics the evolution processes *in vitro*, by combining random mutagenesis and/or recombination of DNA with high-throughput screening methods for selection of the desired protein variants [47]. Using a directed evolution approach, Wang et al. (2000) [50] enhanced the production of lycopene in an engineered *E. coli* strain, by random mutagenesis of the archeal geranylgeranyl diphosphate synthase (GGPS). From 10,000 colonies, eight mutants were obtained, and the final lycopene production was increased by about 2-fold. Protein engineering can also be employed to alter substrate and product specificities in order to improve final product yield or to produce novel compounds. For example, DNA shuffling was used to construct a library of phytoene desaturase (crtI) and lycopene cyclase (crtY) variants using the respective genes from

Erwinia uredovora and *Erwinia herbicola*. The expression of the shuffled enzymes in *E. coli* cells led to the production of carotenoids with different levels of saturation and to the discovery of a novel pathway for the production of torulene [51]. In both cases, protein engineering was successfully used as a tool of synthetic biology, using colorimetric visualization of the colonies as the selection method. Advances in molecular modeling, together with the identification of protein domains that could be used in optimized pathways, and automated high-throughput technologies, will accelerate the design of proteins for the construction of novel or improved platform.

Plasmids for gene expression in yeast

High and low copy episomal plasmids, as well as integrative plasmids, have been used extensively in ME to modify the yeast metabolism. Depending on the goal of the study, each type of plasmids present advantages and disadvantages. Yeast Episomal plasmids (YEp) are commonly employed to achieve high expression of target genes. Due to its autonomous replication origin, they can reach a high copy number inside the host cell and therefore a high expression of the genes carried [52]. Normally, the size limit of YEp allows the transfer of a small number of genes, instead of a whole pathway with several enzymes [53]. Maury et al., (2008) [54] transferred the seven genes encoding the isoprenoid MEP pathway of *E. coli* to *S. cerevisiae*, using episomal plasmids. These vectors could originally trigger the expression of two genes each, but in order to reduce the number of different plasmids used and selection markers, all vectors were further modified to carry four genes each. Quantitative PCR experiments demonstrated the expression of all genes of the MEP pathway. Moreover, the expression of valencene synthase as a reporter, together with lovastatin, a potent MVA pathway inhibitor, resulted in valencene production, demonstrating the functionality of the pathway.

Nevertheless, the continuous maintenance and high-copy replication of these types of plasmids may cause a significant metabolic burden to the cell. Besides, YEps are

unstable if not cultivated in defined media. This restriction severely hampers their interest in industrial applications [55].

The integration of a pathway into the yeast genome allows a more stable expression. This could be accomplished with the use of Yeast Integrative plasmids (YIp). YIp can be maintained only after chromosomal integration at specific loci, and do not need the continuous use of selective media. Since the expression with YIps is different from the one observed with replicative plasmids, overexpression can be achieved by targeting genes into repeating DNA elements, such as ribosomal DNA or delta sites [53].
An important feature of yeast cells in relation with integrative plasmids is its highly active DNA repair system. A linear DNA fragment can be perceived by yeast as broken DNA that needs to be repaired by homologous recombination. This advantage allows the insertion of genes at specific loci by flanking a specific gene, or DNA fragments carried by YIps, with sequences identical to the target site in the genome. The same principle is employed to delete or disrupt genes, or to carry out any change in a specific sequence, like a mutation or promoter replacement [56].

Techniques for DNA assembly

The sequential rounds of optimization during a platform construction require attempting to minimize the time and cost in every step of the process. One of those steps is the cloning phase, a time-consuming and cumbersome process, which traditionally has involved restriction and ligation steps, regardless of the vector chosen to express a target gene. Several cloning methods have been developed over time, based on overlapping sequences instead of restriction sites, reducing the efforts mentioned before. For example, the Uracil-Specific Excision Reaction (USER) is a cloning technique conceived in the early 90s as a ligase independent cloning procedure [57]. Despite that initially it received little attraction, in 2006, Nour-Eldin and colleagues developed a library of USER-compatible vectors by improving the reagents developed by the NEB's USER cloning kit [58]. USER allows the assembly of multiple DNA fragments by the

creation of short, complementary overhangs in both PCR products/genes and destination vector. Each overhang must contain one deoxyuridine (dU) instead of deoxythymidine (dT). After the treatment with USER™ enzymes (a mix of uracil DNA glycosidase and DNA glycosylase-lyase endo VIII, commercially available at NEB), the generation of sticky ends allows the correct annealing of the DNA fragments. USER assembly is convenient for assembly of 2-6 DNA fragments, with efficiency as high as 90% [59]. Another advantage associated with this cloning technique is the library of YIp USER vectors developed by Milkkelsen et al. (2012) [60]. These authors investigated 14 different loci in the genome of *S. cerevisiae* suitable for gene integration. The selected integration sites are located in regions where gene expression is high, centrally in large intergenic regions of minimum 750 bp to reduce the chance that integration influences the fitness of the strain by adversely affecting neighboring genes. To avoid potential genetic instability when the integrated genes had the same promoter or terminator, each site is separated by genetic elements that are essential for wild type growth, preventing strain evolution. The 14 Yip sites selected by the authors facilitate the integration of genes towards the 14 integration elected sites. Each vector contains a USER cloning cassette flanked by two commonly used *S. cerevisiae* terminators (t_{CYC1} and t_{ADH1}). By applying the USER cloning technique, one or two genes of interest (plus a promoter or a bidirectional promoter, respectively) can be simultaneously integrated into the vectors by USER-fusion cloning [58]. This technique supports the expression of a multi-gene pathway, like the production of indoylglucosinolates in yeast, demonstrating the potential and stability of the resulting platform [60].

In addition to the USER-based recombination technique, Daniel Gibson and his colleagues at the J. Craig Venter Institute developed a novel method for the assembly of multiple DNA fragments, that later was used to assemble the complete synthetic 583-kb *M. genitalium* genome [61]. In this work, the assembly of 101 DNA cassettes into four quarters of the *Mycoplasma genitalium* genome was carried out in a two-step thermocycled method [62]. Later on, this method was improved into an isothermal, single-reaction technique able to assemble again the complete synthetic *M. genitalium*

genome [63, 64]. The reaction was carried out at 50ºC for at least 15 minutes, and required the presence of three enzymes: a 5' T5 exonuclease, *Phusion* DNA polymerase, and the *Taq* DNA ligase. The improved method allowed the assembly of 3-4 fragments with only 40 bp of overlapping sequences and with a 90% of efficiency [63]. The size limit for the *in vitro* assembly has not yet been determined, but products as large as 900 kb can be constructed [65]. In our experience, larger numbers of fragments are prone to erroneous junctions reducing the efficiency of the method.

In the latter project leaded by Gibson (2008) [65], the entire synthetic genome assembly of *M. genitalium* was a combination of the *in vitro* recombination described before and an *in vivo* yeast recombination, since DNA fragments larger than 150 kb could not be amplified in *E. coli*. Later, the authors demonstrated that the earlier steps (made originally *in vitro*) could also be assembled in a single step in yeast [61, 63]. The homologous recombination system in yeast can also be used to generate a DNA fragment by overlapping independent DNA parts. The final DNA can be generated by transforming yeast with either double-stranded DNA fragments, like PCR products, or single-stranded oligonucleotides. Ma et al., (1987) [66] reported for the first time the use of yeast for assembling plasmids, constructing new vectors by transformation of yeast with linearized plasmids and DNA fragments from restriction digestions. Today, the technique is routinely employed to build constructs, like plasmids, or to insert different fragments into the genome in the right order. The recombination requires overlaps of more than 30-40 bp, where the precision of assembly increases with the size of the overlapping sequence [67].

Other methods, such as the Circular Polymerase Extension Cloning (CPEC) or the Ligase Cycling Reaction (LCR) also show advantages for the assembly of several fragments. A recent study by Kok et al., (2014) [68] compared four assembly methods - CPEC, LCR, Gibson assembly and yeast recombinational cloning- for the assembly of 12 DNA parts. LCR assembly and yeast recombinational cloning enabled the assembly of all DNA parts, whereas Gibson and CPEC did not result in reliable assemblies of

more than four DNA fragments. The final method of choice will depend on the costs, time, project goals, and by the own experience at the laboratory. Despite the low cost of *de novo* synthesis of complete DNA constructs, the assembly of constructs from single DNA parts or BioBricks [69], obtained by PCR or DNA synthesis, is a very promisory technique that allows the store and reutilization of the DNA parts, saving time and cost, especially when one method has already been standardized.

Substrate channeling

The difficult to achieve commercial productivities can also result from low enzymatic activities and flux unbalancing that, together with interactions between the synthetic pathway and natural environment of the host, affect the final titer, rates and/or yield [70].

Enzyme complexes allow the rearrangement of enzymes in a way that decreases metabolite losses in unintended interactions and increases the pathway flux. Some of the strategies to organize enzymes into complexes include fusion enzymes. The use of bifunctional fusion genes, like *BTS1-DPP1* and *BTS1-ERG20* allowed to improve the synthesis of geranylgeraniol in yeast [71]. Other successful examples include fusion between terpene synthases with *ERG20*, like for the production of the monoterpene sabinene, where the fusion protein SabS1p-ERG20p increased 3.5 fold the monoterpene yield [72]; and patchoulol production, where the fusion of the two genes (*ERG20-PTS)* increased the production of this sesquiterpene by 2-fold [73].

Nevertheless, as gene fusions cannot balance enzyme stoichiometry, other alternatives have emerged to this endeavor, such as the development of synthetic protein scaffolds to organize enzymes into complexes. For instance, protein scaffold was successfully evaluated in *E. coli* strains to increase mevalonate yields [74].

1.2 TERPENOIDS

Several laboratories and biotech companies are currently working on developing new technologies for the construction of more performant cell factories, and thereby, ensure a sustainable source of biocompounds. The efforts to improve yeast platforms are justified by the wide array of applications of natural compounds and their economic implications. One of the families of natural products with a broad spectrum of industrial applications are the isoprenoids. Isoprenoids, also called terpenoids, are the largest and most diverse group of natural compounds found in nature, mostly in plants [75]. Their biochemical role in cells is diverse, ranging from cell membrane components, through functions in subcellular targeting and regulation, to plant defense, communication, and pigmentation [76, 77]. Terpenoids also have attractive commercial applications as flavour and fragrance additives in the food and cosmetic industry. Many others possess pharmaceutical properties, currently used in clinical practice, like the anti-malarial drug artemisinin, or paclitaxel (trade name Taxol®), a chemotherapeutic agent to treat several types of cancer. Recently, attention has also focused in the use of terpenoids as biofuels, due to their potential to serve as advanced biofuel precursors or drop-in components [78, 79].

In nature, terpenoids are synthesized either through the MeValonic Acid pathway (MVA) or by the 1-Deoxy-D-Xylulose-5-Phosphate (DXP) pathway (Figure 1). Both pathways condense precursors, either acetyl-CoA or glyceraldehyde-3-phosphate with pyruvate, respectively, to produce the universal isoprene building unit, isopentenyl diphosphate (IPP). As a general rule, the mevalonate pathway is present mostly in eukaryotes and archaea, whereas the DXP pathway is present in bacteria. However, the existence of an alternative MVA pathway was recently proposed in archaea and some bacteria, where a new enzyme able to produce IPP was discovered [80]. In plants, both pathways are expressed with the MVA pathway enzymes located in the cytosol, while the DXP enzymes present in plastids [6].

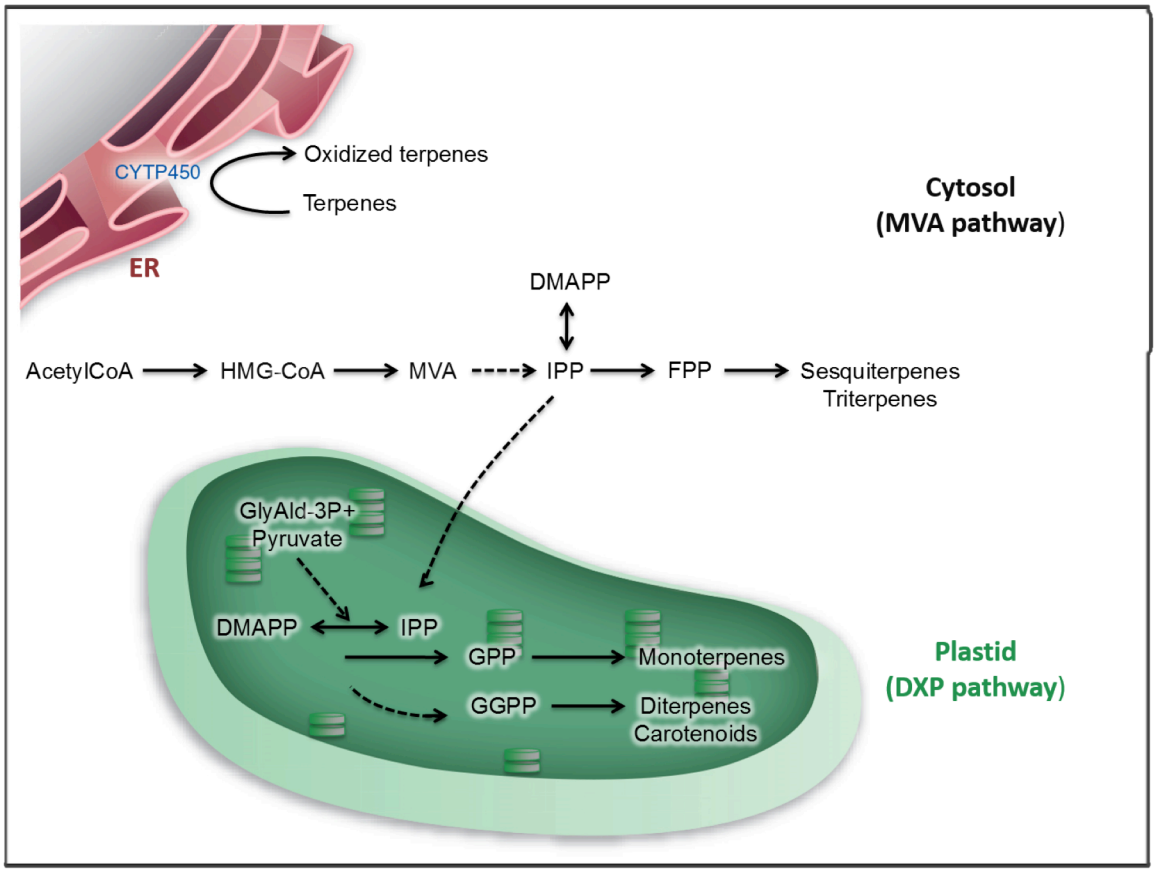

Figure 1. Terpenoids biosynthesis in plants.
Solid and dashed arrows represent single or multiple enzymatic steps respectively. DMAPP, dimethylallyl diphosphate; IPP, isopentenyl diphosphate; GPP, geranyl diphosphate; GGPP, geranylgeranyl diphosphate; GlyAld-3P, glyceraldehide 3-phosphate; FPP, farnesyl diphosphate; HMG-CoA. 3-hydroxy-3-methylglutaryl-CoA; MVA, mevalonate; CYTP450, citochrome-P450 hydroxylase. Modified from Bouweester, (2006) [81].

1.2.1 Metabolic Engineering for isoprenoid production.

Given the wide applications of isoprenoids, their microbial production has been a focus of study in recent decades. Extraction from the native plant sources is often

cumbersome, since these natural compounds tend to accumulate at low quantities over long growth periods, their purification requires separation from a multitude of other compounds of similar structure, and yields are subject to regional, seasonal, and environmental factors [82]. Intensive research work to improve yields of natural compounds in both, the native plant host and plant cell cultures, has been conducted during the past years [83]. Engineered plants for the production of carotenoids [84] or terpenes from essential oils [85] have been successfully obtained. Nevertheless, although most of these plants produced and emitted the final terpenoid, other features were negatively affected, like vitality or growth [86]. In the case of plant cell cultures, these are hampered by long periods of growth and limited biomass production. Chemical synthesis, on the other hand, suffers of several problems due to the complex reaction sequences and the production of enantiomeric mixtures, which involve subsequent separation steps [87]. Additionally, hazardous reagents are often utilized, making many of these syntheses unsafe for food and health applications.

An alternative option to plant extraction and chemical synthesis is the heterologous expression of terpenoid-producing-enzymes in industrial, microbial hosts. Furthermore, with the explosive development of new genetic engineering tools, it is nowadays possible to obtain heterologous products normally found in small amounts in nature [15]. Besides, microbial hosts have demonstrated the ability to reach much higher titers of terpenoids than those reported in plants.

The bacterium *Escherichia coli* and the yeast *Saccharomyces cerevisiae* are the preferred hosts for the production of monoterpenes, sesquiterpenes, diterpenes, triterpenes and tetraterpenes.

1.2.2 The mevalonate pathway in *S. cerevisiae*

In fungi, such as *S. cerevisiae*, terpene biosynthesis starts with the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA via a Claisen condensation mechanism, catalyzed by the enzyme acetoacetyl-CoA thiolase (*ERG10*) [88] (Figure 2).

Then, 3-Hydroxy-3-MethylGlutaryl-CoA Synthase (HMGS/*ERG13*) catalyzes an aldol condensation of another molecule of acetyl-CoA with acetoacetyl-CoA to yield HMG-CoA [89]. Both enzymes are highly regulated by the ergosterol level in yeast. The formation of mevalonic acid from HMG-CoA is catalyzed by the HMG-CoA reductase (HMGR), the rate-limiting reaction in sterol biosynthesis [90]. *S. cerevisiae* encodes two genes with HMGR activity, *HMG1* and *HMG2*. The N-termini of the two enzymes are anchored to the endoplasmic reticulum, while the C-termini, that contain the active sites, are facing the cytoplasm [91]. These two enzymes are regulated by several factors, like feedback control and enzyme degradation [92]. When ERG10, ERG13 and HMGR activity is repressed, production of HMGR is increased, suggesting feedback regulation [93, 94]. Degradation is also controlled by carbon flux within the mevalonate pathway [95]. Terpenoid biosynthesis continues with the conversion of mevalonic acid into IPP in three ATP-dependent steps: a first phosphorylation, catalyzed by the mevalonate-5-phosphotransferase (ERG12) to yield phosphomevalonate; a second phosphorylation, catalyzed by the phosphomevalonate kinase (ERG8) to form mevalonate-5-pyrophosphate; and a decarboxylation by the pyrophosphomevalonate decarboxylase (ERG19) to yield IPP [96]. Successive condensations of IPP and its isomer dimethylallyldiphosphate (DMAPP) by a family of enzymes known as prenyltransferases, result in different length isoprenoid precursors: geranyl diphosphate (GPP) for monoterpenes (C10), farnesyl diphosphate (FPP) for sesquiterpenes (C15), geranylgeranyl diphosphate (GGPP) for diterpenes (C20), two units of FPP for triterpenes (C30) and two units of GGPP for tetraterpenes (C40) [75, 97]

In *S. cerevisiae*, unlike plants, the GPP and FPP formation is catalyzed by the same enzyme, the FPP synthase or ERG20. This enzyme catalyzes two separated condensation reactions: first, IPP and DMAPP is condensed to produce GPP, which does not accumulate in the cytosol but instead, condenses immediately with another IPP molecule to yield FPP. FPP is a branch point in the pathway and is utilized in several biosynthetic pathways. FPP is the substrate for the biosynthesis of sesquiterpenes and GGPP; also, it

dimerizes into squalene in the first step of the sterol biosynthesis, serves as a base molecule in dolichol formations, and provides the substrate for the farnesylation of several proteins [72]. Thus, it is not surprising that ERG20 is a highly regulated enzyme and its deletion was lethal for yeast cells [98].

Finally, GGPP is employed primarily as the substrate for protein prenylation. The GGPP synthase, also known as BTS1, catalyzes the condensation of FPP with another molecule of IPP to form GGPP. The limited demand for GGPP in yeast, explains its low accumulation in *S. cerevisiae* [99].

1.2.3 Pathway engineering in *S. cerevisiae*

Yeast strains do not produce many terpenes, due to the absence of terpene synthases, the family of enzymes that give rise to the vast complexity of terpene structure from the acyclic substrates, prenyl diphosphates, and squalene. However, expression of terpene synthases, derived from plant or other organisms, demonstrate the capability to produce the chemical of interest in yeast cells. This is possible since the heterologous pathway connects to endogenous yeast metabolism, using highly conserved substrates like GPP, FPP, or GGPP [100]. Rather than the expression of individual genes, the flux through the pathway is the most critical issue in pathway engineering in order to get a proper balance between the expression and activities of the different components.

A series of interventions in the MVA pathway of *S. cerevisiae* has been conducted to increase the concentration of precursors for natural or heterologous terpene synthesis. The main approaches include the truncation of key enzymes, like the HMGR1, and the removal or repression of competing enzymes like ERG9, which synthesizes squalene from two molecules of FPP. Several other interventions can be evaluated to increase a particular substrate but to date, those described below are the most popular, mostly in combination with each other.

1.2.4 Manipulation of MVA genes

HMGR

Yeast cells require ergosterol as a structural component of their membranes. Due the similarity between ergosterol and cholesterol of mammalian cells, most of the enzymatic steps of their biosynthesis are shared [101]. Feedback regulation of cholesterol biosynthesis was discovery by Schoenheimer and Breuch, who observed an inverse proportion of cholesterol produced in mice compared with the amount in their diet [102]. After this finding, Siperstein and Guest determined the HMGR as the target of this inhibition [103].

As mentioned before, *S. cerevisiae* encodes two *HMGR* genes, designated *HMG1* and *HMG2*. Like in mammalian cells, these proteins exert a high degree of flux control over the MVA pathway. HMG1p is the primary source of HMGR activity during aerobic growth of *S. cerevisiae* by stimulation of its transcription, which in turn, increase its activity by 10. Simultaneously, aerobic growth represses *HMG2* expression by an unknown mechanism [95]. It is known that HMG2p is regulated at the protein level through Endoplasmic Reticulum-Associated Degradation (ERAD) by the machinery of the HMG-CoA reductase degradation (HRD) pathway. Instead, HMG1p is more stable and its expression is under the translational control of early sterol intermediates formed upstream squalene [104]. Overexpression of the *HMG1* induced endoplasmic reticulum membrane stacks, with no further influence in the sterol metabolism [105]. Nevertheless, Polakowski and co-workers (1997) [106] obtained a 552-residue truncated enzyme, lacking the trans-membrane region, eliminating the regulation and yielding a soluble protein, called tHMG1. Overexpression of this protein dramatically increased squalene accumulation. Nevertheless, a slight improvement in ergosterol/sterol production indicates a second feedback inhibition at ERG9p. More recently, the expression of *tHMG1* has been successfully used for overproducing the sesquiterpenes farnesene [107], miltiradiene [108] and cubebol [109], since an FPP accumulation is also reached by *tHMG* overexpression.

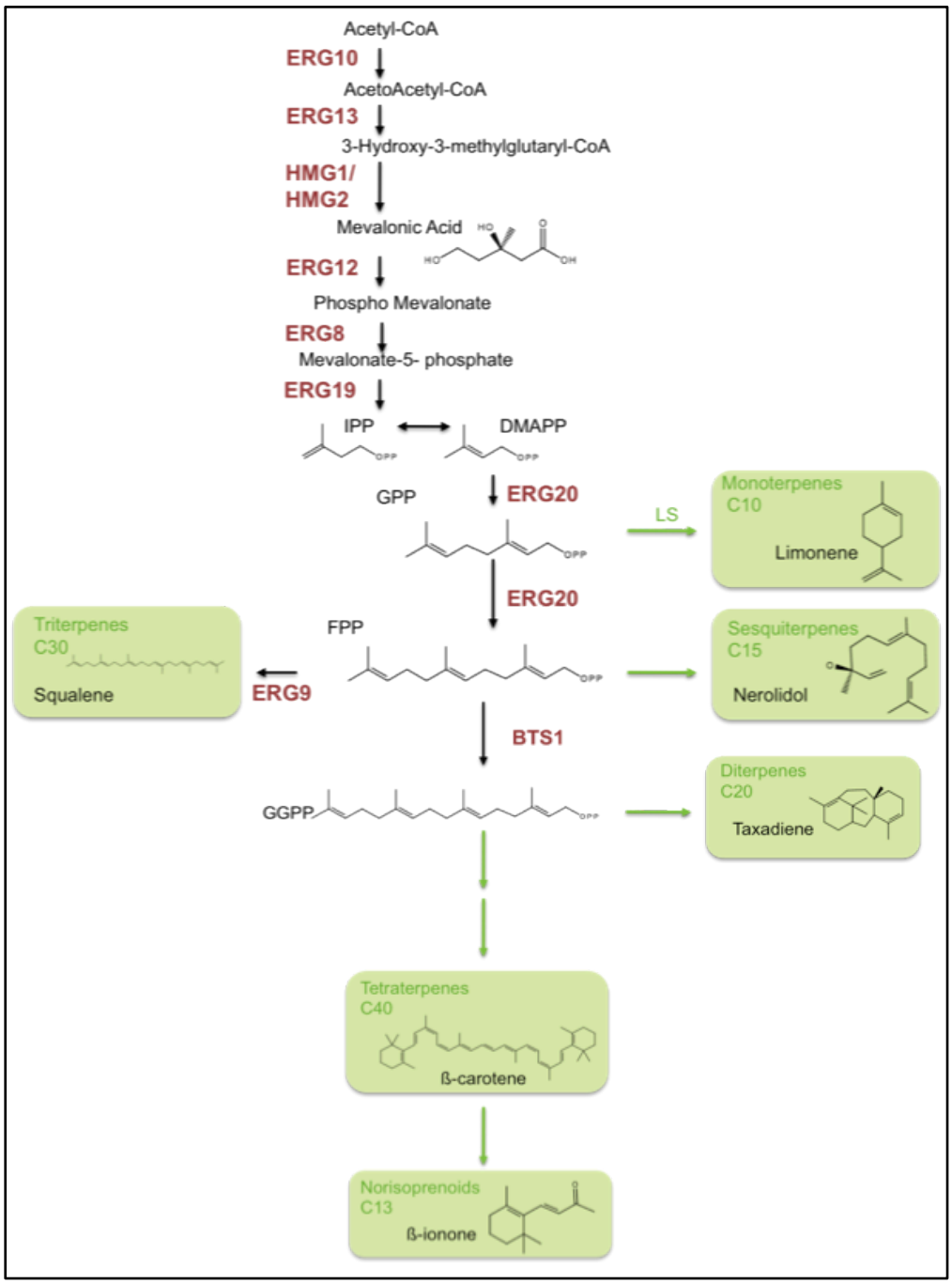

Figure 2. Mevalonate pathway in yeast.
Enzymes are depicted in red. Green boxes represent the terpenoids groups that can be produced in yeast by metabolic engineering.

HMG2p modifications have also been studied, although to a lesser extent. Mutations on the lysine 6 residue to arginine (K6R) in the HMG2p, stabilizes the protein against its degradation. Overexpression of this mutant, named *K6R HMG2* was employed to increase the production of mono- and sesquiterpenes [110] in an already engineered *S. cerevisiae* strain.

ERG9

The first step in sterol biosynthesis is catalyzed by Squalene Synthase (SQS) or ERG9p, which condenses two FPP molecules to form the 30-carbon sterol precursor squalene. Since deletion of *ERG9* is lethal, complete elimination of squalene activity is not feasible, but the replacement of the endogenous promoter by the tunable *MET3* promoter (P_{MET3}) or by the glucose responding promoter of hexose transporter (P_{HXT1}), increased the availability of cytosolic FPP. A problem with P_{MET3} is the consumption of methionine in the culture medium during the fermentation, which decreased the accumulation of FPP and thus terpene production, by relieving the repression on *erg9* [111]. However, a more precise regulation on methionine concentration is needed to improve terpene production by this strategy. On the other hand, the replacement of the native promoter with P_{HXT1} achieved a tighter control of *ERG9* expression, with an efficient down-regulation of the gene under glucose limiting conditions [112].

LPP1, *DPP1*

Both strategies mentioned before resulted in a concomitant increase in farnesol production, a by-product of sesquiterpene production; therefore, they must be simultaneously applied with the deletion of *LPP1* and *DPP1* genes.

LppIp and DppIp are two enzymes identified as phosphatidic acid hydrolases, which are also able to dephosphorylate isoprenoid phosphates [113]. Inhibition of squalene synthase leads to the conversion of FPP to the secretable farnesol (FOH), probably to alleviate the potential toxic effects of FPP accumulation [11, 114]. FOH production was also observed when yeast cells were treated with zaragozic acid, a natural inhibitor of

squalene synthase [115]. Reduction - not complete inhibition - of FOH formation was determined in the double deletion strains (*LPP1*$^{-}$*/DPP1*$^{-}$*),* indicating an additional mechanism for FOH production. Nevertheless, this strategy, together with the expression of tHMG1, significantly increased sesquiterpene synthesis, like α-santalene [112], while its overexpression results in opposite effects on geranylgeraniol production [71].

ERG20

The overexpression of FPPS synthase (ERG20p) has also been employed to increase FPP and the concomitant sesquiterpene production, but the impact mainly depends on the growth conditions and genetic background of the strain used. *ERG20* is normally overexpressed in strains with at least one gene of the mevalonate pathway already engineered. Ro et al. (2006) [116] upregulated the *ERG20* gene in a strain, which has an integrated copy of *tHMGR*, semi-dominant mutant allele *upc2-1* that enhances the transcriptional activity of the mevalonate genes, and downregulated *ERG9*. In this engineered strain, *ERG20* overexpression had little effect on total amorphadiene production, but the specific production increased about 10%. Another example is the production of α-santalene, where the upregulation of NAD^{+}-dependent glutamate dehydrogenase (*GDH2*) and *ERG20,* in combination with other modifications in a yeast strain, improved the α-santalene yield and productivity 4- and 6-fold, respectively [27].

On the other hand, monoterpene production needs GPP as substrate instead of FPP but, so far, few works have focused on generating a pool of available GPP in *S. cerevisiae*. GPP does not accumulate in the cytosol of yeast cells, probably because no specific cellular function has been described in yeast for this compound. Instead, it remains in the catalytic site of ERG20 and is immediately transformed to FPP by the addition of another IPP molecule, limiting monoterpene production [117]. However, yeast mutants excreting geraniol and linalool have been already characterized in the yeast strain FL100, an haploid mating type [118]. The sequencing of the *ERG20* gene (called *ERG20-2*) from this strain demonstrated a single nucleotide mutation, resulting in a Lysine 197 to glutamic acid substitution. This mutation is directly involved in the

terpenoid alcohol formation due to a reduction in the size of the binding pocket of ERG20 [119]. Overexpression of the geraniol synthase from *Ocimum basilicum*, in this mutant strain, increased the geraniol production 2-fold compared to wild type strains [117]. This discovery was followed by a series of new mutants from ERG20 by protein engineering, where at least 10 mutants increased the monoterpenol production [120]. Through comparison of a ERG20 model with structures of substrate-bound form of FPPS, other residues have now been identified, like F96 and A99, which allow, for example, to increase the production in yeast of the monoterpene sabinene 3- and 2-fold, respectively, when these residues where replaced by tryptophan [72].

Pyruvate dehydrogenase bypass

Since sterol biosynthesis via the MVA pathway is initiated with acetyl-CoA, a key metabolite in yeast, it is crucial to secure the supply of sufficient amount of this precursor to the pathway. In yeast, acetyl-CoA is produced and consumed in different compartments, such as cytosol, mitochondria and the peroxisomes, and cannot be transported directly between mitochondria and the peroxisomes without the carnitine/acetyl-carnitine shuttle or glyoxylate cycle [121, 122]. In the cytosol, the pyruvate dehydrogenase bypass converts pyruvate into acetyl-CoA by the action of pyruvate decarboxylase, cytosolic acetaldehyde dehydrogenase and acetyl-CoA synthetase [123]. Acetaldehyde, the first metabolite of this bypass, can also be converted to ethanol by the alcohol dehydrogenase, a flux that increases during growth on glucose due to the Crabtree effect in *S. cerevisiae* [124]. Since *S. cerevisiae* has several alcohol dehydrogenases that catalyze the conversion of acetaldehyde to ethanol, it is difficult to eliminate ethanol production in order to increase acetyl-CoA.

The overproduction of acetaldehyde dehydrogenase ALD6 and the introduction of a heterologous acetyl-CoA synthetase variant (L64IP), together with the expression of *tHMG1*, allowed to increase mevalonate and amorphadiene concentrations, suggesting that engineering this bypass is effective to enhance the flux into the MVA pathway and, moreover, for terpene production [125]. Chen et al. (2013) [121] complemented this

platform by overexpressing *ADH2* that catalyzes the conversion of ethanol to acetaldehyde, deleting either *CIT2* or *MLS1* that prevent the entering of acetyl-CoA to the glyoxylate cycle, and overexpressing *ERG10*, improving the α-santalene titer in 25%, as compared to the work of Shiba et al [125]. Recently, Lian et al. (2014) [126], constructed a yeast platform combining strategies of disrupting competing pathways and introducing heterologous biosynthetic pathways, increasing acetyl-CoA concentration 3 fold and n-butanol titer, used as a reporter, more than 12-fold. Furthermore, Pronk et al. (2014) [127], succeeded to assemble and functionally express a pyruvate dehydrogenase -normally present in the mitochondrial matrix of yeast cells- from *Enterococcus faecalis* in the cytosol of a yeast cell. This enzyme employs an ATP-independent mechanism replacing the ATP-dependent native yeast pathway. These results open the way to improve this yeast strains, making an important contribution to future overproduction of terpenoids and lipids.

Recently, the combination of enzyme discovery, enzyme engineering, and pathway and strain optimization allowed the synthesis of two opiates in yeast [128]. The two opiates required the expression of 21 and 23 enzyme activities respectively, from plants, mammals, bacteria, and yeast itself. Even though these strains are not suitable for production at industrial scale, this work demonstrated the potential of yeast as a chassis for bio-based production of complex chemicals and the power of synthetic biology to coordinate the complete synthesis of a large pathway [129, 130]. Certainly, the dramatic progress that is currently taking place in this area will allow achieving the sustainable and economically-feasible bioproduction of a wide range of natural compounds.

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2. PRODUCTION OF ß-IONONE BY COMBINED EXPRESSION OF CAROTENOGENIC AND PLANT *CCD1* GENES IN *SACCHAROMYCES CEREVISIAE*

2.1 Introduction

Apocarotenoids are a subclass of isoprenoids, which are highly appreciated in the flavoring industry due to their characteristic aromatic notes [1]. In plants, these compounds are produced by the cleavage of carotenoids (C40) by the enzymatic action of CCDs (carotenoid cleavage dioxygenases), a family of oxidative enzymes that specifically cleaves double bonds [2]. Between the different apocarotenoids, ß-ionone is a prominent scent and aromatic molecule present in many flowers and fruits, such as blackberries, peaches and apricots, among others [3]. In odorant terms, ionones (α and ß) are associated with violet, but ß-ionone has also a woody odor character. Despite its low concentration in plants (in the order of ng/kg fresh weight), this compound has the potential to strongly impact the flower aroma due to its significantly low odor threshold (7 ppt in water), only comparable to the rose-like aroma molecule, ß-damascenone [4]. In nature, ß-ionone is obtained by specific cleavage of ß-carotene [3, 5]. This reaction is catalyzed by the action of CCD1, which cleaves carotenoids at the 9–10 position and the 9′–10′ position, in the presence of oxygen [6]. Currently, ß-ionone is used in the food and cosmetic industry, due to its pleasant aroma and its contribution to flavor, but it is also a key intermediate in the synthesis of vitamins A, E and K and therefore has an annual production of several hundreds of tonnes [7].

Since the extraction of aroma compounds from their natural source is an expensive and arduous task, biotechnology represents a very attractive alternative for the sustainable production of flavors and fragrances that can still be considered as “natural” [8].

To date, co-expression of the plant CCD1 enzyme together with the *Xanthophyllomyces dendrorhous* carotenoid enzymes in *Escherichia coli* and *S. cerevisiae,* respectively, has led to the proof-of-principle biotechnological production of ß-ionone. A ß-carotene overproducing *E. coli* strain, together with the episomal expression of the *CCD1* gene from *Petunia hybrida,* was used to demonstrate the activity of the CCD1 enzyme [3]. For ß-ionone production by *S. cerevisiae,* a *crtYB*/*crtI*/*crtE* polycistronic episomal construct, with the three genes necessary for the synthesis of ß-carotene from FPP, were expressed together with the *CCD1* gene from raspberry. However, the low translational efficiency of this system, limited ß-ionone production to a final titer of 0.22 mg/L [9].

In the present study, an alternative *S. cerevisiae* platform was constructed to synthesize ß-ionone by combining two genetic engineering approaches to increase protein expression: USER cloning-compatible integrative vectors and high copy number episomal expression systems (Figure 2-1). We generated a ß-ionone producing microbial cell factory, reaching a maximal concentration of 0.63 ± 0.02 mg/g biomass in shake flask cultures. This platform was constructed by overexpressing a truncated version of the *HMG1* genes (*tHMG1*) and the endogenous GGPP synthase gene *BTS1*, together with the *crtYB* and the *crtI* genes from *Xanthophyllomyces dendrorhous* and the *CCD1* gene from *P. hybrida* (*PhCCD1*) in an FPP overproducing strain (SCGIS22) [10]. In 2 L batch bioreactors, a final concentration of 1 mg/g biomass was reached, equivalent to a 15-fold increase. This corresponds to a titer of more than 5 mg/L, far exceeding the earlier study and representing a starting point for flavor production by a sustainable and efficient process that could also replace current methods [11, 12].

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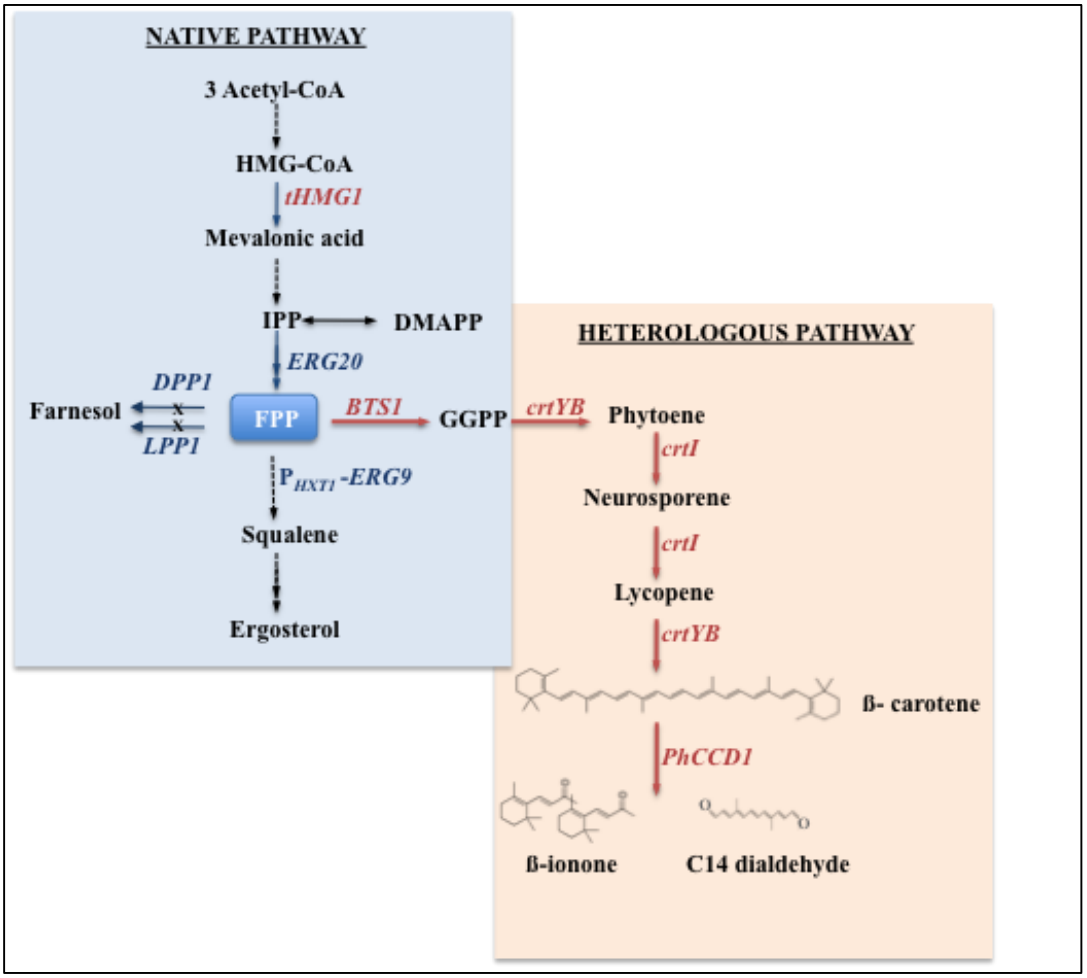

Figure 2-1. Engineered platform for ß-ionone production in yeast.
The blue arrows/genes indicate the modifications of the strain *Saccharomyces cerevisiae* SCIGS22 constructed by Scalcinati et al., 2012. The red arrows/genes show the genes expressed in the present study. In SCIGS22, *ERG20* (encoding FPP synthase) was over-expressed, *DPP1* and *LPP1* (encoding lipid phosphate phosphatases) were deleted and the promoter of *ERG9* (encoding squalene synthase) was replaced for the *HXT1* promoter. In the present study, the *tHMG1* (encoding a truncated HMG-CoA reductase), and *BTSI* (encoding geranylgeranyl diphosphate) genes were overexpressed. The heterologous genes *crtYB* (encoding phytoene synthase/lycopene cyclase), *crtI* (encoding phytoene desaturase) and *PhCCD1* (encoding carotenoid cleavage dioxigenase from *Petunia hybrida*) were both integrated and expressed episomally. HMG-CoA: 3-hydroxy-3-methylglutaryl-CoA, IPP: isopentenyl diphosphate, DMAPP: dimethylallyl diphosphate, FPP: farnesyl diphosphate, GGPP: geranylgeranyl diphosphate

2.2 Material and Methods

2.2.1 Plasmid construction

The genes coding for CrtYB, CrtI and PhCCD1 proteins were synthesized by Genscript (Piscataway, NJ, USA) (Additional file 1). All the sequences were codon optimized for expression in *S. cerevisiae*. The catalytic domain of the HMG-CoA reductase gene (*tHMG1*) together with the TEF1 promoter was PCR amplified using genomic DNA from Scalcinati's strain *S. cerevisiae* SCIGS23 [20] as template and the *BTSI* gene was PCR amplified from genomic DNA of strain *S. cerevisiae* CEN.PK113-5D. Primers used for all amplifications are provided in Additional file 2.

We constructed four plasmids to integrate the *tHMG1* gene and the four genes needed for ß-ionone production, into the yeast genome, using the USER cloning technique [13]. PCR amplification of the DNA fragments was carried out in 35 PCR cycles using the proofreading PfuTurbo Cx Hotstart polymerase (Agilent Technologies, Santa Clara, CA, USA) or PfuX7 [14], following the manufacturer's instructions. The USER vector pXI-5 was amplified by PCR using primer pair 1/2, and the USER vectors pXI-3 and pX-2 were amplified by PCR using primer pair 3/4 followed by digestion with the Nb.BsmI nicking endonuclease for 1 h. The catalytic domain of the HMG-CoA reductase gene (*tHMG1*) [GenBank: NM_001182434] together with the TEF1 promoter was amplified using primer pair 5/6. The *BTS1* gene [GenBank: NM_001183883] was amplified using primer pair 7/8. The genes *crtYB, crtI* and *PhCCD1* were amplified using primers pair 9/10, 11/12 and 13/14 respectively. The bidirectional promoters (*TEF1*/*PGK1*) used for the expression of the genes were amplified from the plasmid pSP-GM2 as a template using the primers 15 and 16. All PCR products were treated with DpnI enzyme to eliminate original vector residues. Purified digested vector (100 ng) was mixed with a molar ratio of the purified PCR products amplified depending on their length. The DNA fragments were mixed with 1 μl of 10× TE buffer (100 mM Tris-HCl, 1 mM EDTA; pH 8.0), 1 U of USER enzyme mix (New England BioLabs) and Milli-Q purified water until 10 μl. The mixture was

incubated for 20 min at 37°C, followed by 20 min at 25°C. Finally, the reaction mix was used to transform chemically competent *E. coli* cells. The resulting plasmids were designated pIRP01 (with tHMG1 under the *TEF1* promoter), pJL01 (with *BTS1* under the *TEF1* promoter and *crtYB* under the *PGK1* promoter), pJL02 (with *crtI* under the *TEF1* promoter) and pJL03 (with *crtI* under the *TEF1* promoter and *PhCCD1* under the *PGK1* promoter). Since the strain used is auxotroph for uracil (*ura3-52*), all vectors contained the *Kluyveromyces lactis* (*Kl*) *URA3* gene flanked by direct repeats (in order to be able to recycle this marker for future transformations).

We also constructed a series of plasmids for episomal expression, using the Gibson assembly technique. All PCRs to obtain DNA fragments suitable for Gibson assembly were carried out in 35 PCR cycles using Phusion High-Fidelity DNA polymerase (Thermo Scientific, Waltham, MA, USA), following the manufacter's instructions. Gibson assembly was performed as previously described [15] with pairs of primers for each fragment to be assembled containing segments of about ~40 bp homologous to the adjacent fragment to be linked. The episomal yeast expression vector p426GPD (Addgene, Cambridge, MA, USA) was amplified with the primer pairs 17/18, 19/20 or 21/22, depending on the gene cloned. *CrtYB*, *crtI* and *PhCCD1* were amplified using primers 23/24, 25/26 and 27/28, respectively. All PCR products were treated with DpnI enzyme to eliminate original vector residues and purified by gel extraction using the Qiaquick Gel Extraction kit from Qiagen, according to manufacturer's instructions. The purified genes fragments and vectors were mixed based on their molar ratios in a final volume of 5 µl containing 100 ng of total DNA. This DNA mix was added to 15 µl of 1.33X master mix (5X isothermal mix buffer, T5 exonuclease 1U/µl, Phusion DNA polymerase 2U/µl, Taq DNA ligase 40 U/µl and Milli-Q purified water) and the reaction mixture was incubated at 50°C for 1 hour. Finally, 10 µl reaction mix were used directly to transform chemically competent *E. coli* cells. The resulting plasmids were designated pEJL04, pEJL05 and pEJL06, containing *crtYB*, *crtI* and *PhCCD1* genes, respectively.

For the construction of another two gene-containing plasmids - pEJL07 and pEJL08 - the pEJL04 plasmid was used as backbone and amplified using primers 29/30, including *crtYb* gene. The *crtI* and *PhCCD1* genes were amplified from pEJL05 and pEJL06 respectively, including in each case the *GPD* promoter and *CYC1* terminator using primer 31/32. Maps of all the plasmids constructed are illustrated in Additional file 3.
All plasmids were verified by sequencing (Macrogen Inc, Seoul, Korea).

2.2.2 Yeast strain construction

The *S. cerevisiae* strain employed in this work has a CEN.PK SCIGS22 background with extra modifications into the genome for the overproduction of FPP [20].
All *S. cerevisiae* strains constructed from this strain are listed in Table 2-1.
Strain SCIGS22a carrying the truncated version of the *HMG1* gene (*tHMG1*) encoding 3-hydroxy-3-methyl-glutaryl-CoA reductase lacking the trans-membrane region, was created from strain SCIGS22 by transformation with the cassette from plasmid pIRP01. The plasmid was digested with enzyme NotI (New England BioLabs, Ipswich, MA, USA), and the fragment purified and transformed into strain SCIGS22, finally called SCIGS22a. The transformation was performed using the standard lithium acetate/single-stranded DNA carrier/PEG procedure [16] and transformants were selected using SC-URA plates. Correct cassette integration into the pXI-5 locus was tested by PCR using primers 33/34. The transformants were grown in YPD medium at 30ºC for 48 hours and then directly spreads onto 5-fluoroorotic acid (5-FOA) plates (50 mg/L) for the recycling of the *KlURA3* marker. Colonies grown on 5-FOA plates were examined by colony-PCR using primers 34/35.
The strain JLS01 carrying the genes *BTS1*, *crtYB* and *crtI* was constructed from strain SCIGS22a by transforming the strain with the cassettes from plasmids pIJL01 and pIJL02. For this purpose, the plasmids pIJL01 and pIJL02 were restricted with enzyme SwaI (New England BioLabs) and the fragments isolated from vector backbones were used for yeast transformation, one cassette at a time. Correct cassette integration into the pXI-3 locus

was checked by PCR using primers 36/37. After the *KlURA3* marker recycling, the cassette from plasmid pIJL02 was used for yeast transformation and again the *KlURA3* marker was recycled for future transformation with episomal plasmids. The correct integration of the cassette in the pX-2 locus was tested by PCR using primers 38/39. Strain JLS02 carrying the genes *BTS1*, *crtYB*, *crtI* and *CCD1* was constructed in the same way, using plasmids pIJL01 and pIJL03.

Strains JLS03, JLS04, JLS05, JLS06 and JLS07 were obtained by transforming the strain JLS02 with the high copy number plasmids pEJL04, pEJL05, pEJL06, pEJL07 and pEJL08, respectively (Table 2-2) containing the *URA3* gene from *S. cerevisiae* as selection marker and the genes *crtYB-crtI* and/or *PhCCD1* under control of the strong constitutive promoter *GPD* (Table 2-1). For all comparative analyses, strains JLS01 and JLS02 were transformed with an empty vector p426.

Table 2-1 - List of *S. cerevisiae* strains used for β-ionone production

Strain	Genotype	Plasmid	Reference
SCIGS22	*MATa MAL2-8^c SUC2 ura3-52 lpp1Δ::loxP dpp1Δ::loxP* P_{ERG9}*Δ::loxP-*P_{HXT1} *gdh1Δ::loxP* P_{TEF1}*-ERG20* P_{PGK1}*-GDH2*	none	20
SCIGS22a	SCIG22 + P_{TEF1}*-tHMG1*	none	This study
JLS01	SCIG22a + P_{TEF1}*-BTSI* P_{PGK1}*-crtYB* P_{TEF1}*-crtI*	none	This study
JLS02	SCIG22a + P_{TEF1}-BTSI P_{PGK1}-crtYB P_{TEF1}-crtI P_{PGK1}*-PhCCD1*	none	This study
JLS03	JLS02	P426 P_{GPD}*crtYB*	This study
JLS04	JLS02	P426 P_{GPD}*crtI*	This study
JLS05	JLS02	P426 P_{GPD}*PhCCD1*	This study
JLS06	JLS02	P426 P_{GPD}*crtYB* P_{GPD}*crtI*	This study
JLS07	JLS02	P426 P_{GPD}*crtYB* P_{GPD}*PhCCD1*	This study

Table 2-2 - Plasmids used for β-ionone production in yeast

Plasmid name	Plasmid description	Reference
pSP-GM2	*URA3*-based expression plasmid carrying a bidirectional P_{TEF1}-P_{PGK1} promoter	[17]
pXI-5	*KlURA3*-based integration plasmid carrying regions for homologous recombination	[17]
pXI-3	*KlURA3*-based integration plasmid carrying regions for homologous recombination	[17]
pX-2	*KlURA3*-based integration plasmid carrying regions for homologous recombination	[17]
P426 GPD	*URA3*-based expression plasmid carrying a P_{GPD} promoter	
pIRP01	P_{TEF1}-*tHMG1*	This study
pIJL01	P_{TEF1}-*BTS1*-P_{PGK1}-*crtYB*	This study
pIJL02	P_{TEF1}-*crtI*	This study
pIJL03	P_{TEF1}-*crtI*-P_{PGK1}-*CCD1*	This study
pEJL04	P_{GPD}-*crtYB*	This study
pEJL05	P_{GPD}-*crtI*	This study
pEJL06	P_{GPD}-*PhCCD1*	This study
pEJL07	P_{GPD}-*crtYB*-P_{GPD}-*crtI*	This study
pEJL08	P_{GPD}-*crtYB*-P_{GPD}-*PhCCD1*	This study

2.2.3 Strain maintenance

For long term storage of the strain, a yeast suspension containing 25% (vol/vol) sterile glycerol was carried out in cryovials and stored at -80°C. Working stocks were maintained on YPD agar plates containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar. Plasmid carrying strains were maintained on synthetic dextrose medium (SCD) agar plates lacking uracil containing 6.9 g/L yeast nitrogen base without amino acids (BD Difco™, BD and Co, Sparks, MD, USA), 0.77 g/L complete supplement mixture without uracil (CSM-URA) (Sunrise Science Products Inc., San Diego, CA, USA), 20 g/l glucose and 20 g/l agar (BD Difco™ BD and Co.).

2.2.4 Growth conditions

Single colonies were inoculated in 3mL pre-cultures in SCD medium without uracil. Then, cultures were grown in 250 mL shake flasks at 20˚C and 180 rpm in a horizontal shaking incubator, with a culture volume of 50 mL with a second phase of dodecane (10%, v/v). All shake-flask cultures were inoculated from pre-cultures grown on the same medium, to an initial OD_{600} of 0.1.

2.2.5 ß-ionone quantification

Culture samples were centrifuged for 2 min at 6000 rpm. The organic phase was dried over anhydrous sodium sulfate. Quantitation was performed by means of a gas chromatography system HP 5890 coupled to a flame ionization detector using a DB-FFAP capillary column (60 m × 0.25 mm id, 0.25 µm film thickness) (J&W Scientific, Agilent Technologies). Injection of the samples was performed in splitless mode at 250ºC. Oven program started at 80ºC for 1 min, then the temperature was raised up in a gradient of 120ºC until 240ºC. Concentrations of β-ionone were calculated by means of a calibration curve in a range of 0.1-50 mg/L using 4-isopropyl-3-methylphenol, 4IP3MP, as internal standard (Sigma-Aldrich, St. Luis, MO, USA). Additionally, mass spectra were obtained using a HP5890A gas chromatograph connected to a HP 5975 C mass spectrometer in electron impact (EI) mode at 70 eV.

2.2.6 Carotenoids analysis

Carotenoid extraction was carried out from cellular pellets according to the acetone extraction method [18], with some modifications, using 50 mL culture volume. The cell pellet was washed once with deonized water and then the cells were broken with 500 µL of 0.5-mm glass beads with 1 ml of acetone for 1 min in a cooling Bead Beater (Bio Spec Products, Bartlesville, OK, USA). After cell disruption, the bead-cell mixture was centrifuged at 14,000 rpm for 5 min, and the clear acetone supernatant was poured off the

cell pellet. This extraction procedure was repeated until the cell pellet became white. The acetone extracts were combined with 1/5th volume of petroleum ether and stirred; then, separation of the two phases was accelerated by means of centrifugation at 14,000 rpm for 5 min. The petroleum ether extract was collected and employed for the total carotenoid quantification.

The total carotenoid composition was calculated by using the 1% extinction coefficient = 2,100 by the formula:

$$\text{Total carotenoid } (\mu g\ /g\ of\ yeast) = \frac{(\text{ml of petrol})(\text{A450})(100)}{(21)\ (\text{yeast dry weight})} \qquad (2.1)$$

The analyses were performed in triplicate, and pigments were normalized relative to the dry weight of the yeast. Carotenoids were separated by RP-HPLC, using a reverse phase C18 column with acetonitrile: methanol: isopropyl (85:10:5 v/v) as mobile phase, with a 1 mL/min flux, under isocratic conditions. The elution spectra were determined with a Shimadzu SPD-M10A diode array detector (Shimadzu Corporation, Kyoto, Japan).

2.2.7 Quantitative real-time PCR

For gene expression analysis, 2 mL of each culture sample were centrifuged at 4°C for 5 min and the pellet was kept in a liquid nitrogen bath for freezing and then stored at -80°C for the next RNA extraction step. RNA isolation was carried out using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions, and stored at -80°C to prevent nucleic acid degradation. Isolated RNA was treated with DNaseI to remove residual genomic DNA. The purity and integrity of RNA was evaluated by electrophoresis in an agarose gel and measuring the A_{260}/A_{280} ratio and concentration in a Nanodrop spectrophotometer. Total RNA (2 ug) was reverse transcribed with a Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, USA), following the manufacturer's instruction. The qPCR was run in a StepOne plus Real-Time PCR instrument (Applied Biosystems, Carlsbad, CA, USA), using the reagent Fast SYBR

Green Master Mix (Applied Biosystems) and specific primers for each gene (Additional file 2). For each strain, 3 clones were analyzed and 3 technical replicates were done for each qPCR measurement. The cycle threshold (CT) values and efficiency values obtained were used for further analysis and calculation of relative expression levels. Each sample was normalized using *TEF1*, as internal control, and then the results from samples JLS03-JLS07 were compared to those in JLS02, as a calibrator sample.

2.2.8 Batch fermentation

Batch cultures were conducted in a 1.6 L working volume of a 2.5 L aerated stirred bioreactor, BioFlo IIc (New Brunswick Scientific). The medium contained 5g/L of $(NH_4)_2SO_4$, 1.7 g/L yeast nitrogen base without amino acids and ammonium sulfate, 0.77 g/L CSM-Ura and 20 g/L glucose. After autoclaving (121°C, 20 min), a filtered-sterilized vitamin solution, prepared according to van Hoek et al. 1998 [19], was added to the medium, as well as 10 % (v/v) dodecane for *in situ* recovery of β-ionone. The fermenter was inoculated with an adequate aliquot of a pre-culture grown in shake flasks prepared in the same medium as described above to give an initial $O.D._{600}$ of 0.1.

During the cultivation, the broth was kept at 20°C, 600 rpm agitation and an air flow rate of 1.0 L min-1. A 20% (w/v) solution of NaOH was employed to maintain the culture pH automatically at 5.0. Samples were collected every one or two hours for kinetics parameters and metabolite measurements. Glucose and ethanol were measure by HPLC as described in Sanchez et al, 2014 [20]. The organic layer was collected at different times during the fermentation for ß-ionone quantification by GC-FID.

2.3 Results

2.3.1 Integration of the *tHMG1* gene and the ß-ionone pathway

The C_{13}-norisoprenoid ß-ionone was synthesized in the FPP-overproducing *S. cerevisiae* strain SCIGS22. This strain combines several strategies to overproduce isoprenoid precursors. It overexpresses *ERG20* (responsible for the production of FPP from IPP and DMAPP) and, since FPP is the precursor of many essential compounds in yeast, the *ERG9* gene (whose gene product synthesizes squalene from FPP) was down-regulated. Additionally, the *LPP1* and *DPP1* genes were deleted in order to minimize farnesol formation – one of the major alternative pathways from FPP.

In this strain, we integrated the cassette pIRP01 carrying a truncated version of the *HMG1* gene, called *tHMG1*, which encodes the enzyme of the mevalonate pathway 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR), lacking the trans-membrane region [21]. This new strain was called *S. cerevisiae* SCIGS22a (Figure 2-1). In the SCIGS22a strain, the integration of the cassettes pIJL01 (*BTSI* and *crtYB* genes) and pIJL02 (*crtI* gene) resulted in orange cells (strain JLS01) (Table 2-1 and 2-2), indicating the synthesis of the colored carotenoids lycopene and ß-carotene. On the other hand, the integration of pIJL01 with pIJL03 (*crtI* and *PhCCD1* genes) resulted in yellow cells with a faint, pleasant violet flavor (strain JL02) (Figure 2-2). Transformants were grown on SC-URA plates without any color loss over time, indicating the genetic stability of the cells. When these strains were grown in a two-phase shake flask culture (with 10% dodecane), final biomass measurements were similar to the original engineered strain (SCIGS22a), indicating that carotenoids and ß-ionone, at least at these concentrations, did not affect cell growth (data not shown).

Carotenoid specific production reached 212 µg/g DCW and 103 µg/g DCW for strains JLS01 and JLS02, respectively. In the strain JLS02, almost all carotenoids were in the form of ß-carotene (77%) and a small percentage in the form of lycopene (2%) (Table 2-3). Additionally, we confirmed ß-ionone production (0.073 ± 0.01 mg/g DCW) in this strain by GC-FID analysis (Table 4). It is worthy to note than when ß-ionone production

was compared after growth at different temperatures, at 20ºC or 30ºC, a 30% higher content was achieved at lower temperature [22].

2.3.2 Transformation with episomal vectors

Accumulation of carotenogenic intermediates (lycopene and ß-carotene), concomitant with the limited production of ß-ionone when one copy of each gene was integrated, suggested that there might be a limitation in channeling the different intermediates into the targeted flow direction. To overcome possible bottlenecks in the pathway, a series of episomal vectors was constructed to generate new transformants, capable of overproducing ß-ionone.

The transformation of strain JLS02 with episomally expressed *crtYB*, *crtI* or *PhCCD1* genes generated the strains JLS03, JLS04 and JLS05, respectively (Table 2-1). Strain JLS03 (carrying an episomal *crtYB gene*) resulted in yellow cells with a strong violet aroma. This strain reached optical densities similar to those reached by the strain JLS02 after 48 hr of cultivation (3.3 OD_{600} vs 3.2 OD_{600}, respectively) (Table 2-2), suggesting that the introduction of this episomal vector did not affect cell growth nor resulted in any metabolic burden by vector replication. After 48 hours of fermentation, most of the carotenoids were in the form of ß-carotene (83.7% of total carotenoids) (Table 2-3) and the ß-ionone specific concentration reached 0.34 ± 0.06 mg/g DCW (Table 2-4), almost 5 times higher than for strain JLS02 (Figure 2-3).

Transformation with the *crtI* (JLS04) or *PhCCD1* (JLS05) carrying plasmids resulted in strains with lighter color and with a lower final biomass (Figure 2-2, Table 2-4). Carotenoid and ß-ionone quantification was not always possible, given the low biomass concentration reached after 48 hr of incubation; these strains were therefore not considered in further analyses.

Two co-expression vectors with the genes *crtYB*/*crtI* (pEJL07) and *crtYB*/*PhCCD1* (pEJL08) under control of identical promoters and terminators were then constructed. The transformation with these vectors resulted in strains JLS06 and JLS07, respectively. Both strains achieved higher ß-carotene concentrations than JLS02. Nevertheless, no significant

differences were observed when compared with JLS03. A different result was observed for ß-ionone. Both JLS06 and JLS07 achieved higher ß-ionone production when compared to JLS02 (6.8- and 8.5-fold, respectively). Additionally, no significant differences in biomass concentration were found between strains JLS -02, -03, -06 and -07 (Figure 2-2 and Table 2-4).

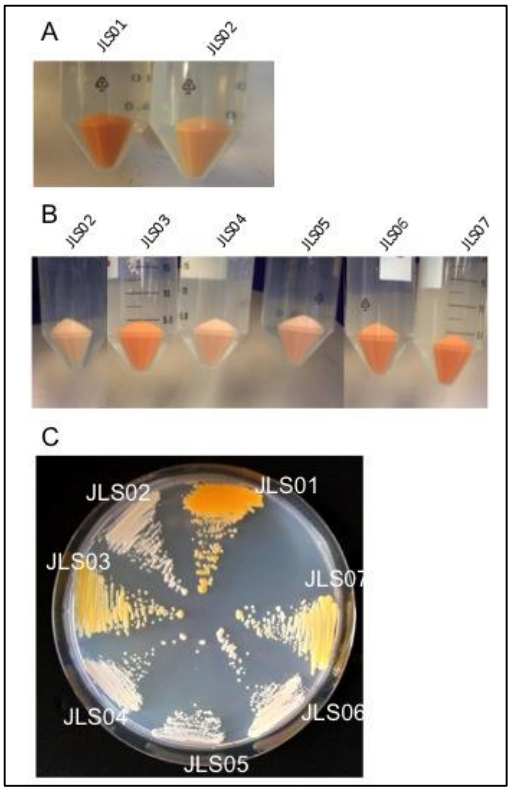

Figure 2-2. Plate color of the different *S. cerevisiae* strains constructed for overproducing β-ionone. (A) Color change from strain JLS01, which only expressed the *BST1* gene from *S. cerevisiae* and the carotenogenic genes *crtYB* and *crtI* from *X. dendrorhous*, to JLS02 that additionally expressed the *P. hybrida* gene *PhCCD1*. (B) Color of the different strains expressing episomal versions of the *crtYb/crtI/CCD1* genes. All the strains expressed the integrated *BTS1*, *crtYB*, *crtI* and *CCD1* genes and were subsequently transformed with episomal vectors containing *crtYB* (JLS3), *crtI* (JLS04), *PhCCD1* (JLS05), *crtYB-crtI* (JLS06) and *crtYB-PhCCD1* (JLS07). All pellets were obtained after 48 hr cultivation in a two-phase culture with 10% dodecane at 20ºC. (C) Representative plate of the constructed strains. Cells were plated after 48 h of cultivation in two-phase culture with 10% dodecane.

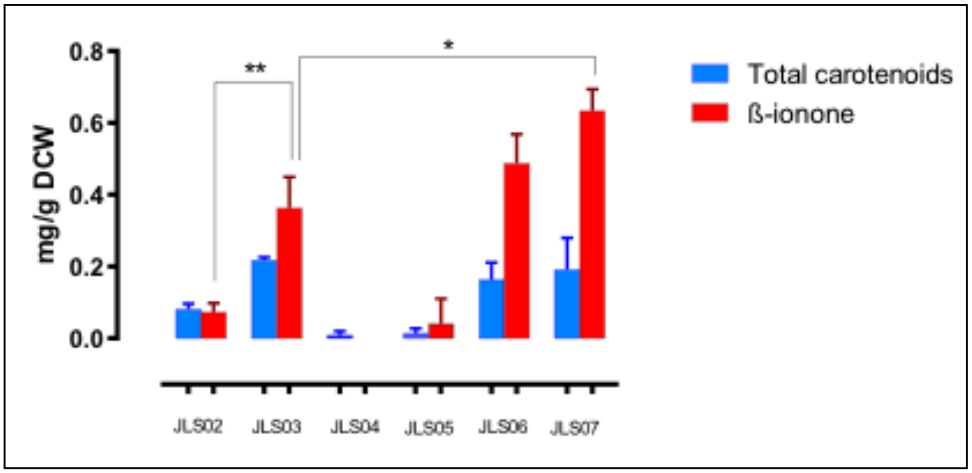

Figure 2-3. Total carotenoids and ß-ionone content of the different ß-ionone producing strains.
Three replicates of each strain were cultivated in SCD-URA medium with 2% glucose and 10% of dodecane for 48 hr. Samples were processed for ß-ionone measurements by GC-FID and total carotenoids by HPLC. *, $p<0.05$, ** $p<0.01$ *t*-test, one-tailed comparison between measurements.

Table 2-3 – Carotenoid biosynthesis by different strains after 48 h cultivation.

Carotenoids	**Carotenoid concentration (ug/g dwc) (% distribution)**					
	JLS02	JLS03	JLS04*	JLS05*	JLS06	JLS07
lycopene	1.6 (2)	--	--	--	--	--
ß-carotene	62.53 (77.2)	181.7 (83.4)	7.37	13.7	140 (86.1)	164.7 (86.1)
Torulene	7.77 (9.6)	19.6 (9)	--	--	8.78 (5.4)	9 (4.7)
Other carotenes	8.91 (11)	16.55 (7.6)	--	--	12.05 (7.4)	17.36 (9.1)
Total	81 ± 8.97	217.9 ± 4.42	7.37 ± 12.73	13.7 ± 13.86	162.6 ± 24.03	191.30 ± 44.25

Values represent the mean of three independent cultures after 48 h of cultivation.
-- not detected.

Finally, production kinetics of JLS02 and JLS07 strains up to 72 hours indicated that the maximal ß-ionone concentration was reached after 48 hr of cultivation in shake flask (Figure 2-4).

qPCR analysis was carried out to correlate gene expression levels of the introduced genes with carotenoid and ß-ionone measurements (Figure 2-5). The strain JLS03 showed a much higher expression of the *crtYB* gene than strain JLS02, with an increment of almost 18-fold. For JLS04 and JLS05, increased expression of *crtI* and *PhCCD1* genes was found, but for *PhCCD1* the increment in expression was lower compared to *crtYB* and *crtI* expression in strains JLS03 and JLS04, respectively (only a 3-fold increase compared to 18-fold for *crtYB* and 7-fold for *crtI*). Finally, for the high copy number vectors with double genes, both strains JLS06 and JLS07 resulted in clearly increased expression of *crtYB*. Surprisingly, only a slight increase in expression of *crtI* (JLS06) and *PhCCD1* (JLS07) genes was measured (Figure 2-5).

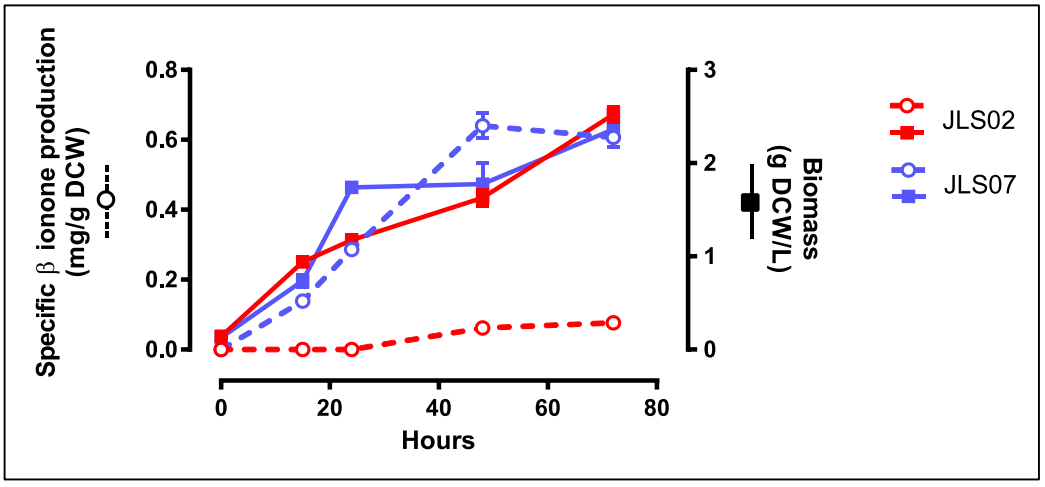

Figure 2-4. Kinetics of ß-ionone production and cell growth of the ß-ionone producing strains JLS02 and JLS07 up to 72 h cultivation.

ß-ionone production (*dashed line*) and biomass (*continuous line*) dynamic of JLS02 (*red*) and JLS07 (*blue*) strains for 72 h shake-flask cultures with a second phase of dodecane. *Value*s represent the mean of three independently grown cultures.

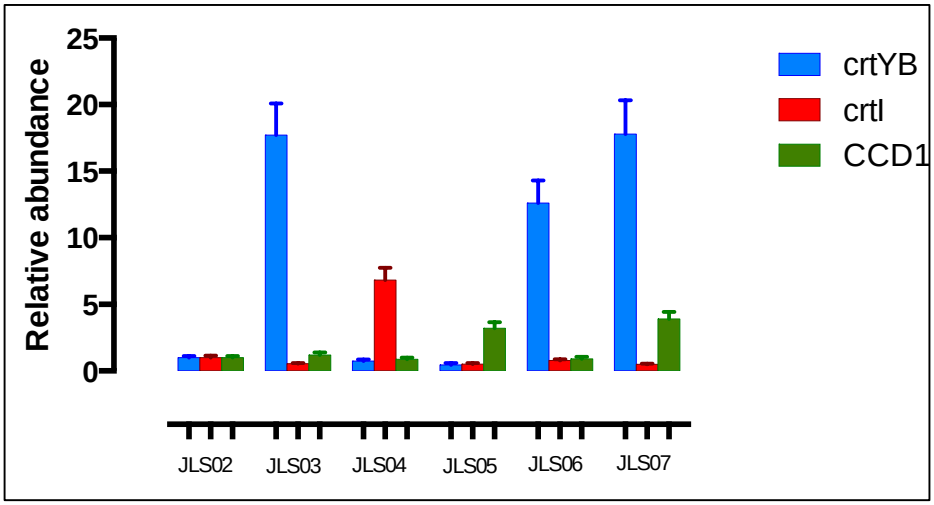

Figure 2-5- qPCR analysis of the carotenogenic *crtYB* and *crtI* genes, and the plant *PhCCD1* gene in the different ß-ionone producing strains.
Three cultures of each strain were inoculated in SC-URA medium with 2% glucose (w/v) and 10% dodecane (v/v), at the same optical density. 48 h later, samples were processed for cDNA synthesis. qPCR experiments were performed to determine the abundance of *crtYB*, *crtI* and *PhCCD1* in each strain relative to *TEF1*, which was used as internal control. Results from strains JLS03-JLS07 were compared to JLS02 as a calibrator sample. The *data* represent the average and the standard deviation of three independently grown cultures.

Table 2-4 – ß-ionone production by different strains after 48 h cultivation.

strain	O.D at 600 nm	ß-ionone (ppm)	ß-ionone (mg/g CDW)	Increase fold[a]
JLS02	3.33 ± 0.14	0.14 ± 0.02	0.073 ± 0.01	-
JLS03	3.22 ± 0.14	0.63 ± 0.13	0.34 ± 0.06	4.7
JLS04	1.43 ± 0.07	0.0	0.0	-
JLS05	1.36 ± 0.08	0.03 ± 0.03	0.04 ± 0.04	-
JLS06	2.76 ± 0.13	0.74 ± 0.06	0.49 ± 0.04	6.84
JLS07	3.06 ± 0.3	0.96 ± 0.08	0.62 ± 0.05	8.5

Values represent the mean ± SD of five independent cultures after 48 h of cultivation.
[a] Increase fold with respect to strain JLS02.

2.3.3 ß-ionone production in 2L bioreactors

Figure 2-6 shows that growth dynamics of strain JLS07 in batch mode in a 2 L aerated bioreactor consisted of an initial glucose-consuming growth, followed by ethanol consumption. The ethanol produced, as a consequence of the Crabtree effect in *S. cerevisiae*, was later consumed. For the glucose-consuming phase, the specific growth rate reached a $\mu = 0.106/h$; and for the ethanol consuming phase, $\mu = 0.05/h$.

At stationary phase (after 51 h of cultivation) an OD = 10.6 was reached; neither glucose nor ethanol were found in the culture. 610 µg/g of carotenoids were determined intracellularly. The extracellular β-ionone concentration reached 5 mg/L.

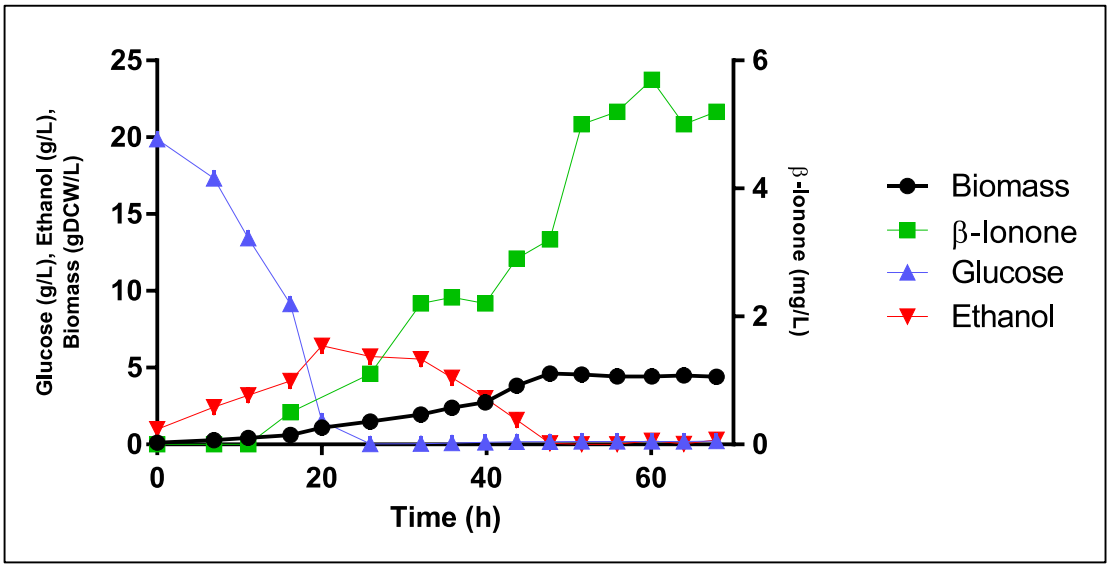

Figure 2-6- Batch culture dynamics of JLS07 strain.
The strain was cultivated in a two-phase culture at 20ºC for 68 hours. Samples were collected every 4 h for determination of kinetic parameters and metabolite measurements.

2.4 Discussion

A ß-ionone producing yeast platform was constructed by expressing the *tHMG1* and *BTS1* genes, the carotenogenic genes *crtYB* and *crtI*, and the gene *PhCCD1* encoding a ß-carotene cleavage enzyme in the engineered *S. cerevisiae* SCIGS22 strain. A maximal ß-ionone concentration of 0.63 mg/g was reached with the strain JLS07 in shake flask cultures and 1 mg/g in batch bioreactors. This corresponds to an 8.5- and 15-fold increase, respectively, compared with our first engineered *S. cerevisiae* JLS02 strain. Additionally, qPCR analysis indicated a significant increase in the expression of *crtYB* and *PhCCD1* genes in this strain (18-fold and 4-fold increase compared to JLS02, respectively), consistent with the highest ß-ionone production. The two-phase culture employed here allowed the continuous removal of ß-ionone, a toxic compound that inhibits cell growth in *S. cerevisiae,* even at 2 ppm (data not shown).

Influence of the expression system on ß-ionone production.

The large number of genes required for the heterologous synthesis of ß-ionone complicates a gene expression strategy exclusively based on plasmids, given the significant number of genetic markers required for strain selection. For the integration of the target genes, two vectors from a USER cloning compatible plasmid collection were used. The advantage of using this collection is that the recombination sites are strategically positioned in the *S. cerevisiae* genome (between essential genes), making the engineered strain stable over time, with minimal risks of gene loss by recombination [17]. In our study, no white colonies were observed when we integrated these genes, strongly indicating the high efficiency of this technique. Furthermore, the color was stable over time, suggesting that the recombinant strains are stable.

Nevertheless, engineering a metabolic pathway by expressing heterologous enzymes normally suffers from flux imbalance, as they typically lack the regulatory mechanisms present in the native metabolism [23]. For determining -and alleviating- possible bottlenecks of the pathway, we expressed multi-copy plasmid derivatives of the native 2

μm plasmid in the original JLS02 recombinant strain, reaching significantly higher ß-ionone concentrations compared to only integrative expression systems. Additionally, we observed coloration of the colonies when *crtYB* (alone or with *crtI/PhCCD1*) was expressed in an episomal vector (JLS03 or JLS07 vs. JLS02). We confirmed an increase in total carotenoids - mostly ß-carotene - by HPLC analysis.

We can further improve our platform, at least in terms of expression, by integrating a higher number of copies of the target genes. Verwaal et al., 2007 [24] reported a 5-fold increase in carotenoids in *S. cerevisiae* using the integrative expression of the *crtYB, crtI* and *BTS1* genes as compared to episomal expression. A similar result was obtained in *E. coli* where the production of ß-carotene was higher using low copy plasmids as compared to high copy plasmids [25]. The major problem with the use of high copy number vectors is associated with the resulting high metabolic burden. To avoid unnecessary enzyme synthesis, the identification of bottlenecks and enzyme efficiency is a key aspect to express genes accordingly. Considering that the plasmid collection used has 11 integrative vectors, with a total capacity to integrate 22 genes in different yeast chromosomes, similar expression levels to those reached by episomal vectors could in principle be obtained, with the additional benefits of stability, and no necessity for selection pressure. Nonetheless, considering that the *S. cerevisiae* SCIGS22 strain has only uracil auxotrophy, each transformation required the removal of the previously used *URA3* marker gene. Given that the 5-FOA elimination method is laborious and time consuming, successive integration - and marker recycling - resulted in an arduous and cumbersome transformation process. The development of a new microbial cell factory containing several auxotrophies as selection markers will allow constructing strains overproducing terpenoids easier and faster. Alternatively, the development of targeted genome editing using engineering nuclease, like the clustered, regularly interspaced, short palindromic repeats (CRISPR) technology, can be used to insert a desired sequence through recombination of exogenous DNA with a specific locus, without drug-resistance marker selection [26].

Influence of gene expression levels.

Four genes were expressed in *S. cerevisiae* in order to achieve ß-ionone biosynthesis. We first expressed an extra copy of the endogenous *BTS1* gene under the strong promoter *TEF1*. The *BTS1* gene is normally expressed at low levels in wild type cells [24], because the P_{BTSI} promoter is one of the weakest constitutive promoters related to terpenoid biosynthesis [27]. Then, we expressed the *crtYB* and *crtI* genes from the ascomycete *X. dendrorhous* to produce ß-carotene. Both genes encode bifunctional enzymes, commonly employed for ß-carotene and astaxanthin production in microorganisms [28]. Finally, the *CCD1 (PhCCD1)* gene from *P. hybrida* was expressed to cleave ß-carotene to ß-ionone, since the respective enzyme successfully produced ß-ionone in *E. coli* [3]. In terms of the genes used here, there is also space for further improvement of the present strain.

One extra copy of the *BTS1* gene may not be sufficient to consume the high FPP levels produced by the SGCI22a strain. Also, in other studies related with ß-carotene production in yeast, a GGPP synthase from *X. dendrorhous* (*crtE*) was expressed, reaching higher concentrations of ß-carotene [27, 28]. Therefore, further *BTS1* over-expression could lead to a higher accumulation of carotenoids and ß-ionone; alternatively, *crtE* over-expression in this platform could allow reaching a similar effect.

The accumulation of ß-carotene - with minimal amounts of lycopene - in our strains suggested a high expression and/or a high activity of the CrtI enzyme (also by the accumulation of torulene, another carotenoid produced by the CrtI enzyme). Verwaal et al, (2007) [24] expressed an extra copy of *crtI* gene in a *S. cerevisiae* strain that carried integrated *crtYB, crtI and crtE* genes, and observed a 10-fold increase in ß-carotene [24]. However, in the present study the overexpression of *crtI* from an episomal vector (measured by qPCR) resulted in cell growth decrease, which hampers to draw an accurate conclusion on the influence of gene overexpression in β-ionone production. The use of a high copy number vector to express this gene may cause an overconsumption of the available FPP, limiting the synthesis of other essential compounds, like ergosterol (considering that also *ERG9* was down regulated in this strain). In the case of strain JLS06, in which there was no increased expression of this gene, we conclude no effect of this

gene at that time. To further evaluate the role of the *crtI* expression level on ß-ionone production, an extra copy of the gene could be integrated or it would be advisable to work with low copy number vectors.

Finally, the significant accumulation of ß-carotene in all constructed strains - JLS03 to JLS07 - suggests that the *PhCCD1* gene might be suboptimally expressed or that the enzyme has low catalytic efficiency. The low growth of the JLS05 strain may indicate the accumulation of inhibitory compounds or the use of other substrates, e.g. lipids, even though the *PhCCD1* expression was not dramatically increased, as compared with the JLS02 strain (Fig. 2-5). In carotenoid accumulating microorganisms, the maize CCD1 enzyme does not exclusively cleave the 9,10 - 9',10' double bonds of carotenoids, but also the 5,6 - 5',6' [29], indicating that CCD1 might not exclusively produce β-ionone. Given that the *PhCCD1* over-expressing strain showed lower growth - and neither carotene nor volatiles were detected - it was not possible to identify the accumulation of toxic or other intermediate compounds. Nevertheless, strain JLS07 (*PhCCD1-crtYB*) reached optical densities similar to strain JLS02, indicating that the overexpression of *PhCCD1* together with *crtYB* did not produce the same effect in the cells. Expression of *CCD1* from different organisms or the use of other enzymes—like CCD4 that can also produce β-ionone from β-carotene—may further increase β-ionone production in yeast. Moreover, CCD4 enzyme seems to be more active than CCD1 in the ß-ionone production. For example, CCD4a and CCD4b enzymes from *Crocus sativus* expressed in *E. coli* strains engineered to accumulate ß-carotene, were 15 and 7.5-fold more active respectively, compared to CCD1 enzyme from the same plant [30]. CCD4 enzymes are targeted to the plastids, whereas CCD1 enzymes are cytosolic [30]. The location of CCD4 enzymes allows them to obtain access to plastid carotenoids, while CCD1 activity is limited to carotenoids out of these organelles - or once these organelles have lost homeostasis.

Other possible strategies include the construction of fusion proteins—to reduce the access of enzymes to other substrates—or the use of an inducible promoter, in order to facilitate cell growth and β-carotene accumulation at the beginning of the fermentation, before inducing β-ionone biosynthesis. We are currently developing a light induction system to

express heterologus genes as an alternative to the use of classical induction with metabolites. The first attempts of this system were evaluated for ß-ionone production in our yeast platform and it will be discussed in Chapter 3.

ß-Ionone titer.

Beekwilder et al. (2014), [9] recently reported the production of ß-ionone in *S. cerevisiae* by heterologous expression of a polycistronic construct, an alternative way to synchronously express a heterologous multigene pathway in *S. cerevisiae* [9]. The maximum concentration reached with this strategy was 0.22 mg/L. The *S. cerevisiae* JLS07 strain developed here increased the ß-ionone production seven-fold, achieving a maximal titer of 1.5 mg/L after 72 hr of cultivation in flask cultures and 5 mg/L after 50 hours in 2 L bioreactors.

Even though the constructed strains overproduced the precursor for the ß-ionone pathway, low optical densities were reached, probably due to the high-level expression of several genes. However, the product yield (Y_{sp}) reached for the strain JLS07 in flask cultures was 5 times higher than the Y_{sp} obtained by the polycistronic system.

Batch fermentation in bioreactors differed from flask cultures. The OD reached a three-fold increase and the ß-ionone concentration was eight-fold higher after 50 hour of cultivation. The higher ß-ionone production during the stationary phase can result from the accumulation of this compound in the dodecane layer. Moreover, the lower expression of the *PhCCD1* gene, compared to the expression of *crtYB* in this strain, could be related with a slow cleavage of ß-carotene (compared to its synthesis) since at 100 h of fermentation ß-ionone continues to accumulate, reaching a concentration of 8 mg/L and 200 ug/g of carotenoids (data not shown). Besides, glucose depletion during this stage can repress the *ERG9* gene expression under the *HXT1* promoter, favoring the accumulation of FPP and the carotenogenic pathway towards ß-ionone synthesis. qPCR analysis of the expression of *ERG9* and carotenogenic genes, together with metabolomics during the fermentation, might help to elucidate this hypothesis.

2.5 Conclusions and future directions

In this study, we constructed a yeast platform for β-ionone production by differential expression of the carotenogenic genes *crtYB* and *crtI*, and the plant gene *PhCCD1*. This platform is capable to produce 1 mg/g DCW in batch cultures, far away from the concentration needed in flavour industry. The fine-tuning of multi-gene expression is the key aspect to reach higher titers, rates and yields of final product. Currently, we are working in the optimization of the strain JLS07 by:

1. Overexpressing genes from the pathway: The same construction from plasmid pIJL02 (*CrtI* and *CCD1*) it will be integrated in another USER compatible locus of the yeast genome (pXI-2 locus). In this work, the overexpression of these two genes in high-copy number plasmids reduced the strain growth. Since these two genes are key for ß-ionone production, the introduction of extra copies could increase the final production without affecting biomass.

2. Expression of the *crtE* gene instead of the extra copy of *BtsI*, since *crtE* is capable to produce more carotenoids compared with *BtsI*.

3. Construction of a fusion enzyme between *crtE* and *ERG20* genes, avoiding FPP consumption in other reactions.

4. Protein engineering of CCD1 enzyme using rational design (homology modeling for CCD1) and directed evolution.

5. Expression of *CCD4* from *Crocus sativus* instead of *PhCCD1*, since the former has higher activity for the production of ß-ionone.

This ß-ionone producing strain is the first step towards the production of higher valuated carotenoids and/or norisoprenoids.

2.6 References

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3. USE OF A LIGHT SWITCHABLE SYSTEM TO INCREASE ß-IONONE SYNTHESIS IN *SACCHAROMYCES CEREVISIAE*.

3.1 Introduction

Saccharomyces cerevisiae is an excellent eukaryotic model system to study protein function and the mechanisms underlying its regulation. One of the most common strategies to control gene expression and therefore, the protein amounts, is the use of different types of promoters. Two classic types of promoters are employed for this purpose; constitutive promoters, such as TEF1, GPD and PGK1, showing variable strength; and inducible promoters, that respond to effectors such as galactose, methionine or copper, among others.

Inducible promoters are one of the easiest and most effective ways to turn on gene expression [1]. Even though these systems seem to control gene expression in a rapid and robust way, their use is hampered by the requirement of cell uptake and subsequent removal from culture, making very difficult to precisely switch on and off the gene expression, limiting their use mostly for *in vivo* studies [2, 3].

Recently, Hughes et al. (2012) [2] reported a novel induction method using light-responsive transcription factors to control DNA transcription. In plants and microorganisms, responses to light are involved in several essential biological processes, such as photosynthesis as well as morphogenesis, triggering differentiation [4]. Photoreceptor molecules, that can perceive either red or blue light in these organisms, are responsible for this powerful *photoresponse* by the activation of gene transcription [5]. As in yeast two-hybrid systems, genes can be induced by a split transcription factor that is reconstructed when two photoreceptors interact among each other, after a light pulse. One of these systems is based on the interaction of *Arabidopsis thaliana* phytochrome B, PhyB, and the Phytochrome Interacting Factor, PIF6, which utilizes red

light (600-700 nm) for the association and far-red light (700-800 nm) for dissociation [2, 6]. Even though these two events have a more rapid stimulation and reversibility than the one reported for galactose induction in yeast [7], the interaction requires the use of an exogenous chromophore or an engineered cell that can synthesize it, increasing time and cost [6]. A second system, recently reported, takes advantage of the interaction of *A. thaliana* CIB1 and cryptochrome 2 (CRY2) proteins, where the latter can be excited by blue light without requiring any exogenous chromophore [8]. In yeast, these two systems were used to regulate gene expression of reporter genes under control of *Gal1* promoter by splitting the transcription factor Gal4 in a ΔGAL4 ΔGAL80 strain. [2].

Other cryptochromes employed in light-switchable systems come from the fungus *Neurospora crassa* [9], one of the most studied eukaryotic organisms with a strong transcriptional response to light. The blue-light responses in *N. crassa* require the proteins White Collar 1 and 2 (WC-1 and WC-2). Both proteins are transcription factors of the fungal GATA zinc finger family, which contains PAS domains required for homo- and heterodimerization [10]. The White Collar Complex (WCC) -resulting from the association of both proteins - binds to consensus GATA and light response elements (LRE) within the promoters of light-regulated genes [11, 12].

Another *N. crassa* photoreceptor is VIVID (VVD). Malzahn et al. (2010) [13], demonstrated that WWC and VVD form highly dynamic homo- and heterodimers through its activated LOV domains (Light-Oxygen Voltage sensing), a subclass of the PAS domain that binds a cofactor capable of undergoing structural changes in response to environmental stimuli, like blue light [14]. In response to blue light, WWC initially forms homodimers that efficiently activates the transcription of *vvd*. VVD accumulates with a delay after light induction, acting as an inhibitor of WWC homodimerization, leading to photoadaptation. This VVD-mediated desensitization of the light-activated WWC, explains how *N. crassa* adapts to photoperiods.

The dynamics of the interaction between WC-1/VVD could be detected by a yeast two-hybrid system, fusing the LOV domains of both proteins to the activation domain

(Gal4AD), and DNA binding domain of Gal4 transcription factor (GalBD) and using a yeast reporter strain [12]. In order to control heterologous genes expression in *S. cerevisiae*, we employed this optogenetic switchable system with WC-1 and VVD1, in a terpene producing CEN-PK strain. This strain is capable of synthesizing the norisoprenoid ß-ionone by constitutive expression of the carotenogenic genes *crtYB* and *crtI* from *Xanthophyllomyces dendrorhous* and the plant gene *CCD1* from *Petunia hybrida* [14]. Since ß-ionone is produced by the enzymatic cleavage of ß-carotene by the latter gene, *CCD1*, we constructed a yeast platform that accumulates ß-carotene in the dark and allows the light-induced production of ß-ionone. To induce *PhCC1* expression, we swapped its original promoter in the JLS02 strain [15] by Gal1 promoter, and to control the gene expression by light, we employed the *N. crassa* genes WC-1 and VVD1 fused to the Gal4BD and Gal4AD of *Gal4* gene (Fig. 3-1). Also, since the transcriptional factor Gal4 operates through an upstream activating sequence (UAS_{GAL}) present in *Gal* gene promoters and that the number of UAS_{GAL} sites leads to a differential activation, we evaluated the strength of this system with a promoter with one (1xUAS) and five (5xGal4UAS) UAS repetitions.

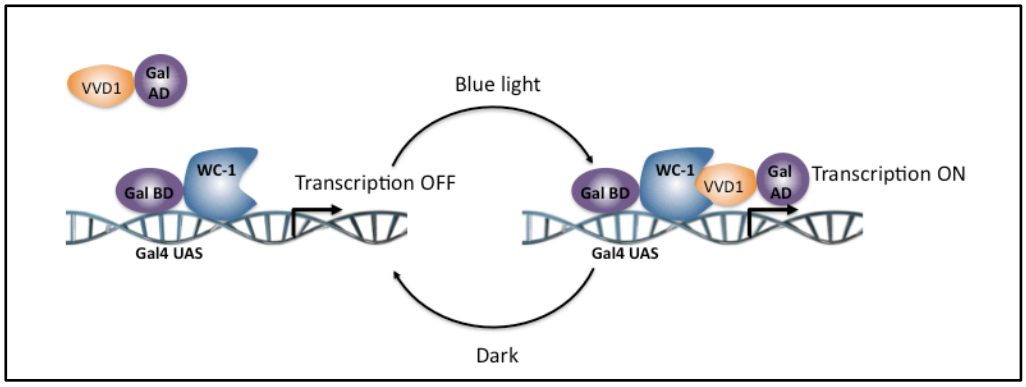

Figure 3-1 - Scheme of the light expression system used for inducible β-ionone production.
A blue-light stimulus enables the interaction of the cryptochromes WC-1 and VVD1 reconstituting the *Gal4* transcription factor, which triggers DNA transcription. WC-1: white collar 1, VVD1: vivid 1, GalAD: Activation domain of Gal4 transcription factor, GalBD: binding domain of Gal4 transcription factor, UAS: upstream activation sequence. Image adapted from *Huges et al*., (2012) [2].

3.2 Material and Methods

3.2.1 Plasmid construction

We constructed three plasmids for the integration of the light expression system into JLS02 genome, using yeast recombinational cloning (YRC) technique [16]. PCR amplification of the DNA fragments was carried out in 35 PCR cycles using Phusion High-Fidelity DNA polymerase (Thermo Scientific, Waltham, MA, USA), following the manufacturer's instructions. The fusions VVD-1/Gal4AD and WC-1_{LOV}/BD (with the promoter and terminator) from PMB1 and PMB2 plasmids were amplified using primers 1/2. The drug resistance genes to nourseothricin, NatMx, and hygromycin B, HphMx, were amplified from pAG25 and pAG32 plasmids with primers 3/4 and 5/6, respectively. For the *PhCCD1* promoter replacement, the drug resistance gene to Kanamycin, KanMx, was amplified using primers 7/8 and the Gal1 promoter with primers 9/10. All PCR products were treated with DpnI enzyme to eliminate methylated plasmid. For YRC, the PCR products were mixed (50 μl of each PCR product) with five μl of the linearized episomal yeast expression vector pRS426GPD (Addgene, Cambridge, MA, USA) treated with EcoRI and XhoI enzymes. Finally, 50 μl of the mix were used to transform competent BY4741 yeast cells. The transformed cells were grown in SC-URA plates. Positive colonies were picked and the plasmid isolated with Zymoprep Yeast Plasmid Miniprep (ZymoReasearch, Irvine, CA, USA). The plasmids recovered were employed to chemically transform competent *E. coli* cells for its amplification (Table 3-2).

The resulted plasmids were designated pIFS01 (WC-1/BD-HphMx), pIFS02 (VVD-1/AC-NatMx), pIFS03 (KanMx-$Gal1_p$) and pIFS04 (KanMx-5xGal4UAS $Gal1_p$). All plasmids were verified by PCR and sequencing (Macrogen, Korea).

Maps of all the constructed plasmids could be found in Additional file 4 and the primers used for all amplifications are provided in Additional file 5.

3.2.2 Yeast strain construction

The *S. cerevisiae* strain used in this work has a CEN.PK background, with auxotrophy for uracyl. All *S. cerevisiae* strains constructed in this work are listed in Table 5.

The strain JLS08, carrying the light expression system was constructed from strain JLS02 (Chapter 2), using plasmids pIFS01 and pIFS02 in three transformations steps:

- First, the *URA3* marker gene was amplified from plasmid p426GPD using primers 11/12, containing 50 bp primer tails complementary to the upstream and downstream regions of *Gal4* gene. The JLS02 strain was then transformed with the PCR-amplified fragment and the transformants were selected on SC-URA plates. Correct integration of the *URA3* into the *Gal4* locus was tested by PCR using primers 13/14. The *URA3* marker was subsequently replaced by the pIFS01 cassette, which contains the fusion of VVD-1/Gal4AC with the *NatMx* marker.
- Second, this resulting fragment was PCR-amplified with primers 15/16 containing the same 50 bp primer tails, complementary to the upstream and downstream regions of *Gal1*. The transformants were selected using YPD plates, containing 100 mg/ml of nourseothricin (Gold Biotechnology, USA). This new integration was checked by PCR with primers 13/14 and negative selection in SC-URA plates.
- Finally, for integration of fusion WC-1/Gal4BD with *HphMx* marker into the *Gal80* locus, the pIFS02 plasmid was PCR-amplified using primers 17/18 and the PCR product transformed into the strains selected before. The transformants were selected using YPD plates containing 0,3 mg/ml of hygromycin B (Sigma). Correct integration was tested by PCR with primers 17/19. This final strain was called JLS08.

The swapping of the CCD1 promoter for the Gal1 promoter with 1xGal4UAS and 5xGal4UAS, resulted in the strains JLS09 and JLS10, respectively. The promoter Gal1 (with different number of UAS) fused to the *KanMx* marker was amplified using primers

20/21 from plasmid pIFS03 and pIFS04, and transformed into strain JLS08. The transformants were selected in YPD plates with 0,4 mg/ml G418. Primers 22/23 were used to test for the correct integration.

Strains JLS08-crtYB, JLS09-crtYB and JLS10-crtYB were obtained after transforming, respectively, strain JLS08, JLS09 and JLS10 with the high copy number plasmid pEJL04 for episomal expression of the *crtYB* gene. These strains were grown in SC-URA media. As a control, parental strains were transformed with empty vector pRS426.

3.2.3 Growth conditions

Single colonies were inoculated in pre-cultures of 3mL of SC medium lacking uracyl. Then, cultures were grown in 250 mL shake flasks at 30ºC or 20˚C and 180 rpm, in a horizontal shaker, with constant light (visible light) or constant darkness. The cultivation volume was 50 mL, with a second phase of dodecane (10%, v/v). Flask cultures were inoculated from pre-cultures grown on the same medium, to an initial OD_{600} of 0.1. Control cultures were run with the same strains, in the same conditions.

3.2.4 Real-time measurements of luciferase activity in yeast cells

Firefly luciferase was employed in the JLS08 cells as a real-time reporter for the light expression system. Strain JLS08 was transformed with a plasmid containing a destabilized version of the firefly luciferase gene, a short-lived version [17], under promoter Gal1 with 1xUAS (pAG413-Gal1-lucCP^{+}), or 5XGALUAS (pEFS01). Transformants were grown in SC-Ura media at 30ºC overnight, at the next day 1 mL of the cells was used to inoculate a flask containing 50 mL of SC-Ura and incubated under constant light or constant darkness conditions. The luminescence was measured during six hours using a Turner TD-20e luminometer. Luminescence of the cell culture was normalized by its OD_{600}.

3.3 Results

3.3.1 Deletion of the *Gal4* and *Gal80* genes

The light-switchable system was developed in the JLS02 strain [15], which has already an active ß-ionone production pathway, together with other modifications in the MVA pathway of the yeast. Since this strain has the *Gal4* and *Gal80* functional genes, both were replaced by two cassettes: *Gal4* was replaced with cassette pIFS01 carrying the fusion between VVD-1 and the activation domain of *Gal4;* and *Gal80* with cassette pIFS02 carrying the fusion between WC-1 and the C-terminal binding domain of *Gal4*. The resulting strain was called JLS08 (Table 3-1).

To evaluate the functionality of the system in this strain, the expression of the reporter gene luciferase under Gal1 promoter, with different number of UAS, was induced by blue light. Figure 3-2 shows the quantification of the luminescence of the strain under continuous light induction (LL) and darkness (DD), at 30ºC. Both promoters induce the expression of the reporter, but when the promoter has 5xGal1 UAS, the expression is almost 5 fold higher, compared to the expression without induction. The system did not show any hardly expression in darkness, suggesting a low expression background. Since luminescence is normalized by cell growth (OD_{600}), results suggest that the expression decreases over time; however, these results are exclusively related to the increment of biomass over time, whereas total expression (without normalization) remained stable (data not shown).

Table 3-1. List of *S. cerevisiae* strains used for inducible β-ionone production

Strain	Genotype	Plasmid	Reference
JLS02	*MATa MAL2-8^c SUC2 ura3-52 lpp1Δ::loxP dpp1Δ::loxP* P_{ERG9}*Δ::loxP-*P_{HXT1} *gdh1Δ::loxP* P_{TEF1}*-ERG20* P_{PGK1}*-GDH2* P_{TEF1}*-tHMG1* P_{TEF1}-BTSI P_{PGK1}-crtYB P_{TEF1}-crtI P_{PGK1}*-PhCCD1*	none	López
JLS08	JLS02 *Gal80Δ::WC-1-HphMx Gal4Δ::VVD-1-NatMx*	none	This study
JLS08-crtYB	JLS08	P426 P_{GPD}*crtYB*	This study
JLS09	JLS08 P_{CCD1}*Δ::KanMx-*P_{Gal1}	none	This study
JLS09-crtYB	JLS09	P426 P_{GPD}*crtYB*	This study
JLS10	JLS08 P_{CCD1}*Δ::KanMx-*P_{Gal1}*5xUAS*	P426 P_{GPD}*PhCCD1*	This study
JLS10-crtYB	JLS10	P426 P_{GPD}*crtYB*	This study

Table 3-2. Plasmids used for inducible β-ionone production in yeast

Plasmid name	Plasmid description	Reference
PMB1	LEU-based expression plasmid carrying the VVD-1 gene fused with the activation domain of Gal4	[12]
PMB2	TRP-based expression plasmid carrying the WC-1LOV domain fused with the DNA binding domain of Gal4	[12]
pAG413-Gal1-lucCP+	PGal1LUC	[15]
pEFS01	PGal1LUC 5xUASGal4	This study
pIFS01	PMB2-HphMx fusion cassette	This study
pIFS02	PMB1-NatMx fusion cassette	This study
pIFS03	PTEF1-crtI-PGa1-CCD1	This study
pIFS04	PTEF1-crtI-PGa1-CCD1 5xUASGal4	This study

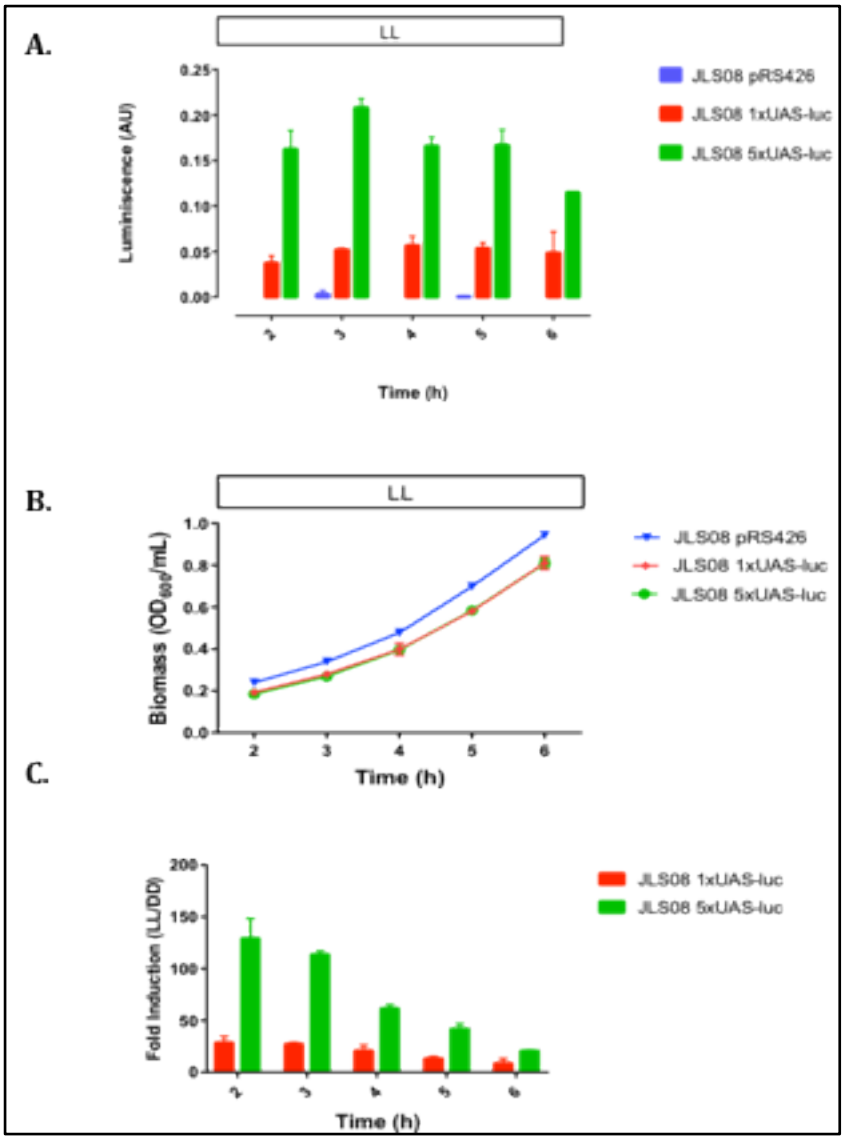

Figure 3-2. Induction of the luciferase reporter gene by WC-1/VVD-1 blue light expression system

(A) Luminescence quantitation of the luciferase reporter gene under Gal1 promoter with 1xUAS (red bars) or 5xUAS (green bars) in JLS08 strain. The expression in all strains was induced by blue light for 6 h. As a control, the same strains were transformed with an empty vector. (B) biomass quantitation of both strains expressing the blue-light induction system. (C) Fold induction of the luciferase reporter gene when the expression is induced by light over darkness (LL/DD).

3.3.2 *phCCD1* Promoter switch and plasmid transformation

In order to induce the expression of the *phCCD1* gene by blue light, we swapped the original constitutive TEF1 promoter of this gene by Gal1 promoter, with one UAS_{GAL} (cassette pIFS03) and with $5xUAS_{GAL}$ (pIFS04). The resulting strains were called JLS09 and JLS10, respectively. Since the maximum accumulation of ß-carotene - the direct precursor of ß-ionone - occurred in strain JLS03, which expressed the carotenogenic gene *crtYB* in a high copy number vector (pEJL04), the strains JLS09 and JLS10 were also transformed with this plasmid, giving rise to the JLS09-crtYB and JLS10-crtYB final strains. Both strains were grown in SC-URA plates and showed the characteristic orange colour of β-carotene, as reported before.

Preliminary assessment of colour changes in these strains was carried out by growing the JLS09-crtYB strain at 30ºC for 48 hours in the dark (DD) or under continuous light (LL). As a control, the JLS08 strain was also grown in the same conditions. No significant changes in colour of the JLS09-crtYB strain in plates was detected although, after centrifugation of both cultures, the pellets showed a higher coloration when the two strains where grown in darkness compared to light (Figure 3-3). This result also confirmed the increase in pigment production when the *crtYB* gene is expressed episomally (JLS09-crtYB vs. JLS08, in both conditions). No ß-ionone production could be detected in these strains.

3.3.3 Cultivation conditions

To set an induction strategy able to produce ß-ionone, the strains JLS09-crtYB and JLS10-crtYB were grown for 48 hours in the dark, followed by 24 hours with light induction. The cultivations were carried out at 20ºC, since we observed that more β-carotene is produced at this temperature [15]. As control, the same strains were grown for 72 hours in the dark to compare the ß-ionone production without induction. In addition, the strain JLS08 was also transformed with the plasmid pEJL04 to compare this new system with the strategy developed before (Chapter 2).

No differences could be found in cell growth (measured as optical density) of both strains with the light expression system (JLS09-crtYB and JLS10-crtYB), compared to the two control strains (JLS08 and JLS08-crtYB). A slight increase in the OD_{600} was, however, observed for all the strains grown 48 hours in the darkness, followed by 24 hours of light (DD/LL condition) (data not shown). Figure 3-4.A shows the pellets of all the strains in the DD/LL and DD (72 h darkness) conditions. Again, we observed higher pigmentation when the strains expressed the *crtYB* gene episomally. There was no clear differentiation in the colour of the cell pellets when the strains have different UAS (JLS08-crtYB and JLS09-crtYB) or were grown in different conditions (DD/LL v/s DD), except for JLS08 where a lighter colour is observed in dark.

ß-ionone measurements showed production by all the strains, but no difference in the concentration could be measured between strains with the light expression system and the control strain JLS08. The strain JLS08-crtYB showed a 10-fold increase in ß-ionone production compared with the other strain, but only in the DD condition.

Finally, since 24 hours might be a too short period for allowing expression of induced-*phCCD1*, the strains were grown for 48 hours in dark, followed by 48 hours in light conditions (DD/LL). As a control, cells were also grown for 96 hours exclusively in darkness (DD). No difference in the growth achieved by both strains was observed (data not shown). Figure 3-5 illustrates the cell pellets and ß-ionone concentrations of all the strains in this condition after 96 hours. Almost no difference in the colour of the pellet of the strain JLS08 was observed between both conditions, but the rest of the cells showed a higher coloration when they were grown only in darkness. This observation was not correlated with ß-ionone production, since the strains carrying the light expression system (JLS08-crtYB and JLS10-crtYB) reached similar concentrations of ß-ionone between both conditions, even compared with strain JLS08. Again, the strain with the constitutive promoter for *phCCD1* and with pEJL04 plasmid showed the highest production.

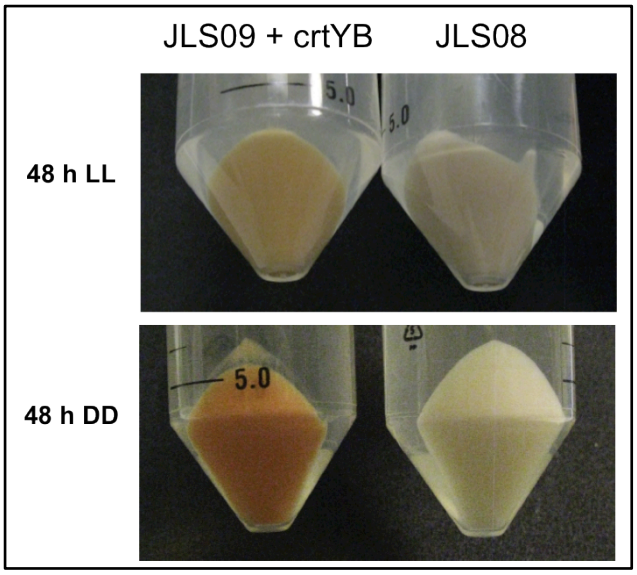

Figure 3-3. Induction of the *phCCD1* gene by WC-1/VVD-1 blue light expression system after 48 hours.
Left. Representative plates of strain JLS09-crtYB (with *crtYB* episomally expressed) grown in continuous light induction (LL) and darkness (DD). Right. Colour change of strain JLS09-crtYB and JLS08 as a control, in same conditions. Photographs were taken after 48 h of cultivation.

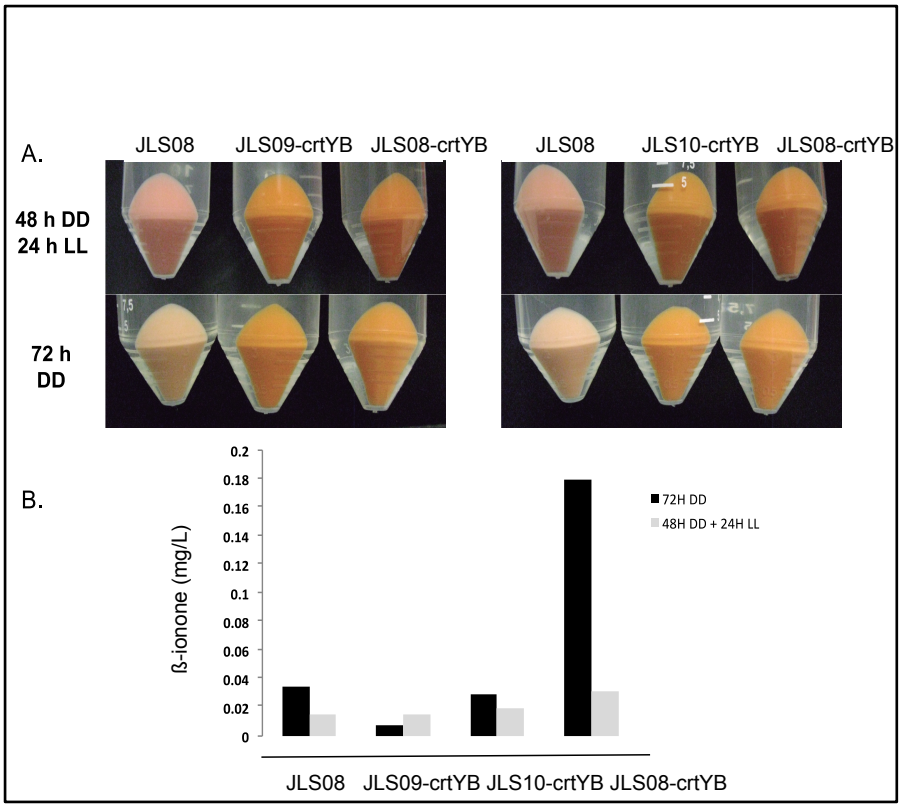

Figure 3-4. Induction of the *phCCD1* gene by WC-1/VVD-1 blue light expression system after 72 hours.
A. Colour of strain pellets at 72 hours under continuous light/darkness (LL/DD) or darkness (DD) conditions. B. ß-ionone concentration of the constructed strains in this condition. *Values* represent the mean of two independently grown cultures (each culture was made in duplicate).

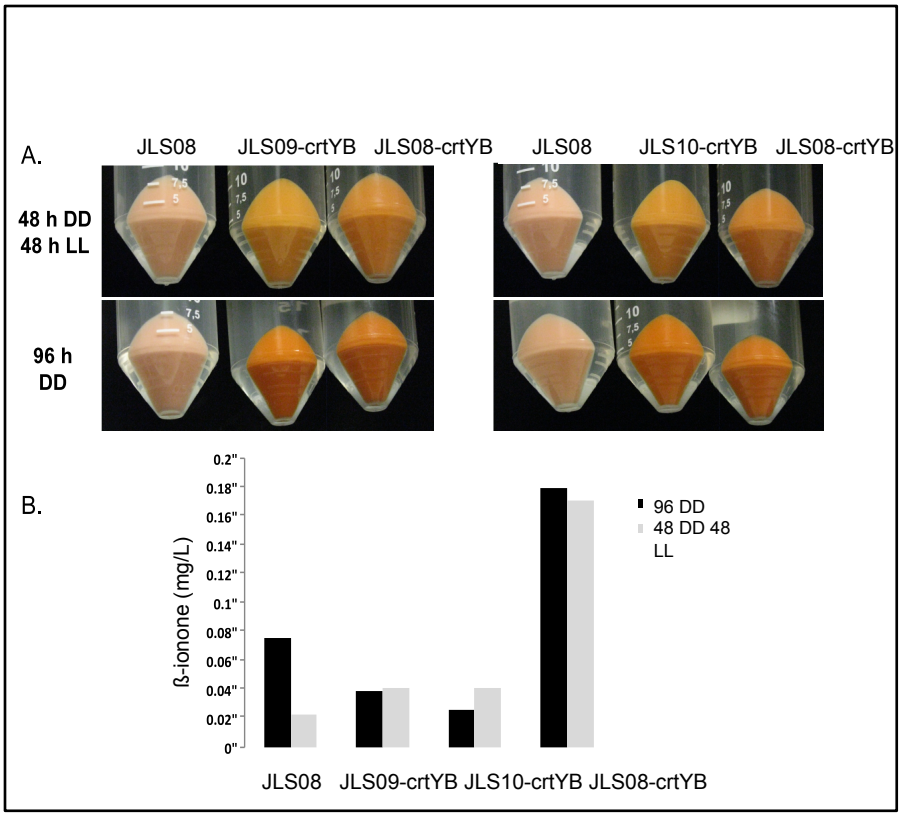

Figure 3-5. Induction of the *phCCD1* gene by WC-1/VVD-1 blue light expression system after 96 hours.

A. Colour of strain pellets at 96 hours under continuous light/darkness (LL/DD) or darkness (DD) conditions. B. ß-ionone concentration of the constructed strains in this condition. *Values* represent the mean of two independently grown cultures (each culture was made in duplicate).

3.4 Discussion

In this chapter, a light expression system was integrated to the ß-ionone producing yeast strain developed in Chapter 2. The original JLS07 strain was able to produce a maximal ß-ionone concentration of 0.63 mg/g in shake flasks and 1 mg/g in batch bioreactors, but accumulates a high amount of ß-carotene, which still can be finally converted to ß-ionone. In order to induce only this last step of the ß-ionone pathway, we integrated a light induction system to the former strain, in which the expression of genes with a Gal1 promoter can be induced by a blue light stimulus. For this purpose, we changed the original *phCCD1* promoter by the Gal1 promoter with either 1xUAS or 5xUAS.

The system worked well for the luciferase reporter gene. We observed low luminescence when the strains were grown in darkness (DD) but, when stimulated with light, the luminescence increased more than 30 fold for the strains containing the promoter with 1xUAS and 150 fold for the promoter with 5xUAS. Until 6 hours of induction, at least, there was a clear differentiation between darkness and light conditions, and the expression was maintained constant over time (data without normalization by biomass).

For the expression of *phCCD1,* we also utilised these two types of promoters and we assayed different induction times. First, we induced strain JLS09-crtYB for 48 hours in continuous light and compared the colour of the strain with the same strain maintained in the darkness. No ß-ionone was measured in the cultures of this strain, but there was a significant loss of pigmentation (observed in cell pellets) after light induction. This suggests a light degradation of some carotenoids by the light, since their photosensitivity is well known. To avoid this effect, we first grew the strain under darkness (for 48 hours) to accumulate ß-carotene, followed by induction of *PhCCD1* expression (continuous light for 24 or 48 hours). No difference in ß-ionone concentration between both strains (JLS09-crtYB and JLS10-crtYB) and the control JLS08 could be determined.

Further experiments need to be conducted to demonstrate the functionality of this system for apocarotenoid biosynthesis. The expression of the genes involved in the ß-ionone production must be quantified using qPCR to determine the level of induction of the *phCCD1* expression, as well as to understand how the light affects the rest of the genes. In addition, we need to measure total carotene content to quantify any loss due light degradation, and, therefore, to correlate it with the decrease of ß-ionone production.
A new set of essays should be carried out to evaluate the kinetics and characteristics of this promising system.

3.5 Conclusions and future directions

This light expression system has several advantages compared with other induction systems of gene expression. No chemical inductor is added to the medium, which implies lower cost and time consumption, when the system is induced. Besides, this system avoids the accumulation of final toxic products that may affect the biomass concentration and the constant gene expression as with constitutive promoters. In the case of ß-ionone, we observed a higher production of this apocarotenoids when the strains were in the stationary phase (Figure 2-6, Chapter 2), and since ß-ionone can be toxic for the cells, a delayed biosynthesis of the compound could improve its final concentration and productivity.

In this study, the light induction system worked well for expression of a reporter gene, in the ß-ionone production platform. Nevertheless, the prolonged light induction seems to degrade the carotenoids, affecting the ß-ionone conversion. A better strategy, with short pulses induction, could avoid this problem. Finally, another interesting platform could contemplate the induction of *crtYB* gene expression instead of *phCCD1*. Indeed, since *crtYB* increased five-fold the β-ionone concentration when was overexpressed (Table 2-4), the induction of this gene by light seems promising.

3.6 References

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4. PRODUCTION OF THE MONOTERPENE CARVONE BY EXPRESSION OF MINT GENES IN *SACCHAROMYCES CEREVISIAE*

4.1 Introduction

Monoterpenes, together with sesquiterpenes, are the main volatile compounds produced in plants [1]. They can act as an indirect type of defense, attracting arthropods that prey on or parasitize herbivores, avoiding further damage in plants [2].

Monoterpenes also have many pharmacological properties. Several studies demonstrated that both, natural monoterpenes and their synthetic derivatives have antifungal, antibacterial, antiviral, antioxidant, anticancer, antiarrhythmic, antinociceptive, anti-inflammatory and antispasmodic properties [3]. For example, the acyclic monoterpene (-)-linalool and the monocyclic monoterpene 1,8 cineole, have strong antibacterial activity against Gram-positive and Gram-negative bacteria [4–6]. Besides these properties, monoterpenes also have industrial applications in foods and cosmetics due to their value as fragrances and additives (e.g. menthol, limonene and linalool, among others). Recently, some aromatic monoterpenes, like p-cymene, have received an increasing attention due to their potential application as components of advanced bio-fuels [7].

Monoterpenes of the *p*-menthane group are the dominant constituents of commercially important essential oils obtained from members of the genera *Mentha*, *Carum*, *Citrus* and *Eucalyptus* [8]. One of the most attractive members is limonene, a monoterpenic olefin that is well known for its citrus-like olfactory properties. This molecule can be further oxidized to several products of interest, including menthol, *perillyl* alcohol and carvone [9].

L-carvone is a monoterpene mostly found in spearmint seeds and caraway [10]. This monocyclic ketone has an *in vitro* bioactivity against a wide spectrum of human pathogenic fungi and bacteria [11], making it attractive as a food preservative. In the United States, it is also employed as insect repellent [12]. Currently, the majority of L-carvone (approximately 2,000 t/year) is obtained by chemical synthesis from (+)-limonene, present at high concentrations in the *Citrus* rind, a by-product of lemon juice production [8].

Monoterpenes are generally obtained from their natural sources, but the increasing development in metabolic engineering could allow the production of significant amounts of these compounds, in a more economical and sustainable way. Limited work has been conducted to optimize a suitable host for its efficient production, compared to other isoprenoids. The latter results because, unlike plants, microorganisms usually do not carry a specific geranyl pyrophosphate synthase (GPPS), resulting in the absence of a geranyl pyrophosphate (GPP) pool available for monoterpene biosynthesis. As mentioned before, in *Saccharomyces cerevisiae* the enzyme farnesyl pyrophosphate synthase (FPPS) is responsible for the formation of both GPP and FPP compounds. Since this enzyme shares both activities, these two metabolites cannot be easily separated. Furthermore, monoterpenes are generally more toxic to microorganisms than sesquiterpenes, mainly due to their lipophilic character, which interferes with membrane components [13].

The isolation of a yeast mutant, exhibiting a 14-fold lower FPPS-specific activity, capable of excreting the prenyl alcohols geraniol and linalool, opened the way for engineering monoterpene production in yeast cells [14]. The mutated gene, called *erg20-2*, showed a single nucleotide change resulting in a $K^{197}E$ substitution [15]. More recently, another variant of this mutant, $K^{197}G$, was employed to produce the monoterpene limonene, reaching a maximal concentration of 0.45 ppm, using a headspace trapping system [16].

In this chapter, we describe the construction of a yeast platform for the production of the monoterpene carvone, in an already engineered *S. cerevisiae.* Carvone was

biosynthesized in a three-step pathway, in which GPP is cyclized to the (-)-limonene, which is then hydroxylated by a cytochrome P450-dependent monooxygenase to trans (-)-carveol and subsequently dehydrogenated to carvone. We generated a carvone-producing strain by overexpressing the genes limonene synthase (*LS*), the p450 limonene hydroxylase (*LH*) and carveol deshydrogenase (*CD*) from *Mentha spicata*, together with the *erg20-2* gene in an engineered yeast strain (Figure 14). Unfortunately, no monoterpenes could be detected so far, indicating that further optimizations are required.

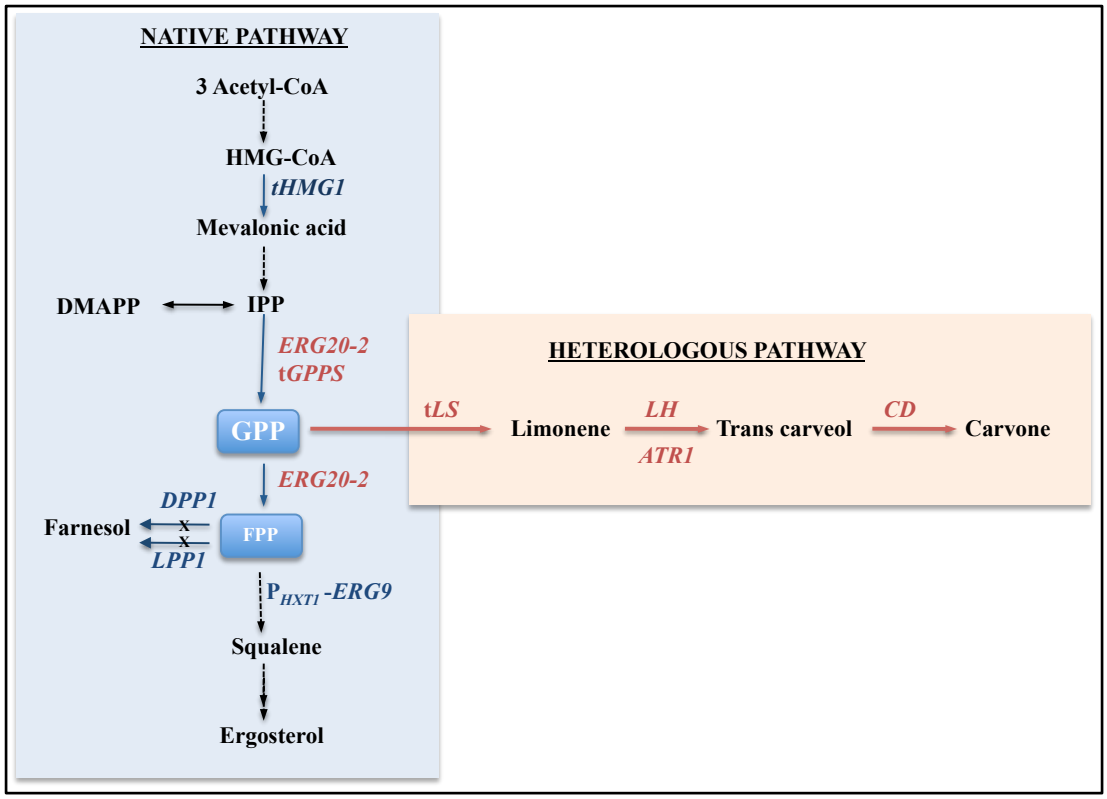

Figure 4-1 - Engineered platform for carvone production in yeast.
The blue arrows/genes indicate the modifications of the strain CEN.PK SCIGS22a (Chapter 2). The red arrows/genes show the genes expressed in the present study. The mutated version of *ERG20* gene, called *ERG20-2*, was overexpressed. The heterologous genes t*GPPS* (encoding GPP synthase without signal peptide), t*LS* (encoding limonene synthase without signal peptide), *LH* (encoding p450 limonene hydroxylase), ATR1 (NADPH- cytochrome P450 reductase) and *CD* (encoding carveol deshydrogenase) were integrated.

4.2 Material and Methods

4.2.1 Plasmid construction

The genes coding for Geranyl Pyrophosphate Synthase (*GPPS*) from *Abies grandis*, Limonene Synthase (*LS*), p450 Limonene Hydroxylase (*LH*) and Carveol Deshydrogenase (*CD*) from *Mentha spicata* and the partner redox NADPH-cytochrome P450 reductase (*ATR1*) from *Arabidopsis thaliana*, were synthesized by Genscript (Piscataway, NJ, USA) (Additional file 6), with codon optimization for expression in *S. cerevisiae.* The *ERG20* gene was PCR amplified from genomic DNA of the strain *S. cerevisiae* CEN.PK113-5D and mutated by fusion PCR to generate the *ERG20-2* gene.

We first constructed two plasmids to integrate the genes needed for carvone production into the yeast genome, using the USER cloning technique [17]. USER assembly was performed as previously described in Chapter 2. The USER vectors pXI-3 and pX-2 were amplified by PCR using primers pair 1/2 followed by digestion with the Nb.BsmI nicking endonuclease. The genes *GPPS*, *LS, LH* and *CD* were amplified using primers pair 3/4, 5/6, 7/8 and 9/10 respectively. Both *GPPS* and *LS* were amplified as truncated versions, *i.e.* without plastid signal peptides, designating them *tGPPS* and *tLS*, respectively (with t for truncated). The resulting plasmids were designated pIKE01 (with *tGPPS* under the *TEF1* promoter and *tLS* under the *PGK1* promoter) and pIKE02 (with *LH* under the *TEF1* promoter and *CD* under the *PGK1* promoter. All vectors contained

the *Kluyveromyces lactis* (*Kl*) *URA3* gene flanked by direct repeats (in order to be able to recycle this marker for future transformations) (Table 4-2).

We also constructed a series of plasmids for episomal expression using the Gibson assembly technique, as previously described in Chapter 2. The episomal yeast expression vector p426GPD (Addgene, Cambridge, MA, USA) was amplified with the primers pairs 11/12, 13/14, 15/16 or 17/18 depending on the gene cloned. *ATR1*, t*GPPS ERG-20-2* and *tLS* were amplified using primers 19/20, 21/22, 23/24 and 25/26 respectively. The resulting plasmids were designated pEKE04, pEKE05 and pEKE06, containing *ATR1*, t*GPPS* and *ERG20-2/tLIMS* genes, respectively.
Finally, we constructed a third integrative plasmid by Gibson Assembly, containing the *ATR1* and *tHMG*1 genes. The pXI-5 USER compatible plasmid was employed as backbone and amplified with primers pair 27/28. *ATR1* was amplified using primers 29/30 and the *tHMG*1 gene, already integrated into the CENPK SCIG22a genome and used as template, was amplified using primers pair 31/32. The assembly was exactly as described before and the resulting plasmid was designated pIKE03.
All plasmids were verified by sequencing (Macrogen Inc, Seoul, Korea). Primers used for all amplifications are provided in Additional file 7 and the maps of all the plasmids constructed are in Additional file 8.

4.2.2 Yeast strain construction

The *S. cerevisiae* strain employed throughout this work has a CEN.PK SCIGS22a background with further genome modifications for the overproduction of FPP [18]. All *S. cerevisiae* strains constructed from this strain are listed in Table 4-1.

Table 4-1. List of *S. cerevisiae* strains used for monoterpene production

Strain	Genotype	Plasmid	Reference
SCIGS22a	MATa MAL2-8c SUC2 ura3-52 lpp1Δ::loxP dpp1Δ::loxP P_{ERG9}Δ::loxP-P_{HXT1} gdh1Δ::loxP P_{TEF1}-ERG20 P_{PGK1}-GDH2 P_{TEF1}-tHMG1	none	[18]
KE01	MATa MAL2-8c SUC2 ura3-52 lpp1Δ::loxP dpp1Δ::loxP P_{ERG9}Δ::loxP-P_{HXT1} gdh1Δ::loxP ERG20Δ::KanMX P_{PGK1}-GDH2 P_{TEF1}-tHMG1	none	This study
KE02	KE01 + P_{TEF1}- tGPPS PPGK1-tLS	none	This study
KE03	KE02 + P_{TEF1}-LH PPGK1-CD	P426 P_{GPD}ATR1	This study
KE04	KE02	P426 P_{GPD}GPPS	This study
KE05	KE03 + PPGK1-ATR1	P426 P_{GPD}ERG20-2 LIMS	This study

4.2.2.1 ERG20 deletion

Strain SCIGS22a carries an extra copy of the ERG20 gene which encodes the *S. cerevisiae* enzyme FPPS. Since our target is to overproduce monoterpenes - and not sesquiterpenes - we deleted the extra copy of this gene by replacing it with the selective marker KanMx, into the genome. For this purpose, we employed a bipartite strategy, in which two overlapping parts of the marker gene were generated, and each fused in a PCR reaction to the upstream and downstream homologous regions of the genome flanking the ERG20 gene. The primers used for the amplification of the first 5' part of the marker gene, including a TEF promoter, were the primers pair 33/34. The 3' part of the gene, including TEF terminator, was amplified using primers 35/36. These two parts overlap by 546 nucleotides. The upstream and downstream regions flanking the ERG20 gene were amplified using the primers 37/38 and 39/40, respectively. For the fusion of the upstream fragment to the 5' part of the KanMx gene, and in the same way, the 3' part of the gene with the downstream fragment, 2 μl of each PCR product were mixed with two outer primers. The fusion PCR was conducted using the Phusion Pol with two steps PCR (16 cycles without primers, and then 35 cycles with the outer primers). Both fused cassettes were employed to simultaneously transform the SCIGS22a - with the standard lithium acetate/single-stranded DNA carrier/PEG procedure - [19] and the

resulting transformants were selected using YPD + G418 plates. The new strain was designated KE01. The correct cassette integration into the ERG20 extra sites was tested by PCR, using the primers pair 37/34.

4.2.2.2 Carvone pathway integration

The *S. cerevisiae* KE03 strain, carrying the genes t*GPPS*, t*LS, LH* and *CD* was constructed from strain KE01 by transforming the strain with the cassettes from plasmids pIKE01, pIKE02 and with the episomal plasmid pEKE04. For this purpose, the plasmids pIJL01 and pIJL02 were restricted with enzyme SwaI (New England BioLabs) and the fragments isolated from vector backbones were used for yeast transformation, one cassette at a time. All transformants were selected using SC-URA plates. Correct cassette integration into the different locus was tested by PCR using the same primers described in Chapter 2.

KE04 strain was constructed with cassettes pIKE01 and PEKE05 to produce only limonene as final product.

Finally, KE05 strain was constructed using cassettes pIKE01, pIKE02, pIKE05 and pEKE06.

Table 4-2 - Plasmids used for monoterpene production in yeast

Plasmid name	Plasmid description	Reference
pSP-GM2	URA3-based expression plasmid carrying a bidirectional PTEF1-PPGK1 promoter	[20]
pXI-5	KlURA3-based integration plasmid carrying regions for homologous recombination	[20]
pXI-3	KlURA3-based integration plasmid carrying regions for homologous recombination	[20]
pX-2	KlURA3-based integration plasmid carrying regions for homologous recombination	[20]
P426 GPD	URA3-based expression plasmid carrying a PGPD promoter	
pIKE01	P_{TEF1}-spLS - P_{PGK1}-spGPPS	This study
pIKE02	P_{TEF1}-LH - P_{PGK1}-CD	This study
pIKE03	P_{TEF1}-ATR1	This study
pEKE04	P_{GPD}-ATR1	This study
pEKE05	P_{GPD}-GPPS	This study
pEKE06	P_{GPD}-ERG20-2 P_{GPD}-LS	This study

4.2.3 Strain maintenance

For long term storage of the strains, a yeast suspension containing 25% (vol/vol) sterile glycerol, was prepared in cryovials, and stored at -80°C. Working stocks were maintained on YPD agar plates containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar. The plasmid carrying strains were maintained on synthetic dextrose medium (SCD) agar plates lacking uracil, containing 6.9 g/L yeast nitrogen base without amino acids (BD Difco™, BD and Co, Sparks, MD, USA), 0.77 g/L complete supplement mixture without uracil (CSM-URA) (Sunrise Science Products Inc., San Diego, CA, USA), 20 g/l glucose and 20 g/l agar (BD Difco™ BD and Co.).

4.2.4 Growth conditions

Single colonies were inoculated in 3mL pre-cultures in SCD medium without uracil. Then, cultures were grown in 250 mL shake flasks at 30°C and 180 rpm in a horizontal shaking incubator, with a culture volume of 50 mL with a second organic phase of

dodecane (10%, v/v). All shake-flasks were inoculated from pre-cultures grown on the same medium, to an initial OD_{600} of 0.1.

4.2.5 Carvone quantification

Culture samples were centrifuged for 2 min at 6000 rpm. The organic phase was dried over anhydrous sodium sulfate. Quantitation was carried out by means of a gas chromatograph HP 5890, coupled to a flame ionization detector using a HP-5 capillary column (60 m × 0.25 mm id, 0.25 μm film thickness) (J&W Scientific, Agilent Technologies). Injection of the samples was performed in splitless mode at 250ºC. Oven program started at 80ºC for 1 min and, then, the temperature was raised up in a gradient of 120ºC until 240ºC. Concentrations of carvone were calculated by means of a calibration curve in a range of 0,1-50 mg/L using 4-isopropyl-3-methylphenol 4IP3MP as internal standard (Sigma-Aldrich, St. Luis, MO, USA).

4.3 Results

Integration of carvone pathway in *S. cerevisiae* strain SCIGS22a

We constructed a yeast platform for the production of monoterpene carvone in the FPP-overproducing strain SCIGS22a, the same strain used for the ß-ionone production. Since this strain was originally developed for sesquiterpene production (from FPP), an extra copy of the *ERG20* gene was integrated into the yeast genome. We deleted the extra copy by replacing *ERG20* with the KanMx marker gene in order to decrease FPP production and accumulate GPP. This new strain was called KE01 (Table 4-1).
In this strain we first integrated the cassette pIKE01 (carrying t*GPPS* and t*LS* genes) and pIKE02 (carrying the *LH* and *CD* genes) (Table 4-2). Then, we expressed the *ATR1* gene episomally to complete the carvone pathway. This new strain was called KE03. When the strains were grown in a two-phase shake flask culture (with 10% dodecane), final biomass measurements (after 48 h) were similar to the originally engineered *S. cerevisiae* strain (SCIGS22a), but no monoterpenes were detected in this strain.

Therefore, we decided to construct a yeast strain that only produce limonene, to discard a limitation with the GPP availability for monoterpene production. We constructed the strain KE02 by expressing only the cassette pIKE01. Unfortunately, no limonene could be detected in the cultures with this strain. The overexpression of the t*GPPS* gene (plasmid pEKE05) neither gave positive results (strain KE04). After 48 hours, no differences in final biomass were detected in both strains when compared with parental strains.
Since the GPP produced by the overexpressed GGPS can be used as substrate by the native *ERG20*, we decided to overexpress the mutated version *ERG20-2* episomally. We finally constructed the strain KE05 which contains the carvone pathway completely integrated with overexpression of the *ERG20-2* and t*LS* genes episomally. Neither limonene nor carvone were detected in these yeast cultures.

4.4 Discussion

In this work, we constructed a yeast platform for carvone production by integrating genes from *Mentha spicata, Arabidopsis thaliana* and *Abies grandies*. The genetic background of our strain contained some of the most common interventions in the MVA pathway used to increase the availability of terpene precursors [18], such as the overexpression of the bottleneck enzyme of the pathway *HMG1* (in its truncated version, *tHMG1*) and the downregulation of ERG9. Both modifications could help to the accumulation of the monoterpene precursor GPP, since isoprenoids accumulation seems to be tightly regulated by certain genes within this pathway. The overexpression of tHMG1 is known for increasing the MVA pathway flux towards terpenoids precursor. For the *ERG9* gene, measurements of six metabolites of the isoprenoid pathway in a wild type *S. cerevisiae* and a *ERG9/COQ1* disrupted strain demonstrated that when both genes were knocked out, the content of GPP, FPP and GGPP was strongly increased by a factor of 1.7; 2.2 and 2.5 compared to the wild type strain [21]. ERG9 repression, in a strain carrying a disrupted E*RG9* gene, also led a significant increase in the GPP content (in the same range of increase), which points towards an impact in GPP levels by this gene.

GPP and limonene as precursors of carvone

In contrast to sesquiterpenes or other larger terpenes, monoterpenes are almost exclusively found in plants [22]. Even though some studies also showed that yeast can produce some GPP, probably as a byproduct of the FPP formation, the levels in the cell are low (almost 30 ng/ml) [23] and no monoterpenes resulted. As an exception, some wine yeast strains can producing monoterpenols, like linalool or terpineol, but only under microaerobic/anaerobic conditions [24]. Besides, physicochemical properties of monoterpenes, such as high volatility, hydrophobicity and high toxicity hamper their heterologous production, compared with other types of chemical compounds.

Heterologous production of limonene in yeast reported a final concentration of 1.5 mg/L in a strain with expression of *tHMG1* gene [25] and 0.5 mg/L in a strain with mutated *ERG20* gene (K197G) [16]. Recently, Willrodt et al., (2014) [22] reached in *E. coli* cells the maximal concentration for limonene production reported in microorganisms (see below). The single expression of the truncated version of both *GPPS* from *Abies grandis* and *LS* from *Mentha spicata* into *E. coli* cells did not produce limonene after 24 h of cultivation, but further improvements allowed the detection of this compound. The addition of the non-toxic solvent diisonoylphtalate allowed the accumulation of low quantities of limonene after 72 h (0.4 mg/L), but the incorporation of the yeast MVA pathway with an inducible promoter and the use of glycerol as carbon source in a fed-batch culture lead to a final concentration of 2.7 ml/l of 50 h [22]. In our study, the attempts to synthesize carvone or limonene were unsuccessful, since no monoterpenes could be detected, even though our platform was constructed in an engineered strain.

The step catalyzed by FPPS is critical for monoterpene biosynthesis. However, since FPPS is essential for yeast survival and has naturally evolved to only produce FPP, the mutants reported did not assure a successful monoterpene production. To avoid any modification in the *erg20* gene, we first integrate the *Gpps* genes from *Abies grandis* into yeast genome (strain KE02) but no carvone was determined, even when a high copy number vector (KE04) was employed. There are several reasons why this could happen but most probably the molecules produced by GPPS can be converted to FPP by ERG20 due to the high affinity of ERG20 for GPP. We then evaluated some of the ERG20 mutants described in the literature. For example, Fisher et al., (2011) [26], generated a set of mutants of ERG20 in the K197 aminoacid. This aminoacid is involved in Mg++ coordination and, together with K254, held the substrate in position. Among the different mutations generated in this position, K197G showed a significant improvement in monoterpenols production; and we then chose to evaluate this variant in our study. However, unlike the former authors, we kept the endogenous copy of *erg20*, since we could not replace it with the mutant variant. This also affected monoterpene production,

since FPPS works as a dimer. In this sense, our yeast platform present homo and hetero dimers, which can still produce FPP. Ignea et al. (2013) [27] also expressed a mutated version of the ERG20 (F96W) gene for sabinene production. Although they could detect sabinene, and improved the production with the expression of ERG20 (F96W), their strains are equally capable of producing sesquiterpenols, indicating that the wild type ERG20 present is sufficient to convert GPP into FPP. A subsequent mutation in the aminoacid 127 (N127W) increased sabinene production, confirming that an efficient monoterpene production requires reduction of the endogenous FPP synthase activity.
Another way to avoid the utilization of GPP by wild type ERG20 is the construction of fusion proteins between the mutated Erg20-2 and the *LS*. This strategy, adopted by Ignea et al., (2013) increased sabinene yield by 3-5 fold, compared with parental strains [27].

The gene *IDI1*, which encodes the isomerase involved in the production of DMAPP, is also a key gene in monoterpene production. It has been reported that tilting the balance in favor of DMAPP, instead of IPP, increases the GPP production and so, the monoterpenes [28]. For example, the integration of *IDI1* or its episomal expression into an engineered yeast, increased dramatically the synthesis of the monoterpene cineol, but no sesquiterpenes [28]. This could be a better strategy to improve our monoterpene platform to evaluate in the future.

Toxicity of monoterpenes

The toxicity of monoterpenes can also be a factor to consider that could have hampered the production of limonene and carvone. Indeed, limonene is toxic for yeast cells, causing severe interferences with cell functions. For example, yeast cultures containing 0.5 to 1.5% (v/v) of limonene inhibit glucose assimilation, affecting cell viability [29]. Brennan et al (2012) [13], showed that limonene, among five monoterpenes, was the most toxic compound for yeast cell growth, with a minimum inhibitory concentration (MIC) of 0.44 mM. Moreover, the attempts to alleviate this toxicity, via cell adaptation, were unsuccessful, with no difference in the MIC after 225 generations. Therefore, yeast

cells must be constantly removing or exporting the limonene produced, affecting the production of derivatives, like carvone. In fact, the only report for carvone production in *E. coli* cells demonstrated that, although the enzymes from the carvone pathway were funtional, and almost 5 mg/L of limonene were detected, nor carveol neither carvone could be detected, probably because of the excretion of limonene [30]. Alonso-Gutierrez et al., 2013 [9], recently constructed an *E. coli* strain containing all the genes of the MVA pathway in a single plasmid, capable to produce 430 mg/L of limonene. This titer is high enough to surpass the exportation problem described by Carter et al. (2003) [30], allowing the synthesis of perillyl alcohol, a high-value limonene-derivative

Optimization of the first steps of the carvone pathway to increase limonene production might allow achieve the synthesis of significant levels of carvone. Also, a substrate channeling strategy could be appropriate to minimize limonene accumulation or export, mimicking plant cells, where cytosolic and membrane enzymes are coupled for efficient biosynthesis of the terpenes [31]. Finally, terpene glycosylation with dedicated glycosyl transferases could lift the cellular toxicity of carvone by increasing its water solubility and subsequent export [32, 33].

Alternative methods for monoterpene extraction

A physical approach to overcome the toxicity of monoterpenes is to remove these compounds from the cultures with a second phase of an organic solvent, like dodecane. Dodecane is the preferred solvent for the extraction of hydrophobic compounds from live cultures due to its low toxicity and good phase separation [13]. However, alternative options for the monoterpene capture have been recently proposed. For example, Jongedijk et al., (2015) [16], tested four methods for the collection of limonene from yeast cultures. When the terpenes produced after 3 days in flasks were collected with SPME or by pentane extraction at fixed time points, limonene was not detected. When the authors employed n-dodecane - diluted in ethyl acetate - only a small quantity of limonene could be detected (0.028 mg/L of (+) limonene and 0.06 mg/L of (-) limonene) in cultures of limonene-producing strains (strain expressing a truncated limonene

synthase). For strains expressing the non-truncated form of limonene synthase, only trace amounts were detected. Finally, the highest amount of limonene was recovered when the culture headspace was constantly refreshed through steel sorbent cartridges containing Tenax^{TM} adsorbent for the collection of volatiles. The volatiles trapped on Tenax cartridges reached 0.12 mg/L for (+) limonene and 0.49 mg/L for (-) limonene, *i.e.* 4 to 8-fold more than the biphasic system [16]. Thus, this system could be an alternative option to explore in the near future to minimize the cell toxicity of these compounds. Besides, the dodecane that we use has several solvent contaminants, like decane and undecane - as well as other unknown compounds -, which affected the final analysis of monoterpenes. This was trickier than ß-ionone quantification (see Chapter 2), because in this case the contaminants have retention times similar to the monoterpenes. The same problem was described by Jongedijk et al., giving to the headspace trapping an extra advantage [16].

Culture conditions

Another parameter that can affect final monoterpene titers are the pH optimum of monoterpene enzymes. Most of the limonene synthase found in plants have a narrow functional pH range, where the optimum pH is around 6.7-7.0, the half maximum velocity is at approximately pH 6.0 and minimal activity below pH 5.5 [34]. Also, the optimum pH reported for *LH* is 7.4; the activity falling to half when the pH is 6.9.

For example, the activity of limonene synthase from *Citrus limon* has an optimal pH of 7.0 [35]. In fact, Behrendorff et al., (2013) [25], evaluated the effect of media conditions on limonene production, by expressing limonene synthase from *Citrus limon* in *S. cerevisiae* strains. When the strains were cultivated in SD media with adjusted pH to 6.3, limonene titer was 0.81 mg/L. On the other hand, without adjusted pH, the production of limonene in this strain was below the limit of quantification. Since pH typically decreases in flask cultures of *S. cerevisiae*, the pH could reduce monoterpene production. This could be easily adapted in the future, possibly improving our yeast platform.

4.5 Conclusions and future directions

A series of strategies were applied for monoterpene production in the yeast *S. cerevisiae*, but neither limonene nor carvone could be detected in cultures with the different strains constructed. Monoterpenes synthesis in heterologous systems is mostly hampered by the toxicity of the compounds produced, as well as by the absence of their direct precursor, GPP, in the cytosol.

The expression of the *IDI1* gene, together with the construction of fusion proteins, could ameliorate the platform developed in this study. In addition, we believe that replacing of wild type ERG20 gene by a mutant version, capable to produce more GPP than FPP, is another critical step for increasing of the monoterpene concentration.

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5. CONCLUSIONS AND PERSPECTIVES

Conclusions

The main objective of this thesis was the construction of an efficient yeast cell factory for the synthesis of isoprenoids. Our first attempt was the production of the apocarotenoid ß-ionone, an enzyme-catalyzed compound derived from the tetraterpenoid β-carotene. As a starting point for ß-ionone production, we used an engineered strain designed for terpenoids production with several interventions on the MVA pathway [1]. This strain has increased FPP flux, the precursor of sesquiterpenes, dolichol, and sterols. The genetic modifications carried out by the Jens Nielsen's group also allow the accumulation of GGPP by the condensation between IPP and the accumulated FPP. An engineered strain with an optimized flux through GGPP, a precursor of carotenoids, was the reason why we chose this strain. Although plants and algae synthesize β-carotene, we decided to express the widely studied *Xanthophyllomyces dendrorhous* carotenogenic pathway. The integration of both *X. dendrorhous* genes, *crtYB* and *crtI*, worked well in our yeast strain since its expression and function in the host was sufficient for the production of ß-carotene (strain JLS01). The operation of the new platform was screened by the "dramatic" change in color, giving orange colonies cultured in plates. The *CCD1* gene from *Petunia hybrida* (*PhCCD1*) was integrated to express a fully functional pathway for the *de novo* synthesis of β-ionone from glucose, resulting in the strain JLS02. Further optimizations of the latter, mainly by increasing the expression levels of the heterologous genes using high copy number plasmids, significantly improved the final titer of ß-ionone (JLS07). This simple strategy incremented the final concentration of ß-ionone in almost 15-fold compared with JLS02, producing 5 mg/L in batch cultures.

The concentrations reached in this study still are not industrially competitive, since at least 100 mg/L in batch fermentation is needed to compete with current, chemical manufacturers (Cataldo et al, in preparation). Furthermore, downstream processing is

laborious and costly, and higher titers and/or productivity is imperious. The theoretical maximum yield is far away from the currently reached yield, using glucose as a carbon source; further genetic changes are currently being conducted to improve β-ionone production.

Even though the integration of *PhCCD1* gene resulted in the production of ß-ionone, the choice of this enzyme would not be the best candidate for ß-ionone production. Indeed, other CCDs enzymes participate in the ß-ionone formation in plants and their expression in our platform could improve the conversion step from β-carotene. We could not rule out the cooperative catalysis of PhCCD1 and other CCDs enzymes to convert efficiently β-carotene into β-ionone. Nevertheless, although genetic optimization has to be done, the obtained strains are capable of much higher titers compared with previous attempts (see for example Beekwilder et al., 2014 [2]).

The construction of this platform has several advantages. Since the final product is colored (as well as their precursors), the platform construction steps are easily followed and any modification could be visually detected by cultivating the strains. Moreover, the integration of the genes in the chosen sites by the USER library is straightforward. In terms of stability, the integrative sites have been chosen to be genetically stable against losses by recombination between integrated cassettes, and no genes were lost during the genetic manipulations of the strain. The only exception – and limitation of this tool - is when the marker gene must to be recycled. Recently, Borodina et al ([3]) published an improved version of the USER library, the EasyClone library, that relies on CreA recombinase to delete dominant marker genes. In addition, the use of the novel and already famous CRISPR/Cas9 technique will allow to skip the recycling step, using marker-less vectors, accelerating further strain construction. The integration at specific genome locus is improved by double strand DNA (dsDNA) breaks, and CRISPR/Cas9 allowed precise and multiplexed genome edition [4, 5].

For the *de novo* biosynthesis of monoterpenes, we attempted to produce carvone, a strong mint flavor with antimicrobial activity, with negative results. I strongly believe

that the insufficient flux between GPP and limonene, probably due to fast conversion to FPP, is the main reason for this failure. The fact that yeast needs FPP as a precursor for other metabolites, rather than GPP, complicates the accumulation of GPP for monoterpene production. Furthermore, monoterpenes are evolutionary plant metabolites, probably within the cyanobacterial origin of plastid, and plant strategies could not be easily transfered to yeast. FPP is essential for the cell since it is a branch point for other pathways and no knockout strategies could be used for increasing GPP without FPP production. The expression of dedicated plant GPP synthases did not improve monoterpene biosynthesis, probably because the GPP produced was also transformed by the native FPP synthase (ERG20). The only strategy, in this case, is the use of a mutant version of *ERG20*. The replacement of the native *ERG20* gene by its truncated version seems to be crucial.

Monoterpenes also present other problems that we do not face before. Its high volatility, toxicity, and problems with its quantification complicated its efficient synthesis. A series of alternative strategies might be evaluated for an efficient platform for its fermentative production. For example, the production of limonene was recently enhanced in *E. coli* to few grams per liter [6], but the conversion to even high-valuable monoterpenes still faces low conversion rates. Because the conversion of limonene uses NADPH to be oxidized into trans-carveol - and $NADP^+$ to further oxidation into carvone - whole-cell biosynthesis is best suited for this task. Nevertheless, monoterpene production in yeast is tightly regulated and the molecular regulatory mechanisms need to be discovered before their production could be significantly enhanced.

Perspectives

Even though the tools and knowledge to rapidly engineer microorganisms are every-day increasing, we have to consider that the inner complexity of the cell metabolism still made time-consuming all the approaches needed for an efficient platform. For example, in our monoterpenes´ objective, its synthesis depends on the coordinated expression of several genes, without the accumulation of some intermediates consumables by yeast enzymes, such as GPP, or toxic for the cells, such as limonene. In plants, the fact of having enzymes in different organelles made this coordination not only temporal but spatially suitable for compound synthesis. Redirecting enzymes towards yeast organelles, such as mitochondrion could in part improve the terpenoids synthesis.

Despite this, the use of microbial cell factories in a more customizable way is today a reality. The knowledge and technologies are mature, and the advance in ME, together with mathematical models, allows a significant and robust degree of manipulation of the microorganism with some remarkable examples of bio-manufacturing compounds. Last year, CRISPR/Cas9 technology was introduced to the scientific community to stand as a cheap, quick and easy to use technology for genome editing. This development is eclipsing other methodologies and accelerating the research in many fields related with the manipulation of genes, mostly in biomedicine. In the field of engineered biosystems it has been already adopted, also accelerating the construction of more cell factories. This type of advances, the decreasing costs in DNA synthesis and the increasing collaboration between academy and industry, strongly encourages our efforts to develop efficient and sustainable cell factories for green chemistry synthesis.

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5. DiCarlo JE, Norville JE, Mali P, Rios X, Aach J, Church GM: **Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems.** *Nucleic Acids Res* 2013, **41**:4336–43.
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A N N E X E S

Additional file 2. Codon optimizad nucleotide sequences.

Codon optimized ***crtYB*** nucleotide sequence

ATGACTGCCTTAGCATACTATCAAATCCACTTAATCTACACCTTGCCTATCTT
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TGGATTATAAGAAATGGTGCTTGGACTTACCCTTCTGCAGAATCAGGTCAAG
GTGTTTTTGGTACATTCTTGGATGTTCCATATGAAGAATACGCATTTTTCGTC
ATCCAAACTGTAATTACAGGTTTGGTCTACGTATTGGCCACCAGACATTTGT
TACCATCATTGGCTTTACCTAAAACTAGATCTTCAGCATTGTCCTTAGCCTTG
AAGGCTTTGATCCCATTGCCTATCATATATTTGTTTACTGCTCATCCATCTCC
TTCACCAGATCCTTTAGTTACCGATCACTATTTCTACATGAGAGCATTGAGTT
TGTTAATTACCCCACCTACTATGTTGTTAGCTGCATTATCTGGTGAATATGCC
TTTGATTGGAAATCAGGTCGTGCTAAGTCCACTATTGCCGCTATAATGATCC
CAACAGTATATTTGATCTGGGTTGATTACGTTGCAGTCGGTCAAGATTCCTG
GAGTATCAATGACGAAAAGATTGTCGGTTGGAGATTAGGTGGTGTATTGCC
AATCGAAGAAGCTATGTTTTTCTTGTTGACAAACTTAATGATTGTTTTAGGTT
TGTCAGCCTGTGATCATACTCAAGCTTTGTATTTGTTGCACGGTAGAACAAT
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Codon optimized ***crtI*** nucleotide sequence

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Codon optimizad ***PhCCD1*** nucleotide sequence

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TATTTGTCTGGTAACTTTGCTCCTACAGATGAAACCCCACCTTTGAACGACTT
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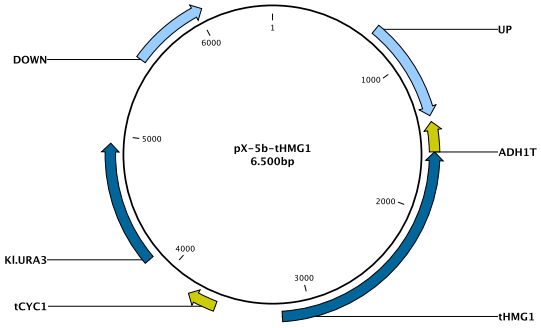
TACCAAAAAGAGTCCCTTACGGTTTCCACGCCTTTTTCGTTACAGAAGAACA
AATACAAGAACAAGCCAAGTTATGA

Additional file 2.- Primers used in the study
Overlapping nucleotides are in bold face

Nº	Name	Sequence (5' to 3')
1	Fw USER pXI-5	ATCCGCTCTAACCGAA AAGGAAGGAG
2	Rv USER pXI-5	GTAGATACGTTGTTGACACTTCTAA
3	Fw USER pXI3/X2	ATCGCGTGCATTCATCCGCTCTAA
4	Rv USER pXI3/X2	ATCGCACGCATTCCGTTGGTAGATA
5	Fw TEF1	CTAA**CTCCTTCCTTTTCGGTTAGAGCGGAU-**GCACACACCATAGCTTCAAAATGTTTC
6	Rv tHMG1	CTTAT**TTAGAAGTGTCAACAACGTATCTAC**TTAGGATTTAATGCAGGTGACGGACC
7	Fw BtsI	**ATCAACGGG**U-AAAAAATGGAGGCCAAGATAGATGA
8	Rv BtsI	**CGTGCGA**U-TCACAATTCGGATAAGTGGTC
9	Fw CrtYB	**AGCGATACG**U-AAAAAATGACTGCCTTAGCATACTATC
10	Rv CrtYB	**CACGCGA**U-TTATTGACCTTCCCAACCAGA
11	Fw CrtI	**ATCAACGGG**U-AAAAAATGGGTAAAGAACAAGATCAAGAC
12	Rv CrtI	**CGTGCGA**U-TTAAAAAGCCAAAACACCTACTGAT
13	Fw PhCCD1	**AGCGATACG**U-AAAAAATGGGTAGAAAAGAATCAGATGAC
14	Rv PhCCD1	**CACGCGA**U-TCATAACTTGGCTTGTTCTTGTATT
15	Fw TEF1	**ACCCGTTGA**U-TTGTAATTAAAACTTAGATTAGATTGCT
16	Rv GPK1	**ACGTATCGC**U-TTGTTTTATATTTGTTGTAAAAAGTAGATAA
17	Fw P426-Crtyb	GTAGTTATGTCTGGTTGGGAAGGTCAATAAGAATTCGATATCAAGCTTATCGATACCGTC
18	Rv P426-Crtyb	GATAGTATGCTAAGGCAGTCATTTTTTTACTAGTTCTAGAATCCGTCGAAACT
19	Fw P426 Crti	CTAGATCAGTAGGTGTTTTGGCTTTTTAAGAATTCGATATCAAGCTTATCGATACCGTC
20	Rv P426 Crti	CTTGTCTTGATCTTGTTCTTTACCCATTTTTTACTAGTTCTAGAATCCGTCGAAACT
21	Fw P426-Ccd1	GAACAAATACAAGAACAAGCCAAGTTATGAGAATTCGATATCAAGCTTATCGATACCGTC
22	Rv P426-Cccd1	CATCTGATTCTTTTCTACCCATTTTTTTACTAGTTCTAGAATCCGTCGAAAC
23	Fw CrtYB	AGTTTCGACGGATTCTAGAACTAGTAAAAAATGACTGCCTTAGCATACTATCAAATC
24	Rv CrtYB	GACGGTATCGATAAGCTTGATATCGAATTCTTATTGACCTTCCCAACCAGACATAACTAC
25	Fw CrtI	AGTTTCGACGGATTCTAGAACTAGTAAAAAATGGGTAAAGAACAAGATCAAGACAAG
26	Rv CrtI	GACGGTATCGATAAGCTTGATATCGAATTCTTAAAAAGCCAAAACACCTACTGATCTAG
27	Fw CCD1	AGTTTCGACGGATTCTAGAACTAGTAAAAAAATGGGTAGAAAAGAATCAGATGAC
28	Rv CCD1	GACGGTATCGATAAGCTTGATATCGAATTCTCATAACTTGGCTTGTTCTTG
29	Fw P426 DG	GTTTTGGGACGCTCGAAGGCTTTAATTTGCGCTCACTGGCCGTCGTTTTACAAC
30	Rv P426 DG	ATTCTTTGAAATGGCGAGTATTGATAATGAGCGCGTAATACGACTCACTATAG
31	Fw pGPD	ATTCGCCCTATAGTGAGTCGTATTACGCGCTCATTATCAATACTCGCCATTTCAAAG
32	Rv tCYC1	CACGACGTTGTAAAACGACGGCCAGTGAGCGCAAATTAAAGCCTTCGAGC
33	XI5UPF1	GCCGTATCTGCTCCATCGAA
34	XI5DWR1	GTCACCGCTATCAGCACAGT
35	URA3F1	ATCCGCTCTAACCGAAAAGGAAGGAG
36	XIUPF1	GCCAATTTGTTGGAGTCCGGATC
37	XIDWR1	GAAAGAATTGGCGACACATTGAG
38	X2UPF1	GGAATCTATGTATTGAGTGACAG
39	X2DWNR1	TTGGAGTTATTCCAAGTGTTAGTATT

Additional file 3. Maps of plasmids construct in this study.

A. pIRP01



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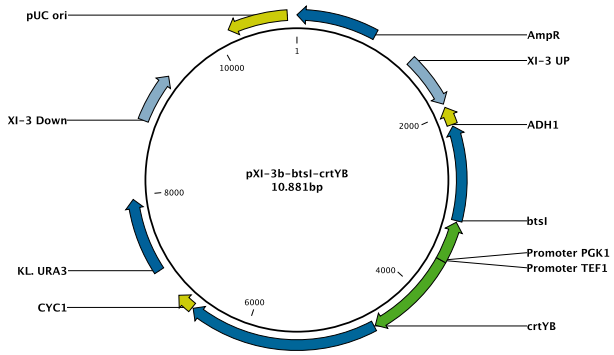

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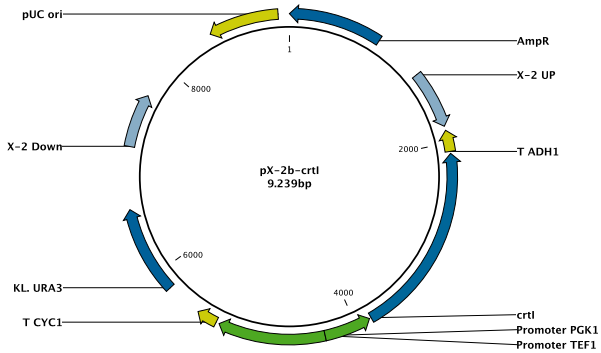

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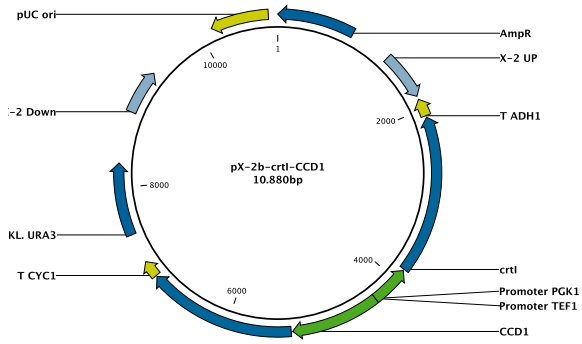

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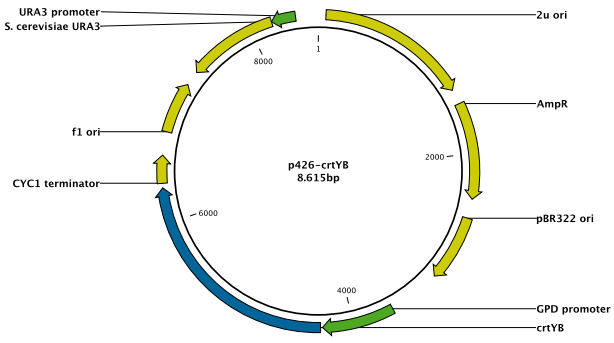

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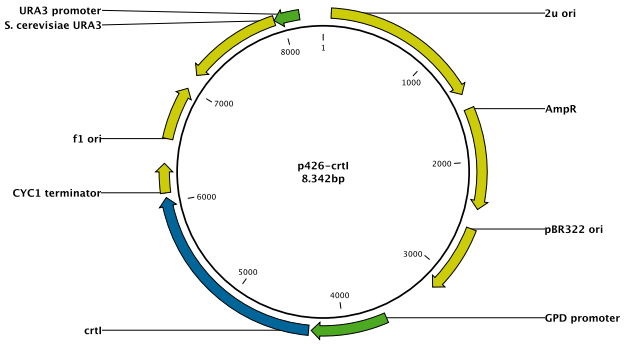
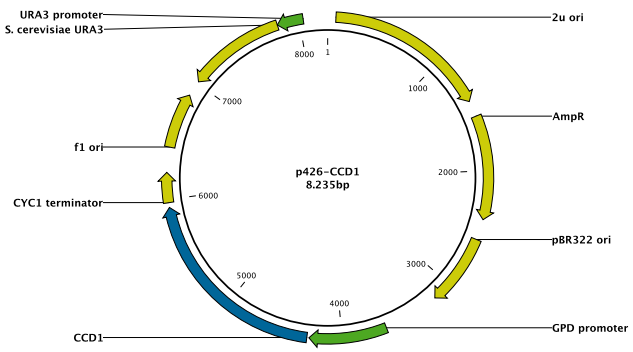


G. pEJL06



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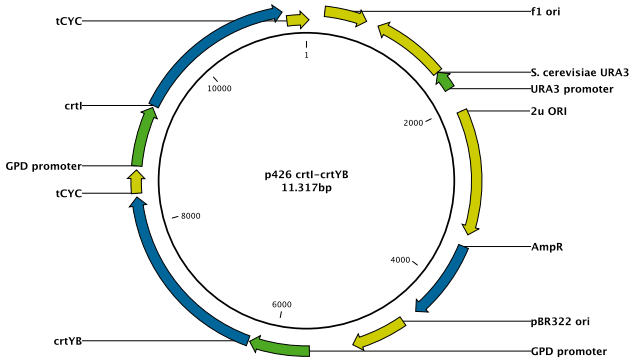

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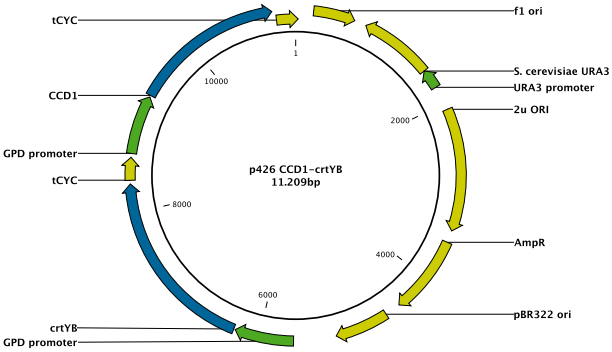
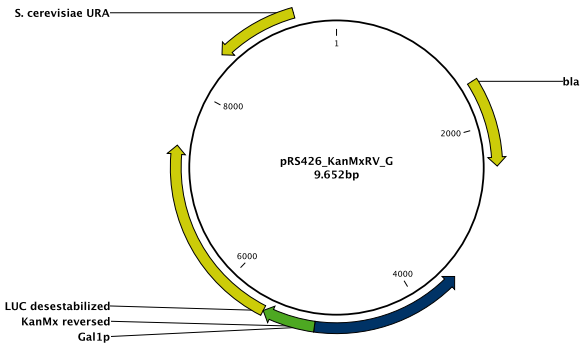


Additional file 4.- Primers used in the study
Overlapping nucleotides are in bold face

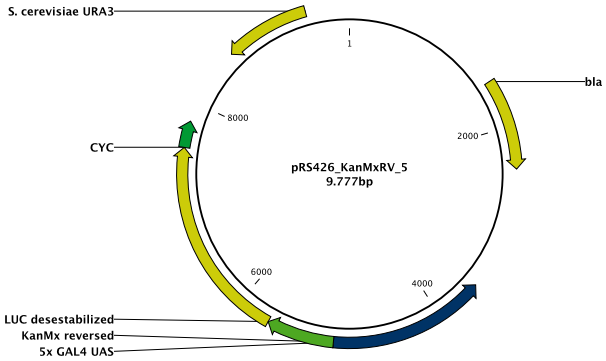
Nº	Name	Sequence (5' to 3')
1	YRP-L ADH1	AGCGGATAACAATTTCACACAGGAAACAGCTAGGCGCATGCAACTTCTTT
2	ADH2 term	GCCGGTAGAGGTGTGGTCAA
3	Nat Fw	GTCGCTCTTATTGACCACACCTCTACCGGCCGGGTTAATTAAGGCGCGCC
4	Nat Rv	GGTAACGCCAGGGTTTTCCCAGTCACGACGATCGATGAATTCGAGCTCGT
5	Hph Fw	GTCGCTCTTATTGACCACACCTCTACCGGCCGGGTTAATTAAGGCGCGCC
6	Hph rv	GGTAACGCCAGGGTTTTCCCAGTCACGACGATCGATGAATTCGAGCTCGT
7	KanMx Fw	AGCGGATAACAATTTCACACAGGAAACAGCATCGATGAATTCGAGCTCGT
8	KanMx Rv	CGGGTTAATTAAGGCGCGCC
9	Gal1 Fw	AAACAGATCTGGCGCGCCTTAATTAACCCGACTAGTACGGATTAGAAGCC
10	Gal1 Rv	GGTAACGCCAGGGTTTTCCCAGTCACGACGGGTAAGCTTAATATTCCCTA
11	Ura Fw	ATTAACGCTGCAGGTAGCAAATCCCCCAGTATTCATGGAACTTCCTTGGACGGC ATCAGAGCAGATTGTA
12	Ura Rv	ATGCACAGTTGAAGTGAACTTGCGGGGTTTTTCAGTATCTACGATTCATTGGTAT TTCACACCGCATAGG
13	check Ura Fw	ATAACACCGTCGTCGAAAGG
14	check Ura Rv	AGGTTACATGGCCAAGATTGA
15	Rec VVd Fw	ATTAACGCTGCAGGTAGCAAATCCCCCAGTATTCATGGAACTTCCTTGGATAGG CGCATGCAACTTCTTT
16	Rec VVd Rv	ATGCACAGTTGAAGTGAACTTGCGGGGTTTTTCAGTATCTACGATTCATTATCGA TGAATTCGAGCTCGT
17	Rec WC Fw	CCAGCGTATACAATCTCGATAGTTGGTTTCCCGTTCTTTCCACTCCCGTCTAGG CGCATGCAACTTCTTT
18	Rec WC Rv	GTTTTTATAACGTTCGCTGCACTGGGGGCCAAGCACAGGGCAAGATGCTTATC GATGAATTCGAGCTCGT
19	Check WC Rv	CGCATGCACGAAAAAGGGAA
20	Kan/Gal1 Fw	TAAAAAAGGAGTAGAAACATTTTGAAGCTATGGTGTGTGCGGCCGGCCTGATC GATGAATTCGAGCTCGT
21	Kan/Gal Rv	TTTCTACACCGTCATCTGATTCTTTTCTACCCATTTTTTTACGTATCGCTGGTAAG CTTAATATTCCCTA
22	Check Gal1Fw	TTGTAATTAAAACTTAGATTAGATTGCT
23	Check Gal1Rv	TCATAACTTGGCTTGTTCTTGTATT

Additional file 5. Maps of plasmids construct in this study.

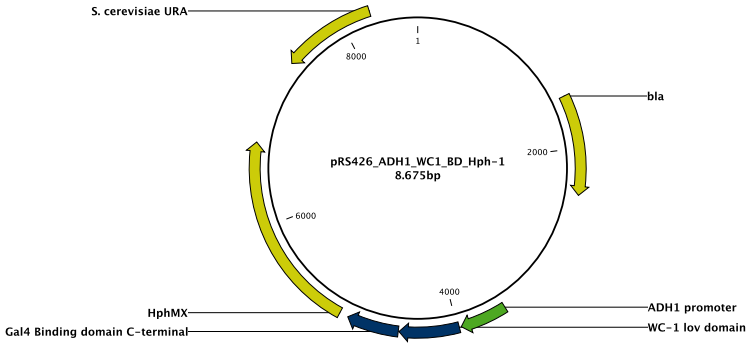
A. pAG413 Gal1-lucCP^{+}



B. pEFS01



C. pIFS01



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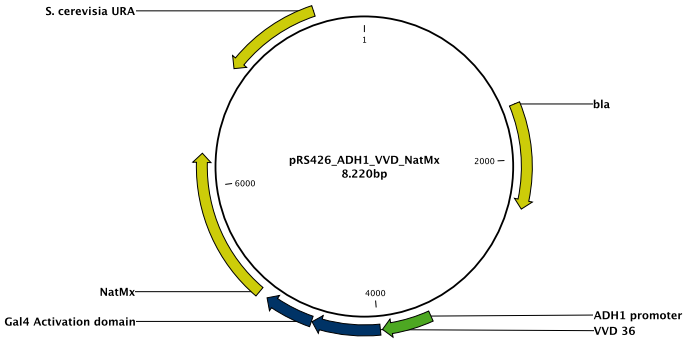

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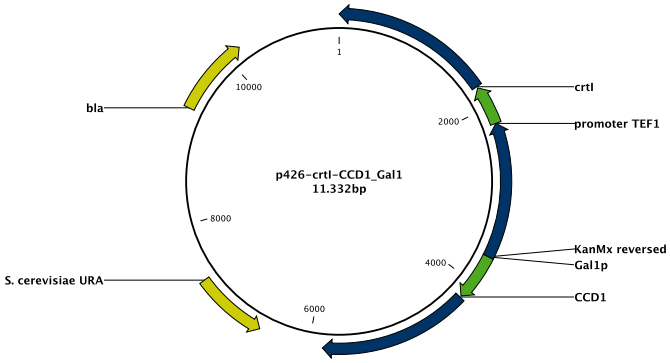

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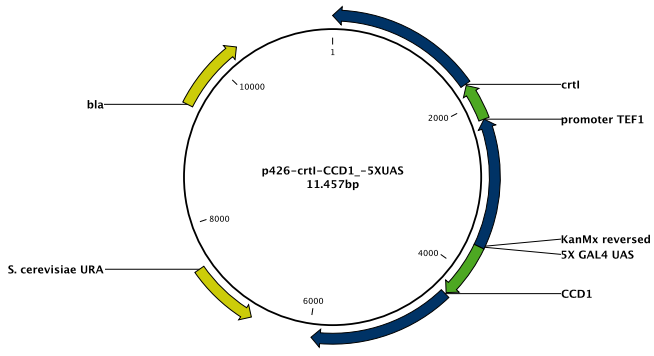

Additional file 6. Codon optimizad nucleotide sequences.

Codon optimized ***GPPS*** nucleotide sequence

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GGCTACAGCAAACGCAAACTGAGCAGCTATAAAAACCCGGCCTCTCGTAGT
TCCAATGCAACCGTGGCACAACTGCTGAACCCGCCGCAAAAAGGCAAAAAA
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TGTTATCGAACGTGCTCGTCGCTACGCACGTTGCGTCGGTCTGCTGTTTCA
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GGCGAACTGTCCTGTTTCGACCCGGTGAAAGCTGCTCCGCTGCTGGGTCTG
GCTGATTATGTGGCATTCCGTCAAAACTGATCTAGA

Codon optimized ***limonene synthase*** nucleotide sequence

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GCTGGGATGTGAATTTTATTCAGAGCCTGCTGTCTGATTACAAAGAAGACAA
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GAAAAAGAAACCGATCAAATCCGCCAACTGGAACTGATTGATGACCTGCAA
CGTATGGGTCTGTCCGATCACTTTCAGAACGAATTTAAAGAAATCCTGAGTT
CCATCTATCTGGATCATCACTACTACAAAAACCCGTTTCCGAAAGAAGAACG
TGACCTGTACAGCACCTCTCTGGCATTTCGCCTGCTGCGTGAACATGGCTT
CCAAGTCGCTCAGGAAGTGTTTGATAGTTTCAAAAACGAAGAAGGTGAATTT
AAAGAAAGTCTGTCCGATGACACCCGCGGCCTGCTGCAACTGTATGAAGCC
AGTTTCCTGCTGACGGAAGGTGAAACCACGCTGGAATCCGCGCGTGAATTT

GCCACCAAATTCCTGGAAGAAAAAGTTAACGAAGGCGGTGTCGATGGCGAC
CTGCTGACCCGTATCGCGTATAGCCTGGATATTCCGCTGCATTGGCGCATC
AAACGTCCGAATGCCCCGGTGTGGATTGAATGGTACCGCAAACGTCCGGAT
ATGAACCCGGTGGTTCTGGAACTGGCGATCCTGGACCTGAATATTGTTCAG
GCCCAATTTCAGGAAGAACTGAAAGAAAGCTTCCGTTGGTGGCGTAATACG
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TTTGGAACACCGGTATTATCGAACCGCGCCAACACGCGTCTGCCCGTATTA
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GCGCTGAACAATTTCGTTGATGACACCAGCTATGATGTCATGAAAGAAAAAG
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CTCTGTATAAATACCACGACCTGGTCCGCTGGTCATCGTTCGTGCTGCGTCT
GGCAGATGACCTGGGTACCTCAGTGGAAGAAGTTTCGCGCGGCGATGTGC
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CTCGCAAACACGTGAAATGGCTGATTGCAGAAGTTTGGAAGAAAATGAATG
CTGAACGTGTCAGTAAAGATTCCCCGTTTGGTAAAGACTTCATCGGCTGTGC
GGTTGATCTGGGCCGCATGGCCCAACTGATGTATCATAATGGCGACGGTCA
CGGCACCCAGCACCCGATTATCCACCAGCAGATGACCCGCACCCTGTTTGA
ACCGTTTGCCTGAGAATTC

Codon optimized ***carveol dehydrogenase*** nucleotide sequence

TCTAGAATGGCGTCGGTGAAAAAACTGGCGGGCAAAGTGGCAATCGTTACG
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CCGTGGCAGAATCAATTGGCGGTCGTCGCTGCTCGTATGTGCATTGTGATA
TCACGGACGAACAGCAAGTTCGTAGCGTCGTGGATTGGACCGCGGCCACG
TACGGTGGTGTTGACGTCATGTTTTGCAACGCAGGTACCGCATCTGCAACC
GCACAGACGGTGCTGGATCTGGACCTGGCACAATTCGATCGTGTGATGCG
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CTCTGAAGGGTGTGGCAATCACGGCTGAACACGTGGCTGAAGCGGTTGCC
TTTCTGGCAAGCGACGAAGCAGCATTCGTGACCGGTCACGATCTGGCTGTG
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Codon optimized ***limonene hidroxylase*** nucleotide sequence

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CCTGCCGCCGTCGCCGCCGAAACTGCCGGTGATCGGCCATCTGCACTTTCT
GTGGGGCGGTCTGCCGCAGCATGTTTTCCGCAGTATTGCACAAAAATATGG
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GGTTGATGTCACCGAAGAAGTTAGTAAAATGTCCTGCGTCGTGGTTTGTCGT
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GCGAAGTCAACGGCTATACCATCCCGGCAAAAACGCGCATCTTCATTAATG
TGTGGGCTATTGGTCGTGATCCGCAGTACTGGGAAGATCCGGACACCTTTC
GCCCGGAACGTTTCGACGAAGTTTCTCGTGATTTTATGGGCAACGACTTTGA
ATTTATCCCGTTCGGTGCAGGTCGCCGTATTTGTCCGGGTCTGCATTTTGGT
CTGGCGAATGTGGAAATTCCGCTGGCCCAACTGCTGTACCACTTCGATTGG
AAACTGCCGCAAGGCATGACGGATGCAGACCTGGATATGACCGAAACGCC
GGGTCTGTCTGGTCCGAAAAAGAAAAATGTGTGTCTGGTTCCGACGCTGTA
TAAATCACCGTAAGAATTC

Codon optimized ***ATR1*** nucleotide sequence

ATGACCTCCGCCTTGTATGCCTCCGATTTGTTTAAGCAATTGAAGTCTATTAT
GGGTACTGACAGTTTATCCGATGATGTTGTTTTAGTAATTGCTACTACATCAT
TGGCATTAGTTGCCGGTTTTGTTGTCTTGTTATGGAAAAAGACCACTGCTGA
TAGAAGTGGTGAATTGAAGCCATTGATGATCCCTAAATCTTTGATGGCAAAG
GACGAAGATGACGATTTGGATTTAGGTTCAGGCAAGACTAGAGTTTCCATAT
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AGCCAGATTTTCTAAATGGTTCACAGAAGAAAACGAAAGAGATATTAAGTTG
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CAGAGTAGTTACTCATGATCCAAGATTCACAACCCAAAAATCAATGGAATCC
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CTTTGGGTACAGGTTTAGCTAGATATGCAGATTTGTTAAACCCTCCAAGAAA
ATCAGCCTTGGTTGCTTTAGCTGCATACGCCACCGAACCATCCGAAGCTGA
AAAATTGAAGCATTTAACATCACCTGACGGTAAAGATGAATATTCCCAATGG
ATTGTCGCTTCACAAAGATCCTTGTTAGAAGTAATGGCCGCTTTTCCAAGTG
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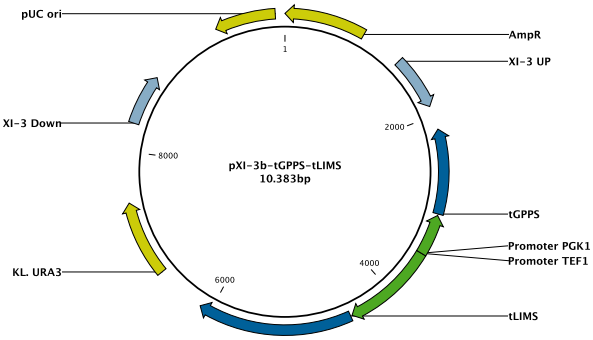
TGGAAAAGGCTGCACAAGTTTGGGATTTGATCAAGGAAGAAGGTTATTTATA
CGTCTGCGGTGACGCTAAGGGTATGGCAAGAGATGTACATAGAACCTTACA
CACTATTGTCCAAGAACAAGAAGGTGTATCTTCATCCGAAGCTGAAGCCATA
GTCAAGAAGTTACAAACCGAAGGTAGATATTTAAGAGATGTCTGGTGA

Additional file 7.- Primers used in the study
Overlapping nucleotides are in bold face

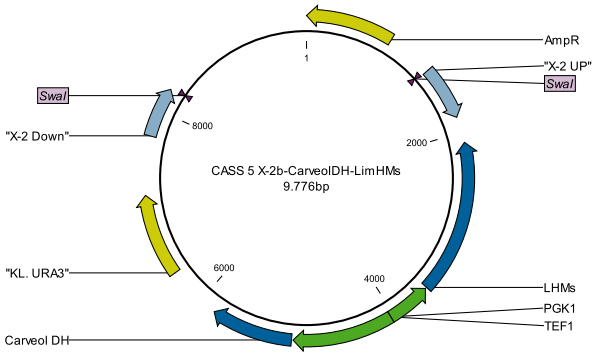
Nº	Name	Sequence (5' to 3')
1	Fw USER pXI3/X	ATCGCGTGCATTCATCCGCTCTAA
2	Rv USER pXI3/X	ATCGCACGCATTCCGTTGGTAGATA
3	Fw tGPPS	**ATCAACGGG**U-AAAAAAATGGTTGAATTCGATTTCAAC
4	Rv tGPPS	**CGTGCGA**U-TCAGTTTTGACGGAATGCCACAT
5	Fw tLS	**AGCGATACG**U-AAAAAAATGGAACGTCGCAGTG
6	Rv tLS	**CACGCGA**U-TCAGGCAAACGGTTCAAACAG
7	Fw LHms	**ATCAACGGG**U-AAAAAAATGGAACTGGACCTGCTGTC
8	Rv LHms	**CGTGCGA**UTTACGGTGATTTATACAGCGTCG
9	Fw CD	**AGCGATACG**UAAAAAAATGGCGTCGGTGAAAAAACTG
10	Rv CD	**CACGCGA**UTTATTTAGCGACAGCCACAAACG
11	Fw P426-ATR1	GTAGATATTTAAGAGATGTCTGGTGAGAATTCGATATCAAGCTTATCGATACCGTC
12	Rv P426-ATR1	CATACAAGGCGGAGGTCATTTTTTTACTAGTTCTAGAATCCGTCGAAACT
13	Fw P426-Gpps	GCTGATTATGTGGCATTCCGTCAAAACTGAGAATTCGATATCAAGCTTATCGATACCGTC
14	Rv P426-Gpps	GTTGAAATCGAATTCAACCATTTTTTTACTAGTTCTAGAATCCGTCGAAACT
15	Fw P426-ERG20-	TTGAACAAAGTTTACAAGAGAAGCAAATAGGAATTCGATATCAAGCTTATCGATACCGTC
16	RV P426-ERG20-	TAGTTACCACTGCGACGTTCCATTTTTTTACTAGTTCTAGAATCCGTCGAAACT
17	Fw P426-tLS	ACCCGCACCCTGTTTGAACCGTTTGCCTGAGAATTCGATATCAAGCTTATCGATACCGTC
18	RV P426-tLS	TAGTTACCACTGCGACGTTCCATTTTTTTACTAGTTCTAGAATCCGTCGAAACT
19	Fw ATR1	AGTTTCGACGGATTCTAGAACTAGTAAAAAAATGACCTCCGCCTTGTATG
20	Rv ATR1	GACGGTATCGATAAGCTTGATATCGAATTCTCACCAGACATCTCTTAAATATCTACCTTC
21	Fw tGPPS	AGTTTCGACGGATTCTAGAACTAGTAAAAAAATGGTTGAATTCGATTTCAACAAATAC
22	Rv tGPPS	GACGGTATCGATAAGCTTGATATCGAATTCTCAGTTTTGACGGAATGC
23	Fw ERG20-2	AGTTTCGACGGATTCTAGAACTAGTAAAAAAATGGCTTCAGAAAAAGAAATTAGG
24	Rv ERG20-2	GACGGTATCGATAAGCTTGATATCGAATTCCTATTTGCTTCTCTTGTAAACTTTG
25	Fw tLS	AGTTTCGACGGATTCTAGAACTAGTAAAAAAATGGAACGTCGCAGTGGTAACTAC
26	Rv tLS	GACGGTATCGATAAGCTTGATATCGAATTCTCAGGCAAACGGTTCAAACAG
27	Fw USER pXI-5	ATCCGCTCTAACCGAA AAGGAAGGAG
28	Rv USER pXI-5	GTAGATACGTTGTTGACACTTCTAA
29	Fw ATR1i	AAAAAAATGACCTCCGCCTTG
30	Rv ATR1i	CAGGTTGTCTAACTCCTTCCTTTTCGGTTAGAGCGGATGAATGCACGCGTCACCAGACATCTCTTAAATATC
31	Fw TEF1	CTAA**CTCCTTCCTTTTCGGTTAGAGCGGAU-**GCACACACCATAGCTTCAAAATGTTTC
32	Rv tHMG1	CTTAT**TTAGAAGTGTCAACAACGTATCTAC**TTAGGATTTAATGCAGGTGACGGACC
33	KanMx-1-fw:	CTGAAGCTTCGTACGCTG
34	KanMx-1-rev:	TCACCATGAGTGACGACTGA
35	KanMx-2-fw:	AATTCCAACATGGATGCTGATTTATATGG
36	KanMx-2-rev:	CTAGTGGATCTGATATCAC
37	Fw UP	TGCCGCAAGTTTCTTG
38	Rv UP	CAGCGTACGAAGCTTCAGATCCTCCGGATTCGCG
39	Fw DOWN	AGTTATTAGGTGATATCAGATCCACTAGTTGTAATTAAACTTAGATTAGATTGCTATG
40	Rev DOWN	GCACACACCATAGCTTCAAAATGTTTCTACTCC

Additional file 8. Maps of plasmids construct in this study.

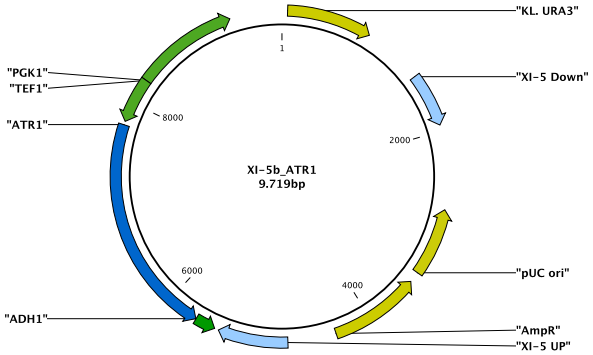
A. pIKE01



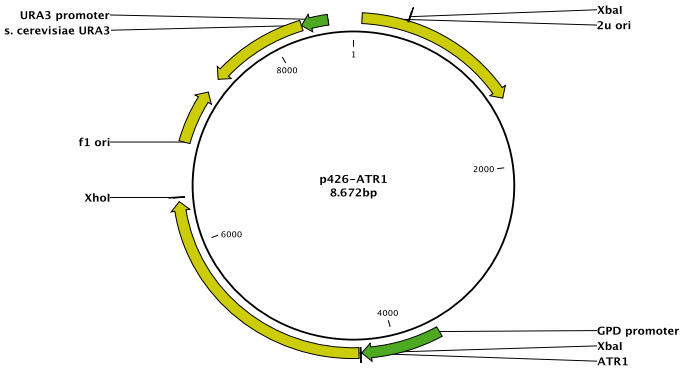
B. pIKE02



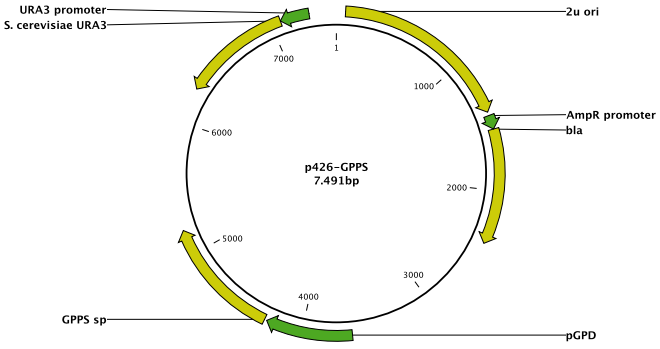
C. pEKE03



D. pEKE04



E. pEKE05



F. pEKE06