

Novel Synthetic Biology Tools for Metabolic Engineering of *Saccharomyces cerevisiae*

SIAVASH PARTOW

Systems and Synthetic Biology
Department of Chemical and Biological Engineering
 CHALMERS UNIVERSITY OF TECHNOLOGY
 Göteborg, Sweden 2012

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Systems and Synthetic Biology

Department of Chemical and Biological Engineering

Chalmers University of Technology

SE-41296 Göteborg

Sweden

Telephone +46 (0)31-772 1000

Cover: Schematic representation of the implementation of synthetic biology tools in metabolic engineering approaches prepared by Siavash Partow as part of this research.

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Dedicate to

Who give me *love* in my life...

*“Although my heart made haste in this desert,
It did not know a single hair, but took to hair-splitting,
In my heart shone a thousand suns,
Yet it never discovered completely the nature of a single atom.”*

-*Ibn Sina (Avicenna)*
980-1037

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ABSTRACT

The most well characterized eukaryote model organism *Saccharomyces cerevisiae* is not only preferred as a microbial cell factory for synthesis of industrial products, e.g. bioethanol, but this eukaryote host system is also defined as a robust scaffold for commercial production of diverse chemicals e.g. isoprenoids. Therefore, a number of tools in different emerging fields e.g. systems biology, evolutionary engineering and synthetic biology have been developed. Synthetic biology offers an alternative approach that is becoming more accessible as a tool for better performing metabolic engineering of yeast. Due to the fact that the regulations of gene dosage and gene transcription are the first two key steps allowing control of metabolic pathways, improve of both gene expression and gene dosage through modulating promoter choice and plasmid copy number were pursued. The strength of seven different constitutive or glucose based promoters, *TEF1*, *PGK1* *TPII*, *HXT7*, *PYK1*, *ADH1* and *TDH3*, was compared at different stages of a batch cultivation using LacZ as reporter. A new divergent promoter was developed, containing two strong and constitutive promoters, *TEF1* and *PGK1*, to support high level gene expression. Furthermore, this bidirectional promoter was used to construct new episomal plasmids, the pSP series, to optimize the endogenous mevalonate (MVA) pathway through gene overexpression and also to construct integration cassettes containing the synthetic methylerithritol phosphate (MEP) pathway genes. The last two studies showed the successful implementation of synthetic biology tools in metabolic engineering in terms of pathway optimization and pathway reconstruction in order to improve sesquiterpene production in *S. cerevisiae*. Optimization of the MVA pathway was performed in two steps, modulating the FPP branch point and modulating the possible nodes which are directly involved or related to the MVA pathway including overexpression of *tHMG1*, *ERG20*, *GDH2* and *upc2-1* and deletion of *GDH1*, *DPP1* and *LPP1*. Combination of all these modifications led to a 4-fold improvement of α -santalene yield over the reference strain. In the second study, the bacterial MEP pathway, containing 8 genes, was reconstructed through stable integration into the yeast genome in two steps. However, a functional MEP pathway was not obtained even after reconstruction of the possible bacterial Fe/S trafficking routes and the bacterial electron transfer system in order to circumvent lack of the enzyme activity. In another approach, improvement of gene dosage via modulating plasmid copy number was investigated. Here, two strategies, individually and in combination, were applied in order to reduce the marker gene at both protein and RNA levels, and their impact on plasmid copy number of pSP-GM1 was investigated. Both methods, destabilization of the marker protein using a ubiquitin/N-degron tag and down-regulation of the marker gene employing weak promoters, elevated the plasmid copy number. Combination of the weak promoter and ubiquitin tag showed a synergistic effect and increased the plasmid copy number by 3 fold. A proof-of-concept study was performed to determine if the enhancement in plasmid copy number could affect patchoulol production when patchoulol synthase was expressed from the modified plasmid. The result showed that while the final biomass concentration was unchanged, patchoulol production reached about 30 mg/L when employing modified plasmid, which was more than 3 times higher compared to when the synthase gene was expressed from the original plasmid.

Key words: *S. cerevisiae*, Synthetic biology, Metabolic engineering, Yeast promoter, MVA pathway, MEP pathway, Fe – S clusters, Multi-copy plasmid.

LIST OF PUBLICATIONS

This thesis is based on the following publications

- I. Siavash Partow, Verena Siewers, Sara Bjørn, Jens Nielsen, Jerome Maury: **Characterization of different promoters for designing a new expression vector in *Saccharomyces cerevisiae*.** *Yeast* (2010) 27: 955-964
- II. Yun Chen⁼, Siavash Partow⁼, Verena Siewers, Sara Bjørn, Jens Nielsen: **Enhancing the copy number of episomal plasmids in *Saccharomyces cerevisiae* for improved protein production.** *FEMS Yeast Research* (2012) 1-10.
= These authors contributed equally to this work
- III. Gionata Scalcinati⁼, Christoph Knuf⁼, Siavash Partow, Yun Chen, Jérôme Maury, Michel Schalk, Laurent Daviet, Jens Nielsen, Verena Siewers: **Dynamic control of gene expressionin *Saccharomyces cerevisiae* engineered for the production of plant sesquiterpene α -santalene in a fed-batch mode.** *Metabolic Engineering* (2012): 14: 91-103.
= These authors contributed equally to this work
- IV. Gionata Scalcinati, Siavash Partow, Verena Siewers, Michel Schalk, Laurent Daviet, Jens Nielsen: **Systematic metabolic engineering applied to plant sesquiterpene production in continuous cultures of *Saccharomyces cerevisiae*.** (Submitted)
- V. Siavash Partow, Verena Siewers, Laurent Daviet, Michel Schalk, Jens Nielsen: **Reconstruction and evaluation of the synthetic bacterial MEP pathway in *Saccharomyces cerevisiae*.** (Submitted)

CONTRIBUTION SUMMARY

A summary of my contribution to each of the above listed publications is provided below:

Paper I: Designed the study, conducted all wet lab experiments, analyzed the data and wrote the manuscript.

Paper II: Designed the study, carried out the experiments including plasmids and strains construction, continuous cultivation, enzyme assay and qPCR, analyzed the data, and wrote the manuscript.

Paper III: Designed the study, assisted the molecular biology experiments, constructed the plasmids and strains, preformed the experimental work, and discussed the results.

Paper IV: Assisted the molecular biology experiments, constructed the plasmids and strains, and discussed the results.

Paper V: Designed the study and conducted all wet lab experiments, analyzed the data and wrote the manuscript.

ABBREVIATIONS COMMONLY USED

S. cerevisiae: *Saccharomyces cerevisiae*

E. coli: *Escherichia coli*

A. thaliana: *Arabidopsis thaliana*

GRAS: Generally Regarded As Safe

DNA: Deoxyribonucleic acid

mRNA: messenger ribonucleic acid

YAC: Yeast artificial chromosome

YEpl: Yeast expression plasmid

YCp: Yeast centromeric plasmid

YIp: Yeast integrative plasmid

3' UTR: 3' Untranslated region

IRES: Internal ribosome entry sites

GFP: Green fluorescent protein

IPP: Isopentenyl diphosphate

DMAPP: Dimethyl allyl diphosphate

MVA pathway: Mevalonate pathway

MEP pathway: 2-C-methyl-D-erythritol 4-phosphate pathway

NADPH: Nicotinamide adenine dinucleotide phosphate hydrogen

FPP: Farnesyl diphosphate

PCN: Plasmid copy number

CIA: cytosolic iron-sulfur protein assembly machinery

ISC: iron-sulfur protein assembly machinery

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CHAPTER 1: INTRODUCTION

1.1.1. Introduction to synthetic biology and its impact on metabolic engineering of yeast

The engineering of biological systems has enormous power to reshape the world in various fields, such as sustainment of all systems, environmental rehabilitation, and manufacturing at all macro- and micro-levels, preventative and curative health issues and general medicine. Synthetic biology is advancing capabilities for engineering biological systems through employing engineering principles and novel biological tools to the process of constructing and implementing human-designed biological systems, and to predictably produce a wide variety of pathways and regulatory networks. These innovations have offered a variety of applications in metabolic engineering of microorganisms aiming to give them new abilities which are not inherent to the microorganism, e.g. production of artemisinic acid in engineered yeast (Ro et al., 2006), production of *n*-butanol in *Saccharomyces cerevisiae* (Steen et al., 2008), enhancements in production of fatty acid derived biofuels by using dynamic sensor-regulator system in *E. coli* (Zhang et al., 2012) and modulation of metabolic flux using synthetic protein scaffolds (Dueber et al., 2009). In the latter study, the authors presented synthetic protein scaffolds to physically emplace metabolic enzymes involved in the mevalonate biosynthetic pathway together. Using these synthetic protein scaffolds a dramatic enhancement was observed in mevalonate production due to substrate tunneling mechanisms by which metabolites moved quickly from one active site to another, without loss by diffusion or degradation (Dueber et al., 2009). These are few examples which have shown the implementation of genetic engineering and synthetic biology in metabolic engineering and have demonstrated the ability of synthetic biology to provide an alternative to traditional methods in order to transplant the genes related to biosynthetic pathways from natural hosts into heterologous hosts such as *E. coli* or *S. cerevisiae*.

Similar to *E. coli*, among the eukaryote host systems, yeast contains the benefits of unicellular organisms i.e., the amenability for genetic manipulations and cell culture. It has also high capability for protein processing i.e., post-translational modifications and protein folding. These benefits are combined with a deep knowledge about yeast physiology, biochemistry and fermentation technologies, and also the lack of endotoxin production, as well as oncogenic or viral DNA-made yeast, *S. cerevisiae*, as suitable organism which has been widely used for heterologous expression of biochemical pathways in the field of pathway engineering and metabolic engineering (Szczebara et al., 2003; Yan, Kohli, & Koffas, 2005; Ro et al., 2006; Dejong et al., 2006). Furthermore, due to its importance in traditional biotechnology such as baking, brewing and wine making, *S. cerevisiae* has been classified as GRAS (generally regarded as safe) and many research activities, historically, have focused on this organism. It was the first eukaryotic organism to have its genome completely sequenced (Goffeau et al., 1996). Besides the aforementioned advantages, *S. cerevisiae* is known as a

eukaryotic model organism because of two important criteria. First, it is a single celled organism with a short generation time (doubling time of 1.25–2 hours) and it can be easily cultured in both rich/complex and minimum/synthetic media. Second, *S. cerevisiae* can be transformed through homologous recombination, allowing for genetic manipulation such as knockout or mutation of native genes, changing the expression level of a desired gene, or insertion of a heterologous gene. These fundamental knowledge bases have led to the development of a number of tools in different emerging fields e.g. systems biology, evolutionary engineering and synthetic biology. Among them, synthetic biology offers an alternative approach that is becoming more accessible as a tool for improved metabolic engineering of yeast. Along this line, there will be discussed important synthetic biology tools developed for controlling enzyme expression levels and the progress in DNA transformation methods in yeast in this chapter. And finally, a short introduction to isoprenoid production through both MVA and MEP pathways will be considered.

1.2. Synthetic biology tools for controlling enzyme expression levels

From an economic point of view, high productivities, titers and yields are essential for microbial production of chemicals. Optimizing pathway flux, reducing toxic intermediates, and balancing stress on the cell are the most important factors required to reach maximal yields. Therefore, pathway optimization and, specifically, modulation of enzyme expression is the focus and is one of the key challenges in most metabolic engineering investigations aiming at production of fine chemicals and pharmaceuticals. Recent advancements in synthetic biology offer a set of novel tools which are useful for controlling enzyme expression levels (Siddiqui et al., 2012). In spite of the diversity, these tools attempt to modulate at process units of central dogma like transcription or translation leading to altered levels of central components, e.g. DNA, RNAs and proteins (**Figure 1**). In 1958 Francis Crick described the Central Dogma. This principle describes a framework which is useful for understanding the way of biological information. In addition to the three major process units (DNA replication, transcription and translation), many other sub-processes have been declared in last two decades, e.g. splicing, which is the process for modifying RNA molecules after transcription, and different types of post-translational processes, which is the chemical modification of protein molecules after translation. Understanding the principle of each process in the central dogma will enhance our ability to design novel tools to control the biological processes, which are performed inside the living cell, at a predictable level. As mentioned before, different and advanced biological tools have been developed for optimizing of biochemical pathways. In the following, the mechanisms of these biological apparatuses will be described with specific consideration of yeast, as the major eukaryotic model organism.

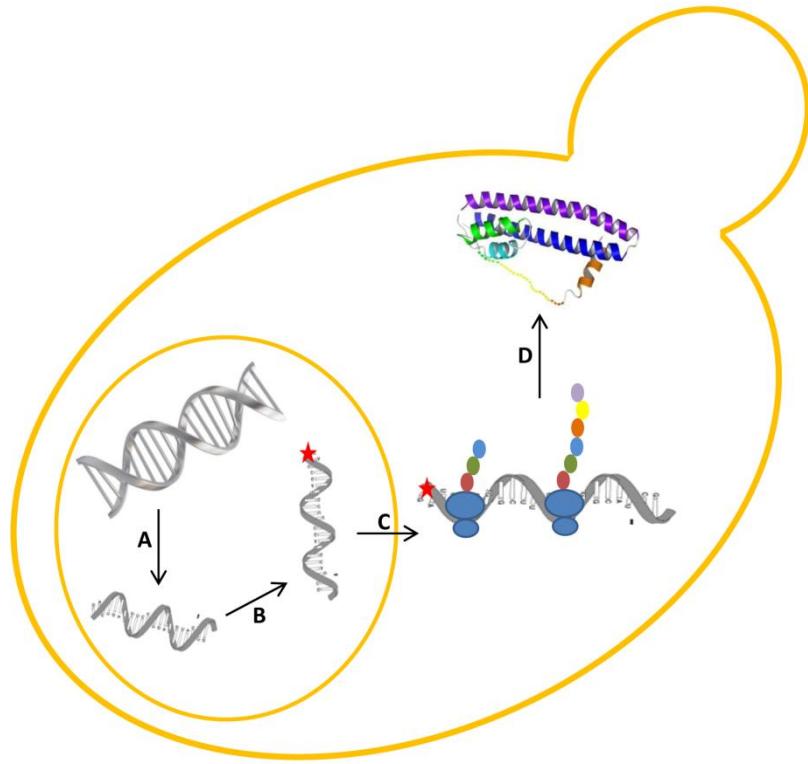


Figure 1: The central dogma; Arrows represent the process units containing (A) transcription process, (B) post-transcription process, (C) translation process and (D) post-translation process.

1.2.1. Synthetic biology tools at DNA level

DNA, which is coding the mystery of life, is the first component in the central dogma. Therefore, several toolsets have been developed for tuning expression of either endogenous or heterologous genes at the DNA level, and especially in yeast. Basically, they aim at altering gene copy number or gene dosage e.g., plasmid DNA and yeast artificial chromosomes (YAC) (Murray A. W, 1983) or offer an accurate control over gene copy number and stability, e.g., integration of heterologous gene via homologous recombination (Shao et al., 2009; Hawkins & Smolke, 2010) (**Figure 2**).

As in *E. coli*, different plasmids have been modified for yeast, while their availability for use in yeast is much more limited than those for *E. coli*. These plasmids have been successfully applied in metabolic engineering investigations (Ro et al., 2006)(Maury et al., 2008). Yeast plasmids are classified into three different classes, YCp, YEp and YIp (**Figure 2**) (Silva & Srikrishnan, 2012). YCp and YEp have been employed for many applications. YCp (yeast centromeric plasmid) vectors contain both an origin of replication and a centromere sequence. These two elements give YCp vectors high segregation stability in selective medium, while maintaining 1-2 copies per cell (Clarke & Carbon 1980). YEp (yeast episomal plasmid) vectors are maintained at more than 10 copies per cell (Romanos et al., 1992). This type of vector harbors either a full version of *S. cerevisiae* native 2 μ sequence or

commonly, a 2μ sequence including both the origin and the stability locus (STB), *REB3* (Futcher & Cox, 1983; Kikuchi 1983). The latter ones are generally more stable in comparison to those which are carrying full 2μ sequence.

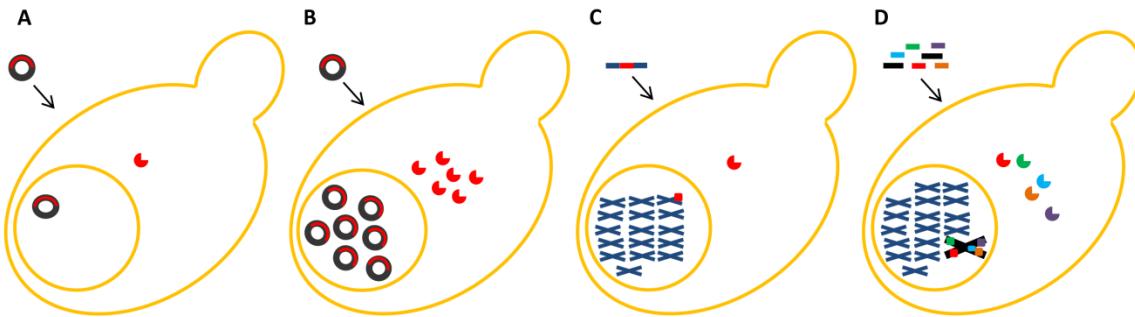


Figure 2: Different synthetic biology tools developed at DNA level. **A:** yeast centromeric plasmid (Ycp); **B:** yeast episomal plasmid (YEp); **C:** yeast integrative plasmid (YIp); **D:** yeast artificial chromosome (YAC)

Besides, the plasmid copy number can be modulated through the engineering of other elements on the plasmid such as the auxotrophic marker. For example, replacing the native promoter region of plasmid auxotrophic markers *URA3* and *LEU2* with the truncated and weak promoters *URA3-d* and *LEU2-d*, respectively, resulted in enhancement of the plasmid copy number (Erhartt & Hollenberg, 1983; Loision et al., 1989). Faulkner and co-workers (1994) had been able to improve the plasmid copy number to 150 and 111 copies per cell by using the *URA3-d* and *LEU2-d* marker, respectively (Faulkner et al., 1994). This type of plasmid with high copy number is recommended for overexpression of a product gene, rather than pathway optimization and metabolic engineering purposes (Jones et al., 2000). Despite over-expression of enzymes, results of such a high copy number may cause the depletion of precursors or resources, which are necessary for growth and production (Glick, 1995).

The third class of yeast vectors are YIps, yeast integrative plasmids, which do not have any replication origin. Therefore, they need to be integrated into the chromosome in order to maintain them in the cell. YIp vectors can be integrated into the genome via homologous recombination occurring between complementary target sites on both plasmid and genome. Different target sites have been developed for YIp vector series e.g., auxotrophic markers which offer integration by single-crossover (Gietz & Sugino, 1988; Cartwright et al., 1994a; Alberti et al., 2007; Sadowski et al., 2007). Table 1 illustrates several vector series in all three classes.

Both YCp and YEp vectors are uncomplicated to use and are ideal for gene overexpression at low or high levels. Although plasmids offer a quick appraisal of the degree of overexpression, which is necessary in a metabolic pathway, the maintenance of two or more YCp (CEN/ARS) and/or YEp (2μ)

vectors for stable existence in a single cell can be difficult. In addition, the use of plasmids is limited to carry small size of DNA molecule. However, this limitation is circumvented by employing yeast artificial chromosomes (YAC) which offer the possibility to transference large DNA molecule (more than several Mbps) (Murray A. W, 1983; Kouprina & Larionov, 2008).

YAC constructs, as YCp and YE_p, require a selective pressure, in order to be maintained in long term cell culture. On the other hand, chromosomal gene integration is efficient in yeast. This natural ability serves a robust expression platform, which allows highly stable maintenance in free-continuous selective pressure. Thus, different methods have been developed, based on this stable integration and they have been successfully employed, either for optimization of endogenous metabolic pathways (Ro et al., 2006) and/or transformation of heterologous pathways (Szczebara et al., 2003; Shao et al., 2009) which will be discussed later in this chapter.

1.2.2. Tools for control of the transcription level

Because most biological processes are regulated at the level of transcription as the first dedicated phase of gene expression (Sikder & Kodadek, 2005), the second toolset for controlling gene expression is, basically, developed for tuning transcription level. Based on these facts, different toolsets have been developed for modulating RNA levels in the cell and are classified into two groups. The first group modulates directly the RNA level during the synthesis process of this molecule performed by RNA polymerase e.g., employs different promoters with promising desired effects, and the second group controls the stability of RNA after being synthetized. The latter is defined as an RNA control device (Liang et al., 2011; Chang et al., 2012).

A: Control of transcription process

Promoters, which are a target for RNA polymerase, are one of the main regulatory elements controlling RNA synthesis and so they can play a significant role in modification of toolsets employed for tuning transcription level and also are potential target application in synthetic biological circuits (Ajo-Franklin et al., 2007; Bashor et al., 2008). Ajo-Franklin and co-workers presented a yeast memory device, which is controlled at the transcription level using two promoters *GAL1/10* and minimal *CYC1* (Ajo-Franklin et al., 2007). In another example, a synthetic feedback loop has been created for modulating the MAP kinase pathway through employing different modulators whose expression was controlled with constitutive or inducible promoters (Bashor et al., 2008).

There are approximately 6000 promoter regions which have been found in *S. cerevisiae*, according to SCPD (The Promoter Database of *S. cerevisiae*: <http://rulai.cshl.edu/SCPD>). These promoters are classified into two categories, constitutive and regulatable (**Figure 3**), however, different promoter

libraries and chimeric promoters have been recently developed, employing DNA manipulation methods (Jensen, 2003; Alper et al., 2005; Zhang et al., 2012). Alper and co-workers have generated a promoter library of constitutive promoter *TEF1*, introducing mutations into the sequence of this promoter via error-prone PCR (Alper et al., 2005). They demonstrated a series of *TEF1* promoters with activity range of 17 to 250% of the original.

Promoters with constant activity have been widely employed for modulating gene expression in *S. cerevisiae*. These promoters usually apply in a simple manner, which makes it not necessary to use additional molecules as inducers or repressors and they provide closely constant levels of gene expression. These features make them favored for the introduction of new pathways in yeast, especially if active pathways are desired during cell growth. Most of the yeast glycolytic pathway genes in *S. cerevisiae* are controlled by constitutive promoters, e.g., *TDH3*, *PGK1*, *PYK1* and *TPI1*. They have been widely used to construct expression cassettes of different plasmids (**Table 1.1**), allowing high expression levels during long-term cell culture.

Although applying strong and constitutive promoters results in radical changes in target gene transcript levels, regulated promoters have an advantage of controlling the expression level of the specific gene in response to changing concentrations of specific molecules, either inducer or repressor. A small number of regulated promoters have been found and employed in yeast. The most important are *GAL1* and *GAL10*, which are induced in the presence of galactose and repressed using glucose as a carbon source (Lohr et al., 1995). *MET25* (Sangsoda et al., 1985), *MET3* (Cherest et al., 1985) and *CUP1* (Etcheverry, 1990) promoters which are responding to the presence of methionine and copper, respectively, are another example of yeast native regulated promoters. In order to redirect the flux of farnesyl diphosphate (FPP) to produce sesquiterpenes, the *MET3* promoter has successfully been employed in down-regulation of *ERG9* encoding squalene synthase (SQS) (Ro et al., 2006; Asadollahi et al., 2008). The *Tet* promoter is an example of a synthetic bacterial regulated promoter which is adapted for use in *S. cerevisiae* and which is induced by the antibiotic tetracycline (Dingermann et al., 1992).

Table 1.1: Vector series for gene expression in *S. cerevisiae* (adapted from Da Silva et al., FEMS Y Re (2012)

Series name	Origins for replication	Selection markers	Promoters	Comments	References
pISXXX	<i>ARS1/CEN4</i> , 2 μ , <i>ARS1</i>	<i>URA3</i>	P_{TEF1} , P_{GAL1} , P_{STE12}	YIplac backbone	Sadowski et al., (2007)
YCp4XX, YE _p 4XX, YRp4XX	<i>ARSH4/CEN6</i> , 2 μ	<i>URA3</i> , <i>TRP1</i> , <i>HIS3</i> , <i>LEU2</i> , <i>LYS2</i>		pBR322 backbone	Ma et al., (1987)
pRS series (YCp, YE _p , YIp)	<i>ARSH4/CEN6</i> , 2 μ	<i>URA3</i> , <i>TRP1</i> , <i>HIS3</i> , <i>LEU2</i> , <i>KanMX</i> , <i>ADE2</i> , <i>MET15</i> , <i>hphNT1</i> , <i>natNT2</i>		pBLUESCRIPT-based vectors	Sikorski & Hieter (1989), Brachmann et al., (1998), Taxis & Knop (2006)
P4XX prom. (pRS variant)	<i>ARSH4/CEN6</i> , 2 μ	<i>URA3</i> , <i>TRP1</i> , <i>HIS3</i> , <i>LEU2</i> ,	P_{CYC1} , P_{ADH1} , P_{TEF1} , P_{GDP1} , P_{MET25} , P_{GAL1} , P_{GALL} , P_{GALS}		Mumberg et al., (1994, 1995)
pCu4XX prom (pRS variant)	<i>ARSH4/CEN6</i> , 2 μ	<i>URA3</i> , <i>TRP1</i> , <i>HIS3</i> , <i>LEU2</i> ,	P_{CTR3} , P_{CTR1} , P_{CUP1}		Labbe & thiele (1999)
p4XX prom.att (Gateway™)	<i>ARSH4/CEN6</i> , 2 μ	<i>URA3</i> , <i>TRP1</i> , <i>HIS3</i> , <i>LEU2</i> ,	P_{TEF1} , P_{GPD1} , P_{MET25} , P_{GAL1}	Four N- and C-epitope tags	Funk et al., (2002)
pVV2XX (Gateway™)	<i>ARS1/CEN4</i>	<i>URA3</i> , <i>TRP1</i>	P_{PGK1} , $P_{tetO-CYC1}$	Four N- and C-epitope tags	Van Mullem et al., (2003)
pJGXXX (Gateway™)	<i>ARSH4/CEN6</i>	<i>URA3</i> , <i>TRP1</i> , <i>HIS3</i> , <i>LEU2</i> ,	P_{GAL1}	Some with V5-6xHis tag	Geiser (2005)
pAG (Gateway™)	<i>ARSH4/CEN6</i> , 2 μ	<i>URA3</i> , <i>TRP1</i> , <i>HIS3</i> , <i>LEU2</i> ,	P_{TDH3} , P_{GAL1}	Seven N- C- epitope tags, integration vectors also available	Alberti et al., (2007)
pCMXXX series (YCplac, YEplac variant)	<i>ARS1/CEN4</i> , 2 μ	<i>URA3</i> , <i>TRP1</i>	$P_{tetO-CYC1}$ with Tta activator	Varied number of tetO boxes	Gari et al., (1997)
pYC, pYES	<i>ARSH4/CEN6</i> , 2 μ	<i>URA3</i> , <i>TRP1</i> , <i>bsd</i> [§]	P_{GAL1}	YES™ vector Collection	Invitrogen,
YCp-SPB series, YE _p -SPB series, YIp-SPB series, YIp-GAL1-SPB	<i>ARS1/CEN4</i> , 2 μ	<i>URA3</i>	P_{PGK1} , P_{GAL1} , P_{GAL10} , P_{PHOS} , P_{CUP1}		Cartwright et al., (1994)
pXP series	<i>ARSH4/CEN6</i> , 2 μ	<i>URA3</i> , <i>TRP1</i> , <i>HIS3</i> , <i>LEU2</i> , <i>ADE2</i> , <i>MET15</i>	P_{PGK1} , P_{TEF1} , $P_{HXT7-391}$, P_{ADH2} , P_{GAL1} , P_{CUP1}	Allows marker recycling (Cre-loxP systems) when use as a template for integration	Fang et al., (2011),
pBEVY, pBEVY-G	2 μ	<i>URA3</i> , <i>TRP1</i> , <i>HIS3</i> , <i>LEU2</i> ,	P_{TDH3} - P_{ADH1} , P_{GAL1} - P_{GAL10}	Bidirectional promoter system	Miller et al., (1998)
pY2x-GAL(1/10)-GPD	2 μ	<i>URA3</i> , <i>TRP1</i> , <i>HIS3</i> , <i>LEU2</i> ,	P_{GAL1} - P_{TDH3} , P_{GAL10} - P_{TDH3}	Bidirectional promoter system	Li et al., (2008)
pESC Vectors	2 μ	<i>URA3</i> , <i>TRP1</i> , <i>HIS3</i> , <i>LEU2</i> ,	P_{GAL1} - P_{GAL10}	Bidirectional promoter system	Agilent Technologies

§ Blasticidin resistance gene.

Regulated promoters have many advantages, e.g., there are easy employment, support well-defined and predictable expression levels and are also a useful tool for verifying the optimal expression level of a particular enzyme in a metabolic network (Westfall et al., 2011; Hawkins & Smolke, 2010). However, inducer or repressor molecules may show pleiotropic effects (Mumberg et al., 1994; Wishart et al., 2005; Labbe & Thiele 1999) or may be consumed by the cell, which both make it complicated system to control the expression level. In addition, the inducer molecules are typically expensive, and using inducible promoters may not be economical for industrial-scale fermentations.

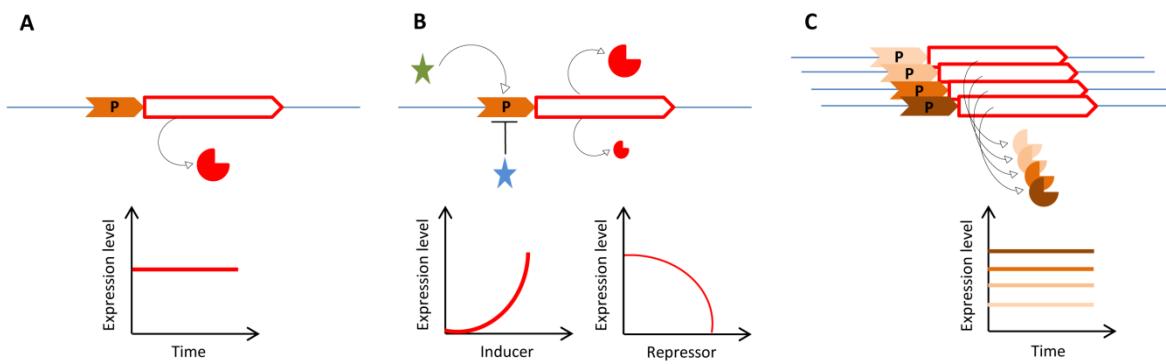


Figure 3: Schematic representation of expression level at transcription, which is controlled by different types of promoters. Promoters are orange chevrons, coding region are red pentagons, pies with different colors represent proteins, blue and green stars represent inducer and repressor molecules, respectively. **A:** constitutive promoter; **B:** regulated promoter; **C:** promoter library

MGPA (multiple-gene-promoter-shuffling) offers a useful tool for metabolic engineering purposes (Lu & Jeffries, 2007). This tool has been implemented to optimize xylose fermentation in yeast. The rate-limiting steps of the pentose phosphate pathway (PPP) have been modulated by employing the combination of multiple promoters with different strengths. The authors have demonstrated that the best ethanol production has been achieved via the optimal expression level of limiting steps of this pathway. Instead of constitutive or inductive expression, in metabolic engineering approaches the combination of different promoters is recommended in order to balance metabolic pathways.

B: RNA control devices

RNA molecules play varying functional roles in living cells e.g., regulation of gene expression through RNA secondary structure, catalytic activity (ribozyme) with functional roles in RNA replication, RNA stability, splicing and translation in both prokaryotes and eukaryotes (Serganov & Patel, 2007) and regulate protein synthesis through antisense-mediated regulation of translation. Due to these facts, recently different synthetic RNA switches with diverse roles including sensing, regulatory, information processing and scaffolding activities have been developed in order to aid programming of biological systems. These synthetic RNA switches are, generally, composed of two

domains. First, is the sensing domain that detects signals inside a cell (input) and second is the actuator domain that alters gene expression. In some cases, a distinct transmitter domain adds to the RNA switch in order to provide better communication between sensing domain and actuator domain (**Figure 4**). Based on the type of regulated process, RNA switches are divided into five different categories: transcription-modulation, splicing-modulation, RNA stability-modulation, RNA interference-modulation, translation-modulation and post translation-modulation switches (Chang et al., 2012). From these, different RNA switches for modulating biological systems at transcription, splicing and RNA stability level have been developed in *S. cerevisiae* (Buskirk et al., 2004; Weigand & Suess, 2007; Win & Smolke, 2007; Win & Smolke, 2008; Babiskin & Smolke, 2011a; Babiskin & Smolke, 2011b). In a recent study, Babiskin and Smolke added the synthetic Rnt1p hairpin, which is a target of RNase III at the 3' UTR of *ERG9*, in order to control its expression (Babiskin & Smolke, 2011b). The ability of this posttranscriptional control device in reducing the expression of *ERG9*, has been demonstrated through comparing the transcription level of *ERG9* in strains carrying this module and wild type (Babiskin & Smolke, 2011b). The authors suggested that this new controlling system has a benefit, to systematically titrate pathway enzyme level while keeping cellular control strategies active.

1.2.3. Tools for control at the protein level

Proteins are the curtai players inside the cell and most biological activities are well-controlled by the functions of different proteins e.g., enzymatic activity, signaling and transporting, and structural proteins. The level of these multi-functional macromolecules plays an important role in modulating biological systems. During the last decade, different protein-based control elements acting through protein degradation have been developed and employed for tuning protein levels (Mateus & Avery, 2000; Hackett et al., 2006; Grilly et al., 2007). These elements usually alter the protein half-lives to provide rigid dynamic regulation over biochemical pathways.

The fundamental importance of these elements is to introduce a degradation tag signal at the *N*- or the *C*- terminus of target protein(s), leading them into the natural degradation machinery of the cell, e.g. ubiquitination in yeast which serves as an exquisite process for control of protein degradation (**Figure 4**). For example, Mateus and Avery (Mateus & Avery, 2000) have constructed a new destabilized green fluorescent protein by fusing the C-terminal residues of yeast G₁ cyclin, Cln2p into yeast-optimized GFP (yEGFP3). The residues of Cln2p contain the PEST motifs of Cln2 and are anticipated to target the protein for ubiquitin (Ub) - dependent degradation. They have shown that the new and modified GFP is efficiently unstable and it can be implemented for monitoring dynamic changes in yeast gene expression (Mateus & Avery, 2000). It has also been shown that modification of the *S. cerevisiae* N-degron signal sequence can influence reporters half-life and bring it down to 2 min

(Hackett et al., 2006). Grilly and coworkers have constructed a synthetic protein degradation network in *S. cerevisiae* (Grilly et al., 2007). They have adapted the prokaryotic *ssrA* tagging system in *S. cerevisiae* by importing an *E. coli* degradation machinery, ClpXP protease. The reduction of the half-life of GFP to as low as 22 min has been observed by employing ClpXP protease (Grilly et al., 2007). In contrast to other tagging systems, aforementioned, (Mateus & Avery, 2000; Hackett et al., 2006), the latter example offers a tunable protein degradation system with less undesired pleiotropic effects in yeast.

In addition to the presented protein toolsets, different internal ribosome entry sites (IRES) have been reported in yeast, which have influence on protein expression (Zhou et al., 2001). Furthermore, a library of internal ribosome entry sites (IRES) have been developed (Zhou et al., 2003). Recent elements may offer a promising device for controlling protein expression in the future and also they can potentially be applied to construct polycistronic gene clusters in eukaryotic systems such as yeast.

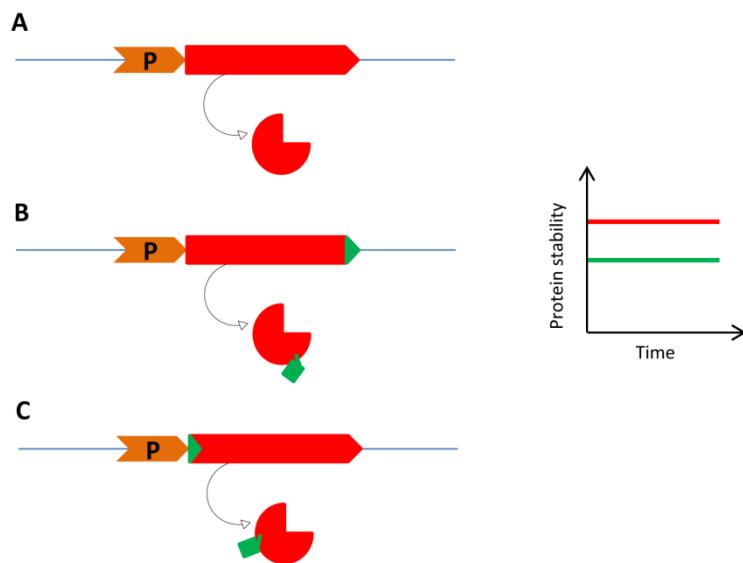


Figure 4: Schematic representation of synthetic biology tools developed for modulating protein stability. Promoters are orange, coding region are red pentagons, red pies represent protein, green boxes are peptide tag at C- and N- terminal, respectively; (A) non-tagged protein; (B) C-tagged protein; (C) N-tagged protein

1.3. Synthetic biology and re-construction of metabolic pathways in *S. cerevisiae*

The first step of most yeast metabolic engineering and synthetic biology studies aiming at design and construction of cell factories indicating non-native and desirable traits, is to reconstruct a completely, or partially, synthetic pathway. So, stable assembly and transfer of heterologous pathways with several enzymatic steps is a major challenge in metabolic engineering. Several methods have been developed which can be used for transferring DNA into *S. cerevisiae* as a desired host. Each of them has benefits and disadvantages. Here, I classify these methods into three categories: (i) Plasmid-based methods, (ii)

YAC-based methods for transferring DNA and (iii) DNA transfer through chromosomal integration. The principle of this classification is based on progression in DNA assembling methods.

1.3.1. Plasmid-based method for transferring DNA

Yeast plasmids offer a simple tool for transferring DNA sequences. Essentially, this method is developed based on restriction and ligation, in order to clone the desired ORF (insert) into a vector. Hence, finding at least one unique restriction site in both plasmid and DNA insert is the first necessary requirement which highlights the first limitation of this method. On the other side, yeast does not naturally express polycistronic operons like prokaryotes, which means that each gene requires its own promoter and terminator flanking sequences. The latter raises the requirement for a different set of promoters and terminators for cassette assembly and it can increase the size of the plasmid. These issues limit the application of either YEp or YCp for transferring a small number of genes e.g. two genes instead of a whole metabolic pathway containing several steps. In addition, maintenance of the plasmid requires selective media which can result in incomplete selection of cells for long-term cultivation. However, in spite of such limitations, episomal plasmids have successfully been employed to reconstruct the bacterial MEP pathway in *S. cerevisiae* (Maury et al., 2008). The seven enzymatic steps of the MEP pathway carried on two episomal plasmids were transformed into yeast.

1.3.2. YAC-based method for transferring DNA

Due to its high efficiency and ease to work with *in vivo* homologous recombination in *Saccharomyces cerevisiae*, different synthetic biology tools were developed for stable transfer of metabolic pathway steps containing large amounts of DNA sequences, e.g., yeast artificial chromosomes (YAC) (Murray A. W, 1983). YACs have been employed in reconstruction of a flavonoid pathway in *S. cerevisiae* (Naesby et al., 2009). Genes from different organisms encoding enzymes of a flavonoid pathway have individually been cloned to make a full expression cassette containing promoter and terminator and, furthermore, all cassettes randomly assembled on Yeast Artificial Chromosomes to construct the flavonoid pathway (Naesby et al., 2009). Kouprina and Larionov (2008) have developed a new protocol which is based on transformation-associated recombination (TAR) in *S. cerevisiae* employing TAR-cloning vector compassing targeting sequences homologous to a desirable region (Kouprina & Larionov, 2008). This method allows for transferring up to 250 kb of selective DNA sequences in size as a circular yeast artificial chromosome (Kouprina & Larionov, 2008). In another example, Gibson and co-workers (2008) have demonstrated assemblage of the *Mycoplasma genitalium* genome (582970 bp) in *S. cerevisiae* in 4 steps using a combination of both *in vitro* enzymatic assembly and *in vivo* TAR-based cloning (Gibson, Benders, Andrews-pfannkoch, et al., 2008). Later, the same research

group reported the successful one-step assembly of the entire synthetic bacterial genome consisting of 25 overlapping DNA constructs in yeast (Gibson et al., 2008). The methods described above are mainly based on *in vivo* DNA assembly which is operated efficiently by yeast because of its high potential for homologous recombination. Recently, an organisms-independent method has been developed, offering an *in vitro* assembly of large DNA sequences (Gibson et al., 2009). In contrast to the two-step thermocycled DNA assembly (Gibson et al., 2008), the one-step isothermal DNA assembly can be efficiently used to construct up to several hundred kilobases of DNA fragment by using a mixture of enzymes including 5' exonuclease, DNA polymerase and DNA ligase in a single reaction (Gibson et al., 2009). In spite of successful assembly and transformation shown by these examples, the maintenance of such systems still requires selective pressure, provided by using selective media.

1.3.3. DNA transfer through chromosomal integration

Via homologous recombination large sizes of DNA can be integrated into the chromosome. Based on this natural ability different methods have been developed, aiming at heterologous DNA transfer into yeast e.g., DNA assembler (Shao et al., 2009). DNA assembler enables design and fast construction of large biosynthetic pathways in *S. cerevisiae* on both plasmids and by integration into the chromosome. Employing this method, Shao and co-workers (2008) have demonstrated rapid assembly of a functional D-xylose utilization pathway consisting of 3 genes ($\approx 9\text{kb}$), a zeaxanthin biosynthetic pathway including 5 genes ($\approx 11\text{kb}$) and combined both pathways ($\approx 19\text{kb}$) with an efficiency of 70-100%, either on plasmid or on a chromosome (Shao et al., 2009). Later, this method has been improved to easily perform genetic manipulations such as site-direct mutagenesis without going through the complicated multi-step procedures, and scar-less gene substitution and deletion which is useful for studying gene function (Shao & Zhao, 2011). The existence of target sites allowing efficient integration via crossing-over and also suitable selection markers for easily isolating correct transformants are two limitations of this method. However, the latter is circumvented by using a reusable selection marker, which allows multiple sequential gene transformation via homologous recombination. *cre/loxP* and *FLP/FRT* are the most famous examples of marker recycling systems with wide applications in yeast (Sauer, 1987; Güldener et al., 1996; Gueldener et al., 2002; Radhakrishnan & Srivastava, 2005).

“Reiterative recombination” is a robust DNA manipulation method developed for direct integration into the yeast chromosome by Wingler and Cornish (Wingler & Cornish, 2011). The key point of this method is based on utilization of recyclable marker and endonuclease-stimulated homologous recombination offering an efficient and simple procedure for sequentially building large libraries of biosynthetic pathways *in vivo* (Wingler & Cornish, 2011). Although this method has been developed

in yeast, it can be used in other organisms which have endogenous or engineered recombination systems.

Nowadays *de novo* DNA synthesis, especially in combination with codon optimization algorithms offer cheap and efficient tools for DNA manipulations. Through the progression in constructing metabolic pathway, using codon optimization algorithms provide inputs, e.g. genes with more efficiently translated heterologous host, subsequently, can improve the activities of pathway enzymes as efficiently as with native gene sequences (Redding-Johanson et al., 2011). Therefore, combining advanced methods for DNA transformation with the latest progression in DNA synthesis can improve not only DNA manipulation, but it may also improve protein activity. **Figure 5** illustrates progress in DNA assembly methods.

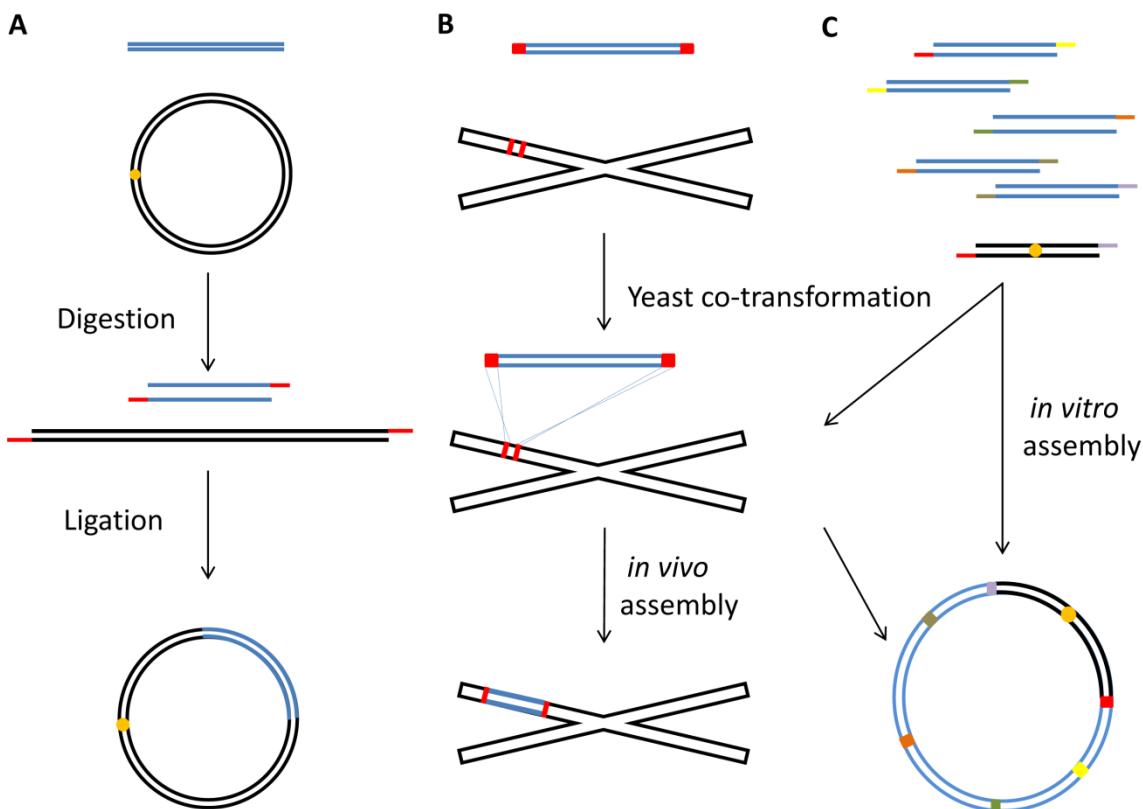


Figure 5: Schematic representation of assembly methods for forming DNA constructs; (A) Plasmid-based based on restriction and ligation; (B) chromosomal integration based *in vivo* homologous recombination; (C) YAC-based method based on both *in vivo* or *in vitro* homologous recombination.

1.4. Biosynthesis of isoprenoids compounds

Isoprenoids are a large group of natural and chemical compounds with more than 50,000 known members. Besides their varied essential biological functions, e.g. cell membrane fluidity (steroids),

respiration (quinones), hormones (abscisic acid), protein regulation (glycosylation), isoprenoids have valued applications e.g. as fragrances, pharmaceuticals and potential biofuels (Kirby and Keasling 2009; Zhang et al., 2011). Although all organisms use isoprenoids for their basic cellular processes, these compounds are found in high variability in plants and play essential roles in specialized processes such as defense, pollinator attraction, communication and involvement in growth and development. However, extraction of these compounds from plants needs a massive amount of raw material and usually suffers from low yield. For example, about six 100-years old Pacific yew trees are needed for producing a sufficient amount of taxol (anti-cancer) required for treatment of one patient (Horwits, 1994). In addition, chemical synthesis and production of these natural compounds can be extremely difficult because of structural complexity which is important for their activity. Due to these facts, the use of microorganisms like *E. coli* or *S. cerevisiae* for producing heterologous isoprenoids is an attractive approach, both environmentally and economically.

Despite the diversity, all isoprenoids are derived from five carbon isoprene units (2-methyl-1, 3-butadiene) and depending on the number of isoprene units in carbon skeleton different groups of isoprenoids are formed (Maury, Asadollahi, & Møller, 2005). Isopentenyl diphosphate (IPP), which is the universal biological precursor for all isoprenoids, is produced via two different metabolic pathways, the mevalonate (MVA) pathway, which is operational in eukaryotic cells and the cytoplasm and mitochondria of plants, and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, which is specific to bacteria, other prokaryotes and the plastids in plants (Maury et al., 2005; Kirby and Keasling 2009).

1.4.1. Mevalonate pathway

As described in Figure 6, the mevalonate pathway is initiated by condensation of two molecules of acetyl-CoA by function of acetoacetyl-CoA thiolase (Erg10). Then, through 5 enzymatic reactions the final product, IPP is produced, which is isomerized to DMAPP by Idi1. The pathway has been targeted in several investigations aiming at heterologous production of different isoprenoids (Ro et al., 2006; Asadollahi et al., 2009; Westfall et al., 2011). In order to increase the isoprenoid production in yeast, the pathway flux is modulated by focusing on increasing the local concentration of pathway enzymes and intermediates through employing different strategies altering the transcriptional level of bottleneck steps, for example, over-expression of 3-hydroxy-3-methylglutaryl-CoA reductase (Hmg1) and down-regulating squalene synthase (Erg9) (Ro et al., 2006; Asadollahi et al., 2008; Asadollahi et al., 2009). However, in a recent investigation, 10 fold enhancement in amorpha-4,11-diene production was achieved by over-expressing every enzyme of the mevalonate pathway to *ERG20* using a strong promoter (Westfall et al., 2011). Furthermore, the combination of these modifications, with improvement of the fermentation process, led to producing more than 40 g L⁻¹ amorpha-4,11-diene

(Westfall et al., 2011). In a different approach, Asadollahi and co-workers tried to increase the sesquiterpene production by addressing targets that are neither involved directly in the pathway nor in supplying the precursor (Asadollahi et al., 2009). Following the result of *in silico* analysis, enhancement of the sesquitrepene was achieved through increasing the pool of NADPH which is consumed by the MVA pathway enzymes (Asadollahi et al., 2009).

Further improvement of pathway yields has been achieved by employing different approaches, such as direct protein fusion and subcellular compartmentalization. In several studies, protein fusion strategies have been employed to redirect flux from the native MVA pathway downstream of the FPP branch point into the heterologous branch, leading to higher production of isoprenoids (Tokuhiro et al., 2009; Ohto et al., 2010; Albertsen et al., 2011). In all of these examples, the farnesyl pyrophosphate synthase (Erg20) was subjected to fusion with synthase enzymes like patchoulol synthase (PTS) or geranylgeranyl diphosphate synthase (Bts1). Tokuhiro and co-workers (2009) demonstrated enhancement of geranylgeranyl diphosphate (GGPP) production by 8-fold while utilizing fused Bts1-Erg20, in comparison to individual expression of Bst1 and Erg20 (Tokuhiro et al., 2009).

Organelle targeting has advantages in providing important cofactors and natural scaffolding or sequestering toxic compounds. Therefore, targeting the specific protein into the specific organelle within the cell is another approach to enhance biosynthetic pathway flux. Farhi and co-workers (2011) have used the mitochondrial targeting signal to localize the plant isoprenoid synthases, TPS1 and ADS in yeast mitochondria. They have also localized the endogenous FPP synthase (Erg20) to mitochondria using the targeting sequences from the COX4 gene fused to the N-terminus of TPS1, ADS and Erg20, individually (Farhi et al., 2011). They have demonstrated 3- and 20-fold increases in valencene and amorpha-4, 11-diene, respectively.

1.4.2. 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway

The MEP pathway was first reported independently by Rohmer and Arigoni (Rohmer et al., 1993; Arigoni et al., 1997). As illustrated in Figure 6, this pathway initiates by condensation of one molecule each of pyruvate and D-glyceraldehyde-3-phosphate through a thiamin diphosphate dependent reaction catalyzed by 1-deoxy-D-xylulose 5-phosphate synthase (Dxs), (Sprenger et al., 1997), followed by the NADPH dependent reduction process being catalyzed by 1-deoxy-D-xylulose 5-phosphate reductoisomerase (Dxr) (Takahashi et al., 1998), generating 2-C-methyl-D-erythritol

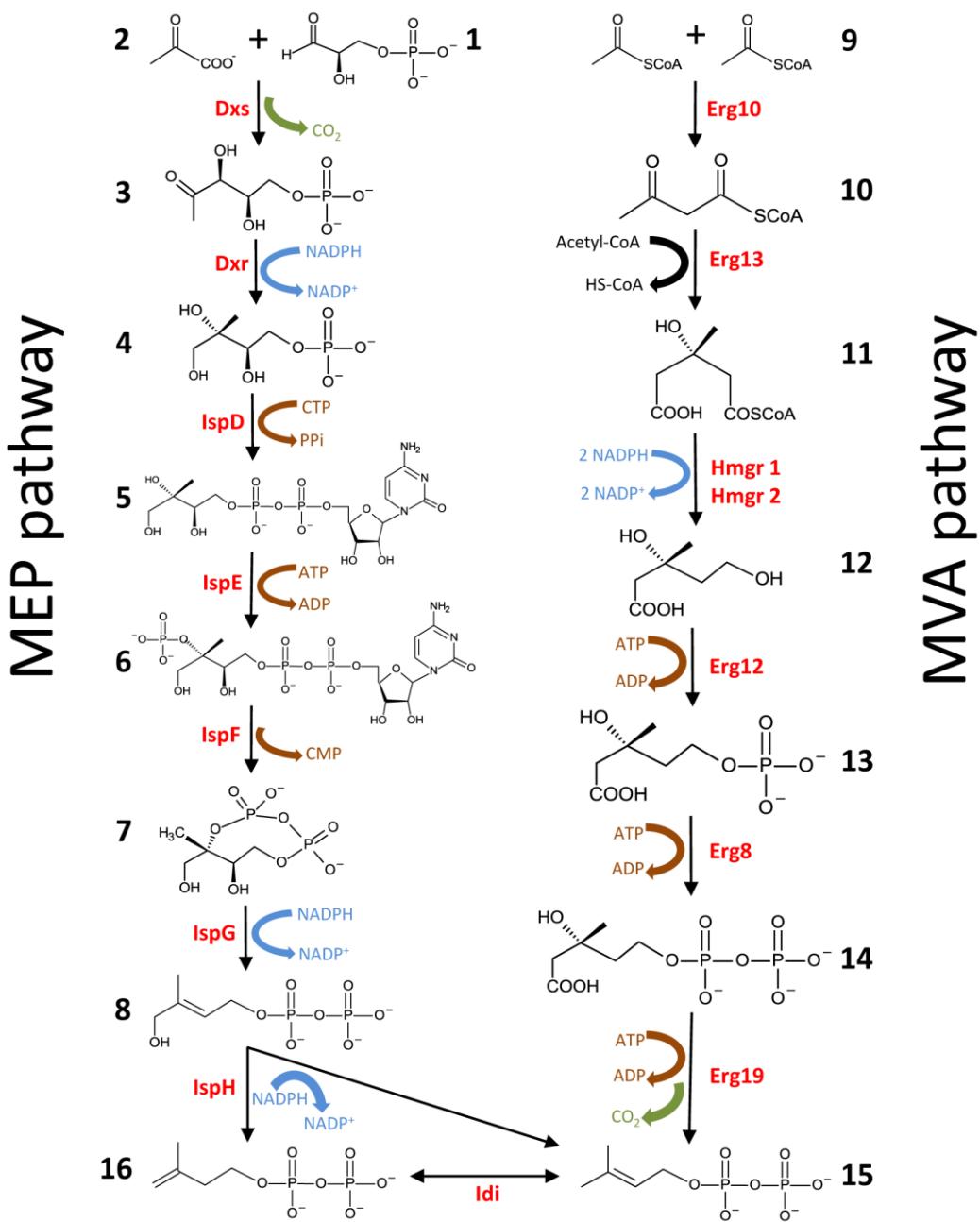


Figure 6: The MEP pathway (left). Enzymes: **Dxs**, 1-deoxy-D-xylulose-5-phosphate synthase; **Dxr**, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; **IspD**, 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase; **IspE**, 4-diphosphocytidyl-2-C-methylerythritol kinase; **IspF**, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; **IspG**, 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase; **IspH**, 1-hydroxy-2-methyl-but enyl 4-diphosphate reductase; Metabolites: **1**, D-glyceraldehyde 3-phosphate; **2**, pyruvate; **3**, 1-deoxy-D-xylulose 5-phosphate; **4**, 2-C-methyl-D-erythritol 4-phosphate; **5**, 4-diphosphocytidyl-2-C-methyl-D-erythritol; **6**, 2-phospho-4-diphosphocytidyl-2-C-methyl-D-erythritol; **7**, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; **8**, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate.

The MVA pathway (right). Enzymes: **Erg10**, acetoacetyl-CoA thiolase; **Erg13**, 3-hydroxy-3-methylglutaryl-CoA synthase; **Hmg1/2**, 3-hydroxy-3-methylglutaryl-CoA reductase; **Erg12**, mevalonate kinase; **Erg8**, phosphomevalonate kinase; **Erg19**, mevalonate pyrophosphate decarboxylase; **Idi**, isopentenyl diphosphate isomerase; Metabolites: **9**, acetyl-CoA; **10**, acetoacetyl-CoA; **11**, 3-hydroxy-3-methylglutaryl-CoA; **12**, mevalonate; **13**, phosphomevalonate; **14**, diphosphomevalonate; **15**, dimethyl allyl diphosphate; **16**, isopentenyl diphosphate.

4-phosphate (MEP). This intermediate is converted into the cyclic 2,4-diphosphate of 2-C-methyl-D-erythritol by the sequential action of the enzymes specified by IspD, IspE and IspF (Rohdich et al., 1999; Lüttgen et al., 2000; Herz et al., 2000) 2-C-methyl-D-erythritol-2,4-cyclodiphosphate is reduced by a reductase encoded by the *ispG* gene (Adam et al., 2002; Querol et al., 2002) followed by the production of IPP and DMAPP by the action of the *ispH* gene product. (Rohdich et al., 2003; Wolff, 2003).

Similar to the MVA pathway in yeast, the MEP pathway has been subjected to metabolic engineering in *E. coli* in order to produce different isoprenoid compounds (Huang et al., 2001; Yuan et al., 2006; Kim & Keasling, 2001; Farmer & Liao 2001). The enhancement in accumulation of taxadiene, which is an intermediate of anticancer drug paclitaxel has been achieved through the over-expression of the first enzyme of the MEP pathway (Huang et al., 2001). Over-expression of both *dxs* and *dxr* led to increased lycopene production in *E. coli* (Kim & Keasling, 2001). The authors suggested that, like Dxs, the second enzyme of the MEP pathway has also an appreciable control coefficient over the flux (Kim & Keasling, 2001). Later, replacing the native promoter of *dxr* with the strong and constitutive promoter T5 from bacteriophage resulted in increasing production of β-carotene by more than 3 fold (Yuan et al., 2006). Farmer and Liao (2001) attempted to increase the availability of MEP pathway precursors pyruvate and glyceraldehyde 3-phosphate (G3P) in order to increase the pathway flux (Farmer & Liao 2001). Although strong competition exists for these substrates which are central metabolites involved in several pathways, such as the tricarboxylic acid cycle, glycolysis and gluconeogenesis and the pentose phosphate pathway, they have shown that lycopene accumulation is controlled by the G3P/pyruvate ratio and not by substrate availability (Farmer & Liao 2001).

In general, all efforts in both *S. cerevisiae* and *E. coli* aimed at increasing isoprenoid production can be divided into two different approaches. In the first approach researchers tried to re-optimize and regulate of the metabolic flux of the endogenous pathway, whereas, introduction of a heterologous pathway to supplement the native pathway was core of the second approach. To address the second one, the MVA pathway has successfully been transferred and optimized in heterologous hosts, e.g. *E. coli* (Martin et al., 2003; Dueber et al., 2009; Ma et al., 2011). However, few records have been found for investigating the MEP pathway in a heterologous host like *S. cerevisiae* (Maury et al., 2008). In the next chapter, both re-optimization of the endogenous MVA pathway and reconstruction of the bacterial MEP pathway in *Saccharomyces cerevisiae* will be considered through the usage of the new synthetic biology tools, also discussed in the next chapter.

CHAPTER 2: RESULTS AND DISCUSSION

High-level expression of exogenous or endogenous genes in microorganisms is often a desired objective with applications in protein production or to over-express pathway enzymes leading to synthesis of, e.g. chemicals or biofuels. Promoter choice and gene copy number are the most important factors to ensure the desired gene transcription levels. Regulation of gene dosage and gene transcription are the first two key steps in biological systems, e.g. allowing control of metabolic pathway function. Both plasmid and chromosomal integration are widely used as tools in this kind of modulation. Like in *E. coli*, different plasmids with varying features have been developed in yeast, while there are not as many different plasmids available as for *E. coli*. They have been employed for many metabolic engineering applications. Yeast episomal plasmids (YEp) usually offer high expression levels. Despite the fact that various YEp series have been developed (**Table 1**), their structure consists of two parts. The expression cassette includes promoter, multi cloning site (MCS) and terminator, and the maintenance section usually consists of two selection markers allowing selection in *E. coli* and yeast, respectively, and also bacterial origin of replication and 2μ sequences providing the stable segregation in both *E. coli* and yeast, respectively. Based on these facts, I proposed to construct a new series of 2μ episomal plasmids which can provide, not only high constant gene expression, but also can improve gene copy number using synthetic biology tools.

The commercially available and widely used plasmid, pESC-URA (Stratagene, La Jolla, CA, USA), was chosen as a vector back-bone for our purposes. pESC-URA harbors the divergent and inducible *GAL1-GAL10* promoter on the expression cassette providing strong protein expression in presence of galactose as carbon source. The maintenance section of this vector consists of the pUC origin and ampicillin resistance (*ble*) ORF, which both are necessary for maintenance in *E. coli*; whereas, 2 micron and f1 origins in addition to the yeast *URA3* ORF are used to replicate and maintain the vector in yeast culture, respectively.

Thus, my experiments were designed to improve gene expression and gene dosage through modulating the elements involved in expression and maintenance sections of pESC-URA, respectively. In this chapter, first I review the final results leading to construct new expression plasmids and further I show the application of these new synthetic biology tools in optimizing the endogenous MVA pathway and in transferring the heterologous MEP pathway into *S. cerevisiae*. Finally, the role of the iron-sulfur cluster maturation process in the functionality of the MEP pathway will be discussed.

2.1. Regulation of expression level via promoter choice

In spite of many advantages offered by employing the bidirectional plasmid pESC-*URA*, e.g. allowing high expression level and evaluation of two ORFs simultaneously, the galactose- dependent of the divergent GAL1/GAL10 promoter highlights the major disadvantage of pESC-*URA* in terms of time and economy, especially when aiming at industrial applications. Because, like most other organisms, *S. cerevisiae* has evolved to preferentially utilize fermentation carbohydrates, typically glucose, as carbon and energy sources (Ronne, 1995). Using glucose as a carbon source not only has economical benefits, this carbon source is 10 times cheaper in bulk as compared to galactose, but it is also possible to produce biomass faster and in higher amounts using glucose as compared to galactose. I initiated this study aiming at developing a glucose based system analogous to the *GAL1/GAL10* system of the pESC vectors.

Several strong constitutive promoters have previously been described and have been shown to be useful for expression of heterologous genes in yeast. In this study, the strength of seven different constitutive or glucose based promoters derived from the following genes - *TEF1* (encoding transcriptional elongation factor EF-1 α) (Cottrelle et al., 1985), *PGK1* (encoding phosphoglycerate kinase) (Ogden et al., 1986; Holland and Holland Biochemistry 1978) *TPI1* (encoding triose phosphate isomerase), *HXT7* (encoding a hexose transporter) (Diderich et al., 1999; Reifenberger et al., 1997), *PYK1* (encoding pyruvate kinase 1) (Nishizawa et al., 1989), *ADH1* (encoding alcohol dehydrogenase 1)(Denis et al.,1983) and *TDH3* (*GPD*) (encoding triose phosphate dehydrogenase) (Bitter et al., 1984) have been compared in different stages of batch culture.

2.1.1. Comparison based on β -galactosidase activity

For this comparison, I used *lacZ* as a reporter gene and constructed 9 different integrative plasmids, in which *lacZ* expression was controlled by either of these promoters. In all cases, the constructed plasmids were integrated into the *ura3-52* locus. Although, in the last decade, different reporter systems have been developed and used for promoter analysis in *S. cerevisiae*, such as green fluorescent protein (Li et al., 2000; Niedenthal et al.,1996), β -lactamase (Cartwright et al., 1994b) and β -D-glucuronidase (Nacken et al., 1996), β -galactosidase encoded by the *lacZ* gene of *E. coli*, is the most commonly employed reporter of gene expression in *S. cerevisiae* and is widely used for different purposes (Flick & Johnston, 1990; Hacker & Magdolen, 1992; Yocom et al.,1998). It was shown that *lacZ*, as a reporter, is not compatible with a high copy number vector, but suitable for expression monitoring in mono copy (Purvis et al., 1987). As I only wanted to compare the strength of different promoters and avoid gene copy number variations, I used *lacZ* on an integrative plasmid pSF01, a derivative of pRS306 (Sikorski & Hieter, 1989), for this comparison. The expression of *lacZ*

controlled by these promoters was assayed 8, 24 and 48 hours after inoculation in shake flasks with 2% glucose. The results are shown in Table 2.1. Since the *TEF1* promoter is one of the strongest constitutive promoters (Gatignol et al., 1990) and since it showed the most stable and highest activity at different time points, I chose to set the P_{TEF1} activity at 8 hours as 100% and compared the activity of the other promoters relative to P_{TEF1} activity at this time point.

Five of these promoters (P_{PGK1} , P_{TPII} , P_{PYK1} , P_{TDH3} and P_{ADH1}) operate the key glycolytic genes and they are generally considered constitutive and strong promoters, in the literature. They did not show a constant activity during the cultivation condition. Therefore, the classification of “constitutive” promoters is often wrongly associated with a “constant” expression rate of the controlled genes. In fact, the expression of the majority of genes is a function of the specific growth rate (Regenberg et al., 2006), nutrition supplementation (Seresht et al., 2011) and environmental condition under which the cells are being cultured, e.g. oxygenation or temperature (Tai et al., 2007).

Table 2.1: Comparison of the promoters used in batch cultivation with 2% glucose.

Time	P_{ADH1}	P_{HXT7}	P_{PGK1}	P_{PYK1}	P_{TPII}	P_{TDH3}	P_{TEF1}
8 hrs	20%	10%	100%	60%	60%	100%	100%
24 hrs	27%	109%	52%	27%	31%	31%	156%
48 hrs	14%	150%	45%	14%	27%	27%	136%

The activities were normalized by setting P_{TEF1} activity at 8 hours to 100%. (Partow et al., 2010)

In conclusion, I observed that the promoter activity varied with the glucose concentration and whether the cells were growing on glucose or ethanol. Taken together, the promoter activities, with the exception of P_{HXT7} , decreased during shake flask cultivation. The overall ranking of the promoters is as described below:

When cells are in exponential phase:

$$P_{TEF1} \sim P_{PGK1} \sim P_{TDH3} > P_{TPII} \sim P_{PYK1} > P_{ADH1} > P_{HXT7}$$

When glucose is exhausted and ethanol is consumed:

$$P_{TEF1} \sim P_{HXT7} > P_{PGK1} > P_{TPII} \sim P_{TDH3} > P_{PYK1} \sim P_{ADH1}$$

Since the aim of this investigation was to construct a dual glucose based expression system to replace the *GAL1/GAL10* promoters in pESC-URA, I needed two promoters with a similar expression profile. As the results of the first comparison (Table 2.1), the *PGK1* and *TDH3* promoters represent options for a promoter that can be combined with P_{TEF1} . Although both of them start with the same activity as P_{TEF1} after 8 hours, their activities decline. After 24 hours, this loss of activity for the *TDH3* promoter

is higher than for the *PGK1* promoter. Previous investigations by Mellor et al. (Mellor et al., 1985) showed that when the *PGK1* gene was cloned into a multicopy plasmid and expressed in yeast, P_{gk1} accumulated to up to approximately 50% of total cell protein. Furthermore, different powerful expression vectors were constructed, based on the promoter region of the *PGK1* gene and these vectors have been used to study the expression of a number of heterologous genes (Tuite et al., 1982; Derynck et al., 1983; Masuda et al., 1994). I therefore chose the *TEF1* and *PGK1* promoters and, thereby constructed a nucleotide sequence containing a bidirectional *TEF1-PGK1* promoter.

2.1.2. Comparison of P_{TEF1} and P_{PGK1} in different contexts

Since the fusion may effect on the individual promoter strength, the activity of P_{TEF1} and P_{PGK1} in the newly bidirectional promoter was compared with the activity of individual P_{TEF1} and P_{PGK1}, respectively, in shake flasks using the same conditions as previously described, in which bidirectional *TEF1-PGK1* promotes were cloned in front of *lacZ* in different orientation and then each construct was integrated into the *ura3-52* locus. The results show that the activity of both the *PGK1* promoter and *TEF1* promoter after fusion to *TEF1* and *PGK1*, respectively, are not significantly different when compared with those of P_{PGK1} and P_{TEF1} alone (Figs. 7A and 7B).

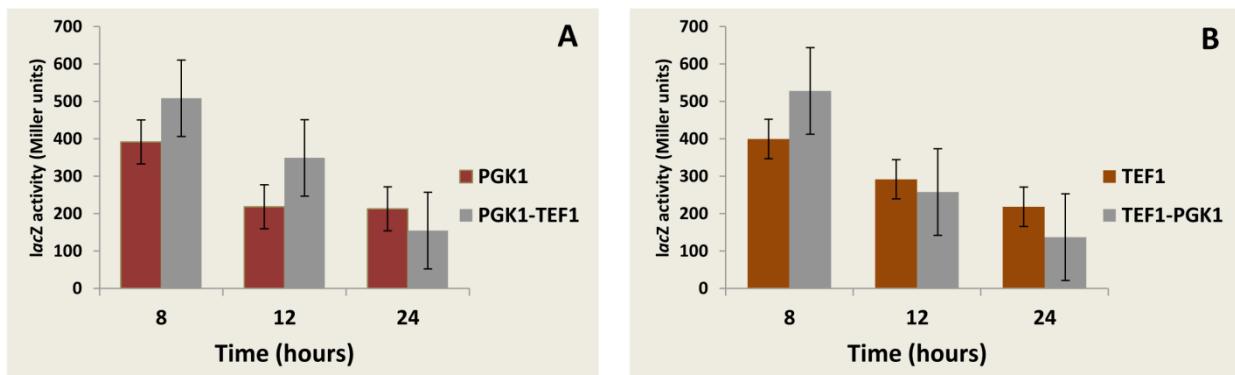


Figure 7: Activity of P_{PGK1} and P_{TEF1} in different contexts; **A**, red columns represents the activity of individual P_{PGK1}, gray columns represents the activity of P_{PGK1} fused to P_{TEF1}; **B**, brown columns represent the activity of individual P_{TEF1}, gray columns represents the activity of P_{TEF1} fused to P_{PGK1}. Error bars represent SEM (standard error of measurement).

Finally, the new divergent promoter, *TEF1-PGK1*, was employed as the basis for construction of 2 different expression vectors, pSP-G1 and pSP-G2 (Figures 8A and 8B), which are useful for evaluating and expressing 2 different genes at the same time. The two different promoter orientations in pSP-G1 and pSP-G2 allow for a greater variety of cloning strategies due to the different promoter – multi cloning site (MCS) combinations. Later, by adding extra cloning sites at the end of each terminator (*CYC1* and *ADH1* terminator) in these plasmids, two further vectors were constructed, pSP-

GM1 and pSP-GM2 (Figure 8C and 8D). These offer the opportunity to clone additional features, e.g. further expression cassettes.

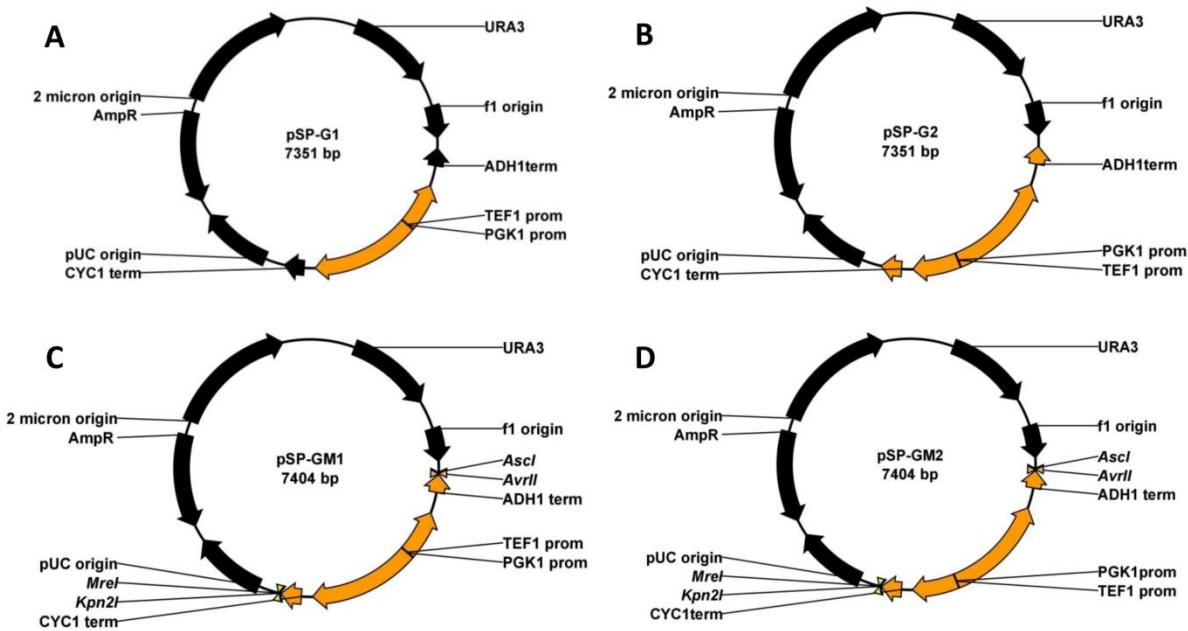


Figure 8: Schematic of pSP series. Details are presented in the text.

2.2. Improvement of gene dosage via modulating the plasmid copy number

Plasmid copy numbers of yeast episomal plasmids (YEps) usually are maintained by employing either the entire *S. cerevisiae* native 2 μ sequence or commonly, a 2 μ sequence including both the origin and the stability locus (STB), *REB3* (Futcher & Cox, 1983; Kikuchi, 1983). However, enhancing plasmid copy number, via modulating the selection marker gene, has been demonstrated by employing defective promoters as for the *LEU2-d* and *URA3-d* alleles leading to poorly express selection marker genes (Beggs, 1978; Erhartt & Hollenberg, 1983; Loision et al., 1989). It was shown that poor expression of the selection marker is a driving force to increase the plasmid copy number to ensure cell survival (Beggs, 1978)(Erhartt & Hollenberg, 1983; Loision et al., 1989). Another possible approach for improving the dosage of the gene on the recombinant plasmid is destabilization of the marker at the protein level using protein-based control elements. These elements act through protein degradation and, usually, alter the protein half-lives (Mateus & Avery, 2000; Hackett et al., 2006; Grilly et al., 2007). We hypothesize that the destabilization of marker protein may indicate the same effect as poorly expressed marker gene on the plasmid copy number.

I examined two strategies individually and in combination, in order to reduce the maker gene at both protein and RNA levels, and their impact on plasmid copy number of pSP-GM1 (Figure 8C). First, a ubiquitin/N-degron tag was fused to the N-terminus of Ura3 (selection marker of pSP-GM1). The *S.*

cerevisiae N-degron signal sequence can lead to impressive destabilization of reporters down to a half-life of 2 min (Hackett et al., 2006). Second, down-regulation of the marker gene *URA3* at the transcriptional level was altered by replacing the *URA3* native promoter with the constitutive weak promoter *KEX2* (Fuller et al., 1989), the conditional promoter of *HXT1* encoding a low affinity hexose transporter (Diderich et al., 1999) and the promoter of the *URA3-d* allele including only 47 nucleotides located upstream of the start codon (Faulkner et al., 1994; Loision et al., 1989), respectively. Further, I combined both strategies, i.e. weak promoter and ubiquitin/N-degron tag and evaluated plasmid copy number in these conditions. Figure 9 illustrates the plasmid constructs.

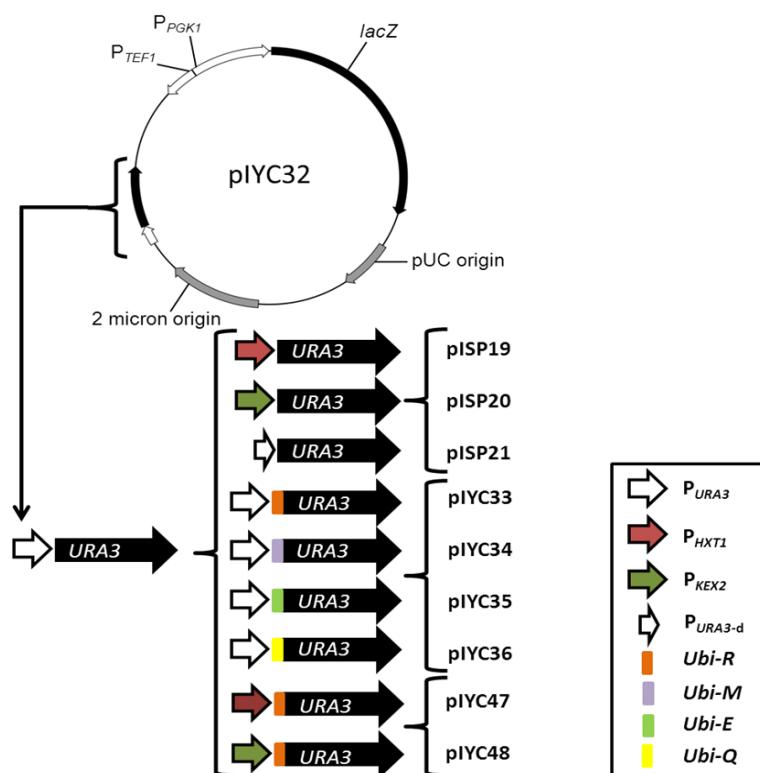


Figure 9: Schematic representation of the plasmid constructs. P_{URA3} , P_{HXT1} , P_{KEX2} , and P_{URA3-d} , promoters employed to control *URA3* expression; *Ubi-R*, *Ubi-M*, *Ubi-E* and *Ubi-Q*, ubiquitin/N-degron tags leading to arginine, methionine, glutamate and glutamine as N-terminal residues of the Ura3 marker protein.

2.2.1. Plasmid copy number determination via LacZ enzyme assay and quantitative PCR in continuous culture

Novel culture strategies have been developed, which allowed the physiological characterization of cells under regulated and defined conditions, aiming at reproducible processes and conclusive

experimental designs. The invention of chemostat cultivations is one of such tools, and its first application goes back to the 1950s (Novic & Szilard, 1950). The unique feature of chemostat cultivation is the ability to grow a cell population under well-defined substrate-limited growth conditions for an indefinite duration. Hence, applying the same growth condition for different engineered strain in continuous culture would raise the potential of this cultivation systems usage for many biotechnological investigations, e.g. plasmid copy number. An aerobic glucose-limited continuous cultivation of *S. cerevisiae* at a fixed dilution rate below the maximum specific growth rate was performed in our experiments. Apart from strain SCISP23 (Table 2.2) carrying the the P_{HXT1} -*URA3* plasmid indicating a lower final biomass formation, no apparent difference in growth characteristics and morphology was observed for the other strains. The observed decrease in biomass production may be the results of the high repression level of *URA3* exerted by the *HXT1* promoter under glucose limitation, not providing sufficient Ura3 protein to maintain higher growth rates or to elevate the dilution rate. This is consistent with the results observed in auxotrophic yeast strains during uracil-limited chemostat culture (Olitta et al., 2010).

Altering the plasmid copy number was verified after applying the above mentioned modifications on plasmid structure (see section 2.3) by two different methods, LacZ enzyme assay and quantitative PCR. The results of these indirect and direct measurements are demonstrated in Table 2.2.

Table 2.2: Comparison of fold changes in LacZ activity and plasmid copy number (PCN)

Strain (Plasmid)	Plasmid description	LacZ activity	PCN	Fold change
SCIYC58 (pIYC32)	P_{URA3} - <i>URA3</i>	1.00	1.00	
SCIYC59 (pIYC33)	P_{URA3} - <i>Ubi-R-URA3</i>	1.74	1.80	
SCIYC60 (pIYC34)	P_{URA3} - <i>Ubi-M-URA3</i>	1.18	1.40	
SCIYC61 (pIYC35)	P_{URA3} - <i>Ubi-E-URA3</i>	1.28	1.20	
SCIYC62 (pIYC36)	P_{URA3} - <i>Ubi-Q-URA3</i>	1.21	1.50	
SCISP23 (pISP19)	P_{HXT1} - <i>URA3</i>	1.91	2.00	
SCISP24 (pISP20)	P_{KEX2} - <i>URA3</i>	1.49	1.60	
SCISP25 (pISP21)	P_{URA3d} - <i>URA3</i>	1.31	1.80	
SCIYC68 (pIYC47)	P_{HXT1} - <i>Ubi-R-URA3</i>	2.61	3.50	
SCIYC69 (pIYC48)	P_{KEX2} - <i>Ubi-R-URA3</i>	3.07	3.00	

The results showed that both the LacZ activity and the plasmid copy number can be further increased by combining (i) destabilization of the marker protein, and (ii) replacing the promoter of the marker gene with a weak promoter. However, fold changes revealed by the Ubi-tagged (*Ubi-M*, *Ubi-E* and *Ubi-Q*) strains are not really significant and less than 50%. Both LacZ and PCN measurements are comparable and show high correlation with little exception such as SCIYC68 showing different fold

change between LacZ activity and plasmid copy number (PCN). Combination of the weak promoter and ubiquitin tag showed a synergistic effect on plasmid copy number and LacZ activity. This synergistic effect induced by the P_{KEX2} -*Ubi-R* was more stable in comparison to what was observed for the P_{HXT1} -*Ubi-R*. SCYC68 displayed higher increase in plasmid copy number than increase of LacZ activity (Table 3). This could, conceivably, relate to the feature of the *HXT1* promoter, in addition to the destabilizing residue arginine, resulting in very low expression of *URA3* and, thereby, more pressure on the cells.

2.2.2. Impact of plasmid copy number on patchoulol production

Patchouli is a type of sesquiterpene obtained by steam distillation of the leaves of *Pogostemon cablin* (patchouli), a plant from the *Lamiaceae* family. This terpenoid derivative is an important ingredient in many fine fragrance products like perfumes, as well as in soaps and cosmetic products. Microbial production of this fragrance compound is of great interest in the perfume industry, as an alternative to extraction from plants. Like other sesquiterpenes, patchoulol is derived from farnesyl diphosphate (FPP), which is an intermediate of the sterol pathway (**Figure 10A**). The patchoulol synthase gene has been isolated before (Munck & Croteau, 1990) and it has been shown that to convert FPP into patchoulol, only a single enzymatic step is sufficient (Asadollahi et al., 2008). To demonstrate the practical application of this new plasmid, the plasmid carrying the P_{KEX2} -*Ubi-R*-*URA3* construct was tested for production of the isoprenoid patchoulol. To ensure that enough FPP precursors are available, a truncated form of HMG-CoA reductase 1 (encoded by *tHMG1*) was also over-expressed from this plasmid. *tHMG1* overexpression has previously been reported to lead to enhanced isoprenoid production in yeast (Ro et al., 2006; Asadollahi et al., 2010). Both *tHMG1* and the patchoulol synthase gene (*PatTps177*) were expressed from the P_{KEX2} -*Ubi-R*-*URA3* plasmid(strain SCIYC76) as well as from the control plasmid (strain SCIYC72) (figure 10B). Patchoulol production and biomass formation were analysed in shake flasks (Figure 10C). While the final biomass concentration was unchanged, patchoulol production reached about 30 mg/L in SCIYC76, more than 3 times compared with control strain SCIYC72. This performance thus demonstrates that the new plasmid could also be beneficial to improve heterologous pathway expression.

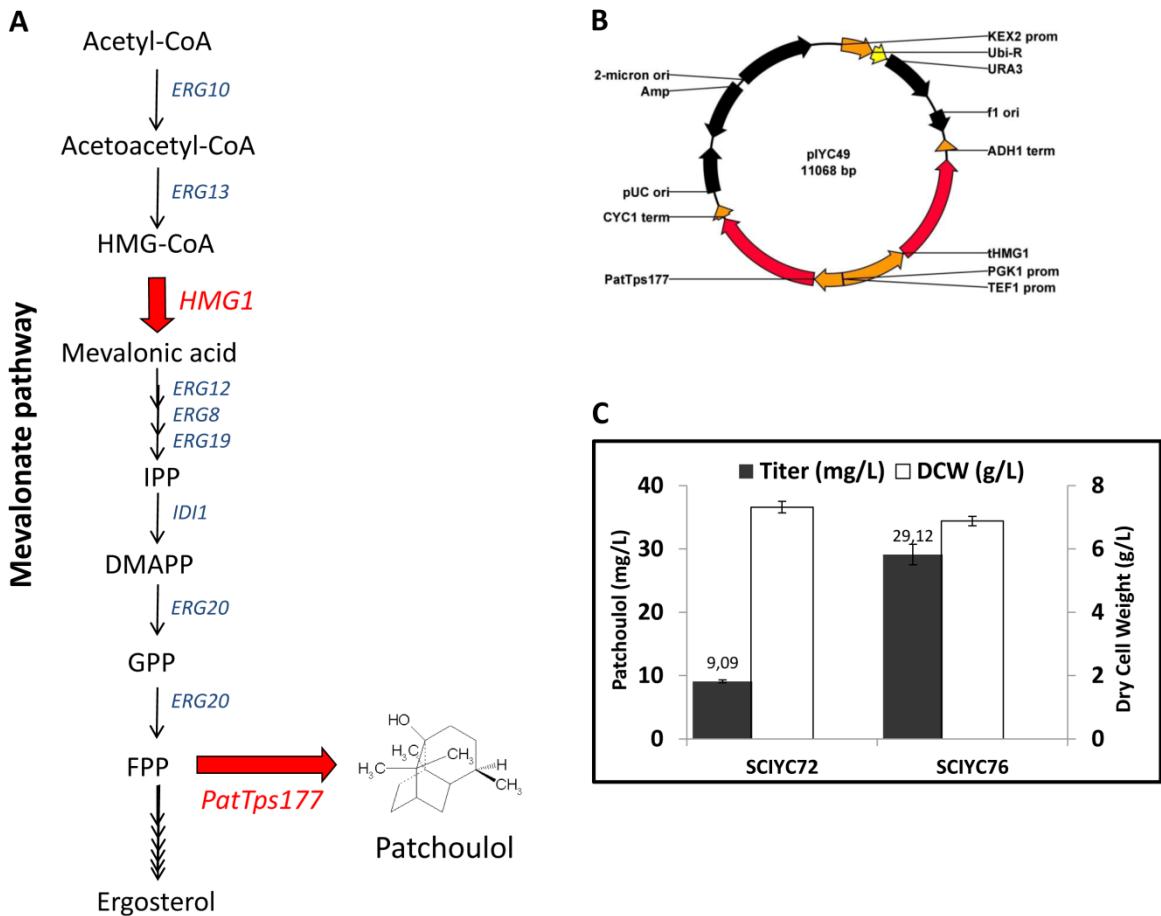


Figure 10: Growth and patchoulol accumulation of strain SCIYC76 and control strain SCIYC72. **A**, mevalonate pathway, red arrows correspond to the over-expressed genes; **B**, map of modified expression plasmid, pIYC49 (P_{KEX2} -*Ubi-R*-*URA3*) which harbors a copy of *tHMG1* and *PatTps177* genes downstream of the *TEF1* and *PGK1* promoter, respectively; **C**, Patchoulol production was evaluated in shake flasks using 2% glucose minimal medium. SCIYC76 contains plasmid pIYC49 (P_{KEX2} -*Ubi-R*-*URA3*) and control strain SCIYC72 contains plasmid pIYC03 (P_{URA3} -*URA3*). The data shown represent the mean +/- SD of three independent cultivations.

2.3. Implementation of synthetic biology tools in metabolic engineering

The goal of metabolic engineering is to optimize and modulate processes within cells by directed modifications of metabolic fluxes employing synthetic biology tools. Many of the described synthetic biology tools and techniques in Chapter 1 have already been applied to engineer yeast strain for production of valuable secondary metabolites, e.g. isoprenoids, in novel and efficient bioprocess that are environmentally friendly. Briefly, altering one or many of the following levels can be subjected in order to over-produce secondary metabolites through interruptions to cellular metabolism: (i) enhancement in the rate of substrate uptake, (ii) reduction of flux to undesirable by-products and enhancement of precursor and cofactor flux, (iii) introduction of a heterologous pathway and optimization of the activity of its constituent enzymes, and (iv) export of the product to the extracellular medium in order to shift the equilibrium towards product formation. In this principle, first

the application of various synthetic biology tools and techniques for metabolic engineering of the mevalonate pathway aiming at producing a sesquiterpenoid, α -santalene, are discussed and, furthermore, the last results of the re-construction of the bacterial MEP pathway and sophisticated challenges in cytosolic Fe/S cluster trafficking to the last two enzymes of this heterologous pathway in *S. cerevisiae* will be considered.

2.4. Metabolic engineering of the MVA pathway

The MVA pathway in yeast endogenously synthesizes different natural isoprenoids compounds which are responsible to control key functions in the cell, e.g. membrane fluidity (ergosterol) and mating response (α -factor). Therefore, yeast is naturally and potentially able to supply many precursors and intermediates which are needed for producing various heterologous isoprenoids, e.g. sesquiterpenoids (Ro et al., 2006; Asadollahi et al., 2008; Asadollahi et al., 2009; Asadollahi et al., 2010). Sesquiterpenoids are a type of isoprenoids produced by the cyclization and further modification of a single farnesyl diphosphate (FPP) intermediate, a branch point of the MVA pathway. α -Santalene, which is the precursor of α -santalol, one of the main components of East Indian sandalwood oil (Baldovini & Joulain, 2011), is a type of sesqiterpene with application in perfumery and aromatherapy industries. Like patchoulol, α -santalene is produced enzymatically in a one-step-conversion from farnesol diphosphate catalyzed by a plant santalene synthase (Schalk, 2011). Here, Re-optimization of the MVA pathway is investigated using synthetic biology tools aiming at enhancing the FPP pool for production of α -santalene. First, different and novel approaches are applied for modulating the FPP branch point. Second, the possible nodes which have direct influence on the MVA pathway flux or necessary co-factor are manipulated and finally, an efficient *S. cerevisiae* strain capable of reaching relevant titers and productivities of α -santalene during an optimized fermentation process is constructed combining all modifications.

2.4.1. Modulating the FPP branch point

Farnesyl diphosphate (FPP) is the universal precursor unit of all sesquiterpenes (C_{15}) (Maury et al., 2005)(Withers & Keasling, 2007). This intermediate is formed by multiple condensations of isopentenyl-diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) and, naturally, it serves as a precursor for production of essential compounds such as dolichol, ubiquinone, isoprenylated proteins and ergosterol (Daum et al., 1998) (**Figure 11**). Therefore, the FPP intracellular concentration is tightly regulated at different levels (Goldstein & Brown, 1990). During normal growth conditions most of the FPP is used for sterol biosynthesis, due to the fact that the cellular demand for sterols is greater compared to the demand for non-sterol FPP derived compounds (Kennedy et al., 1999).

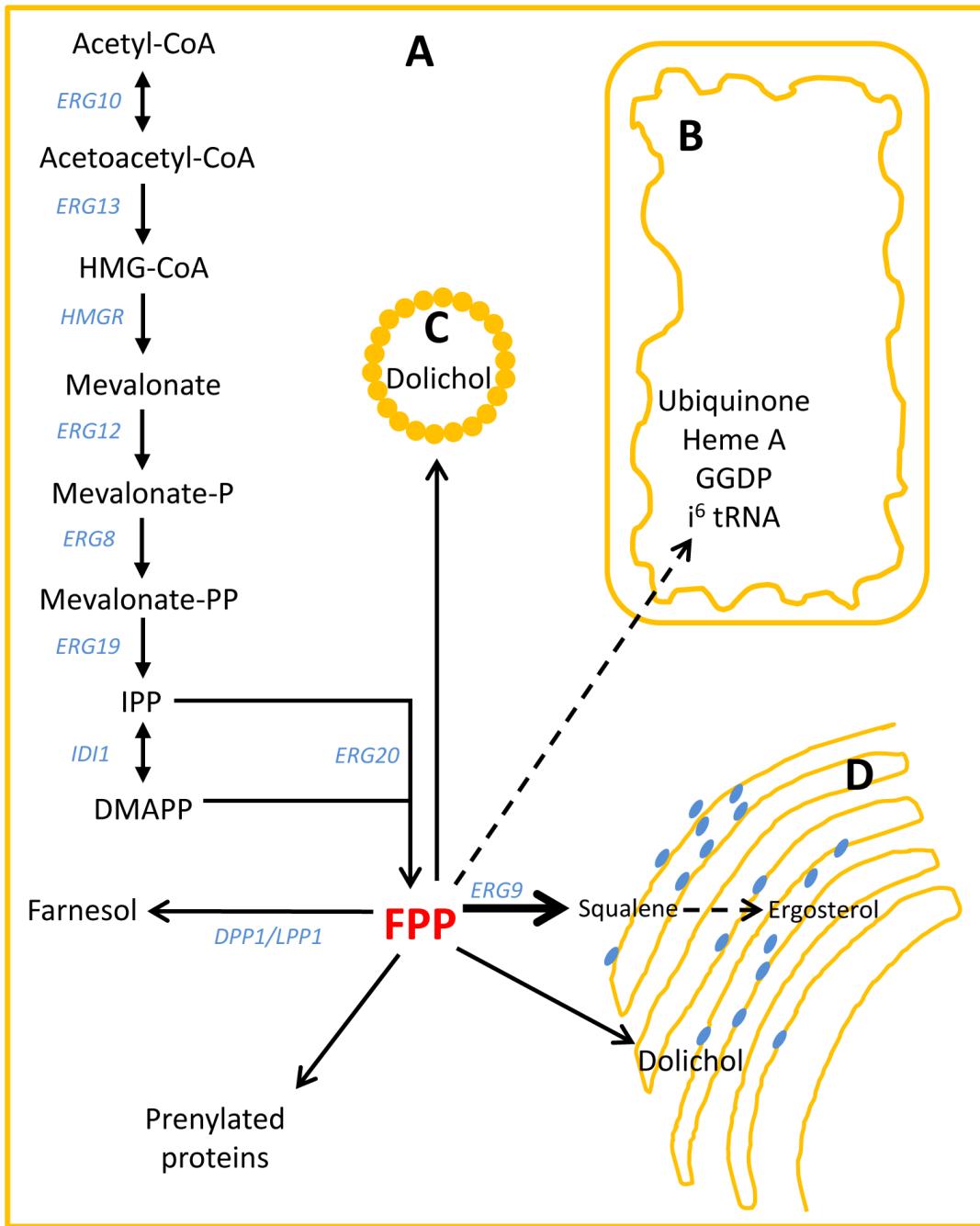


Figure 11: Schematic representation of the farnesyl pyrophosphate (FPP) branch-point in *S. cerevisiae*; **A**, Cytosol; **B**, Mitochondria; **C**, Lipid particle; **D**, Endoplasmic reticulum.

Squalene synthase (Erg9) is the first enzyme of the mevalonate pathway dedicated to sterol biosynthesis. Since disruption of the *ERG9* gene is lethal and produces an ergosterol-dependent mutant (Jennings et al., 1991), several efforts have applied various methods down-regulating this essential genes, in order to increase the FPP pool (Ro et al., 2006; Paradise et al., 2008; Asadollahi et al., 2008). Generally, researchers have replaced the native *ERG9* promoter with the regulatable promoter *MET3*, which is repressed in the presence of methionine (Cherest et al., 1985). However, applying the

regulatable-promoter *MET3* is limited by several parameters such as the cost of methionine for repression and difficulty with controlling the system since the repressing agent, methionine is metabolized by the cells. So, we first hypothesized that the repression effect on the *MET3* promoter may reduce over time. To verify this hypothesis, an integration cassette containing *lacZ* as a reporter downstream of the *MET3* promoter was constructed (**Figure 12A**). The LacZ activity was measured at different time points after addition of 0 mM, 1 mM and 2 mM L-methionine, respectively. The results showed that LacZ activity was increased, about mid-exponential, after methionine addition and rapidly reached the levels measured in the non-repressed culture (**Figure 12B**). These results, thus, demonstrate and confirm the difficulties in controlling promoter activity when cells metabolize the repressing agent.

In the following, down-regulation of *ERG9* was evaluated using two synthetic biology tools, promoter choice and antisense mRNA. The chosen regulatory systems were (i) the low-level constitutive *TEF1* promoter mutant *TEF1M2* selected after an evolutionary engineering approach based on error-prone PCR (Alper et al., 2005; Nevoigt et al., 2007), (ii) the glucose concentration controlled promoter of the hexose transporter gene *HXT1* (Ozcan & Johnston, 1995; Lewis & Bisson, 1991), and (iii) the *HXT2* promoter potentially useful for a gene silencing approach expressing *ERG9* antisense mRNA (Ozcan et al., 1995). These promoters were cloned and integrated in front of *lacZ* and integrated into the yeast chromosome, respectively (**Figure 12A**), and their effect was compared during high and low glucose concentration using *lacZ*, as a reporter (**Figures 12C and 12D**). Further, the impact of different down-regulating methods on ergosterol and α -santalene production was evaluated in a fed-batch process (**Figures 12E and 12F**). Taken together, the results indicate that (i) P_{HXT1} appeared to be a suitable promoter for down-regulating *ERG9* expression under glucose limiting conditions; (ii) The proportion of ergosterol decrease ranged from 50 to 91% using the above mentioned methods (**Figure 12E**) as compared to the native *ERG9* promoter, and (iii) a linear correlation was observed between the reduction in ergosterol content and the increase in α -santalene production. This is a good example of using synthetic biology in metabolic engineering showing the redirection of FPP flux through sesquiterpen production by replacing the native *ERG9* promoter with conditional promoter *HXT1*. Besides, additional modifications for modulating the FPP branch point have been investigated, e.g. deletion of two phosphatases *LPP1* and *DPP1*, which are responsible for most of the cytosolic isoprenoid and lipid phosphate phosphatase activity in *S. cerevisiae* (Toke et al., 1998; Faulkner et al., 1999). However, no significant differences were observed in both α -santalene production and ergosterol content applying single deletion (*lpp1Δ*) or double deletion (*lpp1Δ dpp1Δ*) strains.

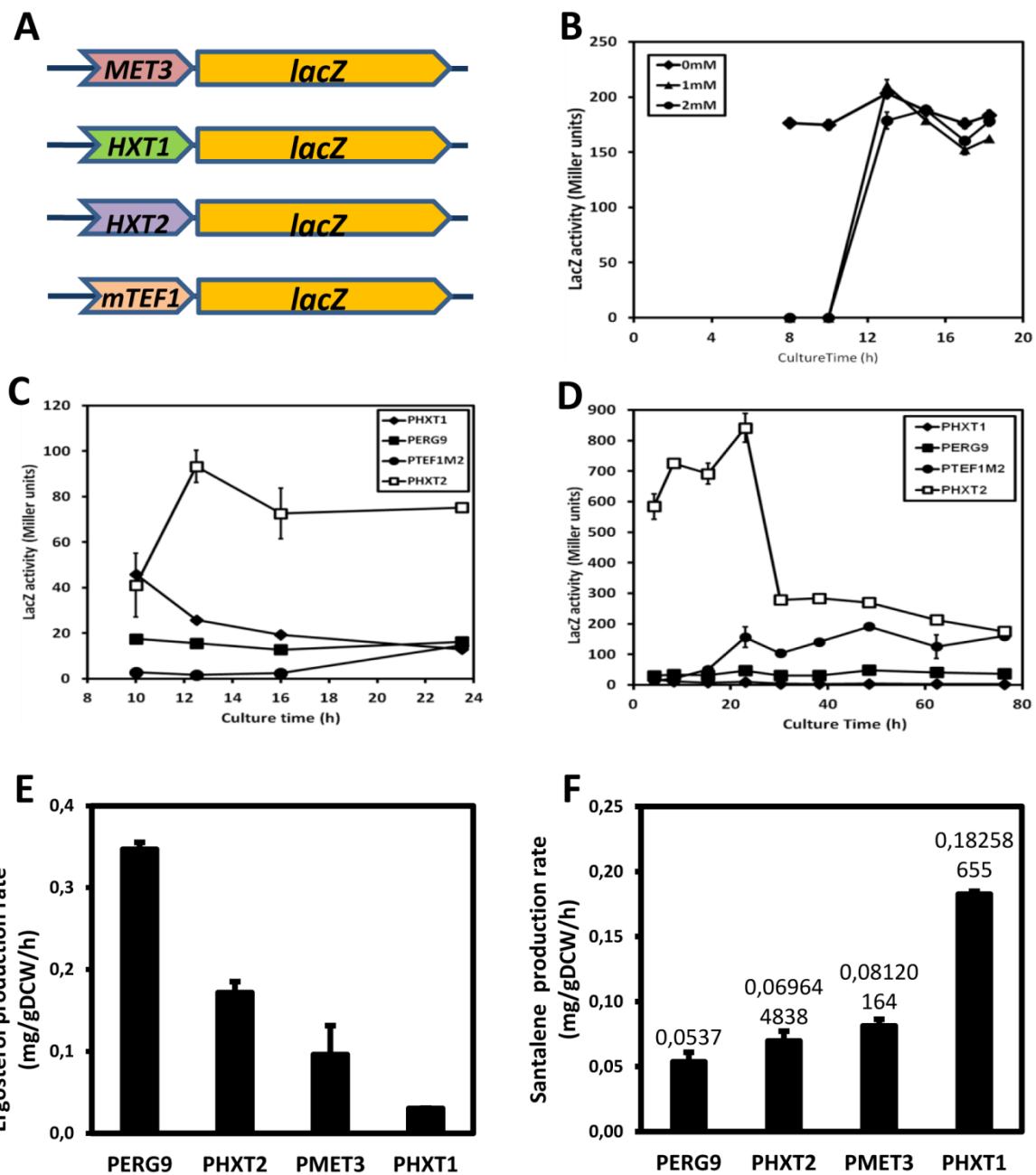


Figure 12: Result of the regulation of FPP branch point. (A) Integrative cassettes include promoter choice (*MET3*, *HXT1*, *HXT2* and mutant *TEF1*) in front of *lacZ* as a reporter gene; (B) LacZ activity under control of P_{MET3} in response to different methionine concentrations, 0 mM (diamonds), 1 mM (triangles) and 2 mM (circles). Strains were cultivated in duplicates, glucose exponential growth phase was between 2 and 16 h of cultivation; (C and D) Characterization of promoter strength, P_{HXT1} (filled diamonds), P_{TEF1M2} (filled circles), P_{HXT2} (empty squares), and P_{ERG9} (filled squares), during shake flask cultivation in glucose exponential growth phase was between 2 and 16 h of cultivation and in fed-batch mode, respectively. (E) Ergosterol production rate ($\text{mg} \cdot \text{g} \text{ biomass}^{-1} \cdot \text{h}^{-1}$); (F) α -santalene and *E,E*-farnesol production rate ($\text{mg} \cdot \text{g} \text{ biomass}^{-1} \cdot \text{h}^{-1}$). Strains were grown in a two-phase partitioned fed-batch glucose limited cultivation mode. The error bars represent the standard deviation for two independent cultivations.

2.4.2. Modulating the possible nodes directly involved or related to the MVA pathway

Manipulating a single gene pathway usually has little effect on metabolite fluxes as individual enzymes generally have only partial flux control in a pathway. Therefore, further improvement of sesquiterpene production was investigated by manipulating several direct and indirect rate-limiting steps of the FPP biosynthesis pathway (early portion of MVA pathway) which is illustrated in **Figure 13A**.

The MVA pathway initiates by condensation of 3 acetyl-CoA molecules to build one molecule of mevalonate which is through sequential phosphorylation and decarboxylation enzymatic steps, forms IPP and DMAPP. The final step in the early portion of the MVA pathway is the conversion of IPP and DMAPP into geranyl and farnesyl diphosphates (GPP and FPP, respectively). These steps are catalyzed by the product of *ERG20* (Maury et al., 2005). The enzyme first combines one molecule of each DMAPP and IPP to make GPP and then by adding one molecule of IPP to GPP produces FPP. Several enzymatic steps and co-factor requirements make the regulation of the mevalonate pathway complex. A reductase (HMG-R), encoded by *HMG1* is a highly regulated enzyme and it is considered to represent the major rate limiting enzyme in the MVA pathway (Basson et al., 1987; Donald et al., 1997; Polakowski & Stahl, 1998).

Several studies have demonstrated an enhancement in isoprenoid production by over-expression of the catalytic domain of the Hmg1 protein encoded by *tHMG1* (Ro et al., 2006; Kirby et al., 2008; Engels et al., 2008; Asadollahi et al., 2010). Over-expression of *ERG20* exposed a slight effect on sesquiterpene production (Ro et al., 2006). Further manipulation has been performed by over-expression of a semi-dominant mutant allele of a global transcription factor regulating sterol biosynthesis in yeast, *upc2-1*, that enhances the activity of Upc2 (Davies et al., 2005) and the impact of this over-expression on isoprenoid production has been demonstrated (Ro et al., 2006; Engels et al., 2008). Enzymes of the MVA pathway are NADPH-dependent e.g. Hmg1 (Maury et al., 2005). Hence, the last modification was implemented in order to increase the pool of NADPH available for Hmg1. Previously, it has been reported that there was an improvement in sesquiterpene production by manipulating the ammonium metabolism in yeast (Asadollahi et al., 2009). Deletion of the *GDH1* encoding NADP-dependent enzyme which consumes a substantial amount of NADPH in the cell (dos Santos et al., 2003) and over-expression of the NADH-dependent enzyme, *GDH2*, has led to an, approximately, 85% increase in the final cubebol titer (Asadollahi et al., 2009).

The impact of the above mentioned modifications on isoprenoid production have previously been investigated individually. Here, different combinations of all these modifications were investigated using chromosomal integration to ensure the genetic stability of the host strain. Unique cloning sites after *ADH1* and *CYC1* terminators of pSP-GM1 allowed simple and efficient construction of the two integration cassettes (**Figure 13B**). The synthetic cassettes were further introduced into the yeast

chromosome through homologous recombination. Modified strains were engineered into a sesquitrepene producing microorganism introducing the expression plasmid pISP15 containing a copy of *tHMG1* and codon optimized *SanSyn* under control of the *PGK1* and *TEF1* promoter, respectively. Name and descriptions of the strains are listed in Table 2.3. In the following, continuous cultures were employed to evaluate the impact of different genetic modifications on α -santalene production. Results are shown in **Figure 14**.

Table 2.3: List of *S. cerevisiae* strains used in this study

Strain	Genotype	Plasmid
SCIGS28	<i>MATa MAL2-8^c SUC2</i>	pISP15
SCIGS29	<i>MATa MAL2-8^c SUC2 lpp1Δ::loxP P_{ERG9}Δ::loxP-P_{HXT1}</i>	pISP15
SCIGS30	<i>MATa MAL2-8^c SUC2 lpp1Δ::loxP dpp1Δ::loxP P_{ERG9}Δ::loxP-P_{HXT1}</i>	pISP15
SCIGS31	<i>MATa MAL2-8^c SUC2 lpp1Δ::loxP dpp1Δ::loxP P_{ERG9}Δ::loxP-P_{HXT1} gdh1Δ::loxP</i>	pISP15
SCIGS24	<i>MATa MAL2-8^c SUC2 lpp1Δ::loxP dpp1Δ::loxP P_{ERG9}Δ::loxP-P_{HXT1} gdh1Δ::loxP P_{TEF1}-ERG20 P_{PGK1}-GDH2</i>	pISP15
SCIGS25	<i>MATa MAL2-8^c SUC2 lpp1Δ::loxP dpp1Δ::loxP P_{ERG9}Δ::loxP-P_{HXT1} gdh1Δ::loxP P_{TEF1}-ERG20 P_{PGK1}-GDH2 P_{TEF1}-tHMG1 P_{PGK1}-upc2-1</i>	pISP15

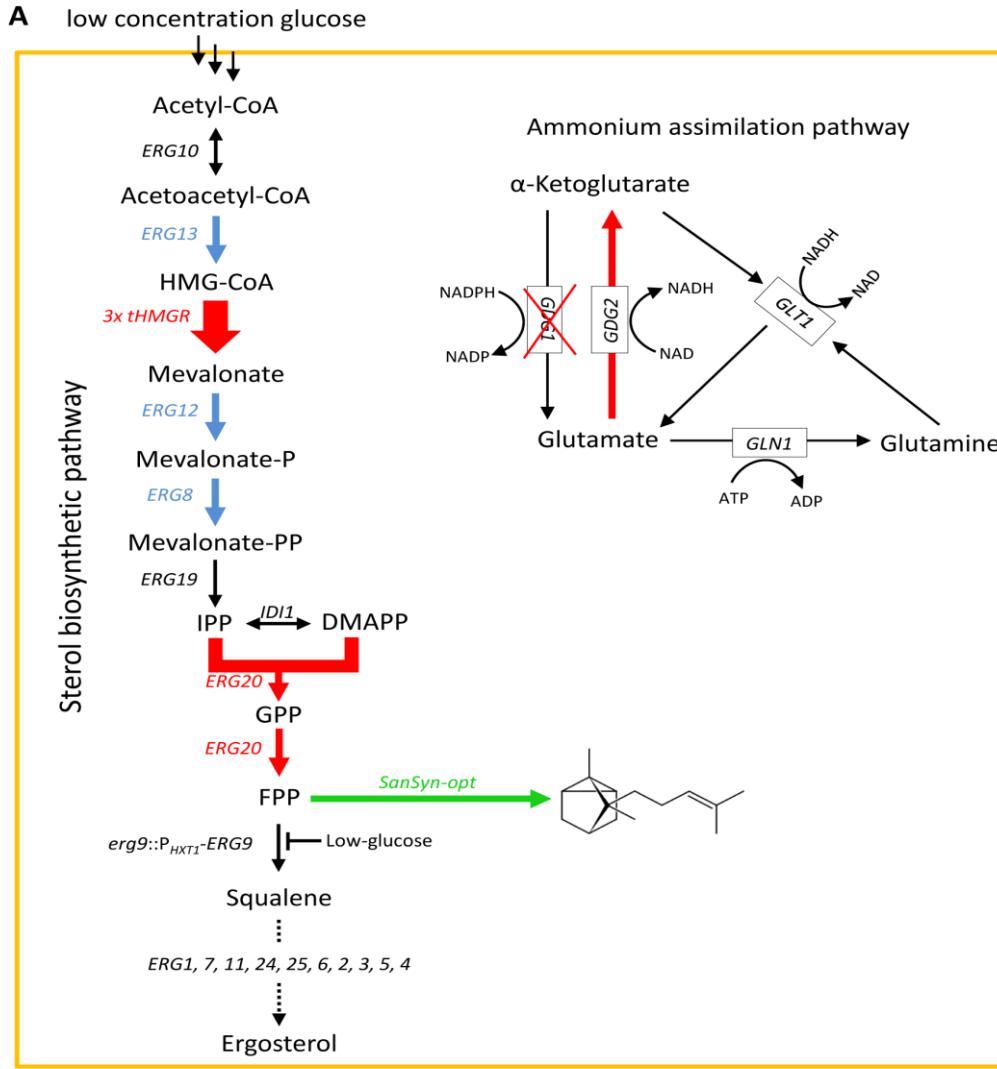


Figure 13: Schematic representation of the engineered α -santalene biosynthetic pathway through the modification of the mevalonate pathway and the ammonium metabolism pathway in *S. cerevisiae*. **A,** The directly up-regulated genes are shown in red and purple; those that are indirectly up-regulated by *upc2-1* expression are in blue; the pathway intermediates IPP, DMAPP and GPP are defined as isopentenyl pyrophosphate, dimethyl allyl pyrophosphate and geranyl pyrophosphate, respectively. Green arrow indicates the enzymatic step leading from farnesyl pyrophosphate (FPP) to α -santalene. **B,** Maps of the integrative constructs used for transferring all modifications into the yeast chromosome and plasmid expression cassette carrying additional copy of *tHMG1* and *SanSyn*.

We showed that the combination of *ERG9* down-regulation and double *lpp1/dpp1* deletion increased α -santalene production by more than 3 times as compared to the wild type strain (SCIGS28) (**Figure 12E**). Further modifications were implemented on SCIGS30 as background strain (Table 2.3). Introducing *GDH1* deletion into SCIGS30 not only showed a decrease in α -santalene production (**Figure 14**), but also strongly affected the growth rate which decreased to 0.18 h^{-1} . The latter effect is consistent with what has been previously reported by Asadollahi and co-workers (Asadollahi et al., 2009); however, they have shown improvement in sesquiterpene production using a *gdh1* mutant. Both growth rate and α -santalene production were enhanced dramatically (strain SCIGS24) while a combination of *ERG20/GDH2* overexpression was introduced into SCIGS31. This can be explained by the fact that deletion of *GDH1* has high impact on the efficiency of ammonium assimilation under these conditions (dos Santos et al., 2003) and this undesirable effect is considerably avoided over-expressing *GDH2* (Asadollahi et al., 2009). Thus, enhancement of α -santalene production could result from growth restoration by over expression of *GDH2* and over-expression of the gene encoding FPP synthase (*ERG20*). However, the later has shown little effect on total sesquiterpene, amorphadiene, production (Ro et al., 2006). To combine all modifications, an additional copy of *tHMG1* and a copy of *upc2-1* were integrated into the chromosome of SCIGS25 resulting in SCIGS25, although combining all these modifications did not show further improvement in α -santalene production in comparison to SCIGS24 (**Figure 14**). This result is consistent with previous reports which have shown that, at high mevalonate concentrations ($>2.5 \text{ mM}$) the reaction rate of *S. cerevisiae* mevalonate kinase (*ERG12*) begins to decrease (Ma et al., 2011). It has also been demonstrated that there is substrate inhibition of the mevalonate kinase of *S. aureus* at high concentrations of mevalonate (Voynova et al., 2004).

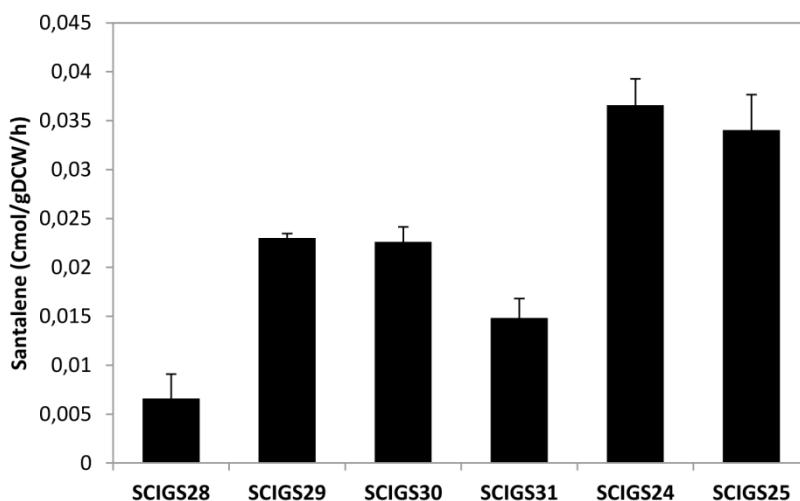


Figure 14: α -(+)-santalene production rate Cmmol (g Biomass) $^{-1}$ h^{-1} in *S. cerevisiae* in a two phase partitioned glucose limited aerobic chemostat. Strains SCIGS28, SCIGS29 ($P_{HXT1}\text{-}ERG9$; $\Delta dpp1$), SCIGS30 ($+\Delta lpp1$), SCIGS31 ($+\Delta gdh1$), SCIGS24 ($+ERG20$; $GDH2$), SCIGS25 ($+upc2-1$, $tHMG1$) cultivated at dilution rate D=0.05 h^{-1} .

2.5. Re-construction of the bacterial MEP pathway in *S. cerevisiae*

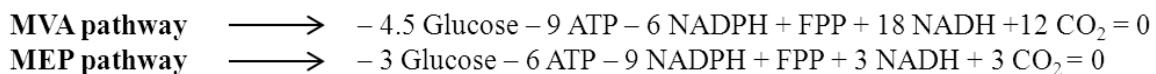
Due to the importance of isoprenoid compounds involved, not only in several metabolic functions, e.g. photosynthesis (chlorophylls), respiration (ubiquinone), hormonal regulation of metabolism (steroids), intracellular interaction (RAS proteins), but also their many industrial applications such as food colorants (carotenoids), pharmaceuticals (artemisinin, taxol, and bisabolol), flavors and fragrances (limonene), synthesis of isoprenoid compounds through the natural metabolic pathways, MVA or MEP pathway, has extensively been considered (Maury et al., 2005; Chang & Keasling, 2006). As aforementioned, by employing synthetic biology tools, such as strong bidirectional promoters and high copy number plasmids, we re-optimized the endogenous MVA pathway in *S. cerevisiae* in order to over-produce α -(+)-santalene. Instead of all these efforts, the bacterial MEP pathway could be a potential target for isoprenoid production, which has not been investigated extensively, in particular in heterologous hosts like *S. cerevisiae*. These findings encouraged me to further evaluate the heterologous MEP pathway in *S. cerevisiae* aiming at generating an efficient yeast cell factory with both the MVA and MEP pathway for producing isoprenoid precursors.

2.5.1. *In silico* analysis of the bacterial MEP pathway

In order to better understand the behavior of the MEP pathway in yeast, the seven enzymatic reactions of the bacterial MEP pathway were evaluated *in silico*, using the yeast genome scale metabolic model, iIN800 (Nookaew et al., 2008). The efficiency of the pathway was compared with the endogenous MVA pathway. The model was optimized for maximum production of farnesyl pyrophosphate (FPP), which is a branch point intermediate in ergosterol biosynthesis, for two different conditions, using the endogenous MVA pathway and using the heterologous MEP pathway, respectively. The result showed that by consuming 1 mol of glucose 0.21 and 0.24 mol farnesyl pyrophosphate could be produced through the MVA and MEP pathway, respectively. According to this analysis, the FPP production through the MEP pathway results in a favourable theoretical yield.

The stoichiometry calculation of glucose, NAD(P)H and ATP consumption to produce one molecule of FPP for both pathways shown in Equation 1.

Equation 1



The stoichiometry showed that, for producing one molecule of farnesyl pyrophosphate from glucose via the MVA pathway six molecules of NADPH and nine molecules of ATP are required, while production via the MEP pathway consumes nine molecules of NAD(P)H and six molecules of ATP.

Provision of sufficient cytosolic NADPH is, therefore, a critical factor for both pathways. In contrast to the MEP pathway, which consumes only 3 molecules of glucose, the MVA pathway consumes 4.5 molecules of glucose for the biosynthesis of one molecule farnesyl pyrophosphate (these values are excluding use of glucose for production of ATP and redox co-factors).

Combining the results derived from the yeast genome scale metabolic model and the stoichiometry calculations, it became evident that the MEP pathway is a more efficient route than the endogenous MVA pathway for isoprenoid production in terms of energy consumption and productivity. This result is consistent with previous reports about the efficiency of the MEP pathway, as compared to the MVA pathway (Ajikumar et al., 2010; Dugar & Stephanopoulos, 2011). This is the rationale for my attempt to express the bacterial MEP pathway in yeast for production of isoprenoids.

2.5.2. Genomic integration of MEP pathway genes

For further evaluation, the four DNA constructs containing the MEP pathway genes, expression elements and selection markers were well designed *in silico*, synthesized *in vitro* (chemically) and integrated into the yeast chromosome applied by a bipartite integration strategy (Erdeniz et al., 1997), respectively. The strains generated in this study are listed in Table 2.4.

Table 2.4: List of strains and plasmids used in evaluation MEP pathway study

Strain	Genotype	Plasmid	Reference
CEN.PK 113-13D	MAT α MAL2-8cSUC2 ura3-52	none	P. Kötter
SCISP06	MAT α MAL2-8cSUC2 ura3-52 dxs dxr ispD ispE ispF ispG ispH idi	none	this work
SCISP16	MAT α MAL2-8cSUC2 dxs dxr ispD ispE ispF ispG ispH idi	pISP08	this work
SCISP28	MAT α MAL2-8cSUC2	pSP-GM1	this work
SCISP29	MAT α MAL2-8cSUC2 dxs dxr ispD ispE ispF ispG ispH idi	pSP-GM1	this work
CEN.PK 113-1C	MAT α MAL2-8c SUC2 trp1-289 ura3-52 his3Δ 1	none	P. Kötter
SCISP12	MAT α MAL2-8c SUC2 trp1-289 ura3-52 his3Δ 1 dxs dxr ispD ispE ispF ispG ispH idi	none	this work
SCISP13	MAT α MAL2-8c SUC2 trp1-289 his3Δ 1 dxs dxr ispD ispE ispF ispG ispH idi	pISP08	this work
SCISP30	MAT α MAL2-8c SUC2 trp1-289	pSP-GM1, pSP-GM3	this work
SCISP31	MAT α MAL2-8c SUC2 trp1-289 dxs dxr ispD ispE ispF ispG ispH idi	pISP08, pISP24	this work
SCISP32	MAT α MAL2-8c SUC2 trp1-289 dxs dxr ispD ispE ispF ispG ispH idi	pISP08, pISP25	this work

The *in vitro* DNA synthesis offers fast, cheap and efficient method for synthesis of large DNA sequences (Kosuri et al., 2010; Matzas et al., 2010). Besides, using synthetic genes with the possibility to manipulate codon bias can take much better control of the expression of heterologous MEP pathway in yeast. From the genetic engineering point of view, the codon bias is one of the first barriers in heterologous protein expression (Gustafsson et al., 2004) and it can prevent the efficient biosynthesis of a recombinant protein, because of altering the correlation between the frequency of the codon and the abundance of its corresponding tRNA, which impairs the translation machinery of the host (Ikemura, 1981). The high efficiency and ease to work with *in vivo* homologous recombination in *S. cerevisiae* allows stable manipulation without requirement of selective pressure for maintenance. In addition, previously, different transcription levels among various chromosomal regions in *S. cerevisiae* have been reported by using *lacZ* as a reporter gene (Flagfeldt et al., 2009). We have shown that the two integration sites, YPRC Δ 15 and YPRC τ 3, on chromosome XVI of *S. cerevisiae* provided potentially higher expression levels than other regions tested (Flagfeldt et al., 2009). Therefore, all genes involved in the bacterial MEP pathway were integrated into these two sites in two steps. **Figure 15** illustrates synthetic constructs and integration methods in greater detail. As can be seen in this figure, the constitutive bidirectional promoter *TEF1-PGK1* was used to support strong transcription level, as I showed high constitutive activity of this promoter in glucose containing media before. In addition, direct repeat DNA sequences of 143 bp introduced at both sides of *K.l.URA3*, and *loxP* sites flanking the *kanMX* cassette allowed recycling of the selectable markers.

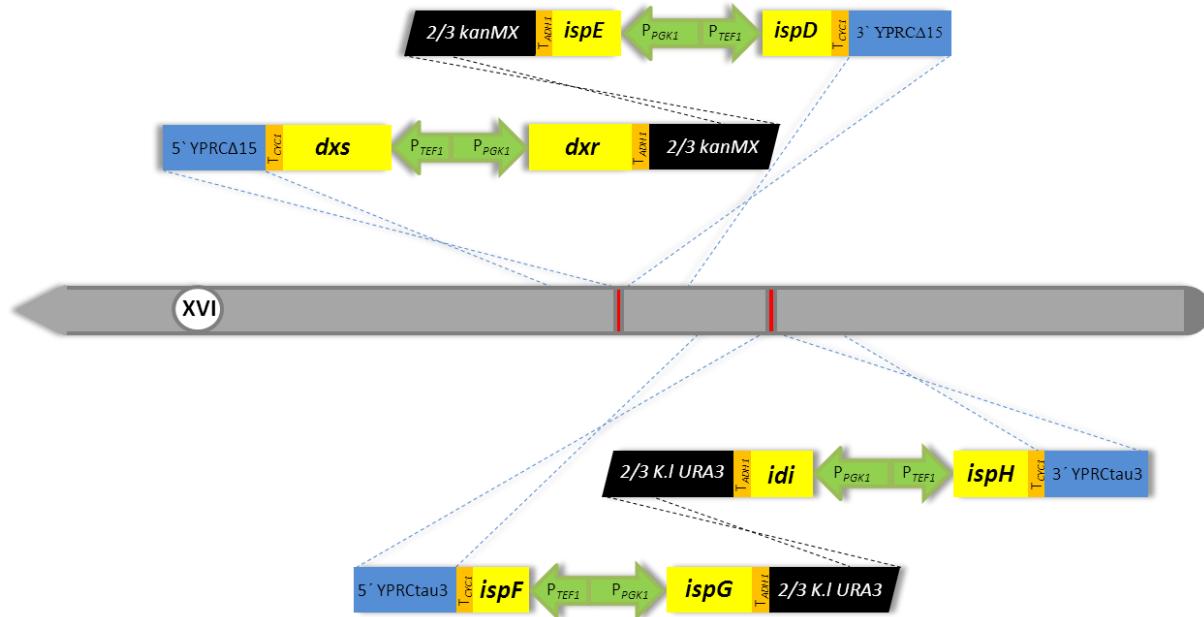


Figure 15: Schematic representation of genetic engineering strategies for genomic integration of the bacterial MEP pathway genes into the yeast genome (chromosome XVI).

In spite of successful integration and transcription, which were confirmed by PCR and RT-PCR, respectively, the bacterial MEP pathway could not complement the lack of endogenous MVA pathway while being repressed in presence of lovastatin, which is a therapeutic agent and is a competitive inhibitor of an early pathway enzyme, HMG-CoA reductase (Alberts et al., 1980) (**Figure 16**). This is in contrast to previously reported findings (Maury et al., 2008). Maury and co-workers reconstructed the bacterial MEP pathway in *S. cerevisiae* by expression of seven enzymatic steps of the pathway from self-replicating, high-copy yeast plasmids. They have reported the ability of the bacterial MEP pathway in producing ergosterol, which is essential compound in *S. cerevisiae*, while the endogenous MVA pathway was inhibited through addition of lovastatin (Maury et al., 2008). We conclude that their result may have derived from incomplete repression of the MVA pathway, even when higher concentrations (2 g L⁻¹) of lovastatin were used, which may result from errors in activation of lovastatin by hydrolysis reducing the actual concentration of the active inhibitor, or the higher-level expression from multi-copy plasmids may have resulted in partial activation of the enzymes resulting in a functional MEP pathway. However, later genetic inhibition of MVA pathway revealed the non-functionality of the MEP pathway to the same level as the chemically inhibited. A brief overview of the enzymatic steps shows that, in general, the MEP pathway requires divalent metal cations such as Mn²⁺, Mg²⁺ or Co²⁺, ATP for providing energy and a reducing agent, such as NADPH (Maury et al., 2005). Providing such requirements should not be limiting for a legitimate activity of the pathway. Metabolite analysis was therefore performed to identify possible bottleneck(s) within the MEP pathway. The detection of intermediates 3 and 5 (**Figure 6**) in the MEP-pathway carrying yeast strains indicated the proper activity of the Dxs, Dxr and the IspD enzymes (data not shown). In addition, non-activity was observed for the last enzyme of the MEP pathway, IspH, while expressed in yeast (Formenti, 2011). We hypothesize that a potential reason for the non-functionality of the MEP pathway in *S. cerevisiae* is the lack of the enzyme activity of IspG and/or IspH, which catalyze the last two reactions of the pathway. Both the IspG and IspH are known to be iron-sulfur cluster proteins (Adam et al., 2002; Querol et al., 2002; Rohdich et al., 2003; Seemann et al., 2005; Altincicek et al., 2002; Gräwert et al., 2010) and it has been reported that the cluster is directly involved in IspH activity (Gräwert et al., 2004). Our hypothesis was supported with findings of the essential role of ErpA, which is an A-type iron-sulfur cluster protein, in the maturation process of IspG, and probably IspH, in *E. coli*.(Loiseau et al., 2007). So, focus turned to the reconstruction of the bacterial Fe-S cluster trafficking routes involved in maturation of IspG and IspH in *S. cerevisiae*.

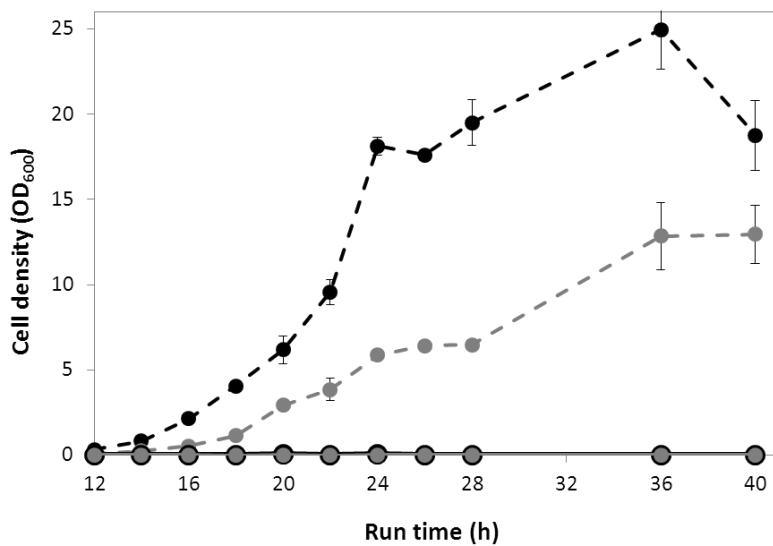


Figure 16: Growth of *Saccharomyces cerevisiae* strains CEN.PK 113-13D (black circles) and SCISP06 (gray circles) in SD minimal medium. Dashed lines represent the growth in 0 g L⁻¹ of lovastatin; solid lines represent the growth in presence of 2 g L⁻¹ of lovastatin. Error bars show the standard deviation from three cultivations.

2.6. Fe-S clusters protein biogenesis in *E. coli* and *S. cerevisiae*

In terms of evolution, Fe-S clusters are thought to be one of the first catalysts in nature. The Fe/S clusters combined within protein structure play several vital functions in living cells, e.g. enzymatic reactions, ribosome biogenesis, regulation of gene expression, respiration, co-factor biosynthesis and so on. Therefore, understanding of the mechanisms leading to assembly of this small inorganic molecule not only is interesting, but it is also necessary to solve many disorders which are connected to defective Fe/S-cluster biogenesis, e.g. Friederich's ataxia, sideroblastic anemia or hereditary myopathy (Campuzano et al., 1996; Camaschella et al., 2007 and Ye and Rouault 2010). The rhombic [2Fe-2S] and cubic [4Fe-4S] are the most common and simplest types of iron-sulfur clusters found in nature. However, the distorted [3Fe-4S] type may be found in several proteins. Unlike bacterial biogenesis, in eukaryotes e.g. yeast, Fe-S clusters biogenesis mainly is performed in mitochondria and this organelle plays a central role in maturation of Fe/S proteins (Lill & Mühlenhoff, 2008; Py & Barras, 2010). The mitochondrial biogenesis is also necessary for maturation of the Fe/S cluster proteins localized in the cytosol and nucleus, which in yeast also involves the cytosolic Fe/S protein assembly (CIA) machinery (Sharma et al., 2010).

Instead of the compartmental localization, the major elements involved in iron-sulfur cluster biogenesis are linked through the evolution from bacteria to eukaryote (*E. coli* / Yeast) comprising a cysteine desulfurase supplying the sulfur (IscS and SufS / Nfs1 and Isd11), an iron sensor/donor (CyaY / Yfh1), electron donor (Fdx / Yah1 and Arh1) and a scaffold protein which forms a platform to

assemble both the rhombic and cubic types (IscU / Iscu1 and Iscu2). In fact, Fe-S clusters are the result of close interaction between scaffold protein with cysteine desulfurase and an iron donor. Finally, the clusters are transferred to the acceptor apo-protein by action of the series of chaperone-like protein (HscA and HscB / Ssq1, Jac1 and Mge1), which occurs in collaboration with the scaffold protein. The clusters can be transferred into the cytoplasm, probably by a mitochondrial ISC export apparatus (Atm1 and Erv1) and, furthermore, through the activity of CIA machinery which contains Nbp35, Cfd1, Nar1, Cia1 and Dre2, the clusters are transferred into cytosolic and nuclear apo-proteins (**Figure 17**). The latter two apparatuses, mitochondrial ISC export and CIA, are not found in bacteria.

There are other elements which are involved in Fe-S clusters biogenesis. However, the function of most of them is not clearly identified, such as A-type proteins (IscA, SufA and ErpA in *E.coli* and Isa1 and Isa2 in yeast mitochondria). The A-type carriers in bacteria can bind both types of Fe-S clusters and can transfer them to apo-proteins *in vitro* (Loiseau et al., 2007; Tan, Lu et al., 2009). Hence, the scaffold function was initially proposed for this type of proteins. However, their inability to interact with cysteine desulfurase rejects this proposed role. Later, it has been shown that Fe/S clusters can be transferred from IscU to IscA (Ollagnier de Choudens et al., 2004). Moreover, purified A-types proteins containing Fe-S clusters have been isolated (Gupta et al., 2009; Zeng et al., 2007). Thus, transferring the Fe/S clusters to apo-targets from a scaffold is a more likely function for the A-type proteins. Indicating the role of ErpA in transferring Fe-S cluster to IspG and probably IspH, was the first report showing a specific target, apo-protein, *in vivo* (Loiseau et al., 2007). And finally, Vinnela and co-workers (2009) proposed different Fe/S trafficking models involved in maturation of *E. coli* enzymes, IspG and IspH, based on A-type carriers (Vinella et al., 2009). Therefore, here the suitable model composed of ErpA with either human IscA (hISCA1)(Song et al., 2009) or IscA from Arabidopsis thaliana (CpIscA)(Abdel-ghany et al., 2005) was re-constructed and expressed in the yeast cytosol, which is harboring the bacterial MEP pathway, and their influence on the functionality of the pathway was investigated.

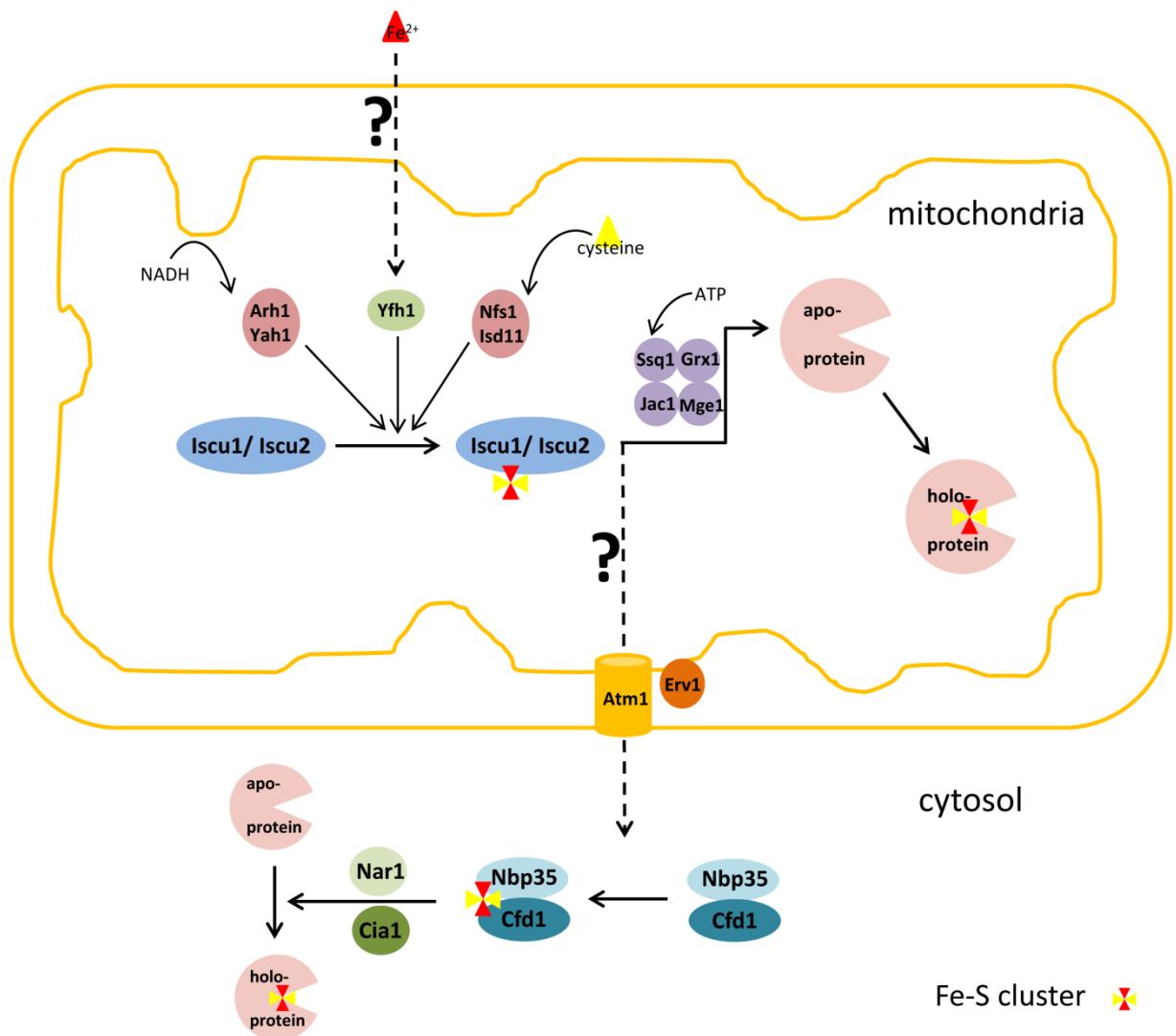


Figure 17: Schematic representation of Fe-S clusters assembly machinery in *S. cerevisiae* (details are discussed in section 2.7). The dashed arrows represent the apparatuses which are not defined completely yet.

2.6.1. Re-construction of the possible bacterial Fe/S trafficking routes and the bacterial electron transfer system

As an attempt to solve the problem of the non-functionality of the MEP pathway in *S. cerevisiae*, the impact of the co-expression of genes involved in transferring of Fe-S cluster into IspG/IspH, *E. coli* electron transfer system and a copy of *ispG* and *ispH* both from *A. thaliana* on the functionality of MEP pathway were investigated. Thus, the coding region of genes including *erpA*, *fpr* and *fldA* from *E. coli* assembled on pISP08 (**Figure 18**) were transformed into SCISP06 generating SCISP16 (Table 2.4). The empty plasmid pSP-GM1 was transformed into CEN.PK113-13D and SCISP06 resulting in SCISP28 and SCISP29, respectively (Table 2.4). A copy of each *ispG* and *ispH* from *A. thaliana* were cloned with *iscA* from either human or *A. thaliana* resulting in pISP24 and pISP25, respectively

(Figure 18). Strains SCISP31 and SCISP32 were constructed by co-transforming pISP08 with either pISP24 or pISP25 into SCISP12, respectively (Table 2.4).

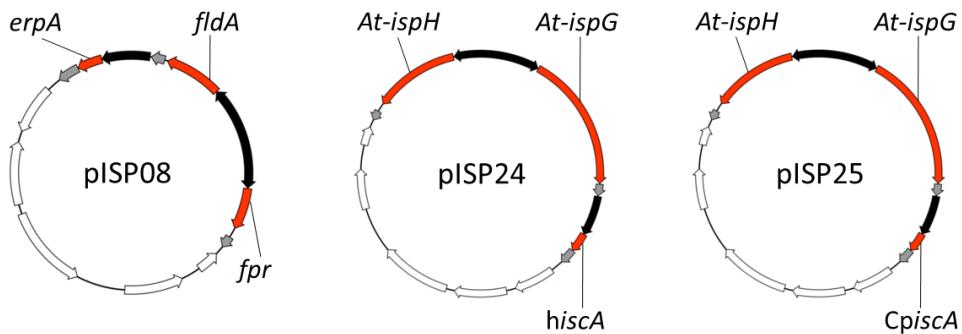


Figure 18: plasmid-based reconstruction of possible Fe/S trafficking routes involved in maturation of bacterial IspG/IspH, bacterial electron transfer systems and plant-derived *ispG/isph* in *S. cerevisiae*.

To rule out any possible additional effect on cell growth using lovastatin for inhibition the MVA pathway, the functionality of the bacterial MEP pathway was investigated by genetically blocking the MVA pathway, which offers promising and absolute inactivation of the MVA pathway. It has been indicated that yeast strains with deficiencies in *ERG13*, *ERG19*, *ERG8*, or *ERG9* are non-viable under normal growth conditions (Servouse et al., 1984; Bergès et al., 1997; Dimster-Denk & Rine, 1996; Tsay & Robinson, 1991; Jennings et al., 1991). Since the MEP pathway contributes to the ergosterol biosynthetic pathway through IPP and DMAPP intermediates, deletion of each *ERG13*, *ERG19* or *ERG8*, which are located upstream of these intermediates, should be more efficient than using lovastatin for blocking the MVA pathway and evaluating the MEP pathway functionality. For our purpose, *ERG13* was a good candidate since supplying the medium with exogenous mevalonate can complement its inactivation. *ERG13* encodes HMG-CoA synthase (Maury et al., 2005), and its disruption results in a strain that requires exogenous mevalonate supplementation for viability (Dimster-Denk & Rine, 1996). The coding region of *ERG13* was replaced by a *KanMx* integration cassette, which was confirmed by PCR (Figure 19). As it is illustrated in Figure 20, the *ERG13* deleted strains could not grow in media lacking mevalonate under aerobic conditions.

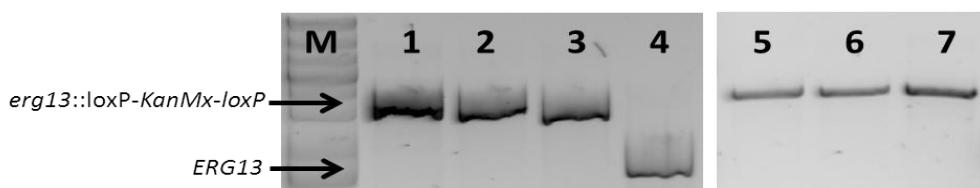


Figure 19: Gel electrophoresis of PCR products to confirm deletion of *ERG13* (1: SCISP28, 2: SCISP29, 3: SCISP16, 4: CEN.PK 113-13D (wild type), 5: SCISP30, 6: SCISP31, 7: SCISP32, M: 1 kb Plus DNA ladder).

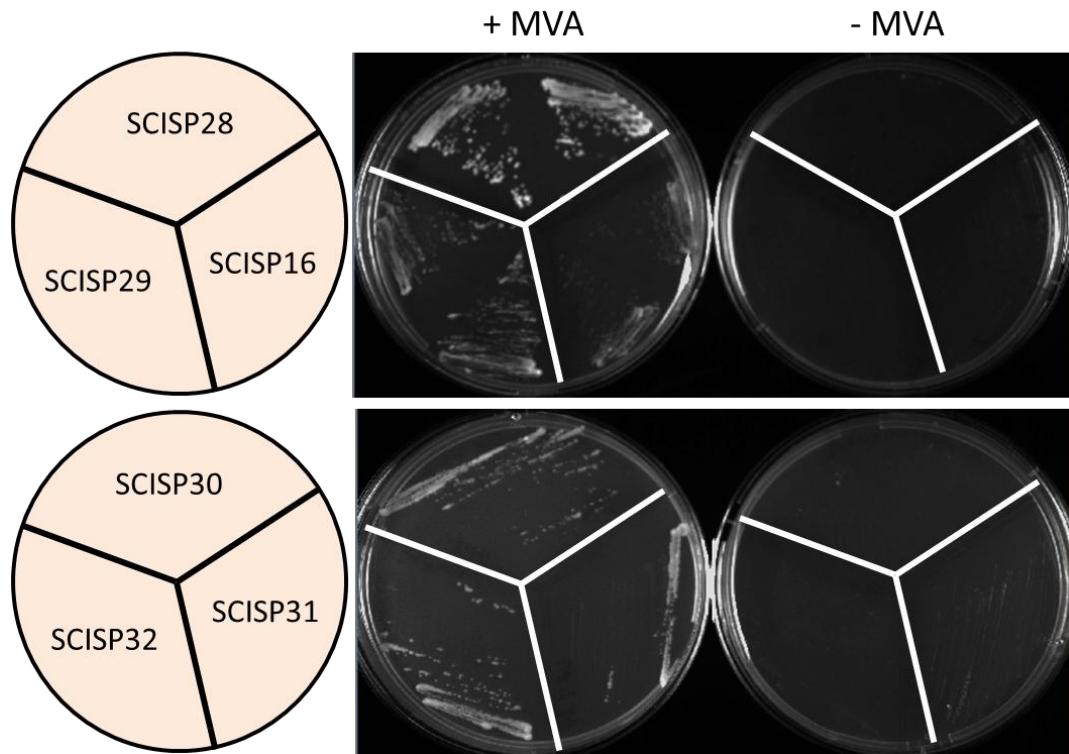


Figure 20: Aerobic cultivation of MEP pathway. upper panel: strains co-expressing *erpA*, *fpr* and *fldA*; lower panel: strains co-expressing *erpA*, *fpr*, *fldA*, *At-IspG*, *At-IspH* with either *CpIscA* or *hISCA*. All strains were *erg13* background.

2.6.2. Evaluation of the bacterial MEP pathway in *S. cerevisiae* under anaerobic condition

Fe-S clusters are sensitive to superoxide (O_2^-) and other oxidative agents (Liochev & Fridovich, 1994; Pantopoulos & Hentze, 1995). The Fe-S cluster contained in IspH is easily destroyed by exposure to molecular oxygen or other oxidative agents (Gräwert et al., 2004). Therefore, to prevent inactivation of the Fe-S clusters in IspG and IspH, all *erg13* strains were also evaluated under anaerobic conditions. Yeast growing in anaerobic condition is ergosterol-dependent as the biosynthesis of ergosterol is disrupted in this condition. For this, exogenous ergosterol was added to the SD media at a final concentration of 1 mg L^{-1} . None of the *erg13* strains showed mevalonate-independent growth (**Figure 21**). This means that, even in anaerobic conditions, the MEP pathway was not able to complement the MVA pathway.

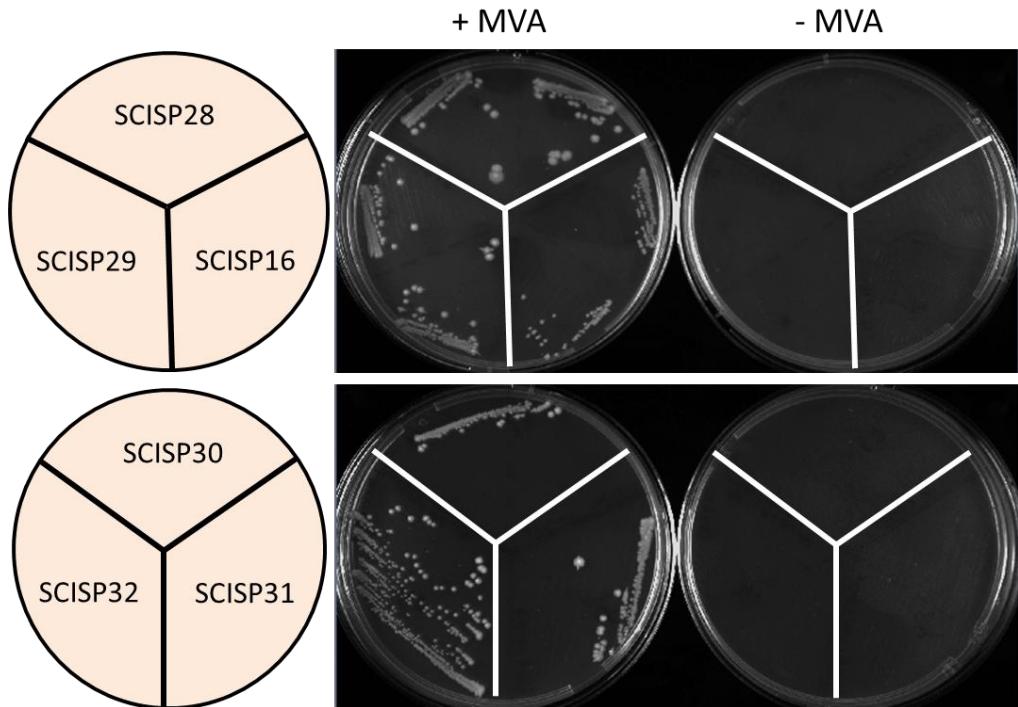


Figure 21: Anaerobic cultivation of MEP pathway. upper panel: strains co-expressing *erpA*, *fpr* and *fldA*; lower panel: strains co-expressing *erpA*, *fpr*, *fldA*, *At-IspG*, *At-IspH* with either *CpIscA* or *hISCA*. All strains were *erg13* background.

Besides demonstrating a strategy for easy integration of eight heterologous genes, here I present different strategies in order to make functional MEP pathway in *Saccharomyces cerevisiae*. My efforts can be approached from different angles, which are considered below. However, the results did not prove any activity of the MEP pathway.

First, I constructed possible bacterial paths (aerobic and anaerobic), which have previously been proposed and shown transferring of Fe-S clusters into IspG and IspH in *E. coli*, in yeast cytosol (**Figure 22**) (Vinella et al., 2009). The authors have suggested that, depending on environmental conditions, e.g. aerobic, anaerobic or stress, Fe-S cluster is transferred from IscU or SufU scaffolds to apoIspG and apoIspH through the combination of A-type carriers, including ErpA, IscA and SufA (Vinella et al., 2009). Hence, *erpA* from *E. coli* was first expressed to build the direct transferring route of Fe/S cluster into IspG and IspH from their scaffold. It was shown that the Fe-S clusters can directly be transferred from IscU to ErpA in *E. coli* (Pinske & Sawers, 2012). I could not obtain functionality of the enzymes. Previously reported data have shown that the cytosolic localization has failed to generate a functional bacterial or human IscU while expressing in yeast (Gerber et al., 2004). Even yeast U-type homolog scaffolds (Isu1 and Isu2) playing a crucial role in maturation of both

cytosolic and mitochondrial Fe-S proteins need to be expressed in the mitochondria to show activity (Gerber et al., 2004).

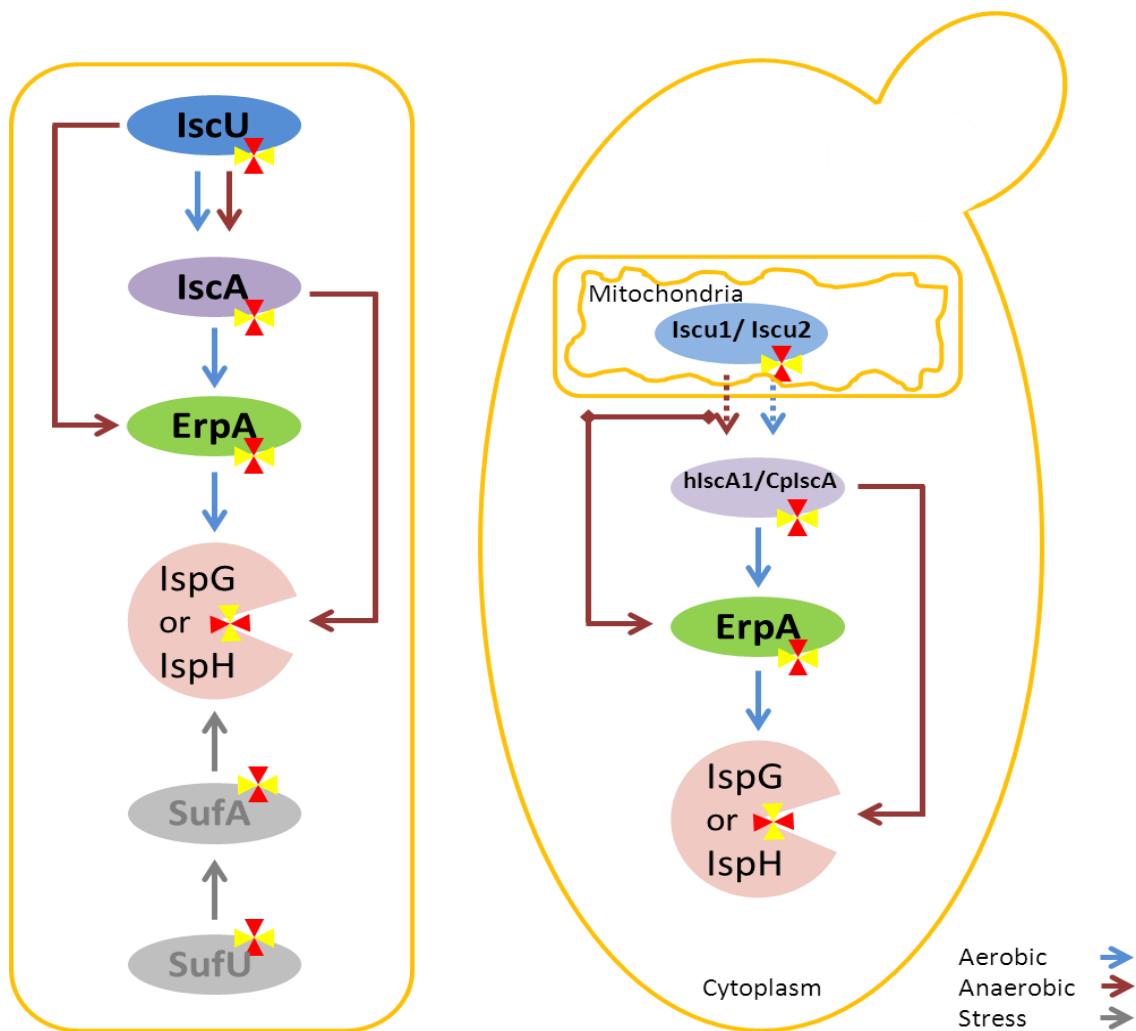


Figure 22: Schematic representation of possible Fe/S trafficking routes involved in maturation of bacterial IspG/IspH in *E. coli* (left) and reconstruction of possible routes preformed in this study in the yeast cytosol (right). Round dot arrows represent unknown mechanisms for transferring the Fe-S clusters from mitochondria to cytosol. For more information see text.

Furthermore, IscA from either human or *A. thaliana* were co-expressed with erpA in order to create the second and third transferring routes of Fe/S clusters into IspG and IspH from scaffold proteins. Previously, localization and activity of human ISCA1 (hISCA1) was shown to be in the cytosol of HeLa cells (Song et al., 2009). The authors have also demonstrated interaction of the small domain of IOP1 (Iron-only hydrogenase-like protein I) with human ISCA1 using yeast two-hybrid systems (Song et al., 2009). CpIscA from *A. thaliana* is involved in Fe-S biogenesis in chloroplasts (Abdel-ghany et al., 2005). The Fe-S cluster in CpIspA indicated stability in presence of oxygen (Abdel-ghany et al., 2005).

Second, the *E. coli* electron transfer system was reconstructed in yeast by co-expressing *fldA* encoding flavodoxin I and *fpr* encoding flavodoxin reductase. Puan and co-workers (Puan et al., 2005) identified *fldA* as an essential gene for isoprenoid biosynthesis in *E. coli*, as it provides reducing equivalents for the Fe/S clusters of IspG and IspH (Jenkins & Waterman, 1994). It has been reported that both enzymes, IspG and IspH, are dependent on NADPH and the flavodoxin/flavodoxin reductase redox system as electron donor for their catalytic activity (Puan et al., 2005; Rohdich et al., 2003; Wolff, 2003; Seemann et al., 2006; Xiao et al., 2008; Xiao et al., 2009). Gräwert and co-workers (Gräwert et al., 2004) have reported that the *in vitro* maximum activity for IspH was obtained with NADPH as co-substrate, together with recombinant flavodoxin and flavodoxin reductase from *E. coli*. Flavodoxin and flavodoxin reductase are FMN and the FAD cofactor containing proteins, respectively, and it has been shown that NADPH is the preferred reducing equivalent of flavodoxin reductase compared to NADH (Jenkins & Waterman, 1994). Over-expression of flavodoxin and flavodoxin reductase might facilitate electron flux from NADPH to IspG and IspH and, therefore, result in increased activity of these enzymes. A similar phenomenon was observed in biosynthesis of hydrocortisone in yeast (Szczecuba et al., 2003). Over-expression of the yeast essential reductase, Arh1 (adrenodoxin reductase homolog), using strong promoter increased the production of hydrocortisone up to 60% (Szczecuba et al., 2003). Both Arh1 and human ADX protein (adrenodoxin) are responsible for transferring electrons from NADPH to the related enzyme. The authors have suggested that the flux of electrons was elevated as a result of the *ARH1* overexpression (Szczecuba et al., 2003).

And finally, due to the above mentioned findings, we also hypothesized that the poor recognition of the bacterial apo-proteins, IspG and IspH, by the eukaryotic Fe/S cluster machinery leads to low amounts of active proteins and subsequently results in inefficient MEP pathway activity. Thus, the co-expression of a copy of each *ispG* and *ispH* both from *A. thaliana* was investigated, in order to increase the pool of expressed enzymes and, subsequently, to overcome the probable lack of IspG and IspH.

Furthermore, my findings indicate that, despite the presence of Fe-S assembly machineries in yeast, the ISC system present in mitochondria and the CIA system for cytosolic Fe-S cluster assembly, (Mühlenhoff et al., 2002; Sharma et al., 2010; Lill & Mühlenhoff, 2005) these may not be suitable for transferring iron-sulfur clusters to IspG and IspH. In addition, different known and unknown elements are involved in transferring the Fe-S clusters from the scaffold to apo-proteins. Some of these elements have been identified and isolated and their collaboration in such transmission has been proved *in vivo* (Loiseau et al., 2007; Vinella et al., 2009). In spite of some differences, these elements show similar biochemical properties and potential functional redundancy. However, such transmission might be a major challenge in order to have a functional MEP pathway in the yeast cytosol.

CHAPTER 3: CONCLUSIONS AND PERSPECTIVES

Pathway optimization and, specifically, modulation of the enzyme expression is subjected and one of the key challenges in the most metabolic engineering investigations aiming at production of fine chemicals and pharmaceuticals. During my PhD study, I have focused on optimizing enzyme activity through the modulation of gene dosage and gene copy number in *Saccharomyces cerevisiae*. Furthermore, a new expression system was developed and implemented in optimizing the endogenous mevalonate pathway and in re-constructing the heterologous MEP pathway, in order to generate a new yeast strain as a microbial cell factory for over-production of isoprenoids.

Due to advantageous use of glucose, in terms of economy and productivity, a new divergent promoter has been developed, containing two strong and constitutive promoters, *TEF1* and *PGK1*. I showed that the two promoters, in the bidirectional construct, have expression profiles similar to the corresponding isolated promoters and can therefore support high level gene expression. Furthermore, the new divergent promoter was used to construct new episomal plasmids, pSP series (Paper I), to optimize the endogenous mevalonate pathway through gene integration (Paper IV) and also to construct the integration cassettes containing the synthetic MEP pathway (Paper V). Besides, the activities of 7 different constitutive and glucose based promoters, P_{TEF1} , P_{TPH1} , P_{TDH3} , P_{ADH1} , P_{PGK1} , P_{HXT7} and P_{PYK1} , were compared with each other and showed varying profiles of activity for each promoter. However, I used P_{PGK1} and P_{TEF1} for constructing the new vector, but depending on the purpose one can use different promoter pairs with comparable or different expression patterns. As we demonstrated in Paper III, using the P_{HXT1} promoter repressed the *ERG9* transcription under glucose limitation in an efficient manner as similar to successful implementation of the synthetic posttranscriptional genetic tool, RNA control modules (Rnt1p), to reduce the transcription level of the *ERG9* gene (Babiskin & Smolke, 2011b). Our results in paper III demonstrated pathway optimization through redirection of the carbon flux through employing synthetic biology tools at transcription level independent of addition of external compounds. In addition, engineering the pSP-G vector by introducing additional restriction sites after both terminator regions to generate pSP-GM allowed us simple construction of integration cassettes containing 4 genes for optimizing the endogenous mevalonate pathway.

Modulating gene copy number at DNA level further improved enzyme activity. In **paper II**, a more than 3-fold improvement of the plasmid copy number was demonstrated through the modulation of the auxotrophic marker (*URA3*) level applying two different biological toolsets, a weak promoter leading to low transcription level and the ubiquitin-tag/N-degron system alerting the protein stability. Combination of both the weak promoter and the ubiquitin-tag/N-degron system indicated positive effect on plasmid copy number. Subsequently, I found a high correlation between the plasmid copy number and patchoulol production. In addition, in this paper, I reported the successful application of

the signal peptide leading to protein-degradation in order to modulate plasmid copy number for the first time generating the new 2 μ m-based episomal plasmid. This expression vector is useful for metabolic engineering projects that aim at high level production of valuable products using yeast as a production platform. Furthermore, the system described here can potentially be applied to other systems using plasmid-based gene expression.

Paper IV and **paper V** represent not only the high capacity of yeast for accepting several genetic manipulations through either chromosomal integration or plasmid transformation, but also it shows the efficiency of the bipartite gene targeting (Erdeniz et al., 1997) in combination with reusable marker for such manipulations. In **paper IV**, the outcomes was showed that introduction of all modifications in combination with a specifically developed continuous fermentation process led to a 4-fold improvement of α -santalene yield over the reference strain.

In the last investigation, I demonstrated a strategy for easy and stable integration of the bacterial MEP pathway, containing 8 genes in two steps into yeast chromosome and I showed the expression of this pathway at the transcription level. However, a Functional MEP pathway was not achieved due to lack of enzyme activity. I found that, despite the presence of Fe-S assembly machineries in yeast, the ISC system present in mitochondria and the CIA system for cytosolic Fe-S cluster assembly, trafficking of Fe-S clusters into the last two enzymes of the MEP pathway is challenging. Inspecting the results presented in **paper V**, I believe that specific physical interaction and compartmentalization would be required for *in vivo* biogenesis and transfer of essential prosthetic groups, here the iron-sulfur clusters for activation of bacterial MEP pathway enzymes in yeast. Therefore, it seems interesting to evaluate IspG and IspH expression in the mitochondria as this may represent a new interesting engineering strategy, which may even be relevant for activation of other bacterial iron-sulfur cluster proteins in yeast.

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Characterization of different promoters for designing a new expression vector in *Saccharomyces cerevisiae*

**Siavash Partow, Verena Siewers, Sara Bjørn, Jens Nielsen,
Jerome Maury**

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Research Article

Characterization of different promoters for designing a new expression vector in *Saccharomyces cerevisiae*

Siavash Partow¹, Verena Siewers¹, Sara Bjørn², Jens Nielsen^{1*} and Jérôme Maury²¹Department of Chemical and Biological Engineering, Chalmers University of Technology, SE-41296 Göteborg, Sweden²Fluxome Sciences A/S, Gymnasievej 5, DK-3660 Stenløse, Denmark**Correspondence to:*Jens Nielsen, Department of Chemical and Biological Engineering, Chalmers University of Technology, Kemivägen 10, SE-41296 Göteborg, Sweden.
E-mail: nielsen@chalmers.se**Abstract**

The widely used pESC vector series (Stratagene, La Jolla, CA, USA) with the bidirectional *GAL1/GAL10* promoter provides the possibility of simultaneously expressing two different genes from a single vector in *Saccharomyces cerevisiae*. This system can be induced by galactose and is repressed by glucose. Since *S. cerevisiae* prefers glucose as a carbon source, and since its growth rate is higher in glucose than in galactose-containing media, we compared and evaluated seven different promoters expressed during growth on glucose (p*TEF1*, p*ADH1*, p*TPII*, p*HXT7*, p*TDH3*, p*PGK1* and p*PYK1*) with two strong galactose-induced promoters (p*GAL1* and p*GAL10*), using *lacZ* as a reporter gene and measuring LacZ activity in batch and continuous cultivation. *TEF1* and *PGK1* promoters showed the most constant activity pattern at different glucose concentrations. Based on these results, we designed and constructed two new expression vectors which contain the two constitutive promoters, *TEF1* and *PGK1*, in opposite orientation to each other. These new vectors retain all the features from the pESC-URA plasmid except that gene expression is mediated by constitutive promoters. Copyright © 2010 John Wiley & Sons, Ltd.

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Keywords: promoter activity; expression vector; bidirectional promoter; yeast**Introduction**

Saccharomyces cerevisiae has been widely used as a host organism for the efficient expression of heterologous proteins. To reach this goal, different expression systems, such as yeast integrative plasmids (YIps) for integration of the desired gene into the yeast genome or yeast episomal plasmids (YEps) for high copy number expression, have been designed and developed. Based on these systems, different plasmids harbouring promoters with different regulation profiles, strengths and various additional features have been constructed (Miller *et al.*, 1998; Li *et al.*, 2008; Hermann *et al.*, 1992, Mumberg *et al.*, 1994, 1995).

Most expression plasmids allow the expression of only one gene. For the expression of entire metabolic pathways, for example, it is desirable to

be able to express more than one gene per plasmid unit. For example, Miller *et al.* (1998) used a constitutively active bidirectional promoter consisting of the promoter of glyceraldehyde 3-phosphate dehydrogenase (p*GPD* = p*TDH3*) and a fragment of the alcohol dehydrogenase 1 promoter (p*ADH1*). The bidirectional expression vectors constructed by Li *et al.* (2008) carry a modified inducible *GAL1* or *GAL10* promoter in one direction and a constitutive *GPD* promoter in the reverse direction.

One widely used expression vector set in *S. cerevisiae* is the pESC series from Stratagene, with the bidirectional *GAL1/GAL10* promoter cassette providing the possibility of expressing two different genes at the same time from a single vector, and its successful applicability has previously been published (e.g. Maury *et al.*, 2008; Asadolahi *et al.*, 2007). Expression systems based on the

GAL1/GAL10 promoters are among the strongest ones (Schneider *et al.*, 1991). However, expression from the *GAL1* and *GAL10* promoters are subject to both galactose induction and glucose repression (Zhang and Rathod, 2002; Lohr *et al.*, 1995; West *et al.*, 1987). This is a major disadvantage, as the preferred carbon sources for yeast are glucose and fructose; in addition, the inducer galactose can be seen as too costly when scaling up synthesis of commercially valuable products is in focus (Haufa *et al.*, 2000). Furthermore, the shift from glucose to galactose causes major metabolic changes (Quintero *et al.*, 2007). In order to develop a glucose-based experimental system analogous to the *GAL1/GAL10* system of the pESC vectors, we initiated this study, aiming at comparing the strength of those two strong galactose-based promoters with different glucose-based promoters. Several strong glucose promoters have previously been described and have been shown to be useful for expression of heterologous genes in yeast. In this study, we compared the strength of seven different constitutive or glucose-based promoters derived from the following genes [*TEF1*, encoding transcriptional elongation factor EF-1 α (Gatignol *et al.*, 1990); *PGK1*, encoding phosphoglycerate kinase (Ogden *et al.*, 1986; Holland and Holland, 1978); *TPII*, encoding triose phosphate isomerase; *HXT7*, encoding a hexose transporter (Diderich *et al.*, 1999; Reifenberger *et al.*, 1997); *PYK1*, encoding pyruvate kinase 1 (Nishizawa *et al.*, 1989); *ADH1*, encoding alcohol dehydrogenase 1 (Denis *et al.*, 1983); and *TDH3* (*YPD*), encoding triose phosphate dehydrogenase (Bitter and Egan 1984)] with the strength of the *GAL1* and *GAL10* promoters (Adams, 1972; St. John *et al.*, 1981; Laughon and Gestland, 1982). Four of these promoters (p*PGK1*, p*TPII*, p*PYK1* and p*TDH3*) are promoters of key glycolytic genes and in the literature they are generally considered strong promoters. Full-length p*ADH1*, p*TEF1* and p*TDH3* have also been utilized to construct the widely used p4XXprom vector series (Mumberg *et al.*, 1996). For this comparison we used *lacZ* as a reporter gene and constructed nine different integrative plasmids, in which *lacZ* expression was controlled by either of these promoters. In all cases the constructed integrative plasmids were integrated into the *URA3* locus.

Based on this analysis, we constructed two new divergent expression cassettes by replacing the

GAL1/GAL10 promoters in pESC–URA with a *TEF1–PGK1* bidirectional promoter cassette in two different orientations. These two new vectors are called pSP-G1 and pSP-G2, respectively.

Materials and methods

Construction of integrative plasmids

The integrative vector pSF011 used in this study was derived from pRS306 (Sikorski and Hieter, 1989). It contains *URA3* as a selectable marker and reporter gene *lacZ* located downstream of a multiple cloning site (MCS) (Figure 1). *lacZ* and the *CYC1* terminator were cloned as described earlier (Flagfeldt *et al.*, 2009). All glucose-based promoters were amplified by PCR from the genome of *S. cerevisiae* CEN.PK 113-7D (*MATa MAL2-8c SUC2*; kindly provided by P. Kötter, University of Frankfurt, Germany). The amplicons of each of the seven glucose-based promoters (p*TEF1*, p*PYK1*, p*HXT7*, p*PGK1*, p*TPII*, p*ADH1* and p*TDH3*) were digested by *NotI–XbaI* and cloned into pSF011 upstream of *lacZ*. The *GAL10* promoter was cloned into pSF011 as a *NotI–BamHI* fragment isolated from pESC–URA (Stratagene, La Jolla, CA, USA). The *GAL1* promoter was first amplified by PCR from pESC–URA, digested by *XbaI–BamHI* and cloned into pSF011. Table 1 shows all the primers used for amplifying the promoters.

Transformation of *S. cerevisiae*

The integrative plasmids were linearized by *NcoI* and transformed into *S. cerevisiae* CEN.PK 113-5D (*MATa MAL2-8c SUC2 ura3-52*; kindly provided by P. Kötter) using a standard transformation procedure (Gietz and Woods, 2002). Transformants were selected on plates containing

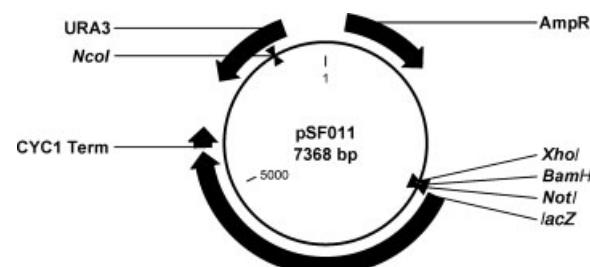


Figure 1. pSF011, integrative plasmid

Table I. Oligonucleotide primers used in this study

Primer name	Sequence (5'-3')
pADH1-top	GTTGTT CTCGAG AGGGGGATCGAAGAAAATGATG
pADH1-bot	GTTGTT <u>GCGGCCGC</u> TGTATATGAGATAGTTGATTG
pHXT7-top	GTTGTT CTCGAG CCGTGAAATGAGGGTATG
pHXT7-bot	GTTGTT <u>GCGGCCGC</u> TTTTGATTTAAATTTAAAAAC
pPGK1-top	GTTGTT CTCGAG GGAAGTACCTCAAAGAATG
pPGK1-bot	GTTGTT <u>GCGGCCGC</u> TTGTTTATATTTGTAAGGAG
pPYK1-top	GTTGTT CTCGAG GAAAGTTTCCGGCAAGCT
pPYK1-bot	GTTGTT <u>GCGGCCGC</u> TGTGATGATGTTTATTG
pTEFI-top	GTTGTT CTCGAG GCACACACCATACTTC
pTEFI-bot	GTTGTT <u>GCGGCCGC</u> TTGTAATTAAAACCTAGATTAG
pTPII-top	GTTGTT CTCGAG CTACGTATGGTCATTCTTC
pTPII-bot	GTTGTT <u>GCGGCCGC</u> TTTAGTTATGATG
pTDH3-top	GTTGTT CTCGAG CAGTTTATCATTATCAACTCGCC
pTDH3-bot	GTTGTT <u>GCGGCCGC</u> GAATCCGTCGAAACTAAGTTCTGGTG
pGAL1-FW	GTTGTT CTCGAG CGTCGTCATCCTTGTAATCC
pGAL1-RE	ATCAACTTCTGTTCCATGTCG
Pgk-fw	GGAAGTACCTTCAAAGAATGG
Tef-fw	<u>CCATTCTTGAAAGGTACTTCC</u> GGCCGGCC GCACACACCATACTTC
Tef-BamHI	GTTGTT GGATCC TTGTAATTAAAACCTAGATTAGATTGC
Tef-NotI	GTTGTT <u>GCGGCCGC</u> TTGTAATTAAAACCTAGATTAGATTGC
Pgk-BamHI	GTTGTT GGATCC TTGTTTATATTTGTTGAAAAAGTAG
Pgk-NotI	GTTGTT <u>GCGGCCGC</u> TTGTTTATATTTGTTGAAAAAGTAG

Restriction sites are indicated in bold face; the underlined sequence corresponds to the overlapping nucleotides.

1.7 g/l yeast nitrogen base without amino acids and ammonium sulfate (Difco Laboratories, Detroit, MI, USA), 5 g/l ammonium sulphate, 0.77 g/l complete supplement mixture (CSM without uracil; MP Biomedicals, Solon, OH, USA), 20 g/l glucose and 20 g/l agar.

Shake-flask cultivation

Baffled, cotton-stopped, 500 ml Erlenmeyer flasks were used for precultures and also for promoter evaluations. The shake flasks contained 100 ml medium with the following composition: 7.5 g/l $(\text{NH}_4)_2\text{SO}_4$, 14.4 g/l KH_2PO_4 , 0.5 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 ml/l trace metal solution, 1 ml/l vitamin solution (Verduyn *et al.*, 1993) and 50 $\mu\text{l}/\text{l}$ synperonic antifoam (Sigma, St. Louis, MO, USA). The pH of the medium was adjusted to 6.5 by adding 2 M NaOH and it was autoclaved separately from the 20% glucose (galactose) solution that was used as a carbon source solution in a final concentration of 2%. Vitamin solution was filter sterilized and aseptically added to the medium after autoclaving. The shake flasks were run in triplicates and incubated at 30 °C and 150 rpm.

Continuous cultivation

Continuous cultivations were carried out in duplicates in well-controlled 2.5 l Braun Biostat manufactured glass bioreactors with a working volume of 2 l. The fermentors were inoculated to initial $\text{OD}_{600} = 0.01$ from the liquid precultures. The medium was identical to that used for shake-flask cultivations. Depending on the promoter being tested, either glucose or galactose was added as carbon source at a concentration of 2%. The pH was maintained at 5 by automatic addition of 2 M KOH. The temperature was kept constant at 30 °C. The airflow was 4 l/min (2 vvm) and was sterilized by filtration and the off-gas passed through a condenser. Agitation was adjusted to maintain the dissolved oxygen tension above 20% of air saturation. The dilution rate was set to 0.1/h during operation of the chemostat and steady state was assumed to be obtained after about 50 h of cultivation.

β -Galactosidase assay

β -Galactosidase activity was assayed as described by Miller (1972). 1 ml *S. cerevisiae* cell culture was spun down and the cell pellet was resuspended

in 1 ml chilled Z buffer (0.06 M Na₂HPO₄, 0.04 M NaH₂PO₄, 0.01 M KCl, 0.001 M MgSO₄·7H₂O, pH 7) and the OD₆₀₀ was determined. 0.1 ml cell solution was diluted in 0.9 ml Z buffer containing 2.7 ml/l β-mercaptoethanol (ME). 100 μl chloroform and 50 μl 0.1% SDS were added and the sample was vortexed for 15 s. The reaction was started by addition of 0.2 ml prewarmed (30 °C) ONPG (*o*-nitrophenyl-β-galactoside) solution (80 mg ONPG in 20 ml Z buffer plus ME) and after the yellow colour had developed the reaction was stopped by adding 0.5 ml 1 M Na₂CO₃. The reaction mix was spun down at maximum speed and the OD₄₂₀ was determined. LacZ activity was expressed in Miller units according to the following equation:

$$\text{Miller units} = 1000 \times \text{OD}_{420}/(T \times V \times \text{OD}_{600})$$

where *T* is the time of reaction and *V* is the volume (ml) of culture used for the assay.

Construction of divergent promoters

The *TEF1* and *PGK1* promoters were fused to each other to construct divergent promoters *TEF1–PGK1* by means of fusion PCR. The PCR was performed with Phusion high-fidelity DNA polymerase (Finnzymes, Espoo, Finland) in two steps. In the first step, each promoter, p*TEF1* and p*PGK1*, was amplified using the primers shown in Table 1 (Tef-fw/Tef-BamHI and Tef-fw/Tef-NotI for amplification of the *TEF1* promoter and Pgk-fw/Pgk-BamHI and Pgk-fw/Pgk-NotI primers for amplification of the *PGK1* promoter). Tef-fw primer carries an overhang at the 5' end which is complementary to the 5' end of the *PGK1* promoter. The PCR products of the first step were used in a second PCR. The second PCR reaction was started without primers so that the *TEF1* and *PGK1* promoters were fused to each other via the overlapping parts. After 15 cycles, primers were added and the programme was run for 30 additional cycles. The *TEF1–PGK1* cassette was digested by BamHI/NotI and cloned in both orientations into pSF011 and pESC-URA. The two new plasmids generated by replacing the *GAL1/GAL10* promoter in pESC-URA were called pSP-G1 and pSP-G2, respectively (Figure 2A, B).

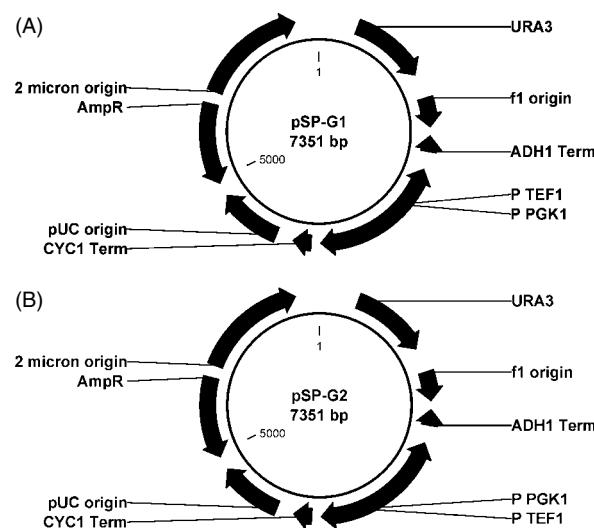


Figure 2. (A) pSP-G1 vector; contains *TEF1–PGK1* bidirectional promoter. (B) pSP-G2 vector; contains *PGK1–TEF1* bidirectional promoter

Results and discussion

Promoter comparison

Several *lacZ* fusions were constructed for comparison of the activity of different promoters, including *TPII*, *ADH1*, *TEF1*, *PGK1*, *TDH3*, *PYK1* and *HXT7* promoters. These fusions were stably integrated in single copy into the genome of *S. cerevisiae* CEN.PK 113-5D, at the *ura3-52* locus. Although in the last decade different reporter systems have been developed and used for promoter analysis in *S. cerevisiae*, such as green fluorescent protein (Li *et al.*, 2000; Niedenthal *et al.*, 1996), β-lactamase (Cartwright *et al.*, 1994) and β-D-glucuronidase (Nacken *et al.*, 1996), β-galactosidase encoded by the *lacZ* gene of *Escherichia coli* is the most commonly employed reporter of gene expression in *S. cerevisiae* and is widely used for different purposes (Yocom *et al.*, 1984; Flick and Johnston, 1990; Hermann *et al.*, 1992). It was shown that *lacZ* as a reporter marker is not compatible with a high copy number vector but suitable for expression monitoring in monocopy (Purvis *et al.*, 1987). As we only wanted to compare the strength of different promoters and avoid gene copy number variations, we used *lacZ* on an integrative plasmid pSF011 for this comparison.

First we compared the glucose-based promoters with each other. The expression of *lacZ* controlled

Table 2. Activities (%) of the promoters used in this study

Time (hours)	pADH1	pHXT7	pPGK1	pPYK1	pTPII	pTDH3	pTEF1	Glucose (g/l)	Ethanol (g/l)
8	20	10	100	60	60	100	100	12.88	0.44
24	27	109	52	27	31	31	156	nd	0.96
48	14	150	45	14	27	27	136	nd	0.42

The activities were normalized by setting pTEF1 activity at 8 h to 100%.

nd, not detected.

by these promoters was assayed 8, 24 and 48 h after inoculation in shake flasks with 2% glucose. The results are shown in Table 2. Since the TEF1 promoter is one of the strongest constitutive promoters (Gatignol *et al.*, 1990), and since it showed the most stable and highest activity at different time points, we chose to set the pTEF1 activity at 8 h as 100% and compared the activity of the other promoters relative to pTEF1 activity at this time point. The results showed that after 8 h pPGK1 and pTDH3 had the same activity as pTEF1. pTPII and pPYK1 showed 60% of the activity of pTEF1. The activity of pADH1 and pHXT7 was 20% and 10% of the pTEF1 activity, respectively.

After 24 h, ADH1, TPII, PYK1 and TDH3 promoters showed a decrease in expression and their activities were in the range 27–31% of the pTEF1 activity at 8 h. Hauf *et al.* (2000) compared TPII, PGK1, ENO1, PYK1, PDC1 and ADH1 promoters with each other and also showed that in ethanol medium pPYK1 was the weakest, and that pTPII and pPGK1 were of similar activity in ethanol medium. Adh1p is responsible for ethanol production during growth on glucose (Young *et al.*, 1982) and ADH1 expression was shown to be reduced when cells enter the ethanol growth phase or during growth on non-fermentable carbon sources (Denis *et al.*, 1983). However, Ruohonen *et al.* (1995) showed that short and middle-sized fragments of the ADH1 promoter kept their activity during the ethanol phase. The activity of pPGK1 also decreased, whereas pHXT7 activity increased continuously until 48 h, when it reached 150% of the initial TEF1 activity (Table 2). Our results obtained for the HXT7 promoter can be explained by previous investigations defining Hxt7p as a high-affinity hexose transporter which is highly expressed at low glucose concentration (<4.4 mM) (Reifenberger *et al.*, 1995; Sedlak and Ho, 2004). At the last time point, after 48 h, the activity of

most promoters did not change significantly compared with the previous measurement, except that pADH1 and pPYK1 activities decreased and both promoters were considered as the weakest promoters in this study.

In conclusion, we observed that the promoter activity varied with the glucose concentration and whether the cells were growing on glucose or ethanol. Taken together, the promoter activities, with the exception of pHXT7 and pTEF1, decreased during shake-flask cultivation and an overall ranking of the promoters is as follows:

When cells are in glucose consuming phase:

$$\begin{aligned} \text{pTEF1} &\sim \text{pPGK1} \sim \text{pTDH3} > \text{pTPII} \sim \text{pPYK1} \\ &> \text{pADH1} > \text{pHXT7} \end{aligned}$$

When glucose is exhausted and ethanol is being consumed:

$$\begin{aligned} \text{pTEF1} &\sim \text{pHXT7} > \text{pPGK1} > \text{pTPII} \sim \text{pTDH3} \\ &> \text{pPYK1} \sim \text{pADH1} \end{aligned}$$

Comparison in different types of cultivation

In order to evaluate the strength of the studied promoters with two strong and well-characterized promoters, i.e. the two galactose inducible promoters, pGAL1 and pGAL10, four promoters with different activity according to the shake-flask results, pTEF1, pTPII, pADH1 and pHXT7, were chosen and compared in batch and continuous cultures.

Comparison in batch cultivation

pTEF1, pTPII, pADH1, pGAL1 and pGAL10 activities could be detected and measured from the beginning of the exponential phase, whereas the activity of the pHXT7 promoter at this step

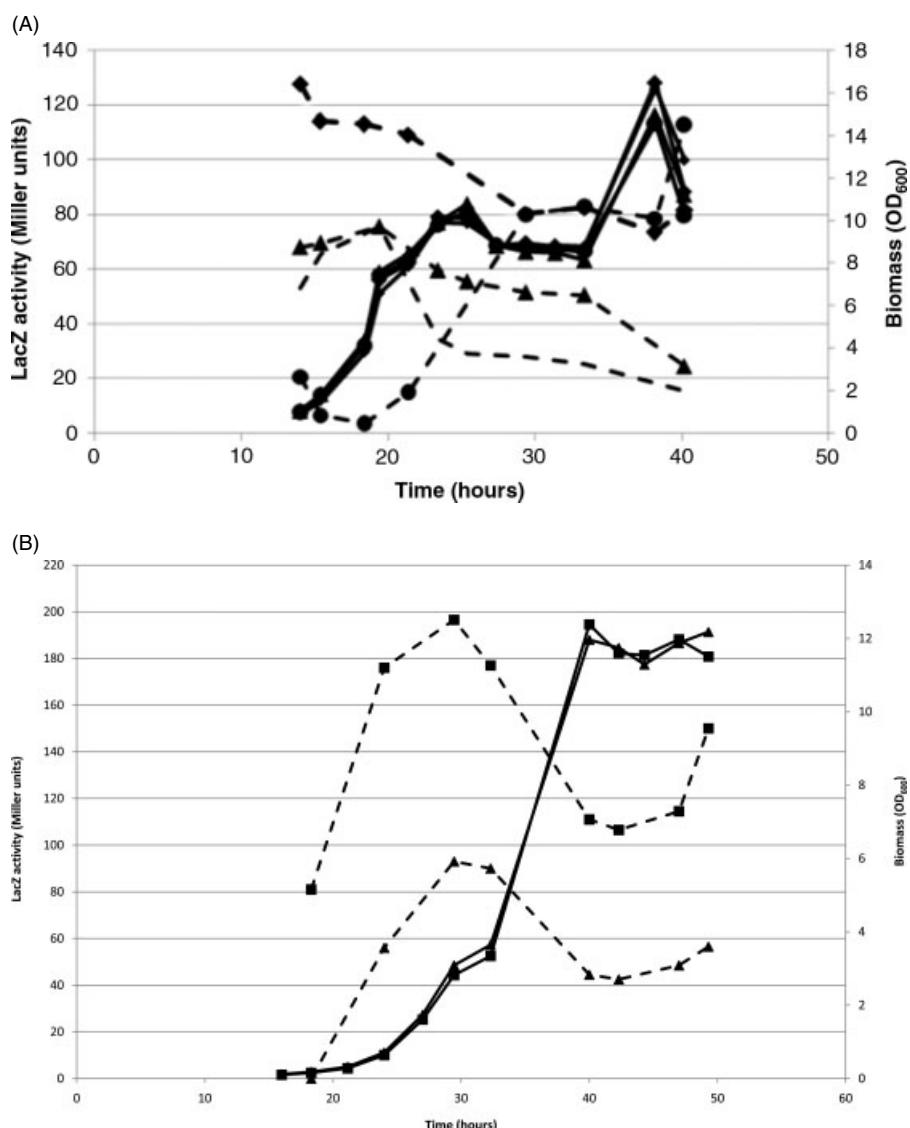


Figure 3. (A) LacZ activity and biomass profile in batch cultivation with glucose based promoters. Dashed lines, β -galactosidase activity; solid lines, biomass (OD_{600}); diamonds, pTEF1; circles, pHXT7; triangles, pTPII; no symbols, pADH1. (B) LacZ activity and biomass profile in batch cultivation with GAL1 and GAL10 promoters. Dashed lines, β -galactosidase activity; solid lines, biomass (OD_{600}); triangles, pGAL1; squares, pGAL10

was not detectable or very low and then increased. At the end of the exponential phase and in the ethanol phase, this promoter had a higher activity than pTEF1, pTPII, pADH1 and pGAL1 (Figure 3A, B). As in the results obtained from comparison of all the glucose-based promoters (Table 2), pTEF1 showed the most stable activity during the exponential and ethanol phases. This promoter also showed a high and stable activity in galactose-containing medium (data not shown).

Therefore, it seems that the activity of pTEF1 is not affected tremendously by glucose concentration or by changes in the carbon source, i.e. to galactose or ethanol, and it can therefore be characterized as a truly constitutive promoter. The activities of pGAL1 and pGAL10 decreased at the end of the exponential phase, when galactose concentration is low and in the ethanol consumption phase. A similar trend could be seen for both pTPII and pADH1 (Figure 3A, B).

Comparison in chemostat

Growth rates of *S. cerevisiae* in glucose- and galactose-containing media are significantly different in batch cultivation. In order to avoid any effect of the growth rate on the promoter strength, the four promoters p*TEF1*, p*HXT7*, p*GAL1* and p*GAL10* were compared in continuous cultures at a fixed dilution rate (0.1/h in both glucose and galactose media) and the β -galactosidase enzyme assay was performed when the cultures were in steady state. The results are shown in Figure 4.

As expected from the shake-flask results, p*HXT7* showed the highest activity among all four promoters in the chemostat (Figure 4). p*TEF1* and p*GAL10* come in second and showed similar activity levels, whereas the *GAL1* promoter was the weakest (it exhibited half of the average activity of the *GAL10* and *TEF1* promoters). The high activity of p*HXT7* in the glucose-limited chemostat is consistent with its role as a high-affinity hexose transporter (Reifenberger *et al.*, 1995; Sedlak and Ho, 2004). Expression from p*HXT7* is maximized when maintaining very low glucose concentrations in the chemostat (Figure 4). This high expression level was not observed in the shake flasks, as glucose is only present in low concentration for a short period before being exhausted (Figure 3A). As observed in the shake flasks (Figure 3B), p*GAL10* is also stronger than p*GAL1* in the chemostat and this is in contrast to previous investigations. Yocom *et al.* (1984) cloned a 914 bp fragment containing the *GAL1/GAL10* divergent promoters in front of *lacZ* into single, multicopy and integrative plasmids and

evaluated the promoter activity in different carbon sources. They showed for all conditions that *GAL1* had a two- to four-fold higher activity than *GAL10*. In another approach, West *et al.* (1987) constructed different chimeric promoter cassettes, including the upstream activating sequence (UAS) from the *CYC1* promoter and fragments of the *GAL1* or *GAL10* promoter, and they used *lacZ* as a reporter gene. Evaluating the efficiency of different regulative elements on *GAL1/GAL10* promoters, they also observed a generally much higher activity for p*GAL1* than p*GAL10*. In the last example, Cartwright *et al.* (1994) used β -lactamase as a secreted reporter in single and multicopy vectors to compare *PGK1*, *GAL1*, *GAL10*, *PHO5* and *CUP1* promoters under varying nutritional conditions. Again, the results showed that the *GAL1* promoter was more active than the *GAL10* promoter. To ensure that there was only a single integration of p*GAL10*, we tested the strains by Southern blot analysis, and this showed a single integration for both the p*GAL1* and the p*GAL10* strain (data not shown).

However, as these two promoters were cloned using different restriction enzymes (p*GAL1* cloned using *Xba*I/*Bam*HI and p*GAL10* cloned using *Not*I/*Bam*HI), the distance between the promoter and the *lacZ* gene was different in both cases. Since our cloning strategy also differed from those used in the other studies, this may have some effect on the translation efficiency.

Since the aim of this investigation was to construct a dual glucose-based expression system to replace the *GAL1/GAL10* promoters in pESC-*URA*, we needed two promoters with a similar expression profile. As the results of the first comparison (Table 2), the *PGK1* and *TDH3* promoters represent options for a promoter that can be combined with p*TEF1*. Although both of them start with the same activity as p*TEF1* after 8 h, their activities decline. After 24 h this loss of activity for the *TDH3* promoter is higher than for the *PGK1* promoter. Previous investigations by Mellor *et al.* (1985) showed that when the *PGK1* gene was cloned into a multicopy plasmid and expressed in yeast, Pgk1p accumulated to up to approximately 50% of total cell protein. Furthermore, different powerful expression vectors were constructed, based on the promoter region of the *PGK1* gene, and these vectors have been used to

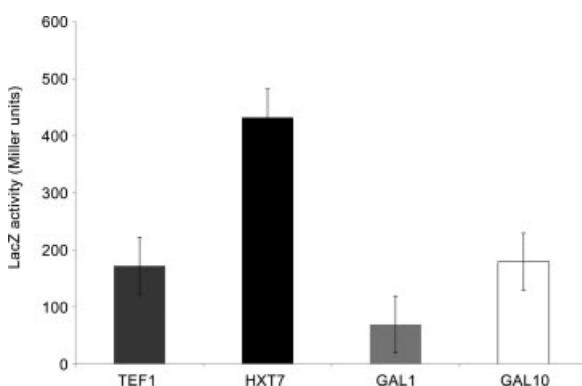


Figure 4. LacZ activity in chemostat. p*TEF1*, grey column; p*HXT7*, black column; p*GAL1*, light grey column; p*GAL10*, white column. Error bars represent SEM

study the expression of a number and heterologous genes (Dernyk *et al.*, 1983; Tuite *et al.*, 1982; Masuda *et al.*, 1994). We therefore chose the *TEF1* and *PGK1* promoters as the basis for construction of a new vector containing two bidirectional promoters.

Comparison of p*TEF1* and p*PGK1* in different contexts

A PCR fusion fragment consists of two PCR fragments which are fused together by using a pair of matched adaptamers, which contain a complementary sequence at their 5' ends (Erdeniz *et al.*, 1997). We used fusion PCR to fuse *TEF1* and *PGK1* promoters in opposite orientation to each other, and thereby constructed a nucleotide sequence containing bidirectional *TEF1–PGK1* promoter. These bidirectional promoter were cloned into pSF011 in front of the *lacZ* gene in both orientations and the resulting integrative vectors were integrated into the genome of *S. cerevisiae* CEN.PK 113-5D at the *ura3-52* locus. To evaluate the activity of p*TEF1* and p*PGK1* in the newly bidirectional promoter, we compared their activities with individual p*TEF1* and p*PGK1*, respectively, in shake flasks using the same conditions as described above. The results show that the activity of the *PGK1* promoter after fusion to p*TEF1* was not significantly different when compared with that of p*PGK1* alone (Figure 5A). p*TEF1* does not show significant change in activity after the fusion with p*PGK1* either (Figure 5B).

Final constructions

After comparison of the different promoters, we constructed four different expression vectors with bidirectional strong promoter, p*TEF1–PGK1*. Two of these constructs, pSP-G1 and pSP-G2 (Figure 2A, B), are useful for evaluating and expressing two different genes at the same time. The two different promoter orientations in pSP-G1 and pSP-G2 allow for a greater variety of cloning strategies due to the different promoter–multi-cloning site (MCS) combinations. Constitutive enzyme (α -amylase) expression with the help of these vectors has been verified (data not shown).

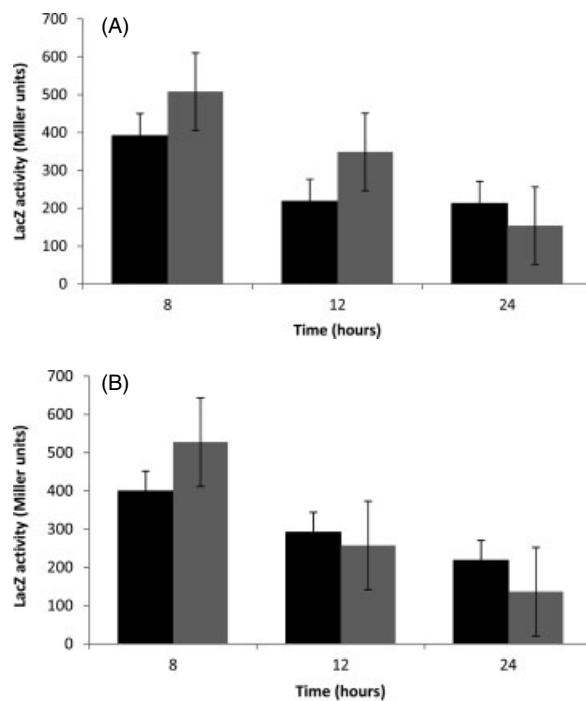


Figure 5. (A) Activity of p*PGK1* in different context. Black columns correspond to the activity of p*PGK1* alone; grey columns correspond to the activity of p*PGK1* fused to p*TEF1*. Error bars represent SEM. (B) Activity of p*TEF1* in different context. Black columns correspond to the activity of p*TEF1* alone; grey columns correspond to the activity of p*TEF1* fused to p*PGK1*. Error bars represent SEM

Conclusion

Here, the activities of seven different constitutive and glucose-based promoters, p*TEF1*, p*TPI1*, p*TDH3*, p*ADH1*, p*PGK1*, p*HXT7* and p*PYK1*, were compared with each other and also compared with the two strong galactose-inducible promoters, p*GAL1* and p*GAL10*. We used *lacZ* as reporter and the integrative plasmid pSF011 for this comparison. We further constructed a bidirectional promoter cassette consisting of p*TEF1–PGK1* and showed that the two promoters, in this context, have expression profiles similar to the corresponding isolated promoters, and can therefore support high level gene expression. We then integrated this bidirectional promoter based on p*TEF1* and p*PGK1* into an expression vector that retains all the features of the pE_{SC}–*URA* plasmid, except that gene expression is mediated by constitutive promoters. Two vectors were constructed with opposite orientation of the bidirectional promoter. Both vectors are very

useful for metabolic engineering projects that aim at high level production of valuable products using yeast as a production platform.

Our results showed varying profiles of activity for each promoter; for example, the *TEF1* promoter showed the most constant activity during fermentation and *pHXT7* represented the strongest one in continuous culture limited by glucose. We used *pPGK1* and *pTEF1* for constructing the new vector but, depending on the purpose, one can use different promoter pairs with comparable or a different expression pattern. For example, the *HXT7* promoter is suggested for fed-batch or continuous cultivation in glucose-limited conditions to reach very high gene expression levels. On the other hand, the full-length *ADH1* promoter would be suitable for conditional expression of genes at high glucose concentrations. It may therefore be a good idea to use different promoter combinations for different experiments, since it is simple to exchange the present promoters with any other promoter used in this study to change the expression level rate of the cloned genes.

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PAPER II

Enhancing the copy number of episomal plasmids in *Saccharomyces cerevisiae* for improved protein production

Yun Chen⁼, Siavash Partow⁼, Verena Siewers, Sara Bjørn,
Jens Nielsen

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= These authors contributed equally to this work

RESEARCH ARTICLE

Enhancing the copy number of episomal plasmids in *Saccharomyces cerevisiae* for improved protein production

Yun Chen, Siavash Partow, Gionata Scalcinati, Verena Siewers & Jens Nielsen

Department of Chemical and Biological Engineering, Chalmers University of Technology, Göteborg, Sweden

Correspondence: Jens Nielsen, Department of Chemical and Biological Engineering, Chalmers University of Technology, Kemivägen 10, SE-412 96 Göteborg, Sweden. Tel.: +46 31 772 3804; fax: +46 31 772 3801; e-mail: nielsenj@chalmers.se

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ubiquitin-tag; promoter; episomal plasmid; protein expression; copy number; *Saccharomyces cerevisiae*.

Abstract

2 μm-based episomal expression vectors are widely used in *Saccharomyces cerevisiae* for recombinant protein production and synthetic pathway optimization. In this study, we report a new approach to increase the plasmid copy number (PCN) and thus improve the expression of plasmid-encoded proteins. This was achieved by combining destabilization of the marker protein with decreasing the marker gene transcription level. Destabilization of the marker protein alone by fusing a ubiquitin/N-degron tag (ubi-tag) to the N-terminus of the Ura3 marker protein could increase the PCN and activity of LacZ expressed from the same vector. When arginine was exposed at the N-terminus of the marker protein after cleavage of ubiquitin, the PCN and LacZ activity were increased by 70–80%. Replacement of the native *URA3* promoter with the *HXT1*, *KEX2* or *URA3-d* promoter resulted in an increase in the PCN and LacZ activity by about 30–100%. Combining the ubi-tag and promoter modification of the marker gene, increased the PCN and LacZ activity by threefold. We also demonstrated that this new expression vectors can be used to increase enzyme activity by improving patchoulool production by threefold.

Introduction

High-level expression of exogenous or endogenous genes in microorganisms is often a desired objective with applications in protein production or to overexpress pathway enzymes leading to synthesis of, for example, chemicals or biofuels. Promoter choice and gene copy number are the most important factors to ensure the desired gene transcription levels. To achieve high copy numbers, episomal plasmid vectors are usually employed. Multi-copy plasmids for gene expression in *Saccharomyces cerevisiae* are derivatives of the native 2 μm plasmid. 2 μm-based vectors have for instance been used for the industrial production of recombinant human albumin and albumin fusion proteins from *S. cerevisiae* at up to 5 g L⁻¹ soluble protein in culture supernatants (Finnis *et al.*, 2005). For the native 2 μm plasmid, it was shown that replication is under strict cell cycle control (Zakian *et al.*, 1979). It is, however, possible to increase the copy number of certain 2 μm-derived vectors by over-expression of *FLP1* or *RAF1* that are involved in regulation of the plasmid amplification process (Murray *et al.*, 1987; Som *et al.*, 1988) or disruption of *UBC4* that plays a role in the pro-

teolytic degradation pathway (Sleep *et al.*, 2001). These strategies would not be applicable to the most frequently used 2 μm expression plasmids, which only contain one FRT site. In this study, we report that the normal 2 μm-based plasmid copy number can be increased by employing a ubiquitin/N-degron-tag (ubi-tag) system combined with modulation of the promoter strength of the selective marker gene.

Ubiquitin is one of the most conserved proteins, and the ubiquitin-proteasome system is responsible for the major cellular proteolytic process in eukaryotic cells (Lee & Goldberg, 1998). Ubiquitin is a 76-residue protein that is covalently conjugated to a lysine residue of the target protein prone to degradation, followed by the formation of a multi-ubiquitin chain and target protein degradation by the 26S proteasome. It has been shown that a single molecule of ubiquitin fused to the N-terminus of a protein is cleaved off *in vivo*, making it possible to generate proteins with N-terminal amino acids (proline being the single exception) other than methionine (Bachmair *et al.*, 1986). A set of certain destabilizing N-terminal amino acid residues along with a proximal lysine residue comprise a degradation signal termed an N-degron. The *in*

vivo half-life of a protein carrying an N-degron signal is mediated by the ubiquitin-dependent N-end rule pathway and can differ with time scales from minutes to several hours that depend on the destabilizing N-terminal residue, which is recognized by the polyubiquitination machinery (Worley *et al.*, 1998; Dantuma *et al.*, 2000). In yeast, the tertiary destabilizing residues glutamine (Gln) and asparagine (Asn) are deaminated by N-terminal amidohydrolase to yield the secondary destabilizing residues glutamate (Glu) and aspartate (Asp). Arg-tRNA-protein transferase then catalyzes the conjugation of the primary destabilizing residue arginine (Arg) to Glu and Asp, respectively (Varshavsky, 1996). We hypothesized that it could be possible to use this system to modulate the stability of a plasmid-encoded selectable marker protein and thus regulate the plasmid copy number. In this study, different types of destabilizing residues, which were supposed to yield varying half-lives of the marker protein, were chosen, and the effect of destabilized marker protein on the plasmid copy number was tested.

The expression level of the selection marker gene is also an important factor to influence the plasmid copy number. It has been reported that using a heterologous gene including its original promoter as a marker, which has a potentially lower expression level than its *S. cerevisiae* counterpart, can be advantageous to achieve high plasmid copy numbers and high protein expression levels (Liu *et al.*, 2012). Down-regulating the transcription level of a native marker gene can also be achieved by employing a regulatable (Compagno *et al.*, 1993) or a truncated promoter (Erhart & Hollenberg, 1983; Loison *et al.*, 1989; Piper & Curran, 1990). The promoter of *KEX2*, encoding a Ca²⁺-dependent serine protease (Fuller *et al.*, 1989) has been reported to be a constitutive promoter with very low activity (Nacken *et al.*, 1996). The conditional promoter of *HXT1* encoding a low affinity hexose transporter shows a low level of activity when a low concentration of a fermentable carbon sources is available, that is, transcription is expected to be repressed during late exponential growth phase and during a glucose-limited chemostat cultivation (Diderich *et al.*, 1999). It has also been demonstrated that the truncated version of the *URA3* promoter, the promoter of the *URA3-d* allele including only 47 nucleotides located upstream of the start codon revealed a lower activity in comparison with the full-length *URA3* promoter (Loison *et al.*, 1989; Faulkner *et al.*, 1994). Thus, in this study, the influence of using these three promoters to guide marker gene expression on the plasmid copy number was evaluated.

Finally, new 2 µm-based vectors were developed by modifying the marker protein expression through promoter exchange and protein destabilization increasing the plasmid copy number by threefold. We also demonstrate

that these new expression vectors can be used to increase enzyme activity for heterologous pathway expression improving patchoulool production by threefold.

Materials and methods

Strains and media

Saccharomyces cerevisiae strain CEN.PK 113-5D (*MATa SUC2 MAL2-8c ura3-52*; kindly provided by P. Kötter, University of Frankfurt, Germany) (Van Dijken *et al.*, 2000) was used as a background strain. All strains used in this study are summarized in Table 1. *Saccharomyces cerevisiae* transformants were selected on synthetic dextrose (SD) medium containing 6.7 g L⁻¹ of yeast nitrogen base w/o amino acids (Difco Laboratories, Sparks, MD), 0.77 g L⁻¹ of complete supplement mixture (CSM, w/o uracil) (MP Biomedicals, Solon, OH) and 2% glucose. Yeast strains were grown in defined minimal medium (Verduyn *et al.*, 1992) with 20 g L⁻¹ glucose. *Escherichia coli* DH5α was used for general cloning procedures, and strains were cultured in Luria Bertani (LB) broth with 80 mg L⁻¹ ampicillin.

Plasmid and strain construction

All plasmids constructed are based on the vector pSP-GM1 carrying constitutive promoters *P_{TEF1}* and *P_{PGK1}* as well as *URA3* as a marker gene. pSP-GM1 was generated from pSP-G1 (Partow *et al.*, 2010, derived from pESC-URA (Agilent Technologies, Santa Clara). To provide additional restriction enzyme recognition sites downstream of each terminator (*T_{ADH1}* and *T_{CYCI}*, respectively), the entire expression cassette of pSP-G1 was amplified by PCR using primers 22 and 23, cut with *Pvu*II and ligated back into the vector backbone to generate pSP-GM1. The primers used for amplification are listed in Table 2.

Genomic DNA was purified from CEN.PK 113-5D using the Fast DNA Spin Kit for Soil (MP Biomedicals). A 390-bp fragment containing the *URA3* promoter (*P_{URA3}*) was amplified from plasmid pSP-GM1 using primers 1 and 2. Four 228-bp DNA fragments (Ubi-X) encoding ubiquitin were amplified from genomic DNA of CEN.PK 113-5D by PCR using primer 3 as forward primer and either primer 4, 5, 6 or 7 as reverse primer, to generate arginine (R), methionine (M), glutamate (E) and glutamine (Q) as N-termini of the Ura3 marker protein and a 11 amino acid linker (Heessen *et al.*, 2003). A 0.7-kb partial coding sequence of *URA3* (*URA3*) was amplified from plasmid pSP-GM1 using primers 8 and 9. Fragment pairs *P_{URA3}/Ubi-R*, *P_{URA3}/Ubi-M*, *P_{URA3}/Ubi-E* and *P_{URA3}/Ubi-Q* were fused via PCR. Each of the resulting

Table 1. List of plasmids and *Saccharomyces cerevisiae* strains used in this study

	Description	Origin
Plasmids		
pSP-G1	$P_{TEF1}\text{-}P_{PGK1}$ bidirectional promoter (2 μm <i>URA3</i>)	Partow et al. (2010)
pIYC32	pSP-GM1 $P_{PGK1}\text{-}lacZ$	This study
pIYC33	pSP-GM1 $P_{URA3}\text{-}Ubi\text{-}R\text{-}URA3 P_{PGK1}\text{-}lacZ$	This study
pIYC34	pSP-GM1 $P_{URA3}\text{-}Ubi\text{-}M\text{-}URA3 P_{PGK1}\text{-}lacZ$	This study
pIYC35	pSP-GM1 $P_{URA3}\text{-}Ubi\text{-}E\text{-}URA3 P_{PGK1}\text{-}lacZ$	This study
pIYC36	pSP-GM1 $P_{URA3}\text{-}Ubi\text{-}Q\text{-}URA3 P_{PGK1}\text{-}lacZ$	This study
pISP19	pSP-GM1 $P_{HXT1}\text{-}URA3 P_{PGK1}\text{-}lacZ$	This study
pISP20	pSP-GM1 $P_{KEX2}\text{-}URA3 P_{PGK1}\text{-}lacZ$	This study
pISP21	pSP-GM1 $P_{URA3d}\text{-}URA3 P_{PGK1}\text{-}lacZ$	This study
pIYC45	pSP-GM1 $P_{HXT1}\text{-}Ubi\text{-}R\text{-}URA3$	This study
pIYC46	pSP-GM1 $P_{KEX2}\text{-}Ubi\text{-}R\text{-}URA3$	This study
pIYC47	pSP-GM1 $P_{HXT1}\text{-}Ubi\text{-}R\text{-}URA3 P_{PGK1}\text{-}lacZ$	This study
pIYC48	pSP-GM1 $P_{KEX2}\text{-}Ubi\text{-}R\text{-}URA3 P_{PGK1}\text{-}lacZ$	This study
pIYC03	pSP-GM1 $P_{TEF1}\text{-}Pat_{OPT}\text{-}P_{PGK1}\text{-}tHMG1$	This study
pIYC49	pIYC46 $P_{TEF1}\text{-}Pat_{OPT}\text{-}P_{PGK1}\text{-}tHMG1$	This study
Strains		
CEN.PK	MAT α <i>SUC2</i> <i>MAL2-8c</i> <i>ura3-52</i>	P. Kötter, University of Frankfurt, Germany
113-5D		
SCISP23	CEN.PK 113-5D, pISP19	This study
SCISP24	CEN.PK 113-5D, pISP20	This study
SCISP25	CEN.PK 113-5D, pISP21	This study
SCIYC58	CEN.PK 113-5D, pIYC32	This study
SCIYC59	CEN.PK 113-5D, pIYC33	This study
SCIYC60	CEN.PK 113-5D, pIYC34	This study
SCIYC61	CEN.PK 113-5D, pIYC35	This study
SCIYC62	CEN.PK 113-5D, pIYC36	This study
SCIYC68	CEN.PK 113-5D, pIYC47	This study
SCIYC69	CEN.PK 113-5D, pIYC48	This study
SCIYC72	CEN.PK 113-5D, pIYC03	This study
SCIYC76	CEN.PK 113-5D, pIYC49	This study

fragments $P_{URA3}\text{-}Ubi\text{-}X$ was then fused by PCR to fragment *URA3*, yielding a cassette $P_{URA3}\text{-}Ubi\text{-}X\text{-}URA3$. All cassettes were cut with *Nde*I and *Nco*I, and cloned into the *Nde*I/*Nco*I sites of pSP-GM1 to construct pIYC24, pIYC25, pIYC26 and pIYC27, respectively. The constructs were checked by sequencing. The *lacZ* gene encoding β -galactosidase was cloned from pSF011 (Flagfeldt *et al.*, 2009) into pSP-GM1 and pIYC24 to pIYC27 using BamHI/Bsp1407I. This resulted in plasmids pIYC32 to pIYC36.

P_{HXT1} (1122 bp) and P_{KEX2} (476 bp) promoters were amplified by PCR using CEN.PK 113-5D genomic DNA as template and primer pairs 16/17 and 18/19, respectively, while pSP-GM1 and primer pair 20/21 were used for amplifying the 47 nucleotides upstream of the *URA3* gene (the *URA3-d* promoter) together with the first 465 bp of its coding region. Two 35 nt flanking sequences were added at both ends of the PCR products of P_{HXT1} and P_{KEX2} through the PCR amplification. These flanking regions were complementary to the region upstream and downstream of the native *URA3* promoter on pSP-GM1. The upstream 35 nt flanking sequence was also added at the upstream region of

the PCR product, which contained the *URA3-d* promoter. Gap repair cloning was employed for replacing the *URA3* promoter on the plasmid with P_{HXT1} , P_{KEX2} and P_{URA3-d} , respectively. *Pst*I/*Nde*I restricted pSP-GM1 and each PCR-amplified promoter were transformed simultaneously into yeast strain CEN.PK 113-5D, using the standard lithium acetate method (Gietz & Woods, 1998). The circular plasmids resulting from homologous recombination between linearized pSP-GM1 and the PCR fragments were isolated from yeast cells using the Zymoprep yeast plasmid mini-prep II kit (Zymo Research, Irvine, CA) and transformed into *E. coli* (DH5 α). The resulting plasmids pISP16 to pISP18 were sequenced for further confirmation. The *lacZ* gene was cloned into pISP16 to pISP18 as described above to obtain plasmids pISP19 to pISP21.

To construct a plasmid with ubiquitin-tag and marker gene promoter replacement, a 327-bp fragment (P_{KEX2}) that contained part of the *KEX2* promoter was amplified from plasmid pISP16 using primers 10 and 11. A 750-bp fragment (P_{HXT1}) containing part of the *HXT1* promoter was amplified from plasmid pISP17 using primers 12 and 13. Two 0.9-kb fragments (K-Ubi-R-*URA3* and H-Ubi-R-

Table 2. List of primers using in this study

Primer #	Sequence (5'-3')
1	AGACGGTCACAGCTTGTGTC
2	GACGAAAATCTGCATTGTTTATTATCTTCGTTCTG
3	ACGAAGATAAATAAAACAAATGCAGATTTCTGCAAGAC
4	GTTGTCGACCAAGCTCCCGGACCCACCTCTAGCCTAGCAC
5	GTTGTCGACCAAGCTCCCCATACCACCTCTAGCCTAGCAC
6	GTTGTCGACCAAGCTCCCTGACCACCTCTAGCCTAGCAC
7	GTTGTCGACCAAGCTCCCCCTACCCACCTCTAGCCTAGCAC
8	GGTCACACCGGTCGCCACCATGTCGAAAGCTACATATAAG
9	CACATCATCACGGTTCT
10	AAAGGAAAGGTTAGCATA
11	GACGAAAATCTGCATATCTGATAATGGGTTAGTAG
12	GCTCGGATTATCTTCATT
13	GACGAAAATCTGCATGATTTACGTATATCAAC
14	CCATTATCAGATATGCAGATTTCTGCAAGAC
15	ATACGTAAAATCATGCAGATTTCTGCAAGAC
16	ATTGTAATGAGTCGACCATACACAGCTTCATGCAGGTCTCATCTGAAATATAATTCC
17	AGTAGCAGCACGTTCTTATATGTTAGCTTCGACATGATTTACGTATATCAACTAGTTGACGATTATG
18	ATTGTAATGAGAGTCGACCATACACAGCTTCAGTAGATACACGTATCTGACA
19	AGTAGCAGCACGTTCTTATATGTTAGCTTCGACATCTGATAATGGGTTAGTAGTT
20	ATTGTAATGAGAGTCGACCATACACAGCTTCATAACCCAACTGCACAGAAC
21	GCATGACAATTCTGCTAACATC
22	GAACAACAGCTGGATAAAGGCGCGCAAACGACCTAGGAATTGGAGCGACCTCATGCTATAC
23	GAACAACAGCTGGATAAACGCCGGCAAACGATCCGGAGGATCTCGAGCGCCAAAAC
24	GCTATGTGGCGCGGTATTAT
25	AAGTTGGCCGCAGTGTATC
26	ATCAAACAGAGAAAAGATGACTCAAAT
27	ATCAAGTAGTCAGTCACATCTCACCG

URA3) were amplified from plasmid pIYC24 using primer pairs 14/9 and 15/9. Fragment pairs P_{KEX2}/K-Ubi-R-*URA3* and P_{HXT1}/H-Ubi-R-*URA3* were fused to each other via PCR. The resulting fragment P_{KEX2}-Ubi-R-*URA3* was then restricted with NdeI and NcoI, and cloned into the NdeI/NcoI sites of pISP17 to construct pIYC45. Fragment P_{HXT1}-Ubi-R-*URA3* was then digested with PstI and NcoI, and cloned into the PstI/NcoI sites of pISP16 to construct pIYC46. The plasmids were confirmed by sequencing. The *lacZ* gene was introduced into pIYC45 and pIYC46 using BamHI/Bsp1407I, to construct plasmids pIYC47 and pIYC48, respectively.

Yeast strain CEN.PK 113-5D was transformed with four different ubi-tag plasmids (pIYC33 to 36) and three different promoter exchange plasmids (pISP19 to 21), respectively. Table 1 lists the names and descriptions of all strains used in this study. Two colonies from each transformation were selected for evaluation.

Batch and chemostat cultivation

Strains were grown in defined minimal medium containing 20 g L⁻¹ glucose. Five hundred milliliters of medium was used as working volume in a 1 L bioreactor (DasGip, Jülich, Germany) operated at 30 °C, 600 r.p.m. agitation

and 1 vvm air flow. The pH was maintained at 5 by controlled addition of 2 M KOH. Bioreactors were inoculated to an OD_{600 nm} = 0.01 from late exponential phase shake flask cultures, and the OD_{600 nm} was measured throughout the cultivation. Dry cell weight (DCW) was measured by filtering 5 mL of culture broth through a 0.45 µm nitro-cellulose filter and measuring the increased weight of the dry filter. Glucose, ethanol, glycerol and acetate were measured using a Summit HPLC (Dionex, Sunnyvale, CA) with an Aminex HPX-87H column (Bio-Rad, Hercules, CA). Carbon dioxide and oxygen levels were measured in the off-gas, and dissolved oxygen was monitored.

For chemostat cultivations, the medium composition was the same as the one used in batch cultivations except that the glucose concentration was 10 g L⁻¹. The dilution rate was adjusted to 0.1 h⁻¹. Samples were taken after more than 5 doubling times in the steady-state phase. Duplicate fermentations were carried out for each strain.

β-galactosidase assay

β-galactosidase activity was measured as described before (Partow *et al.*, 2010). Samples were diluted when needed before re-suspension in Z buffer to avoid short reaction times.

Determination of plasmid copy number by qPCR

The phenol/chloroform DNA extraction method (Hoffman & Winston, 1987) was employed for isolating total DNA from frozen samples. Samples contained 5 mL cell suspension collected at different time points during continuous cultivation and were kept at -20°C for < 1 month.

The *bla* ampicillin resistance gene encoding beta-lactamase and *ACT1* encoding actin were selected as target and reference gene, respectively. Both genes are present in a single copy on the plasmid (pSP-GM1 and its derivatives) and the yeast genome, respectively (Ng & Abelson, 1980; Partow *et al.*, 2010). Oligonucleotide primers for qPCR were designed with Primer 3 (Version 0.4.0; Rozen & Skaletsky, 2000). Criteria used for primer design were a predicted melting temperature T_m of 60°C and an amplicon length between 200 and 250 bp. Optimal primer concentrations were determined by analyzing the C_q values at three different primer concentrations using the same DNA template concentration. Potential primer-dimerization reactions and formation of unspecific amplicons were excluded via melting curve analysis and gel electrophoresis.

The Stratagene Mx3005P instrument (Agilent) was used for qPCR analysis. qPCRs were prepared in duplicates of 20 μL reaction mixtures in Agilent optical 96-well reaction plates with lid. Each reaction well contained 5 μL of template DNA (equal to 5 ng), the concentration of which had been determined by absorbance at 260 nm with a Bio Photometer (Eppendorf, Hamburg, Germany), 10 μL of Brilliant II SYBR[®] Green QPCR Master Mix (Agilent Technologies), and 200 nM each of forward and reverse primer. Negative control reactions were set up by substituting the template DNA with H₂O and routinely showed a high C_q values (usually more than 30), which represented the lower detection limit. The PCR program was set for 40 cycles. Each cycle contained three steps, 95 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min.

The copy number of the plasmid (pSP-GM1) and the yeast genomic DNA standards were calculated using the following equation (Whelan *et al.*, 2003):

$$\begin{aligned} \text{copy number} = & [6.02 \times 10^{23} (\text{mol}^{-1}) \\ & \times \text{DNA amount (g)}] / [\text{DNA length (bp)} \\ & \times 660 (\text{g/mol} \times \text{bp})] \end{aligned}$$

Serial 10-fold dilutions, each ranging from 2×10^3 to 2×10^7 copies of DNA, of both plasmid and genomic DNA were analyzed in triplicates to establish the standard curves. The standard curve is a plot of the threshold cycle (C_q) vs. the log 10 of the concentration. C_q values of all

samples were determined after setting the threshold line based on the amplification plot in a semi-logarithmic scale. The PCR efficiency was calculated from the slope of the standard curve. Absolute plasmid copy numbers (PCN) were determined by relating the C_q values of any unknown sample to the standard curves using the equation of the standard curve trend line (Yu *et al.*, 2005).

Patchoulol production and quantification

For patchoulol production, 20 mL cultures were grown in 100 mL Erlenmeyer flasks by inoculating them to an optical density of 0.02 at 600 nm (OD_{600 nm}). The strains were grown at 30 °C with 180 r.p.m. orbital shaking in defined minimal medium. Ten per cent (v/v) dodecane was added when the culture had reached an OD_{600 nm} of 1 to capture patchoulol in the organic phase. Samples from the organic layer were centrifuged for 5 min at 2600 g and subsequently analyzed by GC-MS to determine the level of patchoulol during the course of fermentation as described previously (Asadollahi *et al.*, 2008).

Results and discussion

Destabilization of the marker protein by fusion to a ubiquitin/N-degron tag

Selective marker proteins are normally stable enough to sustain the enzyme levels required for normal growth under selective conditions. Changing the N-terminal methionine with another amino acid can lead to changes in the *in vivo* half-life of a protein (Bachmair *et al.*, 1986). Modification of the N-terminus of a selective marker protein encoded on an episomal plasmid may therefore increase the plasmid copy number and in consequence also increase the expression of a protein of interest encoded on the same vector. To validate this hypothesis, pSP-GM1, a 2 μm -based plasmid was modified by fusing a DNA sequence encoding a mono-ubiquitin and different N-degron signals to the marker gene (*URA3*). The first codon of the N-degron sequence was chosen to encode either methionine (M), arginine (R), glutamate (E) or glutamine (Q), which represent stabilizing, primary, secondary and tertiary destabilizing residues, respectively, resulting in four ubiquitin/N-degron-tagged (ubi-tag) vectors. To evaluate the influence of this modification on protein expression, reporter gene *lacZ* encoding β -galactosidase was inserted into the multiple cloning regions of these vectors as well as control plasmid pSP-GM1, under the control of the *PGK1* promoter. After each of the 5 plasmids thus designed (pIYC32 – pIYC36, Fig. 1, Table 1) had been introduced into the yeast strain

CEN.PK 113-5D, resulting in strains SCIYC58 – SCIYC62 (Table 1), analysis of the LacZ activity was performed in glucose-limited continuous culture with a dilution rate of 0.1 h^{-1} , to avoid any effect of changes in cell physiology on the plasmid copy number.

No apparent difference in growth characteristics and morphology was observed between the ubi-tag plasmid containing strains and the control strain SCIYC58 (harboring control plasmid pIYC32) (data not shown). However, the β -galactosidase enzyme assay revealed that SCIYC59 (containing pIYC33 with a ubi-R tag) showed significantly higher (74%) LacZ activity, compared to the strain containing the control plasmid (Fig. 2a). In contrast, when methionine, glutamate or glutamine was exposed at the N-terminus of the Ura3 marker, the LacZ activities were only slightly increased compared with the original plasmid (Fig. 2a). This phenotype implies that gene dosage of lacZ was clearly increased only when the N-terminus of fusion Ura3 protein was changed from methionine to arginine, yielding the highest improvement in LacZ activity. This was confirmed by determining the plasmid copy number (PCN) using qPCR (Fig. 2b). As can be seen in this figure and in accordance with the LacZ activity, SCIYC59 pictured a higher plasmid copy number (≈ 7.5 plasmids per cell) compared to the other ubi-tag plasmid containing strains (SCIYC60 to 62) and

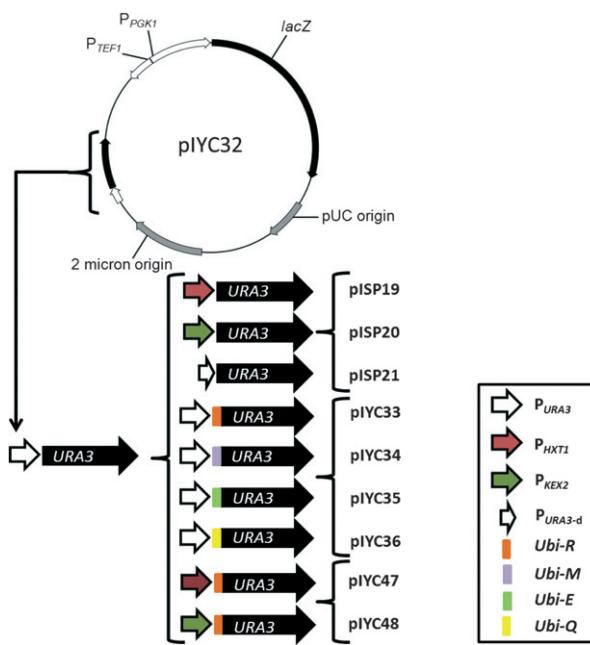


Fig. 1. Schematic representation of the plasmid constructs. P_{URA3} , P_{HXT1} , P_{KEX2} and P_{URA3-d} promoters employed to control $URA3$ expression; *Ubi-R*, *Ubi-M*, *Ubi-E* and *Ubi-Q*, ubiquitin/N-degron tags leading to arginine, methionine, glutamate and glutamine as N-terminal residues of the Ura3 marker protein. See details in the text.

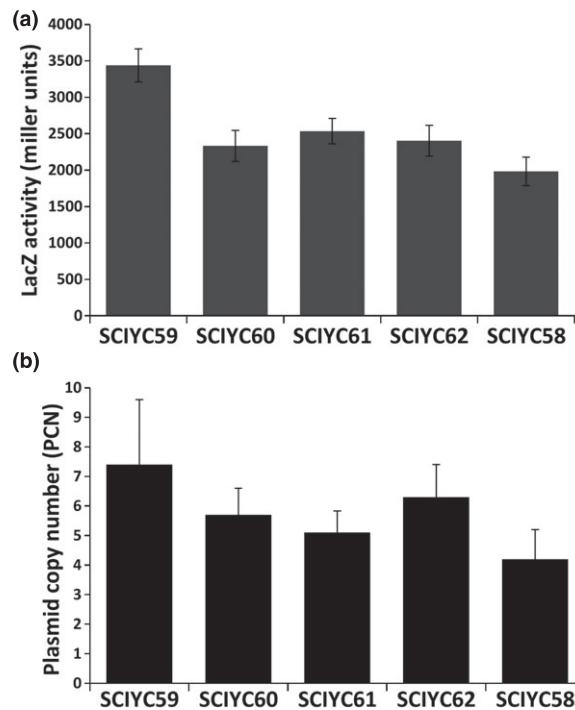


Fig. 2. Effect of different ubiquitin/N-degron-tags on LacZ activity (a) and plasmid copy number (b). Samples were taken from continuous culture. Strains used were SCIYC59 ($P_{URA3}-Ubi-R-URA3$), SCIYC60 ($P_{URA3}-Ubi-M-URA3$), SCIYC61 ($P_{URA3}-Ubi-E-URA3$), SCIYC62 ($P_{URA3}-Ubi-Q-URA3$) and control strain SCIYC58 ($P_{URA3}-URA3$). The data shown represent the mean \pm SD from four data points.

to the control strain (SCIYC58), showing an average of 5.7, 5.1, 6.3 and 4.2 plasmids per cell, respectively. The consistent increase in β -galactosidase activity and plasmid copy number for strain SCIYC59 (Table 3) thus implied that tagging ubiquitin to the plasmid-encoded marker protein with a modified N-terminus affected the stability of the marker protein, leading to changing levels of plasmid copy number and consequently yielding an increase

Table 3. Comparison of fold changes in LacZ activity and plasmid copy number (PCN)

Strain (plasmid)	Plasmid description	Fold change LacZ activity	PCN
SCIYC58 (pIYC32)	$P_{URA3}-URA3$	1.00	1.00
SCIYC59 (pIYC33)	$P_{URA3}-Ubi-R-URA3$	1.74	1.80
SCIYC60 (pIYC34)	$P_{URA3}-Ubi-M-URA3$	1.18	1.40
SCIYC61 (pIYC35)	$P_{URA3}-Ubi-E-URA3$	1.28	1.20
SCIYC62 (pIYC36)	$P_{URA3}-Ubi-Q-URA3$	1.21	1.50
SCISP23 (pISP19)	$P_{HXT1}-URA3$	1.91	2.00
SCISP24 (pISP20)	$P_{KEX2}-URA3$	1.49	1.60
SCISP25 (pISP21)	$P_{URA3-d}-URA3$	1.31	1.80
SCIYC68 (pIYC47)	$P_{HXT1}-Ubi-R-URA3$	2.61	3.50
SCIYC69 (pIYC48)	$P_{KEX2}-Ubi-R-URA3$	3.07	3.00

in target protein expression. It was observed that only arginine, not glutamate or glutamine, exposed at the N-terminus of the marker protein (*Ura3*) and showed a significant higher effect on the copy number. This is probably due to the fact that arginine at the N-terminus of a given protein leads to a shorter half-life than glutamate and glutamine. It has been reported by Bachmair *et al.* (1986) that the *in vivo* half-life of R- β -galactosidase, that is, β -galactosidase containing an arginine at its N-terminus, was the shortest of all constructs tested in their study, approximately 2 min. Although also the E-*Ura3* and Q-*Ura3* modifications may have led to less stable marker proteins, the protein levels could still have been high enough to sustain normal growth and thus did not lead to an increase in plasmid copy numbers. Besides the N-terminal amino acid, other factors can influence the strength of an N-degron signal, such as the positioning of lysine residues in the N-terminal region of the protein that represent possible targets for polyubiquitination (Suzuki & Varshavsky, 1999) or the nature of the amino acid at position 2 involved in binding to ubiquitin ligase Ubr1 (Choi *et al.*, 2010). Optimization of these factors could lead to a further increase in plasmid copy number.

Change of marker gene promoter

To modulate the transcription of the selection marker gene, the promoter region of *URA3* was replaced by the conditional *HXT1* promoter, the constitutive weak *KEX2* promoter and the truncated version of the *URA3* promoter (*URA3-d*) including only 47 nucleotides located upstream of start codon, respectively. As above, the *lacZ* gene was inserted in these vectors generating pISP19 to 21 (Fig. 1, Table 1). Yeast strain CEN.PK113-5D was transformed with these plasmids resulting in strains SCISP23, SCISP24 and SCISP25, respectively (Table 1). All strains were grown and compared in glucose-limited continuous culture with a dilution rate of 0.1 h⁻¹. LacZ enzyme activity assay and qPCR were performed to evaluate the effect of the marker gene promoter on the plasmid copy number.

Unlike for the ubi-tag plasmid containing strains, differences in growth characteristics were observed for the strains harboring the promoter exchange plasmids in chemostat. However, all strains had approximately the same specific growth rate in batch culture ($\mu_{\max} = 0.32$). Strain SCISP23 carrying the *P_{HXT1}-URA3* plasmid indicated a lower final biomass formation (≈ 2.8 g L⁻¹) in comparison with strains SCISP24, SCISP25 and SCIYC58 (≈ 5 g L⁻¹) (data not shown). The observed decrease in biomass production may be the results of the high repression level of *URA3* exerted by the *HXT1* promoter under glucose limitation, not providing sufficient *Ura3* protein to maintain higher growth rates or to elevate the dilution

rate. This is in consistence with the results observed in auxotrophic yeast strains during uracil-limited chemostat culture (Basso *et al.*, 2010).

Figure 3a shows the results of the LacZ enzyme assay. Strains SCISP23 to 25 indicated an increase in the LacZ activity in comparison with the control strain (SCIYC58). The LacZ activity was elevated in strains SCISP23 to 25 by 90%, 50% and 30%, respectively. Similar to the LacZ activity results, an increased plasmid copy number (up to two-fold increase) was detected for strains SCISP23 to 25 in comparison with the control strain (SCIYC58) (Fig. 3b). The fold changes in LacZ activity and plasmid copy number in strains SCISP23 to SCISP25 are shown in Table 3. Thus, all three promoter replacements were able to result in increased copy numbers. Low expression of a selection marker gene is supposed to exert pressure on the cell to increase the plasmid copy number because of the requirement for sufficient levels of its essential product. Our results are consistent with previous studies, which showed an inverse relation between marker gene promoter activity and plasmid copy number (Piper & Curran, 1990). Faulkner *et al.* (1994) had been able to improve the plasmid copy number 3 times by replacing the *URA3* marker with *URA3-d*. In contrast, we only saw a slight increase in copy number using *P_{URA3-d}*. Such differences as well as the very large variations in copy numbers observed for 2 μ m-based plasmids by different groups (e.g., Faulkner *et al.*, 1994; Geymonat *et al.*, 2007; Krogh *et al.*, 2008; Fang *et al.*, 2011) may well be caused by differences in plasmid size and backbone as well as the background strain. It has furthermore been shown that the expression of a plasmid-encoded gene – in our case the *lacZ* reporter – can influence the plasmid copy number (Fang *et al.*, 2011). Strain SCISP23 harboring pISP19 (*P_{HXT1}*) showed the highest increase in LacZ activity and in plasmid copy number in glucose-limited continuous culture. This result is in agreement with the nature of the *HXT1* promoter activity, which is repressed under low glucose conditions (Ozcan & Johnston, 1999).

Combination of ubi-tag and promoter exchange

Destabilization of the marker protein and weakening the transcription of the marker gene both showed an improvement in plasmid copy number and expression of the target protein. In addition, both modifications did not cause significant changes in growth rate and biomass yield (except for *P_{HXT1}-URA3* under glucose limitation conditions) of the plasmid-carrying cells, which means that the metabolic burden caused by a higher plasmid copy number and the decreased activity of the essential marker protein did not impair too much cellular processes, implying the possibility to further increase protein

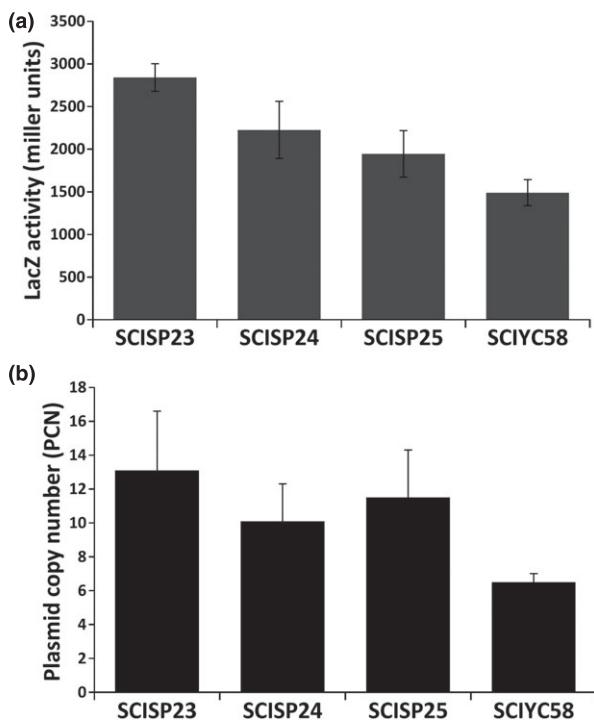


Fig. 3. Effect of promoter exchange on LacZ activity (a) and plasmid copy number (b). Samples were taken from continuous culture. Strains used were SCISP23 (P_{HXT1} -URA3), SCISP24 (P_{KEX2} -URA3), SCISP25 (P_{URA3d} -URA3) and control strain SCIYC58 (P_{URA3} -URA3). The data shown represent the mean \pm SD from four data points.

expression. Thus, each of the two promoter modifications showing highest increase in protein expression (P_{HXT1} -URA3 and P_{KEX2} -URA3) was combined with the ubi-R tag resulting in plasmids pIYC45 and pIYC46 (Table 1). As for the plasmids constructed previously, the lacZ gene was cloned into each of two plasmids under control of the PGK1 promoter, yielding plasmids pIYC47 and pIYC48 (Fig. 1, Table 1). Strain SCIYC68 and SCIYC69 (Table 1) were thus obtained by introducing pIYC47 and pIYC48 into CEN.PK 113-5D.

Strains were characterized in batch fermentation to understand the effects of the modified plasmids on cell physiology. No obvious difference in growth was observed in SCIYC68 with the combined P_{HXT1} -Ubi-R-URA3 construct. However, the P_{KEX2} -Ubi-R-URA3 combination in SCIYC69 led to a longer lag phase and lower biomass yield compared with control strain SCIYC58 (data not shown). The maximum specific growth rate of strain SCIYC69 was decreased to 0.28 h^{-1} , which corresponds to 70% of that of reference strain SCIYC58. This is probably due to the fact that Ura3 levels in this strain were too low to support normal growth.

To identify the effect of the combined modification on plasmid copy number and the expression of the target

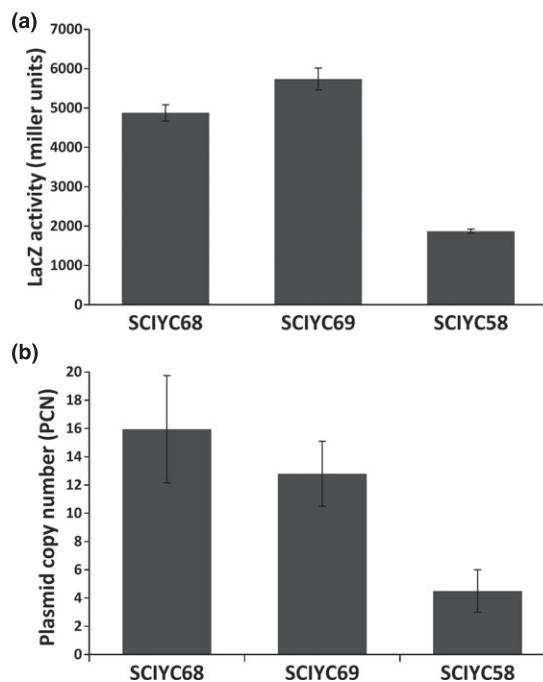


Fig. 4. Effect of combining promoter exchange and destabilization of the marker protein on LacZ activity (a) and plasmid copy number (b). Samples were taken from continuous culture. Strains used were SCIYC68 (P_{HXT1} -Ubi-R-URA3), SCIYC69 (P_{KEX2} -Ubi-R-URA3) and control strain SCIYC58 (P_{URA3} -URA3). The data shown represent the mean \pm SD from four data points.

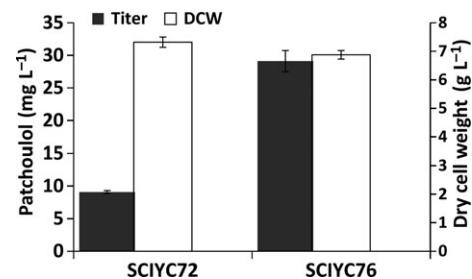


Fig. 5. Growth and patchoulol accumulation of strain SCIYC76 and control strain SCIYC72. Cells were cultured in shake flasks using glucose minimal medium. SCIYC76 contains plasmid pIYC49 (P_{KEX2} -Ubi-R-URA3) and control strain SCIYC72 contains plasmid pIYC03 (P_{URA3} -URA3). The data shown represent the mean \pm SD of three independent cultivations.

protein, chemostat cultures were carried out at a dilution rate of 0.1 h^{-1} . The results of the LacZ assay in continuous cultivation are shown in Fig. 4a. It was found that LacZ activities were increased by 2.5-fold in SCIYC68 and threefold in SCIYC69 compared to the reference strain SCIYC58. This was also reflected by an improvement in plasmid copy number observed in both strains (Fig. 4b).

The absolute plasmid copy number was elevated by 3.5- and 3-fold in SCIYC68 and SCIYC69, respectively. Comparing the fold change in the LacZ activity and the plasmid copy number revealed by strain SCIYC68 and SCIYC69, it was found that SCIYC68 displayed higher increase in plasmid copy number than increase in LacZ activity (Table 3). This could conceivably relate to the feature of the *HXT1* promoter, in addition to the destabilizing residue arginine, resulting in very low expression of *URA3*, and thereby more pressure to the cells. This is consistent with the fact that the biomass yield in SCIYC68 was lower than that of control strain (data not shown).

Application of the modified vector for patchoulol production

Our results showed that both the LacZ activity and the plasmid copy number can be further increased by combining (i) destabilization of the marker protein and (ii) replacing the promoter of the marker gene with a weak promoter. To demonstrate the practical application of this new plasmid, the plasmid carrying the *P_{KEX2}-Ubi-R-URA3* construct was tested for production of the isoprenoid patchoulol. Microbial production of this fragrance compound is of great interest in the perfume industry, as an alternative to extraction from plants. To convert farnesyl diphosphate (FPP), a yeast endogenous metabolite into patchoulol, only a single enzymatic step is needed (Asadollahi *et al.*, 2008). To ensure that enough FPP precursor is available, a truncated form of HMG-CoA reductase 1 (encoded by *tHMG1*) was also over-expressed on this plasmid. *tHMG1* overexpression has previously been reported to lead to enhanced isoprenoid production in yeast (Ro *et al.*, 2006; Asadollahi *et al.*, 2010). Both *tHMG1* and the patchoulol synthase gene were expressed from the *P_{KEX2}-Ubi-R-URA3* (strain SCIYC76) as well as from the control plasmid (strain SCIYC72). Patchoulol production and biomass formation were analyzed in shake flasks (Fig. 5). While the final biomass concentration was unchanged, patchoulol production reached ~30 mg L⁻¹ in SCIYC76, increased more than three times compared with that of control strain SCIYC72. This performance thus demonstrates that this new plasmid could also be beneficial to improve heterologous pathway expression.

Conclusion

In this study, the combination of down-regulation of the selection marker gene (*URA3*) using the weak promoters of *KEX2* and *HXT1* with destabilization of Ura3 using the ubiquitin-tag/N-degron system was applied to a 2 µm-based plasmid. A more than threefold improvement of the plasmid copy number was demonstrated. In

consequence, this led to enhanced protein expression. This new expression vector is useful for metabolic engineering projects that aim at high-level production of valuable products using yeast as a production platform. Furthermore, the system described here can potentially be applied to other systems using plasmid-based gene expression.

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Authors' contribution

Y.C. and S.P. contributed equally to this work.

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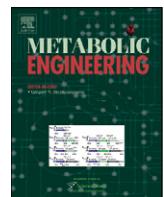
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PAPER III

Dynamic control of gene expression in *Saccharomyces cerevisiae* engineered for the production of plant sesquiterpene α -santalene in a fed-batch mode

Gionata Scalcinati⁼, Christoph Knuf⁼, Siavash Partow, Yun Chen,
Jérôme Maury, Michel Schalk, Laurent Daviet, Jens Nielsen,
Verena Siewers

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= These authors contributed equally to this work



Dynamic control of gene expression in *Saccharomyces cerevisiae* engineered for the production of plant sesquiterpene α -santalene in a fed-batch mode

Gionata Scalcinati ^{a,1}, Christoph Knuf ^{a,1}, Siavash Partow ^a, Yun Chen ^a, Jérôme Maury ^b, Michel Schalk ^c, Laurent Daviet ^c, Jens Nielsen ^{a,*}, Verena Siewers ^a

^a Department of Chemical and Biological Engineering, Chalmers University of Technology, SE-412 96 Göteborg, Sweden

^b Center for Microbial Biotechnology, Department of Systems Biology, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

^c Firmenich SA, Corporate R&D Division, CH-1211 Geneva 8, Switzerland

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ABSTRACT

Microbial cells engineered for efficient production of plant sesquiterpenes may allow for sustainable and scalable production of these compounds that can be used as e.g. perfumes and pharmaceuticals. Here, for the first time a *Saccharomyces cerevisiae* strain capable of producing high levels of α -santalene, the precursor of a commercially interesting compound, was constructed through a rationally designed metabolic engineering approach. Optimal sesquiterpene production was obtained by modulating the expression of one of the key metabolic steps of the mevalonate (MVA) pathway, squalene synthase (*Erg9*). To couple *ERG9* expression to glucose concentration its promoter was replaced by the *HXT1* promoter. In a second approach, the *HXT2* promoter was used to express an *ERG9* antisense construct. Using the *HXT1* promoter to control *ERG9* expression, it was possible to divert the carbon flux from sterol synthesis towards α -santalene improving the productivity by 3.4 fold. Combining this approach together with the overexpression of a truncated form of 3-hydroxyl-3-methyl-glutaryl-CoA reductase (HMGR) and deletion of lipid phosphate phosphatase encoded by *LPP1* led to a strain with a productivity of 0.18 mg/gDCW h. The titer was further increased by deleting *DPP1* encoding a second FPP consuming pyrophosphate phosphatase yielding a final productivity and titer, respectively, of 0.21 mg/gDCW h and 92 mg/l of α -santalene.

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1. Introduction

The production of plant sesquiterpenes by microbial fermentation is an environmentally friendly and attractive alternative to the commonly used chemical synthesis and plant extraction (Chang and Keasling., 2006; Rohlin et al., 2001; Ajikumar et al., 2008; Kirby and Keasling, 2009; Wang et al., 2011). Sesquiterpenes are mainly used by the chemical industry for production of fragrances and aroma chemicals (Daviet and Schalk, 2010). They represent a diverse class of secondary metabolites, the precursors of which are natively present in the metabolic network of *Saccharomyces cerevisiae* (Förster et al., 2003). Sesquiterpenes are naturally produced in *S. cerevisiae* through the mevalonate (MVA) pathway from multiple condensation of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) leading to farnesyl diphosphate (FPP), the universal precursor unit of all sesquiterpenes (C_{15}) (Maury et al., 2005; Withers and Keasling,

2007). Nowadays, most fragrance compounds are produced via plant and microbe extraction (Howes et al., 2004), and by total chemical synthesis or semi-synthesis (Corey et al., 1957; Julia, 1976; Janssens et al., 1992). Traditional methods of extraction are limited by low yields and high costs. Here, microbial biosynthesis offers several advantages: it (i) avoids formation of racemic mixtures providing enantiomerically pure products through enzymatic biocatalysis, (ii) reduces process costs using inexpensive sugar based carbon sources, (iii) increases sustainability avoiding harvesting and extraction from natural sources and thus reducing environmental footprint, (iv) increases yield and productivities using genetic manipulation of the heterologous hosts and (v) is compatible with scalable high density fermentation processes.

α -Santalene is the precursor of α -santalol, one of the main components of East Indian sandalwood oil (Corey et al., 1957; Baldovini et al., 2010). Sandalwood oil is commonly used in cosmetic, perfumery and aromatherapy industries and has recently been identified as a potential chemotherapeutic and chemopreventive agent against skin cancer (Dwivedi et al., 2003). α -santalene is produced enzymatically in a one-step-conversion from farnesol diphosphate catalyzed by a plant santalene synthase (Schalk, 2011). Here, an efficient *S. cerevisiae* strain

* Corresponding author. Fax: +46 31 772 38 01.

E-mail address: nielsenj@chalmers.se (J. Nielsen).

¹ Contributed equally to this research.

Nomenclature

FPP	Farnesyl diphosphate
FOH	(E,E)-farnesol
SQS	squalene synthase

HMGR	3-hydroxy-3-methyl-glutaryl-coenzyme A reductase
MVA	mevalonate
SanSyn	santalene synthase gene
Log P	logarithm (base 10) of partition coefficient

capable of reaching relevant titers and productivities of α -santalene during an optimized fermentation process is constructed. First, yeast was engineered to produce α -santalene by introducing a heterologous santalene synthase gene (*SanSyn*) derived from *Clausena lansium* (wampee) that catalyzes the conversion of FPP to α -santalene (Schalk, 2011). Metabolic engineering has been extensively applied to manipulate metabolic fluxes and enhance the microbial production of sesquiterpene compounds (Ro et al., 2006; Shiba et al., 2007; Takahashi et al., 2007; Asadollahi et al., 2008, 2009, 2010; Kirby et al., 2008; Ma et al., 2011). In order to increase the precursor pool for isoprenoid synthesis enabling efficient conversion to the target compound α -santalene, two of the main regulatory steps of the MVA pathway catalyzed by 3-hydroxyl-3-methyl-glutaryl-CoA reductase (HMGR) and squalene synthase (SQS) were optimized by introducing genetic modifications that enable to channel the flux towards α -santalene synthesis. The conversion of 3-hydroxyl-3-methyl-glutaryl-CoA into mevalonate catalyzed by HMGR is one of the most studied key regulatory steps in the MVA pathway and is considered the main flux controlling step (Scallen and Sanghvi, 1983; Basson et al., 1986). In yeast, two isoforms of HMGR exist encoded by genes *HMG1* and *HMG2* (Basson et al., 1986) and their activity is subject to tight regulation including feedback regulation and cross-regulation (Hampton and Rine, 1994; Hampton et al., 1996; Brown and Goldstein, 1980). HMGR is composed of an interspecies conserved catalytic domain and a variable membrane anchoring region also referred to as sterol sensing domain (SSD). Hmg1 regulation acts at the level of the SSD domain through a complex mechanism leading to protein degradation (Nielsen, 2009). Over-expression of a truncated form of Hmg1 lacking the SSD domain bypasses this post-transcriptional control and results in a constitutively active non-membrane bound form (Donald et al., 1997; Polakowski et al., 1998). This strategy has been

extensively used to increase the flow through the MVA pathway in order to produce isoprenoid derived compounds (Jackson et al., 2003; Ro et al., 2006; Kirby et al., 2008; Asadollahi et al., 2009, 2010; Farhi et al., 2011). The second key step in the MVA pathway is represented by SQS, as this controls the flux of FPP towards sterols or non-sterol sesquiterpenes. FPP is a pivotal intermediate as it is a common precursor for production of essential compounds such as dolichol, ubiquinone, isoprenylated proteins and ergosterol (Daum et al., 1998) and its intracellular concentration is tightly regulated at different levels (Goldstein and Brown, 1990). During normal growth conditions, most of the FPP is converted into ergosterol due to the fact that the cellular demand for sterols is greater compared to the demand for non-sterol FPP derived compounds (Kennedy et al., 1999). Strategies to increase sesquiterpene production based on the disruption of the main FPP consuming reaction catalyzed by squalene synthase produced lethal mutants and restoration of viability requires ergosterol supplementation resulting in an economically not feasible process for industrial purposes (Takahashi et al., 2007). Here, we undertook a genetic engineering approach to balance SQS activity during a fermentation process. Previous attempts to control SQS (encoded by *ERG9*) expression diverting the flow from sterol components to desired FPP-derived compounds were mainly based on replacement of the native *ERG9* promoter with the methionine-repressible *MET3* promoter (Ro et al., 2006; Paradise et al., 2008; Asadollahi et al., 2008, 2009, 2010). However, industrial scale development of this system is limited by the cost of the repressor and its possible consumption by the cell. Instead, we aimed at coupling *ERG9* expression to the glucose concentration in the media.

The production capacity of the engineered strains is evaluated through a fermentation process coupling biochemical production to biomass formation that allows capturing the water insoluble compound during production resulting in an efficient *S. cerevisiae* cell factory for biosynthesis of sesquiterpenoid fragrances.

Table 1
S. cerevisiae strains used in this study.

Strain	Genotype	Plasmid	Reference
CEN.PK113-5D	MAT α <i>MAL2-8c SUC2 ura3-52</i>		P. Kötter, University of Frankfurt, Germany Asadollahi et al., 2008
YIP-M0-04	MAT α <i>MAL2-8c SUC2 ura3-52 P_{ERG9Δ::kanMX-P_{MET3}}</i>		This study
SCICK00	MAT α <i>MAL2-8c SUC2 ura3-52 lpp1Δ::loxP P_{ERG9Δ::loxP-P_{MET3}}</i>		This study
SCICK01	MAT α <i>MAL2-8c SUC2 ura3-52 lpp1Δ::loxP P_{ERG9Δ::loxP-P_{HXT1}}</i>		This study
SCICK03	MAT α <i>MAL2-8c SUC2 ura3-52 lpp1Δ::loxP P_{ERG9Δ::loxP-P_{ERG9}}</i>		This study
SCICK05	MAT α <i>MAL2-8c SUC2 ura3-52 lpp1Δ::loxP P_{ERG9Δ::loxP-P_{ERG9}}</i>		This study
SCICK06	MAT α <i>MAL2-8c SUC2 ura3-52::pSF011-P_{HXT1}</i>		This study
SCICK08	MAT α <i>MAL2-8c SUC2 ura3-52::pSF011-P_{TEF1M2}</i>		This study
SCICK09	MAT α <i>MAL2-8c SUC2 ura3-52::pSF011-P_{HXT2}</i>		This study
SCICK10	MAT α <i>MAL2-8c SUC2 ura3-52::pSF011-P_{MET3}</i>		This study
SCICK11	MAT α <i>MAL2-8c SUC2 ura3-52::pSF011-P_{ERG9}</i>		This study
SCICK12	MAT α <i>MAL2-8c SUC2 ura3-52 lpp1Δ::loxP P_{ERG9Δ::loxP-P_{HXT1}}</i>	pICK01	This study
SCICK13	MAT α <i>MAL2-8c SUC2 ura3-52 lpp1Δ::loxP P_{ERG9Δ::loxP-P_{ERG9}}</i>	pICK01	This study
SCICK14	MAT α <i>MAL2-8c SUC2 ura3-52 lpp1Δ::loxP P_{ERG9Δ::loxP-P_{ERG9}}</i>	pICK01	This study
SCICK15	MAT α <i>MAL2-8c SUC2 ura3-52 lpp1Δ::loxP P_{ERG9Δ::loxP-P_{MET3}}</i>	pICK01	This study
SCICK16	MAT α <i>MAL2-8c SUC2 ura3-52 lpp1Δ::loxP dpp1Δ::loxP P_{ERG9Δ::loxP-P_{HXT1}}</i>	pICK01	This study
SCICK17	MAT α <i>MAL2-8c SUC2 ura3-52 lpp1Δ::loxP dpp1Δ::loxP P_{ERG9Δ::loxP-P_{HXT1}}</i>	pICK01	This study

2. Materials and methods

2.1. Strains and maintenance

Strains used in this study are listed in **Table 1**. Strains were maintained on YPD plates containing 10 g/l yeast extract, 20 g/l casein peptone, 20 g/l glucose and 20 g/l agar. Plasmid carrying strains were selected on synthetic dextrose (SD) agar containing 6.9 g/l yeast nitrogen base w/o amino acids (Formedium, Hünstanton, UK), 0.77 g/l complete supplement mixture (CSM) w/o uracil (MP Biomedicals, Solon, OH, USA), 20 g/l glucose, and 20 g/l agar and counter-selected on SD plates supplemented with 30 mg/l uracil and 750 mg/l 5-fluoroorotic acid (Formedium). Strains containing the *kanMX* cassette were selected on YPD plates containing 200 mg/l G418 (Formedium).

2.2. Plasmid construction

To construct integrative plasmids carrying the *lacZ* gene under control of different promoters plasmid pSF011 (Partow et al., 2010) was used. Promoters *P_{ERG9}*, *P_{MET3}*, *P_{HXT1}*, and *P_{HXT2}*, and were PCR amplified from genomic DNA of *S. cerevisiae* CEN.PK113-5D, and *P_{TEF1M2}* was amplified from plasmid p416TEF1M2 (kindly provided by G. Stephanopoulos, Massachusetts Institute of Technology, Cambridge, MA, USA) using primers 1 to 6 and 50 to 53 (**Table 2**), restricted with *NotI/HindIII* and ligated into *NotI/HindIII* cut pSF011 resulting in formation of plasmids pSF011-*P_{ERG9}*, pSF011-*P_{MET3}* and pSF011-*P_{HXT1}*, pSF011-*P_{HXT2}* and pSF011-*P_{TEF1M2}*, respectively.

To construct the α -santalene expression vector the *SanSyn* gene was amplified by PCR from plasmid Cont2B-27-pET101

Table 2

Oligonucleotide primers used in this study^a.

No.	Name	Sequence (5'→3')
1	HindIII_ERG9_f	CAACAAAAGCTTCCCATCTTCACAAACAATACC
2	NotI_ERG9_r	CAACAAGGCCGCCGCTGTGTCATGTGACGCT
3	HindIII_MET3_f	CAACAAAAGCTTGATAAGTGAGGGGGTCCACAG
4	NotI_MET3_r	CAACAAGGCCGCCGCTTAATTATACTTTATTCTGTATTATACTTACttc
5	HindIII_HXT1_f	CAACAAAAGCTTGAGCTCATCTGAATAATTCC
6	NotI_HXT1_r	CAACAAGGCCGCCGATTTACGTATATACTAGTTGACGATTATqG
7	santa_f	GTTGTTGCCGCCCAAACATGTCACACTAACAGTTCATAG
8	santa_r	GTTGTTTAATTAACATACTCGCAAGCTTAACGGG
9	tHMG1_up	GTTGTTGGATCCAAACATAAGGCTGCAGACCAATTGGTG
10	tHMG1_down	GTTGTTGCTAGCTAGGATTTAATGCAAGGTGACG
11	Int_check_f	GGTCCCGCCACATTCCCC
12	Int_check_r	GGAACTCTGTTGTTCTTGGAG
13	LacZ_r	GGGATCTGCCATTGTCAGAC
14	LPP_up_f	AAGGATGATCTCTGTCATGG
15	LPP_up_r_tail	GATCCCCGGAAATTGCCATGTGTTAGGGCAGCATTTATGC
16	LPP_dw_f	GCAGGGATGCCGCTGACGCACTCCAAGCGGACATTCAAG
17	LPP_dw_r	GAAGTATCTCTTTTCCC
18	Pr-b-Kan'	CATGCCAATTCCGGGGATCCCTTAATATAACTCGTATAATGTATGC
19	Kan3' int	CCATGAGTACGACTGAATCCGG
20	Kan5' int	GCAAAGGTAGCGTTGCAATG
21	dKan3'	GTCAGCGCCGATCCCTGCCGACTCACTATAAGGAGACCG
22	LPP1_verif_up	TAGTGGCACGTTGAAACCTGACAAC
23	LPP1_verif_dw	AATTTCATCGGTATTGCTTCGTC
24	loxP_ERG9_f	CGAAAGTTATTAGGTGATATCAGATCCACTTGAGGTCTCATCTGAATAATTACCG
25	ERG9d_r	GTCGTAGCTGGACGGTTG
26	loxP_HXT1_f	CGAATTATTAGGTGATATCAGATCCACTTGAGGTCTCATCTGAATAATTACCG
27	ERG9_Hxt1_r	GCTGCCCTCATCTGCCGATGCCAATGCAATGTAATAGCTTICCATGATTACGTTACACTAGCTGACGATTATG
28	ERG9_loxP_f	GAGTGAACCTGCTGCCGCTGACTCAGTACAGACATCATTGCTGACGTTACGCTGCAAGGTCGACAAC
29	loxP_r	AGTGGATCTGATATCACCTAATAACTCG
30	Erg9_fr_f	CCTTGCTTACACAGAGTGAACCTGCTGCC
31	Erg9_fr_r	CTTCAGCTCAAAGCTGCCATCTGCAACCG
32	DPP-1-fw	AGGGCACGTTTCAATTGT
33	DPP-1-rev	CAGCGTACCAAGCTCAGAGAAACTCGTACTGAACCAAG
34	DPP-2-fw	GTGATATCAGATCCACTAGCAGACATCATTGCTG
35	DPP-2-rev	AACTCTAAGGCTTCTG
36	KanMx-1-fw	CTGAAGCTTGTACGCTG
37	KanMx-1-rev	TCACCATGAGTGAACGACTGA
38	KanMx-2-fw	TTCCAACATGGATGCTGAT
39	KanMx-2-rev	CTAGTGGATCTGATATCAC
40	Amp_fw	GTGGTTTACATCGAACGTGATC
41	Amp_rv	CATCCATAGTTGCCGTACTGC
42	14up_HXT2_f	GATTGATGACTGTTCTAAAATTATGTCATTCTACACCGCATATGATTACCGATGTAATAACAAAATG
43	ERG9as_HXT2_r	TTATTGAATTCCACAAATTGAAACTATGTTGTTATAAGCTTTGTAA
44	ERG9as_f	GTTTCAATTGTTGAAATTCAATAA
45	CYC1t_ERG9as_r	TTCTTTCCGTTAGAGCGGATCTGGAAATTAGGACAGGGC
46	ERG9as_CYC1t_f	GCCCCCTGCTTAATTCCAGATCCGCTCAACCGAAAGGAA
47	loxP_CYC1t_r	GGTTGTCGACCTGCACTCGAGCGTCCAAAACCTT
48	loxP_f	GTACGCTGAGGTGCAAC
49	14down_loxP_r	GATAACCGCGAAGATTATAATGTTTATCGGTTGCATTCCATGAGTAAGTGGATCTGATATCACCTAATAACTTCG
50	HindIII_HXT2_f	CAACAAAAGCTTCTACCGATGTAATAACAAAATG
51	NotI_Hxt2_r	CAACAAGGCCGCCGCTATGTTGCTTATAAGTCTTTGTAA
52	HindIII_TEF1_M2_f	CAACAAAAGCTGACACACCAGCTAAAG
53	NotI_TEF1_M2_r	CAACAAGGCCGCCCTTTCTAGAAAACCTGGATTAGTTG

^a Restriction enzyme recognition sites are underlined.

(Schalk, 2011), using primers 7 and 8, cut with *NotI/PacI* and ligated into *NotI/PacI* restricted vector pSP-G1 (Partow et al., 2010). Subsequently, *tHMG1* was PCR amplified using genomic DNA of *S. cerevisiae* CEN.PK113-5D as template and primers 9 and 10, cut with *BamHI/NheI* and ligated into the same vector after restriction with the respective enzymes. This resulted in formation of expression plasmid pICK01.

2.3. Strain construction

Strains carrying a genomic integration of *lacZ* under control of different promoters were constructed by transforming CEN.PK113-5D with the *NcoI* restricted integrative plasmids pSF011-P_{ERG9}, pSF011-P_{MET3}, pSF011-P_{HXT1}, pSF011-P_{HXT2} and pSF011-P_{TEF1M2}, respectively, resulting in formation of strains SCICK11, SCICK10, SCICK06, SCICK09 and SCICK08, respectively. Correct integration at the *ura3-52* locus was verified using primers 11 and 12. To test for tandem integration of the plasmid, primers 11 and 13 were used. To exclude additional integrations of the plasmid, strains were subjected to Southern blot analysis.

The *loxP* flanked *kanMX* cassette in strain YIP-M0-04 was excised with help of the Cre recombinase expression plasmid pSH47 as described by Guldener et al. (1996). To delete *LPP1* in this strain a bipartite gene targeting strategy was applied (Erdeniz et al., 1997). The 5' and 3' region of the gene were amplified by PCR using primer pairs 14/15 (fragment 1) and 16/17 (fragment 2), respectively, and genomic DNA of CEN.PK113-5D as template. The 5' and the 3' part of the *kanMX* cassette were amplified from plasmid pUG6 (Guldener et al., 1996) using primer pairs 18/19 (fragment 3) and 20/21 (fragment 4), respectively. Complementary primer tails allowed for the combination of fragments 1 and 3 by fusion PCR. Likewise, fragments 2 and 4 were fused to each other. Cells were transformed with both fusion PCR fragments and integration of the *kanMX* cassette at the *LPP1* locus was tested by PCR using primers 22 and 23. Subsequent excision of the *kanMX* cassette led to formation of strain SCICK00.

To replace the *ERG9* controlling *MET3* promoter in SCICK00 the *ERG9* promoter and the *HXT1* promoter were amplified from genomic DNA by PCR using primer pairs 24/25 and 26/27, respectively. In addition, the *kanMX* cassette was amplified in a PCR containing primers 28 and 29. The marker cassette was combined with either of the two promoters by fusion PCR and the resulting fragments were amplified once more using primers 30 and 31 in order to extend the flanking regions for genomic integration. Transformation of SCICK00 with these fragments and subsequent excision of the *kanMX* cassette resulted in strains SCICK01 and SCICK03, respectively.

For genomic integration of an *ERG9* antisense expression cassette, four PCR fragments were generated containing the *HXT2* promoter (primers 42/43, fragment 5), a fragment of *ERG9* (primers 44/45, fragment 6), the *CYC1* terminator (primers 46/47, fragment 7) and the *kanMX* cassette flanked by *loxP* sites (primers 48/49, fragment 8). The *ERG9* fragment comprised the first 412 bp of the coding sequence and 99 bp upstream of the start codon. Fragments 5, 6 and 7 and fragment 7 and 8 were combined by fusion PCR and used to transform SCICK03. 5' extensions of primers 42 and 49 allowed for integration by homologous recombination at YMRWdelta15 (Flagfeldt et al., 2009). Excision of the *kanMX* cassette resulted in strain SCICK05.

For deletion of *DPP1* in strain SCICK01, bipartite gene targeting was applied as described above. Here, primer pairs 32/33 and 34/35 were used to amplify the 5' and 3' region of *DPP1* and primer pairs 36/37 and 38/39 were employed for amplification of the 5' and 3' part of the *kanMX* cassette. Integration and following excision of the resistance marker led to formation of strain SCICK16.

By transforming SCICK00, SCICK01, SCICK03, SCICK05 and SCICK16 with plasmid pICK01 strains SCICK15, SCICK12, SCICK13, SCICK14 and SCICK17 were constructed.

2.4. Southern blot analysis

Genomic DNA was cut with *HindIII*, separated on a 1% agarose gel and transferred to a Hybond™-N+ membrane (GE Healthcare, Uppsala, Sweden) according to the supplier's instructions. As probe, a fragment of the ampicillin resistance gene in vector pSF011 was amplified by PCR using primers 40 and 41. The AlkPhos Direct Labeling and Detection system (GE Healthcare) was applied using CDP-Star as detection reagent and a ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA, USA) for chemiluminescence imaging.

2.5. Media and growth conditions

For batch cultivations, a previously described (Verduyn et al., 1992) mineral salts medium was used consisting of the following (per liter): $(\text{NH}_4)_2\text{SO}_4$, 5 g; KH_2PO_4 , 3 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.50 g; Antifoam 289 (A-5551, Sigma-Aldrich, St. Louis, MO, USA), 0.05 ml; trace metals, 1 ml and vitamins, 1 ml. The trace metal solution consisted of the following (per liter): EDTA (sodium salt), 15.0 g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.45 g; $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$, 1 g; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.3 g; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.3 g; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.4 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.45 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g; H_3BO_3 , 0.1 g and KI, 0.1 g. The pH of the trace metal solution was adjusted to 4.0 with 2 M NaOH prior to heat sterilization. The vitamin solution contained (per liter): biotin, 0.05 g; p-amino benzoic acid, 0.2 g; nicotinic acid, 1 g; Ca-pantothenate, 1 g; pyridoxine-HCl, 1 g; thiamine-HCl, 1 g and myo-inositol, 25 g. The pH of the vitamin solution was adjusted to 6.5 with 2 M NaOH. The vitamin solution was filter sterilized and stored at 4 °C. This medium was supplemented with 30 g/l glucose. The medium used for shake flask cultivation had the same composition as described above, but the $(\text{NH}_4)_2\text{SO}_4$ concentration was increased to 7.5 g/l, and the KH_2PO_4 concentration to 14.4 g/l. The glucose concentration was 20 g/l and the pH was adjusted to 6.5 prior autoclaving. The feed composition used for fed-batch cultivation had the same composition as described above, but the $(\text{NH}_4)_2\text{SO}_4$; KH_2PO_4 ; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, vitamin solution, and trace metal solution concentrations were increased 10 times; the glucose concentration was 200 g/l.

2.6. Inoculum preparation

The seed cultures for the cultivations were grown at 30 °C in 500-ml shake flasks containing 100 ml of culture with agitation in an orbital shaker at 100 rpm. Pre-cultures were used to inoculate the fermentors to a final dry weight of 1 mg/l. All cultivations were performed in duplicate.

2.7. Shake flask cultivation

Cultivations were carried out in 500 ml baffled Erlenmeyer flasks with four diametrically opposite baffles and side necks for aseptic sampling. The flasks were prepared with 100 ml medium as described above. Cultures were incubated with agitation in an orbital shaker at 100 rpm and the temperature was controlled at 30 °C.

2.8. Fed-batch mode in shake flasks

Shake flasks in fed-batch mode were realized using the FeedBeads polymer-based slow-release technique as previously described (Jeude et al., 2006). Media were prepared as described

above without initial glucose content. Four sterile silicone elastomer disks Ø12 mm (Kühner AG, Basel, Switzerland) containing glucose crystals were added immediately before inoculation to 250 ml Erlenmeyer flasks containing 25 ml of medium.

2.9. Fed-batch operation

The aerobic fed-batch process was performed in 2.5 l Applikon vessels (Applikon, Schiedam, The Netherlands) with a working volume of 1.0 l. Agitation at 600 rpm was maintained using an integrated stirrer (DasGip, Jülich, Germany) and the temperature kept at 30 °C. The rate of aeration was set to 0.6 l/min. The pH of the medium was maintained at 5.0 by automatic addition of 2 N KOH during the batch phase and 10% NH₄OH during the feed phase. The temperature, agitation, gassing, pH and composition of the off-gas were monitored and controlled using the DasGip monitoring and control system. Dissolved oxygen concentration was monitored with an autoclavable polarographic oxygen electrode (Mettler Toledo, Columbus, OH, USA) and kept above 30% via stirrer speed and gas flow rate using the DasGip control system. The effluent gas from the fermentation was analyzed for real-time determination of oxygen and CO₂ concentration by DasGip fedbatch pro® gas analysis systems with the off gas analyzer GA4 based on zirconium dioxide and two-beam infrared sensor.

The fed-batch cultures were initiated as batch cultures using 30 g/l glucose. Feeding with fresh medium commenced only after residual ethanol produced from the glucose consumption phase was completely depleted. A feed strategy was designed keeping the volumetric growth rate constant (Nielsen et al., 2003). An exponential feed rate $v(t)$ (l/h) was calculated according to:

$$v(t) = \frac{Y_{xs}\mu_0}{S_f - S_0} x_0 V_0 \exp(\mu_0 t)$$

where x_0 , S_0 and V_0 were the biomass density (gDCW/l), the substrate concentration (g/l) and the reactor volume (l) at the start of the feed phase, Y_{xs} was the respiratory yield coefficient (g glucose/gDCW); S_f was the concentration of the growth limiting substrate (g glucose/l) in the reservoir; μ_0 was the specific growth rate (h⁻¹) during the feed phase and t the feeding time. According to the equation above the feed was increased exponentially with a specific feed rate of 0.06 h⁻¹. Correct feed addition was obtained programming the fb-pro software (DasGip) and controlled using the DasGip control system. An organic layer of dodecane (Sigma-Aldrich) was added aseptically to a final volume of 10% (v/v) immediately before starting the feed.

2.10. Analytical methods

The cell dry weight was measured by filtering known volumes of the cultures through pre-dried and pre-weighed 0.45-μm-pores size nitrocellulose filters (Supor-450 membrane filters; PALL Life Sciences Ann Arbor, MI). The filters with the biomass were washed with water, dried for 15 min in a microwave oven at 150 W, and weighed again. The optical density at 600 nm was determined using a Hitachi U-1100 spectrophotometer.

Concentrations of glucose, glycerol, ethanol, acetate, succinate and pyruvate were analyzed by an isocratic high-performance liquid chromatography (UltiMate® 3000 Nano, Dionex) with an Aminex HXP-87H ion-exchange column (Bio-Rad, Hercules, CA) at 65 °C using 5 mM H₂SO₄ as mobile phase at a flow rate of 0.6 ml min⁻¹. Glucose, glycerol and ethanol were measured with a refractive index detector (RI-101 Refractive Index Detector, Shodex®), and acetate, succinate and pyruvate were measured with a UV-visible light absorbance detector (UltiMate 3000 Variable Wavelength Detector, Dionex).

2.11. β-Galactosidase activity assay

The enzyme activity assay was performed as described earlier (Flagfeldt et al., 2009).

2.12. Analysis of sesquiterpenes

Sesquiterpene production during the course of fermentation was determined as described previously (Asadollahi et al., 2010). Samples from the organic layer were centrifuged 5 min at 5000 g and the supernatants were analyzed by gas chromatography-mass spectrometry (GC/MS) with a DSQ II single quadrupole mass spectrometer (Thermo Scientific, Waltham, MA). Analytes from 1 μL sample were separated on a SLB-5 ms capillary column (15 m, 0.25 mm i.d., 0.25 μm film thickness; Supelco, Bellefonte, PA, USA) using helium as carrier gas at a flow rate of 1.2 ml min⁻¹. A split/splitless injector was used in the splitless mode. The initial oven temperature was 80 °C and the injector temperature was 250 °C. The oven temperature was increased to 120 °C at a rate of 10 °C/min and subsequently increased to 160 °C at a rate of 3 °C/min. The oven temperature was finally increased to 270 °C at a rate of 10 °C/min and held for 5 min at this temperature. Full mass spectra were generated by scanning *m/z* range within 40–500 for metabolite identification, α-santalene and *E,E*-farnesol were identified comparing mass spectra and retention time with the available authentic standards; *trans*-α-bergamotene by comparison with library spectra using NIST mass spectra search program (<http://chemdata.nist.gov/index.html>). Quantification of α-santalene and *E,E*-farnesol was carried out using standard curves, *trans*-α-bergamotene was quantified with a correction factor determined using α-humulene as internal standard.

2.13. Analysis of total sterol fraction

For the extraction of sterols, a previously described method (Asadollahi et al., 2010) was used with minor modifications. Known volumes of fermentation broth were harvested by centrifuging at 5000 rpm for 10 min. The cell pellet was washed twice with distilled water and the cell suspension was centrifuged for another 10 min at 5000 rpm. The cell pellet was re-suspended in 4 ml of 0.2 N HCl and heated in a water bath at 85 °C for 1 h and then allowed to cool to room temperature. After centrifugation for 10 min at 5000 rpm and removal of the supernatant, the cell pellet was resuspended in 2 ml methanol containing 0.2% (w/v) pyrogallol and 1 ml 4 N KOH and transferred to a 14 ml glass vial sealed with a PTFE lined screw cap, heated again for 2 h in a water bath at 85 °C for saponification and then cooled to room temperature. Sterols were extracted by addition of 5 ml heptane followed by vigorous mixing for 2 min. After 2 h, the *n*-heptane layer was transferred to a new glass vial for HPLC analyses. Quantitative determination of total ergosterol was carried out by a isocratic high-performance liquid chromatograph (UltiMate® 3000 Nano, Dionex) with a reverse phase Develosil column (C30-UG-5; Nomura Chemicals, Aichi, Japan) at 40 °C using 70% MeOH as the mobile phase at a flow rate of 1 ml min⁻¹. The ergosterol concentration was measured with a UV-visible light absorbance detector set at 280 nm (Photodiode Array Detector, Dionex). The amount of ergosterol was determined with Dionex Chromeleon® software using absolute calibration curves.

2.14. Calculation of specific rates and yield coefficients

The maximum specific growth rates, yield coefficients, specific product formation rates and specific substrate consumption rates are expressed as mg or g product substrate per g dry cell weight

per hour and were calculated as previously described (Nielsen et al., 2003). All calculations are limited to the exponential glucose-limited growth phase.

3. Results

3.1. α -Santalene production in *S. cerevisiae*

The α -santalene production in *S. cerevisiae* was initially evaluated introducing the expression plasmid pICK01 containing a copy of *tHMG1* and santalene synthase (*SanSyn*) under control of the *PGK1* and *TEF1* promoter, respectively, into a *lpp1Δ* strain to reduce farnesol formation (Faulkner et al., 1999) resulting in strain SCICK13. Production capacity was tested cultivating the strain in a batch in situ product removal (ISPR) reactor mode, consisting of an aqueous two-phase partitioning system. This strategy had previously been used successfully to increase product recovery in different microbial production processes (Daugulis, 1991; Stark and von Stockar, 2003). Dodecane was selected as organic phase due to its hydrophobicity ($\text{Log } P_{\text{dodecane}}: 6.6$; $\text{Log } P_{\text{santalene}}: 6.2$), low volatility and biocompatibility with *S. cerevisiae* growth (Newman et al., 2006; Asadollahi et al., 2008). Product accumulation in the dodecane layer was monitored by gas chromatography-mass spectrometry (GC/MS). The transformed strain was able to synthesize a low amount of α -santalene. Analysis of the organic layer revealed a major chromatographic peak corresponding in retention time and mass spectrum to the plant-extracted α -santalene and a second minor peak also present in the plant extract, subsequently identified as *trans*- α -bergamotene (Fig. 5). The amount of the sesquiterpenes α -santalene and *trans*- α -bergamotene produced were, respectively, 1.45 ± 0.02 and 0.17 ± 0.01 mg/l.

3.2. MET3 promoter activity during shake flask cultivation

As a next step, we wanted to increase α -santalene production by reducing ergosterol formation. A promoter, which is widely used for lowering the expression of *ERG9* encoding squalene synthase and hereby increasing the FPP pool, is the *MET3* promoter. As mentioned above, this might not be an optimal solution, since the repressing agent, methionine, is metabolized by the cells thus releasing repression. This was demonstrated by fusing *P_{MET3}* to the *lacZ* gene followed by measuring β -galactosidase activity of the resulting strain (SCICK10) cultivated in shake flasks without L-methionine or supplied with 1 mM and 2 mM L-methionine, respectively. The concentrations of the inhibitor were chosen based on the amounts previously used for *ERG9* repression (Asadollahi et al., 2008). As shown in Fig. 1, the β -galactosidase activity was constant in the cultures not containing L-methionine. In the cultures that contained L-methionine, LacZ activity was initially very low. However, at about mid-exponential phase, it started to increase and rapidly reached the levels measured in the non-repressed culture.

These results thus demonstrate the difficulties controlling promoter activity when the repressing agent is metabolized by the cells. We therefore tested, if D-methionine or 2-hydroxy-4-(methylthio)butyric acid could serve as L-methionine analogs to repress the *P_{MET3}* promoter, because they may not be metabolized by yeast or metabolized to a lesser extent. At concentrations of up to 4 mM in the medium neither of the two compounds had the capability to reduce *P_{MET3}* activity (data not shown).

3.3. Evaluation of alternative promoters for controlling squalene synthase activity

Because of the disadvantages of the *MET3* promoter, alternative systems were evaluated to down-regulate *ERG9* expression.

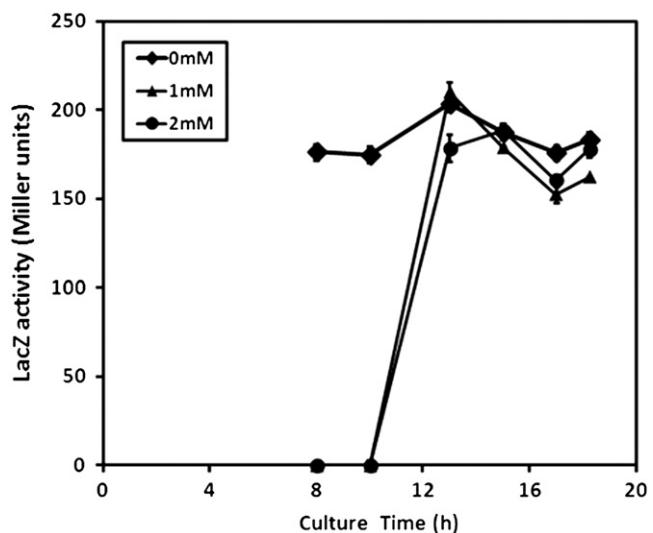


Fig. 1. LacZ activity in strain SCICK10 ($P_{\text{MET3}}-\text{lacZ}$) in response to different methionine concentrations, 0 mM (diamonds), 1 mM (triangles) and 2 mM (circles). Strains were cultivated in duplicates, glucose exponential growth phase was between 2 and 16 h of cultivation.

The chosen regulatory systems were (i) the low-level constitutive *TEF1* promoter mutant P_{TEF1M2} selected after a directed evolution approach based on error-prone PCR (Alper et al., 2005; Nevoigt et al., 2006), (ii) the glucose concentration controlled promoter of the hexose transporter gene *HXT1* (Ozcan and Johnston, 1995; Lewis and Bisson, 1991) and (iii) the *HXT2* promoter potentially useful for a gene silencing approach expressing *ERG9* antisense mRNA (Ozcan and Johnston, 1995). The approach of using promoters, which are regulated by glucose concentration was chosen as a means to achieve moderate expression levels during exponential growth in batch cultivation, i.e. at high glucose concentration and maximal repression during low glucose concentration, e.g. during the feed phase of a glucose-limited fed-batch process. To test whether the chosen promoters show suitable activity levels compared to the native *ERG9* promoter, fusion constructs of P_{TEF1M2} , P_{HXT1} , P_{HXT2} and P_{ERG9} with the *lacZ* reporter gene were integrated into the yeast genome. Strains SCICK06 (P_{HXT1}), SCICK08 (P_{TEF1M2}), SCICK09 (P_{HXT2}) and SCICK11 (P_{ERG9}) were cultivated in shake flasks and LacZ activity was monitored. Strain SCICK11 showed a steady LacZ activity level throughout the cultivation (Fig. 2). β -galactosidase activity in SCICK06 decreased with decreasing glucose concentration reaching the same level as in SCICK11 in late stationary phase, whereas LacZ activity in SCICK09 increased. During exponential growth, SCICK08 exhibited a very low activity, which increased slightly during stationary phase. The different strains displayed similar growth profiles and no differences were observed in biomass formation.

Developing an efficient cultivation method is a key step in designing a cost effective bioprocess. Fed-batch cultivation mode is widely applied during industrial productions and is often a first choice to achieve high productivity (Nielsen et al., 2003). As previously shown, when controlled by its native promoter, *ERG9* transcript levels display context dependency, i.e. the gene shows different expression levels depending on the growth conditions and the carbon source utilized (Kennedy et al., 1999; Kennedy, 2001). To investigate whether the selected promoters could also be employed during a fed-batch based fermentation process, the same strains were cultivated in the presence of glucose feed beads thus simulating the feed phase in a glucose-limited fed-batch cultivation. Under these conditions, glucose is released

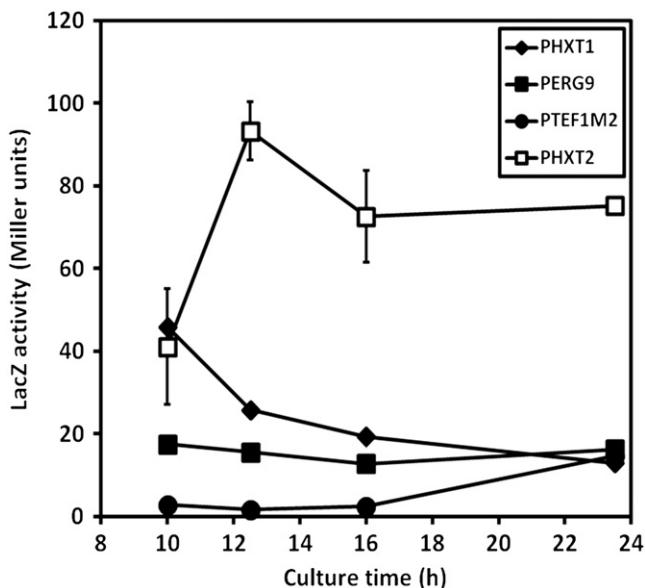


Fig. 2. LacZ activity in strains SCICK06 (P_{HXT1} , filled diamonds), SCICK08 (P_{TEFLM2} , filled circles), SCICK09 (P_{HXT2} , empty squares) and SCICK11 (P_{ERG9} , filled squares) during shake flask cultivation. Strains were cultivated in duplicates, glucose exponential growth phase was between 2 and 16 h of cultivation.

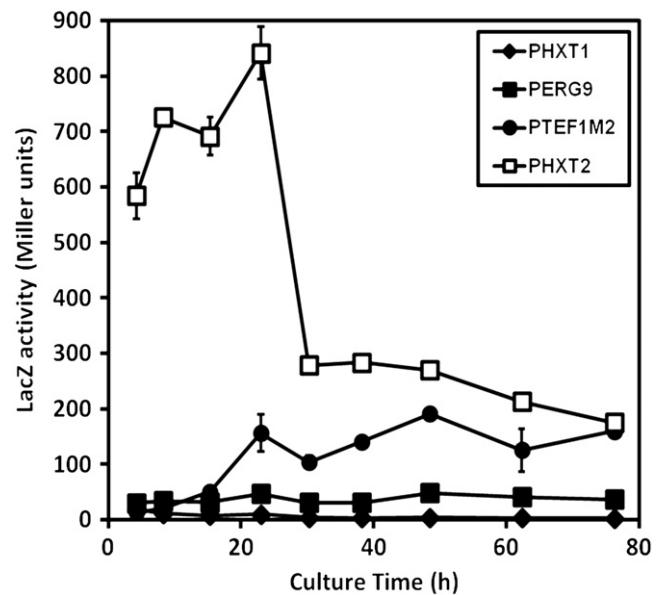


Fig. 4. Characterization of promoter strength during shake flask cultivation in fed-batch mode. β -galactosidase activity in strains SCICK06 (P_{HXT1} , filled diamonds), SCICK08 (P_{TEFLM2} , filled circles), SCICK09 (P_{HXT2} , empty squares) and SCICK11 (P_{ERG9} , filled squares). β -galactosidase activity is the average of values obtained from at least three independent cultivations assayed in duplicates.

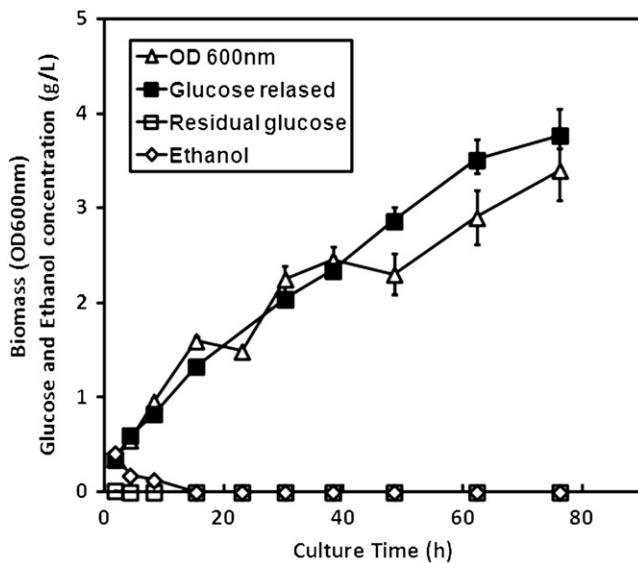


Fig. 3. Shake flask cultivation in fed-batch mode showing absolute glucose release kinetics without cells, residual glucose and ethanol concentration as well as biomass formation. The data represented were obtained for strain SCICK03; all the strains tested exhibited similar profiles.

from silicone elastomers with a controlled kinetics profile (Fig. 3), which allows mimicking a fed-batch fermentation mode comparable to a regular fed-batch. The glucose release (in absence of cells) as well as glucose concentration, cellular growth and by-product formation during the entire cultivation were monitored. This glucose restrained process allows a precise comparison of the different constructs and to fully explore the potential of the glucose sensing promoters. After an initial period of about 5 h of adaptation, where glucose release was larger than the cellular consumption, glucose became the limiting factor for growth and all the glucose released from the elastomers was rapidly converted into biomass with no ethanol overflow metabolism or formation of other by-products (Fig. 3). All strains exhibited a similar growth profile and no significant differences in glucose

consumption (data not shown). The expression of β -galactosidase controlled from the different promoters was assayed at regular intervals and monitored during a cultivation period of 80 h. As in the previous experiment, LacZ activity was steady in SCICK11 (P_{ERG9}) (Fig. 4). As expected, the highest activity was measured for SCICK09 (P_{HXT2}), the lowest for SCICK06 (P_{HXT1}). Surprisingly, β -galactosidase activity in SCICK08 (P_{TEFLM2}) drastically increased during the cultivation finally reaching the same level as for SCICK09 (P_{HXT2}), which indicates that (random) mutagenesis may turn a constitutive promoter – P_{TEFL} had previously shown a constant level of expression throughout different cultivations (Partow et al., 2010) – into a conditional promoter.

According to these results, P_{HXT1} appeared to be a suitable promoter to down-regulate $ERG9$ expression under glucose limiting conditions, whereas P_{HXT2} was chosen to regulate the expression of an $ERG9$ antisense construct. Due to its high activity levels at low glucose concentrations, the idea of employing P_{TEFLM2} for $ERG9$ regulation was discarded.

3.4. Evaluation of santalene production in fed-batch fermentation mode

Based on the results of the promoter characterization study, four strains were constructed. All strains carried a deletion in the phosphatase encoding $LPP1$ gene to reduce the loss of FPP to farnesol (Faulkner et al., 1999). $tHMG1$ and $SanSyn$ were expressed from a high copy number plasmid under control of the $PGK1$ and $TEF1$ promoter, respectively. Strain SCICK13 containing the native $ERG9$ promoter served as a reference strain. In strain SCICK12, the $ERG9$ promoter had been replaced by the $HXT1$ promoter. Strain SCICK14 carried an antisense DNA fragment comprising the 5' region of $ERG9$ and part of its 5' UTR (Bonoli et al., 2006; Olsson et al., 1997), whose expression was controlled by the $HXT2$ promoter and which was integrated into chromosome XIII at a site providing high expression levels (Flagfeldt et al., 2009). For comparison with previous approaches, strain SCICK15, which carried P_{MET3} instead of P_{ERG9} , was used. In order to maintain $ERG9$ repressed in the SCICK15 culture during the

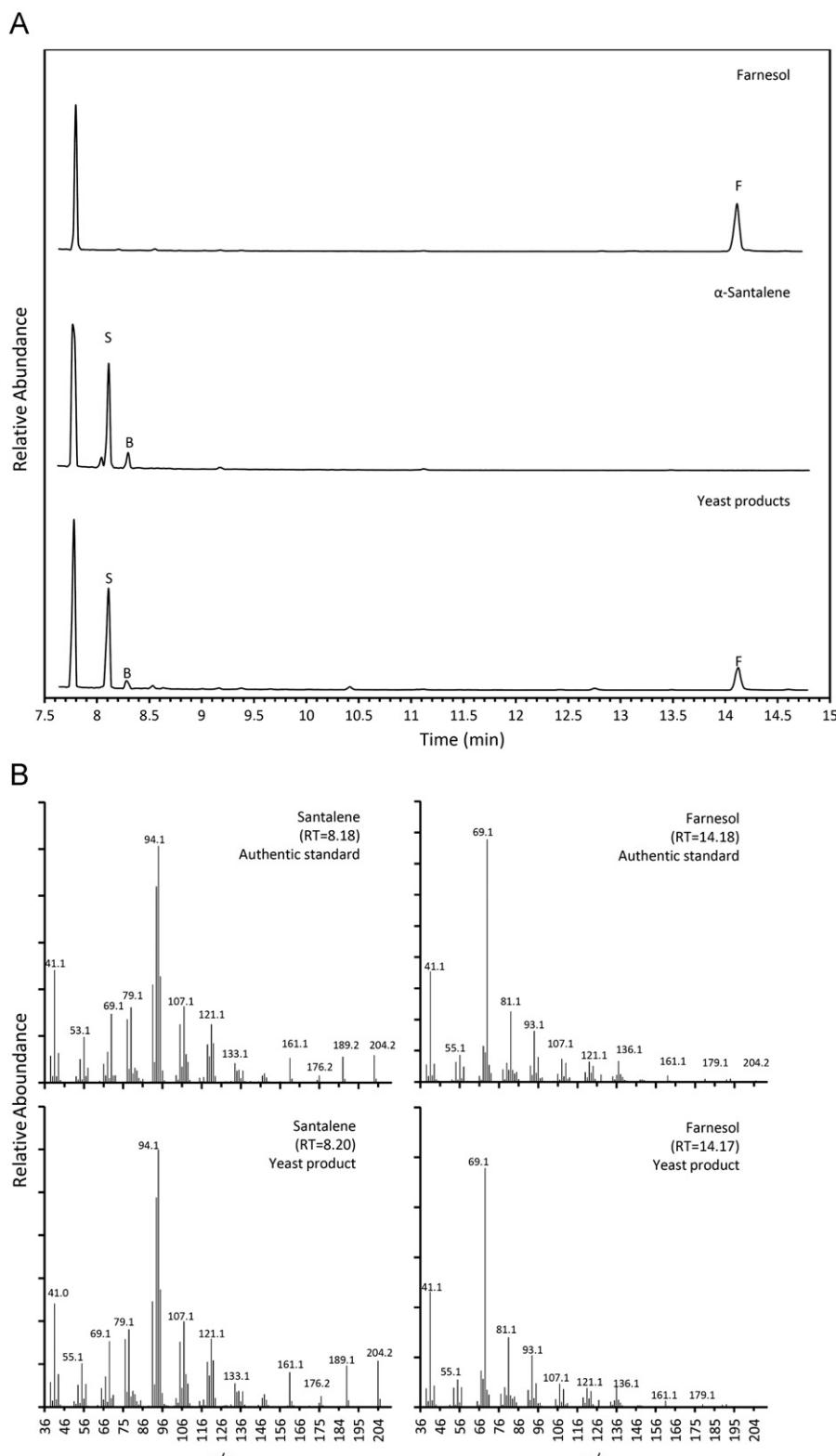


Fig. 5. (A) Total ion chromatograms from GC-MS analysis of authentic standard of farnesol, α -santalene, and an extract of engineered *S. cerevisiae* showing peaks of α -santalene (S), *trans*- α -bergamotene (B) and farnesol (F). The representative ion chromatogram referred to as yeast products was obtained during ISPR fed-batch fermentation of strain SCICK12. (B) Mass spectra and retention times of α -santalene produced from yeast and extracted from plant (left panel) and *E,E*-farnesol produced from yeast and chemical standard (right panel).

fermentation, L-methionine was added at regular intervals every 6 h to a final concentration of 2 mM.

Physiological characterization of the strains was completed in aerobic glucose-limited fed-batch cultures (Table 3). A fed-batch

in situ product removal (ISPR) reactor mode was chosen to evaluate the α -santalene production capacity of these strains engineered to accumulate FPP. Cultivation was started as batch with 30 g/l of glucose. After complete glucose consumption and

Table 3

Physiological parameters obtained during fed-batch cultivation of strains SCICK13, SCICK12, SCICK14, SCICK15 and SCICK17.

Strain	μ (h^{-1})	Y_{SX} (g g^{-1})	r_s (mmol (g biomass) $^{-1} \text{h}^{-1}$)	r_{CO_2} (mmol (g biomass) $^{-1} \text{h}^{-1}$)	r_{O_2} (mmol (g biomass) $^{-1} \text{h}^{-1}$)	RQ	Tot_{Sant} (mg l^{-1})
SCICK13 (P_{ERG9})	0.061 ± 0.006	0.50 ± 0.02	0.58 ± 0.03	1.22 ± 0.06	1.29 ± 0.03	0.95 ± 0.01	49.86 ± 0.23
SCICK12 (P_{HXT1})	0.056 ± 0.005	0.50 ± 0.01	0.58 ± 0.02	1.32 ± 0.01	1.43 ± 0.03	0.92 ± 0.01	75.73 ± 0.34
SCICK14 (P_{HXT2})	0.064 ± 0.006	0.48 ± 0.03	0.64 ± 0.28	1.57 ± 0.01	1.62 ± 0.04	0.97 ± 0.01	49.00 ± 0.34
SCICK15 (P_{MET3})	0.057 ± 0.006	0.49 ± 0.03	0.60 ± 0.01	1.49 ± 0.05	1.53 ± 0.07	0.96 ± 0.01	46.56 ± 0.87
SCICK17 (P_{HXT1})	0.057 ± 0.005	0.49 ± 0.01	0.60 ± 0.01	1.46 ± 0.08	1.48 ± 0.09	0.98 ± 0.01	91.96 ± 0.71

Specific growth rate μ (h^{-1}). Biomass yield Y_{SX} ($\text{g biomass} (\text{g substrate})^{-1}$). Specific carbon dioxide production rate (r_{CO_2}) and substrate (r_s) and oxygen (r_{O_2}) consumption rates (mmol (g biomass) $^{-1} \text{h}^{-1}$). Respiratory quotient RQ measured as r_{CO_2}/r_{O_2} . Referred to the initial 30 h of the glucose-limited feed phase. Tot_{Sant} , α -santalene titer (mg l^{-1}) measured at the end of the feed process. Values represent the mean \pm S.D. of two independent cultivations.

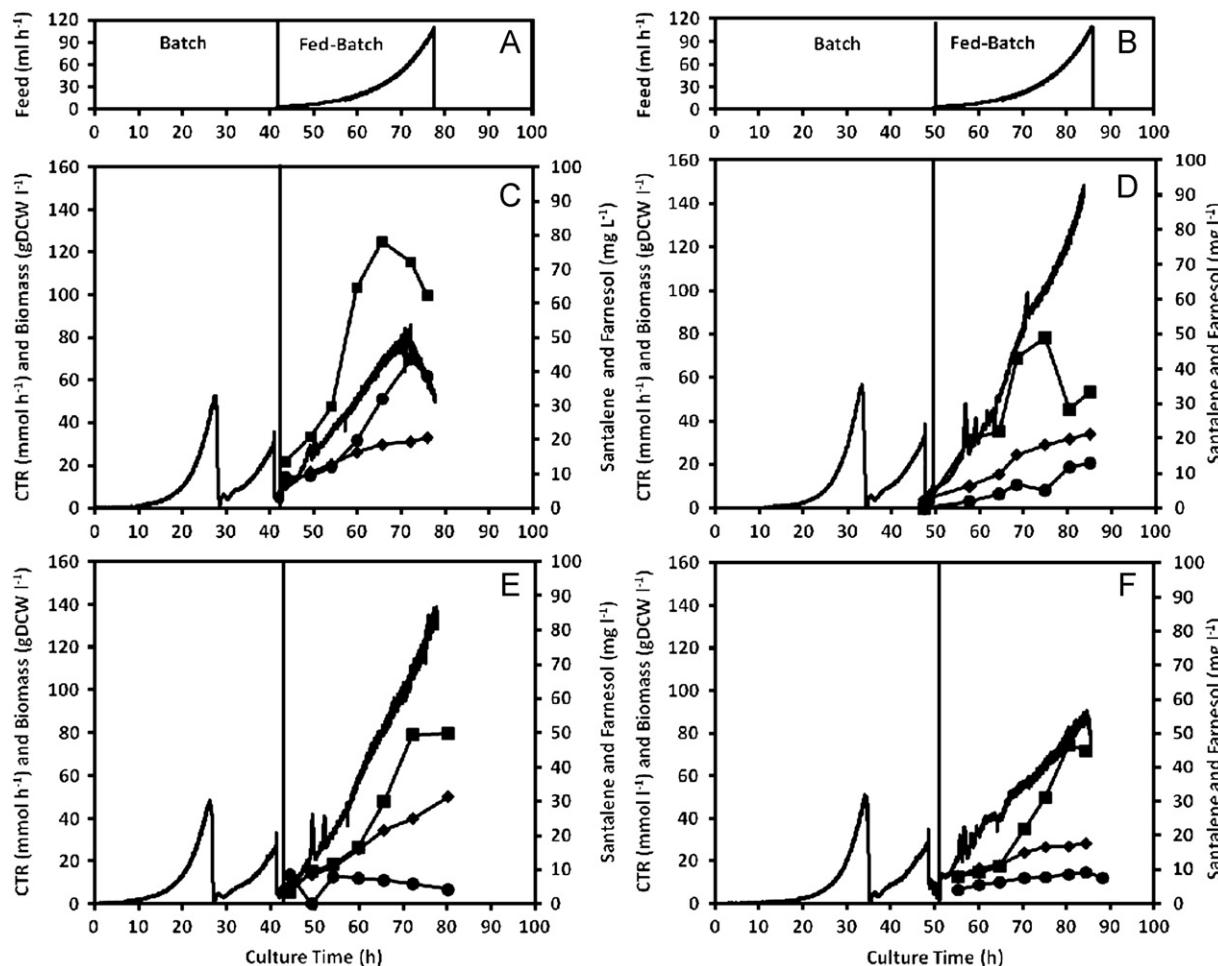


Fig. 6. Time course of an aerobic fed-batch culture with exponential sugar feed of *S. cerevisiae* strains SCICK12 (C) SCKCK14 (D) SCICK13 (E) SCICK15 (F). The feed of glucose (ml h^{-1}) is shown on the upper graph (A) and (B). Typical profile observed for formation of biomass (g l^{-1} , filled diamonds); α -santalene (mg l^{-1} , filled squares); E,E-farnesol (mg l^{-1} , filled cycles) carbon dioxide production CTR (mmol h^{-1} , lines) are represented. Data represent the average of two independent cultures.

after residual ethanol produced during the glucose consumption phase was completely depleted, the organic layer was added to the fermentor and the production phase was started by initiating a feed of fresh concentrated substrate with exponential kinetics for a total feed period of 36 h (Fig. 6). Within the first 30 h of feed the culture metabolism was completely respiratory characterized by complete oxidation of glucose with biomass and carbon dioxide as the major products and complete absence of fermentation products, while the respiratory coefficients remained close to 1 for all strains (Table 3). The period of respiratory growth was followed by a phase where yeast growth was no longer consistent with the feeding profile resulting a shift towards fermentative metabolism accompanied by accumulation of glucose and ethanol

(data not shown). To examine the effect of *ERG9* repression on the sterol pathway, the total cellular sterol content was measured. Both the two P_{ERG9} replacement mutants and the strain expressing the antisense construct showed a lowered sterol content when compared to the strain containing the original *ERG9* promoter. The decrease in ergosterol ranged from 50 to 91%, and strain SCICK12 (P_{HXT1}) showed the lowest sterol content (Fig. 7).

To establish if the lower sterol content reflected an increased availability of FPP precursor for sesquiterpene conversion, product accumulation in the organic layer was measured. Similarly to the results in shake flasks, formation of α -santalene was accompanied by *trans*- α -bergamotene production observed in

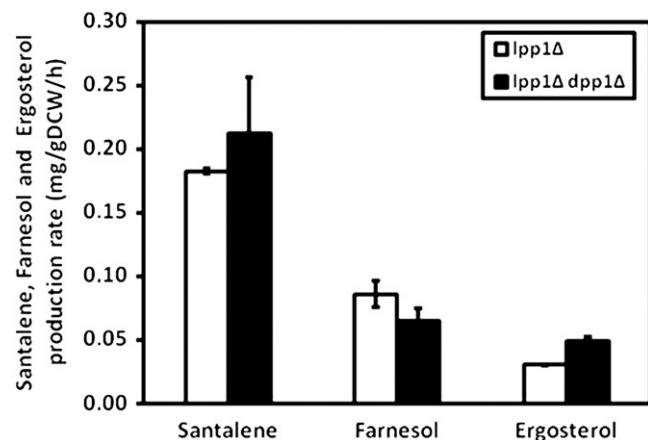
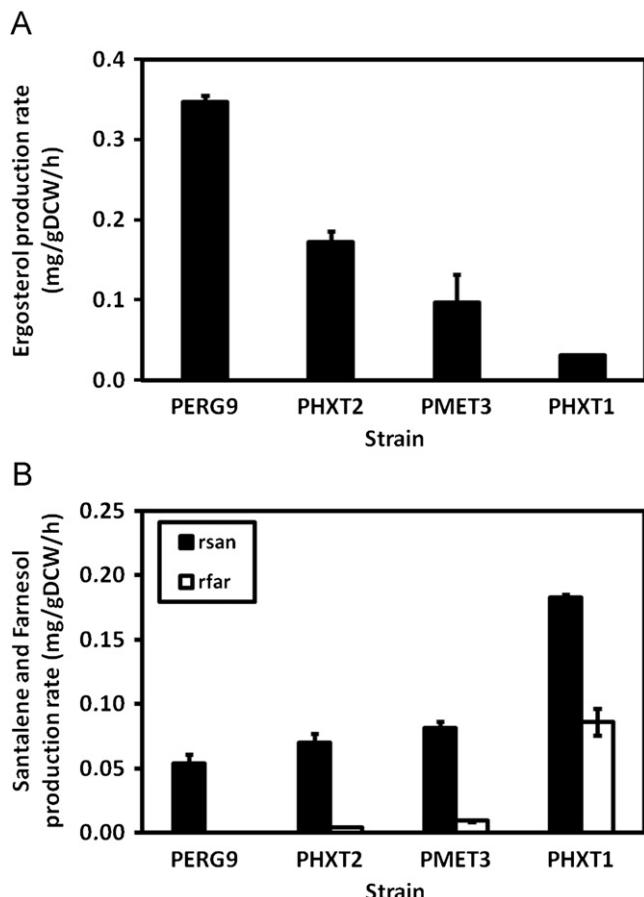


Fig. 8. Effect of single $lpp1\Delta$ and $lpp1\Delta dpp1\Delta$ double deletion on α -santalene, E,E -farnesol and ergosterol production rates ($\text{mg g biomass}^{-1} \text{h}^{-1}$) in strains SCICK12 (P_{PHXT1} $lpp1\Delta$) (empty bars) and SCICK17 (P_{PHXT1} $lpp1\Delta dpp1\Delta$) (filled bars). The error bars represent the standard deviation for two independent cultivations.

(Takahashi et al., 2007). Together with Lpp1, Dpp1 is responsible for most of the cytosolic isoprenoid and lipid phosphate phosphatase activity in *S. cerevisiae* (Toke et al., 1998; Faulkner et al., 1999). As all engineered strains showed an increased conversion of FPP to FOH thus reducing substrate availability for santalene synthase the additional effect of deletion of $DPP1$ on the best producing strain SCICK12 (P_{PHXT1}) was investigated. $DPP1$ was deleted in strain SCICK01 and subsequently transformed with the expression plasmid pLCK01 containing a copy of *tHMG1* and santalene synthase resulting in strain SCICK17. Deletion of $DPP1$ resulted in an increase of the α -santalene specific production rate from 0.18 to 0.21 $\text{mg (g biomass)}^{-1} \text{h}^{-1}$ together with a 24% drop in farnesol accumulation, but showed only a minor effect on the ergosterol content (Fig. 8).

Using the different promoter systems, it was possible to increase the santalene productivity from 0.05 to 0.18 $\text{mg (g biomass)}^{-1} \text{h}^{-1}$. $DPP1$ deletion contributed to reduce the FOH formation and further increase the final santalene titer by 54% (Table 3). Combining these modifications resulted in a strain capable of the highest α -santalene production level of any strain tested with a final titer of 92 mg/liter.

4. Discussion

Previous studies have reported successful examples of expression of different plant derived terpene synthases in the yeast *S. cerevisiae* (Yamano et al., 1994; Jackson et al., 2003; Dejong et al., 2006; Ro et al., 2006; Asadollahi et al., 2008). In this work, yeast was engineered for the first time to produce α -santalene introducing santalene synthase (*SanSyn*) from *C. lansium*. As observed during expression in *Escherichia coli* (Schalk, 2011), α -santalene was the main product formed by this enzyme and only a minor amount of the secondary compound *trans*- α -bergamotene was detected. Catalytic efficiency and specificity of the heterologous synthase are often referred to as key factors in order to achieve relevant titers of the desired compound (Picaud et al., 2005).

Terpene precursors are produced in yeast through the mevalonate pathway (MVA). Due to the variety of essential compounds derived from this pathway, the activity of many enzymes is strictly regulated at different levels (Maury et al., 2005). Yeast was engineered to increase α -santalene production by modulating the expression of two key metabolic steps of the mevalonate pathway (i) down-regulating the squalene synthase gene (*ERG9*)

3.5. Contribution of $DPP1$ deletion to santalene production

$DPP1$ deletion had been used previously in order to reduce farnesol accumulation in a sesquiterpene producing strain

and (ii) over-expressing the truncated version of HMG-CoA reductase (*tHMG1*) to increase the pool of the critical intermediate FPP and enabling the redirection of the carbon flux towards α -santalene.

FPP-derived squalene is a critical precursor of ergosterol, a key component of the yeast cytoplasmic membrane and essential for membrane fluidity. The attempt to increase the FPP pool by *ERG9* deletion resulted in a complete loss of squalene formation and has been shown to be lethal (Jennings et al., 1991). Ergosterol supplementation for restoring viability would be economically unfeasible for industrial applications. Recently, many different techniques have been applied to reduce a specific gene activity as a suitable alternative to complete gene deletions (Mijakovic et al., 2005; Hammer et al., 2006). In this work, several strategies for down-regulation of *ERG9* were evaluated to precisely adjust enzyme activity throughout the entire course of fermentation enabling sufficient squalene production to fulfill the minimum ergosterol requirements to sustain cellular growth without extra-cellular sterol supplementation and improve FPP availability for conversion into α -santalene. Characterization of the promoter activity based on a *lacZ* gene reporter assay allowed the identification of promoters that could provide the optimal level of SQS necessary to optimize α -santalene production. Based on their activity profiles, P_{HXT1} and P_{HXT2} were chosen to promote expression of *ERG9* and antisense *ERG9*, respectively, in a fed-batch process with the aim to couple SQS activity to glucose concentration, i.e. to achieve maximal repression during the feed phase when glucose is limiting. Among the different systems tested, repression of *ERG9* transcription under glucose limitation using the P_{HXT1} promoter was more efficient than induction of antisense RNA controlled by P_{HXT2} or employing the previously used P_{MET3} promoter. The minor effect observed using the antisense strategy is probably due to the fact that the expression of asRNA resulted in incomplete gene repression in yeast (Bonoli et al., 2006). Using a glucose responsive promoter has the additional advantage that no addition of an expensive repressing or inducing agent is needed to control its activity.

Data reported in this work show that engineering the FPP branch point increases the cellular pool of FPP reducing the sterol content and leading to an effective enhancement of flux towards sesquiterpenes. Applying different levels of repression of SQS resulted in a consistent redirection of carbon from ergosterol towards α -santalene and FOH. A linear correlation was observed between the different levels of decrease in sterol content and sesquiterpene (santalene+farnesol) formation indicating that down-regulation of *ERG9* changed the availability of FPP and resulted in diverting the flow to the sesquiterpene compounds. It has been hypothesized that SQS has a lower affinity for FPP compared to most of the other enzymes acting at the FPP branch point resulting in high flux toward the non-sterol branches at low FPP concentration (Scheffler, 2002). The catalytic performance of santalene synthase was sufficient to compete with the non-sterol branches and efficiently drained the FPP pool towards the sesquiterpene compound α -santalene. However, santalene synthase was not able to completely convert the excess of FPP and this resulted in accumulation of small amounts of farnesol. This hypothesis was supported by a small increase in the ergosterol level observed when farnesol production was further reduced by deleting *DPP1* leaving the level of α -santalene mainly unchanged. This suggests that the catalytic capacity of santalene synthase could be saturated and therefore not sufficient to convert the additional FPP created by down-regulation of SQS thus resulting in FOH overflow.

Sterol alteration had no effect on the growth characteristics of the engineered strains probably due to the reduced growth rate ($\mu=0.06 \pm 0.01$) applied during the fed-batch process, which is

far below the maximum specific growth rate measured for these strains under normal batch conditions ($\mu_{max}=0.35 \pm 0.01$). The accumulation of sterol intermediates is known to result in feedback inhibition of the MVA pathway (Maury et al., 2005). Here, the decrease in sterol content achieved by down-regulating *ERG9* could contribute to relieve this regulatory mechanism and further enrich the flow through the MVA pathway leading to high yields of α -santalene.

According to previous reports, farnesol accumulation was observed in strains over-expressing HMGR (Ohto et al., 2009, 2010). Conversion of FPP to farnesol is the preferred alternative route when squalene synthase is inhibited in mouse, rat and dog (Bansal and Vaidya, 1994). Yeast strains blocked at squalene synthase require ergosterol for growth and produce farnesol (Song, 2003). FOH accumulation was previously detected in yeast strains treated with zaragozic acid, a natural inhibitor of SQS (Kuranda et al., 2010). Moreover, the acitivity of HMGR is increased in glucose de-repressed fermentation mode (Quain and Haslam, 1979). Farnesol formation could be explained by the effects of the deregulation of HMGR combined with glucose de-repressed growth conditions that increased the intracellular FPP concentration and shunted the FPP pool towards farnesol via dephosphorylation. Due to the potentially toxic effect of intracellular FPP accumulation (Bansal and Vaidya, 1994), dephosphorylation could act as self-defense mechanism diverting the excess of FPP into FOH that can then be secreted.

In contrast to other organisms where farnesol production is attributed to specific farnesyl pyrophosphatases (Christophe and Popja, 1961; Bansal and Vaidya, 1994), yeast enzymatic activities involved in the FPP dephosphorylation process have not been fully elucidated yet. Several mechanisms have been suggested, among them (i) self-de-phosphorylation by FPP synthase (Erg20) (Chambon et al., 1990); (ii) non-identified specific phosphatase or pyrophosphatase activities (Chambon et al., 1990) and (iii) acid catalyzed non-enzymatic hydrolysis (Muramatsu et al., 2008). Biochemical enzymatic characterizations demonstrated that diacylglycerol pyrophosphate phosphatase encoded by *DPP1* has broad substrate specificity and can utilize isoprenoid phosphate compounds as substrate (Faulkner et al., 1999; Carman and Wu, 2007). Deletion of *DPP1* has been previously used in an attempt to reduce dephosphorylation of FPP to FOH during isoprenoid production resulting in a reduction of 67% in the FOH production from 90 mg/l to 30 mg/l (Takahashi et al., 2007). Together with Dpp1, lipid phosphate phosphatase Lpp1 accounts for most of the cytosolic lipid phosphate phosphatase activity in *S. cerevisiae* (Toke et al., 1998). When two enzymes compete for the same substrate the catalytic efficiency (V_{max}/K_m) may represent a decisive parameter for increasing the flux through a specific enzyme. Previous work showed that simultaneous knock-out of *LPP1* and *DPP1* reduced the rate of hydrolysis of FPP into FOH in vitro to about 10% (Faulkner et al., 1999). Introducing *DPP1* deletion in an *lpp1\Delta* strain further improved α -santalene productivity in the fermentation process at the expense of FOH formation.

An efficient fermentation process strategy that couples biochemical production to biomass formation was utilized for improving the production of α -santalene. Fed-batch fermentation operations are commonly used during industrial production processes to achieve a high yield and productivity of the target product (Nielsen et al., 2003). Limited exponential feed profiles of glucose for *S. cerevisiae* fed-batch cultivations were used to maximize the carbon flux from glucose to biomass and the desired target compound, alleviating glucose repression and Crabtree effect (Pronk et al., 1996). Due to the low water solubility of α -santalene the compound easily gets stripped with the gas bubbles used for aeration, but here the product was

captured using an *in situ* product removal bioreactor set-up. This technique has been intensively used to enhance the production of high value products such as secondary metabolites (Daugulis, 1997). This double phase partitioning system allows an *in situ* product capturing in the bioreactor minimizing loss of volatiles and compounds with low solubility through the gas outlet and reducing potential toxic effect due to product accumulation.

Combining a metabolic engineering strategy together with fermentation optimization, a production process capable of reaching industrial relevant amounts of the compound α -santalene was realized. Final titers of approximately 92 mg l⁻¹ and 131 mg l⁻¹ of α -santalene and total sesquiterpene were reached in 36 h of feed from a synthetic minimal medium. Furthermore, our study shows that through the use of glucose concentration regulated promoters it is possible to dynamically redirect carbon fluxes in the cell during fed-batch fermentation, and this approach may find application also in the production of a wide range of other products by yeast.

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PAPER IV

Systematic metabolic engineering applied to plant sesquiterpene production in continuous cultures of *Saccharomyces cerevisiae*

Gionata Scalcnati, Siavash Partow, Verena Siewers, Michel Schalk,
Laurent Daviet, Jens Nielsen

(Submitted)

Combined metabolic engineering of precursor and co-factor supply to increase α -santalene production by *Saccharomyces cerevisiae*

Gionata Scalcinati¹, Siavash Partow¹, Verena Siewers¹, Michel Schalk², Laurent Daviet², Jens Nielsen^{1§}

¹Department of Chemical and Biological Engineering, Chalmers University of Technology,
SE-412 96 Göteborg, Sweden.

²Firmenich SA, Corporate R&D Division, CH-1211 Geneva 8, Switzerland

[§]Corresponding author

Professor Jens Nielsen

Email addresses:

GS: gionata@chalmers.se

SP: partow@chalmers.se

VS: siewers@chalmers.se

MS: Michel.SCHALK@firmenich.com

LD: Laurent.DAVIET@firmenich.com

JN: nielsenj@chalmers.se

Abstract

Background

Sesquiterpenes are a class of natural products with a diverse range of attractive industrial properties. Due to economic difficulties of sesquiterpene production via extraction from plants or chemical synthesis there is interest in developing alternative and cost efficient bioprocesses. The hydrocarbon α -santalene is a precursor of sesquiterpenes with relevant commercial applications. Here, we construct an efficient *Saccharomyces cerevisiae* cell factory for α -santalene production.

Results

A multistep metabolic engineering strategy targeted to increase precursor and cofactor supply was employed to manipulate the yeast metabolic network in order to redirect carbon toward the desired product. To do so, genetic modifications were introduced acting to optimize the farnesyl diphosphate branch point, modulate the mevalonate pathway, modify the ammonium assimilation pathway and enhance the activity of a transcriptional activator. The approach employed resulted in an overall α -santalene yield of $0.0052 \text{ Cmmol (Cmmol glucose)}^{-1}$ corresponding to a 4-fold improvement over the reference strain. This strategy, combined with a specifically developed continuous fermentation process, led to a final α -santalene productivity of $0.036 \text{ Cmmol (g biomass)}^{-1} \text{ h}^{-1}$.

Conclusions

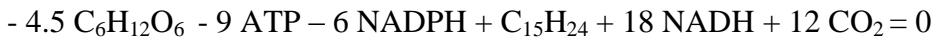
The results reported in this work illustrate how the combination of a metabolic engineering strategy with fermentation technology optimization can be used to obtain significant amounts of the high-value sesquiterpene α -santalene. This represents a starting point toward the construction of a yeast “sesquiterpene factory” and for the development of an economically viable bio-based process that has the potential to replace the current production methods.

Keywords

Metabolic engineering, isoprenoids, sesquiterpenes, continuous culture, *Saccharomyces cerevisiae*.

Background

Isoprenoids are a class of natural compounds with many potential commercial applications (e.g. flavoring agents, fragrances, food colorants, pharmaceutical agents and biofuel precursors), and there has recently been much interest in biotechnological production of these compounds [1-4]. Limitations in raw material accessibility, low yields and high costs of the current isoprenoid production through plant extraction or difficulties with chemical synthesis have caused interest in engineering cell factories that can be used to produce isoprenoids in cost competitive bioprocesses [5-7]. Isoprenoids are natively produced in yeast though the mevalonate (MVA) pathway in which the universal isoprene functional unit isopentenyl diphosphate (IPP) is produced from acetyl-CoA (Figure 1) [8]. The terminal product IPP and its isomer dimethylallyl diphosphate (DMAPP) are subsequently condensed in the prenyl diphosphate pathway generating isoprene derivatives of different chain length (C₅-C₂₀) [9]. The sesquiterpene hydrocarbon α -santalene is a precursor of commercially relevant sesquiterpenes (C₁₅) and it is generated in a one-step conversion from the intermediate building block farnesyl diphosphate (FPP) [10]. Stoichiometry of α -santalene (C₁₅H₂₄) production in *S. cerevisiae* via the MVA pathway in purely oxidative growth conditions can be summarized as:



which demonstrates that α -santalene production involves a net consumption of ATP and NADPH, whereas there is a net production of NADH.

Considerable efforts have been made to engineer yeast for isoprenoid production [8, 11]. Recently, progress has been reported in developing a *S. cerevisiae* strain capable to produce commercially relevant amounts of α -santalene [12], and the aim of the present work was to develop a *S. cerevisiae* production platform for sesquiterpene compounds that could serve as an inexpensive, environmentally compatible alternative to current production methods. We undertook a multistep metabolic engineering strategy combining four different approaches to increase α -santalene production. These included: (i) Modulation and optimization of the FPP branch point (ii) Modulation of the MVA pathway to increase the precursor pool for isoprenoid

synthesis (iii) Increasing the availability of the reductive cofactor NADPH by modifying the ammonium assimilation pathway and (iv) Enhancing the activity of a transcriptional activator of sterol biosynthesis.

(i) In order to minimize the overflow to the biosynthetically related sterols that have the same precursor as α -santalene, FPP, the native promoter (P_{ERG9}) of squalene synthase (SQS) was replaced with a glucose sensing P_{HXT1} promoter [12]. Previous attempts to increase cytosolic FPP availability by down-regulating the $ERG9$ gene resulted in a rapid dephosphorylation of FPP to farnesol (FOH) [13-15]. To minimize the flux towards farnesol two genes, $LPP1$ and $DPP1$, encoding enzymes with FPP dephosphorylation activity have been deleted [12, 16], and we also adapted this approach here (Figure 1).

(ii) As a second part of the strategy we amplified the flux through the MVA pathway by engineering two key enzymatic steps. The mevalonate producing 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR) enzyme is a highly regulated enzyme and is generally believed to exert a high degree of flux control in the MVA pathway. Part of its regulation is via the N-terminal domain of Hmg1 that spans the membrane of the endoplasmic reticulum (ER) and hereby interacts with sterol sensing components of the ER membrane. This feed-back regulation by sterols can be eliminated by expressing a modified form of HMGR lacking the trans-membrane region [17]. Here we used a genetic modification widely used in the past in order to circumvent post-transcriptional regulation of HMGR [18]. The $HMG1$ gene region coding for the catalytic domain was over-expressed resulting in a constitutively active, cytosolic variant of Hmg1. This strategy has been successfully used before for over-producing several different isoprenoids in *S. cerevisiae* [12, 14, 19-21]. The other enzymatic step engineered in the MVA pathway was the one mediated by farnesyl diphosphate synthase (FPPS) (encoded by the essential gene $ERG20$), which catalyses the condensation of IPP units into geranyl diphosphate (GPP) and FPP [22]. IPP condensing enzymes are interspecies conserved and the yeast $ERG20$ gene product evolved towards specific production of FPP rather than GPP [23]. Due to the pivotal nature of the FPP molecule as precursor of many essential compounds such as dolichol, ubiquinone, isoprenylated proteins and ergosterol [24] its synthesis by FPPS is tightly regulated and has been identified as a flux controlling step of the MVA pathway, in particular controlling the intracellular FPP availability and its distribution

into derived products [25,26]. The efficiency of *ERG20* overexpression to increase the level of IPP conversion to FPP and its derivatives depends, however, on the growth conditions employed and the yeast background strain utilized [20, 27]. In this study, the effect of overexpressing *ERG20* on α -santalene production has been investigated.

(iii) The manipulation of the NADH and NADPH cofactor balance in order to overcome limits imposed from the cellular redox constraints is a well-established metabolic engineering strategy [28]. The reaction leading to α -santalene formation results in net production of NADH and consumption of NADPH (see reaction above). A change in the NADH:NADPH ratio in favor of NADPH would therefore be beneficial for product formation. Increasing the availability of reduced cofactor NADPH by deleting the NADPH consuming reaction of glutamate dehydrogenase encoded by *GDH1* has previously been applied to improve product formation [29]. Similarly, activation of an alternative ammonium utilization route in a *gdh1* \square strain by overexpressing the NAD-dependent glutamate dehydrogenase encoded by *GDH2* resulted in an increase of NADH consumption during the anabolic process and in a modification of the yeast cofactor balance [30]. More recently, *in silico* analysis identified the same strategy as an approach to increase sesquiterpene production [31]. Here we evaluated the effect of *GDH1* deletion alone as well as coupled with simultaneous over-expression of *GDH2* on α -santalene production (Figure 1).

(iv). The last strategy we employed involved engineering of a key transcription factor with the objective to generally up-regulate expression of the MVA pathway genes. Upc2 and Ecm22 have been identified as the main transcription factors responsible for the activation of several MVA and ergosterol pathways genes [32]. The point mutation *upc2-1* discovered first for conferring the ability to assimilate extracellular sterols during aerobic cultivation [33] has been demonstrated to result in a constitutively active form of Upc2 [34]. Overexpression of *upc2-1* has been employed to transcriptionally up-regulate the MVA pathway genes during isoprenoid production, but its effect on enhancing the carbon flow through the pathway was modest when used alone [19, 20]. However, when combined together with *ERG9* down-regulation, it produced a clear increase in total isoprenoid production [20, 35]. In the current work, contribution of the *upc2-1* overexpression on the production of α -santalene was tested in combination with the modifications described above.

Although these strategies have been employed before for increasing sesquiterpene production in yeast, they are here for the first time combined in a single strain. All the genetic modifications described above were integrated into the yeast genome to enhance the genetic stability of the production strain during long term cultivation. However, in order to ensure flexibility and to allow the platform strain to be used for production of a range of different isoprenoids, we expressed the synthase gene required for the final conversion of FPP into α -santalene together with an additional copy of *tHMG1* on a multicopy plasmid (Figure 1). The effect of the different metabolic engineering strategies on isoprenoid production was evaluated using an integrated fermentation/downstream recovery process with a two-phase partitioning continuous cultivation set-up (Figure 2). By combining the different strategies we developed a yeast strain and a fermentation process that resulted in high sesquiterpene titers and the results represent a first step toward the long term goal of establishing an efficient sesquiterpene production process.

Results

The primary objective of this study was to enhance the availability of intracellular FPP to increase the production level of the sesquiterpene α -santalene and to evaluate the metabolic response of *S. cerevisiae* to the genetic modifications. A double phase continuous cultivation method was developed as production process to investigate the performances of the engineered strains at glucose-limited conditions.

Characterization of engineered sesquiterpene producing strains in two-phase chemostat cultivation

S. cerevisiae was engineered to produce α -santalene by introducing the expression plasmid pISP15 containing a copy of *tHMG1* and codon optimized *SanSyn* (*SanSyn_{opt}*) under control of the *PGK1* and *TEF1* promoters, respectively (strain SCIGS28). The transformed strain was initially tested for its α -santalene producing capacity in a double-phase chemostat process at a dilution rate of 0.05 h⁻¹ resulting in an α -santalene yield of 0.0013 Cmmol (Cmmol glucose)⁻¹ and a production rate of 0.006 Cmmol (g biomass)⁻¹ h⁻¹ (corresponding to 0.086 mg (g biomass)⁻¹ h⁻¹). All the following strain development strategies were assessed based on the yield and productivity of this control strain (SCIGS28) and are reported in Figure 3 and 4, whereas the titers are given in Figure 5.

Replacement of the native P_{ERG9} promoter with P_{HXT1} was previously proven to efficiently reduce ergosterol production and increase the availability of FPP for the conversion into sesquiterpene products [12]. Here, the same modification was introduced in an $lpp1\Delta$ strain carrying the expression vector resulting in strain SCIGS29. Using P_{HXT1} to control $ERG9$ expression combined with $LPP1$ deletion resulted in an increase in α -santalene yield and productivity of 3- and 3.8-fold, respectively. α -Santalene production was accompanied by the formation of the FPP-derived farnesol (FOH) at a production rate of $0.006 \text{ Cmmol (g biomass)}^{-1} \text{ h}^{-1}$ (Figure 3A). The impact of the additional deletion of $DPP1$ was tested in an attempt to reduce the rate of hydrolysis of FPP into the undesired by-product FOH (strain SCIGS30). This resulted in an almost unchanged flux towards α -santalene formation, but in a reduction of the farnesol yield and productivity by 50% and 44%, respectively.

In a following approach, the impact of perturbing the redox metabolism on α -santalene accumulation was evaluated introducing the deletion of $GDH1$ encoding NADP-dependent glutamate dehydrogenase (strain SCIGS31). In the strain harboring the additional $GDH1$ deletion, no further enhancement in α -santalene productivity was detected. Interestingly, no substantial FOH formation was detected in this strain (Figure 3A and 4).

Subsequently, we monitored the effect of activating an NADH consuming reaction for ammonium assimilation together with the up-regulation of the second MVA pathway flux controlling step FPPS, integrating and over-expressing simultaneously the genes $GDH2$ and $ERG20$ (strain SCIGS24). This combination resulted in a significant increase of sesquiterpene production contributing to attain the maximum α -santalene yield and productivity of $0.0052 \text{ Cmmol (Cmmol glucose)}^{-1}$ and $0.036 \text{ Cmmol (g biomass)}^{-1} \text{ h}^{-1}$, respectively. The additional up-regulation of $GDH2$ and $ERG20$ combined with all previous features produced a 4- and 6-fold improvement, respectively, in α -santalene yield and productivity compared to the control strain (Figure 3A and 4).

The MVA pathway was further engineered by integrating into the yeast genome the mutated transcription factor gene $upc2-1$ and an extra copy of $tHMG1$ (strain SCIGS25). Previously, both strategies, using an additional genome integrated copy of $tHMG1$ next to plasmid-based expression and the over-expression of $upc2-1$ have

displayed little or only a strain-dependent effect on final product production [14, 20]. Similarly, our combined approach did not contribute to increase α -santalene production over the best producing strain obtained, SCIGS24. However, in contrast to the insignificant change in α -santalene productivity strain SCIGS25 exhibited a 2-fold increase in FOH formation yielding a final FOH yield of 0.0024 Cmmol (Cmmol glucose) $^{-1}$ and a productivity of 0.018 Cmmol (g biomass) $^{-1}$ h $^{-1}$. It is therefore worth mentioning that strain SCIGS25 reached the highest total sesquiterpene yield and productivity of 0.0069 Cmmol (Cmmol glucose) $^{-1}$ and 0.052 Cmmol (g biomass) $^{-1}$ h $^{-1}$ (santalene + farnesol), respectively (Figure 3A and 4).

Evaluation of sesquiterpene production strains at different dilution rates

Under the employed conditions, the engineered strains exhibited significant changes in the total amount of sesquiterpene produced. The sesquiterpene productivity level varied almost 10-fold between the strains, from 0.006 to 0.052 Cmmol (g biomass) $^{-1}$ h $^{-1}$. Chemostat cultivation mode offers the advantage of manipulating with accuracy the dilution rate, which at these conditions is equal to the specific growth rate [36]. We therefore decided to investigate the behaviour of the sesquiterpene production strains at two different growth rates. All previous cultivations were performed at a dilution rate of 0.05 h $^{-1}$ and when the control strain was grown at D=0.1 h $^{-1}$, a small decrease in the α -santalene yield was observed (Figure 4) whereas its productivity remained essentially unchanged (Figure 3). The increase in α -santalene production observed for strains SCIGS29 and SCIGS30 at low dilution rate (D=0.05 h $^{-1}$) was also seen at the higher dilution rate of 0.1 h $^{-1}$. α -Santalene productivities measured for these strains were, respectively, 0.041 and 0.043 Cmmol (g biomass) $^{-1}$ h $^{-1}$ representing a 6-fold increase compared to the control strain and almost a 2-fold increase compared to the productivity at D=0.05 h $^{-1}$ (Figure 3B). In contrast, the yield was slightly reduced. Consistently, the *DPPI* deletion resulted in reduced FOH accumulation in strain SCIGS30 compared to the *lpp1Δ* single deletion (strain SCIGS29). The ratios between the α -santalene and the farnesol yield in the two strains of 2.3 and 4.2, respectively, were maintained when the dilution rate was raised to 0.1 h $^{-1}$ (Figure 4). Consistently, the same product proportion was also seen in the productivities (Figure 3). Therefore, the distribution of FPP between the two products remained unchanged when the dilution rate was increased.

Surprisingly, strains SCIGS31, SCIGS24 and SCIGS25 were unable to sustain growth at D=0.1 h⁻¹ and cultures were washed out (see section below).

Strain physiology in batch and chemostat cultivation

In order to evaluate if the modifications applied to increase sesquiterpene production affected yeast physiology a detailed characterization of the recombinant strains was carried out. Control strain SCIGS28 displayed a fully respiratory metabolism (RQ=1.0) under both dilution rates. The principal physiological parameters (e.g. Y_{sx}, r_s, r_{CO₂} and r_{O₂}) were comparable with the wild type strain CEN.PK113-7D [37, 38]. Strains SCIGS29 and SCIGS30 exhibited major alterations in their physiology. An increase in the residual glucose concentration of 6.4 fold at D=0.05 h⁻¹ and 2.5 fold at D=0.1 h⁻¹ was observed for both strains. As direct consequence of the increase in the residual glucose concentration aerobic fermentation set in, resulting in ethanol formation accompanied with acetate accumulation. A marked reduction in the biomass yield from 0.5 to 0.29-0.28 (D=0.05 h⁻¹) and 0.28-0.25 g biomass (g glucose)⁻¹ (D=0.1 h⁻¹) was measured for the two strains (Table 1). However, only a small fraction corresponding to 4% (Cmmol products (Cmmol glucose)⁻¹) of the glucose consumed was fermented to ethanol and acetate. Additionally, a clear increase in the glucose (r_s) and oxygen consumption rate (r_{O₂}) and carbon dioxide production rate (r_{CO₂}) was observed (Table 1). This physiological response was observed at both D=0.05 and 0.1 h⁻¹. Despite several attempts, it was not possible to achieve a steady-state when strains SCIGS31, SCIGS24 and SCIGS25 were grown at D=0.1 h⁻¹. Instead, a progressive decrease of the biomass concentration over time was observed consistent with wash-out kinetics. The following characterization for these strains was therefore conducted only at D=0.05 h⁻¹.

When deletion of *GDH1* was introduced (strain SCIGS31) a considerable fraction of the glucose, 31 mmol l⁻¹, was recovered corresponding to a consumption of only 33% of the total sugar provided. However, it was still possible to reach a steady state. In this strain, the rate of alcoholic fermentation increased to 0.51 mmol (g biomass)⁻¹ h⁻¹ and the metabolism shifted more predominantly to a respiro-fermentative state (RQ=1.62), where 21% of the carbon source was metabolized to the fermentation products ethanol and acetate. These pronounced metabolic changes were probably

related to a limitation in ammonium consumption as a consequence of *Gdh1* inactivation.

Overexpression of *GDH2* is known to partially complement the ammonium assimilation defect in a *gdh1Δ* strain [30] and resulted in a clear reduction of the ethanol and acetate production rate in strains SCIGS24 and SCIGS25 compared to strain SCIGS31. It is worth noticing that in strain SCIGS24, a large decrease in the biomass yield occurred and the specific glucose and O₂ consumption rates and the CO₂ production rate increased respectively to a value of 1.16, 3.21 and 3.85 mmol (g biomass)⁻¹ h⁻¹. The previously described overflow metabolism phenomenon towards fermentation products was also observed in strains SCIGS24 and SCIGS25 and led to a fraction of carbon fermented to ethanol and acetate close to 6% for both strains. All engineered strains except the control strain exhibited overflow metabolism under the tested conditions. The fraction of glucose converted into fermentation products ranged between 0.04 and 0.21 Cmmol products (Cmmol glucose)⁻¹. If strain SCIGS31 (which exhibited a behaviour different from all other strains probably related to the major role played by the ammonium limitation) is excluded from this consideration, it is interesting to notice that the ratios of the different fermentation products measured vary substantially between the strains. A significantly higher ethanol:acetate ratio was observed for strains SCIGS24 and SCIGS25 compared to SCIGS29 and SCIGS30 indicating a redistribution of flux around the pyruvate dehydrogenase (PDH) bypass at the acetaldehyde level. The increased ethanol:acetate ratio was reflected in an increase in the formation of sesquiterpene products, which are derived directly from the cytosolic acetyl-CoA produced through the pyruvate decarboxylase route. On the other hand, the engineered strains showed a clear decrease in biomass yield compared to the control strain suggesting a carbon flux redirection towards other products. The fraction of carbon lost in the drop of biomass yield could not be accounted for in the residual unconsumed glucose or in the fermentation products. Instead, carbon dioxide was the main carbon product. Surprisingly, the increase in ethanol and acetate productivity was not related to any decrease in the respiration rate. The oxygen uptake rate was increased in all engineered strains compared to the control strain and reached the highest value of 4.41 mmol (g biomass)⁻¹ h⁻¹ in strain SCIGS25 suggesting a strong reprogramming of cell metabolism in these strains.

Discussion

In this study, we provide an example of several rounds of metabolic engineering aimed at increasing the production of the commercially relevant sesquiterpene compound α -santalene. The strain improvement strategy was combined with development of a cost effective fermentation process based on a two-phase continuous cultivation mode.

Double-phase chemostat as a tool to study metabolically engineered strains

Continuous cultivation modes have been employed in industrial bioprocesses (e.g. insulin production) and offer several advantages compared to batch conditions [36]. One is that they allow a precise comparison of productivities of selected genetically engineered strains under well-controlled constant conditions and to explore the effect of the growth rate independently of the other parameters.

Being extensively used in bioprocesses to produce aroma compounds, *in situ* product removal (ISPR) (for review see [39]) was applied in this study to maximize the product recovery. Through the combination of ISPR with chemostat cultivations we obtained a production system that offers the advantage of continuous recovery of the product in the fermenter effluent from the selected organic phase which can subsequently be recycled, regenerated and reused in the same process for a prolonged time of cultivation (for review see [40]). The developed set-up is a suitable approach to develop an upscaled industrial process.

Influence of the genetic modifications on strain productivity

Here we examined the impact of different metabolic engineering strategies and their combinations on α -santalene productivity and yield. The control strain was minimally engineered to produce α -santalene functionally expressing a codon optimized santalene synthase (SanSyn) from *C. lansium* and a truncated version of 3-hydroxyl-3-methyl-glutaryl-coenzyme A reductase (HMGR). SanSyn belongs to the class I group of sesquiterpene cyclases. These enzymes catalyze a complex intramolecular cyclization of FPP with very different product specificity and the reaction mechanism often involves several partial reactions [41]. SanSyn has a high specificity for α -santalene as its main product with only minor amounts of *trans*- α -bergamotene formed [12]. Many studies have reported examples of heterologous production of

isoprenoids simply expressing the plant synthase in the desired microbial host. However, the yields obtained are often extremely low [15, 19, 20, 42-45]. Similar to our previous study we decided to construct a reference α -santalene producing strain (SCIGS 29) combining the synthase expression with the expression of the deregulated form of Hmg1 (tHmg1) [12]. The use of *tHMG1* represents an excellent example of bypassing one of the regulatory mechanisms controlling the MVA pathway flux and has been successfully applied in a number of microbial isoprenoid production processes [14, 19-21, 43]. The yield obtained in this control strain was comparable with our previously reported values obtained during a fed-batch process [12] and demonstrates the feasibility and robustness in applying our novel double-phase continuous cultivation. In order to improve the production of the target compound it is necessary to overcome the regulatory mechanisms that have evolved to prevent flux imbalances. In this work, we modulated some of the well-recognized key points that tightly regulate the carbon flux to sesquiterpenes in *S. cerevisiae*. A slight reduction in yield and unchanged productivity observed in the control strain at a higher dilution rate suggests a limitation of the plant synthase in efficiently draining the FPP precursor from the MVA pathway, consistent with the previous hypothesis that at low FPP concentration *SanSyn* competes with the other cellular FPP consuming reactions [12]. A general strategy extensively applied in sesquiterpene bioprocess development [14, 15, 20, 35] consists in down-regulating SQS to increase the intracellular FPP pool. Replacement of the native *P_{ERG9}* promoter with the glucose-sensing *P_{HXT1}* promoter was recently successfully employed to divert the carbon flux to sesquiterpene products instead of sterols [12]. Applying the same *ERG9* modification in this study together with deletion of *LPP1* greatly increased the sesquiterpene productivity and yield under chemostat conditions compared to the control strain. The obtained productivity level appears to increase with the dilution rate employed pointing to a direct relation between the specific growth rate and the overall flux through the MVA pathway and indicating that the efficiency of the *ERG9* modification in the enhanced FPP availability was supported at different specific growth rates. A similar growth dependent relation has been reported for the cellular content of ergosterol [46], which is also derived from FPP.

In the *lpp1Δ* and *lpp1Δ/dpp1Δ* mutants known to exhibit lower FPP phosphatase activity [16, 47], the excess of FPP was redistributed between α -santalene and FOH in

a consistent ratio when different dilution rates were applied. These results suggest the hypothesis that once a threshold level of intracellular flux toward FPP is reached the thermodynamically favourable endogenous dephosphorylation starts and competes with the catalytic capacity of santalene synthase leading to FOH accumulation. On the other hand, the unchanged α -santalene yield coupled with higher productivity achieved at higher dilution rates suggests that the santalene synthase was not fully saturated at low dilution rates and there was excess activity to cope with high FPP flux. This points out that the FOH formation is not only a direct consequence of limited santalene synthase activity but that other cellular mechanisms are likely to be involved. Reduction but not complete inhibition of FOH formation in the *lpp1Δ dpp1Δ* double deletion strain compared to the single *lpp1Δ* deletion strain was consistent with our previous report [12], and confirmed that the *DPP1* encoded lipid phosphate phosphatase has a role in FPP dephosphorylation and together with Lpp1 is involved in the conversion of FPP into FOH. However, these are clearly not the only mechanisms responsible for this conversion as we still observed some FOH production in the double deletion strain, but it is uncertain whether this is the result of the activity of additional phosphatases or caused by non-enzymatic hydrolysis.

Stoichiometry of the pathway reaction for α -santalene formation from glucose reveals a net consumption of 0.4 mol of NADPH and net production of 1.2 mol of NADH per Cmol of α -santalene formed. This fact renders the sesquiterpene production pathway a target for cofactor engineering to improve its productivity. Improving the NADPH availability by modifying the ammonium assimilation pathway has proven to be an effective strategy to increase sesquiterpene production [14]. Interestingly, when the previously employed deletion of *GDH1* to manipulate the cell redox metabolism was introduced a reduction in α -santalene productivity without FOH accumulation was obtained. This modification also strongly affected the strain physiology (see below). Therefore, it is likely that the limitation in ammonium assimilation imposed by the *GDH1* deletion reduces the flux through the MVA pathway below the level necessary to trigger FOH formation and conversion of FPP into α -santalene was sufficient to avoid intracellular FPP accumulation.

Combining the simultaneous overexpression of the NAD-dependent glutamate dehydrogenase and prenyl transferase encoded, respectively, by *GDH2* and *ERG20* positively affected sesquiterpene production. Overexpression of *GDH2* is known to

restore the ammonium assimilation and consequently alter the NADH:NADPH equilibrium favouring the NADPH availability at the expense of NADH produced [14, 30].

The consensus binding motif for the sterol biosynthesis activating transcription factor Upc2 has been found in most of the promoters of the ergosterol pathway genes [35]. Moreover, it was shown that some genes of the MVA pathway including *ERG8*, *ERG12*, *ERG13*, *ERG20* and *HMG1* contain sequences similar to the consensus binding sequence [32, 48]. Expression of *upc2-1* together with an additional copy of *tHMG1* contributed to increase the carbon flux through the MVA pathway and had a beneficial effect on the total sesquiterpene production. The fraction of FOH produced was almost double in this strain and largely contributed to the observed increase of total sesquiterpenes indicating that when the flux toward sesquiterpene is altered through the introduction of genetic modifications the FPP branch point displayed an unexpected flexibility in product distribution.

The optimal solution was obtained through combining all the modifications resulting in the highest sesquiterpene yield (strains SCIGS24 and SCIGS25). Compared to our previous study [12] the engineering strategy employed here led to a 1.8-fold increase in α -santalene final yield (Cmmol α -santalene /Cmmol glucose). These results highlight the importance of combining different engineering strategies to achieve the goal of generating an efficient platform strain for sesquiterpene production. It is noteworthy that comparable sequiterpene productivity was achieved in the strains not fully engineered simply by increasing the operational dilution rate (strains SCIGS29 and SCIGS30) whereas the fully engineered strains were washed out when the same conditions were imposed. Further studies are necessary to elucidate the factors leading to the inability of these mutants to sustain growth at higher dilution rates.

Influence of genetic modifications on strain physiology

In this study, the effect of controlling the diversion of carbon flow from sterol synthesis towards sequiterpene production by modifying the *ERG9* promoter has been investigated during aerobic chemostat glucose limited cultivation conditions. The *lpp1Δ* and *lpp1Δ/dpp1Δ* mutants carrying the *P_{HXT1}-ERG9* construct clearly showed an increase in the residual glucose concentration slightly above the critical concentration that triggers aerobic fermentation, which was reported to lie between

0.5 and 0.8 mM [49, 50] and results in a typical Crabtree response. It is possible that regulating the Erg9 activity using the P_{HXTI} glucose sensitive promoter under strictly glucose limited conditions resulted in its almost complete down-regulation and in an increased biosynthetic demand of the essential compound ergosterol. Ergosterol is the main sterol present in the plasma membranes where it has several essential functions [51]. Yeast is dependent on oxygen for sterol and fatty acid formation. Under strictly anaerobic conditions this compound has to be provided in the media. Reducing its provision results in a decrease of biomass formation and an increase in ethanol formation [52]. Activity of P_{HXTI} has been shown to be induced at an extracellular glucose concentration of 5.6 mM [53] suggesting that the observed increase in the residual glucose concentration in the cultures was necessary to restore a minimal P_{HXTI} activity in order to maintain the ergosterol level necessary to sustain cell growth. The response to the limitation in the essential compound ergosterol could be the reason leading to the observed decrease in biomass yield and increase of the fermentative metabolism. A similar phenomenon in fact was observed in autotrophic yeast strains in uracil-limited chemostat culture [54]. The observed overflow metabolism toward ethanol and acetate formation increases the carbon flux through the PDH bypass possibly resulting in an increase in the cytosolic acetyl-CoA availability that was subsequently more efficiently channelled towards the MVA pathway in the engineered strains enhancing the final sesquiterpene production.

Strain SCIGS31 exhibited a particular physiology and needs to be discussed separately. Deletion of $GDH1$ is known to impair the ammonium assimilation resulting in a lower specific biomass formation rate on different carbon sources (glucose/galactose) and under different growth conditions (batch/chemostat and aerobic/anaerobic) [29, 30], which was confirmed in this study. When deletion of $GDH1$ was introduced, ethanol formation as well as glucose accumulation occurred, resulting in a situation similar to cultivation limited in essential nutrients [54]. Most likely, the combination of the limitation in ammonium assimilation as result of the $GDH1$ deletion together with the possible ergosterol limitation due to the $ERG9$ downregulation produced the observed respiro-fermentative metabolism.

Conclusions

Microbial production of sesquiterpenes is an active research area; advances in pathway engineering and fermentation technologies have a significant impact in accomplishing the aim to develop an economically viable biobased industrial process. In this study, engineering different pathways simultaneously resulted in a robust *S. cerevisiae* production host capable of efficiently producing α -santalene. The engineered strains were evaluated in an optimized double-phase continuous fermentation method leading to a high yield of α -santalene and resulting in a robust production process that could possibly be used for commercial applications. Levels of products observed open up to the possibility to explore new engineering option for increasing the sesquiterpene productivity. The presented systematic metabolic engineering approach represents a gateway toward the creation of yeast platform that can be applied to the production of an array of sesquiterpene products.

Methods

Plasmid construction

An overview of the plasmids constructed in this study is reported in Table 2, the detailed maps of the plasmids is contained in supplementary file 1. The gene coding for α -santalene synthase (*SanSyn_{opt}*) was codon optimized for expression in *S. cerevisiae* and synthesized by DNA 2.0 (Menlo Park, CA, USA) (supplementary file 2), cut with *NotI/PacI* and ligated into *NotI/PacI* restricted vector pICK01 containing *tHMG1* [12] resulting in plasmid pISP15 (Figure 1).

To simultaneously integrate multiple genes into the yeast genome a series of plasmids containing the genes, constitutive strong promoters, terminators, marker gene sequences and the required region for genomic integration were constructed. All endogenous *S. cerevisiae* genes were PCR amplified using genomic DNA of strain CEN.PK113-5D as template. Primers used for amplification are provided in supplementary file 3. All PCRs were performed using high fidelity Phusion™ DNA polymerase (Finnzymes, Vantaa, Finland). The *ERG20* gene [GenBank: NM_001181600] was amplified using primers pair 1/2, subsequently digested with *BamHI/NheI* and ligated into the vector pSP-GM2 [55] restricted with the respective enzymes downstream of the *TEF1* promoter resulting in plasmid pIGS01. A 711 bp upstream flanking region (AD1) selected for genomic integration was amplified using

primers pair 3/4, cut with *MreI/Kpn*2I and ligated into vector pIGS01 restricted with the respective enzymes resulting in plasmid pIGS02. Plasmid pIGS03 was obtained by cloning gene *GDH2* [GenBank: NM_001180275] amplified with primers pair 5/6 into pIGS02 downstream of the *PGK1* promoter using *PacI/NotI* restriction sites. A downstream flanking region of 653 bp (AD2) was amplified with primers 7/8, digested with *AscI/AvrII* and ligated into pIGS03. The resulting plasmid was named pIGS04. To complete the plasmid for integration the *Kluyveromyces lactis* (*Kl*) *URA3* gene [GenBank: Y00454] was amplified with primers 9 and 10 using plasmid pWJ1042 [56] as template, cut with *FseI* and ligated into pIGS04 after restriction with the respective enzyme. The resulting plasmid was designated pIGS05, digested with *MreI/AscI* and the resulting fragment used for integration into the yeast genome as described below. The 5' region of the *Kl URA3* gene was amplified with primers 11 and 12, cut with *AvrII/AscI* and cloned into pIGS03 restricted with the respective enzymes resulting in plasmid pIGS06. Amplification of the catalytic domain of the HMG-CoA reductase gene (*tHMG1*) [GenBank: NM_001182434] was performed using primers pair 13/14, the resulting fragment cleaved with *NheI/BamHI* and cloned downstream of the *TEF1* promoter into *NheI/BamHI* restricted pSP-GM2 resulting in pIGS07. A mutant allele *upc2-1* of the *UPC2* gene [GenBank: NC_001180521] was created by use of primer pair 15/16. To introduce the pleiotropic mutation G888D, the corresponding codon GGT was mutated to GAT generating the amino acid substitution. Subsequently, the PCR amplified *upc2-1* was cloned downstream of the *PGK1* promoter into pIGS07 using *NotI/PacI* resulting in plasmid pIGS08. An 829 bp downstream flanking region (AD3) selected for genomic integration was amplified using primer pair 17/18 cut with *MreI/Kpn*2I and ligated into vector pIGS08 restricted with the respective enzymes resulting in plasmid pIGS09. The 3' region of *Kl URA3* (overlapping with the 5' region described above) was amplified with primers 19 and 20, cut with *AvrII/AscI* and cloned into pIGS09 restricted with the respective enzymes resulting in plasmid pIGS10. All plasmids were verified by sequencing (Sigma-Aldrich, St. Luis, MO). Subsequently, plasmids pIGS06 and pIGS10 were restricted with *MreI/AscI*, the cassettes isolated from the vector backbone and used for yeast transformation (see below).

Yeast strain construction

All *S. cerevisiae* strains constructed in this work have a CEN.PK background with auxotrophy for uracil [57] and are listed in Table 3.

Strain SCIGS03 carrying a *GDH1* [GenBank: NC_001183795] deletion was created from strain SCICK16 using a bipartite gene-targeting technique [55]. Upstream and downstream region of *GDH1* were amplified by PCR from CEN.PK113-5D genomic DNA using primer pairs 23/24 and 25/26. The *loxP-kanMX-loxP* cassette was amplified from plasmid pUG6 [59] as two overlapping fragments using primer pairs 29/30 (5' part) and 31/32 (3' part). By fusion PCR, the upstream region of *GDH1* was combined with the 5' part of the kanMX cassette and the 3' part of the kanMX cassette with the downstream region of *GDH1* and the resulting fragments used to transform SCICK16. Transformation was performed using the standard lithium acetate procedure [58] and transformants were selected using YPD plates containing 200 mg/l G418 (Formedium, Hunstanton, UK). Correct integration of the kanMX cassette into the *GDH1* locus was tested by PCR using primers 27/28. The kanMX marker was subsequently excised by transient transformation with plasmid pSH47 containing the Cre recombinase encoding gene [59] leading to formation of strain SCIGS03. Strain SCIGS06 carrying a genomic integration of genes *ERG20* and *GDH2* under control of the *TEF1* and *PGK1* promoter, respectively, was obtained by transforming strain SCIGS03 with the *MreI/AscI* fragment isolated from plasmid pIGS05. Correct integration into the YORWΔ22 locus on chromosome XV [60] was verified by PCR using primer pairs 33/1 and 5/34. Strain SCIGS12 carrying a genomic integration of genes *ERG20*, *GDH2*, *tHMG1* and *upc2-1*, was constructed by co-transforming strain SCIGS03 with the *MreI/AscI* fragments isolated from plasmids pIGS06 and pIGS10. Correct integration into the YORWΔ22 locus was verified by PCR using primer pairs 33/1, 5/35, 19/15, 35/15, 16/36 and 13/34.

Kl URA3 was replaced in strains SCIGS06 and SCIGS12 with the kanMX marker. The *loxP-kanMX-loxP* cassette was independently amplified from plasmid pUG6 using primers pairs 37/38 for integration in strain SCIGS06 and 39/40 for integration in SCIGS12 containing 71-74 bp primer tails complementary to the target integration sites. Both strains were transformed with the respective PCR-amplified fragment. Transformants were selected on YPD plates containing 200 mg/l G418. *Kl URA3*

replacement was initially tested by replica plating on synthetic complete (SC) medium without uracil and YPD/G418 medium. The *kanMX* marker was subsequently removed [59] leading to strains SCIGS22 and SCIGS23.

Strains SCIGS28, SCIGS29, SCIGS30, SCIGS31, SCIGS24 and SCIGS25 were obtained transforming, respectively, strains CEN.PK113-5D, SCICK01, SCICK16, SCIGS03, SCIGS22 and SCIGS23 with the high copy number plasmid pISP15 (Table 3) containing the *URA3* gene and the genes *SanSyn_{opt}* and *tHMG1* under control of the strong constitutive promoters *TEF1* and *PGK1*, respectively (Table 2).

Strain maintenance

Long term storage of yeast suspensions containing 25% (vol/vol) sterile glycerol was performed in cryovials at -80°C [61]. Working stocks were maintained on YPD agar plates containing 10 g/l yeast extract, 20 g/l casein peptone, 20 g/l glucose and 20 g/l agar. Plasmid carrying strains were maintained on synthetic dextrose medium agar plates lacking uracil containing 6.9 g/l yeast nitrogen base without amino acids (Formedium), 0.77 g/l complete supplement mixture without uracil (Formedium) 20 g/l dextrose and 20 g/l agar.

Media and growth conditions

A mineral salts medium was used for batch cultivations as previously described [62] and had the following composition (per liter): $(\text{NH}_4)_2\text{SO}_4$, 5 g; KH_2PO_4 , 3 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.50 g; Antifoam 289 (A204, Sigma–Aldrich), 0.05 ml; trace metals, 1 ml and vitamins, 1 ml. The trace metal solution consisted of the following (per liter): EDTA (sodium salt), 15.0 g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.45 g; $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$, 1 g; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.3 g; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.3 g; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.4 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.45 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g; H_3BO_3 , 0.1 g and KI, 0.1 g. The pH of the trace metal solution was adjusted to 4.0 with 2 M NaOH prior to heat sterilization. The vitamin solution contained (per liter): biotin, 0.05 g; p-amino benzoic acid, 0.2 g; nicotinic acid, 1 g; Ca-pantothenate, 1 g; pyridoxine-HCl, 1 g; thiamine-HCl, 1 g and myo-inositol, 25 g. The pH of the vitamin solution was adjusted to 6.5 with 2 M NaOH. The vitamin solution was filter sterilized and stored at 4°C. This medium was supplemented with 20 g/l glucose. The feed composition used for continuous cultivation had the same composition as described above, but the glucose concentration was 10 g/l. The medium used for shake flask cultivation has the same composition as described above, but the

$(\text{NH}_4)_2\text{SO}_4$ concentration was increased to 7.5 g/l, and the KH_2PO_4 to 14.4 g/l; the glucose concentration was 20 g/l; the pH was adjusted to 6.5 prior autoclaving.

Inoculum preparation and pre-culture

A single colony from an SC-ura agar plate was selected to inoculate a 500 ml shake flask containing 100 ml mineral salts medium. The seed culture was grown at 30°C in an orbital shaker at 100 rpm to late-exponential phase and used to inoculate the fermenter to a final dry weight of 1 mg/l. All cultivations were performed in triplicate.

Chemostat operation

Aerobic, carbon limited chemostat cultivations were performed in 1.0 l stirrer pro vessels (DasGip, Jülich, Germany) with a working volume of 0.3 l. The temperature was monitored using a platinum RTD temperature sensor and kept at 30°C using a BioBlock integrated heating and cooling thermo well. Agitation was maintained at 600 rpm using an overhead drive stirrer with one Rushton impeller. The air flow rate was kept at 1 vvm by a mass flow controller (DasGip). The pH was maintained constant at 5.0 by automatic addition of 2 M KOH. The fermenters were integrated in a DasGip monitor and control system used to control all fermentation parameters, temperature, agitation, pH, and gas flow. Dissolved oxygen was monitored using an autoclavable polarographic oxygen electrode (Mettler Toledo, Columbus, OH) and maintained above 30% saturation via regulating stirrer speed and gas flow rate. Exhaust gas was cooled, dried and the gas composition was analyzed for real time continuous determination of oxygen and carbon dioxide concentration by a DasGip fed batch pro® gas analysis system with off gas analyzer GA4 based on zirconium dioxide and two-beam infrared sensor. The integrated mass flow sensor allowed on-line monitoring and calculation of oxygen transfer rate (OTR), carbon dioxide transfer rate (CTR) and respiratory quotient (RQ). The chemostat bioreactor was initiated as batch culture with 10 g/l glucose. Only after the residual ethanol produced was completely consumed the feed was started and the fermentation run in a continuous mode. Fermenters were operated at dilution rate 0.05 or 0.1 h^{-1} . A two-phase product partition chemostat was performed by co-feeding medium containing 10 g/l glucose and the organic phase (Figure 2). To obtain a dilution rate of 0.1 h^{-1} , the inlet medium was fed at 27 ml/h and the organic phase at 3 ml/h. To obtain a dilution rate of 0.05 h^{-1} , medium was feed at 13.5 ml/h and the organic phase at 1.5 ml/h resulting in a

constant inlet feed ratio of medium:organic phase of 9:1 (vol/vol). Dodecane (Sigma-Aldrich, St. Luis, MO) was used as organic phase and filter sterilized prior addition. The culture working volume of 0.3 l (0.27 l of medium + 0.03 l of dodecane) was kept constant by automatic withdrawal of broth based on an electric level sensor measurement. The set-up allowed maintaining the correct medium/organic phase ratio inside the fermenter throughout the fermentation time. The correct ratio of 9:1 vol/vol between the two phases was constantly monitored and differed by less than 2% in samples taken directly from the culture and from the effluent line. Steady state was reached after at least 5 residence times, defined by constant values of CTR, OTR and biomass concentration (less than 5% deviation).

Cell mass determination

Cell growth during fermentation was monitored off-line by measuring optical density and dry cell weight and on-line with an optical density transmitter OD4 sensor (DasGip) integrated in the fermenter system. The optical density at 600 nm was determined using a Genesis20 spectrophotometer (Thermo Scientific, Madison, WI, USA). The cell dry weight was measured by filtering known culture volumes through pre-dried and pre-weighed 0.45- μm -pore size nitrocellulose filters (Sartorius Stedim Biotech GmbH, Göttingen, Germany). The filters with the biomass were washed with water, dried for 15 min in a microwave oven at 150 W, and weighed again. The correlation factor between off-line and on-line parameters was determined.

Metabolite analysis

Samples for analysis of extracellular metabolite concentrations were withdrawn from two-phase steady state chemostat cultures and centrifuged for 5 min at 5000 g. The organic layer was discarded and the cultivation broth was filtered through 0.45- μm -pore size nylon filters (VWR international, Radnor, PA, USA) and stored at -20°C until further analysis. Glucose, glycerol acetate, succinate, and pyruvate were quantified by HPLC (UltiMate® 3000 Nano, Dionex, Bannockburn, IL, USA) with an Aminex HXP-87H ion-exchange column (Bio-Rad, Hercules, CA) maintained at 65°C and using 5 mM H₂SO₄ as mobile phase at a flow rate of 0.6 ml min⁻¹. Glucose, glycerol, and ethanol were measured with a refraction index detector (RI-101 Refractive Index Detector, Shodex®), and acetate, succinate, and pyruvate were

measured with a UV-visible light absorbance detector (UltiMate 3000 Variable Wavelength Detector, Dionex).

Analysis of sesquiterpenes

Sesquiterpene production was determined as described previously [12] with minor modifications. Culture samples were centrifuged 15 min at 5000 g and the organic layer was diluted with an equal volume of dodecane containing a defined amount of α -humulene as internal standard. Samples were diluted in heptane and analyzed by gas chromatography-mass spectrometry (Thermo Scientific) equipped with an SLB-5 ms capillary column (30 m, 0.25 mm i.d., 0.25 μ m film thickness; Supelco, Bellefonte, PA, USA). Full mass spectra were generated by scanning the *m/z* range within 40-500 for metabolite identification. Sesquiterpene identification was carried out comparing mass spectra and retention time with authentic standards, concentrations were calculated using a correction factor determined for the internal standard α -humulene relative to α -santalene and *E,E*-farnesol.

Abbreviations

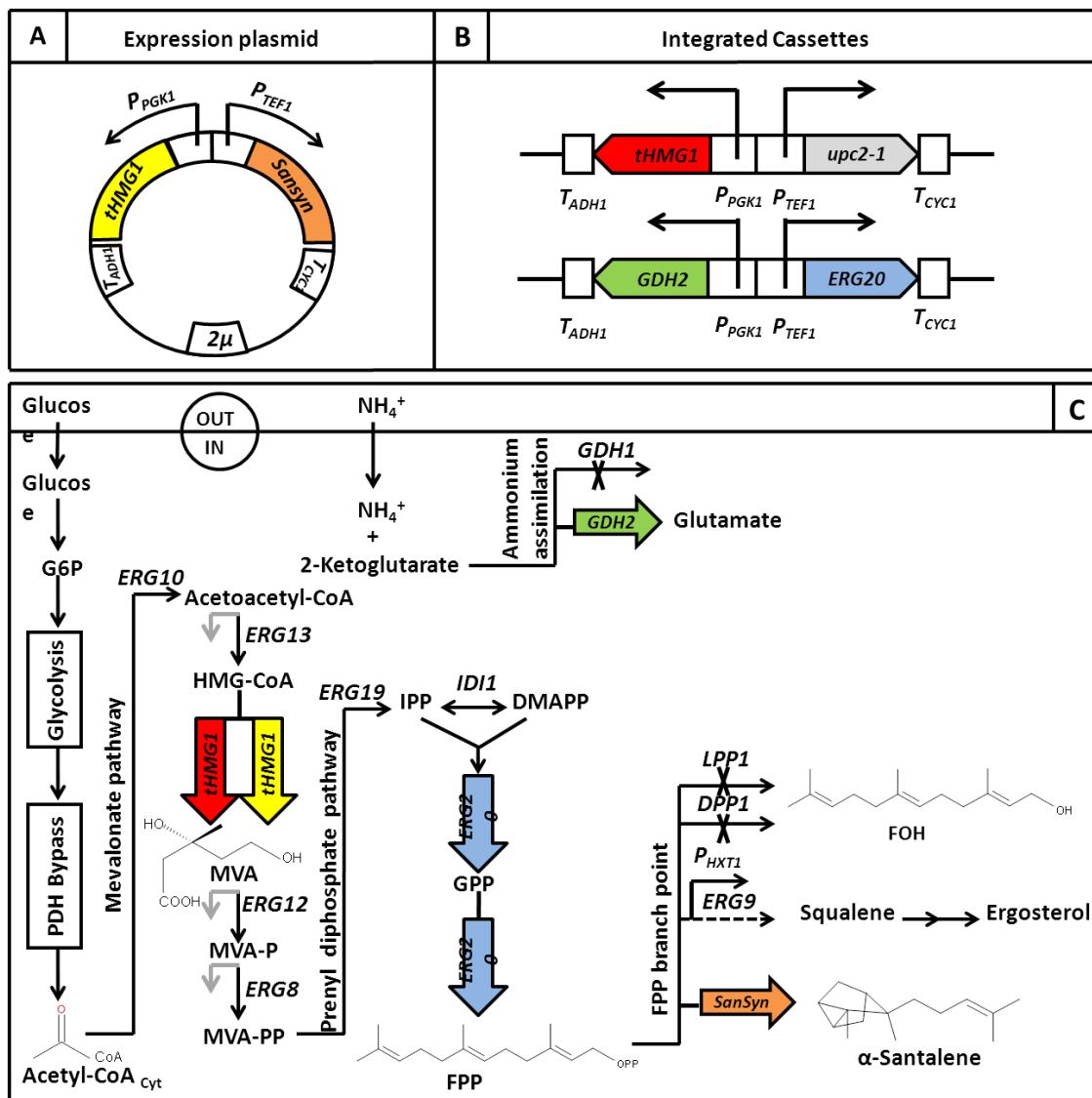
CTR, carbon dioxide transfer rate; DMAPP, dimethylallyl diphosphate; ER, endoplasmic reticulum; FOH, farnesol; FPP, farnesyl diphosphate; FPPS, farnesyl diphosphate synthase; GPP, geranyl diphosphate; HMGR, 3-hydroxy-3-methyl-glutaryl-CoA reductase; IPP, isopentenyl diphosphate; ISPR, *in situ* product removal; MVA, mevalonate; OTR, oxygen transfer rate; PDH, pyruvate dehydrogenase; RQ, respiratory quotient; SanSyn, santalene synthase; SQS, squalene synthase;

Authors' contributions

J.N. and G.S. participated in the design of the study. J.N. and V.S. supervised the project. G.S. performed the experimental work. S.P. assisted in the molecular biology experiments. M.D. and L.D. assisted in the GC/MS analysis of sesquiterpenes. G.S. analyzed the data and wrote the manuscript. All the authors discussed the results, edited and approved the final manuscript.

Acknowledgements

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Figures

Figure 1 - Genetic engineering approach for increasing α -santalene production.

(A) Expression plasmid pISP15 containing *tHMG1* encoding truncated HMG-CoA reductase, a codon optimized santalene synthase gene (*SanSyn_{opt}*) *P_{TEF1}* and *P_{PGK1}* promoters as well as *T_{ADH1}* and *T_{CYC1}* terminator sequences. (B) Integrated cassettes, rectangles containing arrows represent the promoters and their directionality, pentagons the genes and empty squares the terminators. (C) Scheme of the engineered mevalonate, prenyl phosphate and FPP branch point; overexpressed and deleted genes are highlighted. Pathway intermediates: G6P: glucose-6-phosphate, Acetyl-CoA_{cyt}: cytosolic acetyl-CoA, HMG-CoA: 3-hydroxy-3-methylglutaryl-CoA, MVA: mevalonate, MVA-P: phosphomevalonate, MVA-PP: diphosphomevalonate, IPP: isopentenyl diphosphate, DMAPP: dimethylallyl diphosphate, GPP: geranyl diphosphate, FPP: farnesyl diphosphate, FOH: farnesol.

Overexpressed genes are *tHMG1* (encoding truncated HMG-CoA reductase), *ERG20* (encoding FPP synthase), *GDH2* (encoding NAD-dependent glutamate dehydrogenase), and *SanSyn_{opt}* (encoding α -santalene synthase). Deleted genes are *GDH1* (encoding NADP-

dependent glutamate dehydrogenase), *LPP1* and *DPP1* (both encoding lipid phosphate phosphatases). The promoter of the *ERG9* gene (encoding squalene synthase) is replaced with *P_{HXT1}*. Genes whose promoters contain Upc2 binding sites are indicated with a grey arrow: *ERG13* (encoding HMG-CoA synthase), *ERG12* (encoding mevalonate kinase), and *ERG8* (encoding phosphomevalonate kinase). Additional genes indicated are *ERG10* (encoding acetoacetyl-CoA thiolase), *ERG19* (encoding diphosphomevalonate decarboxylase) and *IDI* (encoding IPP isomerase).

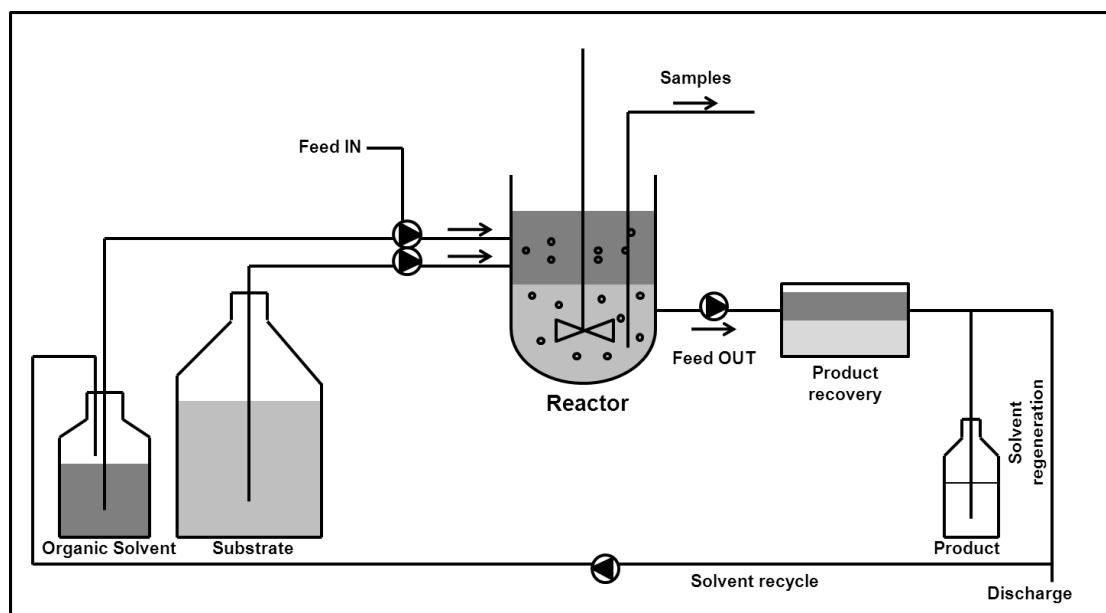


Figure 2 - Set-up of the *in situ* product removal (ISPR) chemostat cultivation process.

A stirred tank reactor is operated in continuous cultivation mode as double phase system feeding culture medium (light grey) and organic solvent (dark grey). The product is continuously captured in the organic phase due to its high hydrophobicity. In an integrated downstream step the two phases of the effluent are partitioned in a settler. Subsequently, the product is recovered from the organic phase, which can then be further recycled in the same process. The exhausted medium is discarded.

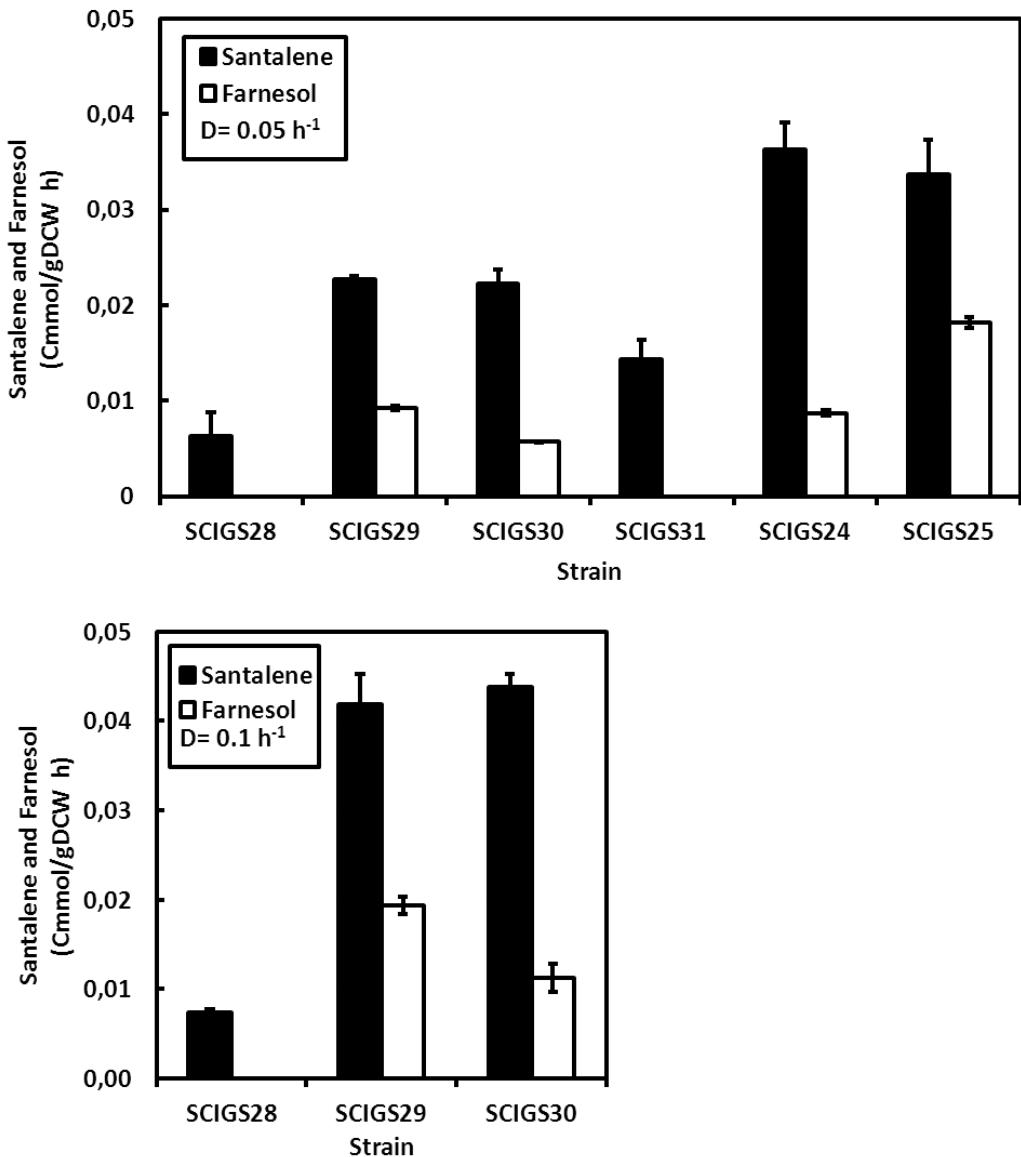


Figure 3 - Sesquiterpene productivity in a two-phase partitioned glucose-limited aerobic chemostat.

α -Santalene and farnesol production rate in Cmmol (g biomass) $^{-1} \text{ h}^{-1}$ (the C-molar weight of α -santalene and farnesol are, respectively, 13.62 and 14.82 g Cmol $^{-1}$). **(A)** Strains SCIGS28 ($tHMG1\uparrow$), SCIGS29 (+ $P_{HXT1}\text{-}ERG9$, $lpp1\Delta$), SCIGS30 (+ $dpp1\Delta$), SCIGS31 (+ $gdh1\Delta$), SCIGS24 (+ $ERG20\uparrow$, $GDH2\uparrow$), SCIGS25 (+ $upc2-1\uparrow$, $tHMG1\uparrow$) cultivated at dilution rate $D=0.05 \text{ h}^{-1}$. **(B)** Strains SCIGS28 ($tHMG1\uparrow$), SCIGS29 (+ $P_{HXT1}\text{-}ERG9$; $lpp1\Delta$), SCIGS30 (+ $lpp1\Delta$) cultivated at dilution rate $D=0.1 \text{ h}^{-1}$. Error bars represent the standard deviation from three independent cultivations.

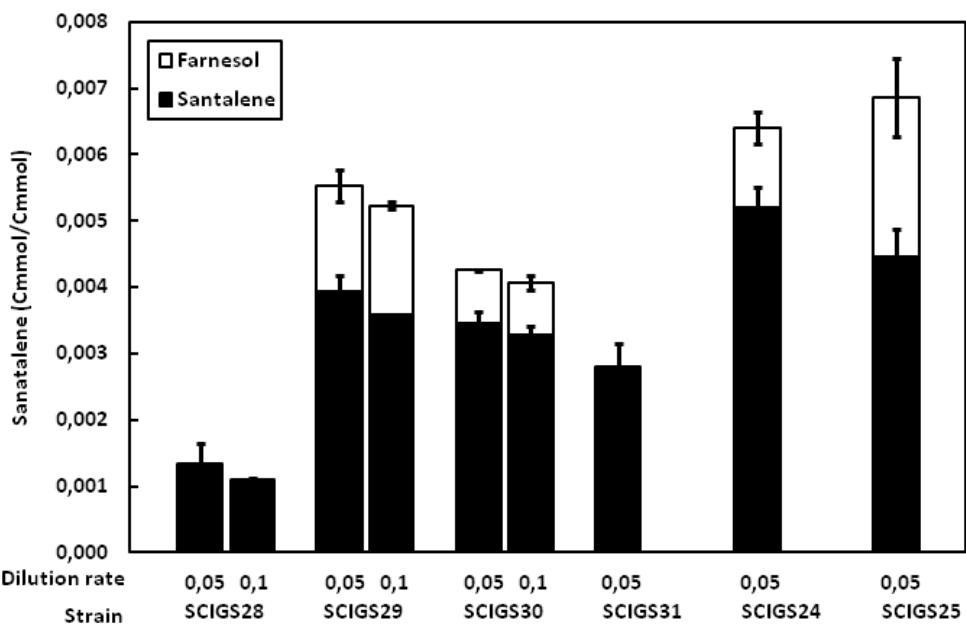


Figure 4 - Sesquiterpene yield in a two-phase partitioned glucose limited aerobic chemostat.

α -Santalene and farnesol yield in Cmmol (Cmmol glucose) $^{-1}$. Strains SCIGS28 ($tHMG1\uparrow$), SCIGS29 (+ P_{HXT1} - $ERG9$, $lpp1\Delta$), SCIGS30 (+ $dpp1\Delta$) were cultivated at dilution rate D=0.05 h $^{-1}$ and D=0.1 h $^{-1}$. Strains SCIGS31 (+ $gdh1\Delta$), SCIGS24 (+ $ERG20\uparrow$, $GDH2\uparrow$), SCIGS25 (+ $upc2-1\uparrow$, $tHMG1\uparrow$) were cultivated at dilution rate D=0.05 h $^{-1}$. Error bars represent the standard deviation from three independent cultivations.

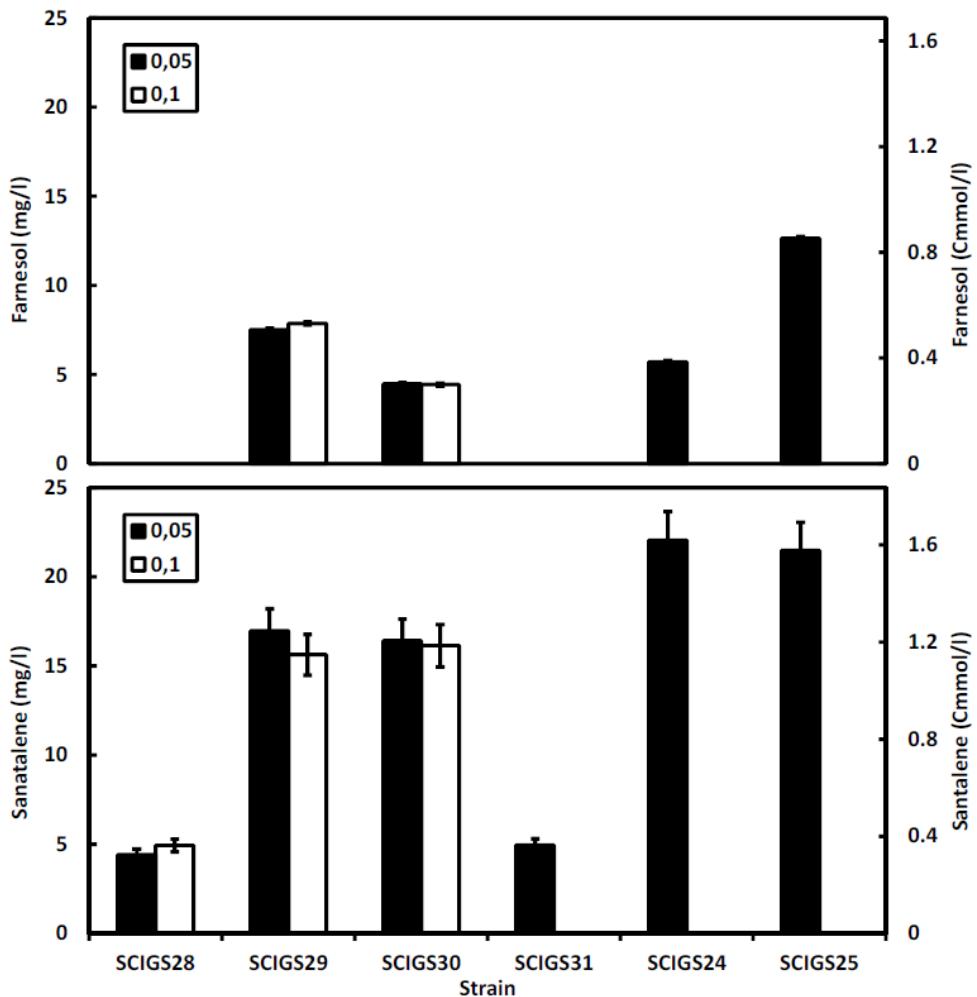


Figure 5 - α -Santalene titer in a two-phase partitioned glucose limited aerobic chemostat.

α -Santalene (bottom) and farnesol (top) titers express in mg l⁻¹ and Cmmol l⁻¹ (the C-molar weight of α -santalene and farnesol are respectively 13.62 and 14.82 g Cmol⁻¹). Strains SCIGS28 (*tHMG1*↑), SCIGS29 (+ *P_{HXT1}-ERG9*, *lpp1*Δ), SCIGS30 (+ *dpp1*Δ) were cultivated at dilution rate D=0.05 h⁻¹ and D=0.1 h⁻¹. Strains SCIGS31 (+ *gdh1*Δ), SCIGS24 (+*ERG20*↑, *GDH2*↑), SCIGS25 (+ *upc2-1*↑, *tHMG1*↑) were cultivated at dilution rate D=0.05 h⁻¹. Error bars represent the standard deviation from three independent cultivations.

Tables

Table 1 - Physiological parameters measured during double-phase chemostat cultures of strains SCIGS28, SCIGS29, SCIGS30, SCIGS31, SCIGS24 and SCIGS25.

μ_{max} , specific growth rate (h^{-1}); D , dilution rate (h^{-1}); Y_{xs} , biomass yield (g biomass (g substrate) $^{-1}$); specific consumption rates of glucose(r_s), and oxygen (r_{O_2}) (mmol (g biomass) $^{-1}\text{h}^{-1}$); specific production rates of carbon dioxide (r_{CO_2}), ethanol (r_{etho}),and acetate (r_{acet}) (mmol (g biomass) $^{-1}\text{h}^{-1}$). RQ, respiratory quotient r_{CO_2}/r_{O_2} ; C_s , residual glucose concentration (mM); $C_{balance}$, carbon recovery (%); Tot_{sant} total amount of α -santalene produced (mg $24\text{h}^{-1}\text{l}^{-1}$). Values represent the mean \pm S.D. of three independent cultivations.

Strain	D	Y_{xs}	r_s	r_{CO_2}	r_{O_2}	r_{etho}	r_{acet}	RQ	C_s	$C_{balance}$	Tot_{sant}
	(h^{-1})	(g g $^{-1}$)	(mmol g biomass $^{-1}\text{h}^{-1}$)					(r_{CO_2}/r_{O_2})	(mM)	(%)	(mg $24\text{h}^{-1}\text{l}^{-1}$)
SCIGS28	0.051 ± 0.002	0.50 ± 0.01	0.57 ± 0.01	1.12 ± 0.08	1.12 ± 0.06	0	0	1.00 ± 0.02	0.18 ± 0.02	100.3 ± 2.1	11.8 ± 0.3
	0.10 ± 0.01	0.50 ± 0.01	1.11 ± 0.03	2.67 ± 0.15	2.49 ± 0.07	0	0	1.07 ± 0.05	0.16 ± 0.01	101.9 ± 1.1	5.2 ± 0.2
SCIGS29	0.050 ± 0.003	0.29 ± 0.01	0.97 ± 0.04	3.17 ± 0.04	2.95 ± 0.15	0.082 ± 0.001	0.024 ± 0.001	1.07 ± 0.07	1.16 ± 0.04	96.9 ± 3.2	37.6 ± 0.2
	0.10 ± 0.01	0.28 ± 0.01	1.95 ± 0.05	6.40 ± 0.18	5.51 ± 0.20	0.128 ± 0.004	0.039 ± 0.008	1.16 ± 0.22	0.39 ± 0.01	95.0 ± 0.9	20.4 ± 0.1
SCIGS30	0.051 ± 0.001	0.28 ± 0.02	1.09 ± 0.05	3.60 ± 0.09	3.41 ± 0.22	0.099 ± 0.015	0.024 ± 0.003	1.05 ± 0.09	1.15 ± 0.04	94.7 ± 0.4	39.4 ± 0.4
	0.10 ± 0.02	0.25 ± 0.01	2.26 ± 0.09	7.70 ± 0.19	6.37 ± 0.29	0.161 ± 0.007	0.032 ± 0.005	1.21 ± 0.14	0.40 ± 0.01	93.5 ± 1.2	20.1 ± 0.1
SCIGS31	0.051 ± 0.001	0.33 ± 0.01	0.86 ± 0.01	2.31 ± 0.08	1.42 ± 0.23	0.501 ± 0.077	0.047 ± 0.007	1.62 ± 0.11	30.67 ± 0.78	105.4 ± 4.9	6.1 ± 0.1
SCIGS24	0.051 ± 0.001	0.24 ± 0.01	1.16 ± 0.03	3.85 ± 0.05	3.21 ± 0.07	0.185 ± 0.003	0.020 ± 0.008	1.20 ± 0.02	2.53 ± 0.09	93.7 ± 5.3	26.9 ± 0.2
SCIGS25	0.048 ± 0.003	0.21 ± 0.01	1.26 ± 0.02	4.41 ± 0.04	3.82 ± 0.04	0.195 ± 0.005	0.027 ± 0.007	1.16 ± 0.03	2.91 ± 0.14	93.8 ± 2.9	24.9 ± 0.3

1

2 **Table 2 - Plasmids used in this study**

3

Plasmid name	Plasmid description	Reference
pSP-GM2	<i>URA3</i> -based expression plasmid carrying a bidirectional P_{TEFI} - P_{PGK1} promoter	[55]
pICK01	P_{TEFI} - <i>SanSyn</i> , P_{PGK1} - <i>tHMG1</i>	[12]
pISP15	P_{TEFI} - <i>SanSyn_{opt}</i> , P_{PGK1} - <i>tHMG1</i>	this study
pIGS01	P_{TEFI} - <i>ERG20</i>	this study
pIGS02	P_{TEFI} - <i>ERG20</i> , AD1	this study
pIGS03	P_{TEFI} - <i>ERG20</i> , P_{PGK1} - <i>GDH2</i> , AD1	this study
pIGS04	P_{TEFI} - <i>ERG20</i> , P_{PGK1} - <i>GDH2</i> , AD1, AD2	this study
pIGS05	P_{TEFI} - <i>ERG20</i> , P_{PGK1} - <i>GDH2</i> , AD1, AD2, <i>KIURA3</i>	this study
pIGS06	P_{TEFI} - <i>ERG20</i> P_{PGK1} - <i>GDH2</i> , AD1, 5' <i>KIURA3</i>	this study
pIGS07	P_{TEFI} - <i>tHMG1</i>	this study
pIGS08	P_{TEFI} - <i>tHMG1</i> , P_{PGK1} - <i>upc2-1</i>	this study
pIGS09	P_{TEFI} - <i>tHMG1</i> , P_{PGK1} - <i>upc2-1</i> , AD3	this study
pIGS10	P_{TEFI} - <i>tHMG1</i> , P_{PGK1} - <i>upc2-1</i> , AD3, 3' <i>KIURA3</i>	this study

4

1 Table 3 - List of *S. cerevisiae* strains used in this study

Strain	Genotype	Plasmid	Reference
CEN.PK113-5D	MATa <i>MAL2-8^c</i> <i>SUC2 ura3-52</i>	none	P. Kötter, University of Frankfurt, Germany
SCIGS28	MATa <i>MAL2-8^c</i> <i>SUC2 ura3-52</i>	pISP15	this study
SCICK01	MATa <i>MAL2-8^c</i> <i>SUC2 ura3-52</i> <i>lpp1Δ::loxP P_{ERG9Δ}::loxP-P_{HXT1}</i>	none	[12]
SCIGS29	MATa <i>MAL2-8^c</i> <i>SUC2 ura3-52</i> <i>lpp1Δ::loxP P_{ERG9Δ}::loxP-P_{HXT1}</i>	pISP15	this study
SCICK16	MATa <i>MAL2-8^c</i> <i>SUC2 ura3-52</i> <i>lpp1Δ::loxP dpp1Δ::loxP</i> <i>P_{ERG9Δ}::loxP-P_{HXT1}</i>	none	[12]
SCIGS30	MATa <i>MAL2-8^c</i> <i>SUC2 ura3-52</i> <i>lpp1Δ::loxP dpp1Δ::loxP</i> <i>P_{ERG9Δ}::loxP-P_{HXT1}</i>	pISP15	this study
SCIGS03	MATa <i>MAL2-8^c</i> <i>SUC2 ura3-52</i> <i>lpp1Δ::loxP dpp1Δ::loxP</i> <i>P_{ERG9Δ}::loxP-P_{HXT1} gdh1Δ::loxP</i>	none	this study
SCIGS31	MATa <i>MAL2-8^c</i> <i>SUC2 ura3-52</i> <i>lpp1Δ::loxP dpp1Δ::loxP</i> <i>P_{ERG9Δ}::loxP-P_{HXT1} gdh1Δ::loxP</i>	pISP15	this study
SCIGS06	MATa <i>MAL2-8^c</i> <i>SUC2 ura3-52</i> <i>lpp1Δ::loxP dpp1Δ::loxP</i> <i>P_{ERG9Δ}::loxP-P_{HXT1} gdh1Δ::loxP</i> <i>P_{TEF1-ERG20} P_{PGK1-GDH2}</i> <i>KIURA3</i>	none	this study
SCIGS22	MATa <i>MAL2-8^c</i> <i>SUC2 ura3-52</i> <i>lpp1Δ::loxP dpp1Δ::loxP</i> <i>P_{ERG9Δ}::loxP-P_{HXT1} gdh1Δ::loxP</i> <i>P_{TEF1-ERG20} P_{PGK1-GDH2}</i>	none	this study
SCIGS24	MATa <i>MAL2-8^c</i> <i>SUC2 ura3-52</i> <i>lpp1Δ::loxP dpp1Δ::loxP</i> <i>P_{ERG9Δ}::loxP-P_{HXT1} gdh1Δ::loxP</i> <i>P_{TEF1-ERG20} P_{PGK1-GDH2}</i>	pISP15	this study
SCIGS23	MATa <i>MAL2-8^c</i> <i>SUC2 ura3-52</i> <i>lpp1Δ::loxP dpp1Δ::loxP</i> <i>P_{ERG9Δ}::loxP-P_{HXT1} gdh1Δ::loxP</i> <i>P_{TEF1-ERG20} P_{PGK1-GDH2} P_{TEF1-tHMG1} P_{PGK1-upc2-1}</i>	none	this study
SCIGS12	MATa <i>MAL2-8^c</i> <i>SUC2 ura3-52</i> <i>lpp1Δ::loxP dpp1Δ::loxP</i> <i>P_{ERG9Δ}::loxP-P_{HXT1} gdh1Δ::loxP</i> <i>P_{TEF1-ERG20} P_{PGK1-GDH2} P_{TEF1-tHMG1} P_{PGK1-upc2-1} KIURA3</i>	none	this study
SCIGS25	MATa <i>MAL2-8^c</i> <i>SUC2 ura3-52</i> <i>lpp1Δ::loxP dpp1Δ::loxP</i> <i>P_{ERG9Δ}::loxP-P_{HXT1} gdh1Δ::loxP</i> <i>P_{TEF1-ERG20} P_{PGK1-GDH2} P_{TEF1-tHMG1} P_{PGK1-upc2-1}</i>	pISP15	this study

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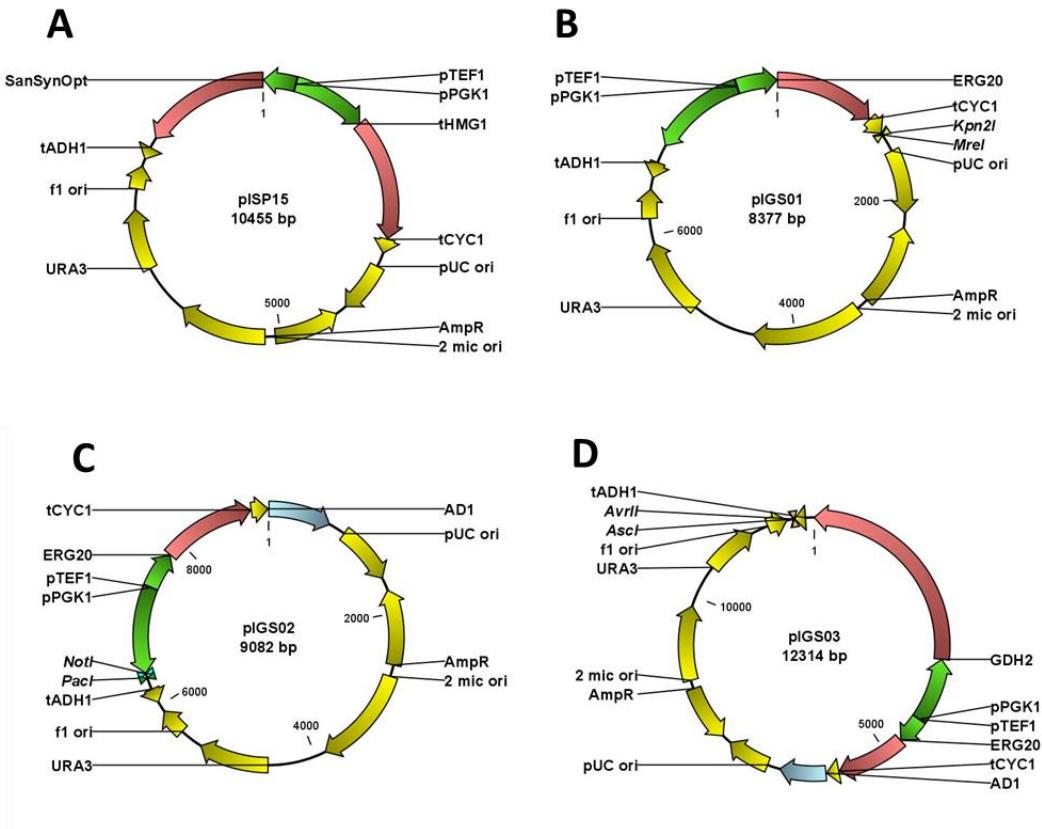
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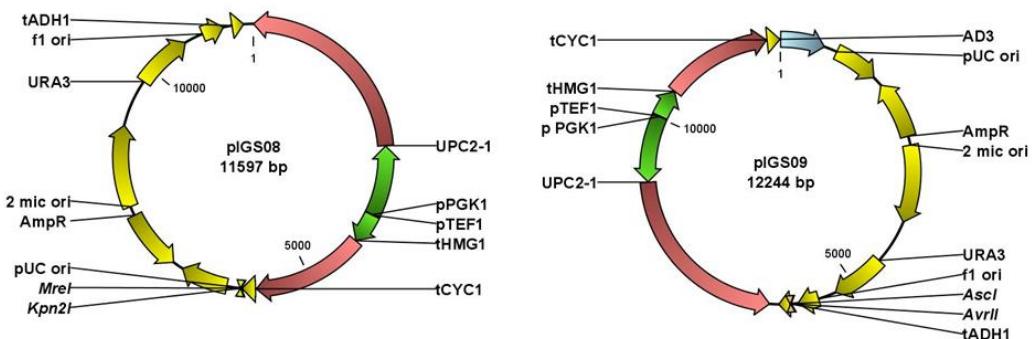
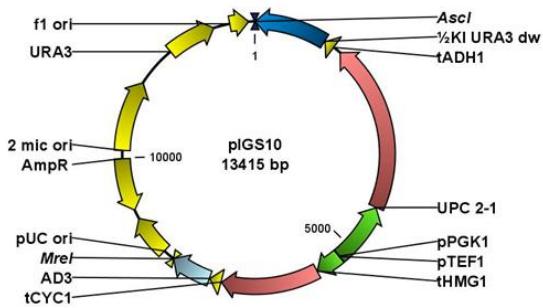
Additional files

Additional file 1 – Maps of plasmids constructed in this study

(A) pISP15, (B) pIGS01, (C) pIGS02, (D) pIGS03, (E) pIGS04, (F) pIGS05, (G) pIGS06, (H) pIGS07, (I) pIGS08, (L) pIGS09, (M) pIGS10.



I

**M**

Additional file 2 –Codon optimized santalene synthase nucleotide sequence

ATGTCTACTCAACAAGTCTCATCCGAGAACATAGTTAGAAATGCTGCGAACTTTC
ATCCTAACATATGGGTAACCATTCTGACTTGTCTCAAACAAATTGACTCT
TGGACCCAACAGCACCAAGGAACCTAACAGGAAAGATTGCCTTGATTGATA
TCTGATGCTAACAAACCAGCGAAAGATTGCCTTGATTGATA
TGGGTGTTGCCTACCATTGAAAAGGAAATTGATGATGCTTAGAAAAGATTGG
TCATGATCCTTCGATGACAAAGATGATTGTACATCGTTCACTATGTTCAGAC
TCCTCGTCAACACGGTATCAAGATCTCCTGTGATGTGTTGAAAAGTTCAA
TGACGATGAAAGTTCAAGGCCAGCCTGATGAATGACGTCCAAGGTATGCTATC
ATTGTACGAAGCAGCTCATTGGCAATCCATGGGAAGATATCTGGATGAAGCA
ATAGTGTAACTACTCACTAACAGTCCACAGTTCTAACCTACAGTC
TACATTGCTAACAGATAAGACACTCTTGAGAGTTCCATTGAGGAAAGCTGTC
CCAAGATTGGAGTCTAGGTATTCCTGATATCTACTCCAGAGATGACTTACACG
ACAAAAACACTCTAACATTGCTAAACTAGATTCAACATCCTACAGGCTATGCA
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CTGCCTCGAAACATTGGTAGACGATACCTCGATGCCTATGGAACCTTGATGAA
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TGCCAGAACATGAAGTTATCTTCAAAACACTCATTGATGTAACTCTGAGGC
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CTAGAAGCGCTGACTTCATGTACGGCAATGCACAAGATAGACTACACACTAAC
TATGATGAAAGATCAAGTCGATTGGTACTAAAGATCCAGTTAACAGTTAGATGAT
TAG

PAPER V

Reconstruction and evaluation of the synthetic bacterial MEP pathway in *Saccharomyces cerevisiae*

**Siavash Partow, Verena Siewers, Laurent Daviet, Michel Schalk,
Jens Nielsen**
(Submitted)

**Reconstruction and evaluation of the synthetic bacterial MEP pathway in
*Saccharomyces cerevisiae***

Siavash Partow¹, Verena Siewers¹, Laurent Daviet², Michel Schalk², Jens Nielsen^{1*}

¹ Department of Chemical and Biological Engineering, Chalmers University of Technology,
SE-412 96 Göteborg, Sweden

² Firmenich SA, Corporate R&D Division, CH-1211 Geneva 8, Switzerland

* Corresponding author

Department of Chemical and Biological Engineering,
Chalmers University of Technology, Kemivägen 10,
SE-412 96 Göteborg, Sweden
E-mail: nielsenj@chalmers.se
Tel: +46 31 772 3804
Fax: +46 31 772 3801

ABSTRACT

Isoprenoids, which are a large group of natural and chemical compounds with a variety of applications as e.g. fragrances, pharmaceuticals and potential biofuels, are produced via two different metabolic pathways, the mevalonate (MVA) pathway and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. Here, we attempted to replace the endogenous MVA pathway in *Saccharomyces cerevisiae* by a synthetic bacterial MEP pathway integrated into the genome to benefit from its superior properties in terms of energy consumption and productivity at defined growth conditions. It was shown that the growth of MVA pathway deficient *S. cerevisiae* strain could not be restored by the heterologous MEP pathway even when accompanied by the co-expression of genes *erpA*, *hISCA1* and *CpIscA* involved in the Fe-S trafficking routes leading to maturation of IspG and IspH and *E. coli* genes *fldA* and *fpr* encoding flavodoxin and flavodoxin reductase believed to be responsible for electron transfer to IspG and IspH.

INTRODUCTION

In connection with the production of many natural products the transfer of complete biosynthetic pathways from native to heterologous organisms is an attractive approach, as it may allow for use of industrially compatible strains and for further pathway engineering[1,2,3]. Although this approach imposes a number of challenges such as gene codon optimization, correct protein folding and proper enzyme function, there are several examples where whole biochemical pathways have been transferred successfully such as expressing the mevalonate (MVA) pathway in *Escherichia coli* [4], transferring a complex mammalian hydrocortisone biosynthetic pathway containing 8 genes into yeast [5], and reconstruction of the early four steps of the flavonoid biosynthetic pathway in *Saccharomyces cerevisiae* in order to convert phenylpropanoid acids into flavanones [6].

The MVA pathway in yeast and most other eukaryotes and the 2-C-methyl-D-erythritol (MEP) pathway in most bacteria and plant plastids are responsible for production of isoprenoids, which represent an important class of biochemical compounds [7]. The MEP pathway was first reported independently by Rohmer and Argoni [8,9]. This pathway initiates by condensation of one molecule each of pyruvate and D-glyceraldehyde-3-phosphate through a thiamin diphosphate dependent reaction catalyzed by 1-deoxy-D-xylulose 5-phosphate synthase (Dxs) [10], followed by an NADPH dependent reduction process being catalyzed by 1-deoxy-D-xylulose 5-phosphate reductoisomerase (Dxr) [11], generating 2-C-methyl-D-erythritol 4-phosphate (MEP). This intermediate is converted into the cyclic 2,4-diphosphate of 2-C-methyl-D-erythritol by the sequential action of the enzymes specified by IspD, IspE and IspF [12,13,14]. 2-C-methyl-D-erythritol-2,4-cyclodiphosphate is reduced by a reductase encoded by the *ispG* gene [15,16] followed by the production of IPP and DMAPP by the action of the *ispH* gene product [17,18]. Unlike the MVA pathway, the MEP pathway

has not been investigated extensively, in particular in heterologous hosts. *S. cerevisiae* is widely used as a platform for heterologous expression of biochemical pathways [5,19,20], due to its well-characterized physiology and the availability of molecular biology tools. Maury and co-workers reported the reconstruction of the bacterial MEP pathway in *S. cerevisiae* by expression of seven enzymatic steps of the pathway from self-replicating, high-copy yeast plasmids [21]. By inhibiting the endogenous MVA pathway through addition of lovastatin, it was shown that the MEP pathway was active and could ensure production of ergosterol, which is essential for yeast. However, transferring entire biochemical pathways using episomal plasmids is not recommended for industrial applications due to poor genetic stability. In addition, maintenance of plasmids requires selective pressure provided by selective media which increase the costs. In contrast, gene integration offers a stable manipulation without requirement of selective pressure provided through the media.

In this work, we show by using genome-scale modeling that transferring the entire bacterial MEP pathway into *S. cerevisiae* gives a higher theoretical maximum yield of the isoprenoid precursor compared with biosynthesis via the endogenous MVA pathway. In order to activate this pathway in yeast eight enzymatic steps of the bacterial MEP pathway were integrated into the chromosome of *S. cerevisiae*. Following expression of the heterologous MEP pathway, we found that the IspG and the IspH enzymes are potential bottlenecks of the MEP pathway in *S. cerevisiae* and activating them requires the successful transfer of Fe-S clusters to these two enzymes and a suitable electron transfer system. So, both possible Fe-S trafficking routes responsible for maturation of IspG and IspH and a bacterial electron transfer system were reconstructed in the yeast cytosol by co-expression of the bacterial gene *erpA* with *iscA* from either human or *Arabidopsis thaliana* and flavodoxin and flavodoxin reductase, respectively. These genetic modifications were accompanied with over-expression of a copy of each *IspG*

and *IspH* from *A. thaliana*. However, introducing the above mentioned manipulations did not result in a functional MEP pathway in *S. cerevisiae*.

Based on this study we suggest that specific physical interaction or compartmentalization is required for *in vivo* biogenesis and transfer of essential prosthetic groups, in this case transfer of iron-sulfur clusters, into apoIspG and apoIspH and consequently activation of the bacterial MEP pathway.

RESULTS

***In silico* evaluation of MVA and MEP pathway in *S. cerevisiae*.**

Seven genes responsible for the enzymatic steps of the bacterial MEP pathway (Figure 1) were introduced into the yeast genome scale metabolic model iIN800 [22]. The efficiency of the MEP pathway was evaluated and compared with the endogenous MVA pathway using the model. The model was optimized for maximum production of farnesyl pyrophosphate (FPP), which is a branch point intermediate in ergosterol biosynthesis, for two different conditions, using the endogenous MVA pathway and using the heterologous MEP pathway, respectively. The result showed that by consuming 1 mol of glucose 0.21 and 0.24 mol farnesyl pyrophosphate could be produced through the MVA and MEP pathway, respectively. According to this analysis the FPP production through the MEP pathway results in a favorable theoretical yield.

The stoichiometry calculation of NAD(P)H and ATP consumption for both pathways showed that for producing one molecule of farnesyl pyrophosphate from glucose via the MVA pathway six molecules of NADPH and nine molecules of ATP are required, while production via the MEP pathway consumes nine molecules of NAD(P)H and six molecules of ATP. Provision of sufficient cytosolic NADPH is therefore a critical factor for both pathways. In contrast to the MEP pathway, which consumes only 3 molecules of glucose, the MVA

pathway consumes 4.5 molecules of glucose for the biosynthesis of one molecule farnesyl pyrophosphate (these values are excluding use of glucose for production of ATP and redox co-factors).

Combining the results derived from the yeast genome scale metabolic model and the stoichiometry calculation, it became evident that the MEP pathway is a more efficient route than the endogenous MVA pathway for isoprenoid production in terms of energy consumption and productivity. Therefore we decided to investigate this pathway in *S. cerevisiae*, which has been widely used as a platform for heterologous expression of isoprenoids [19,20].

Genomic integration of MEP pathway genes.

Since the efficiency of *in vivo* homologous recombination in *S. cerevisiae* is high, a bipartite integration strategy was applied [23]. Eight codon optimized MEP pathway genes including *dxs*, *dxr*, *ispD*, *ispE*, *ispF*, *ispG*, *ispH* and *idi* were organized in four different synthetic fragments (Figure 2A). Each fragment contained two genes located on each side of the bidirectional promoter $P_{TEFI}\text{-}P_{PGK1}$, which had shown high constitutive activity in glucose containing media before [24], and in front of either the *ADH1* or the *CYC1* terminator. Furthermore, each fragment harbored a part (ca. 2/3) of a gene coding for a selectable marker (*kanMX* or *K.l.URA3*) and flanking regions which corresponded to the desired integration site on the chromosome and which were necessary for integration via homologous recombination. In order to recycle the selectable markers, direct repeat DNA sequences of 143 bp were introduced at both sides of *K.l.URA3*, whereas *loxP* sites flanked the *kanMX* cassette.

Previously, different transcription levels among various chromosomal regions in *S. cerevisiae* have been reported by using *lacZ* as a reporter gene [25]. We have shown that the two

integration sites, YPRC Δ 15 and YPRC τ 3, on chromosome XVI of *S. cerevisiae* provided potentially higher expression levels than other regions tested [25]. Therefore, all genes involved in the bacterial MEP pathway were integrated into these two sites in two steps (Figure 2A). The selectable markers, *kanMX* and *K.l.URA3*, were looped out. Integration and transcription of the MEP genes was confirmed by PCR and RT-PCR, respectively (data not shown). Table 1 lists the strains which were constructed during this work. Strains SCISP06 and SCISP12 were obtained through the integration of MEP pathway genes into the chromosome of CEN.PK 113-13D and CEN.PK 113-1C, respectively.

The functionality of the bacterial MEP pathway was tested by blocking the endogenous MVA pathway which is essential for *S. cerevisiae* because of its supply of ergosterol involved in regulation of membrane fluidity [26]. The MVA pathway was inhibited using lovastatin (mevinolin) [27] which is a therapeutic agent and acts as a competitive inhibitor of an early pathway enzyme, HMG-CoA reductase. Surprisingly, no growth was observed in both wild type (CEN.PK 113-13D) and SCISP06 (yeast strain with integrated MEP pathway) in presence of 2 g L⁻¹ lovastatin (Figure 3), respectively, which showed that the MEP pathway could not complement the MVA pathway, and which is in contrast to the previous report [21].

Re-construction the possible bacterial Fe/S trafficking routes and the bacterial electron transfer system

The detection of intermediates 3 and 5 (Figure 1) in the MEP-pathway carrying yeast strains indicated proper activity of the Dxs, Dxr and the IspD enzymes (data not shown). In addition, no-activity was observed for the last two enzymes of the MEP pathway, IspG and IspH, when expressed in yeast in a previous study [28]. IspG and IspH are known to be iron-sulfur cluster proteins [15,16,17,29,30,31] and it has been reported that this cluster is directly involved in IspH activity [32]. The essential role of ErpA, which is an A-type iron-sulfur cluster protein,

in the maturation process of IspG, and probably IspH, in *E. coli* has been investigated [33]. Furthermore, Puan and co-workers [34] identified *fldA* as an essential gene for isoprenoid biosynthesis in *E. coli*, as it provides reducing equivalents for the Fe/S clusters of IspG and IspH. *fldA* encodes flavodoxin I, which together with *fpr* encoded flavodoxin reductase composes an *E. coli* electron transfer system [35]. As an attempt to solve the problem of the non-functionality of the MEP pathway in *S. cerevisiae*, the impact of the co-expression of genes involved in transferring Fe-S clusters to IspG/IspH apoproteins and of the described *E. coli* electron transfer system was investigated. The coding region of genes *erpA*, *fpr* and *fldA* from *E. coli* were cloned on a single plasmid, pISP08, (Figure 2B) that was transformed into SCISP06 generating SCISP16 (Table1). The empty plasmid pSP-GM1 was transformed into CEN.PK113-13D and SCISP06 resulting in SCISP28 and SCISP29, respectively (Table 1).

To rule out any possible additional effect on cell growth using lovastatin for inhibition of the MVA pathway the functionality of the bacterial MEP pathway was investigated by deletion of *ERG13*, an essential gene in the MVA pathway. *ERG13* encodes HMG-CoA synthase [7], and its disruption results in a strain that requires exogenous mevalonate supplementation for viability [36]. The coding region of *ERG13* in strains SCISP16, SCISP28 and SCISP29 was replaced by a *kanMX* integration cassette, which was confirmed by PCR (Figure 4A). As it is illustrated in figure 4B, the *ERG13* deleted strains could not grow in media lacking mevalonate under aerobic conditions.

Since bacterial IspG and IspH have not shown any activity in yeast [28], we asked whether a eukaryotic version of both IspG and IspH would be active in yeast; the codon optimized plant genes of IspG and IspH from *A. thaliana* were chemically synthesized. In addition, an Fe-S trafficking model has previously been proposed that describes the transfer of Fe-S clusters to IspG and IspH in *E. coli* (figure 5) [37]. The authors suggested that depending on the

environmental conditions e.g. aerobic, anaerobic or stress, the Fe-S cluster is transferred from IscU or SufU scaffolds to apoIspG and apoIspH through the combination of A-type carriers including ErpA, IscA and SufA [37]. Assuming that this model can be transferred from *E. coli* to yeast the expression of *iscA* may fill the gap in this proposed model [37]. Therefore, one copy of each *IspG* and *IspH* from *A. thaliana* was cloned into expression plasmids with *iscA* from either human or *A. thaliana* resulting in pISP24 and pISP25, respectively (Figure 2B). Previously, localization and activity of human ISCA1 (hISCA1) was shown in mitochondria as well as in the cytosol of HeLa cells [38]. The authors have also demonstrated interaction of the small domain of IOP1 (Iron-only hydrogenase-like protein I) with human ISCA1 using yeast two-hybrid systems [38]. CpIscA from *A. thaliana* is involved in Fe-S biogenesis in chloroplasts [39]. The Fe-S cluster in CpIspA indicated stability in presence of oxygen [39]. Strains SCISP31 and SCISP32 were constructed by co-transforming pISP08 with either pISP24 or pISP25 into SCISP12, respectively (Table 1). Like for SCISP16, no grow was observed in the absence of exogenous mevalonate when *ERG13* was disrupted in both SCISP31 and SCISP32 (Figure 4C).

Fe-S clusters are sensitive to superoxide (O_2^-) and other oxidative agents [40,41]. In addition, the Fe-S cluster of IspH is easily destroyed by exposure to molecular oxygen or other oxidative agents [32]. Therefore, to prevent inactivation of the Fe-S clusters in IspG and IspH, all *erg13* strains were also evaluated under anaerobic conditions. Yeast growing in anaerobic condition is ergosterol-dependent as the biosynthesis of ergosterol is disrupted in this condition. For this, exogenous ergosterol was added to the SD media at a final concentration of 1 mg L⁻¹. None of the *erg13* strains showed mevalonate-independent growth (Figure 4D and 4E). This means that even in anaerobic conditions, the MEP pathway was not able to complement the MVA pathway.

DISCUSSION

In the current study, the seven enzymatic reactions of the bacterial MEP pathway were evaluated *in silico*, using the yeast genome scale metabolic model, iIN800 [22]. iIN800 consists of 1446 metabolic reactions and 1013 metabolites [22]. In comparison to the first *S. cerevisiae* model by Förster and co-workers [42], iIN800 covers lipid metabolism in great detail. Applying mathematical models has previously been demonstrated to be predictive and beneficial for metabolic engineering approaches e.g. to identify new target genes enhancing the biosynthesis of sesquiterpenes in yeast [43], and to schematically represent the effect of the interaction of protease supplementation and type of sugar on amino acid metabolisms in brewer's yeast [44]. Here, *in silico* analysis of the bacterial MEP pathway expressed in yeast revealed the benefits of this heterologous pathway in terms of energy consumption and yield compared to the endogenous MVA pathway. Our result is in consistence with previous reports about the higher efficiency of the MEP pathway in converting sugars or glycerol into terpenoids compared to the MVA pathway [45,46]. This is the rationale for our attempt to express the bacterial MEP pathway in yeast for production of isoprenoids.

The first step of most yeast metabolic engineering and synthetic biology studies involves reconstruction of a complete or partially synthetic pathway. Although several methods have been developed [3,47], *in vitro* DNA synthesis offers a fast, cheap and efficient method for synthesis of large DNA sequences [48,49]. Besides, using synthetic genes with the possibility to manipulate codon bias can take much better control of the expression of heterologous MEP pathway genes in yeast. From the genetic engineering point of view, the codon bias is one of the first barriers in heterologous protein expression [50] and it can prevent the efficient biosynthesis of a recombinant protein because of altering the correlation between the

frequency of the codon and the abundance of its corresponding tRNA, which impairs the translation machinery of the host [51]. The high efficiency and ease to work with *in vivo* homologous recombination in *S. cerevisiae* allows stable manipulation without requiring selective pressure for maintenance. Here we developed a strategy for easy integration of eight heterologous genes. The four DNA constructs containing the eight MEP pathway genes, including expression elements and selection markers were designed *in silico*, synthesized *in vitro* and integrated into the yeast chromosome via homologous recombination. The functionality of this pathway in *S. cerevisiae* was evaluated by the attempt to block the endogenous MVA pathway. Inhibition of the MVA pathway can be achieved using an inhibitor or by deletion of the essential genes of this pathway [26,36,52-55]. Unlike previously reported [21], chemical as well as genetic inhibition of MVA pathway revealed the non-functionality of the MEP pathway. We conclude that the previous result [21] may have derived from incomplete repression of the MVA pathway, even when higher concentrations (2 g L⁻¹) of lovastatin were used, which may result from errors in activation of lovastatin by hydrolysis reducing the actual concentration of the active inhibitor, or the higher-level expression from multi-copy plasmids may have resulted in partial activation of the enzymes resulting in a functional MEP pathway. High concentrations of lovastatin could also have caused side effects which mask the operation of the MEP pathway. Thus, the inhibition of Hmg1 and Hmg2 using lovastatin has shown to result in an altered transcriptional response including the up-regulation of genes related to plasma membrane proteins, protein catabolism and ribosome biosynthesis and down-regulation of *MAF1* [56,57] which encodes a repressor of RNA polymerase III [58]. Previously, Kaminska and co-workers have shown an increase in tRNA levels when Maf1 is diminished [59] and consequently the demand for DMAPP, which is involved in tRNA biosynthesis, was increased [60]. Such requirement may be higher than what is provided by the MEP pathway. Gene deletion in contrast offers absolute inactivation

of the MVA pathway. It has been indicated that yeast strains with deficiency in *ERG13*, *ERG19*, *ERG8*, or *ERG9* are nonviable at normal growth conditions [26, 36, 52-55]. Since the MEP pathway contributes to the ergosterol biosynthetic pathway through IPP and DMAPP intermediates, deletion of *ERG13*, *ERG19* or *ERG8* which are located upstream of these intermediates should be more efficient than using lovastatin for blocking the MVA pathway and evaluating the MEP pathway functionality. For our purpose *ERG13* is a good candidate since supplying the medium with exogenous mevalonate can complement its deletion. However, the inability to delete *ERG13* showed that the MEP pathway could not complement the MVA pathway deficiency.

We hypothesize that a potential reason for the non-functionality of the MEP pathway in *S. cerevisiae* is the lack of the enzyme activity of IspG and/or IspH, which catalyze the last two reactions of the pathway. It has been reported that both enzymes, IspG and IspH, are dependent on NADPH and the flavodoxin/flavodoxin reductase redox system as electron donor for their catalytic activity [17,18, 34,61-63]. Gräwert and co-workers [32] have reported that the *in vitro* maximum activity for IspH was obtained with NADPH as co-substrate, together with recombinant flavodoxin and flavodoxin reductase from *E. coli*. Flavodoxin and flavodoxin reductase are FMN and FAD cofactor containing proteins, respectively, and it has been shown that NADPH is the preferred reducing equivalent of flavodoxin reductase compared to NADH [35]. Overexpression of flavodoxin and flavodoxin reductase might facilitate electron flux from NADPH to IspG and IspH and therefore result in increased the activity of these enzymes. A similar phenomenon was observed in biosynthesis of hydrocortisone in yeast [5]. Overexpression of the essential endogenous reductase Arh1 (adrenodoxin eeductase homolog) using a strong promoter increased the production of hydrocortisone up to 60% [5]. Both Arh1 and human ADX protein (adrenodoxin) are

responsible for transferring electrons from NADPH to the related enzyme. The authors suggested that the flux of electrons was elevated as a result of *ARHI* overexpression [5]. Furthermore, both IspG and IspH are known as iron-sulfur cluster proteins which are harboring cubic type of Fe/S clusters, and it has been suggested that these [4Fe–4S] clusters participate in the electron transfer process [15-17, 29-31]. Despite the presence of Fe-S assembly machineries in yeast - the ISC system is present in the mitochondria and the CIA system is used for cytosolic FeS cluster assembly [64-66] - and these systems may not be suitable to transfer iron-sulfur clusters to IspG and IspH. Recently, a suitable model has been proposed demonstrating the Fe/S trafficking paths leading to IspG and IspH maturation in *E. coli* [37]. The essential role of ErpA in maturation of the IspG and the IspH enzymes in *E. coli* has been indicated [33]. It was also shown that the Fe-S clusters can directly be transferred from IscU to ErpA in *E. coli* [67]. Based on these findings, the functionality of the bacterial MEP pathway was evaluated in presence of the cytosolic expression of bacterial genes *erpA*, *fpr* and *fldA* in yeast, but still we could not obtain functionality of the enzymes. Previously reported data have shown that the cytosolic localization has failed to generate a functional bacterial or human IscU while expressed in yeast [68]. Even yeast U-type homolog scaffolds (Isu1 and Isu2) playing a crucial role in maturation of both cytosolic and mitochondrial Fe-S proteins need to be expressed in mitochondria to show activity [68]. As illustrated in figure 5, the Fe-S cluster is transferred to the last two enzymes of the MEP pathway through A-type proteins (IscA, ErpA and SufA) in three different conditions (aerobic, anaerobic and stress) [37]. A-type iron-sulfur carriers (ATCs) have initially emerged in most bacteria before being acquired by eukaryotes and a few archaea by means of horizontal gene transfer [37]. Tan and co-workers have suggested that both IscA and SufA are required for assembly of cubic Fe-S clusters in *E. coli* under aerobic condition [69]. We further constructed possible bacterial paths (aerobic and anaerobic) which are involved in

transferring Fe-S clusters to IspG and IspH in the yeast cytosol (Figure 5). Co-transformation of *erpA* with either human *ISCA1* or plant derived *CpIscA* in addition to plant *ispG* and *ispH* and bacterial genes, *fpr* and *fldA* did not result in a functional MEP pathway in both aerobic and anaerobic conditions although human ISCA1 had been shown to have partial cytosolic activity in HeLa cells [38]. *S. cerevisiae* also contains two types of A-type carriers, Isa1 and Isa2, which are localized in the mitochondrial matrix and in the mitochondrial intermembrane space, respectively[70,71]. The mitochondrial localization is necessary for the functionality of both the Isa1 and the Isa2 protein in yeast [70]. The contribution of these proteins in the maturation process of IspG and IspH in an *E. coli* strain which has a deficiency in *erpA*, *iscA* and *sufA* has been demonstrated [37]. However, it has been demonstrated recently that only Isa1 can be functionally replaced by the bacterial A-type ISC proteins, ErpA, IscA and SufA [72].

In conclusion, we believe that specific physical interaction and compartmentalization would be required for *in vivo* biogenesis and transfer of essential prosthetic groups, here the iron-sulfur clusters for activation of bacterial MEP pathway enzymes in yeast. Therefore, it seems interesting to evaluate IspG and IspH expression in the mitochondria as this may represent a new interesting engineering strategy, which may even be relevant for activation of other bacterial iron-sulfur cluster proteins in yeast.

Materials and Methods

Strain and plasmid construction.

Sequences of all *E. coli* MEP pathway genes including *dxs* (AAC73523), *dxr* (AAC73284), *ispD* (AAC75789), *ispE* (AAC74292), *ispF* (AAC75788), *ispG* (AAC75568), *ispH* (AAC73140) and *idi* (AAC75927) were used to construct four different integrative fragments

(Supplementary file), in which each gene was placed behind a *TEF1* or *PGK1* promoter and in front of a *CYC1* or *ADH1* terminator, respectively (Figure 2). Gene sequences were codon optimized for expression in *S. cerevisiae* and the four fragments were synthesized by DNA2.0 (Menlo Park, CA, USA).

The synthetic fragments were integrated into *S. cerevisiae* CEN.PK 113-13D (*MATα MAL2-8c SUC2 ura3-52*) and CEN.PK 113-1C (*MATα MAL2-8c SUC2 trp1-289 ura3-52 his3Δ 1*) (kindly provided by P. Kötter, University of Frankfurt, Germany) chromosome XVI (sites YPRCΔ15 and YPRCτ3) by using a standard transformation procedure [73] and a bipartite gene targeting strategy [23]. *kanMX* as a selectable marker was looped out by methods described previously[74] and *Kluyveromyces lactis* (*K.l.*)*URA3* was looped out by selection on SD plates supplemented with 30 mg L⁻¹ uracil and 750 mg L⁻¹ 5-fluoroorotic acid (5-FOA), respectively. The strains harboring all MEP genes will in the following be referred to as SCISP06 and SCISP12 (Table 1).

The genes *erpA*, *fldA* and *fpr* were amplified by PCR using *E. coli* DH5α genomic DNA as a template and primers listed in table 2. The *NotI/SacI* restricted *fldA* fragment and *BamHI/XhoI* restricted *fpr* fragment were cloned into pSP-GM1, a derivative of pSP-G1 [24, 75]. A P_{TDH3}-*erpA-T_{PGK1}* cassette was constructed by fusion PCR performed with Phusion high-fidelity DNA polymerase (Finnzymes, Espoo, Finland) and primer pair PGK1T-MreI-rev / TDH3-Kpn2I-fw, restricted by *Kpn2I/MreI* and cloned into pSP-GM1 containing the *fldA* and *fpr* genes. This resulted in construction of plasmid pISP08 (Figure 3B). pISP08 was transformed into strain SCISP06 resulting in formation of strain SCISP16 (Table 1).

Sequences of plant genes encoding IspG (AAN87171.1) and IspH (AAO15446.1) from *Arabidopsis thaliana* were synthesized by DNA2.0 (Menlo Park, CA, USA). The sequence of *iscA* from *Homo sapiens*, *hISCA* (NP_112202.2) was synthesized by GenScript (Piscataway,

NJ, USA). The sequence of *iscA* from *A. thaliana*, *CpIscA* (Q9XIK3.2) was used to construct the expression cassette, P_{TDH3} -*CpIscA*- T_{PGK1} , and this cassette was synthesized by GenScript. All synthetic genes were codon optimized for expression in *S. cerevisiae*.

The *Bam*H/*Xho*I restricted *At-IspG* fragment and *Not*I/*Sac*I restricted *At-IspH* fragment were cloned into pSP-GM3, a derivative version of the pSP-GM1 plasmid [75]. The *Pvu*II restricted fragment of pSP-GM1 including the *TEF1-PGK1* promoter region was cloned into *Pvu*II restricted pESC-HIS (Startagene, La Jolla, USA) to construct pSP-GM3. A truncated fragment of *hISCA1* encoding a protein without the mitochondrial signal peptide was amplified by PCR using the synthetic *hISCA1* as a template and primers listed in table 2. A P_{TDH3} -*hISCA1*- T_{PGK1} cassette was constructed by fusion PCR performed with Phusion high-fidelity DNA polymerase (Finnzymes, Espoo, Finland) and primer pair PGK1T-MreI-rev / TDH3-Kpn2I-fw. Both *Kpn*2I/*Mre*I restricted P_{TDH3} -*hISCA1*- T_{PGK1} and P_{TDH3} -*CpIscA*- T_{PGK1} fragments were cloned into pSP-GM3 containing the *At-IspG* and *At-IspH* genes, respectively, resulting in pISP24 and pISP25. In the next step, pISP08 was co-transformed with either pISP24 or pISP25 (Figure 3B) into strain SCISP12 resulting in generation of strain SCISP31 and SCISP32, respectively. To create the control strain, empty plasmids, pSP-GM1 and pSP-GM3, were cloned into wild type (CEN.PK113-13D), SCISP06 and SCISP12 resulting in strains SCISP28, SCISP29 and SCISP30, respectively. The strains used in this study are listed in table 1.

In order to delete *ERG13*, upstream and downstream flanking regions of the target gene were PCR amplified. These upstream and downstream flanking regions were fused to the 5' and the 3' part of the *kanMX* cassette amplified from plasmid pUG6 [74] by fusion PCR performed with Phusion high-fidelity DNA polymerase (Finnzymes). In the next step, the fused PCR fragments 1 and 2 including upstream flanking region of *ERG13* + *loxP* - 2/3 *kanMX* and 2/3

kanMX - loxP + downstream flanking region of *ERG13*, respectively, were used for deletion by bipartite gene targeting [23]. Deletion of *ERG13* was verified by diagnostic PCR. For this purpose, PCR primers were designed to bind 400 to 500 bp up- and downstream of the start and stop codon, respectively, (Table 2). All PCR products were sequenced.

Media composition

The transformants were selected on minimal medium plates containing 1.7 g L⁻¹ yeast nitrogen base w/o amino acids and ammonium sulfate (Formedium, Hunstanton, England), 5 g L⁻¹ ammonium sulfate, 0.77 g L⁻¹ complete supplement mixture (CSM w/o uracil or CSM w/o uracil and histidine) (MP Biomedicals, Solon, OH, USA), 20 g L⁻¹ glucose and 20 g L⁻¹ agar. In media containing G418, 0.86 g L⁻¹ L-glutamic acid monosodium salt monohydrate was used instead of ammonium sulfate. Filter sterilized G418 disulfide salt (Sigma-Aldrich, St. Louis, MO) was added to the media before plating to a final concentration of 200 mg L⁻¹. Mevalonic acid lactone (Sigma-Aldrich) was prepared as a 500 mg L⁻¹ stock solution in 2N NaOH, incubated at 37°C for 30 min, filter sterilized, and then added to the media to reach a final concentration of 10 mg L⁻¹. For anaerobic cultivations 125 µL ergosterol was added to the media from a stock solution (1 g L⁻¹) that was prepared as described previously [76]. Lovastatin (Sigma) was hydrolyzed in ethanolic sodium hydroxide (15% (v/v) ethanol, 0.25% (w/v) NaOH) at 60°C for 1 h. After cooling down to room temperature, it was added to shake flasks at a final concentration of 2 g L⁻¹.

Batch cultivation

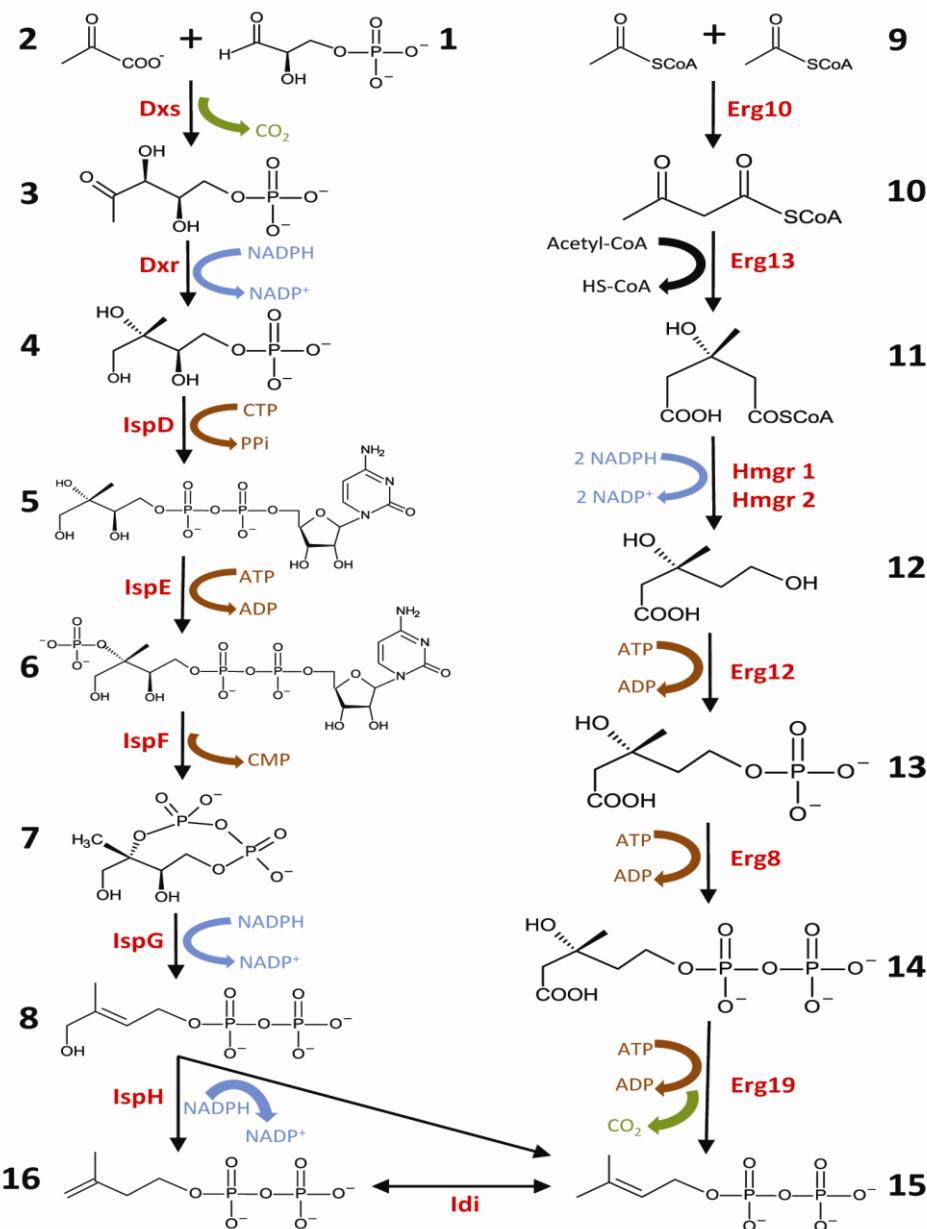
Cotton-stopped, 50 mL Erlenmeyer flasks were used for evaluation of the MEP pathway functionality using lovastatin. The shake flasks contained 10 mL medium with the above mentioned composition. 50 mL falcon tubes containing 5 mL medium were used for seed cultures. Both seed tubse and shake flasks were incubated at 30°C and agitated in an orbital

shaker at 180 rpm. Pre-cultures were used to inoculate the shake flasks to a final dry weight of 1 mg L⁻¹. Cell growth was monitored by measuring the optical density at 600 nm using a Genesis20 spectrophotometer (Thermo Scientific, Madison, WI, USA). All cultivations were performed in triplicate.

Acknowledgements

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MEP pathway



MVA pathway

Figure 1: The MEP pathway (left). Enzymes: **Dxs**, 1-deoxy-D-xylulose-5-phosphate synthase; **Dxr**, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; **IspD**, 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase; **IspE**, 4-diphosphocytidyl-2-C-methylerythritol kinase; **IspF**, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; **IspG**, 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase; **IspH**, 1-hydroxy-2-methyl-but enyl 4-diphosphate reductase; Metabolites: **1**, D-glyceraldehyde 3-phosphate; **2**, pyruvate; **3**, 1-deoxy-D-xylulose 5-phosphate; **4**, 2-C-methyl-D-erythritol 4-phosphate; **5**, 4-diphosphocytidyl-2-C-methyl-D-erythritol; **6**, 2-phospho-4-diphosphocytidyl-2-C-methyl-D-erythritol; **7**, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; **8**, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate.

The MVA pathway (right). Enzymes: **Erg10**, acetoacetyl-CoA thiolase; **Erg13**, 3-hydroxy-3-methylglutaryl-CoA synthase; **Hmg1/2**, 3-hydroxy-3-methylglutaryl-CoA reductase; **Erg12**, mevalonate kinase; **Erg8**, phosphomevalonate kinase; **Erg19**, mevalonate diphosphate decarboxylase; **Idi**, isopentenyl diphosphate isomerase; Metabolites: **9**, acetyl-CoA; **10**, acetoacetyl-CoA; **11**, 3-hydroxy-3-methylglutaryl-CoA; **12**, mevalonate; **13**, phosphomevalonate; **14**, diphosphomevalonate; **15**, dimethyl allyl diphosphate; **16** isopentenyl diphosphate.

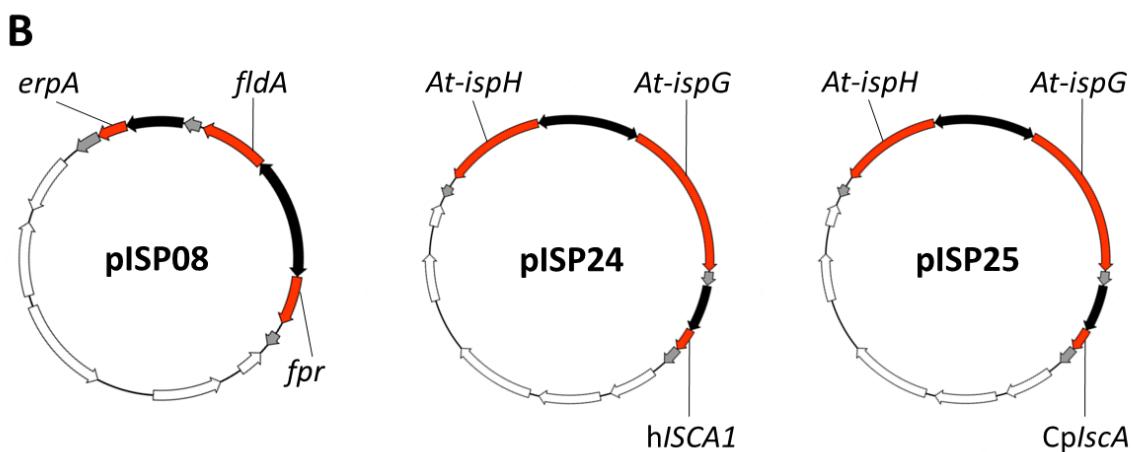
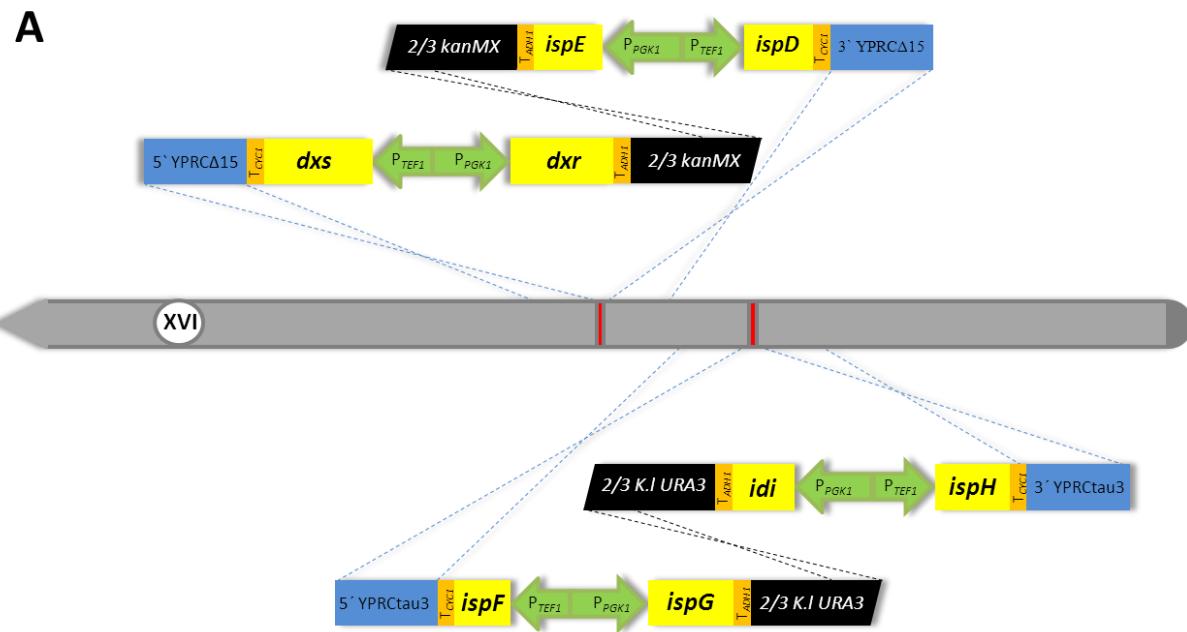


Figure 2: Schematic representation of genetic engineering strategies for **A**) genomic integration of the bacterial MEP pathway genes into the yeast genome (chromosome XVI), and **B**) plasmid-based reconstruction of possible Fe/S trafficking routes involved in maturation of bacterial IspG/IspH, bacterial electron transfer systems and plant-derived *ispG/ispH* in *S. cerevisiae*. For details see text.

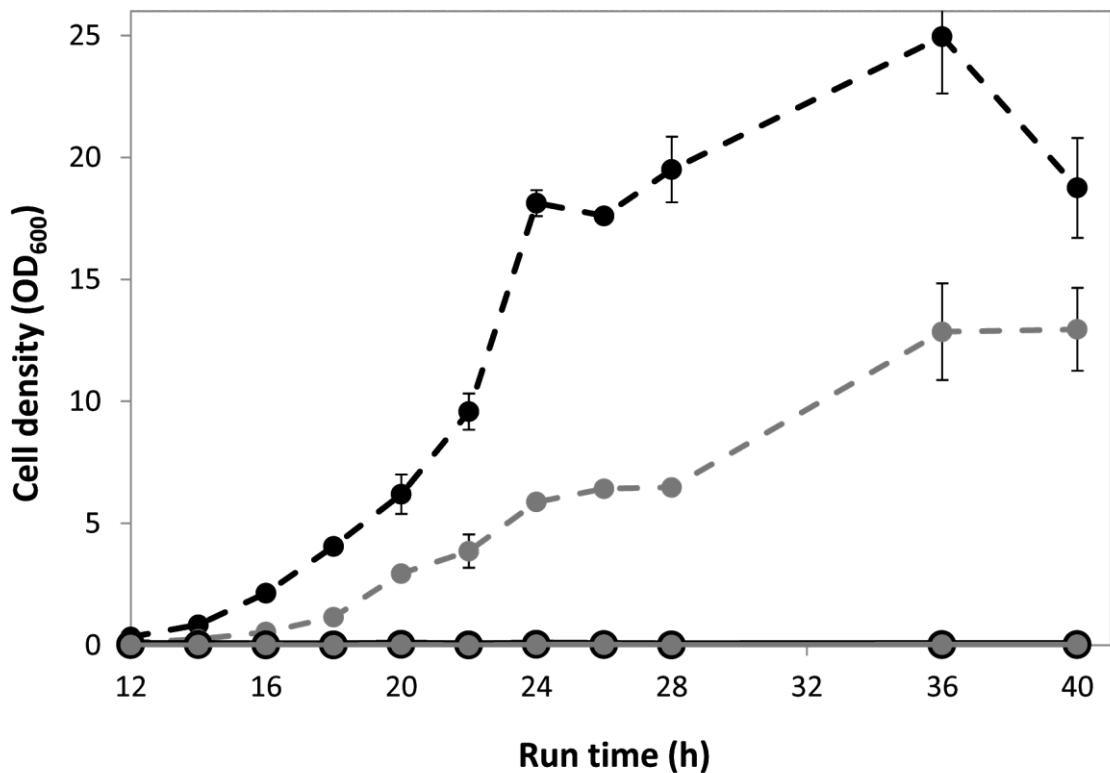


Figure 3: Growth of *S. cerevisiae* strains CEN.PK 113-13D (black circles) and SCISP06 (gray circles) in SD minimal medium. Dashed lines represent the growth in 0 g L⁻¹ of lovastatin; solid lines represent the growth in presence of 2 g L⁻¹ of lovastatin. Error bars show the standard deviation from three cultivations.

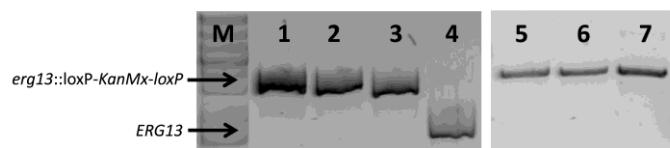
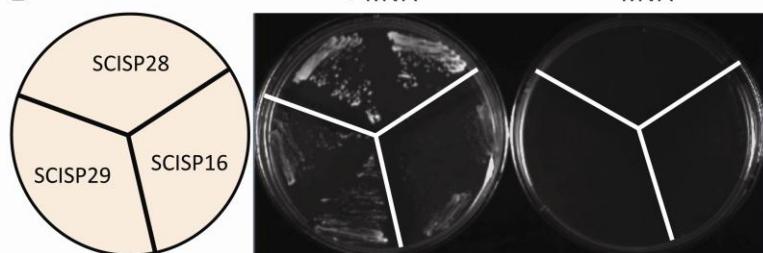
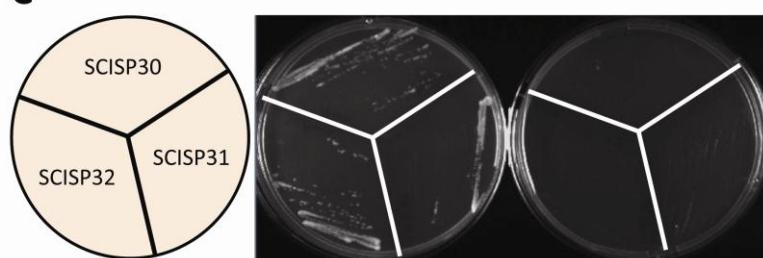
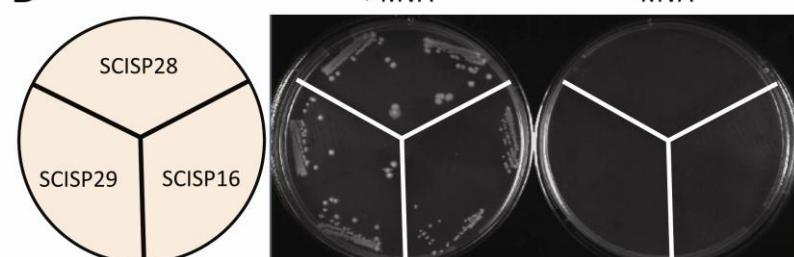
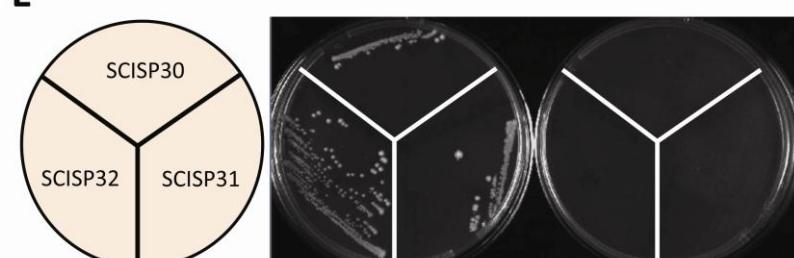
A**B****C****D****E**

Figure 4: **A)** Gel electrophoresis of PCR products to confirm deletion of *ERG13* (1: SCISP28, 2: SCISP29, 3: SCISP16, 4: CEN.PK 113-13D (wild type), 5: SCISP30, 6: SCISP31, 7: SCISP32, M: 1 kb Plus DNA ladder (Fermentas, Maryland, USA); **B)** Aerobic cultivation of MEP pathway strains co-expressing *erpA*, *fpr* and *fldA*; **C)** Aerobic cultivation of MEP pathway strains co-expressing *erpA*, *fpr*, *fldA*, *At-IspG*, *At-IspH* with either *CpIscA* or *hISCA1*; **D)** Anaerobic cultivation of MEP pathway strains co-expressing *erpA*, *fpr* and *fldA*; **E)** Anaerobic cultivation of MEP pathway strains co-expressing *erpA*, *fpr*, *fldA*, *At-IspG*, *At-IspH* with either *CpIscA* or *hISCA1*. All strains carried an *ERG13* deletion and were plated on medium with or without 10 mg L⁻¹ mevalonate (MVA).

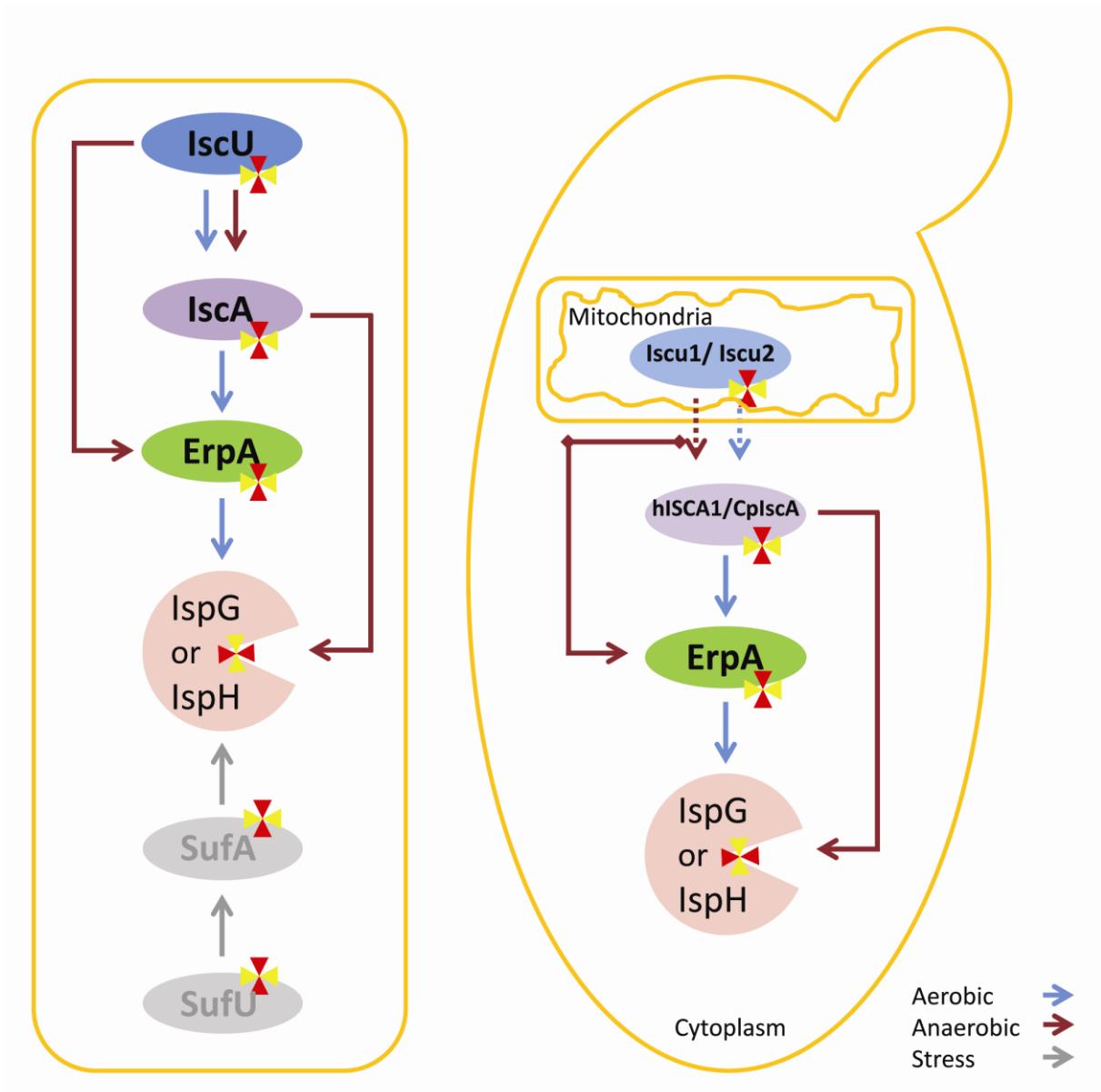


Figure 5: Schematic representation of possible Fe/S trafficking routes involved in maturation of bacterial IspG/IspH in *E. coli* (left) and reconstruction of possible routes preformed in this study in the yeast cytosol (right). Dashed arrows represent unknown mechanisms for transferring the Fe-S clusters from mitochondria to cytosol. For more information see text.

Table 1: List of strains and plasmids used in this study

Strain	Genotype	Plasmid	Reference
CEN.PK 113-1D	<i>MATα MAL2-8c SUC2 ura3-52</i>	None	P. Kötter ¹
SCISP06	<i>MATα MAL2-8c SUC2 ura3-52 dxs dxr ispD ispE ispF ispG ispH idi</i>	None	this work
SCISP16	<i>MATα MAL2-8c SUC2 dxs dxr ispD ispE ispF ispG ispH idi</i>	pISP08	this work
SCISP28	<i>MATα MAL2-8c SUC2</i>	pSP-GM1	this work
SCISP29	<i>MATα MAL2-8c SUC2 dxs dxr ispD ispE ispF ispG ispH idi</i>	pSP-GM1	this work
CEN.PK 113-1C	<i>MATα MAL2-8c SUC2 trp1-289 ura3-52 his3Δ1</i>	None	P. Kötter ¹
SCISP12	<i>MATα MAL2-8c SUC2 trp1-289 ura3-52 his3Δ1 dxs dxr ispD ispE ispF ispG ispH idi</i>	None	this work
SCISP13	<i>MATα MAL2-8c SUC2 trp1-289 his3Δ1 dxs dxr ispD ispE ispF ispG ispH idi</i>	pISP08 ²	this work
SCISP30	<i>MATα MAL2-8c SUC2 trp1-289</i>	pSP-GM1, pSP-GM3	this work
SCISP31	<i>MATα MAL2-8c SUC2 trp1-289 dxs dxr ispD ispE ispF ispG ispH idi</i>	pISP08, pISP24 ³	this work
SCISP32	<i>MATα MAL2-8c SUC2 trp1-289 dxs dxr ispD ispE ispF ispG ispH idi</i>	pISP08, pISP25 ⁴	this work

¹ University of Frankfurt, Germany.

² pISP08 contains *erpA*, *fpr* and *fldA*.

³ pISP24 contains *hISCA1*, *At-IspG* and *At-IspH*.

⁴ pISP25 contains *CpIscA*, *At-IspG* and *At-IspH*.

Table 2: List of oligonucleotide primers used in this study^a

Primer name	Sequence
Oligonucleotide primers for verification of gene integration and transcription	
DXS up	ATGTCCTTGATATTGCTAAATATCC
DXS down	TAGGCCAACCAAGCCTTATC
DXR up	TGAAGCAGCTAACTATCTTGGGT
DXR down	TCGTTAAGAAGCTAGCTCATAACTTC
ispD up	CACGACACACTTAGATGTGTG
ispD down	TCAAGTGTCTGGATG
ispE up	ATGAGAACTCAATGCCCTCC
ispE down	ATAACATTGCCCTATGAAGAGG
ispF up	ATGAGAATAGGTACCGGTTCG
ispF down	TTCGTAGCCTTGATTAGCAATG
ispG up	CACAACCAAGCCCCAATACA
ispG down	TCATTTCTCCACCTGTGGAC
ispH up	TGCAAATATTATTGGGAATCC
ispH down	TCAATCGACCTCACGTATATCC
idi up	ATGCAGACTGAACACGTTATCTG
idi down	TTAATTGGGTGAATGCTGACAG
URA up	GATGATGTAGTTCTGGTTAAATC
URA down	TTAGCTTGACATGATTAAGCTCA
KanMx up	TAGGTCTAGAGATCTGTTAGCTGC
KanMx down	ATTAAGGGTCTCGAGAGCTCG
Oligonucleotide primers for gene deletions	
KanMx-1-fw	CTGAAGCTTCGTACGCTG
KanMx-1-rev	TCACCATGAGTGACGACTGA
KanMx-2-fw	TTCCAACATGGATGCTGAT
KanMx-2-rev	CTAGTGGATCTGATATCAC
ERG13-1-fw	GTTGGTGTGGTATTAAAGGA
ERG13-1-rev	<u>CAGCGTACGAAGCTCAGGGACTTGTCAATCAGAGTT</u>
ERG13-2-fw	<u>GTGATATCAGATCCACTAGCAACCTGTAAATTGGTCAC</u>
ERG13-2-rev	CGTAAGATCTTCTAAATTGTG
Oligonucleotide primers for verification of gene deletions	
ERG13-up-fw	TACGAGTGTGTTGAAAGTAG
ERG13-down-rev	CATTATGAAGGGGGTTCAG
Oligonucleotide primers for amplification of the bacterial genes	
fpr-BamHI-fw	GTGTTGGATCCCAGGAGAAAAACATGGCTGA
fpr -XhoI-rev	GTGTTCTCGAG CGTTTATCGATAAGTAACCGCT
fldA-Not1-fw	GTGTTGCGGCCGCGAGGTATTCACTCATGGCT
fldA-SacI-rev	GTITGITGAGCTCCATCACATCAGGCATTGAGA
ErpA -fw	ATGAGTGTGACGTAGCACT
ErpA-rev	TTAGATACTAAAGGAAGAACCGCA
PGK1T-fus-fw (erpA)	<u>TGCGGTTCTCCTTAAAGTATCTAAGGTGTTGCTTCTTATCCGA</u>
PGK1T-MreI-rev	GTGTTCGCCGGCGGGTCGAGAATTTCGAGTT
TDH3-Kpn2I-fw	GTGTTCCGGACAGTTATCATTATCAATACTCGCC
TDH3-fus-rev (erpA)	<u>AGTGCTACGTCACTCATGAATCCGTCGAAACTAAGTTCTGGTG</u>
Oligonucleotide primers for amplification of the human gene	
hisca-fw	ATGGCCGCCTTGACCTTGACT
hisca-rev	<u>TCAGATGTGAAGGATTCACCG</u>
PGK1T-fus-fw (iscA)	<u>CGGTGAATCCTCAACATCTGAGGTGTTGCTTCTTATCCGA</u>
TDH3-fus-rev (iscA)	<u>AGTCAAGGTCAAGGCCATGAATCCGTCGAAACTAAGTTCTGGTG</u>

Underlined characters correspond to flanking sequences used for fusion PCR; bold characters correspond to restriction sites.

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Supplementary file:

Sequences of the four synthetic integrative cassettes including all *E. coli* MEP pathway genes which are codon optimized for efficient expression in *S. cerevisiae*.

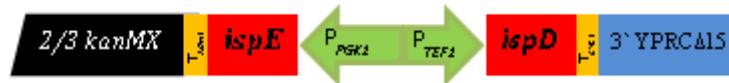
Construct 1:



CGCCGGCGACCAGATGTTACCTAATTCTGGTGAATTAGAGAAGTACAGAAGTTACTATTAATCCCACCATAGAAAT
 TTGTATAGGAAAGTAGTTATTGGAGTTATTGGATATACTGTGAAACTATTCTGAAATTGTAATCTAAGATGCTCTTCT
 TATTCTATTAAGAAAATGATTTCATATTATTATTATTTGGCATTACTCTCATCTTCCCTCTA
 AGAAGCTCCCTTCTTATAAGGATAACAAAACCAAAAGGAATATTGGTCAGATGAATGGACGCGAATGCAAGACAG
 AAGTCAAATCACGTCAAGACAAGAAAGAAAGAAAAACTAACACATTAATGTTAGTTAAAATTCAAATCCGAAAC
 AACAGAGCATAGGGTTTCGCAAACAAACTAAATATGCTAGAATAAAATTAACTATCATCTATTGACTAGTATTCTAT
 ATGACGTAATAAAATCTCGAGCGTCCAAAACCTCTCAAGCAAGGTTTCAGTATAATGTTACATCGTACACGCGTCTG
 TACAGAAAAAAAGAAAATTGAAATATAAAACGTTCTAATACTAACATAACTATAAAAAAATAATAGGGACCTAG
 ACTTCAGGTTGCTAACTCTTCTTTCGTTAGAGCGGATTAGGCCAACCAAGCCTTATCTAGCTTCCATTCCAGCA
GCGTCTAACCAAGCTAGCCCTCATCTCTTGTGAGTTCCGTGGAATGAAAAAATCGGTAACCGATATTAAGAAC
 AGGAACGGGCTTACGATGGGCATCAAACTTCATTGACTCCCGAACCTGCTCCACCCATAATGGCGTTCTTCTACAGT
 TACCAAGGCTCGTGTGAAGCTGCCATTCAAGGATCAAAGCTCATCCAAGGGTTGACAATCTCATATCGACTAATG
 TTGCTTAAGTGATTCCGCAACTTCGAGCTCCGGCATTAAAGGTACCAAAAGTTAGAATTGCCAATTCTCACCTCTCC
 TTTAACTATACTTACCAATTGGTAGTTTCCAATGGGTTAATCCACTCCTACGGCTTACCCCTAGGGTACCTTAC
 AGCGATGGCGTCTTAACTATGATATCCTGTGTATAACATCTGCCACTCGTTTCTGACGGTGTCTGATTTAC
 CATTCTGGAATACATCTAAGTACGAAAGATCAAAGGCACCTGGTAGTTGACCATCAGCACCAACTATGCCGAC
 GATCGATTGCAAACACACAGGCAACTTGAATTGCTACATCATGTTAGAACCTGATCATAAGCTTTGCAAAAGGTT
 GAATAATGGCGACAATTGGTTATGCCACCGATTGCCAACCCAGCAGCAAATGTTACAGCGTGTCTGCGATGG
 CAACGTAAAATACCTGCTGGAAACTCCTAGAGAATTCTACCATACCAGAACCTCTCATGGCAGGAGTAATTGCC
 ATCAATTATTATCCTTGGCGGTTTCGATAACCAGTCACCGAAGATTTACTATAAGATGGCAAACCCACAGATGA
 CTCGGTAAGCATCCAGAGCTGGATCGAATTGGAACGGCGTAAAGGTAATGGATTTTCAAGCCGCTCATAA
 CCACGACCTCTTAGTCATGATATGTTAGGAAATTGTTGGCCCTTCAGATCTCTCATGTTTCAAAGTTGTGATCA
 AATACATCGTCCCCATCCACCGGTCTATGTAATTGAAACCAACTCCTCAAACAAATGTACCTGGAACCCACCAT
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 CGAAACAGAGTCCAACAGATACTTCAACTCATCACACAACCTAGGCAAACCTTCTTAGGCAATAAACGTAATTCT
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GGCGCGATGCGCGGGAGTCCGAGAAAAATCTGAAGAGTAAAAAAGGAGTAGAACACATTGAAAGCTATGGTGTGCGGCC
GGCCGGAAGTACCTCAAAGAATGGGTCTTATCTGTTGCAAGTACACTGAGCAGGATAATAATAGAAATGATAATA
TACTATAGAGATAACGTCATGACTTCCACTGTAACTGCTTGTAGTTGCAAGTACACTGAGCAGGATAATAATAGAAATGATAATA
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TATAACATCTGCATAATAGGCATTGCAAGAATTACTCGTAGTAAGGAAAGAGTGAGGAACATCGCATACCTGCATT
AAGATGCCATTGGCGCGAATCCTTATTTGGCTCACCCCTCATACTATTACAGGGCAGAAAAGGAAGTGTCCC
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CAAGGTCTAGCGACGGCTCACAGGTTGTAACAAGCAATCGAAGGTTGGAATGGGGGAAAGGGTTAGTAC
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ATCGTGTGACAACAAACAGCCTGTTCTCACACACTCTTCTTAACCAAGGGGTTAGTTAGTAGAACCTCGTGA
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CGGCCGATGAAGCAGCTAACTATCTGGGTTGACTGGTTCTATCGGTTGCTCACTCTGATGTTAGAACCGACAATC
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CGTACGCTGTTATGGATGATGAAAGCCTGGCAAAGTTACTGAAAGACGATGTTACAACAAACAAGGGAGTAGAACTGAA
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Construct 2:



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Construct 3:



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Construct 4:



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Engineering biological systems has enormous power to reshape the world in various fields, such as creating a sustainable society, environmental rehabilitation, and scalable manufacturing of chemicals, preventative and curative health issues and general medicine. Synthetic biology is advancing capabilities for engineering biological systems through employing engineering principles and novel biological tools to the process of constructing and implementing human-designed biological systems, and to predictably produce a wide variety of pathways and regulatory networks.



The most well characterized eukaryote model organism *Saccharomyces cerevisiae* is not only preferred as a microbial cell factory for synthesis of industrial products, e.g. bioethanol, but this eukaryote host system is also defined as a robust scaffold for commercial production of diverse chemicals e.g. isoprenoids. Therefore, a number of tools in different emerging fields e.g. systems biology, evolutionary engineering and synthetic biology have been developed. Synthetic biology offers an alternative approach that is becoming more accessible as a tool for better performing metabolic engineering of yeast. Regulations of gene dosage and gene transcription are the first two key steps allowing controlling metabolic pathways improve both gene expression and gene dosage through modulating promoter choice and plasmid copy number and were investigated in this study. A new divergent promoter was developed, containing two strong and constitutive promoters to support high level gene expression. Furthermore, this bidirectional promoter was used to construct new episomal plasmids, the pSP series, to optimize the endogenous mevalonate pathway through gene integration and also to construct integration cassettes containing the synthetic methylerithritol phosphate (MEP) pathway genes. However, a functional MEP pathway was not achieved even after reconstructing the possible bacterial Fe/S trafficking routes and the bacterial electron transfer system in order to circumvent lack of the enzyme activity. In the last approach improvement of gene dosage via modulating plasmid copy number was investigated. Here, two strategies individually and in combination were applied in order to increase plasmid copy number of pSP-GM1 through reducing the marker gene at both protein and RNA level. Both methods, destabilization of marker protein using a ubiquitin/N-degron tag and down-regulation of the marker gene employing weak promoters, elevated the plasmid copy number. This thesis showed the successful implementation of synthetic biology tools in metabolic engineering in terms of pathway optimization and pathway reconstruction in order to improve sesquiterpene production in *S. cerevisiae*.

SIAVASH PARTOW

Systems and Synthetic Biology
Department of Chemical and Biological Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
Göteborg, Sweden 2012