THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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

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Siavash Partow

PhD thesis
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.

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 TPI1*, *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 maker 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** *Saccharomy cescerevisiae* **engineered for the production of plant sesquitepene 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.*
(Accepted in Microbial Cell Factory)

V.   Siavash Partow, Verena Siewers, Laurent Daviet, Michel Schalk, Jens Nielsen: **Reconstruction and evaluation of the synthetic bacterial MEP pathway in** *Saccharomyces cerevisiae.* (Submitted)

CONTRIBIUTION 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

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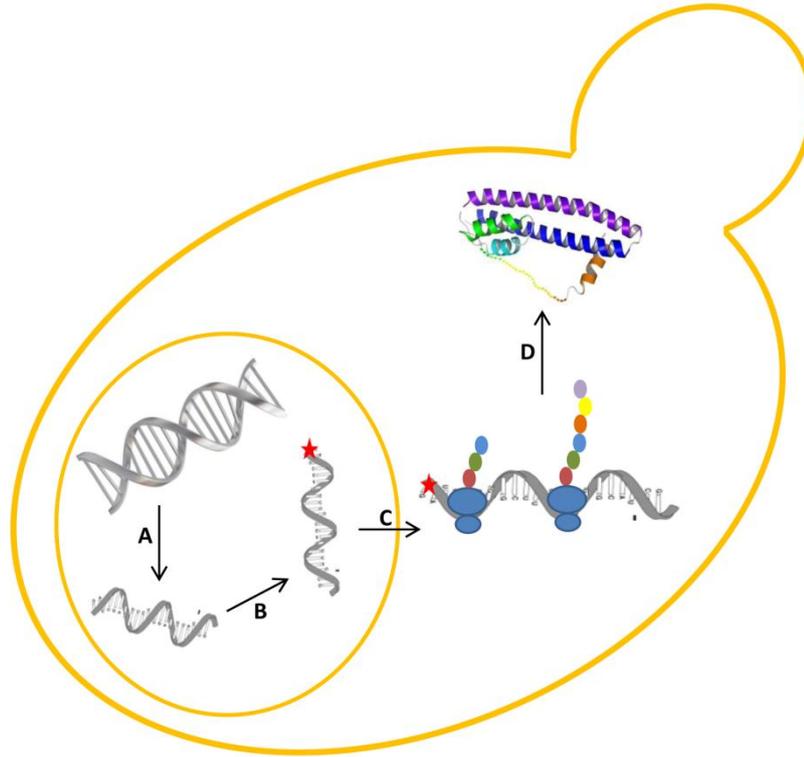
Figure 1: The central dogma; Arrows represent the process units containing (**A**) transcription process, (**B**) post-transcription process, (**C**) translation process and (**D**) post-translation process.

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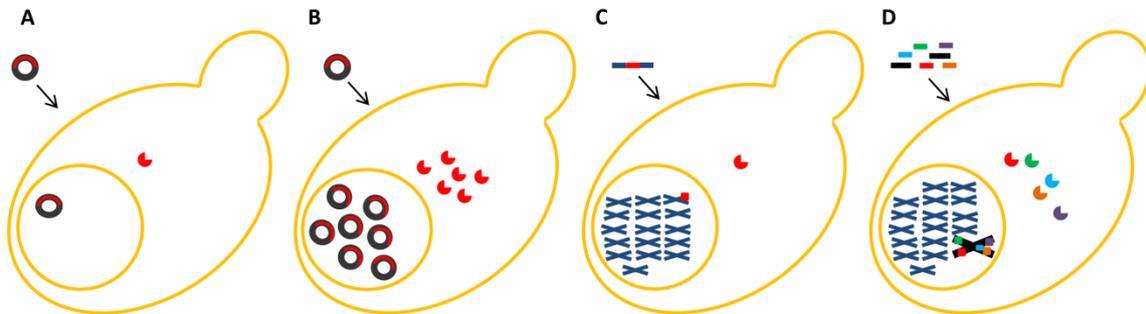
Figure 2: Different synthetic biology tools developed at DNA level. **A**: yeast centromeric plasmid (YCp); **B**: yeast episomal plasmid (YEp); **C**: yeast integrative plasmid (YIp); **D**: yeast artificial chromosome (YAC)

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 YEp, 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	*ARS1/CEN4*, 2μ, *ARS1*	*URA3*	P_{TEF1}, P_{GAL1}, P_{STE12}	YIplac backbone	Sadowski et al., (2007)
YCp4XX, YEp4XX, YRp4XX	*ARSH4/CEN6*, 2μ	*URA3, TRP1, HIS3, LEU2, LYS2*		pBR322 backbone	Ma et al., (1987)
pRS series (YCp, YEp, YIp)	*ARSH4/CEN6*, 2μ	*URA3, TRP1, HIS3, LEU2, KanMX, ADE2, MET15, hphNT1, natNT2*		pBLUESCRIPT –based vectors	Sikorski & Hieter (1989), Brachmann et al., (1998), Taxis & Knop (2006)
P4XX prom. (pRS vaiant)	*ARSH4/CEN6*, 2μ	*URA3, TRP1, HIS3, LEU2,*	P_{CYC1}, P_{ADH1}, P_{TEF1}, P_{GDP1}, P_{MET25}, P_{GAL1}, P_{GAL1}, P_{GALS}		Mumberg et al., (1994, 1995)
pCu4XX prom (pRS variant)	*ARSH4/CEN6*, 2μ	*URA3, TRP1, HIS3, LEU2,*	P_{CTR3}, P_{CTR1}, P_{CUP1}		Labbe & thiele (1999)
p4XX prom.att (Gateway™)	*ARSH4/CEN6*, 2μ	*URA3, TRP1, HIS3, LEU2,*	P_{TEF1}, P_{GPD1}, P_{MET25}, P_{GAL1}	Four N- and C-epitope tags	Funk et al., (2002)
pVV2XX (Gateway™)	*ARS1/CEN4*	*URA3, TRP1*	P_{PGK1}, $P_{tetO-CYC1}$	Four N- and C-epitope tags	Van Mullem et al., (2003)
pJGXXX (Gateway™)	*ARSH4/CEN6*	*URA3, TRP1, HIS3, LEU2,*	P_{GAL1}	Some with V5-6xHis tag	Geiser (2005)
pAG (Gateway™)	*ARSH4/CEN6*, 2μ	*URA3, TRP1, HIS3, LEU2,*	P_{TDH3}, P_{GAL1}	Seven N- C- epitope tags, integration vectors also available	Alberti et al., (2007)
pCMXXX series (YCplac, YEplac variant)	*ARS1/CEN4*, 2μ	*URA3, TRP1*	$P_{tetO-CYC1}$ with Tta activator	Varied number of tetO boxes	Gari et al., (1997)
pYC, pYES	*ARSH4/CEN6*, 2μ	*URA3, TRP1, bsd§*	P_{GAL1}	YES™ vector Collection	Invitrogen,
YCp-SPB series, YEp-SPB series, YIp-SPB series, YIp-GAL1-SPB	*ARS1/CEN4*, 2μ	*URA3*	P_{PGK1}, P_{GAL1}, P_{GAL10}, P_{PHO5}, P_{CUP1}		Cartwright et al., (1994)
pXP series	*ARSH4/CEN6*, 2μ	*URA3, TRP1, HIS3, LEU2, ADE2, MET15*	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),
pBEVV, pBEVY-G	2μ	*URA3, TRP1, HIS3, LEU2,*	P_{TDH3}-P_{ADH1}, P_{GAL1}-P_{GAL10}	Bidirectional promoter system	Miller et al., (1998)
pY2x-GAL(1/10)-GPD	2μ	*URA3, TRP1, HIS3, LEU2,*	P_{GAL1}-P_{TDH3}, P_{GAL10}-P_{TDH3}	Bidirectional promoter system	Li et al., (2008)
pESC Vectors	2μ	*URA3, TRP1, HIS3, LEU2,*	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.
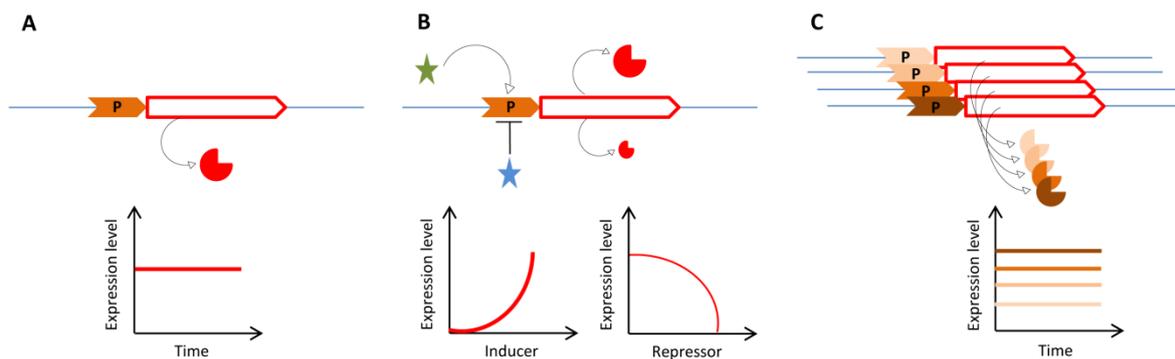
Figure 3: Schematic representation of expression level at transcription, which is controlled by different types of promoters. Promoters are orang chevrons, coding region are red pentagons, pies with different colors represent proteins, blue and green stars represent inducer and repressor molecules, respectively. **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 curtail 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 G_1 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.
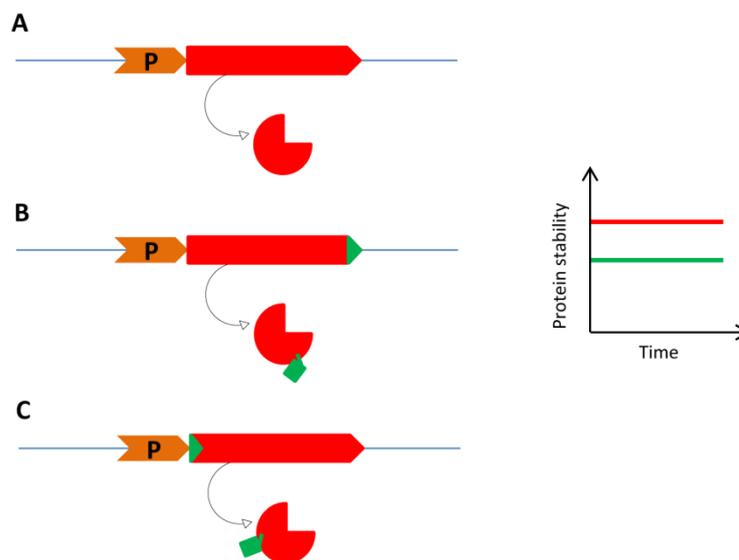
Figure 4: Schematic representation of synthetic biology tools developed for modulating protein stability. Promoters are orange, coding region are red pentagons, red pies represent protein, green boxes are peptide tag at C- and N- terminal, respectively; **(A)** non-tagged protein; **(B)** C-tagged protein; **(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. cerevisiea* 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, Shoa and co-workers (2008) have demonstrated rapid assembly of a functional D-xylose utilization pathway consisting of 3 genes (≈9kb), a zeaxanthin biosynthetic pathway including 5 genes (≈11kb) and combined both pathways (≈19kb) 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.
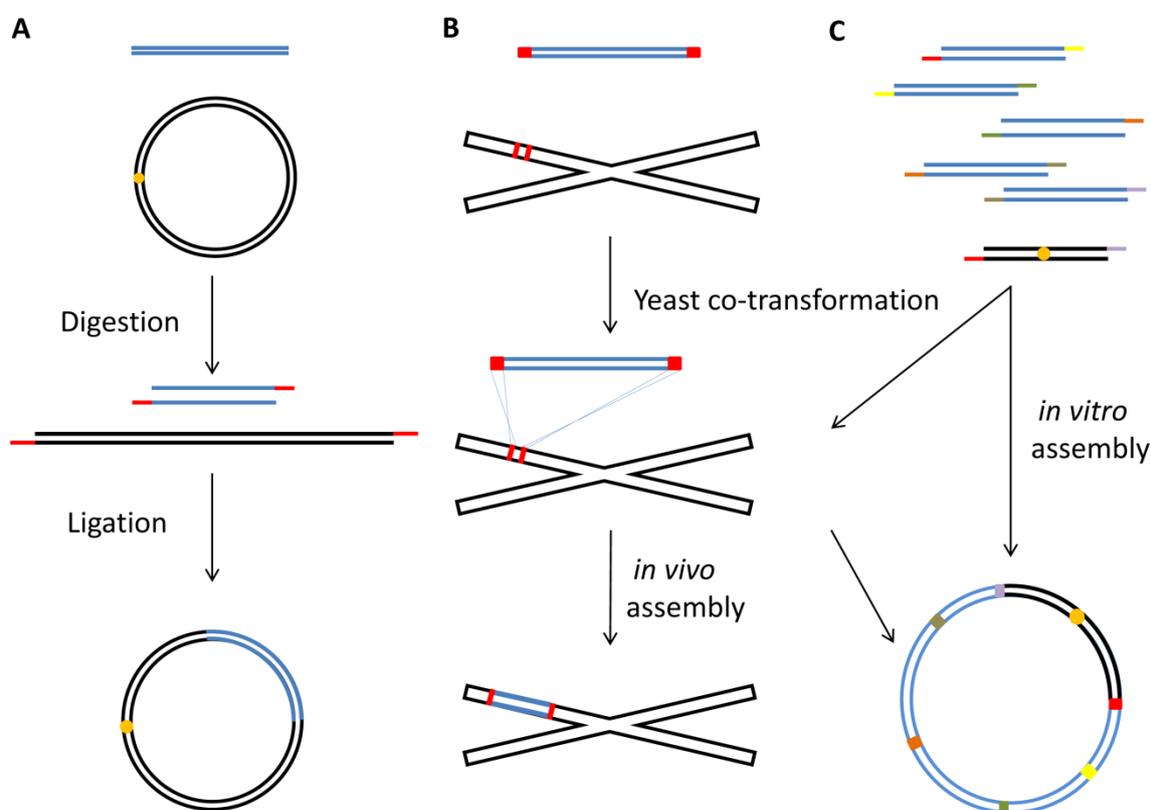
Figure 5: Schematic representation of assembly methods for forming DNA constructs; **(A)** Plasmid-based based on restriction and ligation; **(B)** chromosomal integration based *in vivo* homologous recombination; **(C)** YAC-based method based on both *in vivo* or *in vitro* homologous recombination.

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-glyceralaldehyde-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
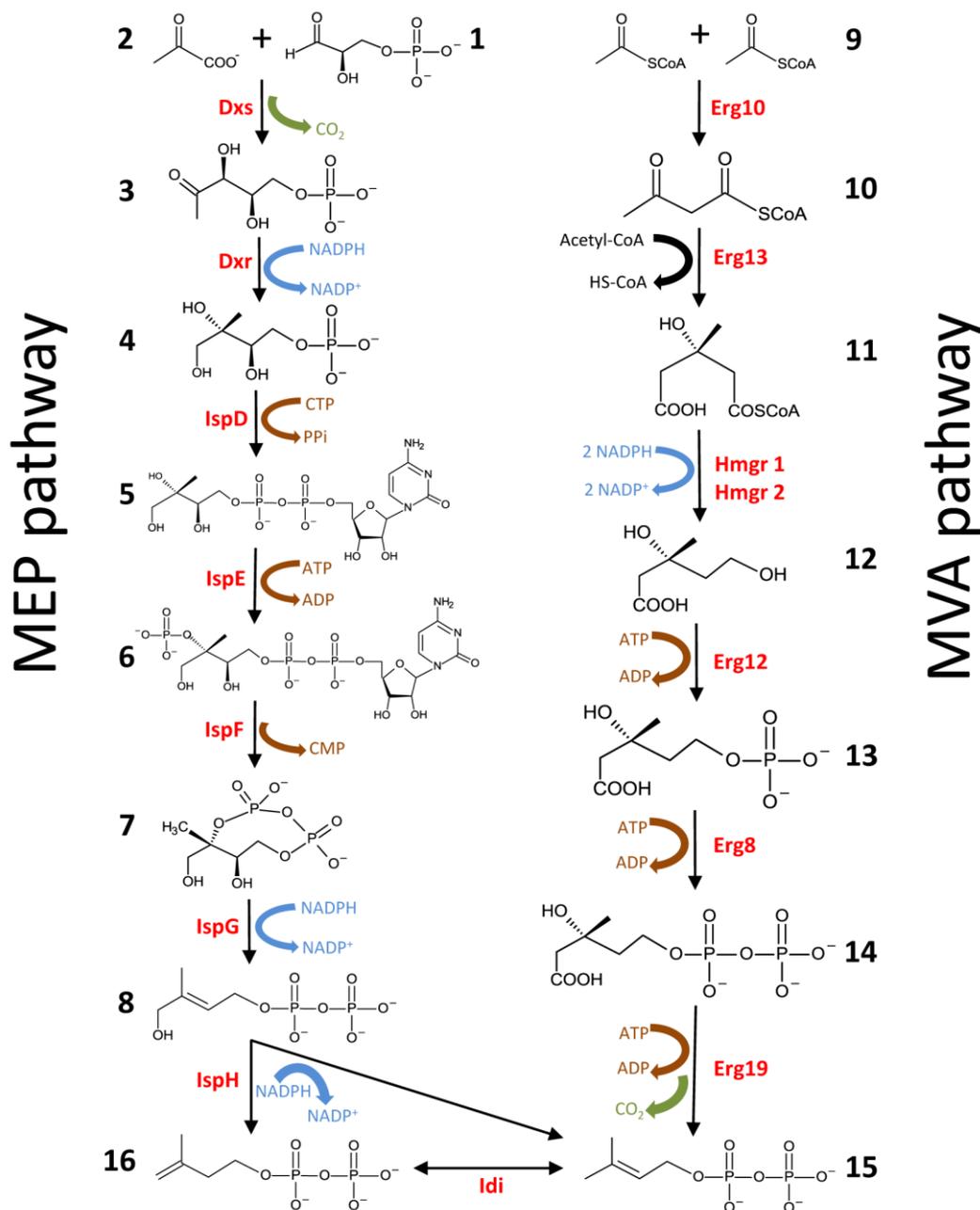
Figure 6: The MEP pathway (left). Enzymes: **Dxs,** 1-deoxy-D-xylulose-5-phosphate synthase; **Dxr,** 1-deoxy-D-xylulose 5-phosphate reductoisomerase; **IspD,** 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase; **IspE,** 4-diphosphocytidyl-2-C-methylerythritol kinase; **IspF,** 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; **IspG,** 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase; **IspH,** 1-hydroxy-2-methyl-butenyl 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-optimizae 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.

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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; Yocum 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_{TPI1}, 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_{TPI1}	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} ~ P_{PGK1} ~ P_{TDH3} > P_{TPI1} ~ P_{PYK1} > P_{ADH1} > P_{HXT7}

When glucose is exhausted and ethanol is consumed:

P_{TEF1} ~ P_{HXT7} > P_{PGK1} > P_{TPI1} ~ P_{TDH3} > P_{PYK1} ~ 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, 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 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).
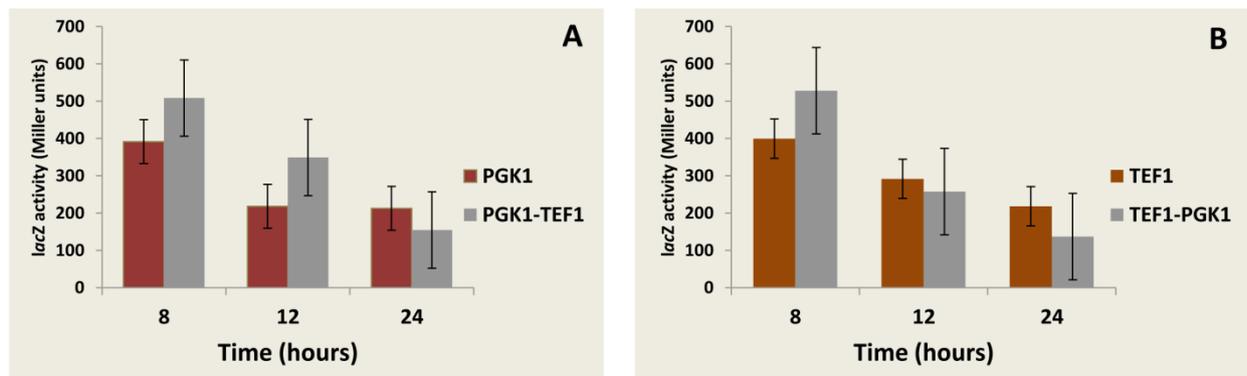
Figure 7: Activity of P_{PGK1} and P_{TEF1} in different contexts; **A**, red columns represents the activity of individual P_{PGK1}, gray columns represents the activity of P_{PGK1} fused to P_{TEF1}; **B**, brown columns represent the activity of individual P_{TEF1}, gray columns represents the activity 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.
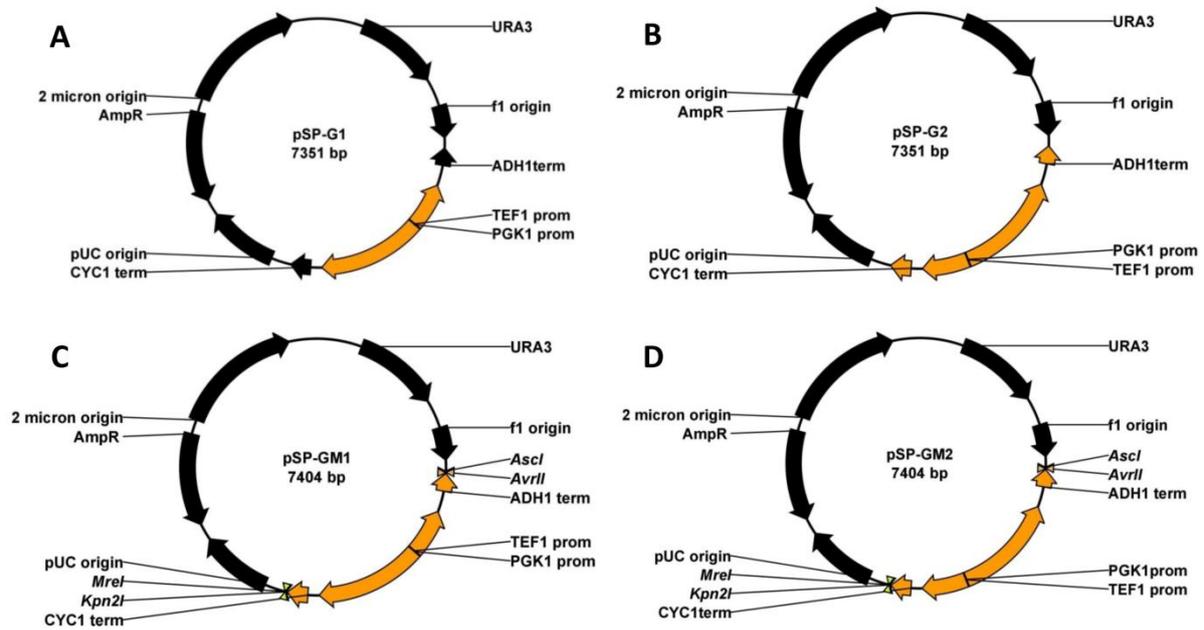
Figure 8: Schematic of pSP series. Details are presented in the text.

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 2µ sequence or commonly, a 2µ 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* allels 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)(Erhart & 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.*

*cerevisia*e 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 stategies, i.e. weak promoter and ubiquitin/N-degron tag and evaluated plasmid copy number in these conditions. Figure 9 illustratesthe plasmid constructs.
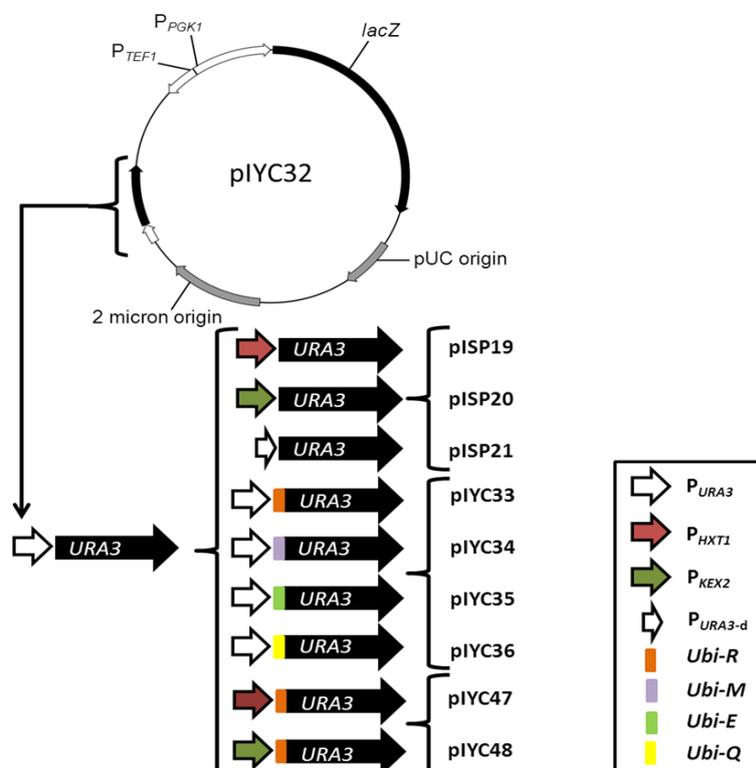
Figure 9: Schematic representation of the plasmid constructs. P_{URA3}, P_{HXT1}, P_{KEX2}, and P_{URA3-d}, promoters employed to control *URA3* expression; *Ubi-R*, *Ubi-M*, *Ubi-E* and *Ubi-Q*, ubiquitin/N-degron tags leading to arginine, methionine, glutamate and glutamine as N-terminal residues of 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 *HXT*1 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)

		Fold change	
Strain (Plasmid)	Plasmid description	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_{URA3d}-*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

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 patchouli 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.
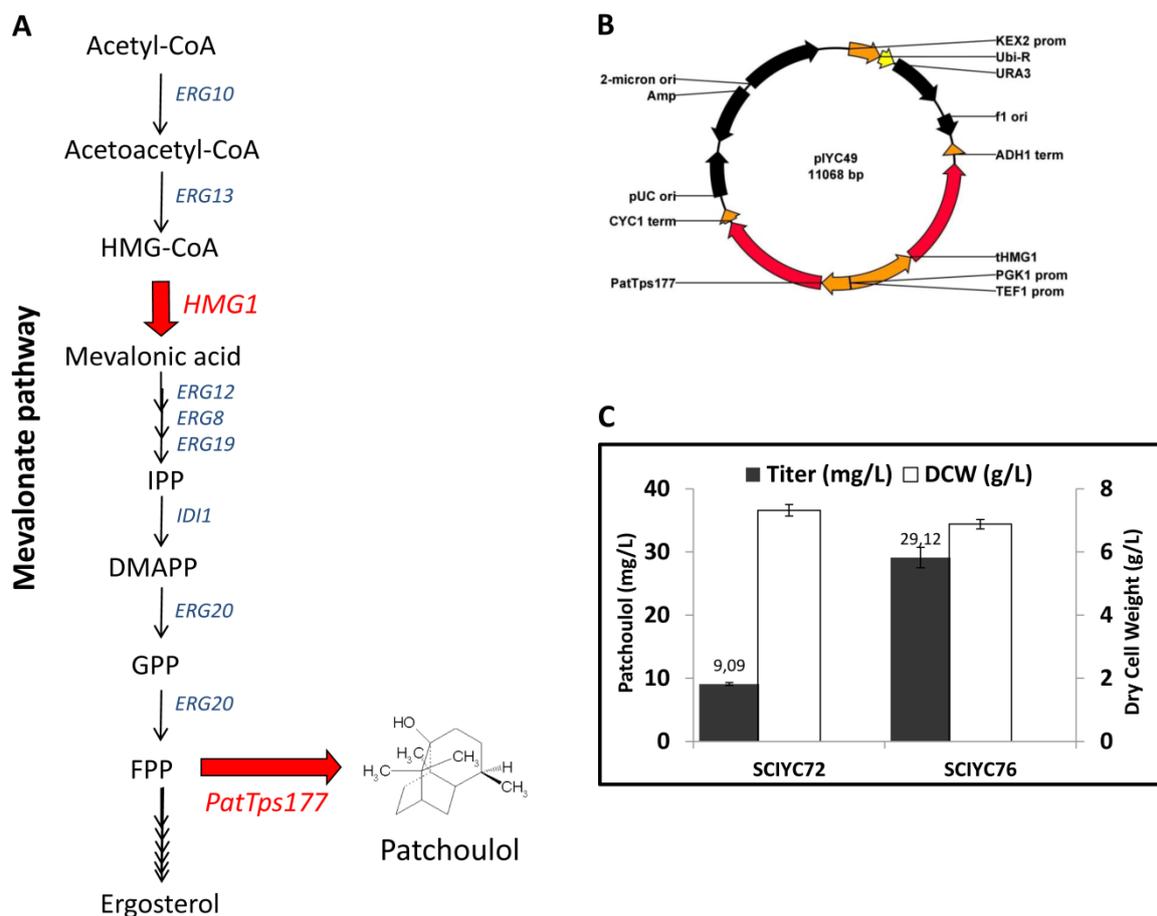
Figure 10: Growth and patchoulol accumulation of strain SCIYC76 and control strain SCIYC72. **A**, mevalonate pathway, red arrows correspond to the over-expressed genes; **B**, map of modified expression plasmid, pIYC49 (P*KEX2*-*Ubi-R-URA3*) which harbors a copy of *tHMG1* and *PatPs177* genes downstream 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 (a-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 dimethyllallyl 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).
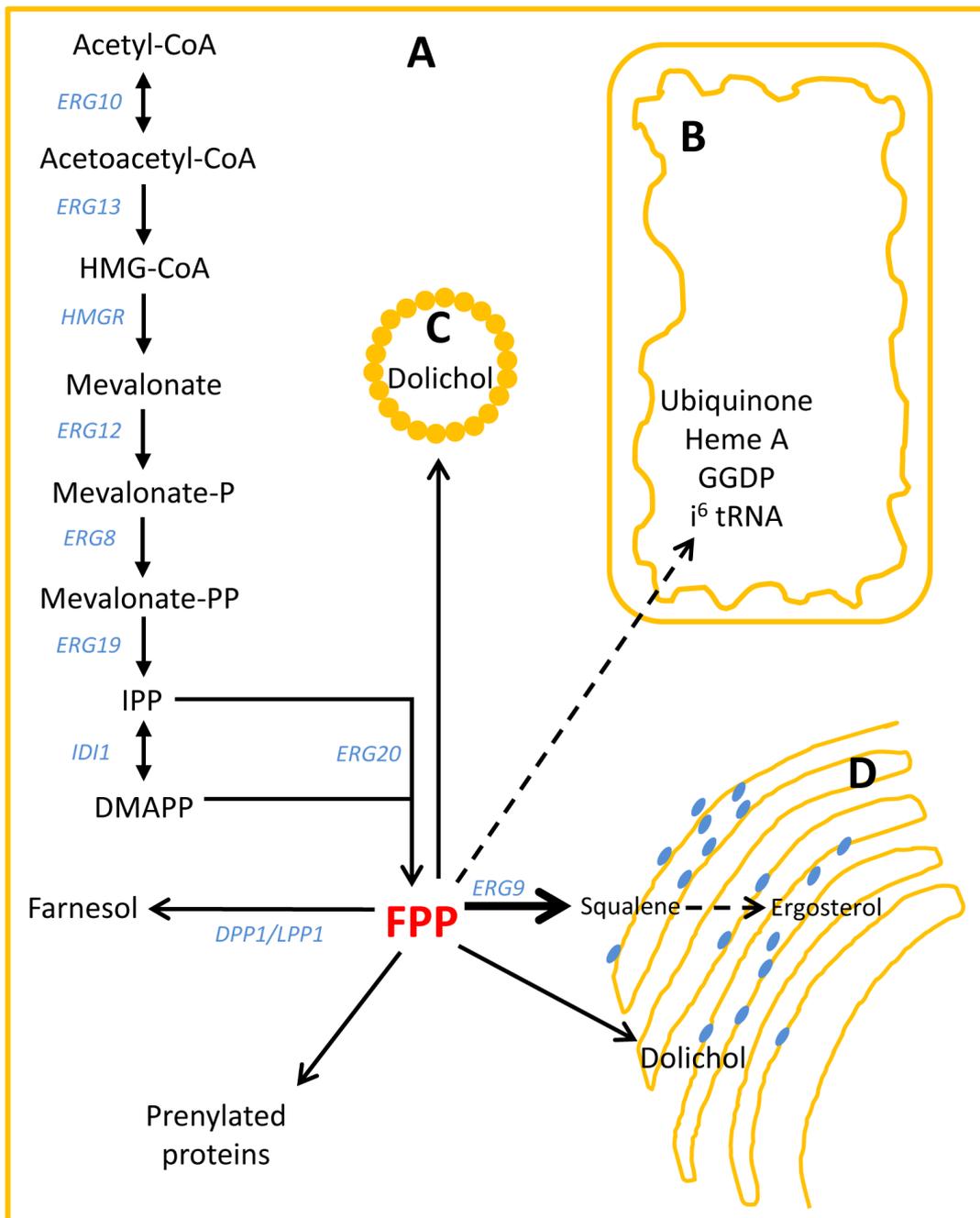
Figure 11: Schematic representation of the farnesyl pyrophosphate (FPP) branch-point in *S. cerevisiae*; **A**, Cytosol; **B**, Mitochondria; **C**, Lipid particle; **D**, Endoplasmic reticulum.

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.
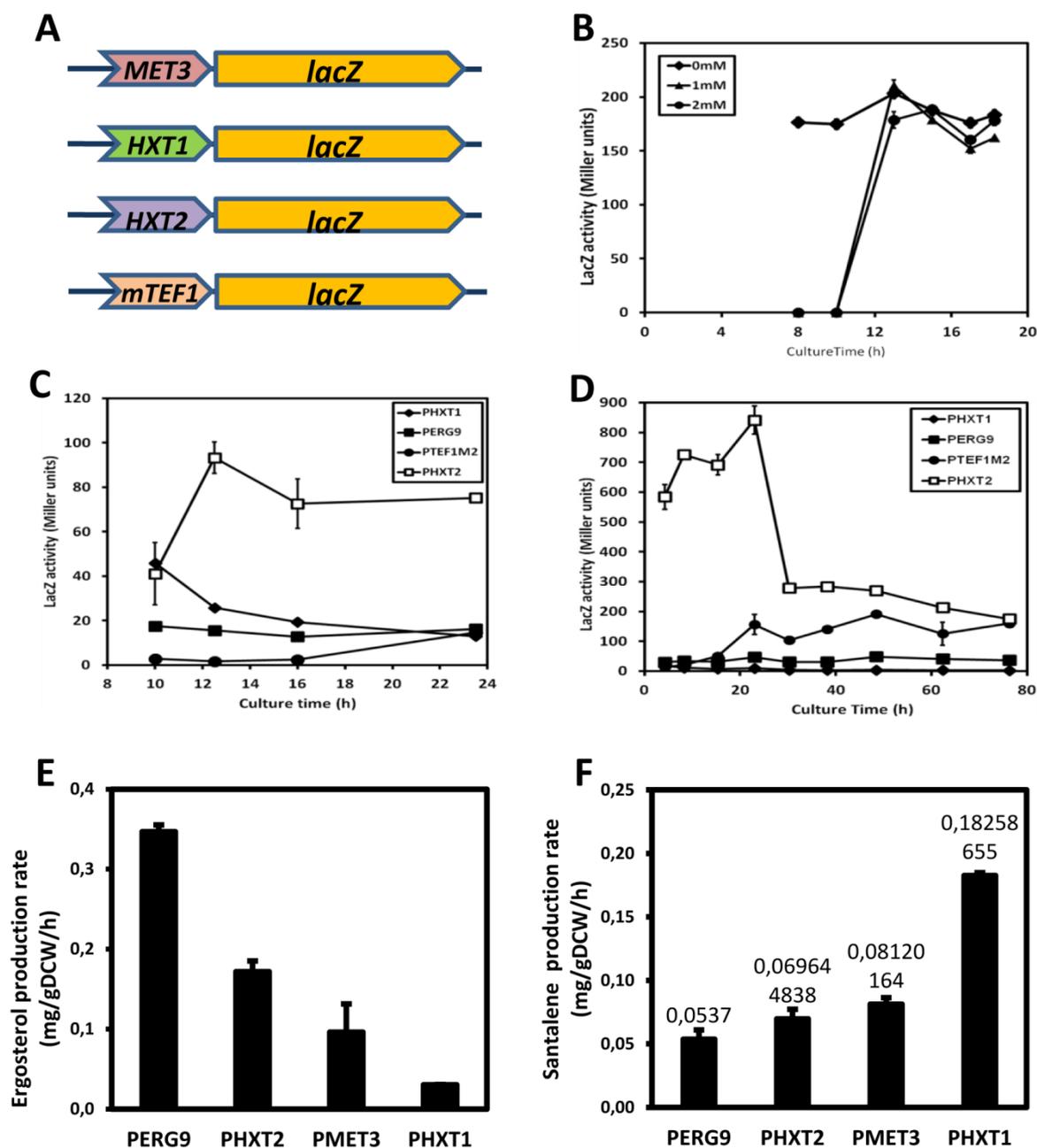
Figure 12: **Result of the regulation of FPP branch point**. (**A**) Integrative cassettes include promoter choice (*MET3, HXT1, HXT2* and mutant *TEF1*) in front of *lacZ* as a reporter gene; (**B**) LacZ activity under control of P_{MET3} in response to different methionine concentrations, 0 mM (diamonds), 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 (mg*g biomass^{-1}•h^{-1}); (**F**) α-santalene and *E,E*-farnesol prodcution rate (mg•g biomass^{-1}•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 **Figure13A**.

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 requirments 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 an 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	*MATa MAL2-8ᶜ SUC2*	pISP15
SCIGS29	*MATα MAL2-8ᶜ SUC2 lpp1Δ::loxP P$_{ERG9}$Δ::loxP-P$_{HXT1}$*	pISP15
SCIGS30	*MATα MAL2-8ᶜ SUC2 lpp1Δ::loxP dpp1Δ::loxP P$_{ERG9}$Δ::loxP-P$_{HXT1}$*	pISP15
SCIGS31	*MATα MAL2-8ᶜ SUC2 lpp1Δ::loxP dpp1Δ::loxP P$_{ERG9}$Δ::loxP-P$_{HXT1}$ gdh1Δ::loxP*	pISP15
SCIGS24	*MATα MAL2-8ᶜ SUC2 lpp1Δ::loxP dpp1Δ::loxP P$_{ERG9}$Δ::loxP-P$_{HXT1}$ gdh1Δ::loxP P$_{TEF1}$-ERG20 P$_{PGK1}$-GDH2*	pISP15
SCIGS25	*MATα MAL2-8ᶜ 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*	pISP15
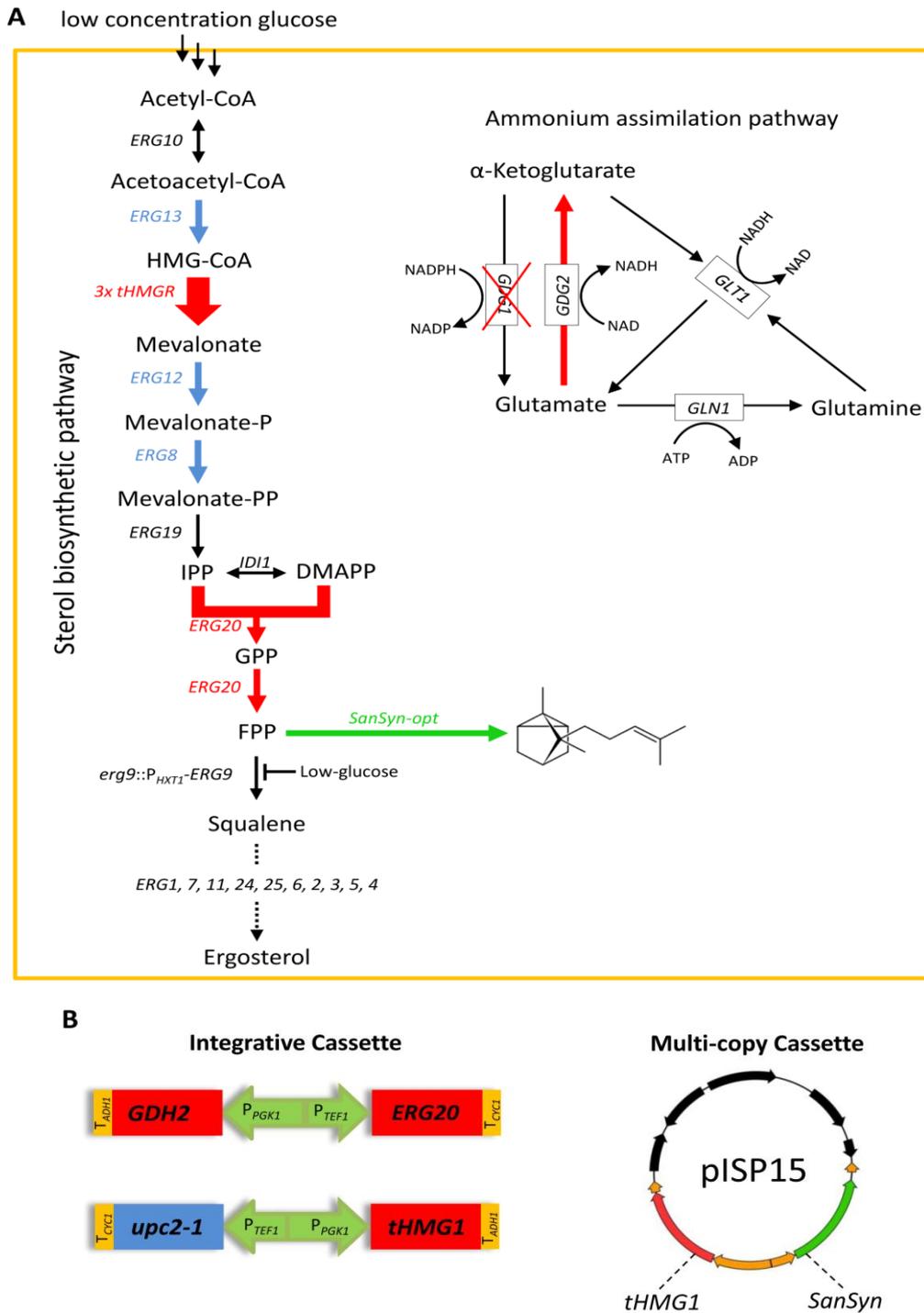
Figure 13: Schematic representation of the engineered α-santalene biosynthetic pathway through the modification of the mevalonate pathway and the ammonium metabolism pathway in *S. cerevisiae*. **A,** The directly up-regulated genes are shown in red and purple; those that are indirectly up-regulated 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 (stain 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, amorphiadiene, 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 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).
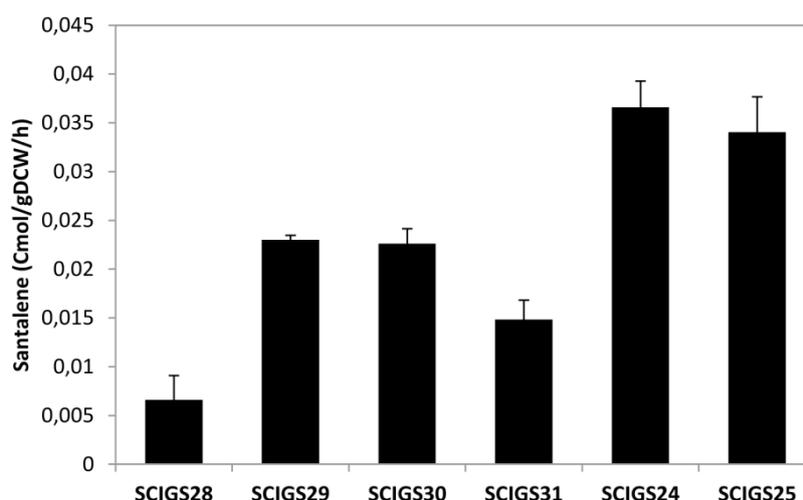
Figure 14: α-(+)-santalene production rate Cmmol (g Biomass)$^{-1}$ h^{-1} in *S. cerevisiae* in a two phase partitioned glucose limited aerobic chemostat. Strains SCIGS28, SCIGS29 (*P$_{HXT1}$-ERG9; Δdpp1*), SCIGS30 (+*Δlpp1*), SCIGS31 (+*Δgdh1*), SCIGS24 (+*ERG20; GDH2*), SCIGS25 (+*upc2-1, tHMG1*) 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

$$\text{MVA pathway} \longrightarrow -4.5\,\text{Glucose} - 9\,\text{ATP} - 6\,\text{NADPH} + \text{FPP} + 18\,\text{NADH} + 12\,CO_2 = 0$$

$$\text{MEP pathway} \longrightarrow -3\,\text{Glucose} - 6\,\text{ATP} - 9\,\text{NADPH} + \text{FPP} + 3\,\text{NADH} + 3\,CO_2 = 0$$
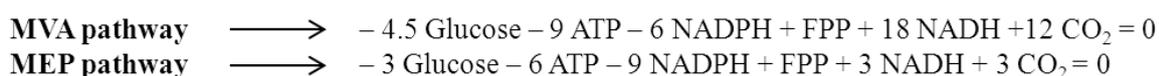
The stoichiometry showed that, for producing one molecule of farnesyl pyrophosphate from glucose via the MVA pathway six molecules of NADPH and nine molecules of ATP are required, while production via the MEP pathway consumes nine molecules of NAD(P)H and six molecules of ATP.

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	MATαMAL2-8cSUC2 ura3-52	none	P. Kötter
SCISP06	MATαMAL2-8cSUC2 ura3-52 dxs dxr ispD ispE ispF ispG ispH idi	none	this work
SCISP16	MATαMAL2-8cSUC2 dxs dxr ispD ispE ispF ispG ispH idi	pISP08	this work
SCISP28	MATαMAL2-8cSUC2	pSP-GM1	this work
SCISP29	MATαMAL2-8cSUC2 dxs dxr ispD ispE ispF ispG ispH idi	pSP-GM1	this work
CEN.PK 113-1C	MATa MAL2-8c SUC2 trp1-289 ura3-52 his3Δ 1	none	P. Kötter
SCISP12	MATa MAL2-8c SUC2 trp1-289 ura3-52 his3Δ 1 dxs dxr ispD ispE ispF ispG ispH idi	none	this work
SCISP13	MATa MAL2-8c SUC2 trp1-289 his3Δ 1 dxs dxr ispD ispE ispF ispG ispH idi	pISP08	this work
SCISP30	MATa MAL2-8c SUC2 trp1-289	pSP-GM1, pSP-GM3	this work
SCISP31	MATa MAL2-8c SUC2 trp1-289 dxs dxr ispD ispE ispF ispG ispH idi	pISP08, pISP24	this work
SCISP32	MATa 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, YPRCΔ15 and YPRCτ3, 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.
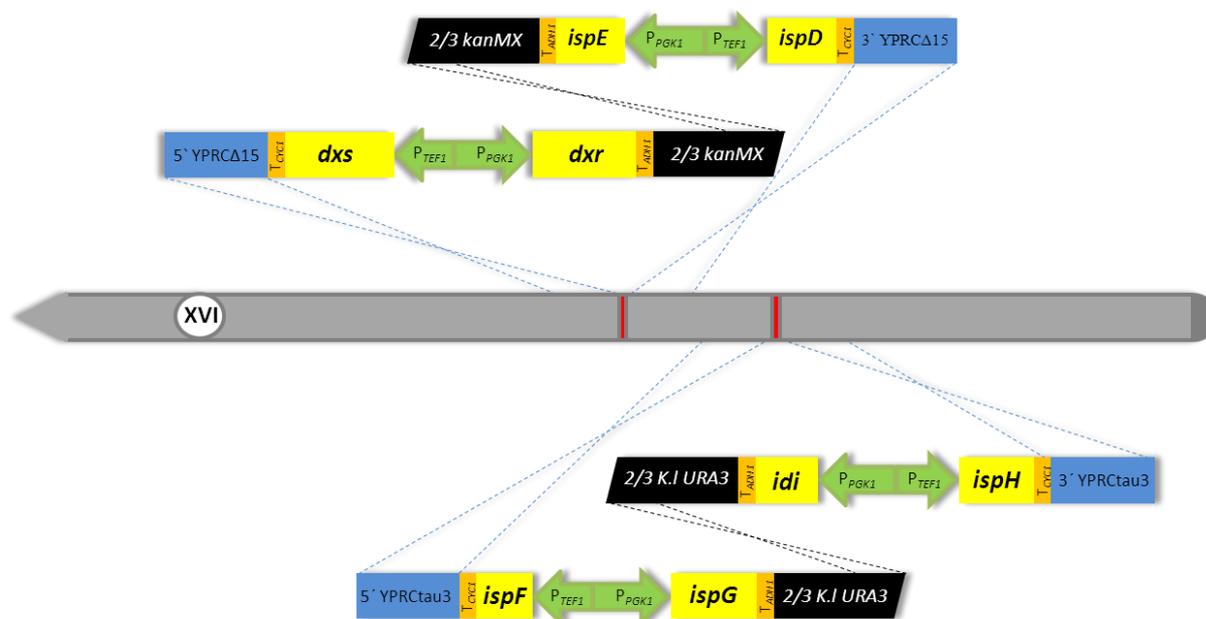

Figure 15: Schematic representation of genetic engineering strategies for genomic integration of the bacterial MEP pathway genes into the yeast genome (chromosome XVI).

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 Mn^{2+}, Mg^{2+} or Co^{2+}, 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.*
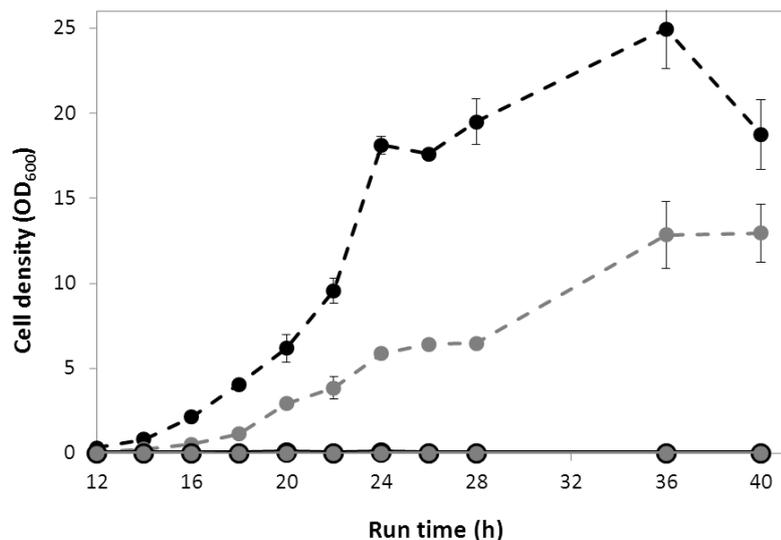
Figure 16: Growth of *Saccharomyces cerevisiae* strains CEN.PK 113-13D (black circles) and SCISP06 (gray circles) in SD minimal medium. Dashed lines represent the growth in 0 g L^{-1} of lovastatin; solid lines represent the growth in presence of 2 g L^{-1} of lovastatin. Error bars show the standard 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 /Nfs1and Isd11), an iron sensor/doner (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.
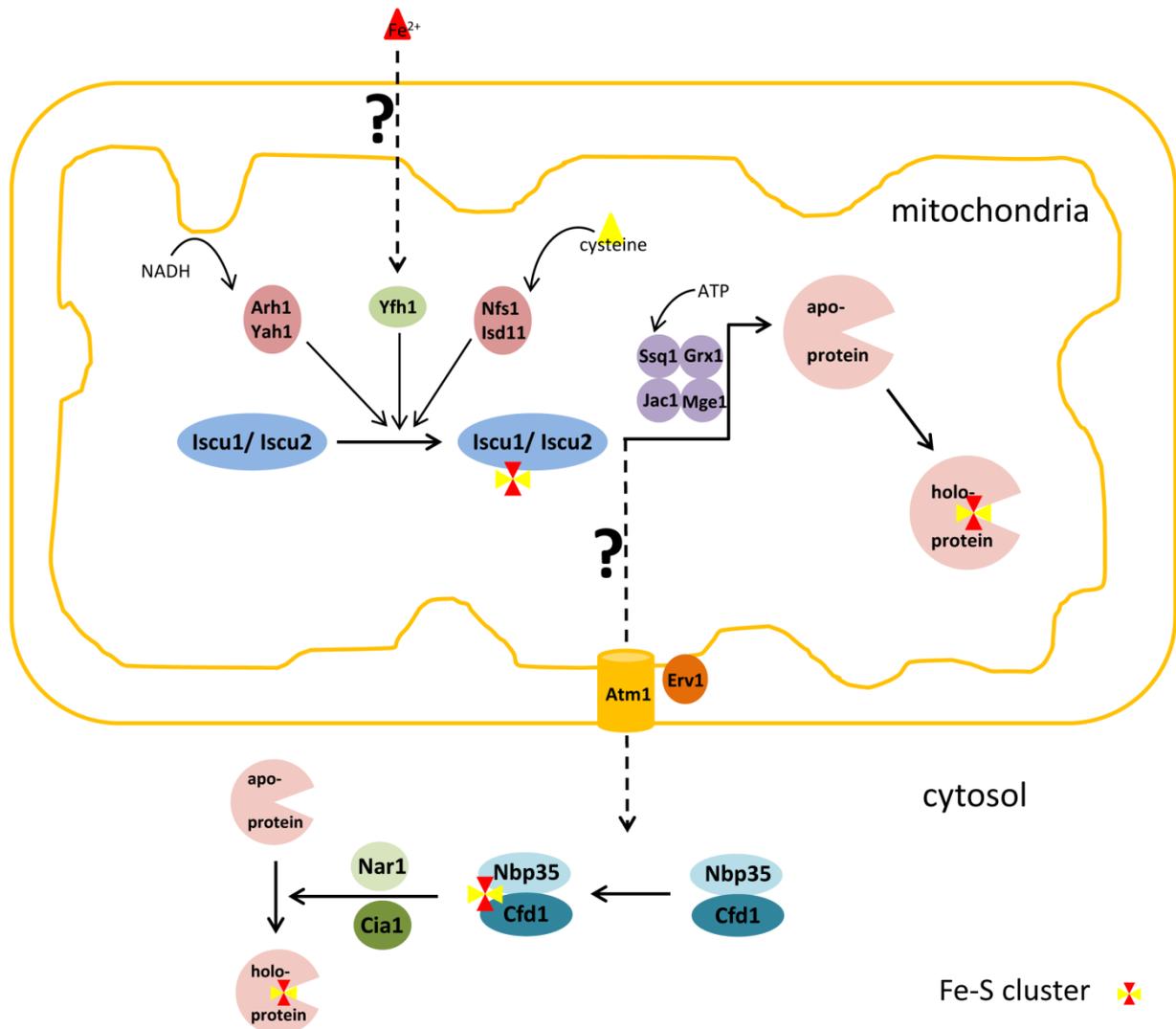
Figure 17: Schematic representation of Fe-S clusters assembly machinery in *S. cerevisiae* (details are discussed in section 2.7). The dashed arrows represent the apparatuses which are not defined completely yet.

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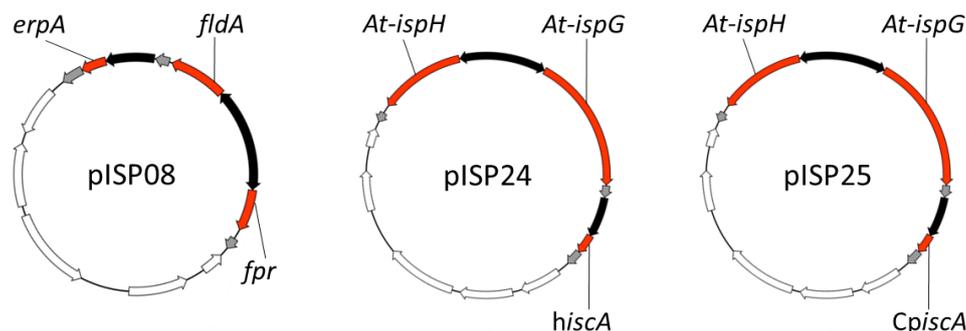
Figure 18: plasmid-based reconstruction of possible Fe/S trafficking routes involved in maturation of bacterial IspG/IspH, bacterial electron transfer systems and plant-derived *ispG/ispH* in *S. cerevisiae*.

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.
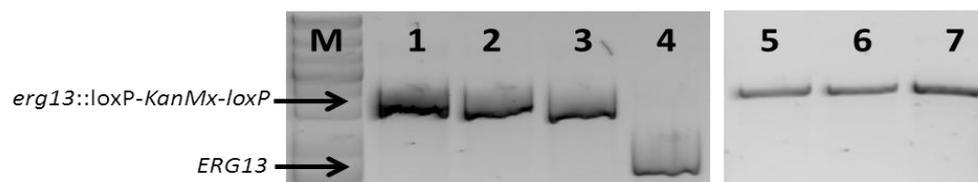
Figure 19: Gel electrophoresis of PCR products to confirm deletion of *ERG13* (1: SCISP28, 2: SCISP29, 3: SCISP16, 4: CEN.PK 113-13D (wild type), 5: SCISP30, 6: SCISP31, 7: SCISP32, M: 1 kb Plus DNA ladder.
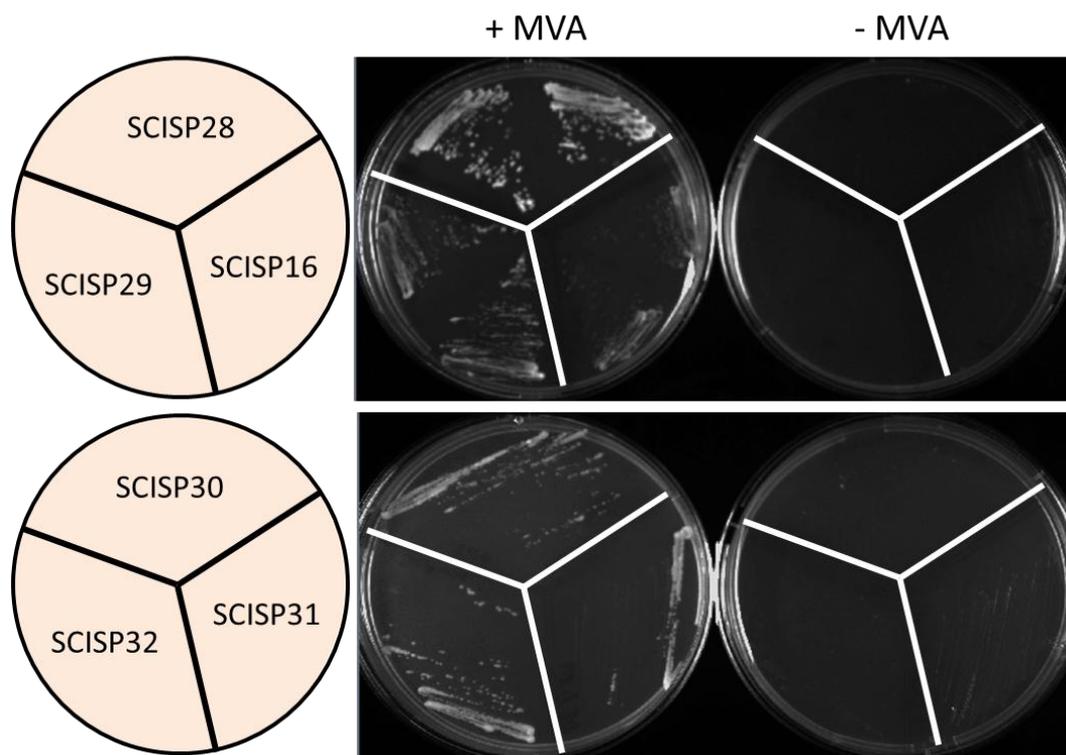
Figure 20: Aerobic cultivation of MEP pathway. upper panel: strains co-expressing *erpA, fpr and fldA*; lower panel: strains co-expressing *erpA, fpr, fldA*, *At-IspG* , *At-IspH* with either *CpIscA* or *hISCA*. All strains were e*rg13* background.

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.
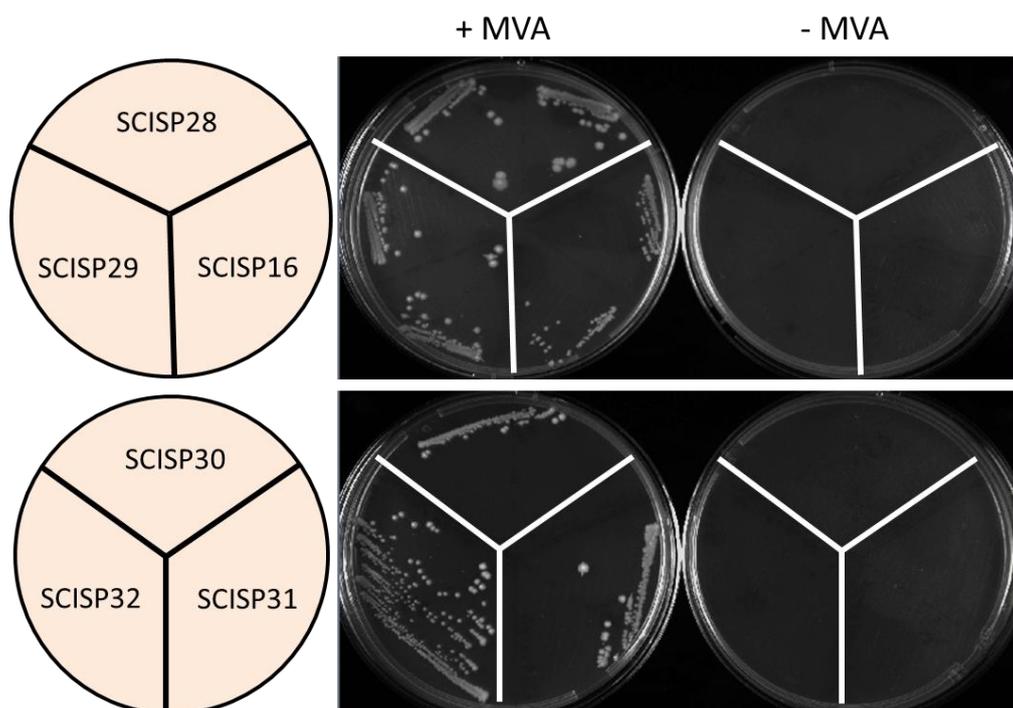
Figure 21: Anaerobic cultivation of MEP pathway. upper panel: strains co-expressing *erpA, fpr and fldA*; lower panel: strains co-expressing *erpA, fpr, fldA*, *At-IspG* , *At-IspH* with either *CpIscA* or *hISCA*. All strains were e*rg13* background.

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)**.**
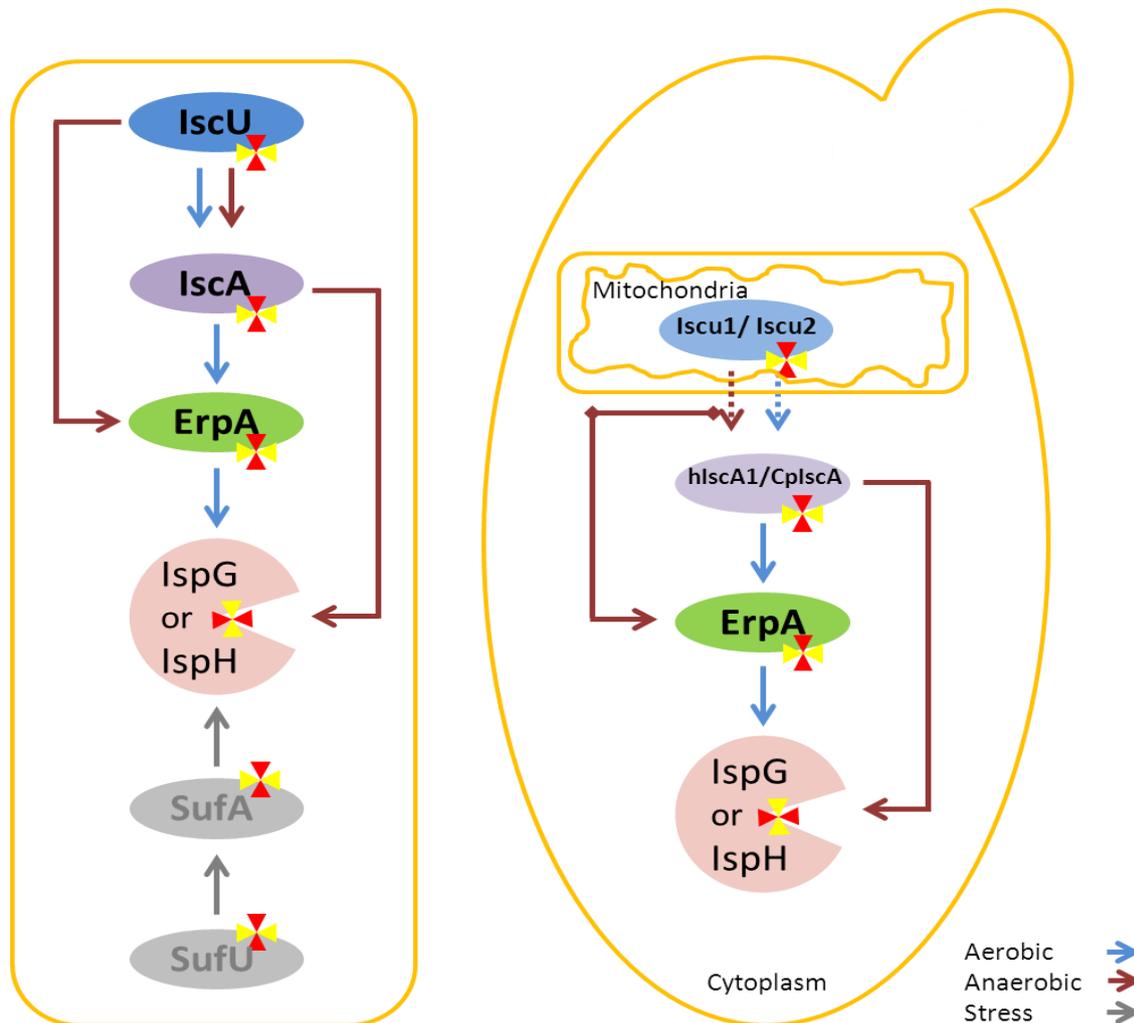
Figure 22: Schematic representation of possible Fe/S trafficking routes involved in maturation of bacterial IspG/IspH in E. coli (left) and reconstruction of possible routes preformed in this study in the yeast cytosol (right). Round dot arrows represent unknown mechanisms for transferring the Fe-S clusters 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 (**I**ron-**o**nly hydrogenase-like **p**rotein **I**) with human ISCA1 using yeast two-hybrid systems (Song et al., 2009). Cp*iscA* 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 (Szczebara 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% (Szczebara 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 (Szczebara 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_{TPI1}, 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 2μm-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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<div align="right">

Siavash Partow

July, 2012

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