THESIS FOR DEGREE OF DOCTOR OF PHILOSPHY

Metabolic Engineering of *Saccharomyces cerevisiae* for Sesquiterpene Production

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Systems & Synthetic Biology

Department of Chemical and Biological Engineering

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Cover: Schematic representation of the integrated metabolic engineering, systems biology, Synthetic biology and evolutionary engineering approach for the construction of a “yeast cell factory”

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Dedicated to

My family, the support of my life…

My love, the inspiration of my life…

"Cyclops, you asked my noble name, and I will tell it; but do you give the stranger's gift, just as you promised. My name is Nobody. Nobody I am called by mother, father and by all my comrades"

Odyssey, Chapter 9 line 366

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ABSTRACT

Industrial biotechnology aims to develop robust “microbial cell factories”, to produce an array of added value chemicals presently dominated by petrochemical processes. The exploitation of an efficient microbial production as sustainable technology has an important impact for our society. Sesquiterpenes are a class of natural products with a diverse range of attractive industrial proprieties. Due to economic difficulties of their production via traditional extraction processes or chemical synthesis there is interest in developing alternative and cost efficient bio-processes. Microbial cells engineered for efficient production of plant sesquiterpenes may allow for a sustainable and scalable production of these compounds. *Saccharomyces cerevisiae* is one of the most robust and characterized microbial platforms suitable to be exploited for bio-production. The hydrocarbon α-santalene is a precursor of sesquiterpenes with relevant commercial application and was selected as case study. Here, for the first time a *S. cerevisiae* strain capable of producing high levels of α-santalene was constructed through a multidisciplinary system level metabolic engineering approach. First, a minimal engineering approach was applied to address the feasibility of α-santalene production in *S. cerevisiae*. Successively, a rationally designed metabolic control strategy with the aim to dynamically modulate a key metabolic step to achieve optimal sesquiterpene production was applied, combined with the engineering of the main regulatory checkpoint of targeted pathway. It was possible to divert the carbon flux toward the sesquiterpene compound, and the resulting strain shows a 88-fold improvement in α-santalene productivity. A second round of strain optimization was performed using a multistep strategy focused to increase precursors and co-factor supply to manipulate the yeast metabolic network in order to further redirect the carbon toward the desired product. This approach results in an overall increase of 1.9-fold in α-santalene productivity. Furthermore, strain improvement was integrated with the development of an efficient fermentation/ downstream recovery process, resulting in a 1.4-fold improvement in productivity and a final α-santalene titer of 193 mg l^{-1}. Finally, the substrate utilization range of the selected platform was expanded to use xylose as alternative carbon source for biorefinery compatibility, via pathway reconstruction and an evolutionary strategy approach, resulting in a strain capable of rapid growth and fast xylose consumption. The results obtained illustrate how the synergistic application of multilevel metabolic engineering and bioprocess engineering can be used to obtain a significant amount of high value sesquiterpene in yeast. This represents a starting point toward the construction of a yeast “sesquiterpene production factory” and for the development of an economically viable bio-based process that has the potential to replace the current production methods.

Keywords: Metabolic Engineering, Systems Biology, Synthetic Biology, Evolutionary engineering, Microrefinery, Cell factory, *Saccharomyces cerevisiae*.

PREFACE

This dissertation represents the tangible results of my PhD study, carried out at the Systems and Synthetic Biology group (Sys^{2}Bio), Department of Chemical and Biological Engineering, Chalmers University of Technology in the period between 2008 and 2012, under supervision of Professor Jens Nielsen. I believe the results obtained in this thesis are just a small drop in a sea considering the potential applications of the constantly emerging field I had the privilege to work in during this research period.

When I first came to Chalmers in July 2008 the Department of Chemical and Biological engineering did not host a Systems and Synthetic Biology group, but every accomplishment starts with the decision to try, so under the guidance of a phenomenal group leader and surrounded by a selected group of finest scientist we start from scratch and embrace the challenge to create what today I consider a group for excellence in systems level metabolic engineering. In life there's always an easy way out but I choose the less travelled road; I lost sight of days, I lost sight of time, I could have been there for hours days or months just figuring things out, but that did not matter comparing to how exiting and motivating it was and in the end the hard work paid off.

The title page of this thesis quotes sentences form the ancient Greek poems ΟΔΥΣΣΙΑ (= Odyssey). My father use to read me the story of the epic voyage of Ulysses (= Odysseus) when I was a child; just as Ulysses journey the path that brings me to this doctoral dissertation was rich of uncertain, unforeseen difficulties, overwhelming hurdles, failure, frustration but even joy, success, happiness, maturation and friendship. Approaching the end of my dissertation, I now reached my Ithaca and I am holding the hunting bow ready to shoot the arrow through iron axe-helve sockets twelve in line to finish this amazing story I thought I dream it only. I do not yet know what future holds in store for me but I am ready once again to chase my dream…

Gionata Scalcinati

June, 2012

LIST OF PUBBLICATIONS

This thesis is based on the following publications & patent.

Patent Application:

I. Scalcinati G, Knuf C, Schalk M, L. Daviet L, Siewers V, Nielsen J. **Modified microorganisms and use thereof for terpene production**. *United States Provisional patent* application filed on June 27, 2011 and *PCT Patent Application* EP11171612.2 filed on June 28, 2011.

Publications:

I: Scalcinati G, Knuf C, Partow P, Chen Y, Maury J, Schalk M, Daviet L, Nielsen J, Siewers V. **Dynamic control of gene expression in *Saccharomyces cerevisiae* engineered for the production of plant sesquiterpene α-santalene in fed batch mode.** *Metabolic Engineering*. 2012. 14 (2): 91-103.

II: Scalcinati G, Partow S, Siewers V, Schalk M, Daviet L, Nielsen J. **Combined metabolic engineering of precursors and co-factor supply to increase α-santalene production by *Saccharomyces cerevisiae*.** *Submitted*

III: Scalcinati G and Nielsen J. **Optimization of fed batch process for production of a sesquiterpene biofuel-like precursor α-santalene by *Saccharomyces cerevisiae*.** *Submitted*

IV: Scalcinati G, J.M. Otero JM, Van Vleet J, Jeffries TW, Olsson L, Nielsen J. **Evolutionary engineering of *Saccharomyces cerevisiae* for efficient aerobic xylose consumption.** *FEMS Yeast research*, DOI: 10.1111/j.1567-1364.2012.00808.x.

During this doctoral research additional publications have been co-authored that are not included in this thesis:

V. Chen Y, Partow S, Scalcinati G, Siewers V, Nielsen J. **Enhancing the copy number of episomal plasmids in *Saccharomyces cervisiae* for improved protein production.** *FEMS Yeast Research*. DOI: 10.1111/j.1567-1364.2012.00809.x

VI. Papini M, Nookaew I, Scalcinati G, Siewers V, Nielsen J. **Phosphoglycerate mutase knock-out mutant *Saccharomyces cerevisiae*: Physiological investigation and transcriptome analysis.** *Biotechnology Journal*. 2010. 5 (10):1016–1027.

VII. Hou J, Scalcinati G, Oldiges M, Vemuri GN. **Metabolic Impact of Increased NADH Availability in *Saccharomyces cerevisiae*.** *Applied Environmental Microbiology*. 2009. 76 (3): 851–859.

AUTHOR'S[1] PAPER CONTRIBUTION

A summary of the author's contribution to the publications on which this thesis is based is provided below:

Paper I

JN, VS and GS designed the study. JN and VS supervised the project. CK and GS performed the experimental work. SP and JM assisted the molecular biology experiments. YC assisted the strain physiology experiments. MD and LD assisted the GC/MS analysis of sesquiterpenes. GS analyzed the data and wrote the manuscript. All the authors discussed the results, edited and approved the final manuscript.

Paper II

JN and GS designed the study. JN and VS supervised the project. GS performed the experimental work. SP assisted the molecular biology experiments. MS and LD assisted the GC/MS analysis of sesquiterpenes. GS analyzed the data and wrote the manuscript. All the authors discussed the results, edited and approved the final manuscript.

Paper III

JN and GS designed the study. GS performed the experimental work. GS analyzed the data and wrote the manuscript. JN and GS discussed the results, edited and approved the final manuscript.

Paper IV

JMO, GS, JVV, JN, LO participated in the design of the study. JMO and GS performed the experimental work. JMO and GS wrote the manuscript. JVV, TJ, LO, and JN edited the manuscript. All the authors have read and approved the final manuscript.

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Abbreviations & Nomenclature

asRNA: antisense RNA

B. subtilis: Bacillus subtilis

C. lansium: Clausena lansium

C. glutamicum: Corynebacterium glutamicum

CTR: Carbon transfer rate mmol l^{-1}

D: Dilution rate h^{-1}

$D_{crit:}$: Critical Dilution rate h^{-1}

DNA: Deoxyribonucleic acid

DO: Dissolved oxygen

DXP: 1-deoxyxylulose-5-phosphate

E. coli: Escherichia coli

ER: Endoplasmic reticulum

ERG9: Squalene synthase gene

FPPS: Farnesyl diphosphate synthase

FPP: (*E,E*)-Farnesyl diphosphate

FOH: (*E,E*)-Farnesol

FAO: Food and Agriculture Organization of the United Nations

gDCW: Grams dry cell weight of biomass

GO: Gene ontology

HMG1: HMG-CoA reductase gene

HMGR: 3-hydroxy-3-metyl-glutaryl-coenzyme A reductase

LogP: Logarithm (base 10) of partition coefficient

Mb: Mega base; a million of bases

miRNAs: micro RNAs

MVA: Mevalonate

NAD^+: Nicotinamide adenine dinucleotide

NADH: Nicotinamide adenine dinucleotide hydrogen

$NADP^+$: Nicotinamide adenine dinucleotide phosphate

NADPH: Nicotinamide adenine dinucleotide phosphate hydrogen

NPP: Nerolidyl diphosphate

OPP^-: Diphosphate anion

OTR: Oxygen transfer rate mmol l^{-1}

V_{max}: Maximum reaction rate

K_m: Michaelis constant

P_{ERG9}: Squalene synthase native promoter

PPP: Pentose phosphate pathway

PUFAs: Polyunsaturated fatty acids

P. stipitis: Pichia stipitis

rasiRNAs: Repeat associated small interfering RNAs

RQ: Respiratory quotient

Rs: Indian rupee

S. cerevisiae: Saccharomyces cerevisiae

SF: Shake flask

SanSyn: Santalene synthase gene

$SanSyn_{Opt}$: Santalene synthase-codon optimized gene

siRNAs: small interfering RNAs

SQS: Squalene synthase

SNS: Santalene synthase

SSD: Sterol sensing domain

$: United States Dollars

TFs: Transcription factors

tHMG1: Truncated version of HMG-CoA reductase gene

tHmg1: Truncated version of HMG-CoA reductase

μ_{max}: Maximum specific growth rate

CHAPTER 1 Introduction

1.1 Toward a bio-based economy- the rapidly evolving field of Industrial biotechnology

Biotechnology is reshaping industrial production, and the past 20 years have witnessed an exponential increase of bio-based products and bio-energy in the global economy (Enriquez, 2009). The chemical industry is actively searching for alternative routes to petroleum-based processes influenced by environmental sustainability trends and the need to freeing the dependency from non-renewable resources. The concept *"bio-product"* has been known since the origin of the fermentative solution for production of bread, beer, wine or cheese (Russo *et al.,* 1995). The movement toward a more green society has driven unprecedented research focus on the *"bio-route"* in order to diversifying away from petrochemical feedstock and in an effort toward a more sustainable development (Otero *et al*., 2007, Stephanopoulos, 2010). *Industrial biotechnology*[2] rapidly penetrates in the chemical manufacturing world as concrete sustainable, renewable and ecologically friendly alternative, allowing developing new biological products exploiting biological systems, using fermentation technology processes to convert agricultural basic raw material (e.g. corn syrup) into a wide range of products. The technologies involved in the industrial biotechnology process are nowadays self evident and sufficiently mature to reach the final stage of full commercialization. Already in 2005, 7% of chemical sales depended on biotech, with $77 billion in revenue within the chemical sector (source: McKinsey, SRI) making industrial biotechnology a realty.

Efficient development in cell factory design is a crucial aspect in the success of industrial biotechnology. Over the years, tremendous progress has been made to turning biological systems into *"biorefineries*[3]*"* capable of converting inexpensive raw material into valuable chemicals. Microbial cellular metabolism has synthetic potential and chemical features that rarely can be achieved by a chemist under the same physical conditions (e.g. temperature and pressure). Therefore the field has largely focused on the creation of efficient microbial, self regenerating, factories to produce chemicals, fuels and material.

Current industrial biotechnology major market segments are represented by specialty chemicals (31%) base chemicals (25.3%) consumer chemicals (22.5%) and active pharma ingredients (21.2%) (Festel, 2010). McKinsey & Company forecasted that the global biotech industry

[2] ***Industrial Biotechnology:*** *The application of biotechnology for the processing and production of chemicals, material and energy (Otero et al., 2007).*

[3] ***Biorefinery:*** *Conversion of renewable resources into bio-products (chemicals and materials) and/or energy, via biocatalysis using microbial fermentation or enzyme catalysis. (Bohlmann 2005; Kamm et al., 2004).*

revenue has the potential to generate upwards of $300 billion by the year 2020 (McKinsey SRI). The market driving forces for the biorefineries establishment are attributed mainly to biofuel (ethanol and biodiesel), however, the projected growth showed how the greatest impact will be in fine chemicals production (The economist, 2010; Dornburg *et al.,* 2008). In the following, the use of industrial biotechnology for production of isoprenoids compounds a widespread group of molecules with a variety of potential applications heavily targeted for biorefinery is examined.

1.2 Isoprenoids origins and definitions

Isoprenoids (often called *terpenoids*) are a ubiquitous class of natural compounds (over 40,000 different compounds) with many potential commercial applications that have not been fully explored, e.g. fragrances (linalool, geraniol, menthol etc.), cosmetics (squalane), disinfectants (camphor, α-pinene), flavoring agents, food colorants (zeaxanthines, astaxanthine), food supplements (vitamins A, E, K), functional foods (α-humulene), bio-pesticides, nutraceutical and pharmaceutical agents (taxol, artemisinin). They represent a very diverse class of secondary metabolites and they satisfy distinct biological functions like pheromones, defensive agents, photosynthetic pigments, attractants, repellents, toxins, antibiotics, anti-feedants, electron transporting chain quinones, structural membrane components (McGravey *et al.,* 1995). They have many different physico-chemical proprieties, lipophilic or hydrophilic, volatile or non-volatile, cyclic or acyclic, chiral or achiral, reflected in their complexity, due to the multitude of biological activities they fulfill (Bohmann *et al.,* 2008). They are naturally produced in sub-sequential head-tail heteropolymeryzation condensation of isoprene functional units, isopentenyl diphosphate IPP, in all organisms and classified based on the content of isoprene units as: hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}). The isoprene universal building block IPP is naturally synthesized via two independent pathways: the mevalonate (MVA) pathway and the 1-deoxyxylulose-5-phosphate (DXP) pathway (Kuzuyama *et al.,* 2003). These two biosynthetic pathways are taxonomically distributed, the MVA pathway is found in *Eukarya*, *Archaea* (a modified version) and a few bacteria whereas the DXP pathway in *Bacteria* and photosynthetic *Eukarya*. Some bacteria and plants have been shown to have both pathways, and the existence of an alternative MVA pathway was recently discovered (Lombard *et al.,* 2010) (Fig. 1.1). The MVA pathway starts with the condensation of three units of acetyl-CoA into the intermediate mevalonate that successively undergoes phosphorylation and decarboxylation resulting in formation of IPP. The DXP pathway starts with the production of DXP from pyruvate and glyceraldehyde-3P that is then rearranged into MEP that reacts with cytidine 5'-triphosphate. The resulting reaction product is phosphorylated, cyclized and in the final two steps IPP and DMAPP are formed (see

Fig. 1.1 for details). The two pathways are compartmentalized differently depending on the organism and may occur in the cytosol, peroxisome, outer phase of the endoplasmic reticulum and plastid (Lange *et al.,* 2000).

From the current state of the art, several isoprenoid products are successfully produced or road-ready and expected to be produced in the near future by a biotech process, and a small-subset of relevant examples is provided in Table 1.1.

Table 1.1. Examples of key production platforms of isopenoid bio-products

Product	Formula	Company	Application	Source
Isoprene	C_5H_8	*Genencore*	*Rubber*	*Withed et al., 2010*
Artemisinic acid	$C_{15}H_{22}O_5$	*Amyris/Sanofi-Aventis*	*Antimalarial drugs precursor*	*GEN News, 2008*
Farnesene (Biofene^{TM})	$C_{15}H_{24}$	*Amyris/Tate &Lyle*	*Biodiesel*	*GEN News, 2010*
Squalane	$C_{30}H_{62}$	*Soliance/Amyris*	*Cosmetic*	*Katie, 2010*

In this study, particular focus was dedicated to the sesquiterpenes, a class of compounds originated from the common precursor farnesyl diphosphate FPP derived from the assembly of three IPP units (Maury *et al.* 2005).Sesquiterpenes are one of the largest isoprenoids groups (over 7000 different compounds) (Misawa, 2011). C_{15}-branched sesquiterpenes are receiving increasing attention as they may not only serve as precursor chemicals for production of perfumes and pharmaceuticals but also as precursors for a new generation of biofuels that can be used as diesel and jet fuels (Peralta-Yahya *et al.,* 2011; Zhang *et al.,* 2011; Rude *et al.,* 2009; Lee *et al.,* 2008). The portfolio of fuel candidate compounds in fact has been greatly expanded lately, with special attention dedicated to the *drop-in biofuel "class of bio-fuel that can easily replace gasoline or diesel in existing engines"* (Craig *et al.* 2012), highlighting branched and cyclic sesquiterpenes as potential jet fuel precursors based on their physicochemical proprieties (Peralta-Yahya *et al.,* 2011; Renninger *et al.,* 2008).

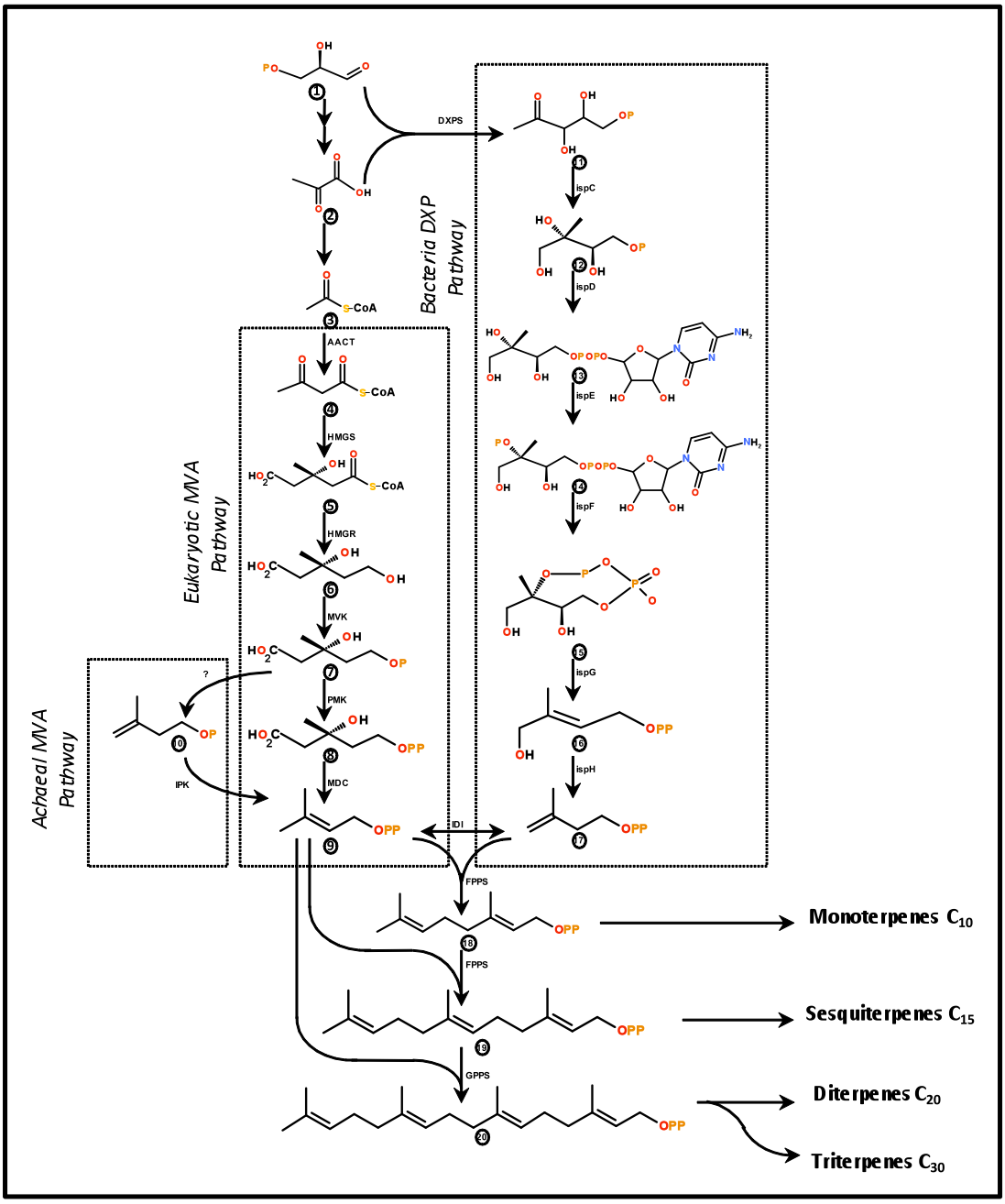


Figure 1.1. Eukaryal mevalonate (MVA) pathway, modified archeal mevalonate (MVA) pathway and bacterial methylerythritol phosphate (MEP) pathway. (1) glyceraldehyde-3-phosphate, (2) pyruvate, (3) acetyl-CoA, (4) acetoacetyl-CoA, (5) 3-hydroxy-3-methylglutaryl-CoA, (6) mevalonate, (7) mevalonate-5-phosphate, (8) mevalonate-5-diphosphate, (9) isopentenyl pyrophosphate, (10) isopentenyl phosphate, (11) 1-deoxyxylulose-5-phosphate, (12) 2-C-metyl-D-erythritol-4-phosphate, (13) 4-diphosphocytidyl-2-C-methyl-D-erythritol, (14) 4-diphosphocytidyl-2-C-methyl-D-erythritol-2-phosphate, (15) 2-C-methylerythritol-2,4-cyclopyrophosphate, (16) 1-hydroxy-2-methyl-2-(E)-butenyl-4-pyrophosphate, (17) dimethyallyl diphosphate, (18) geranyl diphosphate, (19) farnesy diphosphate, (20) greanylgeranyl diphosphate. (ACCT) Acetyl-CoA thiolase, (HMGS) HMG-CoA synthase, (HMGR) HMG-CoA reductase, (MVK) mevalonate kinase, (PMK) phosphomevalonate kinase, (?) phosphomevalonate decarboxylase (*not identified yet*), (IPK) isopentenyl phosphate kinase (MDC) mevalonate pyrophosphate decarboxylase, (IDI) isopentenylpyrophosphate isomerase, (FPPS) farnesyl diphosphate synthase, (GPPS) geranylgeranyl diphosphate synthase, (DXS) DXP synthase, (IspC) DXP reductoisomerase, (IspD) 2-C-metyl-D-erythritol-4-phosphate cytidyltransferase, (IspE) 4-diphosphocytidyl-2-C-methyl-D-erythritol-2-phosphate kinase, (IspF) 2-C-methylerythritol-2,4-cyclopyrophosphate, synthase, (IspG) 1-hydroxy-2-methyl-2-(E)-butenyl-4-pyrophosphate synthase, (IspH) 4-hydroxy-3-methylbut-2-enyl diphosphate reductase.

1.3 Market drivers toward microbial production of sesquiterpenes

As introduced in the previous chapter, the demand for microbial production of chemicals as an alternative to petrochemical based synthesis is increasing due to economical, environmental and geopolitical factors (Dellomonaco *et al.,* 2010; Stephanopoulos *et al.,* 2007). Microbial productions are gaining popularity especially for biosynthesis of added value compounds (Hong *et al.,* 2012; Kim *et al.,* 2012) due mainly to the small margin achievable from commodity production. Isoprenoids and isoprene derivative represent nowadays a $650 million global market (Sims, 2012). Recently, their role as biomaterial resource has been rediscovered leading to renewed interest in this class of molecules (Bohmann *et al.,* 2008). The complexity of isoprenoid is often the main drawback for the industrial scale production. Nowadays, most of the isoprene derived compounds are produced via plant extraction and by total or semi-synthesis (Teisserire 1994). Extraction from natural resources is limited by raw material accessibility, low yields, high process costs and often lead to a complex mixture of products (Koepp *et al.,* 1995); complete chemical synthesis generally involve multistep transformation resulting in an inefficient, expensive process and may not result in enantiomeric pure products (Miyaoka *et al.,* 2002, Mukaiyama *et al.,* 1999, Danishefsky *et al.,* 1996,). The production of isoprenoids by microbial fermentation is an environmentally friendly and attractive alternative to the traditional methods and offers several advantages, among them it (i) avoids formation of racemic mixtures providing pure isomer products through enzymatic biocatalysis; (ii) reduces process cost using inexpensive sugar based carbon sources, (iii) increases sustainability avoiding harvesting and extraction from natural sources and thus reducing environmental footprint, lowering CO_2 emissions and toxic waste e.g. solvents and metal catalysts (iv) increases yield and productivities using genetic manipulation of the heterologous host and (v) is compatible with scalable high density fermentation processes. This has caused interest in engineering cell factories that can be used to produce isoprenoids in a cost competitive fashion (Khalil *et al.,* 2010; Koffas et al., 2009; Fortman *et al.,* 2008).

1.4 The new era of systems level metabolic engineering-from local to global

Metabolic engineering[2] is a constantly evolving field and has driven for years the construction of recombinant microorganism for the production of target compounds. Metabolic engineers have relied for long time on traditional and intuitive approaches to bioengineer microbial cells to produce desired chemicals. However, through the years it appears clear that the hierarchical complexity of cell regulation requires a systems level approach moving from local to global applications. The need of and holistic access to the cellular network leads to the synergistic application of related emerging disciplines: *systems biology*, *synthetic biology* and *evolutionary*

engineering (Box 1.1) opening new opportunity for cellular engineering and creating the intertwining that produced the modern multi-disciplinary field of metabolic engineering (Nielsen *et al.,* 2012, Lee *et al.,* 2011[a]). The integration and impact of these different disciplines for metabolic engineering is briefly introduced in the following, with the techniques mostly applied through this research study being addressed.

Box. 1.1.

[1]Evolutionary engineering:
The application of a selection procedure to obtain a desired phenotype [¥].

[2]Metabolic engineering:
Use of genetic engineering modifications to manipulate cell factories with the objective to improve their proprieties for industrial application [‡].

[3]Synthetic Biology:
Design and construction of new biological components, functions, and genetic circuits de novo or redesign existing biological systems [†].

[4]Systems Biology:
To obtain new insight into the molecular mechanism occurring in living cells for predicting the function of biological systems through the combination of mathematical modeling and experimental biology [#].

Sources: [‡]Bailey et al., 1991 & Stephanopoulos et al., 1991; [#]Nielsen et al., 2001; [†]Keasling et al., 2008; [¥]Sauer et al., 2001

1.4.1 Evolutionary engineering[1]

Evolutionary approaches have been widely used to improve the properties of industrial cell factories: the creation of novel metabolic functions, expanding substrate utilization range, improve the growth rate, improve tolerance towards multiple compounds, improve biocatalysis and many other favorable phenotypes (Cakar *et al.,* 2010). Directed evolutionary methods refer to selection procedures based on the use of specific environmental pressures through iterative genetic diversification with the final goal of strain improvement (Chatterjee *et al.,* 2006). These methods exploit natural selective pressure rationally applied and offer a non-invasive alternative to the classical mutagenesis technique. Among the existing multitude of adaptive evolutionary approaches the most popular are (i) extended chemostat cultivation (Jensen *et al.,* 2005; Sauer *et al.,* 2001;) and (ii) repetitive batch cultivation (Barrick *et al.,* 2009; Kuyper *et al.,* 2005), performed under selective conditions. Evolutionary engineering has been frequently combined with metabolic engineering from the early days of industrial biotechnology as simple methods to overcome cellular complexity because of the capacity to address multi-gene traits (e.g. resistance to toxic compounds) that can be difficult to solve with rational approaches. The common limitation of this approach is the dependency on the screening method and the random outcome and the inability to elucidate the mechanisms that confer the adaptive fitness. However, recent advances in high-throughput techniques and DNA sequencing efforts have facilitated the identification of genetic modifications driving identified phenotypes and hereby

greatly enhanced the application of this technique In this study, evolutionary engineering was applied to expand the spectrum of usable carbon sources of the selected cell factory in order to open the possibility to efficiently use alternative feedstocks like lignocellulose as raw material (Ritter, 2008). Due to its global abundance and renewability lignocellulose is an attractive starting material for bio-production of value added products (*Chapter 3.5*).

1.4.2 Synthetic Biology

Synthetic biology[2] can be envisaged as the extension of engineering principles to genetic engineering by biologists involving the design/redesign of devices and circuits for controlling biological systems (Endy, 2005). The impact of synthetic biology on metabolic engineering is rapidly reshaping the industrial biotechnology field (Keasling, 2012). The dramatic decrease in the cost of whole genome sequencing and long-chain DNA synthesis has led to the development of modern synthetic biology tools and methodology bringing new prospects and un-restricted access to microbial pathway engineering (Smolke *et al.,* 2012, May, 2009). Synthetic biology has influenced the bioresearch field by making cell factory development faster and more efficient allowing wider exploration of the biosynthetic potential of microbial production and advancing our metabolic engineering capabilities (Keasling, 2010). The diverse set of tools emerged for pathway engineering increase the capability to achieve specific cellular functions (Canton *et al.,* 2008). It is generally accepted that pathway engineering requires a balanced expression of single and multiple genes avoiding wasteful and potentially toxic intermediate accumulation and preventing “robbing” of the cell of key precursors. Additionally, traditional overexpression technique may result in high protein levels resulting in unwanted metabolic burden. Therefore, an optimization strategy should be carefully designed, and synthetic biology can be used to introduce synthetic sensors like dynamic control element able to sense cellular metabolic state and regulate the expression of specific functions (Farmer *et al.,* 2000, Zhang *et al*., 2011) and hereby shed light on the importance of the dynamic aspect of pathway engineering (Holtz *et al.,* 2010).

In this study, a synthetic biology concept was applied combining a static engineering module with dynamic control for pathway engineering. Remodeling of the cellular network was conducted using an environment–responsive promoter to dynamically control the gene expression of a regulatory branch point in response to an extracellular signal molecule concentration and modulating the flux between the target pathway and three branches (see *Chapter 3.2.3*). An attempt to create a dynamic driving force along the engineered pathway was performed modifying cellular cofactor availability (see *Chapter 3.5*). In this work, a synthetic

pathway for expanding substrate range capability was also re-constructed in the production host (see *Chapter 3.6*).

1.4.3 Systems Biology

Systems biology[3] aims to get insight into the complexity of cellular functions offering the opportunity to understand and optimize cellular processes through the combined use of high-throughput experimental methods (top-down approach) and computational models (bottom-up approach). The ability to obtain a quantitative analysis of the whole cellular system is strategically useful during the design of a novel cell factory (Nielsen *et al.,* 2007). Advances in high-throughput technique allow rapid cellular phenotype characterization affecting the ability to engineer cell metabolism. The systems biology toolkits (*x-omics*) routinely applied for this purpose include: genomics, transcriptomics, metabolomics, fluxomics (Petranovic *et al.*, 2009). On the other hand, the availability of detailed mathematical models expands analytical access to strain engineering; the predictive capacity of *in silico* analysis of metabolic flux distribution is crucial in guiding the strain improvement identifying potential targets for modification required to achieve desired performances (Patil *et al.,* 2004 Stephanopoulos *et al.,* 1999). Moreover, the capability of exploring multiple possible flux distribution scenarios using computational analysis saves time and costs required for *in vivo* experimentation, selecting the best set of modifications out of large number of potential combinatorial changes and further delineating strain construction strategies (Burgard *et al.,* 2003; Patil *et al.,* 2005). Sophistication in bioinformatics for system level data handling greatly contribute to the integration of the different "*x-omics"* dataset enhancing the application of this techniques and changing the way in which metabolic engineering is executed.

In this study, systems biology was applied at two levels: (i) Transcriptome analysis, one of the most developed and implemented "*x-omics"* tools for metabolic engineering (Jewett *et al*., 2005), was employed to further elucidate metabolism and physiology of the mutant obtained through evolutionary techniques (see *Chapter 3.5*); (ii) A non-intuitive systematic strategy obtained from previously performed *in silico* analysis using a genome scale metabolic model (Asadollahi *et al.,* 2009) was applied to manipulate the cellular cofactor balance of the constructed cell factory in an attempt to empower flux toward the target product (see *Chapter 3.3*).

Although the above mentioned disciplines are quite different the high level of interconnection allows their simultaneous application for bioengineering purposes. In the past decade, multidisciplinary system level metabolic engineering approaches have started to have a strong

impact in the biological production of sesquiterpene derived compounds and the number of reports of engineered microorganisms producing sesquiterpene compounds has risen dramatically making the microbial production of these series of compounds an industrial reality (Fig 1.2).

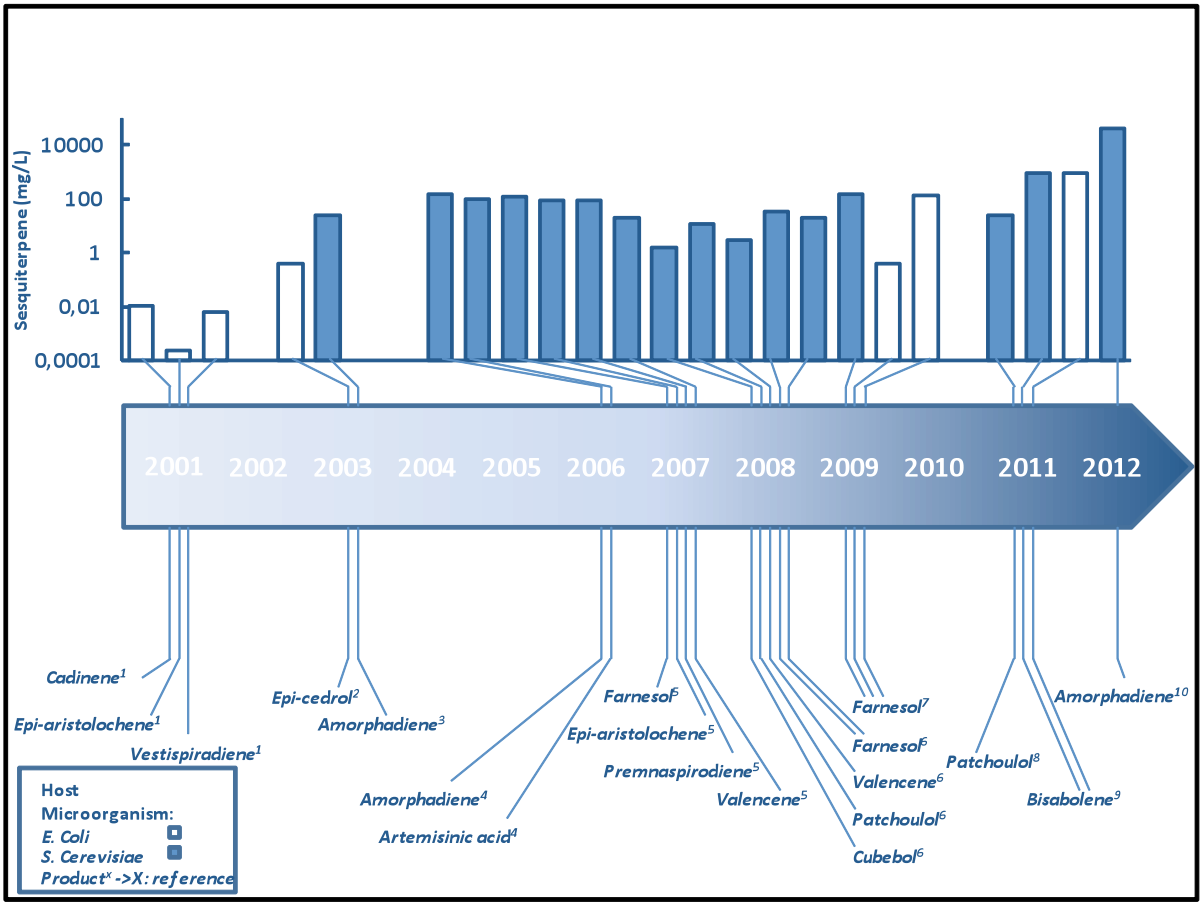


Figure 1.2. Microbial production timeline for some relevant plant sesquiterpene products. Synthetic biology advanced the classic metabolic engineering approach leading to dramatic improvement in final titers achievable. The list of examples provided is by no means exhaustive and it is intended to provide an overview of the context referred. Reference data, Martin *et al.,* 2001; Jackson *et al.,* 2003; Martin *et al.,* 2003; Ro *et al.,* 2006; Takahashi *et al.,* 2007; Asadollahi *et al.,* 2008; Wang *et al.,* 2011a; Albertsen *et al.,* 2011; Peralta-Yahya *et al.,* 2011; Westfall *et al.,* 2012.

Today, the creation of "*superbugs*" requires a dynamic interaction and application of all these disciplines (Nielsen *et al.,* 2011). Among several successful examples of how this combined approach has impacted industrial biotechnology the yeast-based production of the anti-malaria drug precursors amorpha-4,11-diene and artemisinic acid represent a remarkable achievement (Westfall *et al.,* 2011) (Fig 1.2). Another salient example is the bacterial production of taxol precursors taxadiene and taxadien-5α-ol (Ajikumar *et al.,* 2010).

CHAPTER 2 Development of a "*Microrefinery*[4]"

2.1 Industrial Biotechnology process overview

Development of a biotechnological process involves different phases (i) target product identification (ii) selection of a suitable production host (iii) production strategy design and (iv) production process design, including the cost and accessibility of the raw material (e.g. the carbon source) (Fig. 2.2). During the early design stage it is important to take into consideration the entire process and integrate together the different steps avoiding pitfalls moving from one stage to another. Typically, process optimization proceeds via several rounds of cyclic optimization. The result of the metabolic engineering efforts are evaluated by available screening techniques, bottlenecks are being identified and another round of optimization takes place.

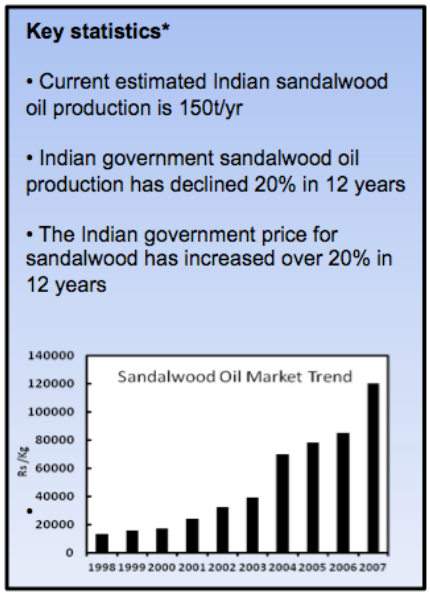


Figure 2.1 Key statistics on the natural source of the target compound of this study α-santalene. *Adapted from Essential Oils the new crop industries handbook RIRDC 2004 (Rs=17$)

2.2. Target product of this study, sesquiterpene hydrocarbon α-santalene ($C_{15}H_{24}$)

Natural products are the most valuable fragrances, but limited access to many of these compounds has led the perfume industry to look for artificial substitutes (Chapuis *et al.,* 2004). The woody fragrance sandalwood for examples is one of the most expensive perfumery raw materials and its components are extremely difficult to synthesize (Davies 2009). α-Santalene (CAS Number: 512-61-8; IUPAC Name: [(-)-1,7-dimethyl-7-(4-methyl-3-pentenyl)-tricyclo (2.2.1.0 (2,6)) heptane]) (Table 2.2) is the precursor of the hydroxylated α-santalol one of the main components of the East Indian saldalwood oil (Corey, 1957; Baldovini, 2010). The extracted essential oil is among the most precious and highly prized world's fragrances. α-Santalol together with ß-santalol are

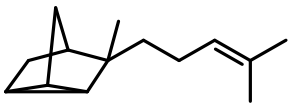
α-(+)-santalene	
	
Molecular Formula	$C_{15}H_{24}$
Molar Mass(g mol^{-1})	*204.35*
Density (Kg l^{-1})	*0.944*
Molar energy density (kJ mol^{-1})	*9148*
Mass energy density (MJ Kg^{-1})	*44.8*
Boiling point (°C at 760 mmHg)	*247.6*

Table 2.2 Chemical structure and properties of the target compound of this study, α-santalene.

[4] ***Microrefinery:*** *Microbial system conversion of renewable resources into bio-products chemicals and fuel (LS9, INC).*

the main olfactory components of the sandalwood oil that can contain up to 90% of this sesquiterpene alcohol (60-50%-α, 30-20%-ß) and confer the sweet-woody, warm, animal and milky-nutty scent employed for centuries in religious and cultural contexts (Howes *et al.,* 2004; Schalk, 2011; Brunke *et al.,* 1995). Sandalwood essential oil is mainly extracted from tree and roots of the two plant species Indian sandalwood (*Santalum album*) and Australian sandalwood (*Santalum spicatum*). In the past decade, the sandalwood oil price has skyrocketed due to intensive harvesting that rendered the Indian tree an endangered species and governmentally protected (FAO 1995) and the constant increase in demand (Fig 2.1). India is the major supplier of sandalwood oil, but the international scenario is quickly changing (Misra 2009). Nowadays, the market price is estimated to lie between $1.200-2.700/ kg depending on the quality (http://www.alibaba.com/). However, because the content of α/ß santal-ol/ene determines the oil market price (Nautiyal 2011), the 100% pure santalene α-(+) isomer price could be up to 10 fold higher. Besides its commercial use in cosmetic, perfumery and aromatherapy industries sandalwood oil finds application as chemotherapeutic and chemopreventing agent against skin cancer (Dwivedi *et al.,* 2003) and for its antimicrobial (Jirovetz *et al.,* 2006) and antiviral proprieties (Benecia *et al.,* 1999).

2.3 Selection of production host: *yeast as suitable platform for sesquiterpene production*

The choice of microbial host is dictated by many factors and often requires a trade-off; here are discussed some of the aspects that need to be considered in order to fulfill the industrial demands. Among desirable features of the selected microorganism are (i) the metabolic capability toward the desired product; (ii) high substrate utilization rate and ability to grow fast on minimally supplemented media and synthesize all the required macromolecules for growth from inexpensive C source and N, P, S salts avoiding the supplement of complex nutrients; (iii) tolerance to inhibitory compounds potentially present in the industrial fermentation media (e.g. hydrolyzate tolerance) or intermediate metabolites and side products produced along the process; (iv) robustness toward the target compound itself; if the selected organism can tolerate a certain concentration of final product this limit cannot be exceeded without resulting in toxic effects; (v) resistance to adverse environmental conditions; ideally the suitable host should tolerate elevated temperatures (thermo-tolerance), low pH (acid-tolerance) and high osmotic pressure (osmo-tolerance) reducing cooling costs, probability of contamination and osmotic pressure derived from elevated concentrations of nutrients or products; (vi) capacity to efficiently perform regardless of environmental changes during the production process; (vii) genetic tractability, considering capacity to integrate and efficiently express heterologous DNA and high transformation efficiency; (viii) genetic stability during extended cultivation periods; (iv) the

availability of metabolic engineering tools and (x) genome wide characterization including access to the "*x-omics*" analysis tools.

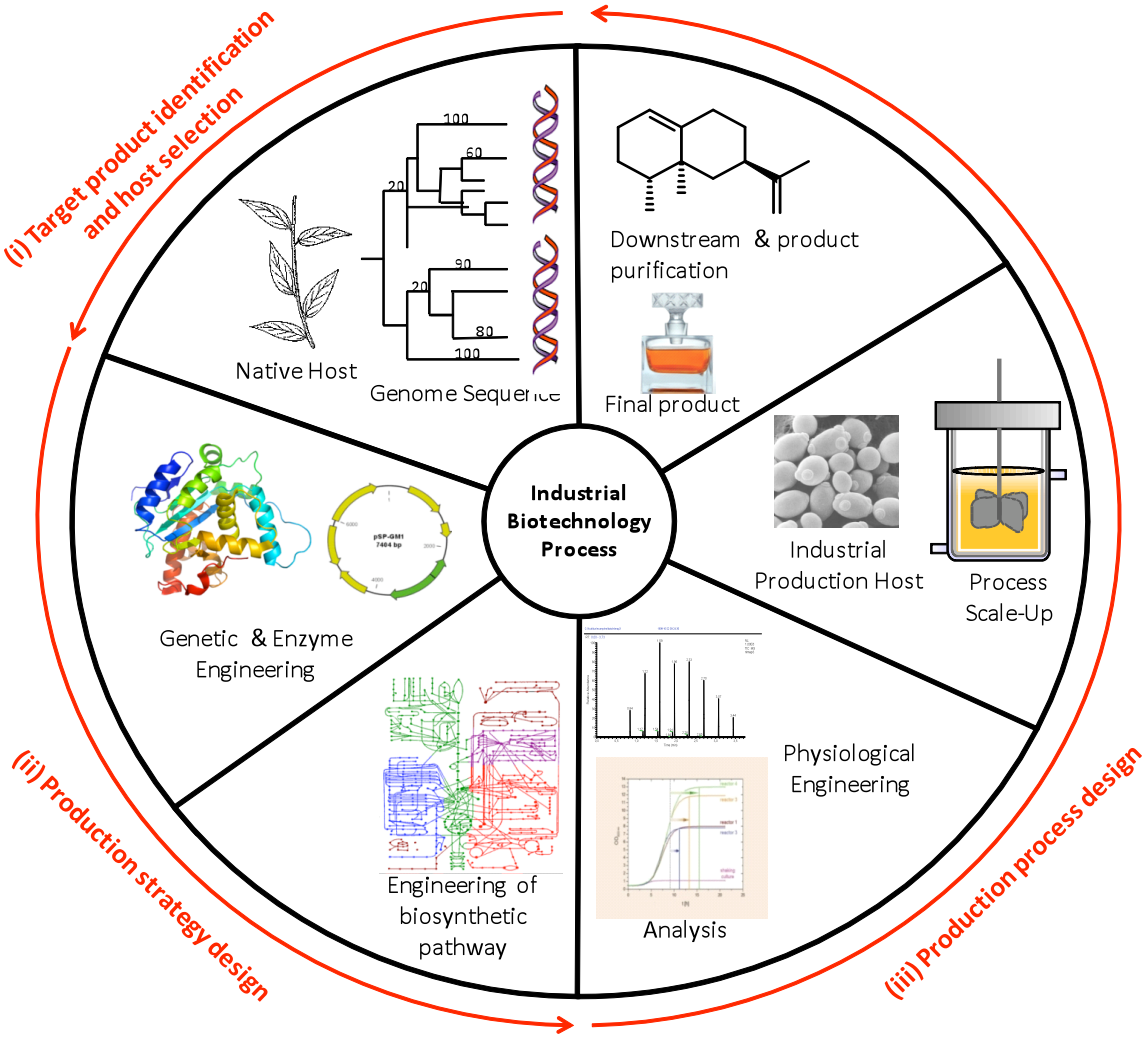


Figure 2.2. Industrial biotechnology process overview. The first step consists in the identification of the compound to be produced and the selection of a suitable production host. Second, a production strategy design including genetic, enzyme and biosynthetic pathway engineering is developed. Third, fermentation and downstream process are performed to produce the final target. Process efficiency is obtained through several cycles of optimization of the different steps proposed.

Considering the number of variables involved host choice is clearly not one solution problem. Often the decision lies between engineering recombinant microorganisms or exploring the potential of native producer microorganism (Alper *et al.,* 2009). Depending on the target compound non-recombinant microorganisms may have high process capability and a high level of toxicity resistance but the lack of tools and detailed physiology knowledge could require costly and time demanding research efforts in order to establish an efficient process. Traditionally applied *"model organisms"* (e.g. *E. coli, S. cerevisiae, A. niger, B. subtilis, C.*

glutamicum) are on the other hand well characterized and easy to manipulate but they might lack the required industrial robustness. The sophistication of systems and synthetic biology tools have largely improved the capacity to manipulate model microorganisms and accelerate the process to achieve efficient *"microrefineries"* expanding their potential of model organism and making them more attractive platforms (Enyeart *et al.,* 2011) (see *Chapter 1.4.2 & Chapter 1.4.3* for details). In this study the *S. cerevisiae* laboratory strain CEN.PK113-7D, which is widely applied for industrial biotechnology applications (van Dijken *et al.,* 2000), was selected as starting point for the development of sesquiterpene bio-production. For *S. cerevisiae*, there are well-characterized genetic manipulation protocols, detailed physiology records, advanced metabolic engineering tool set to perform precise gene expression, it has been extensively characterized with high-throughput approaches (genomics, transcriptomics, proteomics, metabolomics, fluxomics); computational methods (e.g. genome scale models) are available for guiding *in silico* experimental design and data analysis. It has a generally regarded as safe (GRAS) status and has been widely applied in successful industrial processes. *S. cerevisiae* was identified as best ergosterol producer among over 69 yeast species (Dulaney *et al.,* 1954), and the CEN.PK background strains displays a high ergosterol content during growth on glucose (Daum *et al.,* 1999). Ergosterol is produced in yeast through the sterol pathway from the final product of the MVA pathway, FPP, from which sesquiterpenes are also derived (see *Chapter 1.2* & Fig 1.1). Recently, the whole genome sequence of CEN.PK113-7D was completed and SNP analysis revealed that the strain specific high sterol biosynthetic capacity may be due to genetic variation of several genes in the MVA pathway (*ERG8*, *ERG9*, *ERG12*, *HMG1* and *ERG20*) compared to the reference strain with lower ergosterol content (Otero *et al*., 2010). Additionally, greater variability was found in the promoter region of the same genes (http://www.sysbio.se/cenpk). The combination of all these characteristics can be capitalized upon to enable the industrial application of *S. cerevisiae* CEN.PK113-7D as production host and favored the choice as production host in this study.

2.4 *Production strategy design-Pathway engineering*

Once product and host have been selected a production strategy needs to be designed. Typically, production strategy optimization is an iterative process where the simultaneous regulation and timing of the expression of multiple heterologous and native genes is required for the redirection of the metabolic flux towards the target compound. Common pathway engineering operations include (i) re-engineering of existing pathways, (ii) combination of existing pathways with exogenous or synthetic novel functions, (iii) *de novo* assembly of new pathways.

An important aspect concerns the optimization of endogenous pathways compared to the import of heterologous functions (Alper *et al.,* 2009). Target compounds of this study, sesquiterpene derivatives are naturally produced by *S. cerevisiae* network thus, through engineering strategy was focused on optimize the yeast native MVA pathway. Different approaches were performed aimed to maximize the flux through the MVA pathway, increase the flux to the MVA pathway and redirect the flux at the branch point of the MVA pathway. On the other hand, a *"bioprospecting*[5]" approach was used to import a novel pathway to expand the substrate capability of the designed cell factory.

Cellular networks have evolved the ability to rapidly sense and respond to environmental changes. When perturbations are introduced in an attempt to increase flux toward a desired path there is a risk to produce unexpected and unwanted responses as a result of flux imbalance that could result in host instability. Metabolic engineering side effects that limit the final yield can be ascribed to (i) poor understanding of the complex cellular regulation; (ii) unbalanced consumption of cellular resources (e.g. cofactors imbalance, precursors pools) (iii) metabolic burden of heterologous protein; (iv) accumulation of toxic intermediate, (v) toxicity or inhibitory effect of the final product, metabolites and heterologous enzymes, (vi) negative feedback loop; (v) poor expression of desired new component.
Therefore, a number of tools have been developed to control and coordinate fluxes through different branches in the cellular network such that there is maintained a balance between the resources required for cell growth and the precursors for target compounds. Engineering of biological systems can be realized at multiple levels: gene number, transcription, post-transcription, post-translation (Young *et al.,* 2010; Boyle *et al.,* 2009; Nevoigt *et al.,* 2008).

2.4.1 Engineering DNA and gene copy number

The DNA-level manipulation toolset for pathway engineering comprises plasmids vectors, and chromosomal integrations methods (Siddiqui *et al.,* 2012; Siewers *et al.,* 2010). Plasmid vectors are the most common and widely applied gene expression tools for metabolic engineering. Recently, commercial cloning vectors available for yeast use have been reviewed in detail (Da Silva *et al.,* 2012). Among the desirable features required for an expression vector are segregation stability and the stability in the host for many generations under low selective pressure (Keasling *et al.,* 1999). Through this study, three classes of plasmids have been employed based on the YEp, YCp and YIp vector series according to their destination of use.

[5] ***Bioprospecting:*** *"Searching and borrowing useful genes from other organisms to confer a specifically desired phenotype" (Alper et al., 2009)*

The YEp vectors, based on the 2□ sequence are maintained at high copy number (< 7) in the cell (Chen *et al.,* 2012) and were applied to achieve high level of expression of the gene encoding the enzyme catalyzing the final reaction toward the target product to ensure that this step would not limit the entire process. (see *Chapter 3.1.1*). Differently, YCp vectors, based on the *CEN/ARS* autonomous replication and centromeric sequence are maintained at low copy number (1-2) in the cell (Fang *et al.,* 2011). Due to the great level of segregation stability provided and low metabolic burden they were employed for the reconstruction of synthetic pathways (see *Chapter 3.5*). YIp integrative vectors on the other hand, do not replicate autonomously and represent a versatile tool for rapid chromosomal integration; here they were used to perform the promoter replacement studies (See *Chapter 3.2.3*).
Alternatively, classic PCR fragment-based genomic integration was applied in chromosome engineering for gene deletion and stable gene expression during pathway optimization. For gene overexpression applications, chromosomal integration offer the most stable solution. The integration locus may however affect the expression level. In this study, previously characterized integration sites were used in order to ensure the desired level of expression (Flagfeldt *et al.,* 2009). Multiple rounds of targeted sequential integration strategies based on recyclable selectable markers for selection were employed for deletion/overexpression procedures (see *Chapter 3.3*).

In an ideal context the platform strain would provide high genetic stability and ensure the flexibility to allow the production of a range of different sequiterpene derivative compounds. In order to combine these features in this study the functions required to redirect the carbon flux toward the target pathway were integrated into the yeast genome, whereas the steps for the final conversions were expressed on plasmids using the techniques described above.

2.4.2. Engineering transcription

Promoters represent a key determinant to transcriptionally control gene expression. Promoter replacement techniques are an effective tool to control the gene expression at the transcriptional level. Mainly two classes of promoter are utilized for pathway engineering, constitutive and regulatable (inducible/repressible) expression systems. Strong constitutive promoters have been widely applied to reach high levels of expression of target genes. However, in some cases only small changes of expression are required; therefore the selection of proper promoter systems is a critical choice to achieve the desired expression level in the cultured cell. In order to achieve optimal transcription, several systems-orientated approaches have been used to create synthetic promoter libraries of constitutive promoter with a wide range

of strength (Blount *et al.,* 2012; Braatsh *et al.,* 2008; Nevoigt et al., 2006; Alper *et al.,* 2005, Solem *et al.,* 2002). Regulatable promoters instead are required when it is necessary to time the gene expression during a determined process phase. Ideally a linear and uniform response to the inducer/repressor concentration is preferable to achieve tight regulation. Some inducible system in fact are affected by non uniform cell response that produces population heterogeneity and may subsequently lead to a detrimental effect on cell growth affecting the overall productivity (Keasling *et al.,* 2007; Keasling *et al.,* 1999).

In many cases transcript levels display context dependency. Different growth conditions, medium and carbon source lead to different expression levels. For this reason, many studies focus on characterizing and standardizing panels of promoters under multiple environmental conditions to fine tune gene expression for pathway engineering applications (Sun *et al.,* 2012; Lee *et al.,* 2011[b]; Kelly *et al.,* 2009). In this study, both constitutive and regulatable promoters have been applied and a simple screening method to titrate the promoter activities under the desired condition has been developed.

Alternatively to promoter engineering, transcription factors - due to their global regulation role - have been targeted in many studies for transcription level engineering using rational (Nielsen, 2001; Blom *et al.,* 2000) and global approaches (Auslander *et al.,* 2012; Alper *et al.,* 2006). In this thesis a modified version of a transcription factor known to regulate the targeted MVA pathway was over-expressed to override the native host regulatory system.

2.4.3 Engineering translation-RNA processing

Driven by the development of inexpensive and rapid DNA synthesis procedures, *de novo* gene synthesis for pathway engineering has become an economically feasible routine in many laboratories. The novel synthesized genes are transferred into specific host strain to confer new functionality; the expression of exogenous functions can be optimized at the translational level. Recently, a great number of post transcriptional tools based on RNA control systems have been developed e.g. asRNAs, miRNAs, siRNAs, rasiRNAs, riboregulators and riboswitches (Bayer *et al.,* 2005; Zamore *et al.,* 2005; Isaacs *et al.,* 2004; Patel *et al.,* 1997). In this study codon optimization methods and the use of antisense RNAs (asRNAs) have been applied. Codon optimization successfully succeed in improving the rate of translation in many cases of foreign gene expression in a heterologous host and appears to be particularly important when the expressed function are sheared between microorganisms distantly related (e.g. as in the case of this study *C. lansium* plant genes expressed in yeast *S. cerevisiae*). Several algorithms exist to formulate codon optimization, however, unique design principles are yet not available. In the future, application of synthetic biology to such guiding principles may play an important role in

generation of guidelines to overcome this crucial problem (Welch *et al.,* 2009). Antisense RNAs are a class of RNA regulatory molecules that control gene expression post-transciptionally (Good, 2003). Antisense-based strategies consist of the use of an antisense RNA to bind a target RNA sequence and e.g. inhibit translation. The expression of antisense copies of genes has been used especially for plants genetic manipulations as an alternative to gene knockout (Bourque, 1995), but only few applications of this technique are reported in the yeast *S. cerevisiae* (Bonoli *et al* 2006; Olsson *et al.,* 1997). In this study, an RNA-mediated strategy was employed using a selected antisense DNA fragment comprising the 5' region of the target gene and part of its 5'UTR, controlled under a specific promoter to express an mRNA antisense construct for silencing the target gene (see *Chapter 3.2.3*).

2.4.4 Engineering post translation

Protein engineering for pathway engineering is a vast area of research that recently gained benefit from the application of computational techniques (Keith *et al.,* 2007). A large number of protein-level regulatory mechanisms exist to control protein function, activity, stability and localization. Much of the effort in protein manipulation methods focuses on modifying protein properties (e.g. V_{max}, K_m, cofactor/substrate/product specificity) to improve catalytic performances (Leonard *et al.,* 2010, Watanabe *et al.,* 2007; Yoshikuni *et al.,* 2006). In contrast, to target catalytic proprieties, simple examples of protein level engineering are based on modifying protein regulatory functions and their localization (Steen *et al.,* 2010; Cho et al., 1995). In this study, a key regulatory enzyme of the targeted pathway was re-localized expressing a truncated form of the protein deleted in the periplasmic membrane anchor domain resulting in a cytolsolic soluble form that bypasses the endogenous regulatory feedback loops (see *Chapter 3.2.2*)

Beyond these reported approaches a number of elegant protein-based solutions for pathway engineering have been recently demonstrated e.g. direct protein fusion strategies (Albertsen *et al.,* 2010), synthetic scaffold systems (Dueber *et al.,* 2009), protein shell systems (Lee *et al.,* 2011[c]) and tag localization in cellular sub-compartment (Farhi *et al.,* 2011), focused to localize engineered functions and spatially organize pathways. Although these technique represent an active growing branch of pathway engineering and they have been successfully applied in several cases, they are not the primary focus of this thesis and will therefore not be further discussed.

2.5 Production process design-Industrial microbial fermentation

Microbial high density fermentation capabilities make industrial-scale sesquiterpene production attractive in a prospective of a viable biotechnological production process. The development of an efficient bioreactor operation has great impact in the optimization of a competitive bioprocess (particularly in the case of low-value products), process engineering plays a critical role in the establishment of a low-cost process (Leib *et al.,* 2001). Essentially three different reactor configurations are applied in industrial production processes: (i) batch, (ii) fed-batch (including its variant repeated fed-batch) and (iii) continuous (Nielsen *et al.,* 2003) (Fig 2.3). The different operations modes are briefly discussed below referring specifically to yeast *S. cerevisiae* cultivation cases; only the stirred tank reactor, which is the workhorse of the fermentation industry, is considered.

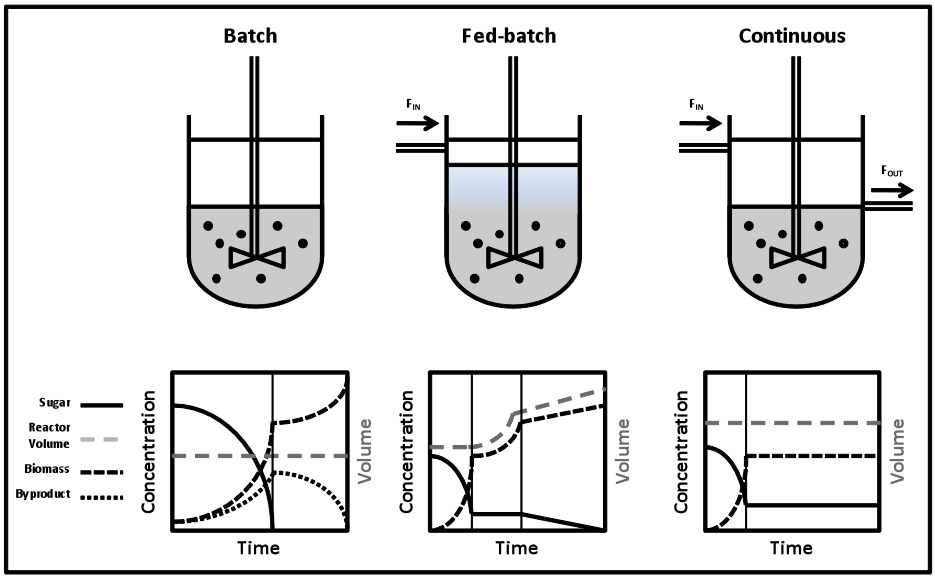


Figure 2.3. Simplified scheme of the three principal cultivation modes employed during a biotechnological process. Batch (F_{IN}= F_{OUT}= *0*); Fed-batch (F_{IN}≠ *0;* F_{out}= *0*) and continuous (F_{IN}=F_{OUT}≠ *0*) process details are described in the text. The different phases which the cell undergoes during the process are highlighted. Adapted from (Nielsen *et al.,* 2003; Stephanopoulos *et al.,* 1998.; Weusthuis *et al.,* 1994; Heijnen *et al.,* 1992).

2.5.1 Batch Cultivation

The batch method is the simplest cultivation technique, pH and dissolved oxygen (DO) are controlled, carbon source (generally sugar) and the required nutrients are provided in excess at the beginning of the cultivation and the fermentor working volume is constant during the entire process (F_{IN}= F_{OUT}= *0*). Typical exponential growth is achieved that proceeds at the maximum rate attainable (μ_{max}). When glucose is used as substrate in aerobic conditions yeast metabolism is respiro/fermentative where glucose is mainly fermented to ethanol. After complete sugar

consumption, the diauxic shift occurs and the fermentation byproducts accumulated in the first phase (ethanol, acetate and glycerol) are re-consumed. The diauxic growth is the result of carbon catabolite repression. Due to the easy set-up batch culture is an essential tool for preliminary screening of strain physiology.

2.5.2 Fed-Batch Cultivation

The majority of industrial processes are nowadays carried out using fed-batch cultivation methods. The process initiates as batch and after a suitable amount of biomass is obtained a feed of fresh concentrated medium is applied but no volume is withdrawn from the fermentor resulting in an increase of the working volume with time ($F_{IN} \neq 0$; $F_{OUT} = 0$). The feed strategy applied influences the overall process performances. Typical glucose based feed configurations are based on a first phase were the feed is kept exponential and a second phase when high cell concentration is reached with constant feed rate to avoid potential limitations (Pham *et al.,* 1998). Ideally the process proceeds maintaining the sugar concentration below the critical level preventing the Crabtree effect, maintaining a respiratory metabolism and avoiding the switch to fermentative metabolism. Advances in fermentation technology produced a multitude of strategies focused on proper control of the feed addition in order to avoid the detrimental effects due to over/under feeding (Lee *et al.,* 1999). An improved variant of the fed-batch consist in a repeated fed batch system were at the end of the fed-batch process a certain volume of culture is periodically withdrawn from the system ($F_{IN} \neq F_{OUT} \neq 0$) (Heijnen *et al.,* 1992). The main advantage of using fed-batch in a large scale process is the high final titer achievable. During this study, an optimized fed-batch production process was designed for sesquiterpene bio-production. Additionally, a feed control method for optimizing the production process was developed (see *Chapter 3.4.2*).

2.5.3 Continuous Cultivation

In continuous cultivation mode, also commonly called *chemostat*, the process starts as a batch similarly to the fed-batch set up. Thereafter follows constant addition of fresh media at a fixed rate and continual removal of spent medium at the same rate, maintaining the working volume constant ($F_{IN} = F_{OUT} \neq 0$). After some time the cells will reach a “steady state” growth condition. Cell growth is usually controlled using a single limiting nutrient (generally the carbon source). In a glucose limited chemostat yeast metabolism is fully respiratory and sugar is completely oxidized to biomass and carbon dioxide as the major products, while fermentation products are absent. Under ideal conditions the growth rate is equal to the dilution rate (D) imposed, and the chemostat cultivation therefore allows to change the operational specific growth rate

(independently of the other parameters) by varying the feed flow to the reactor. The maximum *D* applicable (D_{crit}) corresponds to the μ_{max} (obtained in batch) and for higher dilution rates a wash out occurs ($D > D_{crit}$). Typical industrial yeast continuous culture applications are carried out at $D = 0.1\ h^{-1}$ or greater to allow a productivity advantage versus batch culture (Heijnen *et al.,* 1992). Chemostat cultivation methods have been applied in this study as a tool to investigate the sesquiterpene productivity of the genetically engineered strains constructed, and a novel chemostat set-up production method that allowed for continuous product recovery and suitable for industrial scale up was developed (see *Chapter 3.4.5*).

2.6. Techno-economical analysis of sesquiterpene microbial production

Development of a cost competitive bio-production requires a detailed analysis of the production process performances. The *titer*[6]*, yields*[7] and *productivities*[8] of the target compound are an important set of parameters to monitor for optimization of the fermentation process (Nielsen *et al.,* 2002). During the development of a microbial production process different aspects including physicochemical proprieties of the target compounds and the formation pathway have to be carefully analyzed. Because the final costs of the process depend in large amount on the conversion of the substrate, one of the first parameters to take into consideration is the maximal theoretical yield Y_{sp}. This value cannot be overcome and corresponds to the highest possible product amount achievable from a certain amount of substrate and it can be expressed as Cmol product Cmol substrate^{-1}. Y_{sp} for α-santalene from different carbon sources can be calculated as follow: $Y_{sp} = \kappa_s/\kappa_p$ from a simple energy balance assuming that all the energy content of the substrate (electrons) ended up in the product, where the degree of reduction (DOR) of substrate (κ_s) and the product (κ_p) gives Y_{sp}. The reduction level express in 1 C-atom bases and Y_{sp} of the target compound α-santalene from different substrates is reported in table 2.2.

[6] ***Titer:*** *Final measure of the product concentration*

[7] ***Yield:*** *Efficiency of substrate conversion to product*

[8] ***Productivity:*** *Volumetric production rate, mass of compound produced per unit weight of cell per unit time*

Table 2.2. α-santalene maximal theoretical yield and pathway yield under different carbon sources

	Compound	Formula	Formula (1 C-atom)	Degree of reduction per carbon κ	Y_{sp} (Cmol Cmol^{-1})	Y^p (Cmol Cmol^{-1})
Substrate	Glucose	$C_6H_{12}O_6$	CH_2O	4	0.86	0.56
	Xylose	$C_5H_{10}O_5$	CH_2O	4	0.86	0.56
	Ethanol	C_2H_6O	$CH_3O_{1/2}$	6	1.29	0.83
Product	α-Santalene	$C_{15}H_{24}$	$CH_{5/8}$	4.625	-	-

The calculation of Y_{sp} is based only on the substrate/product analysis and it is independent of the metabolic network. However, in the early process stage it is useful to determine the economic feasibility of the process simply based on the substrate cost and product income determining the maximum usable energy contained in the substrate that can be transferred to the product.

Analysis of the metabolic pathway allows determining the stoichiometric equation for product formation and its redox balance to evaluate the efficiency of the product synthesis through a specific pathway. In the case of α-santalene production in *S. cerevisiae* from different substrates (glucose eq. 1; xylose eq. 2; ethanol eq. 3) via the MVA pathway at purely *oxidative growth*[9] it can be summarized as follow:

$$- CH_2O - 1/3\ ATP - 2/9\ NADPH + 5/9\ CH_{8/5} + 2/3\ NADH + 4/9\ CO_2 = 0 \quad (1.1)$$

$$- CH_2O - 1/5\ ATP - 19/45\ NADPH + 5/9\ CH_{8/5} + 13/15\ NADH + 4/9\ CO_2 = 0 \quad (1.2)$$

$$- CH_3O_{1/2} - 1\ ATP - 1/3\ NADPH + 5/6\ CH_{8/5} + 1/2\ NADH + 1/6\ CO_2 = 0 \quad (1.3)$$

Pathway analysis results in a α-santalene product yield of Y^p= 0.56 Cmol Cmol^{-1} for glucose and xylose and Y^p= 0.83 Cmol Cmol^{-1} for ethanol, respectively, corresponding to a reduction of 35% (glucose & xylose) and 36% (ethanol) compared to the maximum yield achievable (Table 2.2).

In all the three cases NADPH and ATP is required for product formation and an excess of NADH is produced. If it is assumed that neither ATP nor cofactors NADH and NADH can accumulate in the cell, an energy balance can be calculate accounting for the required amount of substrate to compensate the pathway's redox imbalance.

[9] *Calculations are made assuming that during oxydative conditions the formation of cytosolic acetate produced in the reaction catalyzed by acetaldehyde dehydrogenase (ACDH) uses NAD· as exclusive cofactor leading to the formation of 1 molecule of NADH per molecule of acetate produced (Frick et al., 2005).*

CHAPTER 3 Results & Discussion

3.1 Construction of a yeast "sesquiterpene cell factory": α-santalene case study

The main objective of this research was the construction of an efficient *S. cerevisiae* cell factory capable to produce industrially relevant titers of the sequiterpene hydrocarbon α-santalene, a precursor for commercially interesting compounds.

3.1.1 Minimal engineering of yeast for sesquiterpene production: expression of a heterologous plant gene in S. cerevisiae

The first limit in the construction of a yeast cell factory for sesquiterpene production relies on the ability to efficiently express a heterologous plant sesquiterpene synthase. The target compound of this study, α-santalene, is produced in a one step reaction from FPP enzymatically catalyzed by plant santalene synthase. α-Santalene structurally related sequiterpene compounds are widely present and conserved in plant species, and analysis of *Clausena lansium* (wampee) leaves identified a high content of α-santalol (Zhao *et al.,* 2004; Pino *et al.,* 2006). The santalene synthase gene (*SanSyn*) employed in this study was identified through a cDNA library screening from *C. lansium* and was specifically selected due to its previously demonstrated high specificity of 92% towards production of α-santalene (Schalk, 2011). Santalene synthase (*SNS*) belong to the class I group of sesquiterpene cyclases that are among the most studied terpene synthase (Christianson *et al.,* 2008). These enzymes catalyze a complex intermolecular cyclization of FPP with very different product specificity and the reaction mechanism often involves several partial reactions (Fig 3.1). Conversions of the linear FPP into cyclic derivatives are not trivial as it may appear and involve limited numbers of mechanisms dictated from the FPP *trans*-geometry of the double bond and result in the production of diverse classes of sesquiterpenes; FPP cyclization to α-santalene occurs via an enzyme bound nerolidyl diphosphate intermediate (NPP). The substrate is bound in the enzyme's hydrophobic pocket that determines the stereochemistry of the product. The reaction is initiated by the carbocation formation via loss of the diphosphate group (OPP^-), which is kept in complex with Mg^{2+}, and subsequent rearrangements define the final product and determine the specificity of the enzyme. Fast OPP^- release can stop the reaction and result in alternative products (Fig. 3.1) (Jones *et al.,* 2011; Christianson *et al.,* 2008; McCaskill *et al.,* 1997).

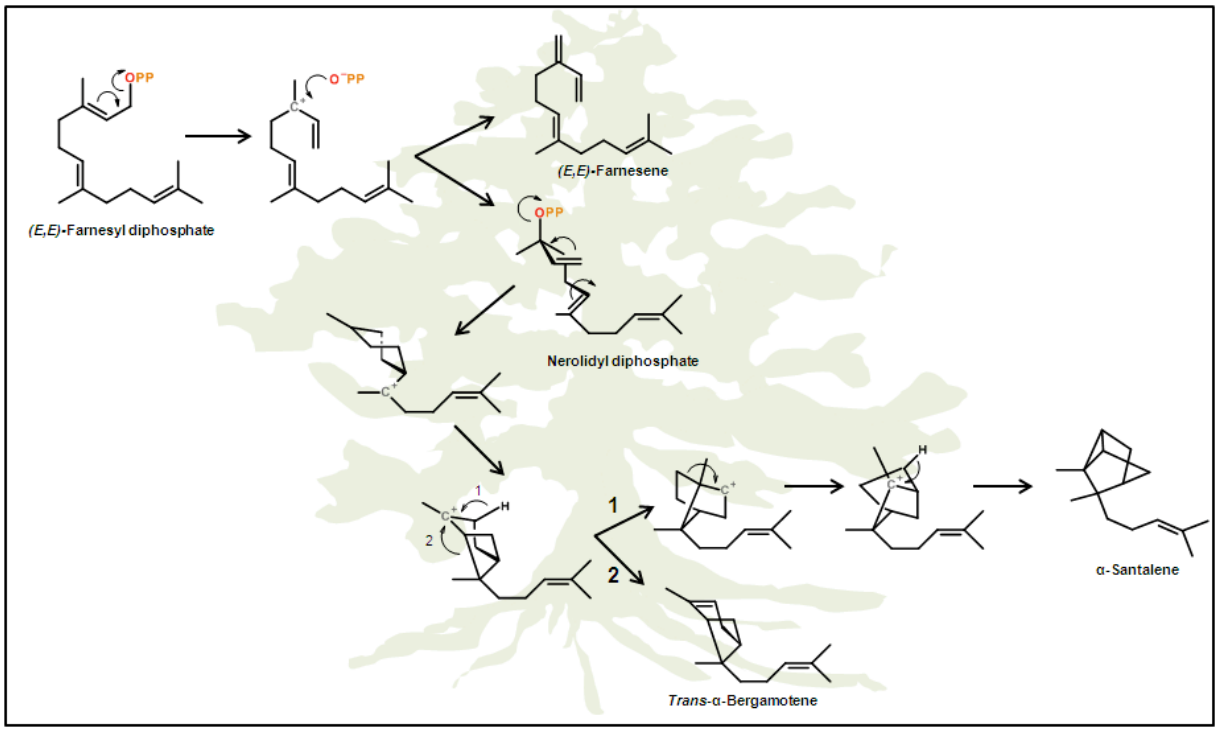


Figure 3.1. Detailed reaction mechanism of plant santalene synthase (*SNS*). Electrophilic attack on the central double bound of the substrate **(*E,E*)-farnesyl diphospahte** produces an allylic carbocation that can evolve into formation of linear product **(*E,E*)-farnesene** or one of the cyclic derivatives **α-santalene** and ***trans*-α-bergamotene** via a nerolidyl diphosphate intermediate (NPP). Adapted from McCaskill *et al.,* 1997; Christianson *et al.,* 2008 and Jones *et al.,* 2011.

In order to ensure high santalene synthase levels an expression vector with suitable transcriptional promoter/terminator was chosen and constructed (Partow *et al.,* 2010). Introducing *SanSyn* yeast was minimally engineered for the first time to produce α-santalene. Product analysis revealed that α-santalene was the main product detected with 1.45 mg l^{-1} and only a minor amount, 0.17 mg l^{-1}, of the secondary product *trans*-α-bergamotene was found. During bio-production the product purity and quality is a major driver to meet commercial demands. The structure of the sesquiterpene produced estimated by GC/MS was identical (~98% purity) to the one produced in plant (Fig. 3.2).

Many studies have reported successful examples of heterologous production of isoprenoids by simply expressing plant synthase genes in a desired microbial host. Not surprising the resulting yield of this simple straightforward approach was often extremely low (ranging between 0.038 and 6.7 mg l^{-1}) (Farhi *et al.,* 2011; Wang *et al.,* 2011b; Asadollahi *et al.,* 2008; Paradise *et al.,* 2008; Ro *et al.,* 2006; DeJong *et al.,* 2005; Jackson *et al.,* 2003; Madsen *et al.,* 2001).

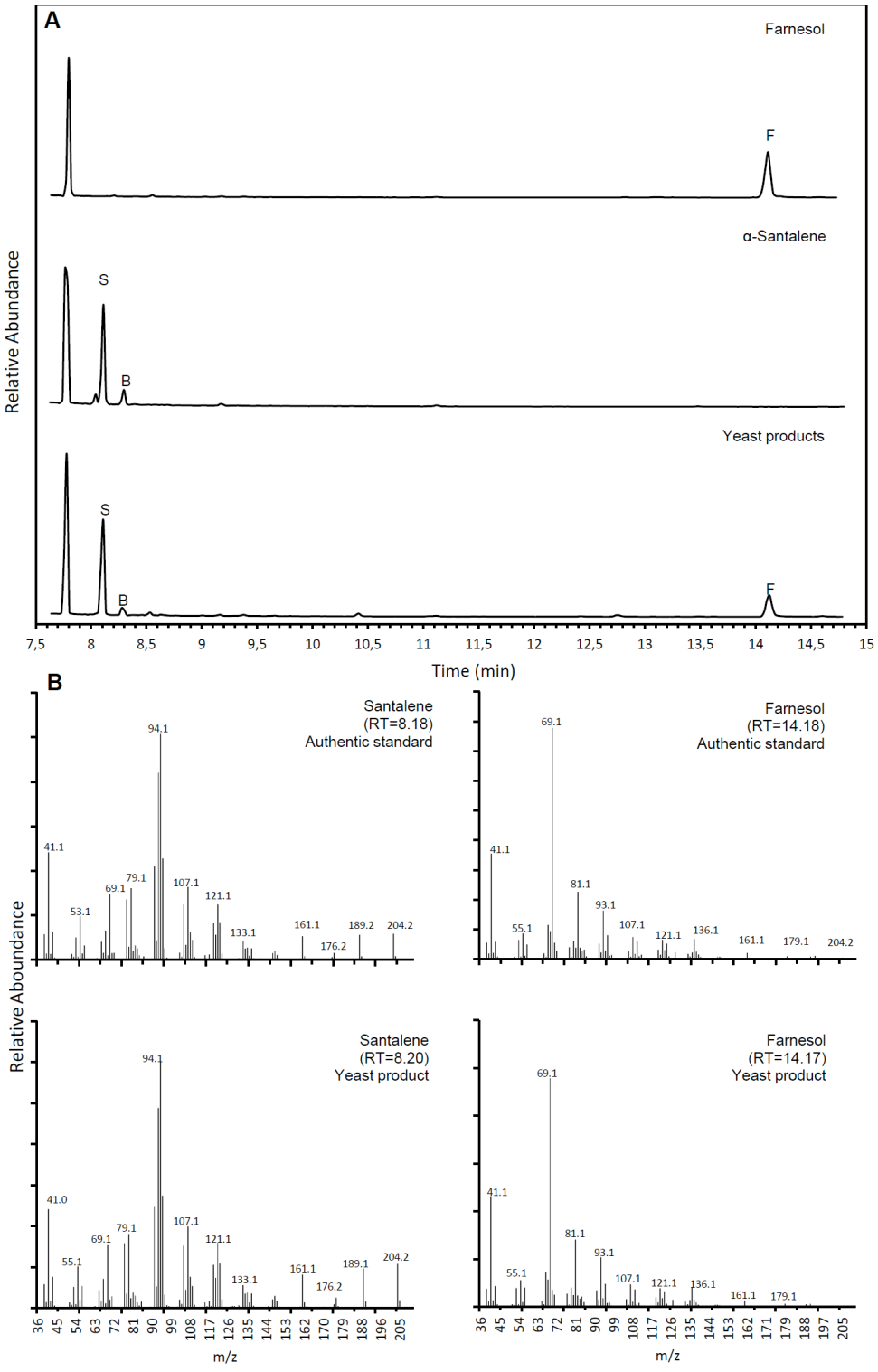


Figure 3.2. (A) Total ion chromatograms from GC-MS analysis of authentic standard of farnesol, α-santalene, and an extract of engineered *S. cerevisiae* showing peaks of α-santalene (S), *trans*-α-bergamotene (B) and *E,E*-farnesol (F). The representative ion chromatogram referred to as yeast products was obtained during ISPR fed batch fermentation (for cultivation methods details see *Chapter 2.5*). **(B)** Mass spectra and retention times of α-santalene produced from yeast and extracted from plant (left panel) and *E,E*-farnesol produced from yeast and chemical standard (right panel).

The catalytic efficiency (V_{max}/K_m) and the specificity are often referred to as key factors during heterologous production (Picaud *et al.,* 2005). Subsequently, during this study a codon-optimized artificial santalene synthase ($SanSyn_{Opt}$) for optimal expression in *S cerevisiae* was designed. Expression of the codon-optimized $SanSyn_{opt}$ led to comparable specificity and only modest increase in efficiency compared with the wild type version, suggesting that although the codon bias has an important role, the level of expression depends on multiple proprieties and other factors may be critical (e.g. mRNA stability, sequence that control the initiation of the translation, nucleotide sequence surrounding the N-terminal region, tRNA levels) (Gustafsson *et al.,* 2004).

3.2 *Rationally designed metabolic control engineering approach*

A second bottleneck that often limits the production of a heterologous compound is the capacity to increase the precursor pool in order to enable efficient conversion to the target compound. Yeast has a very limited secondary metabolism and terpenes are produced exclusively through the mevalonate pathway (see *Chapter 1.2*). Due to the variety of essential compounds produced in the MVA pathway, the activity of many enzymes of this pathway is strictly regulated at different levels (Maury *et al.,* 2005). A rationally designed metabolic control engineering approach was employed to maximize flux through the MVA pathway and obtain optimal sesquiterpene production. This approach relies on the deep knowledge available of yeast biology and MVA pathway regulation. Two of the well recognized regulatory steps of the MVA pathway catalyzed by 3-hydroxy-3-metyl-glutaryl-coenzyme A reductase (HMGR) and squalene synthase (*SQS*) were optimized by introducing genetic modifications that enable to channel increased flux towards α-santalene synthesis.

3.2.1 Engineering the regulatory checkpoint of the MVA pathway.

α-Santalene production was increased combining (i) de-regulating the MVA pathway overexpressing a truncated version of HMG-CoA reductase (*tHMG1*) and (ii) dynamic control of the MVA pathway branch point by down regulating the squalene synthase gene (*ERG9*) (Fig. 3.3).

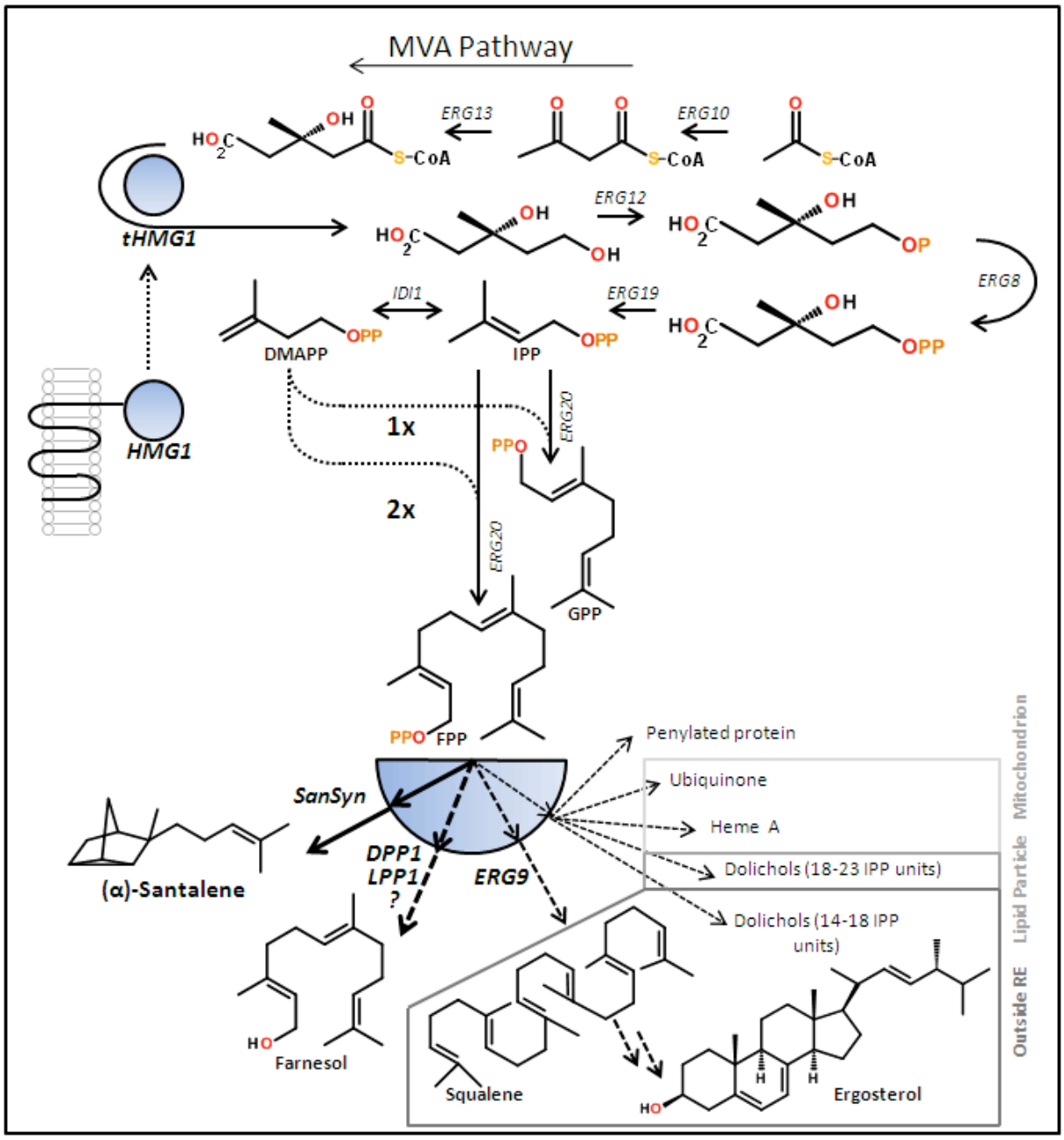


Figure 3.3. Metabolic engineering strategy for overproducing α-santalene. Two key checkpoints in the MVA pathway were engineered. (i) The rate controlling step catalyzed by HMGR was de-regulated to maximize the flux through the MVA pathway overexpressing a truncated non-membrane bound version of HMG1 that represents a constitutively active form of HMGR. (ii) Enzymatic activities acting at the FPP branch point were modulated to redirect carbon flux towards the desired target compound; the main FPP consuming reaction SQS was down-regulated using a promoter replacement technique and two activities competing for FPP, Lpp1 and Dpp1, were disrupted.

3.2.2 De-regulation of the MVA pathway to increase the critical precursor pool

As previously mentioned, because of its crucial roles in supply of several essential compounds the MVA pathway has evolved a hierarchical control architecture. De-regulation is therefore necessary to increase flux trough this pathway to increase the precursor pool for isoprenoid synthesis. The conversion of 3-hydroxy-3-metyl-glutaryl-CoA into mevalonate catalyzed by HMGR is probably the most studied enzyme of all and it is considered to exert a high degree of MVA flux control (Scallen *et al.,* 1983; Basson *et al.,* 1986). In yeast two isoform of HMGR exist and their activity is subject to extensive regulation including feedback regulation and cross-regulation (Hampton *et al.,* 1996, 1994; Brown *et al.,* 1980). HMGR is composed of an interspecies conserved catalytic domain and a variable membrane anchoring N-terminal region referred to as sterol sensing domain (SSD) that spans the membrane of the endoplasmic reticulum (ER) and interact with sterol sensing components of the ER membrane. Part of Hmg1 regulation acts through a complex mechanism leading to protein degradation at the level of the N-terminal domains (SSD domain) (Nielsen, 2009). Overexpression of the truncated form containing only the catalytic domain and lacking the regulatory domain bypasses this post transcriptional circuit and results in a constitutively active soluble form that is non-membrane bound (Fig. 3.3) (Polakowski *et al.,* 1998; Donald *et al.,* 1997). The use of the deregulated form of Hmg1 (tHmg1) represents an excellent example of bypassing the regulatory mechanisms controlling the MVA flux and has been successfully applied to a series of microbial production processes to increase the flow through the pathway (Fahri *et al.,* 2011; Asadollahi *et al.,* 2010, 2009; Kirby *et al.,* 2008; Ro *et al.,* 2006; Jackson *et al.,* 2003).

Previous studies demonstrated that a high level of expression is required to ensure a high MVA flux (Ro *et al.,* 2006), and this strategy was therefore applied by constructing a high copy number expression vector containing *tHMG1* and *SanSyn* under control of strong promoters and this resulted in a 2 fold increase in sesquiterpene production yielding 3.1 mg l^{-1} α-santalene and 0.33 mg l^{-1} trans-α-bergamotene.

3.2.3 Dynamic control of MVA pathway branch point

The second MVA flux controlling step is represented by *SQS* that regulates the FPP flux distribution between sterols, e.g. lanosterol, erosterol, and non-sterols, e.g. dolichols, ubiquinone, heme A, prenyated proteins, and sesquiterpene derived products. FPP is a pivotal intermediate and its intracellular concentration is carefully regulated by a flow diversion mechanism. Under normal growth conditions the cellular sterol demand is higher than that of non-sterols, and most of the FPP is converted into ergosterol and *SQS* is therefore the main

FPP consuming reaction (Kennedy *et al.,* 1999). In order to minimize the overflow to biosynthetically related sterols optimization of FPP branch point flux distribution is necessary. Deletion of the *ERG9* gene encoding *SQS* produces lethal mutants because of the essential role of ergosterol in maintaining the membrane fluidity (Jennings *et al.,* 1991) and restoration of an *erg9Δ* mutantion would require ergosterol supplementation that would have consequences on the economic feasibility of the entire process (Takahashi *et al.,* 2007). Therefore a suitable approach to increase the FPP availability for conversion into α-santalene is to reduce the flux through *SQS* enabling sufficient squalene to satisfy the minimum amount of ergosterol necessary to fulfill cellular growth. Precise adjustment of an essential enzymatic activity avoiding unbalance represented a challenging task to overcome. A variety of tools has been developed to modulate yeast gene expression (see *Chapter 2.4*). Among the several genetics techniques available as alternative to complete gene deletion in order to reduce specific gene activity (Hammer *et al.,* 2006; Mjiakovic *et al.,* 2005) promoter replacement and the use of repressible/inducible promoter systems (Kaufmann *et al.,* 2011) represents an efficient strategy to transcriptionally fine tune gene expression. Previous attempts to regulate SQS activity were mainly based on replacement of the native *ERG9* promoter (P_{ERG9}) with a methionine-repressible promoter system (P_{MET3}) (Asadollahi *et al.,* 2008; Paradise *et al.,* 2008; Ro *et al.,* 2006).

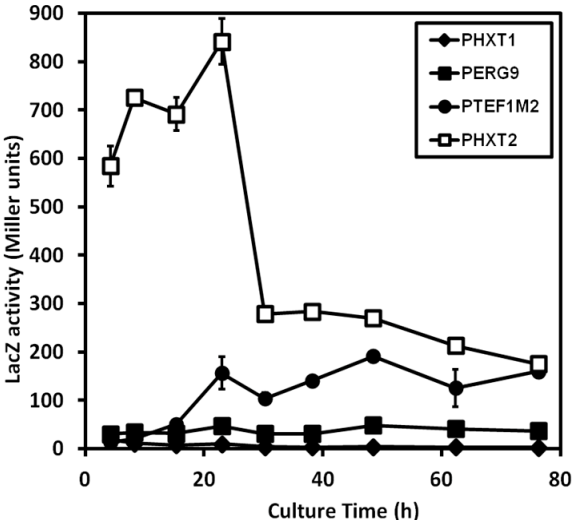


Figure 3.4. Characterization of candidate promoter strength during shake flask cultivation in fed-batch mode. ß-Galactosidase activity with the different tested promoters is the average of values obtained from at least three independent cultivations assayed in duplicates.

Promoter	Description	Reference
PHXT1	*glucose concentration controlled promoter of the hexose transporter gene HXT1*	*Ozcan et al., 1995 Lewi et al., 1991*
PHXT2	*the HXT2 promoter for gene silencing approach expressing ERG9 antisense mRNA*	*Ozcan et al., 1995*
PTEF1M2	*Low-level constitutive TEF1 promoter mutant was selected after directed evolution approach based on error prone PCR*	*Alper et al., 2005 Nevoigt et al., 2006*

Table 3.1. Candidate promoter systems and their brief function description evaluated to promote the activity of SQS.

Ideally the level of repression should be proportional to the concentration of the inducer provided; indeed a careful evaluation of P_{MET3} activity conducted during this study using *lacZ*

reporter system revealed severe difficulties in controlling the promoter activity mainly related to the ability of the cell to metabolize the repressing agent methionine. Moreover this system suffers from several limitations for the industrial scale development due to the cost of the repressor itself.

Using a metabolic control engineering approach a dynamic controller able to sense the physiological state of the cell (fermentative/ fully respiratory metabolism) and regulate the expression of *SQS* was constructed using a promoter replacement technique. A series of alternative promoter systems was preliminary screened for their activity using the reporter *LacZ* gene assay in order to identify a suitable promoter that could provide optimal expression of SQS.

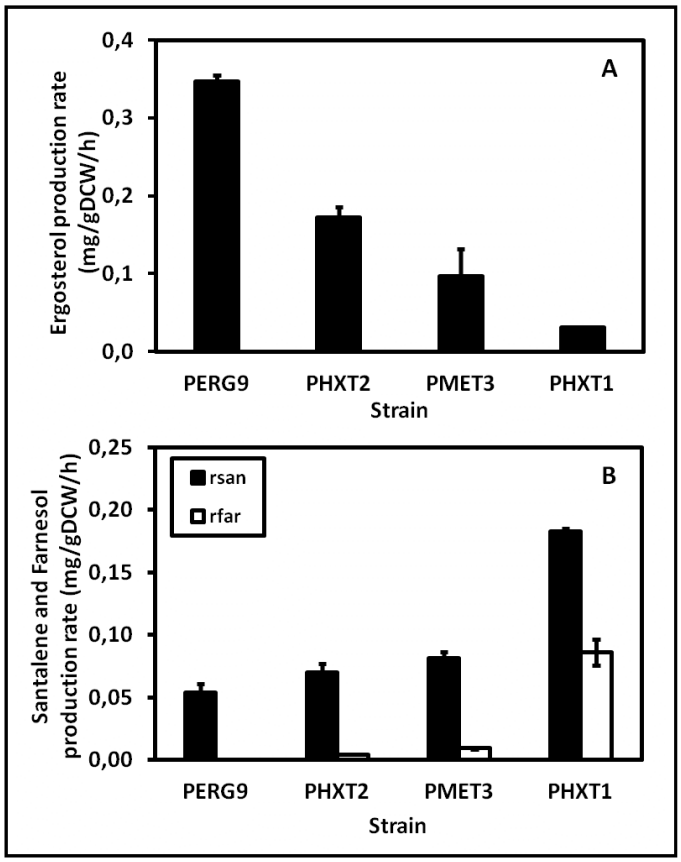


Figure 3.5. (A) Ergosterol production rate (mg g biomass^{-1} h^{-1}). **(B)** α-santalene and *E,E*-farnesol production rate in *S. cerevisiae* strains engineered for sesquiterpene production. P_{ERG9} native *ERG9* promoter serve as reference strain; in P_{HXT1} the *ERG9* promoter had been replaced with *HXT1* promoter; P_{HXT2} carried an antisense DNA fragment comprising the 5'region of ERG9 and part of its 5' UTR, whose expression is controlled by *HXT2* promoter; P_{MET3} carried the methionine repressible promoter instead of the native P_{ERG9}, for this strain L-methionine was added at regular intervals in order to maintain *ERG9* repressed. Strains were growth in two phase partitioned fed-batch glucose limited cultivation mode. The error bars represent the standard deviation for two independent cultivations.

The *ERG9* gene expression level is context dependent and changes based on the growth condition and carbon source utilized (Kennedy *et al.,* 2001, 1999). An efficient screening method was developed capable to simulating the growth condition typical of an industrial production process. The promoters were evaluated in a glucose limited process where the carbon source (glucose) is released using silicone elastiomers with controlled kinetics. This screening method mimics a typical fed-batch process widely applied during industrial production (see *Chapter 2.5.2*) and allowed precise comparison of the candidate promoters under the selected condition.

Two regulatory systems P_{HXT1} and P_{HXT2} were chosen based on the promoter characterization study according to their expression profile. Sequiterpene producing strains containing the expression vector (expressing *tHMG1* and *SanSyn*) were engineered at the SQS level using

P_{HXT1} and P_{HXT2} to promote the expression of *ERG9* and antisense *ERG9*, respectively, during a low glucose level typical of a fed-batch cultivation mode with the aim to couple SQS activity to glucose concentration and achieve maximal repression during the feed phase when glucose is limiting. The selected replacement mutants together with wild type and previously widely applied P_{MET3} system were evaluated for their sesquiterpene production capacity and the total sterol content (Fig 3.5). Applying different levels of repression of *SQS* result in a redirection of carbon flux from ergosterol towards sequiterpene products. From the obtained data it was clear that engineering the FPP branch point modulating *SQS* activity resulted in an increase in FPP that was redistributed toward sesquiterpene products. The best α-santalene producer expression systems selected was P_{HXT1} that is regulated by limiting nutriment (extracellular glucose concentration) and allows timing the promoter strength changing the cultivation condition, separating growth phase (excess of extracellular glucose -> fermentative metabolism) and production phase (limited glucose -> respiratory metabolism) maintaining the repression at the set level during the desired phase without addition of repressing agent. Moreover, using this technique the cellular response is uniformly distributed in the population avoiding the creation of suboptimal population with different expression levels.

The sesquiterpene pool produced from the engineered mutant tested was composed of α-santalene as major product, *trans*-α-bergamotene and *E,E*-farnesol (FOH). The amount of *trans*-α-bergamotene was produced proportionally to α-santalene and corresponds to 12% of the total α-santalene confirming that this compound is a secondary product of *SNS* (as previously anticipated). Substantial accumulation of FOH, a FPP-derived product was observed in all the engineered strains. FOH is the result of the dephosphorylation of FPP. In yeast, specific enzymatic activity involved in this process has not yet been elucidated. *SQS* has a lower affinity for FPP (K_m=2.5 □M V_{max}=0.46 pmol min^{-1} mg^{-1}) compared to most of the other enzymes acting at the FPP branch point (K_m= 0.42-0.55 □M V_{max}=0.86-2.1 pmol min^{-1} mg^{-1}). This produces a high flux toward non-sterol products when the intracellular FPP concentration is low (Scheffler *et al.,* 2002). Obtained results suggest that *SNS* was capable to compete with the non-sterol branches and drain FPP toward sesquiterpene compounds. However, when the level of FPP was increased by down-regulating *SQS* the catalytic performances of *SNS* were not able to completely convert the excess of FPP to α-santalene and there was therefore overflow with dephosphorylation of FPP resulting in formation of FOH. The FOH overflow reduces substrate availability for *SNS*, and in order to reduce the competition for the same substrate a complementary strategy was investigated in this study. Two identified phosphatases responsible for most of the cytosolic isoprenoid phosphatase activity, Lpp1 and Dpp1, were deleted. The single (*lpp1Δ*) and double deleted mutant (*lpp1Δ dpp1Δ*) further improved α-

santalene production at the expense of FOH formation. Reduction of FOH formation confirms the role of these two lipid phosphatase in FPP dephosphorylation, the incomplete reduction of FOH formation, however, suggests that other cellular mechanisms are involved in this conversion. This first round of strain engineering resulted in the production of a base strain capable of a final α-santalene productivity and titer equal to 0.015 Cmmol $gDCW^{-1}$ h^{-1} (= 0.21 mg $gDCW^{-1}$ h^{-1}) corresponding to 92 mg l^{-1}.

3.3 Combined metabolic engineering strategy of precursor and co-factor supply for sesquiterpene production.

One of the major challenges in order to develop an efficient bioprocess is identifying the rate limiting regulatory steps and designing a system wide process avoiding imbalances of pathway intermediates and co-factors. A second round of strain optimization was performed where a multistep metabolic engineering strategy was designed combining four different approaches (*Box 3.1*).

Box. 3.1. Multistep metabolic engineering strategy for the construction of a S. cerevisiae "sesquiterpene cell factory"

(i) *Modulation and optimization of the FPP branch point (P_{HXT1}ERG9 & ΔLPP1 ΔDPP1).*

(ii) *De-regulation of MVA pathway to increase the precursor pool for isoprenoid synthesis (↑tHGM1 & ↑ERG20).*

(iii) *Increasing the availability of the reductive cofactor NADPH modifying the ammonium assimilation pathway (ΔGDH1 & ↑GDH2).*

(iv) *Enhancing the activity of a transcriptional activator of sterol biosynthesis (↑upc2-1).*

(i) The first approach was focused on minimizing the overflow to biosynthetically related sterols that have the same precursor as target product and was explored in details during the 1st round of strain engineering (*Chapter 3.2.2*). The best producer was selected (P_{HXT1}) and utilized during this stage. Previous attempts to increase cytosolic FPP availabilty resulted in a rapid dephosphorylation of FPP to FOH, and to minimize FOH overflow Lpp1 and Dpp1 activities were deleted (*Δlpp1 & Δdpp1*) **(ii)** A second part of the strategy consisted of amplifying the flux through the MVA pathway, and this was realized combining the previously described over-expression of a constitutively active cytosolic variant of Hmg1p (*tHMG1*) with the over-expression of a second enzyme in the MVA pathway, farnesyl diphosphare synthase (FPPS), catalyzing the multiple condensation of IPP units into FPP (Fig. 1.1). Due to the pivotal nature of the FPP molecule its synthesis and distribution into derived products is strictly regulated by FPPS (Chambon *et al.,* 1990; Grabinska *et al.,* 2002). The effect of *ERG20* (gene encoding FPPS) over-expression was therefore investigated in addition to over-expression of *tHMG1*. **(iii)** The manipulation of the

NADH and NADPH co-factor balance in order to overcome limits imposed from the cellular redox constraints is a well-established metabolic engineering strategy (Hou *et al.,* 2010). The reaction leading to α-santalene formation results in net production of NADH and consumption of NADPH (see *Chapter 2.6*). A change in the NADH:NADPH ratio in favor of NADPH would therefore be beneficial for product formation. *In silico* analysis identified the deletion of the NADPH consuming reaction of glutamate dehydrogenase (encoded by *GDH1*) as a target strategy to increasing the availability of the reduced co-factor NADPH (Asadollahi *et al.,* 2010). Activation of an alternative ammonium utilization route in a *GDH1* deleted strain by over-expressing the NAD-dependent glutamate dehydrogenase (encoded by *GDH2*) resulted in an increase of NADH consumption during the anabolic process and in a modification of the yeast co-factor balance (dos Santos *et al.,* 2003). Here, the effect of *GDH1* deletion alone as well as coupled with simultaneous over-expression of *GDH2* on α-santalene production was evaluated.
(iv) The last strategy employed involved engineering of a key transcription factor with the objective to generally up-regulate expression of the MVA pathway genes. *Upc2* and *Ecm22* have been identified as the main transcription factors responsible for the activation of several MVA and ergosterol pathways genes (Vik *et al.,* 2001). The point mutation *upc2-1* discovered first for conferring the ability to assimilate extracellular sterols during aerobic cultivation (Lewis *et al.,* 1988) has been demonstrated to result in a constitutively active form of Upc2 (Davies *et al.,* 2005). Over-expression of *upc2-1* has been employed to transcriptionally up-regulate the MVA pathway genes during isoprenoid production.

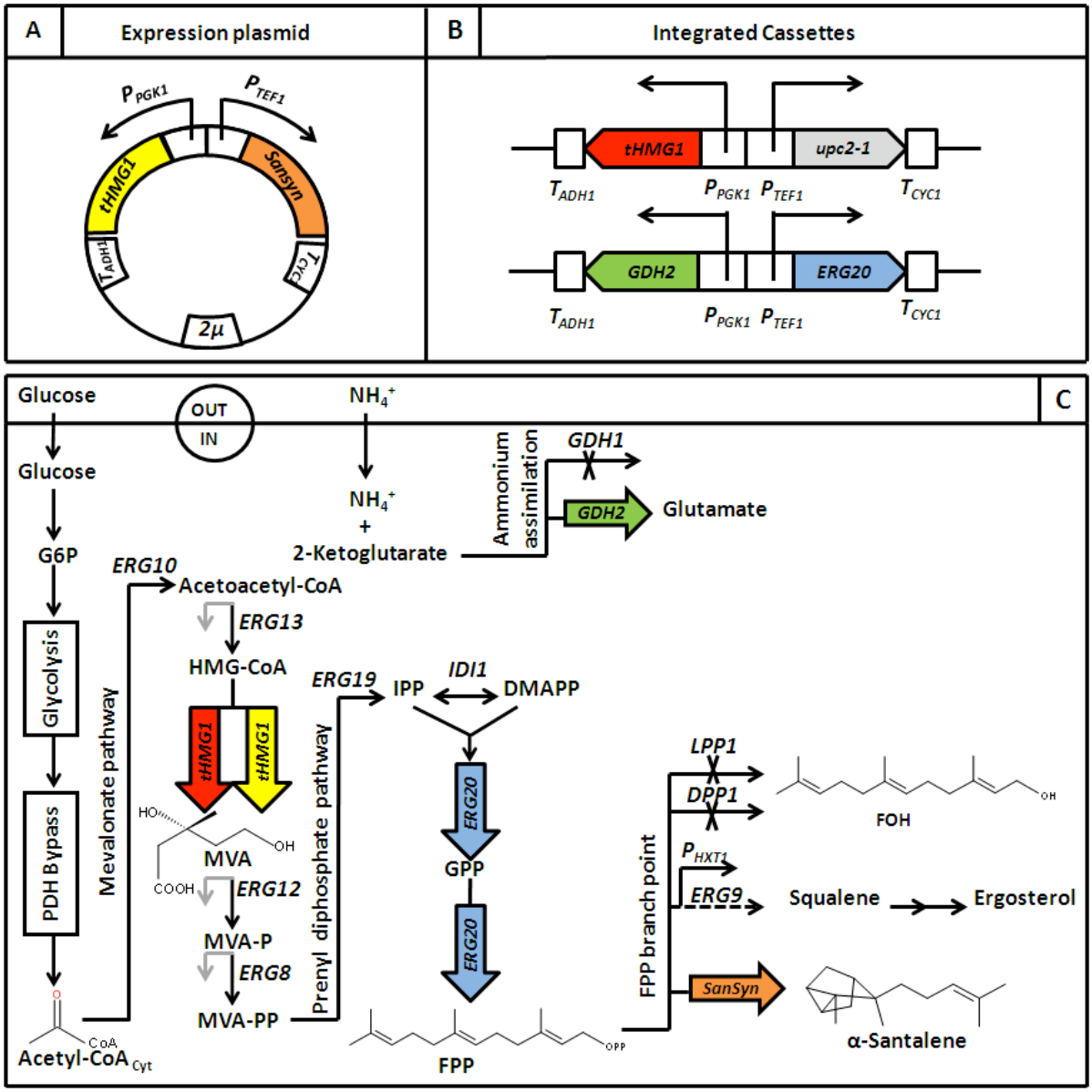
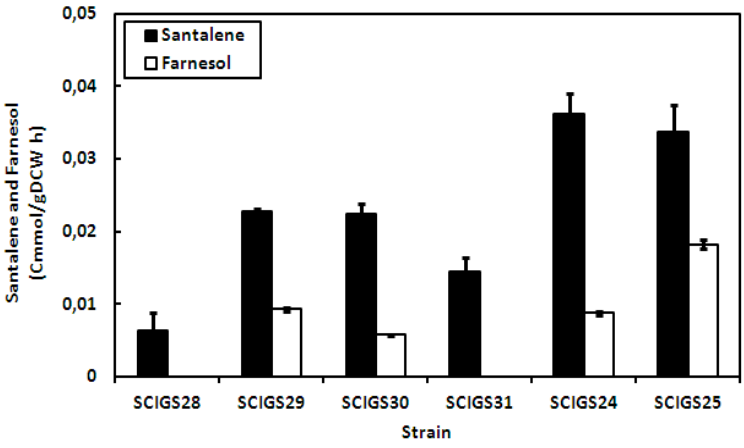


Figure 3.6. Overview of the multistep genetic engineering approach for increasing α-santalene production. (A) Expression plasmid containing *tHMG1* encoding truncated HMG-CoA reductase, a codon optimized santalene synthase gene (*SanSyn*$_{opt}$) P_{TEF1} and P_{PGK1} promoters as well as T_{ADH1} and T_{CYC1} terminator sequences. (B) Integrated cassettes, rectangles containing arrows represent the promoters and their directionality, pentagons the genes and empty squares the terminators. (C) Scheme of the engineered mevalonate, prenyl phosphate and ammonium assimilation pathways and FPP branch point; overexpressed and deleted genes are highlighted. Overexpressed genes are *tHMG1* (encoding truncated HMG-CoA reductase), *ERG20* (encoding FPP synthase), *GDH2* (encoding NAD-dependent glutamate dehydrogenase), and *SanSyn*$_{opt}$ (encoding α-santalene synthase). Deleted genes are *GDH1* (encoding NADP-dependent glutamate dehydrogenase), *LPP1* and *DPP1* (both encoding lipid phosphate phosphatases). The promoter of the *ERG9* gene (encoding squalene synthase) is replaced with P_{HXT1}. Genes whose promoters contain Upc2 binding sites are indicated with a grey arrow: *ERG13* (encoding HMG-CoA synthase), *ERG12* (encoding mevalonate kinase), and *ERG8* (encoding phosphomevalonate kinase). Additional genes indicated are *ERG10* (encoding acetoacetyl-CoA thiolase), *ERG19* (encoding diphosphomevalonate decarboxylase) and *IDI* (encoding IPP isomerase).



Strain	Genome Description	Expression plasmid
SCIGS28	P_{ERG9}-ERG9	$SanSyn_{opt}$ ↑tHMG1 ↑
SCIGS29	P_{HXT1}-ERG9 dpp1Δ	$SanSyn_{opt}$ ↑tHMG1 ↑
SCIGS30	P_{HXT1}-ERG9 dpp1Δ lpp1Δ	$SanSyn_{opt}$ ↑tHMG1 ↑
SCIGS31	P_{HXT1}-ERG9 dpp1Δ lpp1Δ gdh1Δ	$SanSyn_{opt}$ ↑tHMG1 ↑
SCIGS24	P_{HXT1}-ERG9 dpp1Δ lpp1Δmgdh1Δ ERG20 ↑GDH2 ↑	$SanSyn_{opt}$ ↑tHMG1 ↑
SCIGS25	P_{HXT1}-ERG9 dpp1Δ lpp1Δ gdh1Δ RG20 ↑GDH2 ↑ upc2-1 ↑tHMG1 ↑	$SanSyn_{opt}$ ↑tHMG1 ↑

Figure 3.7. Sesquiterpene productivity in a two-phase partitioned glucose-limited aerobic chemostat. α-Santalene and farnesol production rate in Cmmol (g biomass)$^{-1}$ h^{-1} (the C-molar weight of α-santalene and farnesol are, respectively, 13.62 and 14.82 g Cmol^{-1}) cultivated at dilution rate D=0.05 h^{-1}. Error bars represent the standard deviation from three independent cultivations (top). List of *S. cerevisiae* strains engineered (bottom).

All genetic modifications described were chromosomally integrated to enhance genetic stability during long term cultivations and avoid constant application of a selective pressure for maintaining the construct. In order to obtain high level expression the genes were targeted via sequential integration strategy into suitable expression loci previously characterized to confer high gene expression (Flagfeldt *et al.,* 2009). Platform flexibility was maintained by expressing the synthase gene for the final conversion of FPP into α-santalene with the previously described multi-copy expression vector. This allows to utilize the constructed platform for a range of different isoprenoids. The contribution of the different strategies and their combination on isoprenoid production was evaluated. The impact of different combinations of the designed metabolic engineering strategies on sesquiterpene productivity is summarized in figure 3.7. Replacing the native *ERG9* promoter (P_{ERG9}-> P_{HXT1}) and deleting *LPP1* (strain SCIGS29) increased the yield and productivity of α-santalene 3- and 3.8- fold, respectively, over the control strain (strain SCIGS28). Additional *DPP1* deletion (strain SCIGS30) reduced the rate of hydrolysis of FPP into the by-product FOH 2-fold leaving an unchanged flux towards α-santalene. Subsequent *GDH1* deletion (strain SCIGS31) led to no further enhancement in α-santalene productivity, but interestingly no FOH was formed in this strain. When combined with *GDH2* and *ERG20* over-expression (strain SCIGS24) a 4- and 6-fold improvement in α-santalene yield and productivity, respectively, was respectively. Finally, including the overexpression of *upc2-1* and having an extra copy of *tHMG1* (strain SCIGS25) resulted in an insignificant change in α-santalene formation but a 2-fold increase in FOH production. The optimal solution was obtained through combining all the modification (except *upc2-1*) resulting in the highest α-santalene yield of 0.0052 Cmmol Cmmol^{-1} and productivity of 0.036 Cmmol gDCW^{-1}h^{-1} (Strains SCIGS24). The engineering strategy

allowed a 6-fold increase in α-santalene productivity compared to the control strain under the tested conditions and highlights the importance of this systematic approach.

3.4 Development of an efficient fermentation process

During the realization of a microbial bioprocess, the overall aspect of the entire process should be taken in consideration and strain construction should be performed in parallel with optimization of fermentation and recovery procedures. The selected cultivation method and consequently downstream steps are critical choices that affect the economic feasibility of the final system. During this study, the strain improvement strategy was integrated with the development of a cost effective fermentation process resulting in an efficient fermentation strategy that couples biochemical production to biomass formation and improves α-santalene production.

3.4.1 Fed batch in situ product removal (ISPR) integrated bio-process

Bioreactor operations have strong impact on design of an efficient bioprocess and influence in a significant way the production performances. Fed batch fermentation is commonly used during industrial production processes to achieve high titer, yield and productivity of the target compound (Nielsen *et al.,* 2003). Limited exponential feed profiles were used to maximize the carbon flux from glucose to biomass and the desired compound, alleviating glucose repression and Crabtree effect (Pronk *et al.,* 1996). Here the feed rate was designed using an exponential policy chosen so that the volumetric rate of biomass production was constant and equal to the specific growth rate. Exponential feed rate v(t) (l h^{-1}) was calculated according to equation 3.1.

$$v(t) = \frac{Y_{xs}\,\mu_0}{s_f - s_0}\, x_0 V_0 \exp(\mu_0 t) \qquad (3.1)$$

where x_0, s_0 and V_0 were the biomass density (gDCW l^{-1}), the substrate concentration (g l^{-1}) and the reactor volume (l) at the start of the feed phase, *Yxs* was the yield coefficient (g glucose $gDCW^{-1}$); s_f was the concentration of the growth limiting substrate (g glucose l^{-1}) in the reservoir; μ_0 the was the specific growth rate (h^{-1}) during the feed phase and *t* the feeding time (Nielsen *et al.,* 2003).

Due to the low water solubility of the isoprene products this compound can easily be stripped with the gas bubble used for aeration. An *in situ* product removal was therefore applied to maximize the product recovery. This technique is extensively used in bioprocesses to produce

secondary metabolites (Stark *et al.,* 2003), and it consists of a double phase partitioning system that allows constant product removal from the cells and trapping the product into the solvent phase minimizing product loss through the gas outlet and reducing potential toxic effects due to product accumulation in the aquous phase. Dodecane was selected as organic phase due to its hydrophobicity ($logP_{dodecane}$: 6.6; $LogP_{santalene}$: 6.2), low volatility and biocompatibility with *S. cerevisiae* (Asadollahi *et al.,* 2008; Newman *et al.,* 2006). The organic layer was added before initiating the feed addition corresponding to the start of the production phase. This set-up allows performing extractive fermentation directly in the bioreactor resulting in an integrated production/recovery process. Analysis of process performance shows how the culture metabolism was completely respiratory within the first 30 h characterized by complete oxidation of glucose to biomass and carbon dioxide. This period was followed by a phase where yeast growth was no longer consistent with the feeding profile producing a shift towards fermentative metabolism accompanied by accumulation of glucose and ethanol and characterized by a reduced sesquiterpene production rate. As consequence this result in a suboptimal condition affecting productivity. The α-santalene final productivity obtained was 0.15 Cmmol $gDCW^{-1}$ h^{-1}.

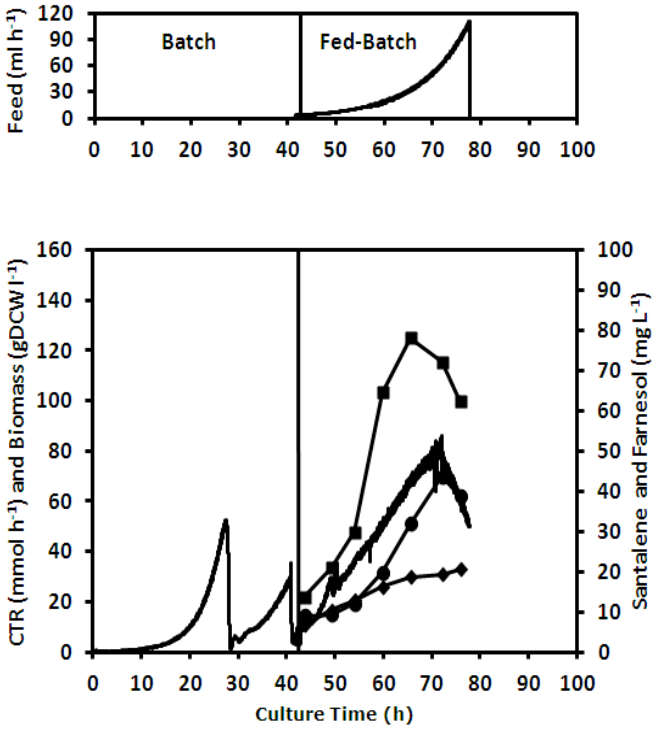


Figure 3.8. Time course of an aerobic fed-batch culture with exponential sugar feed of *S. cerevisiae* strains. Cultivation was started as batch with 1 Cmol l^{-1} (30 g l^{-1}) of glucose as carbon source, after complete glucose consumption and residual ethanol produced during the glucose consumption phase was completely depleted production phase was started feeding concentrated substrate (7.4 Cmol l^{-1} glucose as carbon source) with exponential kinetics for a feed period of 36 h. The feed of glucose (ml h^{-1}) is shown on the upper graph. Typical profile observed for formation of biomass (g l^{-1}, filled diamonds); α-santalene (mg l^{-1}, filled squares); *E,E*-farnesol (mg l^{-1}, filled cycles) carbon dioxide production CTR (mmol h^{-1},lines) are represented. Data represent the average of two independent cultures.

3.4.2 Optimization of ISPR fed-batch process

During fed-batch operation the feed rate represents a key parameter in controlling the overall process performances. From the result obtained in an initial stage of process optimization it was clear that an operational strategy allowing optimal feeding policy was required to overcome the problem related to the detrimental effect observed resulting from the overfeeding. Advances in

fermentation technology allow to design an optimal strategy to proper control the process. Ideally a glucose based feed should proceed maintaining the sugar concentration below the critical value preventing the metabolism switch to fermentative mode during the entire process. A biomass coupled production process was designed applying RQ control together with the previously developed glucose limited exponential feed. In the engineered strain, the extracellular glucose concentration triggers the switch between the flux toward ergosterol biosynthesis and product formation, and it is therefore critical to carefully control the cell metabolism to obtain the best productivity. Instant RQ measurement was used as indicator of the cellular metabolic state in order to fine-tune the feed profile to the cellular demand and maintain a fully respiratory state.

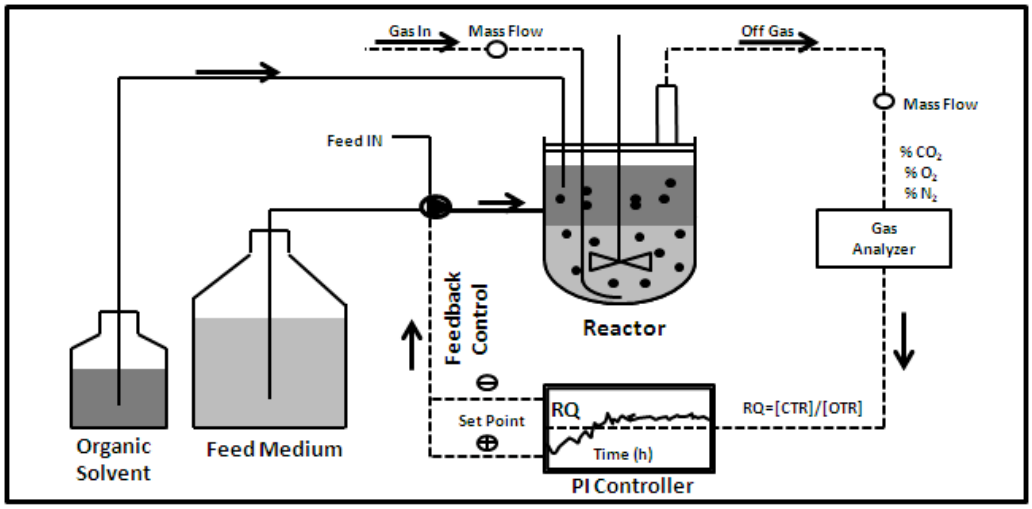


Figure 3.9. The configuration of the *in situ* product removal (ISPR) fed-batch RQ controlled cultivation process. A stirred tank reactor is operated in fed-batch cultivation mode as double phase system adding organic solvent on top of the culture and feeding concentrated culture medium. The product is continuously captured in the organic phase due to its high hydrophobicity. Feed delivery is designed with an exponential policy and it is controlled through a feed-back loop. Fermentation exhaust gas analysis allows the on line determination of the respiratory quotient, the instant RQ measurement modulated the feed addition by a PI controller in order to maintain the desired set point.

The result of the respiratory quotient control on the feed profile is reported in figure 3.10. The applied RQ feedback control method allows operating below the critical value alleviating the glucose repression resulting in a robust feed strategy. Optimal feed policy resulted in the maximum feed rate sustainable from the cell without byproduct formation. Extracellular sesquiterpene analysis resulted in the detection of four different products, beside the target compound α-santalene (representing 63 Cmol % of total sesquiterpene), the known side product FOH, the minor product *trans*-α-bergamotene and *E,E*-farnesene. Apparently, in the tested conditions SNS displayed different levels of specificity confirming a high degree of plasticity that is often reported for this class of enzymes. This method resulted in a process

capable of producing 12 Cmmol l^{-1} (163 mg l^{-1}) of α-santalene in 30 h with a product to substrate yield of 0.0037 Cmmol $Cmmol^{-1}$ and a productivity of 0.023 Cmmol $gDCW^{-1}$ h^{-1}.

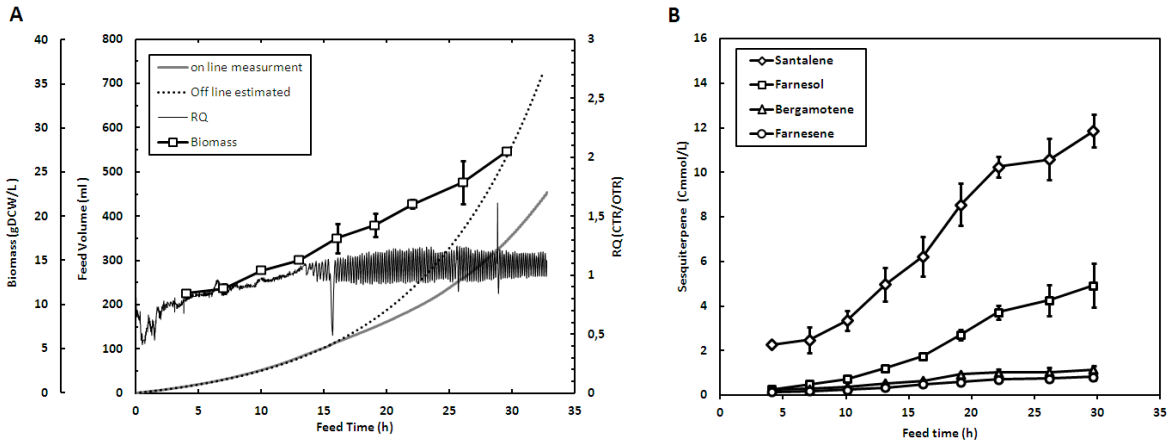


Figure 3.10. Development of an RQ based feed-control ISPR aerobic glucose limited fed-batch cultivation. Production phase of sesquiterpene producing *S. cerevisiae* strain SCIGS24. On-line measurement of CTR and OTR allows instantaneous calculation of RQ value allowing to control the feed addition regulating the fed-batch was performed using glucose with a concentration of 7.4 Cmol l^{-1} (222 g l^{-1}) as a carbon source in the feed media. Profiles reported represent the time course of **(A)** biomass formation (gDCW), RQ value (CTR/OTR), comparison between exponential calculated feed profile and experimental value (ml) and **(B)** α-santalene, E,E-farnesol, trans-α-bergamotene and *E,E*-farnesol (Cmmol l^{-1}) product accumulation as function of feeding time. Data presented are representative of three independent cultures; the error bars represent the standard deviation for three independent cultivations

3.4.3 Effect of ethanol as alternative carbon source to increase the precursor pool

The C_2 carbon ethanol represents an attractive carbon source for secondary metabolite production. However, because the raw material is often the dominating operative cost in industrial bio-production of value-added chemicals (Otero *et al.,* 2007) - although the market price of ethanol decreased substantially due to the advent of bio-ethanol - a fully ethanol based process would probably not be commercially valuable. Yeast *S. cerevisiae* has the ability to simultaneously co-consume glucose-ethanol under fully respiratory conditions (Geurts *et al.,* 1980). The ratio between the two C-sources determines change in the metabolic flux in the central carbon metabolism. If it is maintained below limits (0.57:0.43 Cmol $Cmol^{-1}$) glucose would be employed from the cell mainly for biosynthesis whereas all the cytosolyc acetyl-CoA would be derived from ethanol that would then be used in the TCA cycle (de-Jong-Gubbels *et al.,* 1995; van Gulik *et al.,* 1995). A mixed glucose/ethanol feed was applied to the previously designed fed batch process. Under this condition sesquiterpenes are obtained directly from the cytosolic acetyl-CoA produced from ethanol. Ethanol has a positive effect on process performances yielding a 0.076 Cmmol $gDCW^{-1}$ h^{-1} total sesquiterpene productivity equal to 49% increase compared to the process with glucose alone and a final α-santalene titer of 14.2

Cmmol l^{-1} (193.4 mg l^{-1}). Using this multiple carbon source strategy it was possible to use ethanol as direct precursor for sesquiterpene and glucose for biomass intermediate biosynthesis and increase the supply of acetyl-CoA precursor as a rapid approach to increase the final product titer.

3.4.5 Double phase chemostat as tool for study metabolically engineered strains

Although fed-batch is by far the favored fermentation process in industrial production, continuous cultivation modes find application in several industrial bioprocesses (e.g. insulin production). Among the advantages offered by a chemostat system is the possibility to precisely compare the productivities of selected genetically engineered strains under well controlled constant growth condition and to explore the effect of specific growth rate independently of other cultivations parameters. Combining chemostat cultivation with ISPR would be an effective way to evaluate different strains and also a potential scaffold for an industrial process. This system allows continuous recovery of the product in the fermenter effluent from the selected organic phase which can subsequently be recycled, regenerated and reused in the same process for prolonged time of cultivation (Fig 3.11).

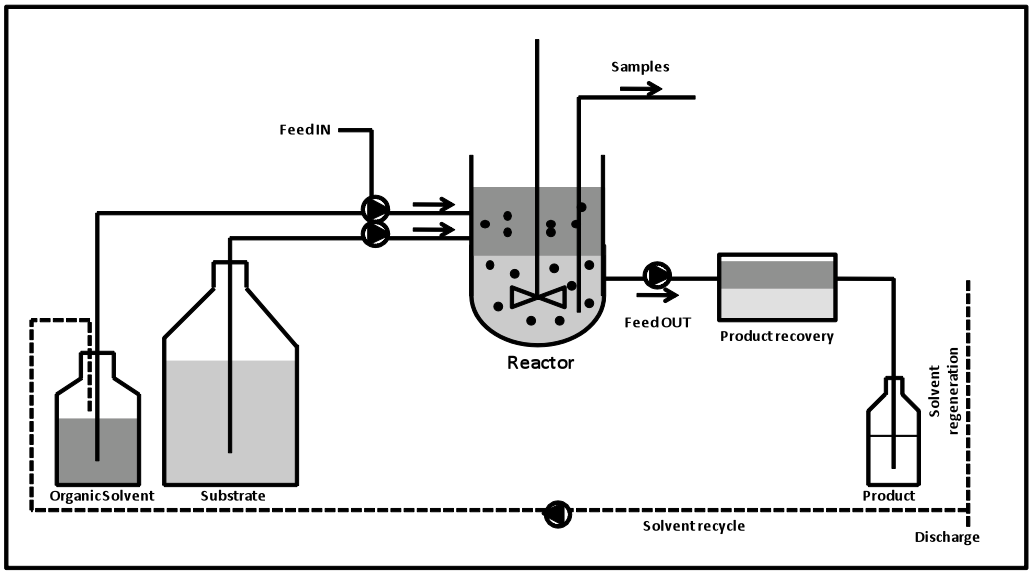


Figure 3.11. Set-up of the *in situ* product removal (*ISPR*) chemostat cultivation process. A stirred tank reactor is operated in continuous cultivation mode as double phase system feeding culture medium and organic solvent. The product is continuously captured in the organic phase due to its high hydrophobicity. In an integrated downstream step the two phases of the effluent are partitioned in a settler. Subsequently, the product is recovered from the organic phase, which can then be further recycled in the same process. The exhausted medium is discarded.

The effect of the specific growth rate that at these conditions is equal to the dilution rate (D) on the sesquiterpene production of the engineered mutants was investigated in this study (Fig

3.12). Increasing the dilution rate (D=0.05 h^{-1} -> D=0.1 h^{-1}), led to essentially unchanged productivity and yield of the control strain (SCIGS28) suggesting that at higher dilution rates a limitation of the plant synthase in efficiently draining the FPP precursor from the MVA pathway when the FPP node was not manipulated to increase FPP availability. In the engineered strains (SCIGS29 & SCIGS30) instead a slightly reduced α-santalene yield and a double productivity was obtained corresponding to 0.041 and 0.043 Cmmol $gDCW^{-1}$ h^{-1}. This showed a clear dependency of the productivity on the specific growth rate. The productivity level obtained appears to be linearly correlated with the dilution rate employed pointing to a direct relation between the specific growth rate and the flux through the MVA pathway .The same proportion was maintained between α-santalene and the side product FOH under the two conditions indicating that the distribution of the excess of FPP precursor between the two products appears to be independent of the specific growth rate. This result suggests the hypothesis that once a threshold level of intracellular flux toward FPP is reached the thermodynamically favourable endogenous dephosphorylation starts to compete with the catalytic capacity of *SNS* leading to FOH accumulation. On the other hand, the unchanged santalene yield coupled with high productivity achieved at higher dilution rates suggests that *SNS* was not fully saturated at low dilution rate and there was an excess activity to cope with higher FPP fluxes. This points out that FOH formation is not only a direct consequence of limited *SNS* activity but other cellular mechanisms are likely involved. Surprisingly, the highly engineered strains (SCIGS31, SCIGS24 & SCIGS25) were unable to grow at the higher specific growth rate applied. It is noteworthy that comparable sesquiterpene productivity was achieved in the strain not fully engineered simply by increasing the operational dilution rate whereas the fully engineered strains were washed out when the same condition were imposed. Detailed analysis reveals that the introduced genetic modifications profoundly affect the strain physiology, in particularly the carbon flux distribution around the PDH bypass node, possibly affecting cytosolic acetyl-CoA availability and its redistribution into the engineered MVA pathway. Further analyses are necessary to elucidate factors leading to this particular physiological state that is most likely linked to the inability of the fully engineered mutant to sustain growth at higher dilution rates.

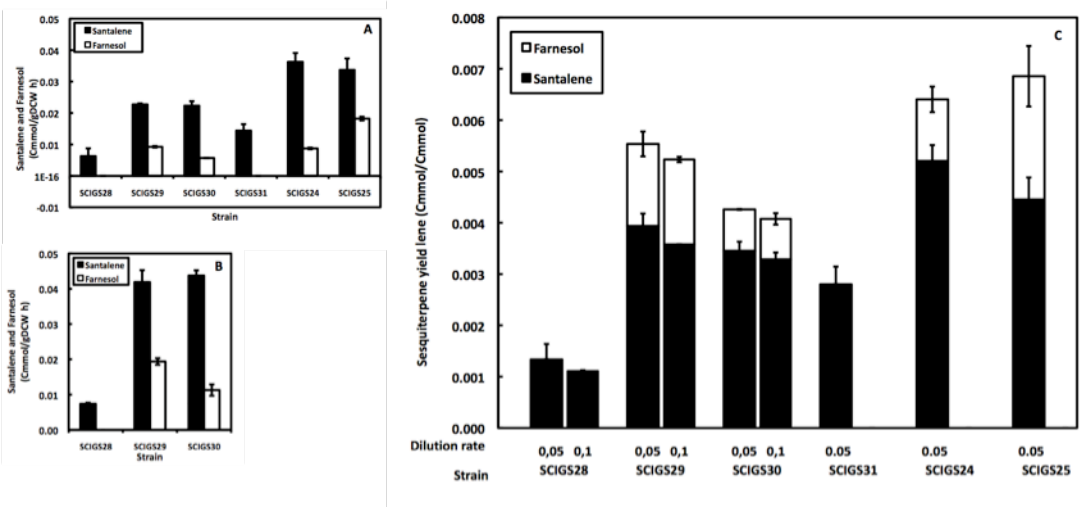


Figure 3.12. Sequiterpene yield and productivities in a two-phase partitioned glucose-limited aerobic chemostat. **(A)** α-santalene and farnesol production rate in Cmmol (g biomass)$^{-1}$ h^{-1} of strains SCIGS28 (*tHMG1↑*), SCIGS29 (+ P_{HXT1}*-ERG9, lpp1Δ*), SCIGS30 (+ *dpp1Δ*), SCIGS31 (+ *gdh1Δ*), SCIGS24 (+ *ERG20↑, GDH2↑*), SCIGS25 (+ *upc2-1↑, tHMG1↑*) cultivated at dilution rate D=0.05 h^{-1} and **(B)** strains SCIGS28, SCIGS29 and SCIGS30 cultivated at dilution rate D=0.1 h^{-1}. **(C)** α-santalene and farnesol yield in Cmmol (Cmmol glucose)$^{-1}$ of strains SCIGS28, SCIGS29, SCIGS30, SCIGS31, SCOGS24 and SCIGS25 cultivated at dilution rate D=0.05 h^{-1} and strain SCIGS28, SCIGS29 and SCIGS30 cultivated at D=0.1 h^{-1}. The C-molar weights of α-santalene and farnesol are, respectively, 13.62 and 14.82 g Cmol^{-1}. Error bars represent the standard deviation from three independent cultivations.

3.5 Intracellular product accumulation and potential toxicity

The previously shown dependency of the productivity on the growth rate was reflected in the inability to sustain a high dilution rate during chemostat cultivation and the impossibility to maintain a constant growth rate during the fed-batch process. This affected the product formation rate resulting in a suboptimal process. In order to investigate the possible causes of the observed growth impediment the intracellular content of sequiterpene was assayed. Only two of the four sesquiterpenes produced by the cell, α-santalene and α-*trans*-bergamotene, were detected intracellularly with α-santalene as the main compound. Because cyclic terpenes can accumulate in the membrane (Sikkema *et al.,* 1994) it is reasonable to assume that this structural difference might be the reason of the yeast capacity to specifically retain these compounds inside the cell. The export of α-santalene from the cell seems to be the main cause of the inhibitory effect that limits its productivity. Up to date the mechanism of secretion of hydrocarbons are not known. The saturation kinetics on time observed for α-santalene might suggest that yeast utilizes a specific transporter to secrete this compound. Transcriptome analysis performed in presence of high sesquiterpene concentration was characterized by up-regulation of multidrug transporters related genes in particular a pleiotropic drug resistant (PDR) network was over-expressed, consistent with several parallel studies of yeast terpene producer

(Verwaal *et al.,* 2010; Ro *et al.,* 2008). Excessive α-santalene intracellular accumulation and an intra-membrane derived toxicity related effect due to the loss of membrane integrity (Sikkema *et al.,* 1995) can explain the observed physiology.

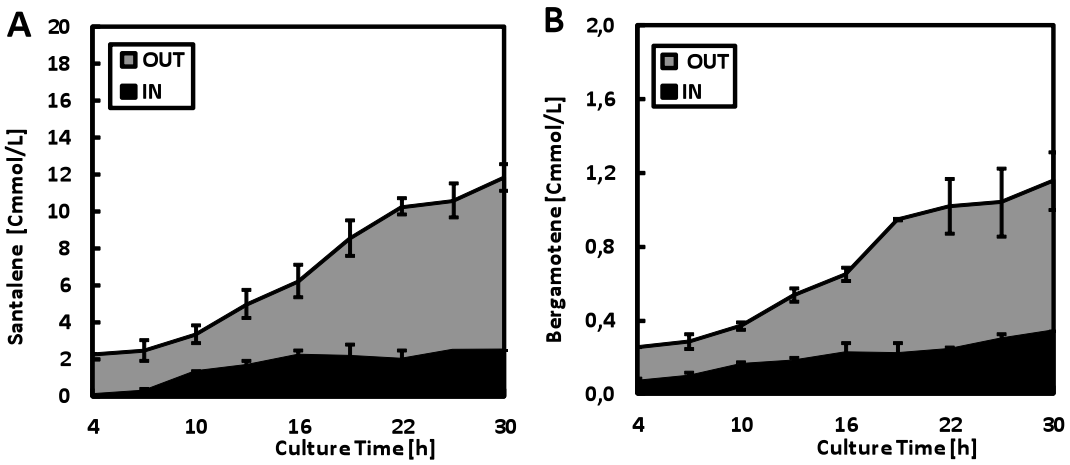


Figure 3.13. Typical extracellular (grey area) and intracellular (black area) sesquiterpene accumulation profiles during RQ based double phase aerobic glucose or glucose/ethanol limited fed batch cultivation of strain SCIGS24. (A) α-santalene; (B) *trans*-α-bergamotene. Typically the intracellular fraction of α-santalene and *trans*-α-bergamotene were between 15-18% and 17-29% of the total amount detected respectively. The two compounds displayed different intracellular accumulation profile respect to the extracellular concentration; intracellular α-santalene reached saturation around the value of 2 Cmmol·. Error bars represent the standard deviation from three independent cultivations.

3.6 Expanding the substrate utilization range—toward a biorefinery

Techno-economical analysis of α-santalene production reveals how the raw material is often the dominating operative cost and the design of a cost effective *S. cerevisiae* production platform would rely on the accessibility to inexpensive carbon sources. Common industrial carbon sources often consist of sugar mixtures (Olsson *et al.,* 2000 Dahod *et al.,* 2010). Lignocellulosic biomass is among the most promising feedstocks that can provide sugar substrates for bio-based production (Stephanopoulos, 2010). Xylose is one of the main components of lignocellulosic feedstocks and is the second most abundant monosaccharide after glucose. Due to the inability of wild type *S. cerevisiae* to efficiently utilize xylose as a sole carbon source, large efforts have been invested form the scientific community to expand the substrate range capability of *S. cerevisiae* (Van Vleet *et al.,* 2009; Matsushika *et al.,* 2009; van Maris *et al.,* 2007; Hahan-Hägerdal *et al.,* 2007). Development of a cell factory for broader biomass-coupled production would be favored without loss of carbon to overflow metabolites (ethanol, glycerol, xylitol) particularly in the case of growth associated production processes like α-santalene production that require simultaneous formation of biomass and target product. Here, *S.*

cerevisiae was metabolically engineered to consume xylose as an exclusive substrate maximizing carbon flux to biomass production. Through the combination of (i) genetic modifications (plasmid introduction) and (ii) application of selective pressure (shake flask repetitive cultivations). (i) The oxido-reductive pathway of the native xylose-metabolizing yeast *Pichia stipitis* consisting of three essential enzymes for xylose uptake (xylose reductase XR, xylitol dehydrogenase XDH and xylulokinase XK) was reconstructed through plasmid-based expression in *S. cerevisiae*. (Fig 3.14). The constructed mutant demonstrated the ability to consume xylose aerobically although with a very poor growth.

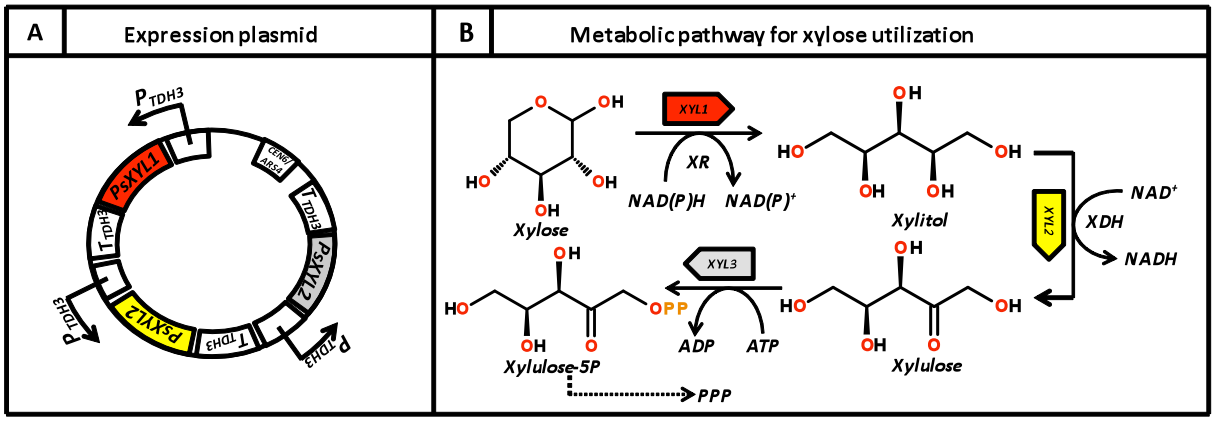


Figure 3.14. Reconstruction of *P. stipitis* xylose assimilation pathway in *S. cerevisae*. **(A)** centromeric plasmid pRS314-X123 for expression of *XYL1* encoding xylose reductase (XR), *XYL2* encoding xylitol dehydrogenase (XDH) and *XYL3* encoding xylulokinase (XK) all derived from *P. stipitis* cloned under the glyceraldehyde-3-phosphate dehydrogenase (*TDH3*) constitutive promoter and terminator (Haiying *et al.,* 2007). **(B)** Xylose utilization occurs in three steps; first xylose is reduced to xylitol via XR (NADPH primarily consuming reaction), then xylitol is oxidized to xylulose via XDH (NADH producing reaction), in the final conversion xylulose is phosphorylated to xylulose-5-P via XK (ATP consuming reaction); xylulose-5-P is further chandelled into glycolytic intermediates via the pentose phosphate pathway (PPP).

(ii) Successively, a directed evolution strategy was applied to select a spontaneous mutant with improved xylose utilization rate using a repetitive batch cultivation technique. A mutant with higher specific growth rate on xylose was selected by serial transfer of cells in batch shake flask cultivation with minimal medium supplemented with xylose as the sole carbon source. This approach targeted strain selection based on biomass formation rate directly coupled to xylose consumption rate. A strain capable of rapid growth and fast aerobic xylose metabolism was obtained in 21 days of selection period highlighting the efficiency and simplicity of the methods and the high level of adaptability of *S. cerevisiae* when strong selective pressure is applied at laboratory conditions (Fig 3.16). A total of 74 cell generations were used to obtain a 15-fold increase in xylose consumption and a 52-fold increase in biomass production for the final selected evolved strain The rapid adaptation observed confirm how early stage evolution plays a critical role in the adaptation process and show how adaptation often occurs in few steps and

often involve only a limited number of mutations. The evolved strain exhibited a clearly respiratory response toward xylose, its consumption was entirely oxidative with a high carbon fraction converted to biomass (62% Cmol $Cmol^{-1}$) and negligible amount of byproducts.

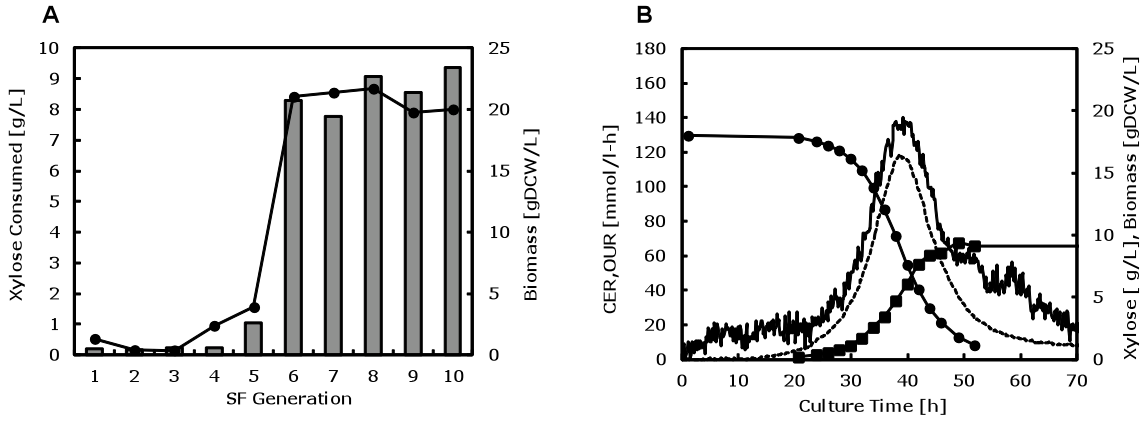


Figure 3.16. (A) Comparison in xylose consumption (bars) and biomass formation (line) during repetitive growth of *S. cerevisiae* in shake flask (SF) cultures on synthetic medium with 20 g l^{-1} xylose. Shake flask generation represents the number of specific shake flasks in series of repetitive cultivation performed to select for mutants with higher specific growth rate and xylose utilization rates. The serial cultivation covers 10 cycles for a period of 500 h (21 days) after four batch cultures an improvement in xylose consumption was detected. **(B)** Time course of aerobic batch culture on defined minimal medium supplemented with 20 g l^{-1} xylose of the evolved strain, carbon evolution rate (CER) (dashed line), oxygen uptake rate (OUR) (solid line) (mM h^{-1}), and xylose (circle), ethanol (diamond) (g l^{-1}); and biomass (square) (gDCW l^{-1}) concentrations as functions of cultivation time. Xylose was completely consumed within 60 h with a specific growth rate of 0.18 h^{-1} and biomass and carbon dioxide as the major fermentation product. Data represent the average of three independent cultures.

Transcriptional profiling was employed to further elucidate the observed physiology. Transcriptome data support the physiological observation at the global and metabolic level. Over-represented gene ontology (GO) process terms in the evolved strain were related to function or features linked to respiratory processes. Transcription factor (TFs) enrichment analysis identified factors primarily involved in carbon catabolite repression response including transcriptional activators of genes involved in non-fermentative metabolism (Fig 3.17). Analysis of gene expression at the metabolic pathway level reveals that a strong up-regulated glyoxylate pathway plays an important role in enabling the observed respiratory metabolism. As extension of the glyoxylate pathway up-regulation of cytosolic isocitrate dehydrogenase likely provides a source of NADPH required to satisfy the biomass requirement. Xylose consumption in *S. cerevisiae* through the oxido-reductive *P. stipitis* pathway has been often dominated by extensive xylitol overflow ascribed as direct result of a redox imbalance of NAD(P) cofactors between the XR and XDH and referred as the major drawback of the XR-XDH strategy. Absence of xylitol formation in the evolved mutant under oxidative conditions may be interpreted as a result of complete xylitol oxidation.

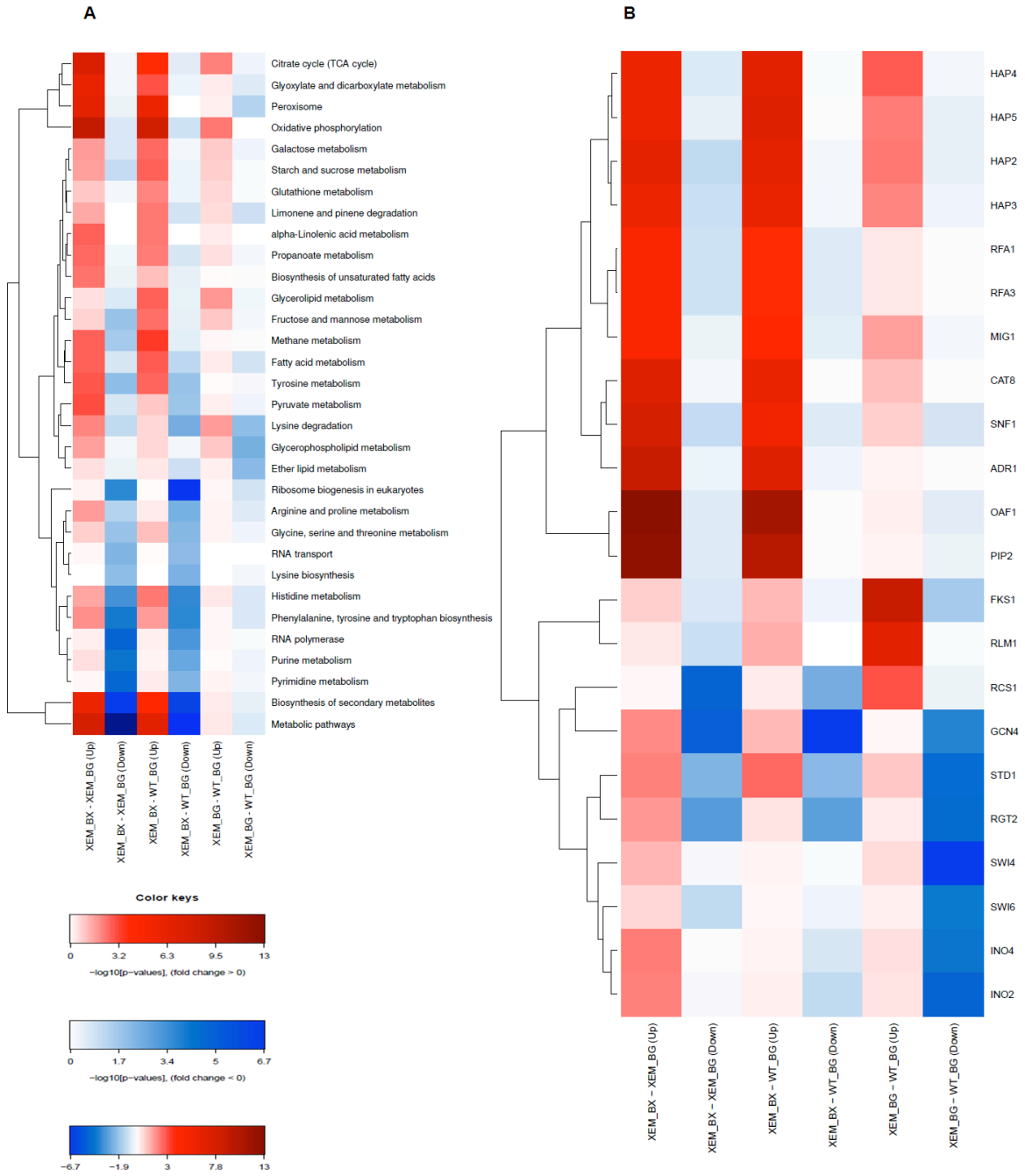


Figure 3.17. Integrated analysis of gene ontology (GO) process terms **(A).** "Glyoxylate and dicarboxylate metabolism", "peroxisome" and "oxidative phosphorylation" were among the most represented functional categories with respect to metabolism of the evolved strain on xylose compared to un-evolved or evolved strain growing on glucose. Transcription factor (TF) analysis **(B).** The main carbon catabolite repressor regulator SNF1 and several of its known targets, the carbon source responsive ADR1 and the four subunits of the global respiratory regulator HAP were the identified over-represented TFs in the evolved strain on xylose compared to un-evolved or evolved strain growing on glucose. The cluster frequency is presented on the y-axis. Compared conditions are the evolved strain cultivated on batch xylose (XEM_BX), evolved strain cultivated on batch glucose (XEM_BG) the unevolved strain cultivated on batch glucose (WT_BG). Color key indicates the different expression in log-fold change ($P_{adjusted}$ <0.01).

The result obtained in this study suggest that up-regulation of isocytrate deydrogenase ensures sufficient NADPH production necessary for the *P. stipitis* NADPH-preferring XR to drive the xylose catabolism, whereas the NADH surplus produced by XDH is reduced through the respiration eliminating the $NADP^+/NAD^+$ imbalance.

CHAPTER 4 Conclusions & Future Prospects

4.1 Conclusions

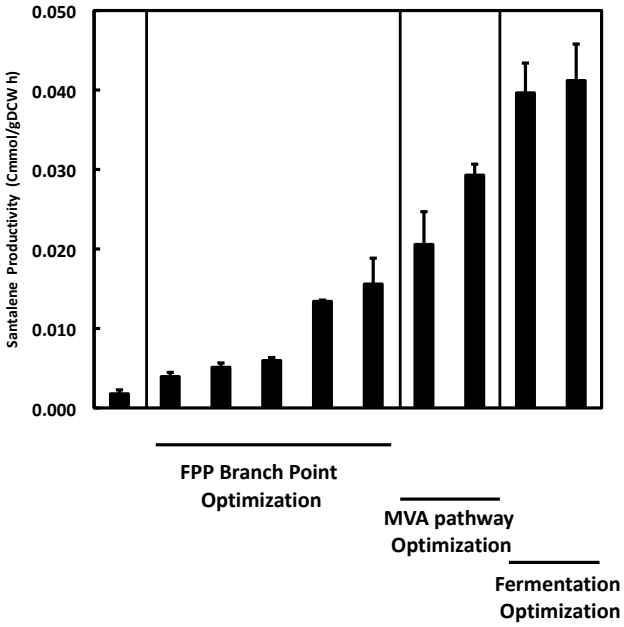


Figure 4.1 α-santalene productivity progression (Cmmol $gDCW^{-1}$ h^{-1}) achieved during this study compared to the reference strain minimally engineered (*Chapter 3.1.1*), applying different strategies: FPP branch point optimization (*Chapter 3.2*) MVA optimization (*Chapter 3.3)* and fermentation optimization (*Chapter 3.4*)

The main objective of this research was the construction of an efficient *S. cerevisiae* cell factory capable of producing industrially relevant titers of the sequiterpene α-santalene, a precursor of commercially interesting compounds. A rationally designed metabolic engineering approach was employed to addresses the feasibility of sesquiterpene α-santalene production in *S. cerevisiae.* The sesquiterpene production was optimized using a novel strategy to dynamically modulate gene expression around the key FPP branch point. Using this strategy it was possible to divert carbon flux toward the desired compound. Subsequently, a multistep metabolic engineering approach to increase precursor and cofactor supply was used to further optimize isoprenoid accumulation. This involved introducing genetic modifications that enabled channeling an increased flux through the isoprenoid pathway. This was combined with fermentation optimization and an integrated product recovery process developed improving yield, productivity and titer (Fig 4.1). Finally, the substrate range of the producing platform was expanded through a complementary evolutionary engineering approach to obtain a cell factory capable of exclusively consuming xylose as a carbon source. The results presented in this study represent a gateway towards the

creation of an industrial microbial platform that can be applied to the production of an array of sesquiterpene products.

4.2 Perspectives

The future feedstock of chemical manufacturing will not be oil but glucose. As we are approaching the "*artificial biotechnology era*" (Gibson *et al.,* 2010; Gibson 2008; Lartigue *et al.,* 2009), one can imagine a future where specialized "microrefineries" will produce a portfolio of renewable chemical compounds from waste raw material, accomplishing the society the demand to produce via a responsible and sustainable way and hereby reduce the production cost.

Our society is reaching a turning point; an intriguing question to ask for the future is if the creation of custom build-cell factories for the production of a wide spectrum of strategic chemicals would be a feasible realistic goal in a near future. The answer is not simple and a number of challenges lie ahead. The continuous advance in metabolic engineering and its complementary related disciplines allow a high degree of manipulation on model industrial microorganism producing spectacular examples of successful biological production of needed chemicals (Westfall *et al.,* 2011; Ajikumar *et al.,* 2010). However, the ability to rapidly engineer microbial cells is still in its infancy and most of their immense potential remains unexplored. The time consuming development of a toolbox of genetic components to control gene expression has slowed down the capacity to manipulate cell features, due to technology limitations and the unpredictable complexity of the cell system. Although the scientific community strives to develop open technology platforms for designing and cataloging biological parts (Hayes *et al.* 2001) that culminated in the creation of registries of "standard" biological parts for synthetic biology, like for example "BioFAB" (Biofab.org) and the "Registry of Standard biological Parts" (partsregistry.org) collecting several thousand of genetic elements (Baker *et al.,* 2006), most of these parts are still uncharacterized or incompatible and unreliable (Kwok, 2010; Kelly *et al.,* 2009) resulting mainly in host-dependent application and therefore difficult to use universally. Additionally, several of the essential design principles for engineering microbes are still missing (Morton 2005; Knight 2005). Similarly, it would be necessary to acquire the same high level of standardization for mathematical design of biological models, in order to empower accuracy and robustness in the predictive capacity of modeling; a notable example in the effort to uniform the rules of model language is the Systems Biology Markup Language (SMBL) (Hucka *et al.,* 2003).

As every rapidly moving area the current efforts in microorganism manipulation could rise a number of ethical, safety, legal and security issues (not discussed here) that could limit near

term commercial opportunities and compromise their marketing success (Dana *et al.,* 2012; Tait *et al.,* 2012; Erickson *et al.,* 2011; Palombi 2009, Venter *et al.,* 2004). Nevertheless, steady progress has been made in pursuing the formidable challenge of bio-manufacturing compounds. The time is ripe, the field is clearly moving from the discovery phase to the application and implementations, and the achievements accomplished so far are encouraging, even though it is clear that major efforts are still required before biological engineering to provide tailored products will became routine.

The current approaches mainly rely on optimization or transfer of a specific activity or an existing pathway between the natural producers selected production organisms, this approach is limited by the number of modification that the engineered strain can tolerate. However, the limits in the creation of custom-build cell factories are only temporary. In the future, the scale down in the price of gene synthesis (today the cost for DNA fragment is less than $0.1/Mb) (Wetterstrand, 2012) would allow to bypass the traditional time consuming DNA manipulation routinely replacing it with automated synthesis (Carr *et al.,* 2009). The increased effort from the scientific community to develop engineered toolsets and cataloged parts (Baker *et al.,* 2006) would allow to fast design and construct cells with new desired characteristics and features, resulting in a more standardized and automated strain engineering process that would lead to a more systematic practice of metabolic engineering rather than the specific case-by-case engineering which we are nowadays assisting. Inspired by the natural cooperative of microbial population recent advances in cell-to-cell communication (Weiss et al., 2005; You *et al.,* 2004) bright light to the opportunity to engineer microbial consortia rather than single organism. Albeit in its young phase co-culture engineering may help in performing multi-task processes in a more efficient manner than a single organism culture (Brenner *et al.,* 2008).

To conclude it is worth to mention how the pioneering work in genome engineering conducted at the J. Craig Venter institute (Rockville, MD, USA) that recently lead to the extraordinary breakthrough in synthetic engineering (Gibson *et al.,* 2010), paved the way to a radical new approach consisting of a *de novo* artificially assembled cell with an entirely *in silico* designed and synthetically synthesized genome that could be programmed to produce fuels, chemicals and medicine. Another interesting approach holding the promise to impact in the future the chemical industry is the use of cell-free systems. Albeit still in its early stage this technology could offer a flexible way to produce chemicals without using intact cells, and bypassing the constraints related to the cell wall (Hodgman *et al.,* 2012). These inspirational goals could reshape in a long term prospective the metabolic engineering field.

While challenges remain, metabolic engineering has and would continue to have a tremendous impact in our society; the stage for the *green technology era* based on engineered biosystems is set.

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PAPER I

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

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

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ABSTRACT

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

1. Introduction

The production of plant sesquiterpenes by microbial fermentation is an environmentally friendly and attractive alternative to the commonly used chemical synthesis and plant extraction (Chang and Keasling., 2006; Rohlin et al., 2001; Ajikumar et al., 2008; Kirby and Keasling, 2009, Wang et al., 2011). Sesquiterpenes are mainly used by the chemical industry for production of fragrances and aroma chemicals (Daviet and Schalk, 2010). They represent a diverse class of secondary metabolites, the precursors of which are natively present in the metabolic network of *Saccharomyces cerevisiae* (Förster et al., 2003). Sesquiterpenes are naturally produced in *S. cerevisiae* through the mevalonate (MVA) pathway from multiple condensation of isopentenyl diphosphate (IPP) and dimethyllallyl diphosphate (DMAPP) leading to farnesyl diphosphate (FPP), the universal precursor unit of all sesquiterpenes (C_{15}) (Maury et al., 2005; Withers and Keasling, 2007). Nowadays, most fragrance compounds are produced via plant and microbe extraction (Howes et al., 2004), and by total chemical synthesis or semi-synthesis (Corey et al., 1957; Julia, 1976; Janssens et al., 1992). Traditional methods of extraction are limited by low yields and high costs. Here, microbial biosynthesis offers several advantages: it (i) avoids formation of racemic mixtures providing enantiomerically pure products through enzymatic biocatalysis, (ii) reduces process costs using inexpensive sugar based carbon sources, (iii) increases sustainability avoiding harvesting and extraction from natural sources and thus reducing environmental footprint, (iv) increases yield and productivities using genetic manipulation of the heterologous hosts and (v) is compatible with scalable high density fermentation processes.

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

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Nomenclature

FPP	Farnesyl diphosphate
FOH	(*E,E*)-farnesol
SQS	squalene synthase
HMGR	3-hydroxy-3-metyl-glutaryl-coenzyme A reductase
MVA	mevalonate
SanSyn	santalene synthase gene
Log *P*	logarithm (base 10) of partition coefficient

capable of reaching relevant titers and productivities of α-santalene during an optimized fermentation process is constructed. First, yeast was engineered to produce α-santalene by introducing a heterologous santalene synthase gene (*SanSyn*) derived from *Clausena lansium* (wampee) that catalyzes the conversion of FPP to α-santalene (Schalk, 2011). Metabolic engineering has been extensively applied to manipulate metabolic fluxes and enhance the microbial production of sesquiterpene compounds (Ro et al., 2006; Shiba et al., 2007; Takahashi et al., 2007; Asadollahi et al., 2008, 2009, 2010; Kirby et al., 2008; Ma et al., 2011). In order to increase the precursor pool for isoprenoid synthesis enabling efficient conversion to the target compound α-santalene, two of the main regulatory steps of the MVA pathway catalyzed by 3-hydroxyl-3-methyl-glutaryl-CoA reductase (HMGR) and squalene synthase (SQS) were optimized by introducing genetic modifications that enable to channel the flux towards α-santalene synthesis. The conversion of 3-hydroxyl-3-methyl-glutaryl-CoA into mevalonate catalyzed by HMGR is one of the most studied key regulatory steps in the MVA pathway and is considered the main flux controlling step (Scallen and Sanghvi, 1983; Basson et al., 1986). In yeast, two isoforms of HMGR exist encoded by genes *HMG1* and *HMG2* (Basson et al., 1986) and their activity is subject to tight regulation including feedback regulation and cross-regulation (Hampton and Rine, 1994; Hampton et al., 1996; Brown and Goldstein, 1980). HMGR is composed of an interspecies conserved catalytic domain and a variable membrane anchoring region also referred to as sterol sensing domain (SSD). Hmg1 regulation acts at the level of the SSD domain through a complex mechanism leading to protein degradation (Nielsen, 2009). Over-expression of a truncated form of Hmg1 lacking the SSD domain bypasses this post-transcriptional control and results in a constitutively active non-membrane bound form (Donald et al., 1997; Polakowski et al., 1998). This strategy has been extensively used to increase the flow through the MVA pathway in order to produce isoprenoid derived compounds (Jackson et al., 2003; Ro et al., 2006; Kirby et al., 2008; Asadollahi et al., 2009, 2010; Farhi et al., 2011). The second key step in the MVA pathway is represented by SQS, as this controls the flux of FPP towards sterols or non-sterol sesquiterpenes. FPP is a pivotal intermediate as it is a common precursor for production of essential compounds such as dolichol, ubiquinone, isoprenylated proteins and ergosterol (Daum et al., 1998) and its intracellular concentration is tightly regulated at different levels (Goldstein and Brown, 1990). During normal growth conditions, most of the FPP is converted into ergosterol due to the fact that the cellular demand for sterols is greater compared to the demand for non-sterol FPP derived compounds (Kennedy et al., 1999). Strategies to increase sesquiterpene production based on the disruption of the main FPP consuming reaction catalyzed by squalene synthase produced lethal mutants and restoration of viability requires ergosterol supplementation resulting in an economically not feasible process for industrial purposes (Takahashi et al., 2007). Here, we undertook a genetic engineering approach to balance SQS activity during a fermentation process. Previous attempts to control SQS (encoded by *ERG9*) expression diverting the flow from sterol components to desired FPP-derived compounds were mainly based on replacement of the native *ERG9* promoter with the methionine-repressible *MET3* promoter (Ro et al., 2006; Paradise et al., 2008; Asadollahi et al., 2008, 2009, 2010). However, industrial scale development of this system is limited by the cost of the repressor and its possible consumption by the cell. Instead, we aimed at coupling *ERG9* expression to the glucose concentration in the media.

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

Table 1
S. cerevisiae strains used in this study.

Strain	Genotype	Plasmid	Reference
CEN.PK113-5D	*MAT***a** *MAL2-8^c SUC2 ura3-52*		P. Kötter, University of Frankfurt, Germany
YIP-M0-04	*MAT***a** *MAL2-8^c SUC2 ura3-52* P_{ERG9}Δ::*kanMX*-P_{MET3}		Asadollahi et al., 2008
SCICK00	*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{MET3}		This study
SCICK01	*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{HXT1}		This study
SCICK03	*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{ERG9}		This study
SCICK05	*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{ERG9} YMRWdelta15Δ::P_{HXT2}-*ERG9*as-T_{CYC1}-*loxP*		This study
SCICK06	*MAT***a** *MAL2-8^c SUC2 ura3-52*::pSF011-P_{HXT1}		This study
SCICK08	*MAT***a** *MAL2-8^c SUC2 ura3-52*::pSF011-P_{TEF1M2}		This study
SCICK09	*MAT***a** *MAL2-8^c SUC2 ura3-52*::pSF011-P_{HXT2}		This study
SCICK10	*MAT***a** *MAL2-8^c SUC2 ura3-52*::pSF011-P_{MET3}		This study
SCICK11	*MAT***a** *MAL2-8^c SUC2 ura3-52*::pSF011-P_{ERG9}		This study
SCICK12	*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{HXT1}	pICK01	This study
SCICK13	*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{ERG9}	pICK01	This study
SCICK14	*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{ERG9} YMRWdelta15Δ::P_{HXT2}- as*ERG9*-T_{CYC1}-*loxP*	pICK01	This study
SCICK15	*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{MET3}	pICK01	This study
SCICK16	*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP dpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{HXT1}		This study
SCICK17	*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP dpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{HXT1}	pICK01	This study

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2. Materials and methods

2.1. Strains and maintenance

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

2.2. Plasmid construction

To construct integrative plasmids carrying the *lacZ* gene under control of different promoters plasmid pSF011 (Partow et al., 2010) was used. Promoters P_{ERG9}, P_{MET3}, P_{HXT1}, and P_{HXT2}, and were PCR amplified from genomic DNA of *S. cerevisiae* CEN.PK113-5D, and P_{TEF1M2} was amplified from plasmid p416TEF1M2 (kindly provided by G. Stephanopoulos, Massachusetts Institute of Technology, Cambridge, MA, USA) using primers 1 to 6 and 50 to 53 (Table 2), restricted with *Not*I/*Hin*dIII and ligated into *Not*I/*Hin*dIII cut pSF011 resulting in formation of plasmids pSF011-P_{ERG9}, pSF011-P_{MET3} and pSF011-P_{HXT1}, pSF011-P_{HXT2} and pSF011-P_{TEF1M2}, respectively.

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

Table 2
Oligonucleotide primers used in this study[a].

No.	Name	Sequence (5′→3′)
1	HindIII_ERG9_f	CAACAAAAGCTTCCCATCTTCAACAACAATACC
2	NotI_ERG9_r	CAACAAGGCGGCCGCTGTGTGTGTGTGATATGTGACGT
3	HindIII_MET3_f	CAACAAAAGCTTGTATAAGGTGAGGGGGTCCACAG
4	NotI_MET3_r	CAACAAGGCGGCCGCGTTAATTATACTTTATTCTTGTTATTATTATACtttc
5	HindIII_HXT1_f	CAACAAAAGCTTTGCAGGTCTCATCTGGAATATAATTCC
6	NotI_HXT1_r	CAACAAGCGGCCGCGATTTTACGTATATCAACTAGTTGACGATTATqG
7	santa_f	GTTGTTGCGGCCGCAAAACAATGTCAACTCAACAAGTTTCATCAG
8	santa_r	GTTGTTTTAATTAACTAATCGTCAAGCTTAACGGG
9	tHMG1_up	GTTGTTGGATCCAAAACAATGGCTGCAGACCAATTGGTG
10	tHMG1_down	GTTGTTGCTAGCTTAGGATTTAATGCAGGTGACG
11	Int_check_f	GGTTCCGCGCACATTTCCCC
12	Int_check_r	GGAACTCTTGTTGTTCTTTGGAG
13	LacZ_r	GGGATCTGCCATTGTCAGAC
14	LPP_up_f	AAGGATGATCTCTGTCATGG
15	LPP_up_r_tail	GATCCCCGGGAATTGCCATGTGTTAGGGCAGCATTTATGC
16	LPP_dw_f_tail	GCAGGGATGCGGCCGCTGACGCACTCCAAGCGGACATTCAAG
17	LPP_dw_r	GAAGTATGTCTCTTTTCGCC
18	Pr-b-Kan′	CATGGCAATTCCCGGGGATCCCCTTAATATAACTTCGTATAATGTATGC
19	Kan3′int	CCATGAGTGACGACTGAATCCGG
20	Kan5′int	GCAAAGGTAGCGTTGCCAATG
21	dKan3′	GTCAGCGGCCGCATCCCTGCCGACTCACTATAGGGAGACCG
22	LPP1 verif_up	TAGTTGCCACGTGAAACCTGACAAC
23	LPP1 verif_dw	AATTTCATCGGTATTTTGGCTTCGG
24	loxP_ERG9_f	CGAAGTTATTAGGTGATATCAGATCCACTGCCCATCTTCAACAACAATACCG
25	ERG9d_r	GTCGTAGTCGTGGACGGTTTGC
26	loxP_HXT1_f	CGAAGTTATTAGGTGATATCAGATCCACTTGCAGGTCTCATCTGGAATATAATTCC
27	ERG9_Hxt1_r	GCTGCCTTCATCTCGACCGGATGCAATGCCAATTGTAATAGCTTTCCCATGATTTTACGTATATCAACTAGTTGACGATTATG
28	ERG9_loxP_f	GAGTGAACCTGCTGCCTGGCGTGCTCTGACTCAGTACATTTCATAGCCCAGTACGCTGCAGGTCGACAACC
29	loxP_r	AGTGGATCTGATATCACCTAATAACTTCG
30	Erg9_fr_f	CCTTGCTTACACAGAGTGAACCTGCTGCCTGGC
31	Erg9_fr_r	CTTCAGCTTCAAAGCTGCCTTCATCTCGACCG
32	DPP-1-fw	AGGGCACGTTATCAATTGT
33	DPP-1-rev	CAGCGTACGAAGCTTCAGAGAAACTCGTACTGAACCAAG
34	DPP-2-fw	GTGATATCAGATCCACTAGTACAGACATCATTTCGTCGA
35	DPP-2-rev	AACTTCTAAGGCTTTCGTGT
36	KanMx-1-fw	CTGAAGCTTCGTACGCTG
37	KanMx-1-rev	TCACCATGAGTGACGACTGA
38	KanMx-2-fw	TTCCAACATGGATGCTGAT
39	KanMx-2-rev	CTAGTGGATCTGATATCAC
40	Amp_fw	GTGGGTTACATCGAACTGGATC
41	Amp_rv	CATCCATAGTTGCCTGACTGC
42	14up_HXT2_f	GATTTGATGACTGTTTCTCAAAACTTTATGTCATTTTCTTACACCGCATATGATTCTACCGATGTAATACAAAAATG
43	ERG9as_HXT2_r	TTATTGAATTCCACAAATTGAAACTATGTTGCTTTATAAGTCTTTTTGTAA
44	ERG9as_f	GTTTCAATTTGTGGAATTCAATAA
45	CYC1t_ERG9as_r	TTCCTTTTCGGTTAGAGCGGATCTGGAAATTAGGACAGGGGC
46	ERG9as_CYC1t_f	GCCCCTGTCCTAATTTCCAGATCCGCTCTAACCGAAAAGGAA
47	loxP_CYC1t_r	GGTTGTCGACCTGCAGCGTACCTTCGAGCGTCCCAAAACCTT
48	loxP_f	GTACGCTGCAGGTCGACAACC
49	14down_loxP_r	GATAACCGCGAAGATTTATAATGGTTTATCGGTTGCATTTTCCATGAGTAAGTGGATCTGATATCACCTAATAACTTCG
50	HindIII_HXT2_f	CAACAAAAGCTTTTCTACCGATGTAATACAAAAATG
51	NotI_Hxt2_r	CAACAAGGCGGCCGCTATGTTGCTTTATAAGTCTTTTTGTAA
52	HindIIITEF1_M2_f	CAACAAAAGCTTGCACACACCACGGCTCTAAAG
53	NotI_TEF1_M2_r	CAACAAGCGGCCGCTTTTCTAGAAAACTTGGATTAGATTGC

[a] Restriction enzyme recognition sites are underlined.

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(Schalk, 2011), using primers 7 and 8, cut with *Not*I/*Pac*I and ligated into *Not*I/*Pac*I restricted vector pSP-G1 (Partow et al., 2010). Subsequently, *tHMG1* was PCR amplified using genomic DNA of *S. cerevisiae* CEN.PK113-5D as template and primers 9 and 10, cut with *Bam*HI/*Nhe*I and ligated into the same vector after restriction with the respective enzymes. This resulted in formation of expression plasmid pICK01.

2.3. Strain construction

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

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

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

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

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

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

2.4. Southern blot analysis

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

2.5. Media and growth conditions

For batch cultivations, a previously described (Verduyn et al., 1992) mineral salts medium was used consisting of the following (per liter): $(NH_4)_2SO_4$, 5 g; KH_2PO_4, 3 g; $MgSO_4 \cdot 7H_2O$, 0.50 g; Antifoam 289 (A-5551, Sigma-Aldrich, St. Louis, MO, USA), 0.05 ml; trace metals, 1 ml and vitamins, 1 ml. The trace metal solution consisted of the following (per liter): EDTA (sodium salt), 15.0 g; $ZnSO_4 \cdot 7H_2O$, 0.45 g; $MnCl_2 \cdot 2H_2O$, 1 g; $CoCl_2 \cdot 6H_2O$, 0.3 g; $CuSO_4 \cdot 5H_2O$, 0.3 g; $Na_2MoO_4 \cdot 2H_2O$, 0.4 g; $CaCl_2 \cdot 2H_2O$, 0.45 g; $FeSO_4 \cdot 7H_2O$, 0.3 g; H_3BO_3, 0.1 g and KI, 0.1 g. The pH of the trace metal solution was adjusted to 4.0 with 2 M NaOH prior to heat sterilization. The vitamin solution contained (per liter): biotin, 0.05 g; p-amino benzoic acid, 0.2 g; nicotinic acid, 1 g; Ca-pantothenate, 1 g; pyridoxine-HCl, 1 g; thiamine-HCl, 1 g and myo-inositol, 25 g. The pH of the vitamin solution was adjusted to 6.5 with 2 M NaOH. The vitamin solution was filter sterilized and stored at 4 °C. This medium was supplemented with 30 g/l glucose. The medium used for shake flask cultivation had the same composition as described above, but the $(NH_4)_2SO_4$ concentration was increased to 7.5 g/l, and the KH_2PO_4 concentration to 14.4 g/l. The glucose concentration was 20 g/l and the pH was adjusted to 6.5 prior autoclaving. The feed composition used for fed-batch cultivation had the same composition as described above, but the $(NH_4)_2SO_4$; KH_2PO_4; $MgSO_4 \cdot 7H_2O$, vitamin solution, and trace metal solution concentrations were increased 10 times; the glucose concentration was 200 g/l.

2.6. Inoculum preparation

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

2.7. Shake flask cultivation

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

2.8. Fed-batch mode in shake flasks

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

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

2.9. Fed-batch operation

The aerobic fed-batch process was performed in 2.5 l Applikon vessels (Applikon, Schiedam, The Netherlands) with a working volume of 1.0 l. Agitation at 600 rpm was maintained using an integrated stirrer (DasGip, Jülich, Germany) and the temperature kept at 30 °C. The rate of aeration was set to 0.6 l/min. The pH of the medium was maintained at 5.0 by automatic addition of 2 N KOH during the batch phase and 10% NH_4OH during the feed phase. The temperature, agitation, gassing, pH and composition of the off-gas were monitored and controlled using the DasGip monitoring and control system. Dissolved oxygen concentration was monitored with an autoclavable polarographic oxygen electrode (Mettler Toledo, Columbus, OH, USA) and kept above 30% via stirrer speed and gas flow rate using the DasGip control system. The effluent gas from the fermentation was analyzed for real-time determination of oxygen and CO_2 concentration by DasGip fedbatch pro® gas analysis systems with the off gas analyzer GA4 based on zirconium dioxide and two-beam infrared sensor.

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

$$v(t) = \frac{Y_{xs}\mu_0}{s_f - s_0} x_0 V_0 \exp(\mu_0 t)$$

where x_0, s_0 and V_0 were the biomass density (gDCW/l), the substrate concentration (g/l) and the reactor volume (l) at the start of the feed phase, Y_{xs} was the respiratory yield coefficient (g glucose/gDCW); s_f was the concentration of the growth limiting substrate (g glucose/l) in the reservoir; μ_0 the was the specific growth rate (h^{-1}) during the feed phase and t the feeding time. According to the equation above the feed was increased exponentially with a specific feed rate of 0.06 h^{-1}. Correct feed addition was obtained programming the fb-pro software (DasGip) and controlled using the DasGip control system. An organic layer of dodecane (Sigma-Aldrich) was added aseptically to a final volume of 10% (v/v) immediately before starting the feed.

2.10. Analytical methods

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

Concentrations of glucose, glycerol, ethanol, acetate, succinate and pyruvate were analyzed by an isocratic high-performance liquid chromatography (UltiMate® 3000 Nano, Dionex) with an Aminex HXP-87H ion-exchange column (Bio-Rad, Hercules, CA) at 65 °C using 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 ml min^{-1}. Glucose, glycerol and ethanol were measured with a refraction index detector (RI-101 Refractive Index Detector, Shodex®), and acetate, succinate and pyruvate were measured with a UV–visible light absorbance detector (UltiMate 3000 Variable Wavelength Detector, Dionex).

2.11. β-Galactosidase activity assay

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

2.12. Analysis of sesquiterpenes

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

2.13. Analysis of total sterol fraction

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

2.14. Calculation of specific rates and yield coefficients

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

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

3. Results

3.1. α-Santalene production in S. cerevisiae

The α-santalene production in *S. cerevisiae* was initially evaluated introducing the expression plasmid pICK01 containing a copy of *tHMG1* and santalene synthase (*SanSyn*) under control of the *PGK1* and *TEF1* promoter, respectively, into a *lpp1*Δ strain to reduce farnesol formation (Faulkner et al., 1999) resulting in strain SCICK13. Production capacity was tested cultivating the strain in a batch in situ product removal (ISPR) reactor mode, consisting of an aqueous two-phase partitioning system. This strategy had previously been used successfully to increase product recovery in different microbial production processes (Daugulis, 1991; Stark and von Stockar, 2003). Dodecane was selected as organic phase due to its hydrophobicity (Log $P_{dodecane}$: 6.6; Log $P_{santalene}$: 6.2), low volatility and biocompatibility with *S. cerevisiae* growth (Newman et al., 2006; Asadollahi et al., 2008). Product accumulation in the dodecane layer was monitored by gas chromatography-mass spectrometry (GC/MS). The transformed strain was able to synthesize a low amount of α-santalene. Analysis of the organic layer revealed a major chromatographic peak corresponding in retention time and mass spectrum to the plant-extracted α-santalene and a second minor peak also present in the plant extract, subsequently identified as *trans*-α-bergamotene (Fig. 5). The amount of the sesquiterpenes α-santalene and *trans*-α-bergamotene produced were, respectively, 1.45 ± 0.02 and 0.17 ± 0.01 mg/l.

3.2. MET3 promoter activity during shake flask cultivation

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

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

3.3. Evaluation of alternative promoters for controlling squalene synthase activity

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

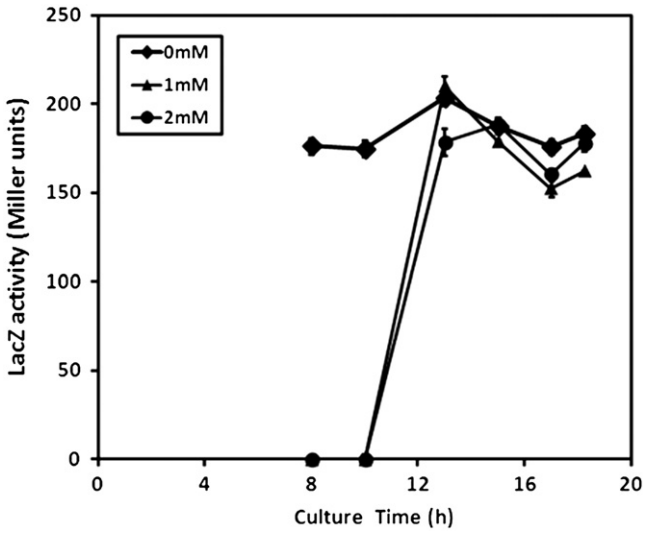

Fig. 1. LacZ activity in strain SCICK10 (P_{MET3}-*lacZ*) in response to different methionine concentrations, 0 mM (diamonds), 1 mM (triangles) and 2 mM (circles). Strains were cultivated in duplicates, glucose exponential growth phase was between 2 and 16 h of cultivation.

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

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

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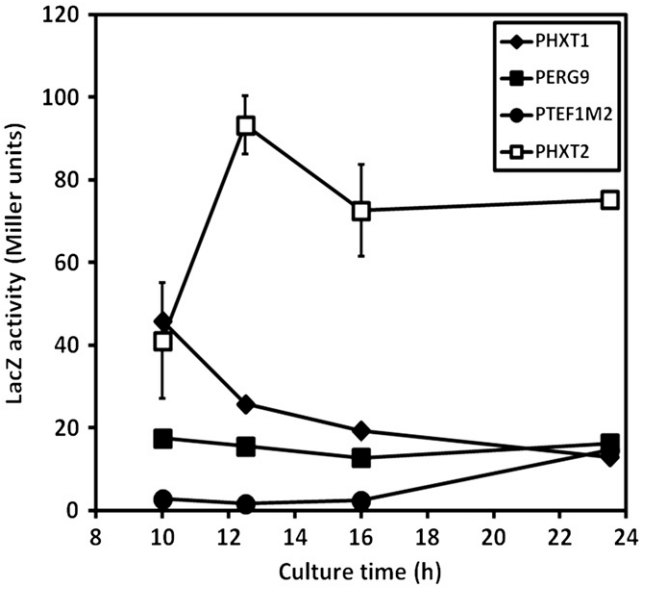

Fig. 2. LacZ activity in strains SCICK06 (P_{HXT1}, filled diamonds), SCICK08 (P_{TEF1M2}, filled circles), SCICK09 (P_{HXT2}, empty squares) and SCICK11 (P_{ERG9}, filled squares) during shake flask cultivation. Strains were cultivated in duplicates, glucose exponential growth phase was between 2 and 16 h of cultivation.

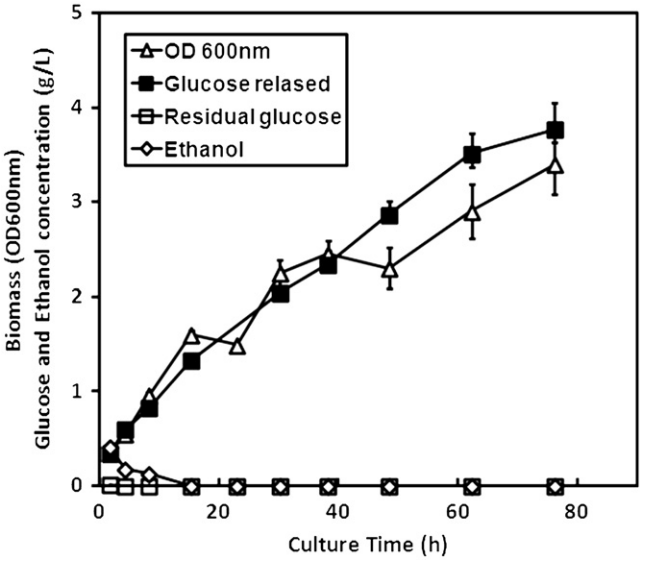

Fig. 3. Shake flask cultivation in fed-batch mode showing absolute glucose release kinetics without cells, residual glucose and ethanol concentration as well as biomass formation. The data represented were obtained for strain SCICK03; all the strains tested exhibited similar profiles.

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

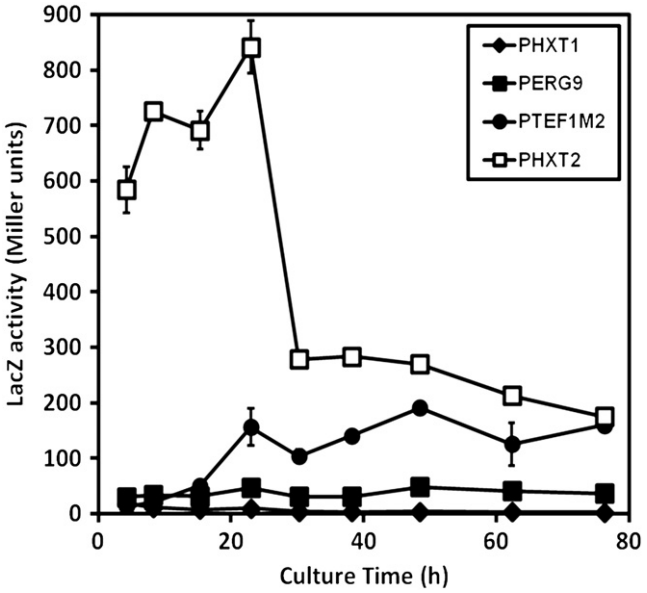

Fig. 4. Characterization of promoter strength during shake flask cultivation in fed-batch mode. β-galactosidase activity in strains SCICK06 (P_{HXT1}, filled diamonds), SCICK08 (P_{TEF1M2}, filled circles), SCICK09 (P_{HXT2}, empty squares) and SCICK11 (P_{ERG9}, filled squares). β-galactosidase activity is the average of values obtained from at least three independent cultivations assayed in duplicates.

consumption (data not shown). The expression of β-galactosidase controlled from the different promoters was assayed at regular intervals and monitored during a cultivation period of 80 h. As in the previous experiment, LacZ activity was steady in SCICK11 (P_{ERG9}) (Fig. 4). As expected, the highest activity was measured for SCICK09 (P_{HXT2}), the lowest for SCICK06 (P_{HXT1}). Surprisingly, β-galactosidase activity in SCICK08 (P_{TEF1M2}) drastically increased during the cultivation finally reaching the same level as for SCICK09 (P_{HXT2}), which indicates that (random) mutagenesis may turn a constitutive promoter – P_{TEF1} had previously shown a constant level of expression throughout different cultivations (Partow et al., 2010) – into a conditional promoter.

According to these results, P_{HXT1} appeared to be a suitable promoter to down-regulate *ERG9* expression under glucose limiting conditions, whereas P_{HXT2} was chosen to regulate the expression of an *ERG9* antisense construct. Due to its high activity levels at low glucose concentrations, the idea of employing P_{TEF1M2} for *ERG9* regulation was discarded.

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

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

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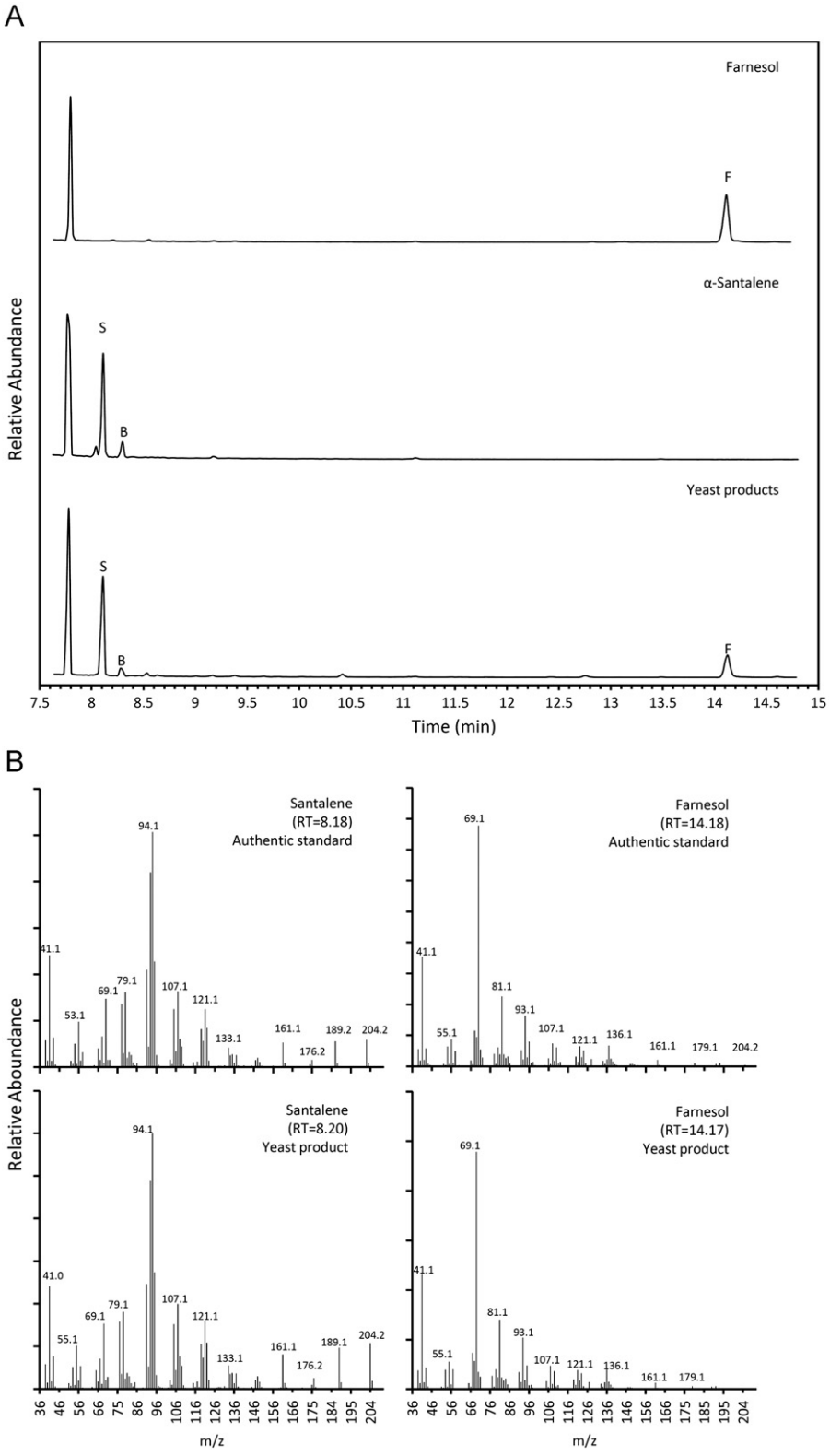

Fig. 5. (A) Total ion chromatograms from GC–MS analysis of authentic standard of farnesol, α-santalene, and an extract of engineered *S. cerevisiae* showing peaks of α-santalene (S), *trans*-α-bergamotene (B) and farnesol (F). The representative ion chromatogram referred to as yeast products was obtained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

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

Physiological characterization of the strains was completed in aerobic glucose-limited fed-batch cultures (Table 3). A fed-batch in situ product removal (ISPR) reactor mode was chosen to evaluate the α-santalene production capacity of these strains engineered to accumulate FPP. Cultivation was started as batch with 30 g/l of glucose. After complete glucose consumption and

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Table 3
Physiological parameters obtained during fed-batch cultivation of strains SCICK13, SCICK12, SCICK14, SCICK15 and SCICK17.

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

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

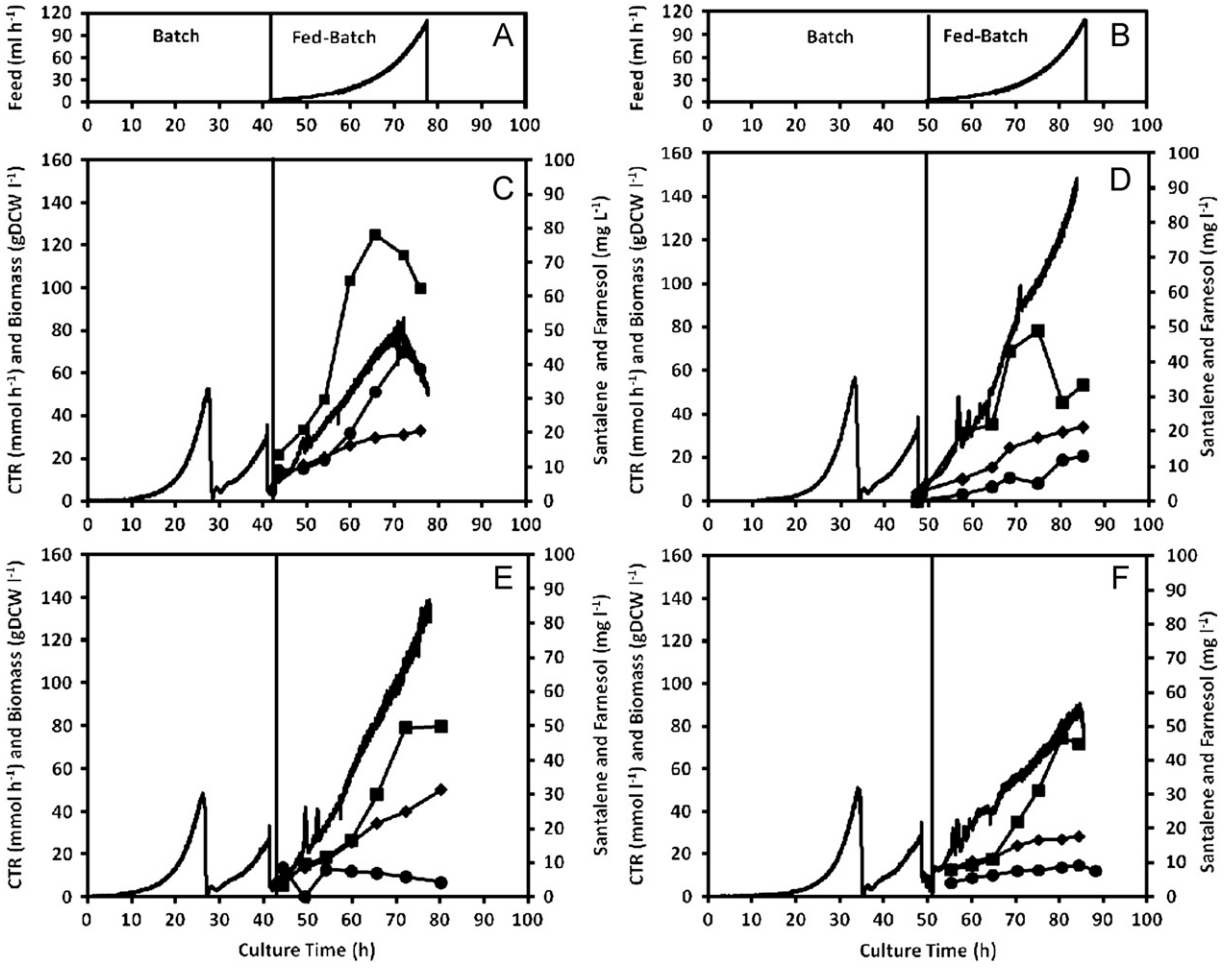

Fig. 6. Time course of an aerobic fed-batch culture with exponential sugar feed of *S. cerevisiae* strains SCICK12 (C) SCKCK14 (D) SCICK13 (E) SCICK15 (F). The feed of glucose (ml h^{-1}) is shown on the upper graph (A) and (B). Typical profile observed for formation of biomass (g l^{-1}, filled diamonds);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

after residual ethanol produced during the glucose consumption phase was completely depleted, the organic layer was added to the fermentor and the production phase was started by initiating a feed of fresh concentrated substrate with exponential kinetics for a total feed period of 36 h (Fig. 6). Within the first 30 h of feed the culture metabolism was completely respiratory characterized by complete oxidation of glucose with biomass and carbon dioxide as the major products and complete absence of fermentation products, while the respiratory coefficients remained close to 1 for all strains (Table 3). The period of respiratory growth was followed by a phase where yeast growth was no longer consistent with the feeding profile resulting a shift towards fermentative metabolism accompanied by accumulation of glucose and ethanol (data not shown). To examine the effect of *ERG9* repression on the sterol pathway, the total cellular sterol content was measured. Both the two P_{ERG9} replacement mutants and the strain expressing the antisense construct showed a lowered sterol content when compared to the strain containing the original *ERG9* promoter. The decrease in ergosterol ranged from 50 to 91%, and strain SCICK12 (P_{HXT1}) showed the lowest sterol content (Fig. 7).

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

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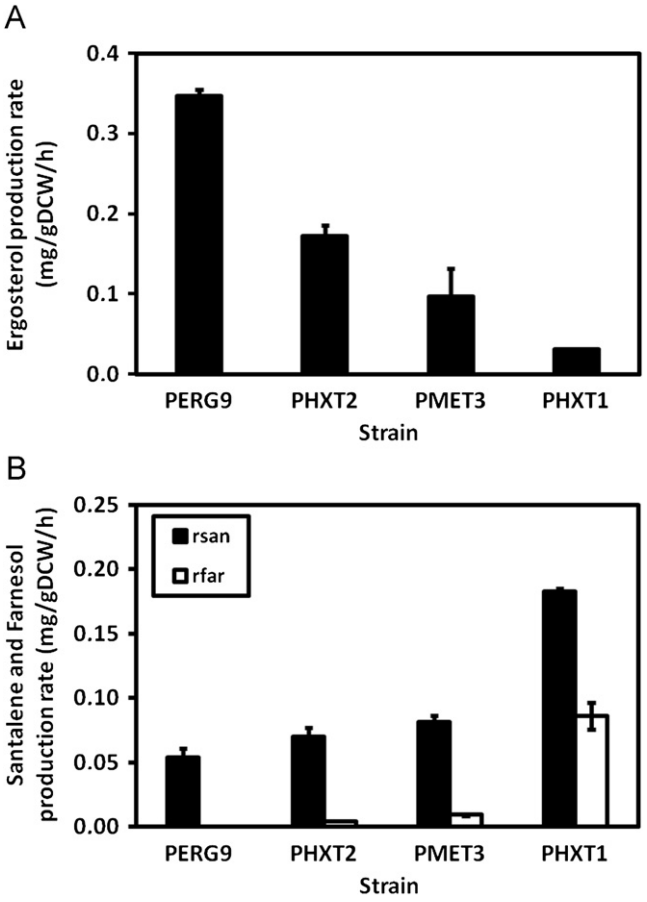

Fig. 7. (A) Ergosterol production rate (mg g biomass^{-1} h^{-1}). (B) α-santalene and *E,E*-farnesol production rate (mg g biomass^{-1} h^{-1}) in strains SCICK13 (P_{ERG9}), SCICK14 (P_{HXT2}-as), SCICK15 (P_{MET3}) and SCICK12 (P_{HXT1}). Strains were grown in a two-phase partitioned fed-batch glucose-limited cultivation mode. The error bars represent the standard deviation for two independent cultivations.

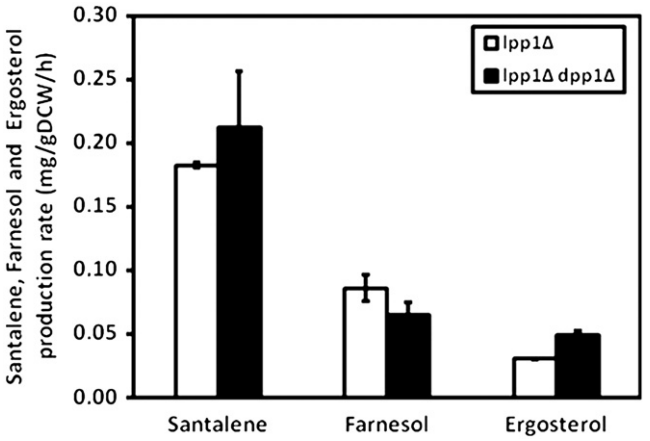

Fig. 8. Effect of single *lpp1*Δ and *lpp1*Δ *dpp1*Δ double deletion on α-santalene, *E,E*-farnesol and ergosterol production rates (mg g biomass^{-1} h^{-1}) in strains SCICK12 (P_{HXT1} *lpp1*Δ) (empty bars) and SCICK17 (P_{HXT1} *lpp1*Δ *dpp1*Δ) (filled bars). The error bars represent the standard deviation for two independent cultivations.

all strains. The amount of *trans*-α-bergamotene detected was proportionally consistent to the amount of α-santalene in all cases and corresponded to about 12% of the total α-santalene produced confirming that this compound is a secondary product of santalene synthase. A linear correlation was observed between the reduction in ergosterol content and the increase in sesquiterpene production. Different levels of *ERG9* repression diverted FPP towards santalene formation redirecting the flux distribution from the sterol pathway to sesquiterpene production with a santalene productivity ranging between 0.054 and 0.183 mg (g biomass)$^{-1}$ h^{-1} (Figs. 6 and 7). Strain SCICK12 (P_{HXT1}) was the best santalene producer with a 3.4 fold improvement in santalene productivity compared to SCICK13 (P_{ERG9}).

As previously described, *ERG9* down-regulation results in accumulation of farnesol (Takahashi et al., 2007; Asadollahi et al., 2008, 2010). Substantial accumulation of farnesol, an FPP-derived by-product, was detected in all strains (Fig. 7). Farnesol formation was inversely correlated with the ergosterol content and correlated to the santalene accumulation pattern observed, suggesting that *ERG9* down-regulation and HMGR over-expression combined with glucose de-repressed growth conditions resulted in an increased availability of FPP for α-santalene synthesis.

3.5. Contribution of DPP1 deletion to santalene production

DPP1 deletion had been used previously in order to reduce farnesol accumulation in a sesquiterpene producing strain (Takahashi et al., 2007). Together with Lpp1, Dpp1 is responsible for most of the cytosolic isoprenoid and lipid phosphate phosphatase activity in *S. cerevisiae* (Toke et al., 1998; Faulkner et al., 1999). As all engineered strains showed an increased conversion of FPP to FOH thus reducing substrate availability for santalene synthase the additional effect of deletion of *DPP1* on the best producing strain SCICK12 (P_{HXT1}) was investigated. *DPP1* was deleted in strain SCICK01 and subsequently transformed with the expression plasmid pICK01 containing a copy of *tHMG1* and santalene synthase resulting in strain SCICK17. Deletion of *DPP1* resulted in an increase of the α-santalene specific production rate from 0.18 to 0.21 mg (g biomass)$^{-1}$ h^{-1} together with a 24% drop in farnesol accumulation, but showed only a minor effect on the ergosterol content (Fig. 8).

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

4. Discussion

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

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

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

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

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

Sterol alteration had no effect on the growth characteristics of the engineered strains probably due to the reduced growth rate ($\mu = 0.06 \pm 0.01$) applied during the fed-batch process, which is far below the maximum specific growth rate measured for theses strains under normal batch conditions ($\mu_{max} = 0.35 \pm 0.01$). The accumulation of sterol intermediates is known to result in feedback inhibition of the MVA pathway (Maury et al., 2005). Here, the decrease in sterol content achieved by down-regulating *ERG9* could contribute to relieve this regulatory mechanism and further enrich the flow through the MVA pathway leading to high yields of α-santalene.

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

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

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

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

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

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PAPER II

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

Scalcinati G, Partow S, Siewers V, Schalk M, Daviet L, Nielsen J.

Submitted

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

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Abstract

Background

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

Results

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

Conclusions

The results reported in this work illustrate how the combination of a metabolic engineering strategy with fermentation technology optimization can be used to obtain significant amounts of the high-value sesquiterpene α-santalene. This represents a starting point toward the construction of a yeast “sesquiterpene factory” and for the

development of an economically viable bio-based process that has the potential to replace the current production methods.

Keywords

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

Background

Isoprenoids are a class of natural compounds with many potential commercial applications (e.g. flavoring agents, fragrances, food colorants, pharmaceutical agents and biofuel precursors), and there has recently been much interest in biotechnological production of these compounds [1-4]. Limitations in raw material accessibility, low yields and high costs of the current isoprenoid production through plant extraction or difficulties with chemical synthesis have caused interest in engineering cell factories that can be used to produce isoprenoids in cost competitive bioprocesses [5-7]. Isoprenoids are natively produced in yeast though the mevalonate (MVA) pathway in which the universal isoprene functional unit isopentenyl diphosphate (IPP) is produced from acetyl-CoA [8]. The terminal product IPP and its isomer dimethylallyl pyrophosphate (DMAPP) are subsequently condensed in the prenyl diphosphate pathway generating isoprene derivatives of different chain length (C_5-C_{20}) [9]. The sesquiterpene hydrocarbon α-santalene is a precursor of commercially relevant sesquiterpenes (C_{15}) and it is generated in a one-step conversion from the intermediate building block farnesyl diphosphate (FPP) [10]. Stoichiometry of α-santalene ($C_{15}H_{24}$) production in *S. cerevisiae* via the MVA pathway in purely oxidative growth conditions can be summarized as:

$$-4.5\ C_6H_{12}O_6 - 9\ ATP - 6\ NADPH + C_{15}H_{24} + 18\ NADH + 12\ CO_2 = 0$$

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

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

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

(ii) As a second part of the strategy we amplified the flux through the MVA pathway by engineering two key enzymatic steps. The mevalonate producing 3-hydroxy-3-

methyl-glutaryl-CoA reductase (HMGR) enzyme is a highly regulated enzyme and is generally believed to exert a high degree of flux control in the MVA pathway. Part of its regulation is via the N-terminal domain of Hmg1 that spans the membrane of the endoplasmic reticulum (ER) and hereby interacts with sterol sensing components of the ER membrane. This feed-back regulation by sterols can be eliminated by expressing a modified form of HMGR lacking the trans-membrane region [17]. Here we used a genetic modification widely used in the past in order to circumvent post-transcriptional regulation of HMGR [18]. The *HMG1* gene region coding for the catalytic domain was over-expressed resulting in a constitutively active, cytosolic variant of Hmg1. This strategy has been successfully used before for over-producing several different isoprenoids in *S. cerevisiae* [12, 14, 19-21]. The other enzymatic step engineered in the MVA pathway was the one mediated by farnesyl diphosphate synthase (FPPS) (encoded by the essential gene *ERG20*), which catalyses the condensation of IPP units into geranyl diphosphate (GPP) and FPP [22]. IPP condensing enzymes are interspecies conserved and the yeast *ERG20* gene product evolved towards specific production of FPP rather than GPP [23]. Due to the pivotal nature of the FPP molecule as precursor of many essential compounds such as dolichol, ubiquinone, isoprenylated proteins and ergosterol [24] its synthesis by FPPS is tightly regulated and has been identified as a flux controlling step of the MVA pathway, in particular controlling the intracellular FPP availability and its distribution into derived products [25,26]. The efficiency of *ERG20* overexpression to increase the level of IPP conversion to FPP and its derivatives depends, however, on the growth conditions employed and the yeast background strain utilized [20, 27]. In this study, the effect of overexpressing *ERG20* on α-santalene production has been investigated.

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

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

down-regulation, it produced a clear increase in total isoprenoid production [20, 35]. In the current work, contribution of the *upc2-1* overexpression on the production of α-santalene was tested in combination with the modifications described above.

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

Results

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

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

S. cerevisiae was engineered to produce α-santalene by introducing the expression plasmid pISP15 containing a copy of *tHMG1* and codon optimized *SanSyn* ($SanSyn_{opt}$) under control of the *PGK1* and *TEF1* promoters, respectively (strain

SCIGS28). The transformed strain was initially tested for its α-santalene producing capacity in a double-phase chemostat process at a dilution rate of 0.05 h^{-1} resulting in an α-santalene yield of 0.0013 Cmmol (Cmmol glucose)$^{-1}$ and a production rate of 0.006 Cmmol (g biomass)$^{-1}$ h^{-1} (corresponding to 0.086 mg (g biomass)$^{-1}$ h^{-1}). All the following strain development strategies were assessed based on the yield and productivity of this control strain (SCIGS28) and are reported in figure 3 and 4, whereas the titers are given in figure 5.

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

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

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

The MVA pathway was further engineered by integrating into the yeast genome the mutated transcription factor gene *upc2-1* and an extra copy of *tHMG1* (strain SCIGS25). Previously, both strategies, using an additional genome integrated copy of *tHMG1* next to plasmid-based expression and the over-expression of *upc2-1* have displayed little or only a strain-dependent effect on final product production [14, 20]. Similarly, our combined approach did not contribute to increase α-santalene production over the best producing strain obtained, SCIGS24. However, in contrast to the insignificant change in α-santalene productivity strain SCIGS25 exhibited a 2-fold increase in FOH formation yielding a final FOH yield of 0.0024 Cmmol (Cmmol glucose)$^{-1}$ and a productivity of 0.018 Cmmol (g biomass)$^{-1}$ h^{-1}. It is therefore worth mentioning that strain SCIGS25 reached the highest total sesquiterpene yield and productivity of 0.0069 Cmmol (Cmmol glucose)$^{-1}$ and 0.052 Cmmol (g biomass)$^{-1}$ h^{-1} (santalene + farnesol), respectively (Figure 3A and 4).

Effect of the dilution rate on sesquiterpene production

Under the employed conditions, the engineered strains exhibited significant changes in the total amount of sesquiterpene produced. The sesquiterpene productivity level varied almost 10-fold between the strains, from 0.006 to 0.052 Cmmol (g biomass)$^{-1}$ h^{-1}. Chemostat cultivation mode offers the advantage of manipulating with accuracy the dilution rate that at these conditions is equal to the specific growth rate [36]. We therefore decided to investigate the effect of the growth rate on sesquiterpene production. All previous cultivations were performed at a dilution rate of 0.05 h^{-1} and when the control strain was grown at D=0.1 h^{-1}, a small decrease in the α-santalene yield was observed (Figure 4) whereas its productivity remained essentially unchanged (Figure 3). The increase in α-santalene production observed for strains SCIGS29 and SCIGS30 at low dilution rate (D=0.05 h^{-1}) was also seen at the higher dilution rate of 0.1 h^{-1}. α-Santalene productivities measured for these strains were, respectively, 0.041 and 0.043 Cmmol (g biomass)$^{-1}$ h^{-1} representing a 6-fold increase compared to the control strain and almost double the productivity obtained when growing the strains at D=0.05 h^{-1} (Figure 3B). In contrast, the yield was slightly reduced. This showed a clear dependence of the productivity on the specific growth rate applied (Figure 4). Consistently, the *DPP1* deletion resulted in reduced FOH accumulation in strain SCIGS30 compared to the *lpp1*Δ single deletion (strain SCIGS29). The ratios between the α-santalene and the farnesol yield in the two strains of 2.3 and 4.2, respectively, were maintained when the dilution rate was raised to 0.1 h^{-1} (Figure 4). Consistently, the same product proportion was also seen in the productivities (Figure 3). Therefore, the distribution of FPP between the two products appears to be independent of the specific growth rate.

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

Strain physiology in batch and chemostat cultivation

In order to evaluate if the modifications applied to increase sesquiterpene production affected yeast physiology a detailed characterization of the recombinant strains was carried out. Control strain SCIGS28 displayed a fully respiratory metabolism (RQ=1.0) under both dilution rates. The principal physiological parameters (e.g. Y_{sx}, r_s, r_{CO2} and r_{O2}) were comparable with the wild type strain CEN.PK113-7D [37, 38]. Strains SCIGS29 and SCIGS30 exhibited a major alteration in their physiology. An increase in the residual glucose concentration of 6.4 fold at D=0.05 h^{-1} and 2.5 fold at D=0.1 h^{-1} was observed for both strains. As direct consequence of the increase in the residual glucose concentration aerobic fermentation set in, resulting in ethanol formation accompanied with acetate accumulation. A marked reduction in the biomass yield from 0.5 to 0.29-0.28 (D=0.05 h^{-1}) and 0.28-0.25 g biomass (g glucose)$^{-1}$ (D=0.1 h^{-1}) was measured for the two strains (table 3). However, only a small fraction corresponding to 4% (Cmmol products (Cmmol glucose)$^{-1}$) of the glucose consumed was fermented to ethanol and acetate. Additionally, a clear increase in the glucose (r_s) and oxygen consumption rate (r_{O2}) and carbon dioxide production rate (r_{CO2}) was observed (table 3). This physiological response was observed at both D=0.05 and 0.1 h^{-1} thus appearing to be independent of the dilution rate.

Despite several attempts, it was not possible to achieve a steady-state when strains SCIGS31, SCIGS24 and SCIGS25 were grown at D=0.1 h^{-1}. Instead, a progressive decrease of the biomass concentration over time was observed consistent with wash-out kinetics. The following characterization for these strains was therefore conducted only at D=0.05 h^{-1}.

When deletion of *GDH1* was introduced (strain SCIGS31) a considerable fraction of the glucose, 31 mmol l^{-1}, was recovered corresponding to a consumption of only 33% of the total sugar provided. However, it was still possible to reach a steady state. In

this strain, the rate of alcoholic fermentation increased to 0.51 mmol (g biomass)$^{-1}$ h^{-1} and the metabolism shifted more predominantly to a respiro-fermentative state (RQ=1.62), where 21% of the carbon source was metabolized to the fermentation products ethanol and acetate. These pronounced metabolic changes were probably related to the limitation in ammonium consumption as a consequence of Gdh1 inactivation.

Overexpression of *GDH2* is known to partially complement the ammonium assimilation defect in a *gdh1*Δ strain [30] and resulted in a clear reduction of the ethanol and acetate production rate in strains SCIGS24 and SCIGS25 compared to strain SCIGS31. It is worth noticing that in strain SCIGS24, a large decrease in the biomass yield occurred and the specific glucose and O_2 consumption rates and the CO_2 production rate increased respectively to a value of 1.16, 3.21 and 3.85 mmol (g biomass)$^{-1}$ h^{-1}. The previously described overflow metabolism phenomenon towards fermentation products was also observed in strains SCIGS24 and SCIGS25 and led to a fraction of carbon fermented to ethanol and acetate close to 6% for both strains. All engineered strains except the control strain exhibited overflow metabolism under the tested conditions. The fraction of glucose converted into fermentation products ranged between 0.04 and 0.21 Cmmol products (Cmmol glucose)$^{-1}$. If strain SCIGS31 (that exhibited a behaviour different from all other strains probably related to the major role played by the ammonium limitation) is excluded from this consideration, it is interesting to notice that the ratios of the different fermentation products measured vary substantially between the strains. A significantly higher ethanol:acetate ratio was observed for strains SCIGS24 and SCIGS25 compared to SCIGS29 and SCIGS30 indicating a redistribution of flux around the pyruvate dehydrogenase (PDH) bypass at the acetaldehyde level. The increased ethanol:acetate ratio was reflected in an

increase in the formation of sesquiterpene products, which are derived directly from the cytosolic acetyl-CoA produced through the pyruvate decarboxylase route. On the other hand, the engineered strains showed a clear decrease in biomass yield compared to the control strain suggesting a carbon flux redirection towards other products. The fraction of carbon lost in the drop of biomass yield could not be accounted for in the residual unconsumed glucose or in the fermentation products. Instead, carbon dioxide was the main carbon product. Surprisingly, the increase in ethanol and acetate productivity was not related to any decrease in the respiration rate. The oxygen uptake rate was increased in all engineered strains compared to the control strain and reached the highest value of 4.41 mmol (g biomass)$^{-1}$ h^{-1} in strain SCIGS25 suggesting a strong reprogramming of cell metabolism in these strains.

Discussion

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

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

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

Being extensively used in bioprocesses to produce aroma compounds, *in situ* product removal (ISPR) (for review see [39]) was applied in this study to maximize the

product recovery. Through the combination of ISPR with chemostat cultivations we obtained a production system that offers the advantage of continuous recovery of the product in the fermenter effluent from the selected organic phase which can subsequently be recycled, regenerated and reused in the same process for a prolonged time of cultivation (for review see [40]) The developed set-up is a suitable approach to develop an upscaled industrial process.

Influence of the genetic modifications on strain productivity

Here we examined the impact of different metabolic engineering strategies and their combinations on α-santalene productivity and yield. The control strain was minimally engineered to produce α-santalene functionally expressing a codon optimized santalene syntase (SanSyn) from *C. lansium* and a truncated version of 3-hydroxyl-3-methyl-glutaryl-coenzyme A reductase (HMGR). SanSyn belongs to the class I group of sesquiterpene cylcases. These enzymes catalyze a complex intramolecular cyclization of FPP with very different product specificity and the reaction mechanism often involves several partial reactions [41]. Many studies have reported examples of heterologous production of isoprenoids simply expressing the plant synthase in the desired microbial host. However, the yields obtained are often extremely low [15, 19, 20, 42-45]. Similar to our previous study we decided to construct a reference α-santalene producing strain (SCIGS 29) combining the synthase expression with the expression of the deregulated form of Hmg1 (tHmg1) [12]. The use of *tHMG1* represents an excellent example of bypassing one of the regulatory mechanisms controlling the MVA pathway flux and has been successfully applied in a number of microbial isoprenoid production processes [14, 19-21, 43]. The yield obtained in this control strain was comparable with our previously reported values obtained during a fed-batch process [12] and demonstrates the feasibility and robustness in applying our

novel double-phase continuous cultivation. In order to improve the production of the target compound it is necessary to overcome the regulatory mechanisms that have evolved to prevent flux imbalances. In this work, we modulated some of the well-recognized key points that tightly regulate the carbon flux to sesquiterpenes in *S. cerevisiae*. A slight reduction in yield and unchanged productivity observed in the control strain at a higher dilution rate suggests a limitation of the plant synthase in efficiently draining the FPP precursor from the MVA pathway, consistent with the previous hypothesis that at low FPP concentration *SantSyn* competes with the other cellular FPP consuming reaction [12]. A general strategy extensively applied in sesquiterpene bioprocess development [14, 15, 20, 35] consists in down-regulating SQS to increase the intracellular FPP pool. Replacement of the native P_{ERG9} promoter with the glucose-sensing P_{HXT1} promoter was recently successfully employed to divert the carbon flux to sesquiterpene products instead of sterols [12]. Applying the same *ERG9* modification in this study together with deletion of *LPP1* greatly increased the sesquiterpene productivity and yield under chemostat conditions compared to the control strain. The obtained productivity level appears to be linearly correlated with the dilution rate employed pointing to a direct relation between the specific growth rate and the overall flux through the MVA pathway and indicating that the efficiency of the *ERG9* modification in the enhanced FPP availability was supported at different specific growth rates. A similar growth dependent relation has been reported for the cellular content of ergosterol [46], which is also derived from FPP.

In the *lpp1*Δ and *lpp1*Δ/*dpp1*Δ mutants known to exhibit lower FPP phosphatase activity [16, 47], the excess of FPP was redistributed between α-santalene and FOH in a consistent ratio when different dilution rates were applied. These results suggest the hypothesis that once a threshold level of intracellular flux toward FPP is reached the

thermodynamically favourable endogenous dephosphorylation starts and competes with the catalytic capacity of santalene synthase leading to FOH accumulation. On the other hand, the unchanged α-santalene yield coupled with higher productivity achieved at higher dilution rates suggests that the santalene synthase was not fully saturated at low dilution rates and there was excess activity to cope with high FPP flux. This points out that the FOH formation is not only a direct consequence of limited santalene synthase activity but that other cellular mechanisms are likely to be involved. Reduction but not complete inhibition of FOH formation in the *lpp1*Δ *dpp1*Δ double deletion strain compared to the single *lpp1*Δ deletion strain was consistent with our previous report [12], and confirmed that the *DPP1* encoded lipid phosphate phosphatase has a role in FPP dephosphorylation and together with Lpp1 is involved in the conversion of FPP into FOH. However, these are clearly not the only mechanisms responsible for this conversion as we still observed some FOH production in the double deletion strain.

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

to trigger FOH formation and conversion of FPP into α-santalene was sufficient to avoid intracellular FPP accumulation.

Combining the simultaneous overexpression of the NAD-dependent glutamate dehydrogenase and prenyl transferase encoded, respectively, by *GDH2* and *ERG20* positively affected sesquiterpene production. Overexpression of *GDH2* is known to restore the ammonium assimilation and consequently alter the NADH:NADPH equilibrium favouring the NADPH availability at the expense of NADH produced [14, 30].

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

The optimal solution was obtained through combining all the modifications resulting in the highest sesquiterpene yield (strains SCIGS24 and SCIGS25). Compared to our previous study [12] the engineering strategy employed here allows an increase of 1.8 fold in α-santalene final yield (Cmmol α-santalene /Cmmol glucose). These results highlight the importance of a systematic approach to achieve the ultimate goal of and economically feasible sesquiterpene microbial production. It is noteworthy that

comparable sequiterpene productivity was achieved in the strains not fully engineered simply by increasing the operational dilution rate (strains SCIGS29 and SCIGS30) whereas the fully engineered strains were washed out when the same conditions were imposed. Further studies are necessary to elucidate the factors leading to the inability of these mutants to sustain growth at higher dilution rates.

Influence of genetic modifications on strain physiology

In this study, the effect of controlling the diversion of carbon flow from sterol synthesis towards sequiterpene production by modifying the *ERG9* promoter has been investigated during aerobic chemostat glucose limited cultivation conditions. The *lpp1*Δ and *lpp1*Δ/*dpp1*Δ mutants carrying the P_{HXT1}-*ERG9* construct clearly showed an increase in the residual glucose concentration slightly above the critical concentration that triggers aerobic fermentation, which was reported to lie between 0.5 and 0.8 mM [49, 50] and results in a typical Crabtree response. It is possible that regulating the Erg9 activity using the P_{HXT1} glucose sensitive promoter under strictly glucose limited conditions resulted in its almost complete down-regulation and in an increased biosyntheic demand of the essential compound ergosterol. Ergosterol is the main sterol present in the plasma membranes where it has several essential functions [51]. Yeast is dependent on oxygen for sterol and fatty acid formation. Under strictly anaerobic conditions this compound has to be provided in the media. Reducing its provision results in a decrease of biomass formation and an increase in ethanol formation [52]. Activity of P_{HXT1} has been shown to be induced at an extracellular glucose concentration of 5.6 mM [53] suggesting that the observed increase in the residual glucose concentration in the cultures was necessary to restore a minimal P_{HXT1} activity in order to maintain the ergosterol level necessary to sustain cell growth. The response to the limitation in the essential compound ergosterol could be

the reason leading to the observed decrease in biomass yield and increase of the fermentative metabolism. A similar phenomenon in fact was observed in autotrophic yeast strains in uracil-limited chemostat culture [54]. The observed overflow metabolism toward ethanol and acetate formation increases the carbon flux through the PDH bypass possibly resulting in an increase in the cytosolic acetyl-CoA availability that was subsequently more effectively channelled towards the MVA pathway in the engineered strains enhancing the final sesquiterpene production. Strain SCIGS31 exhibited a particular physiology and needs to be discussed separately. Deletion of *GDH1* is known to impair the ammonium assimilation resulting in a lower specific biomass formation rate on different carbon sources (glucose/galactose) and under different growth conditions (batch/chemostat and aerobic/anaerobic) [29, 30], which was confirmed in this study. When deletion of *GDH1* was introduced, ethanol formation as well as glucose accumulation occurred, resulting in a situation similar to cultivation limited in essential nutrients [54]. Most likely, the combination of the limitation in ammonium assimilation as result of the *GDH1* deletion together with the possible ergosterol limitation due to the *ERG9* downregulation produced the observed respiro-fermentative metabolism.

Conclusions

Microbial production of sesquiterpenes is an active research area; advances in pathway engineering and fermentation technologies have a significant impact in accomplishing the aim to develop an economically viable biobased industrial process. In this study, engineering different pathways simultaneously resulted in a robust *S. cerevisiae* production host capable of efficiently producing α-santalene. The engineered strains were evaluated in an optimized double-phase continuous fermentation method leading to a high yield of α-santalene and resulting in a robust

production process that could possibly be used for commercial applications. Levels of products observed open up to the possibility to explore new engineering option for increasing the sesquiterpene productivity. The presented systematic metabolic engineering approach represents a gateway toward the creation of yeast platform that can be applied to the production of an array of sesquiterpene products.

Methods

Plasmid construction

An overview of the plasmids constructed in this study is reported in table 1, the detailed maps of the plasmids is contained in supplementary file 3. The gene coding for α-santalene synthase (*SanSyn*$_{opt}$) was codon optimized for expression in *S. cerevisiae* and synthesized by DNA 2.0 (Menlo Park, CA, USA) (supplementary file 2), cut with *Not*I/*Pac*I and ligated into *Not*I/*Pac*I restricted vector pICK01 containing *tHMG1* [12] resulting in plasmid pISP15 (Figure 1).

To simultaneously integrate multiple genes into the yeast genome a series of plasmids containing the genes, constitutive strong promoters, terminators, marker gene sequences and the required region for genomic integration were constructed. All endogenous *S. cerevisiae* genes were PCR amplified using genomic DNA of strain CEN.PK113-5D as template. Primers used for amplification are provided in supplementary file 1. All PCRs were performed using high fidelity Phusion™ DNA polymerase (Finnzymes, Vantaa, Finland). The *ERG20* gene [GenBank: NM_001181600] was amplified using primers pair 1/2, subsequently digested with *Bam*HI/*Nhe*I and ligated into the vector pSP-GM2 [55] restricted with the respective enzymes downstream of the *TEF1* promoter resulting in plasmid pIGS01. A 711 bp upstream flanking region (AD1) selected for genomic integration was amplified using primers pair 3/4, cut with *Mre*I/*Kpn*2I and ligated into vector pIGS01 restricted with

the respective enzymes resulting in plasmid pIGS02. Plasmid pIGS03 was obtained by cloning gene *GDH2* [GenBank: NM_001180275] amplified with primers pair 5/6 into pIGS02 downstream of the *PGK1* promoter using *Pac*I/*Not*I restriction sites. A downstream flanking region of 653 bp (AD2) was amplified with primers 7/8, digested with *Asc*I/*Avr*II and ligated into pIGS03. The resulting plasmid was named pIGS04. To complete the plasmid for integration the *Kluyveromyces lactis* (*Kl*) *URA3* gene [GenBank: Y00454] was amplified with primers 9 and 10 using plasmid pWJ1042 [56] as template, cut with *Fse*I and ligated into pIGS04 after restriction with the respective enzyme. The resulting plasmid was designated pIGS05, digested with *Mre*I/*Asc*I and the resulting fragment used for integration into the yeast genome as described below. The 5´ region of the *Kl URA3* gene was amplified with primers 11 and 12, cut with *Avr*II/*Asc*I and cloned into pIGS03 restricted with the respective enzymes resulting in plasmid pIGS06. Amplification of the catalytic domain of the HMG-CoA reductase gene (*tHMG1*) [GenBank: NM_001182434] was performed using primers pair 13/14, the resulting fragment cleaved with *Nhe*I/*Bam*HI and cloned downstream of the *TEF1* promoter into *Nhe*I/*Bam*HI restricted pSP-GM2 resulting in pIGS07. A mutant allele *upc2-1* of the *UPC2* gene [GenBank: NC_001180521] was created by use of primer pair 15/16. To introduce the pleiotropic mutation G888D, the corresponding codon GGT was mutated to GAT generating the amino acid substitution. Subsequently, the PCR amplified *upc2-1* was cloned downstream of the *PGK1* promoter into pIGS07 using *Not*I/*Pac*I resulting in plasmid pIGS08. An 829 bp downstream flanking region (AD3) selected for genomic integration was amplified using primer pair 17/18 cut with *Mre*I/*Kpn*2I and ligated into vector pIGS08 restricted with the respective enzymes resulting in plasmid pIGS09. The 3´ region of *Kl URA3* (overlapping with the 5´region described above) was amplified with primers 19 and

20, cut with *Avr*II/*Asc*I and cloned into pIGS09 restricted with the respective enzymes resulting in plasmid pIGS10. All plasmids were verified by sequencing (Sigma-Aldrich, St. Luis, MO). Subsequently, plasmids pIGS06 and pIGS10 were restricted with *Mre*I/*Asc*I, the cassettes isolated from the vector backbone and used for yeast transformation (see below).

Yeast strain construction

All *S. cerevisiae* strains constructed in this work have a CEN.PK background with auxotrophy for uracil [57] and are listed in table 2.

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

pIGS05. Correct integration into the YORWΔ22 locus on chromosome XV [60] was verified by PCR using primer pairs 33/1 and 5/34. Strain SCIGS12 carrying a genomic integration of genes *ERG20*, *GDH2*, *tHMG1* and *upc2-1*, was constructed by co-transforming strain SCIGS03 with the *Mre*I/*Asc*I fragments isolated from plasmids pIGS06 and pIGS10. Correct integration into the YORWΔ22 locus was verified by PCR using primer pairs 33/1, 5/35, 19/15, 35/15, 16/36 and 13/34.

Kl URA3 was replaced in strains SCIGS06 and SCIGS12 with the *kanMX* marker. The *loxP-kanMX-loxP* cassette was independently amplified from plasmid pUG6 using primers pairs 37/38 for integration in strain SCIGS06 and 39/40 for integration in SCIGS12 containing 71-74 bp primer tails complementary to the target integration sites. Both strains were transformed with the respective PCR-amplified fragment. Transformants were selected on YPD plates containing 200 mg/l G418. *Kl URA3* replacement was initially tested by replica plating on synthetic complete (SC) medium without uracil and YPD/G418 medium. The *kanMX* marker was subsequently removed [59] leading to strains SCIGS22 and SCIGS23.

Strains SCIGS28, SCIGS29, SCIGS30, SCIGS31, SCIGS24 and SCIGS25 were obtained transforming, respectively, strains CEN.PK113-5D, SCICK01, SCICK16, SCIGS03, SCIGS22 and SCIGS23 with the high copy number plasmid pISP15 (Table 2) containing the *URA3* gene and the genes $SanSyn_{opt}$ and *tHMG1* under control of the strong constitutive promoters *TEF1* and *PGK1*, respectively (Table 1).

Strain maintenance

Long term storage of yeast suspensions containing 25% (vol/vol) sterile glycerol was performed in cryovials at -80ºC [61]. Working stocks were maintained on YPD agar plates containing 10 g/l yeast extract, 20 g/l casein peptone, 20 g/l glucose and 20 g/l agar. Plasmid carrying strains were maintained on synthetic dextrose medium agar

plates lacking uracil containing 6.9 g/l yeast nitrogen base without amino acids (Formedium), 0.77 g/l complete supplement mixture without uracil (Foremedium) 20 g/l dextrose and 20 g/l agar.

Media and growth conditions

A mineral salts medium was used for batch cultivations as previously described [62] and had the following composition (per liter): $(NH_4)_2SO_4$, 5 g; KH_2PO_4, 3 g; $MgSO_4 \cdot 7H_2O$, 0.50 g; Antifoam 289 (A204, Sigma–Aldrich), 0.05 ml; trace metals, 1 ml and vitamins, 1 ml. The trace metal solution consisted of the following (per liter): EDTA (sodium salt), 15.0 g; $ZnSO_4 \cdot 7H_2O$, 0.45 g; $MnCl_2 \cdot 2H_2O$, 1 g; $CoCl_2 \cdot 6H_2O$, 0.3 g; $CuSO_4 \cdot 5H_2O$, 0.3 g; $Na_2MoO_4 \cdot 2H_2O$, 0.4 g; $CaCl_2 \cdot 2H_2O$, 0.45 g; $FeSO_4 \cdot 7H_2O$, 0.3 g; H_3BO_3, 0.1 g and KI, 0.1 g. The pH of the trace metal solution was adjusted to 4.0 with 2 M NaOH prior to heat sterilization. The vitamin solution contained (per liter): biotin, 0.05 g; p-amino benzoic acid, 0.2 g; nicotinic acid, 1 g; Ca-pantothenate, 1 g; pyridoxine-HCl, 1 g; thiamine-HCl, 1 g and myo-inositol, 25 g. The pH of the vitamin solution was adjusted to 6.5 with 2 M NaOH. The vitamin solution was filter sterilized and stored at 4°C. This medium was supplemented with 20 g/l glucose. The feed composition used for continuous cultivation had the same composition as described above, but the glucose concentration was 10 g/l. The medium used for shake flask cultivation has the same composition as described above, but the $(NH_4)_2SO_4$ concentration was increased to 7.5 g/l, and the KH_2PO_4 to 14.4 g/l; the glucose concentration was 20 g/l; the pH was adjusted to 6.5 prior autoclaving.

Inoculum preparation and pre-culture

A single colony from an SC-ura agar plate was selected to inoculate a 500 ml shake flask containing 100 ml mineral salts medium. The seed culture was grown at 30ºC in

an orbital shaker at 100 rpm to late-exponential phase and used to inoculate the fermenter to a final dry weight of 1 mg/l. All cultivations were performed in triplicate.

Chemostat operation

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

was fed at 27 ml/h and the organic phase at 3 ml/h. To obtain a dilution rate of 0.05 h^{-1}, medium was feed at 13.5 ml/h and the organic phase at 1.5 ml/h resulting in a constant inlet feed ratio of medium:organic phase of 9:1 (vol/vol). Dodecane (Sigma-Aldrich, St. Luis, MO) was used as organic phase and filter sterilized prior addition. The culture working volume of 0.3 l (0.27 l of medium + 0.03 l of dodecane) was kept constant by automatic withdrawal of broth based on an electric level sensor measurement. The set-up allowed maintaining the correct medium/organic phase ratio inside the fermentor throughout the fermentation time. The correct ratio of 9:1 vol/vol between the two phases was constantly monitored and differed by less than 2% in samples taken directly from the culture and from the effluent line. Steady state was reached after at least 5 residence times, defined by constant values of CTR, OTR and biomass concentration (less than 5% deviation).

Cell mass determination

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

Metabolite analysis

Samples for analysis of extracellular metabolite concentrations were withdrawn from two-phase steady state chemostat cultures and centrifuged for 5 min at 5000 g. The

organic layer was discarded and the cultivation broth was filtered through 0.45-µm-pore size nylon filters (VWR international, Radnor, PA, USA) and stored at -20ºC until further analysis. Glucose, glycerol acetate, succinate, and pyruvate were quantified by HPLC (UltiMate® 3000 Nano, Dionex, Bannockburn, IL, USA) with an Aminex HXP-87H ion-exchange column (Bio-Rad, Hercules, CA) maintained at 65ºC and using 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 ml min^{-1}. Glucose, glycerol, and ethanol were measured with a refraction index detector (RI-101 Refractive Index Detector, Shodex®), and acetate, succinate, and pyruvate were measured with a UV-visible light absorbance detector (UltiMate 3000 Variable Wavelength Detector, Dionex).

Analysis of sesquiterpenes

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

Competing interests

GS, SP, VS and JN declare they have no competing interests. MS and LD are employees of Firmenich SA.

Authors' contributions

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

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Figures

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

(A) Expression plasmid pISP15 containing *tHMG1* encoding truncated HMG-CoA reductase, a codon optimized santalene synthase gene ($SanSyn_{opt}$) P_{TEF1} and P_{PGK1} promoters as well as T_{ADH1} and T_{CYC1} terminator sequences. **(B)** Integrated cassettes, rectangles containing arrows represent the promoters and their directionality, pentagons the genes and empty squares the terminators. **(C)** Scheme of the engineered mevalonate, prenyl phosphate and ammonium assimilation pathways and FPP branch point; overexpressed and deleted genes are highlighted. Pathway intermediates: G6P: glucose-6-phosphate, Acetyl-CoA_{cyt}: cytosolic acetyl-CoA, HMG-CoA: 3-hydroxy-3-methylglutaryl-CoA, MVA: mevalonate, MVA-P: phosphomevalonate, MVA-PP: diphosphomevalonate, IPP: isopentenyl diphosphate, DMAPP: dimethylallyl diphosphate, GPP: geranyl diphosphate, FPP: farnesyl diphosphate, FOH: farnesol. Overexpressed genes are *tHMG1* (encoding truncated HMG-CoA reductase), *ERG20* (encoding FPP synthase), *GDH2* (encoding NAD-dependent glutamate dehydrogenase), and $SanSyn_{opt}$ (encoding α-santalene synthase). Deleted genes are *GDH1* (encoding NADP-dependent glutamate dehydrogenase), *LPP1* and *DPP1* (both encoding lipid phosphate phosphatases). The promoter of the *ERG9* gene

(encoding squalene synthase) is replaced with P_{HXT1}. Genes whose promoters contain Upc2 binding sites are indicated with a grey arrow: *ERG13* (encoding HMG-CoA synthase), *ERG12* (encoding mevalonate kinase), and *ERG8* (encoding phosphomevalonate kinase). Additional genes indicated are *ERG10* (encoding acetoacetyl-CoA thiolase), *ERG19* (encoding diphosphomevalonate decarboxylase) and *IDI* (encoding IPP isomerase).

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

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

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

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

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

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

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

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

Tables

Table 1 - Plasmids used in this study

Plasmid name	Plasmid description	Reference
pSP-GM2	*URA3*-based expression plasmid carrying a bidirectional P_{TEF1}-P_{PGK1} promoter	[55]
pICK01	P_{TEF1}-*SanSyn*, P_{PGK1}-*tHMG1*	[12]
pISP15	P_{TEF1}-$SanSyn_{opt}$, P_{PGK1}-*tHMG1*	this study
pIGS01	P_{TEF1}-*ERG20*	this study
pIGS02	P_{TEF1}-*ERG20*, AD1	this study
PIGS03	P_{TEF1}-*ERG20*, P_{PGK1}-*GDH2*, AD1	this study
pIGS04	P_{TEF1}-*ERG20*, P_{PGK1}-*GDH2*, AD1, AD2	this study

pIGS05	P_{TEF1}-*ERG20*, P_{PGK1}-*GDH2*, AD1, AD2, *KlURA3*	this study
pIGS06	P_{TEF1}-*ERG20* P_{PGK1}-*GDH2*, AD1, 5´*KlURA3*	this study
pIGS07	P_{TEF1}-*tHMG1*	this study
pIGS08	P_{TEF1}-*tHMG1*, P_{PGK1}-*upc2-1*	this study
pIGS09	P_{TEF1}-*tHMG1*, P_{PGK1}-*upc2-1*, AD3	this study
pIGS10	P_{TEF1}-*tHMG1*, P_{PGK1}-*upc2-1*, AD3, 3´*KlURA3*	this study

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Table 2 - List of *S. cerevisiae* strains used in this study

Strain	Genotype	Plasmid	Reference
CEN.PK113-5D	*MAT***a** *MAL2-8^c SUC2 ura3-52*	none	P. Kötter, University of Frankfurt, Germany
SCIGS28	*MAT***a** *MAL2-8^c SUC2 ura3-52*	pISP15	this study
SCICK01	*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{HXT1}	none	[12]
SCIGS29	*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{HXT1}	pISP15	this study
SCICK16	*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP dpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{HXT1}	none	[12]
SCIGS30	*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP dpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{HXT1}	pISP15	this study
SCIGS03	*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP dpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{HXT1} *gdh1*Δ::*loxP*	none	this study
SCIGS31	*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP dpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{HXT1} *gdh1*Δ::*loxP*	pISP15	this study
SCIGS06	*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP dpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{HXT1} *gdh1*Δ::*loxP* P_{TEF1}-*ERG20* P_{PGK1}-*GDH2 KlURA3*	none	this study
SCIGS22	*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP dpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{HXT1} *gdh1*Δ::*loxP* P_{TEF1}-*ERG20* P_{PGK1}-*GDH2*	none	this study
SCIGS24	*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP dpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{HXT1} *gdh1*Δ::*loxP* P_{TEF1}-*ERG20* P_{PGK1}-*GDH2*	pISP15	this study
SCIGS23	*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP dpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{HXT1} *gdh1*Δ::*loxP* P_{TEF1}-*ERG20* P_{PGK1}-*GDH2* P_{TEF1}-*tHMG1* P_{PGK1}-*upc2-1*	none	this study
SCIGS12	*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP dpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{HXT1} *gdh1*Δ::*loxP* P_{TEF1}-*ERG20* P_{PGK1}-*GDH2* P_{TEF1}-*tHMG1* P_{PGK1}-*upc2-1 KlURA3*	none	this study
SCIGS25	*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP dpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{HXT1} *gdh1*Δ::*loxP* P_{TEF1}-*ERG20* P_{PGK1}-*GDH2* P_{TEF1}-	pISP15	this study

	tHMG1 P_{PGK1}*-upc2-1*		

Table 3 - Physiological parameters measured during double-phase chemostat cultures of strains SCIGS28, SCIGS29, SCIGS30, SCIGS31, SCIGS24 and SCIGS25.

μ_{max}, specific growth rate (h^{-1}); *D*, dilution rate (h^{-1}); *Ysx*, biomass yield (g biomass (g substrate)$^{-1}$); specific consumption rates of glucose(r_s), and oxygen (r_{O2}) (mmol (g biomass)$^{-1}h^{-1}$); specific production rates of carbon dioxide (r_{CO2}), ethanol (r_{etoh}),and acetate (r_{acet}) (mmol (g biomass)$^{-1}h^{-1}$). RQ, respiratory quotient r_{CO2}/r_{O2}; C_s, residual glucose concentration (mM); $C_{balance}$, carbon recovery (%) Values represent the mean ±S.D. of three independent cultivations.

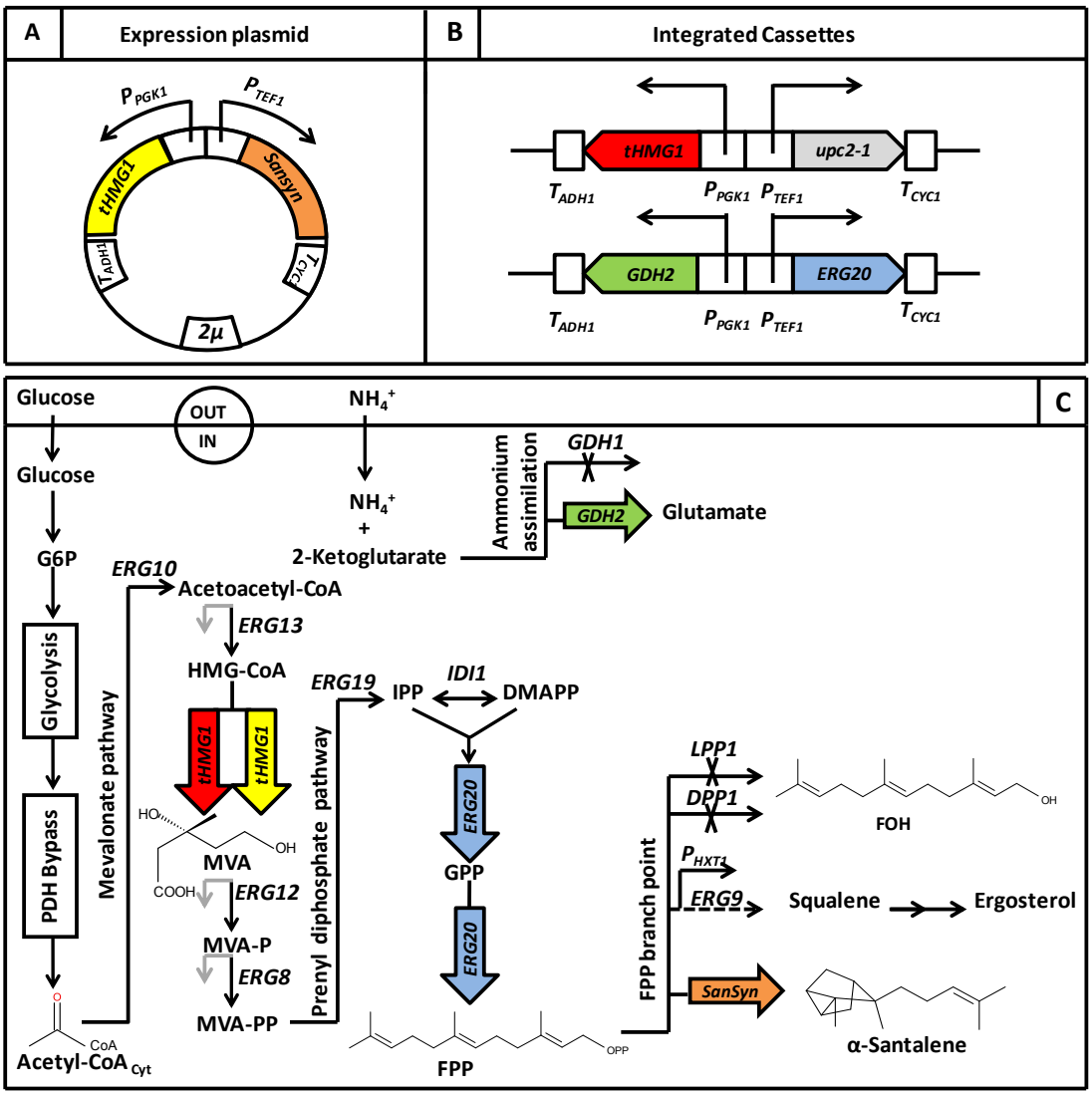
Strain	*D*	Y_{xs}	r_s	r_{CO2}	r_{O2}	r_{etoh}	r_{acet}	RQ	C_s	$C_{balance}$
	(h^{-1})	(g g^{-1})	(mmol g biomass^{-1} h^{-1})					(r_{CO2}/r_{O2})	(mM)	(%)
SCIGS28	0.051 ±0.002	0.50 ±0.01	0.57 ±0.01	1.12 ±0.08	1.12 ±0.06	0	0	1.00 ±0.02	0.18 ±0.02	100.3 ±2.1
	0.10 ±0.01	0.50 ±0.01	1.11 ±0.03	2.67 ±0.15	2.49 ±0.07	0	0	1.07 ±0.05	0.16 ±0.01	101.9 ±1.1
SCIGS29	0.050 ±0.003	0.29 ±0.01	0.97±0.04	3.17 ±0.04	2.95 ±0.15	0.082 ±0.001	0.024 ±0.001	1.07 ±0.07	1.16 ±0.04	96.9 ±3.2
	0.10 ±0.01	0.28 ±0.01	1.95 ±0.05	6.40 ±0.18	5.51 ±0.20	0.128 ±0.004	0.039 ±0.008	1.16 ±0.22	0.39 ±0.01	95.0 ±0.9
SCIGS30	0.051 ±0.001	0.28 ±0.02	1.09 ±0.05	3.60 ±0.09	3.41 ±0.22	0.099 ±0.015	0.024 ±0.003	1.05 ±0.09	1.15 ±0.04	94.7 ±0.4
	0.10 ±0.02	0.25 ±0.01	2.26 ±0.09	7.70 ±0.19	6.37 ±0.29	0.161 ±0.007	0.032 ±0.005	1.21 ±0.14	0.40 ±0.01	93.5 ±1.2
SCIGS31	0.051 ±0.001	0.33 ±0.01	0.86 ±0.01	2.31 ±0.08	1.42 ±0.23	0.501 ±0.077	0.047 ±0.007	1.62 ±0.11	30.67 ±0.78	105.4 ±4.9
SCIGS24	0.051 ±0.001	0.24 ±0.01	1.16±0.03	3.85 ±0.05	3.21 ±0.07	0.185 ±0.003	0.020 ±0.008	1.20 ±0.02	2.53 ±0.09	93.7 ±5.3
SCIGS25	0.048 ±0.003	0.21 ±0.01	1.26 ±0.02	4.41 ±0.04	3.82 ±0.04	0.195 ±0.005	0.027 ±0.007	1.16 ±0.03	2.91 ±0.14	93.8 ±2.9

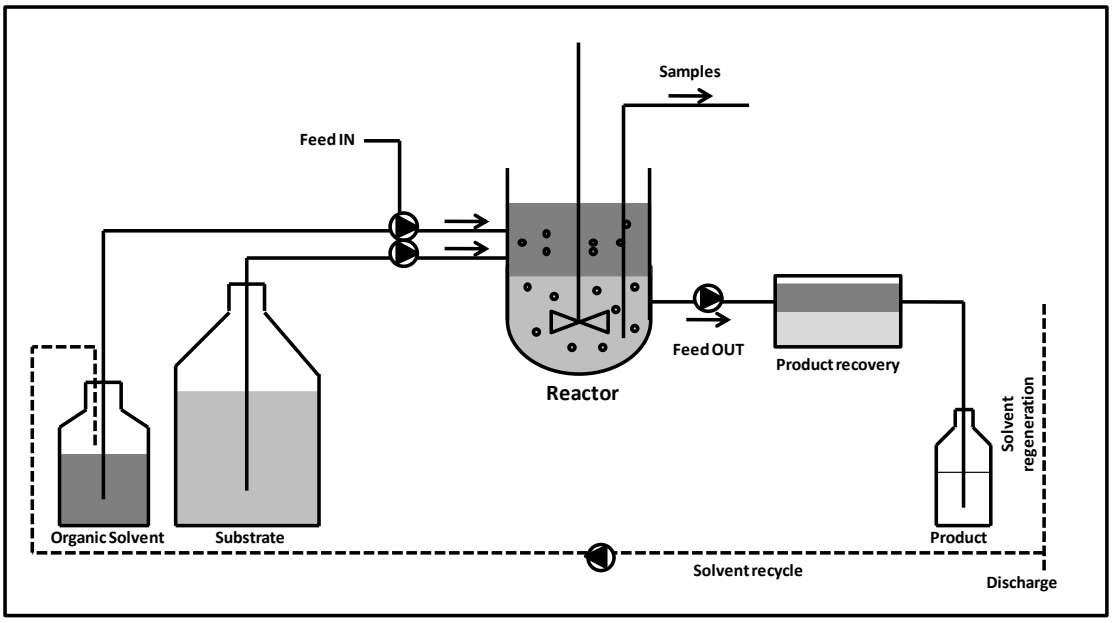
Additional files

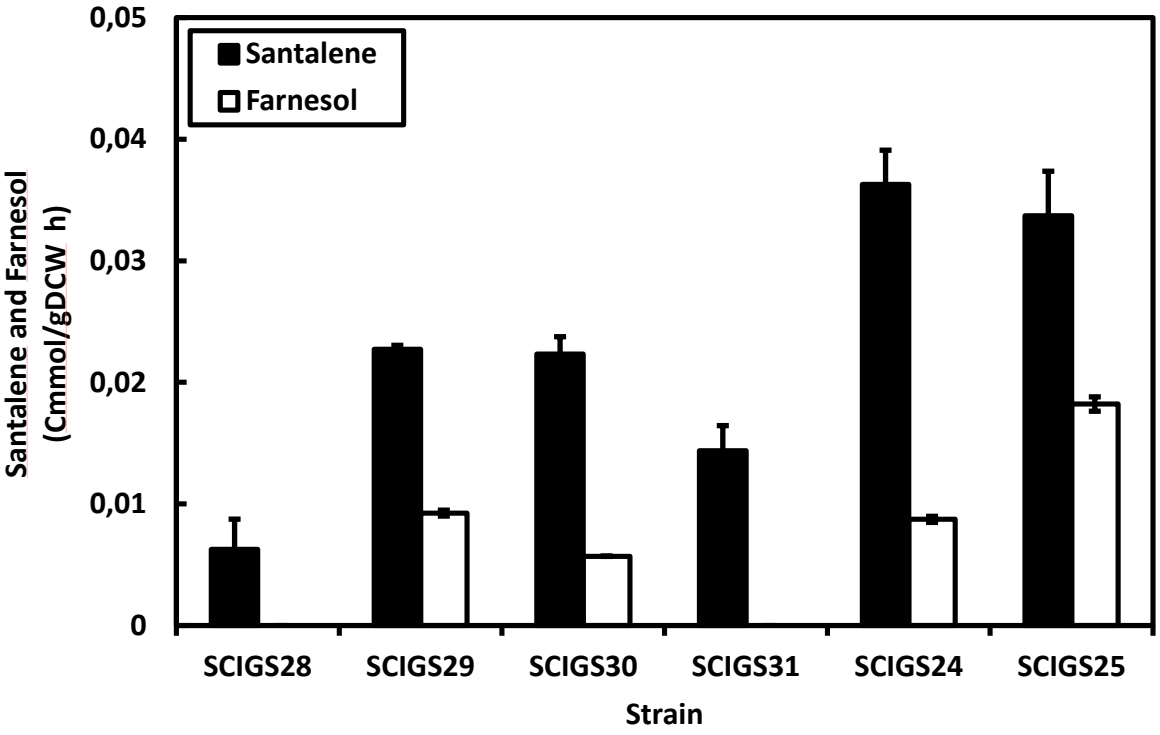
Additional file 1 – Primers used in this study
Restriction sites are indicated in bold face, overlapping nucleotides are underlined, and the modified codon is indicated in italic.

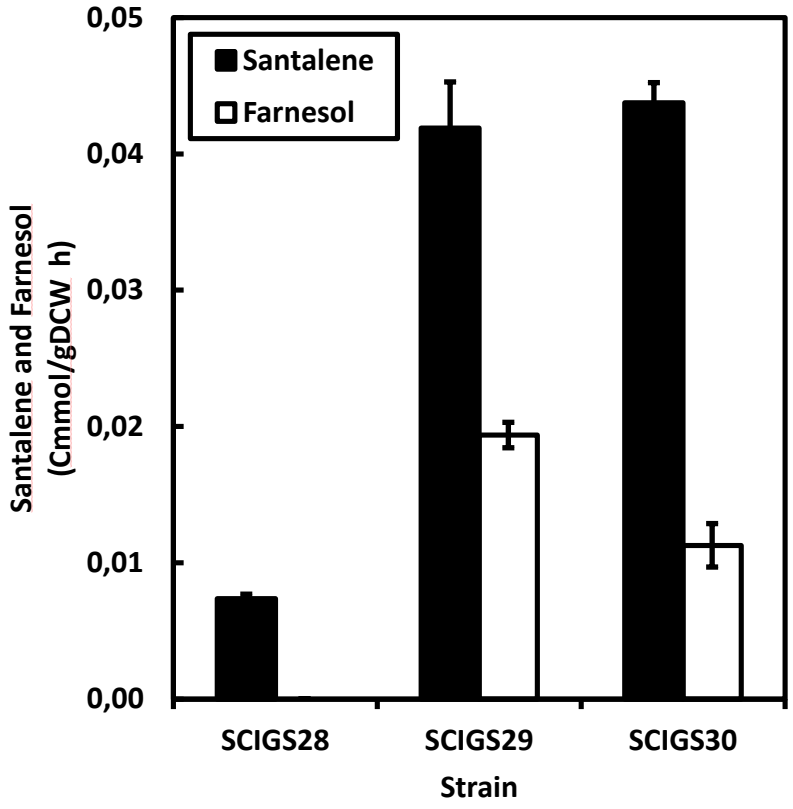
Additional file 2 –Codon optimized santalene synthase nucleotide sequence

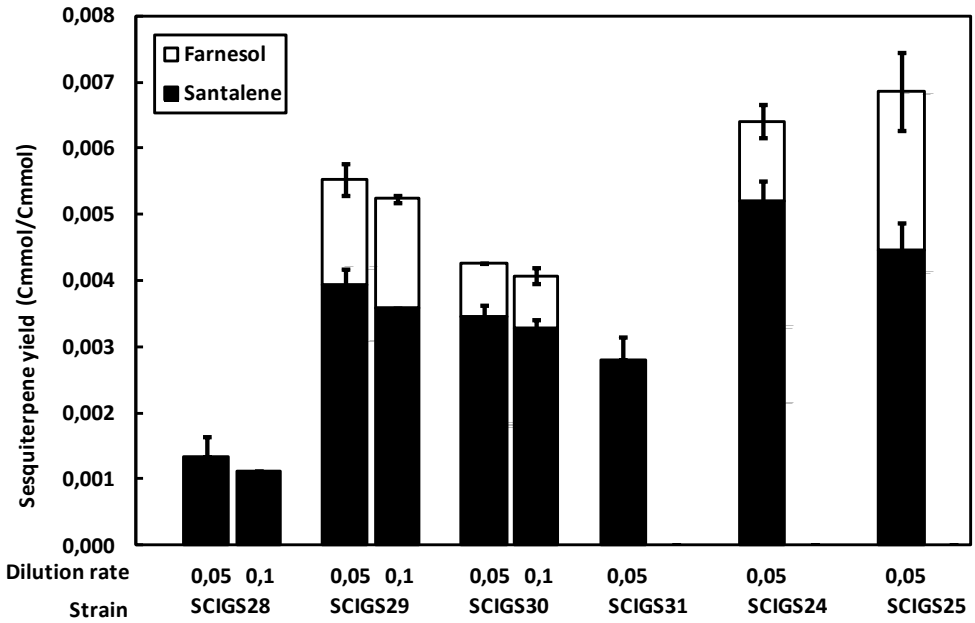
Additional file 3 – Maps of plasmids constructed in this study
(A) pISP15, **(B)** pIGS01, **(C)** pIGS02, **(D)** pIGS03, **(E)** pIGS04, **(F)** pIGS05, **(G)** pIGS06, **(H)** pIGS07, **(I)** pIGS08, **(L)** pIGS09, **(M)** pIGS10.

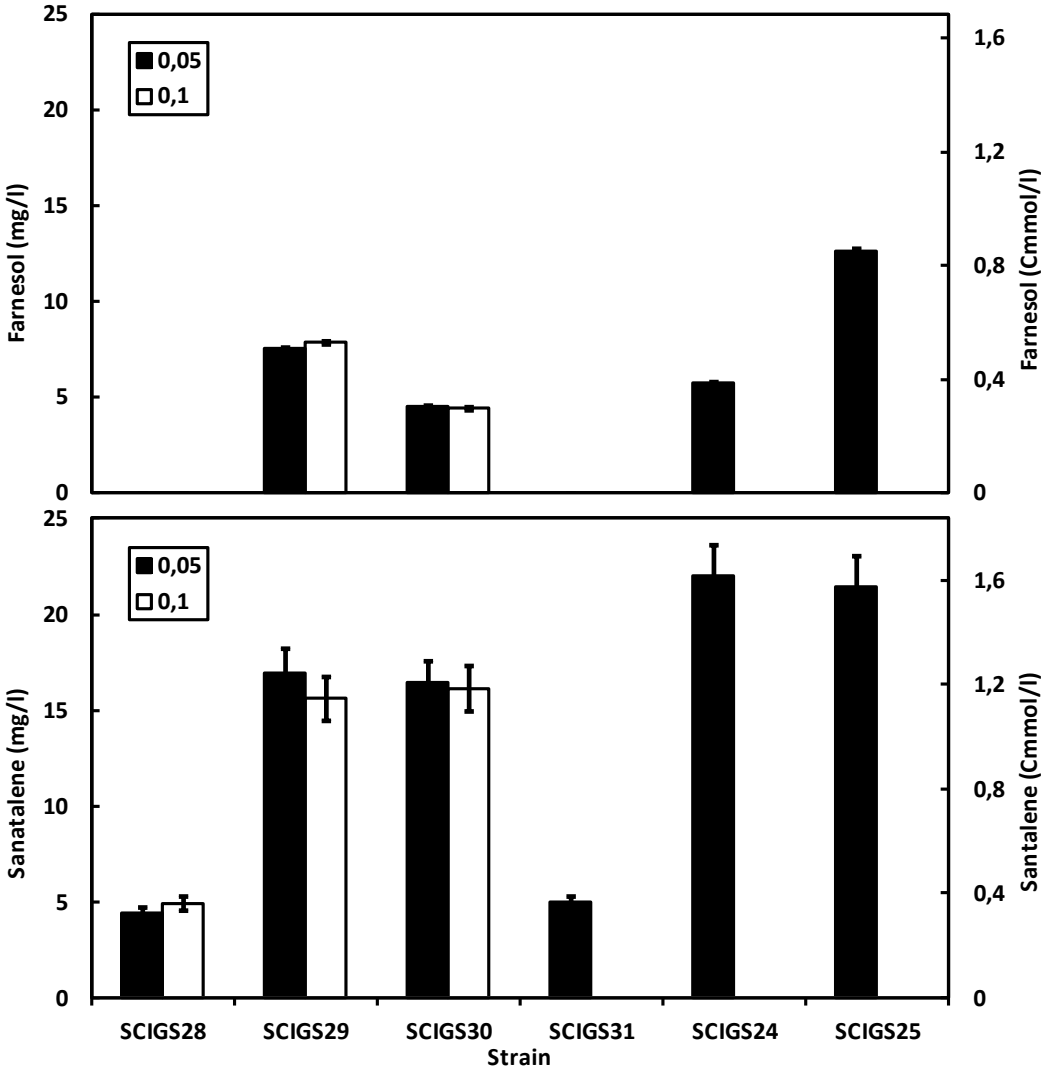

A
Expression plasmid
B
Integrated Cassettes
tHMG1
SanSyn
2µ
upc2-1
GDH2
ERG20
C
Glucose
OUT
IN
Glycolysis
G6P
PDH Bypass
Acetyl-CoA Cyt
Mevalonate pathway
ERG10
Acetoacetyl-CoA
ERG13
HMG-CoA
MVA
ERG12
MVA-P
ERG8
MVA-PP
ERG19
Prenyl diphosphate pathway
IPP
IDI1
DMAPP
GPP
FPP
Ammonium assimilation
GDH1
Glutamate
2-Ketoglutarate
FPP branch point
LPP1
DPP1
FOH
ERG9
Squalene
Ergosterol
SanSyn
α-Santalene


Samples
Feed IN
Feed OUT
Product recovery
Reactor
Solvent regeneration
Organic Solvent
Substrate
Product
Solvent recycle
Discharge


0,05
0,04
0,03
0,02
0,01
0
Santalene
Farnesol
Santalene and Farnesol (Cmmol/gDCW h)
SCIGS28
SCIGS29
SCIGS30
SCIGS31
SCIGS24
SCIGS25
Strain


0,05
0,04
0,03
0,02
0,01
0,00
Santalene
Farnesol
Santalene and Farnesol (Cmmol/gDCW h)
SCIGS28
SCIGS29
SCIGS30
Strain


0,008
0,007
0,006
0,005
0,004
0,003
0,002
0,001
0,000
Sesquiterpene yield (Cmmol/Cmmol)
Farnesol
Santalene
Dilution rate
Strain
0,05 0,1
SCIGS28
0,05 0,1
SCIGS29
0,05 0,1
SCIGS30
0,05
SCIGS31
0,05
SCIGS24
0,05
SCIGS25


0,05
0,1
Farnesol (mg/l)
Farnesol (Cmmol/l)
Sanatalene (mg/l)
Santalene (Cmmol/l)
SCIGS28
SCIGS29
SCIGS30
SCIGS31
SCIGS24
SCIGS25
Strain

PAPER III

Optimization of fed batch process for production of a sesquiterpene biofuel-like precursor **α**-santalene by *Saccharomyces cerevisiae*.

Scalcinati G and Nielsen J.

Submitted.

Optimization of fed batch process for production of sesquiterpene biofuel-like precursor α-santalene by *Saccharomyces cerevisiae*

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Abstract

The development of an efficient microbial production as sustainable technology has an important impact for our society. Advanced biofuel are an emerging target product for develop of novel cell factories. Various sequiterpene compounds have been proposed based on their propriety as suitable biofuel precursor alternative. In the present study, we used a previous engineered *S. cerevisiae* sesquiterpene producing strains to develop an efficient and scalable fed-batch production process. By using these strains as platform host we perform a process engineer and identified some of the process related bottlenecks. A respiratory metabolic control was applied to a rationally designed exponential feed policy. This strategy allowed to control feed delivery and successfully optimize the productivity, representing a valuable tool for implement the production process. Additionally we investigate the effect of ethanol as alternative carbon source to increase the precursor's pool. Detailed process analysis identified the excretion of cyclic sequiterpene as the major process limiting step.

Keyword: Fed-batch fermentation; Isoprenoids; Biofuel; *Saccharomyces cerevisiae*

Introduction

The demand for microbial production of chemicals as alternative to petrochemical based synthesis is increasing due to economical, environmental and geopolitical factors (Dellomonaco et al., 2010; Stephanopoulos et al., 2007). Microbial productions are gaining popularity especially for the biosynthesis of added values compounds (Hong et al., 2012; Kim et al., 2012). Recently, the role of isoprenoids as biomaterial resource has been rediscovered leading to a renewed interest in this class of molecules (Bohlmann et al., 2008). Isoprenoids represent an example of industrially relevant product targeted for bio-production, particularly C_{15}-branched sesquiterpenes beside their application has cosmetics, fragrance, flavouring, nutraceutical and pharmaceutical agents (Leonard et al., 2010; Daviet et al., 2010), are receiving attention as precursors of new generation biofuel for diesel and jet fuels (Peralta-Yahya et al., 2011; Zhang et al., 2011; Rude et al., 2009; Lee et al., 2008). In the last decade, the bio-based production of isoprenoids compound that can complement traditional producing methods has progress dramatically (Westfall et al., 2011; Ro et al., 2006). Advances in metabolic engineering enabled the availability of new technology tools and methods for optimizing metabolic pathways leading to specific strategy for cell bioengineering (Chandran et al., 2011; Keasling et al., 2010). Specifically the application of systems and synthetic biology accelerates the process to achieve the ultimate goal of an efficient "microrefinery" (Keasling et al., 2012; Siddiqui et al., 2012; Nielsen et al., 2011). In yeast sesquiterpenes (C_{15}) are synthesized from cytosolic acetyl-CoA though the mevalonate (MVA) pathway in sub-sequential condensation of three isoprene functional unit isopentenyl diphosphate (C_5) resulting into the universal sesquiterpene building block farnesyl diphosphate (Fig. 1B) (Maury et al., 2005).

Conversions of the liner FPP into cyclic derivative involve limited numbers of mechanisms and result in the production of diverse classes of sesquiterpenes; example of FPP cyclization

products via nerolidyl diphosphate intermediate (NPP) are the structurally related sesquiterpene classes: cedranes, santalanes, bisabolanes and bregamotanes (McCaskill et al., 1997). The portfolio of fuel candidate compound has been greatly expanded lately. Recent researches highlight branched and cyclic sesquiterpene hydrocarbons (e.g. bisabolanes and farnesanes) as potential jet fuel precursor's based on their physicochemical proprieties (Peralta-Yahya et al., 2011; Renninger et al., 2008). Previously, we engineered *S. cerevisiae* through a multistep metabolic engineering approach for the production of cyclic hydrocarbon sesquiterpene α-santelene with suitable characteristics as fuel like precursor (Scalcinati et al., 2012a; Scalcianti et al., 2012b).

Here, we used our yeast sesquiterpene producing platform to develop an efficient fermentation process. Bioreactor operations have strong impact on design an efficient bioprocess and influence in a significant way the production performances. Economically feasible bio-production requires the development of a production process that allow to achieve high titer, yields and productivities of targets compounds. Fed-batch cultivation is by far the most employed fermentation process for industrial production (Nielsen et al., 2003). Among the advantage of using fed-batch process for large scale process are the high final tiers achievable and the minimization of overflow metabolism. During fed batch operation the feed rate represent key parameter in controlling the overall process performances. Typical fed batch configurations of high values products are based on a first phase were the feed is kept exponential and a second phase when high cell concentration is reached with constant feed rate to avoid potential limitation, however this often result in suboptimal conditions affecting productivity and viability of the cell (Pham et al., 1998).

Operational strategies that allow optimal feeding policy are required to overcome problems related to the detrimental effect due to the over/under feeding (Woehrer et al., 1981). Advance in fermentation technology produce a multitude of strategy focused on properly control the

fed batch process (Lee et al., 1999). Ideal glucose based fed batch proceed maintaining a sugar concentration below the critical values preventing the consequent Crabtree effect, if this value is exceeded yeast metabolism rapidly switch to fermentative mode and the fermentation products ethanol, acetate formation set in producing a reduction in the biomass yield.

In our previous study we elucidate the specific growth rate dependence of sesquiterpene productivity of our engineered overproducing mutants (Scalcinati et al., 2012b). An optimal process design would then require simultaneous formation of biomass and target compound and would be favored without loss of carbon to fermentative over-flow metabolites.

Here, we develop a biomass-coupled production process applying a RQ control to a limited glucose exponential fed batch; we use instant RQ measurement as indicator of the metabolic state to fine tuning the feed profile to the cellular demand in order to maintain fully respiratory state and achieve an optimal feed strategy. This strategy was combined with a consolidated double-phase partitioning set-up resulting in an integrated fermentation recovery process (Figure 1A).

Additionally, the effect of ethanol as alternative carbon source on sesquiterpene production was investigated. The C_2 carbon ethanol represents an attractive carbon source for secondary metabolite production. However because the raw material is often the dominating operative cost of added values chemicals industrial bio-production (Otero et al., 2007) a fully ethanol based process would probably not be commercially viable. Yeast *S. cerevisiae* has the ability to co consume glucose-ethanol under fully respiratory conditions (Geurts et al., 1980), if the ratio between the two C-source is maintained below certain limits (0.57:0.43 Cmol $Cmol^{-1}$) glucose would be employed from the cell mainly for biosynthesis whereas all the cytosolyc acetyl-CoA would be derived from ethanol that would then be used in the TCA cycle (de Jong-Gubbels et al., 1995; van Gulik et al., 1995). We decide therefore to investigate the effect of a mixed glucose/ethanol feed applied to the designed fed-batch process.

In this study, we present progress in production of sesquiterpene α-santalene, development in fermentation method result in the creation of an efficient production system for α-santalene capable of an overall yield, productivity and titers of 0.0043 Cmmol (Cmmol substrate)$^{-1}$, 0.051 Cmmol (gDCW h)$^{-1}$ and 14.2 Cmmol l^{-1}.

Materials and methods

Strains and maintenance

S. cerevisiae strains used in this study have been previously engineered for α-sanatlene production. They are SCIGS24 (*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP dpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{HXT1} *gdh1*Δ::*loxP* P_{TEF1}-*ERG20* P_{PGK1}-*GDH2*) and SCIGS25 (*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP dpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{HXT1} *gdh1*Δ::*loxP* P_{TEF1}-*ERG20* P_{PGK1}-*GDH2* P_{TEF1}-*tHMG1* P_{PGK1}-*upc2-1*) both strains carry the plasmid pISP15 (2μ *URA3*) expressing a copy of the truncate *HMG1* (*tHMG1*) and codon optimized *Sansyn* (*SanSynOpt*) under control of the *PGK1* and *TEF1* promoter, respectively (Scalcinati et al., 2012b). Strains were maintained on synthetic dextrose (SD) plate containing 6.9 g l^{-1} yeast nitrogen base w/o amino acids (Formedium, Hunstanton, UK), 0.77 g l^{-1} complete supplement mixture (CSM) w/o uracil (MP Biomedicals, Solon, OH, USA), 20 g l^{-1} glucose, and 20 g l^{-1} agar. Stock cultures were kept in 30% (v/v) glycerol at -80ºC for long term storage (Sherman et al., 1986).

Media and growth conditions

A previously described (Verduyn et al., 1992) mineral salts medium was used consisting of the following (per liter): $(NH_4)_2SO_4$, 5 g; KH_2PO_4, 3 g; $MgSO_4$•7H2O, 0.50 g; Antifoam 289 (A-5551, Sigma–Aldrich), 0.050 ml; trace metals, 1 ml and vitamins, 1 ml. The trace metal solution consisted of the following (per liter): EDTA (sodium salt), 15.0 g; $ZnSO_4$•$7H_2O$, 0.45 g; MnCl2•$2H_2O$, 1 g; $CoCl_2$•$6H_2O$, 0.3 g; $CuSO_4$•$5H_2O$, 0.3 g; Na_2MoO_4•$2H_2O$, 0.4 g; $CaCl_2$•$2H_2O$, 0.45 g; $FeSO_4$•$7H_2O$, 0.3 g; H_3BO_3, 0.1 g and KI, 0.10 g. The pH of the trace

metal solution was adjusted to 4.0 with 2 M NaOH prior to heat sterilization. The vitamin solution contained (per liter): biotin, 0.05 g; p-amino benzoic acid, 0.2 g; nicotinic acid, 1 g; Ca-pantothenate, 1 g; pyridoxine-HCl, 1 g; thiamine-HCl, 1 g and myo-inositol, 25 g. The pH of the vitamin solution was adjusted to 6.5 with 2 M NaOH. The vitamin solution was filter sterilized and stored at 4°C. This medium was supplemented with 30 g l^{-1} glucose. The feed media had the same composition as described above, but the $(NH_4)_2SO_4$; KH_2PO_4; $MgSO_4$•7H2O, vitamin solution, and trace metal solution, concentration were increased 10 times. The carbon source used was 100% glucose alone or a mixture of 57% Cmol glucose and 43% Cmol ethanol. For the cultivations on glucose the target concentration was 6.66 Cmol l^{-1} (200 g l^{-1}); for mixed carbon source cultivation was 3.80 Cmol l^{-1} (114.0 g l^{-1}) glucose and 2.86 Cmol l^{-1} (65.98 g l^{-1}) ethanol, yielding a target final carbon concentration of 6.66 Cmol l^{-1}. Both the sugar solutions were added by sterile filtration using a cellulose acetate filter (0.20 μm pore size Minisart®-Plus Satorius AG).

Fed-batch operation

The aerobic fed-batch process was performed in 2.5 l Applikon vessels (Applikon, Schiedam, The Netherlands) with an initial working volume of 1.0 l. Agitation at 800 rpm was maintained using an integrated stirrer (DasGip, Jülich, Germany) and the temperature was monitored using platinum RTD temperature sensor and kept at 30°C. The rate of aeration was set to 1 vvm by mass flow controller. The pH of the medium was maintained at 5.0 by automatic addition of 2 N KOH during the batch phase and 6 N KOH during the feed phase. The temperature, agitation, gassing, pH and composition of the off-gas were monitored and controlled using the integated DasGip monitoring and control system. Dissolved oxygen concentration was monitored with an autoclavable polarographic oxygen electrode (Mettler Toledo, Columbus, OH, USA) and kept above 30% via stirrer speed and gas flow rate using the DasGip control system. The effluent gas from the fermentation was cooled, dried and

analyzed for real-time determination of oxygen and CO_2 concentration by DasGip fedbatch pro® gas analysis systems with the off gas analyzer GA4 based on zirconium dioxide and two-beam infrared sensor. The integrated mass flow sensor allowed on-line monitoring and calculation of oxygen transfer rate (OTR) (mmol h^{-1}), carbon dioxide transfer rate (CTR) (mmol h^{-1}) and respiration quotient (RQ) calculate dividing CTR by OTR. The seed cultures for the cultivations were grown at 30˚C in 500-ml shake flasks containing 100 ml of culture with agitation in an orbital shaker at 100 rpm. Pre-cultures were used to inoculate the fermentors to a final dry weight of 1 mg l^{-1}. All cultivations were performed in triplicate. The fed-batch cultures were initiated as batch cultures using 30 g l^{-1} glucose. Feeding with fresh medium commenced only after residual ethanol produced from the glucose consumption phase was completely depleted. An exponential feed rate ν (*t*) (liter h^{-1}) was calculated as previously described (Scalcinati et al., 2012a). The feed was initially designed to increase exponentially with a specific feed rate of 0.1 h^{-1}. Correct feed rate addition was obtained using a modified single loop, set-point control method programming the fermenter fb-pro software (DasGip) and controlled using the DasGip control system (Fig. 1A). Feed rate was additionally controlled based on the respiratory culture response monitoring RQ values as indirect measure of the cell metabolic state. On line precise measurement of the exit gas composition allows to adjust the feed rate through a proportional integral (PI) controller maintaining the RQ at the desired set point of 1.0 during feed with glucose alone and 0.82 for glucose ethanol (0.57: 0.43 Cmol $Cmol^{-1}$) mixed feed. An organic layer of dodecane (Sigma-Aldrich St. Luis, MO) was added aseptically to a final volume of 10% (v/v) immediately before starting the feed.

Analytical methods

Cell growth during fermentation was monitored off-line by measuring optical density and dry cell weight and on-line with an optical density transmitter OD4 sensor (DasGip, Jülich,

Germany) integrated in the fermenter system. The optical density at 600 nm was determined using a Genesis20 spectrophotometer (Thermo scientific, Madison WI, USA). The cell dry weight was measured by filtering known volumes of the cultures through pre-dried and pre-weighed 0.45-µm-pores size nitrocellulose filters (Supor-450 membrane filters; PALL Life Sciences Ann Abor, MI). The filters with the biomass were washed with water, dried for 15 min in a microwave oven at 150 W, and weighed again.

Concentrations of glucose, glycerol, ethanol, acetate, succinate, and pyruvate were analysed by an isocratic high-performance liquid chromatograph (UltiMate® 3000 Nano, Dionex, Bannockburn IL, USA) with an Aminex HXP-87H ion-exchange column (Bio-Rad, Hercules, CA) at 65˚C using 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 ml min^{-1}. Glucose, glycerol, and ethanol were measured with a refraction index detector (RI-101 Refractive Index Detector, Shodex®), and acetate, succinate, and pyruvate were measured with a UV-visible light absorbance detector (UltiMate 3000 Variable Wavelength Detector, Dionex)

Analysis of sesquiterpenes

Sequiterpene production during the course of fermentation was determined as described previously (Scalcinati et al., 2012b) with minor modifications. For determine extracellular sesquiterpene concentration culture samples were centrifuged 5 min at 5000 g the organic layer collected, diluted with an equal volume of 2-ethylacetate (Sigma, St Luis, MO, USA) containing defined amount of α-humulene as internal standard and subsequently analyzed by gas chromatography-mass spectrometry (GC-MS). For determine the total intracellular sesquiterpene concentration a repeated extractive process was used. A known volume of cultures was harvested by centrifuging at 5000 rpm for 20 min. The cell pellet was washed twice with distilled water and the cell suspension was centrifuged for another 10 min at 5000 rpm. The cell pellet was re-suspended in 500 µl of 2-ethylacetate and mixed with 250 mg of acid washed glass beads (Sigma, St Luis, MO, USA) in a glass GC vial PTFE lined screw cap,

heated and mixed in a vortex termomixer at 60 ºC for 20 min and then centrifuged 15 min at 5000 rpm. The 2-ethylacetate layer was transferred to a clean GC vials and the pellet undergoes to a new extractive cycle. A total of five cycles of extraction were necessary to completely recover all the intracellular sesquiterpenes. The pooled extracts were diluted with an equal volume of 2-ethylacetate containing defined amount of α-humulene as internal standard and subsequently analyzed by GC-MS. The GC/MS sesquiterpene quantitative determination was performed with a DSQ II single quadrupole mass spectrometer (Thermo Scientific, Waltham, MA) equipped with a SLB-5 ms capillary column (15 m, 0.25 mm i.d., 0.25 µm film thickness; Supelco, Bellefonte, PA, USA) using helium as carrier gas at a flow rate of 1.2 ml min^{-1}. The initial oven temperature was 80°C and the injector temperature was 250°C. The oven temperature was increased to 120°C at a rate of 10°C min^{-1} and subsequently increased to 160°C at a rate of 3°C min^{-1}. The oven temperature was finally increased to 270°C at a rate of 10°C min^{-1} and held for 5 min at this temperature. Full mass spectra were generated by scanning m/z range within 40-500. Sesquiterpene identification was performed comparing mass spectra and retention time with authentic standards, concentrations were calculated using a correction factor determined for the internal standard α-humulene relative to α-santalene, *trans*-α-bergamotene, *E,E*-farnesene and *E,E*-farnesol.

Results

Performance of RQ controlled feeding strategy on double phase fed batch cultivation

Large scale commercial productions of microbial products are mainly realized using fed-batch process (Nielsen et al., 2003). Recently we demonstrate the feasibility of sesquiterpene α-santalene production in *S. cerevisiae.* (Scalcinati et al., 2012a). In order to increase sesquiterpene yield, yeast strains were previously engineered through a multistep metabolic engineered strategy, overexpressing key regulatory gene of the MVA pathway farnesyl

diphosphate synthase (*ERG20*↑) and a constitutive active form of 3-hydroxy-3-methyl-glutaryl-CoA reductase (*tHMG1*↑); down-regulating precursor consuming reaction squalene synthase (↓*ERG9*); deleting gene catalyzing by-product formation (*ΔLPP1, ΔDPP1*); increasing cofactors availability (*ΔGDH1,* ↑*GDH2*); all this modification were integrated in the genome to ensure genetic stability and lead to strain SCIGS24. Additional the overexpression of a mutated global transcription factor (*upc2-1*) resulted in strain SCIGS25 (Scalcinati et al 2012b). Here, we undertook our previously engineered *S. cerevisiae* sesquiterpene producing platform to design an efficient ISPR aerobic fed-batch production process (Fig. 1A). The cultivation process was initiated as batch with 1 Cmol l^{-1} (30 g l^{-1}) glucose as carbon source, production phase was started feeding concentrate substrate after the ethanol produced during the first glucose batch phase was completely consumed and no other carbon sources were left in the media. Fed-batch was carried out as two phase cultivation mode applying an organic overlay that allow *in situ* product recovery (Fig. 1A). Feed rate was initially designed with exponential policy chosen so that the volumetric rate of biomass production was constant and equal to a specific growth rate of 0.1 h^{-1}. Additional RQ control strategy was applied to the traditional exponential feed profile in order to maintain a fully respiratory metabolism during the entire feed process maximizing the biomass formation and avoiding the fermentation byproduct accumulation. Complete oxidation of glucose to carbon dioxide and water through respiratory metabolism would lead to a respiratory coefficient RQ equal to 1. On-line measurement of CTR and OTR allow instantaneous calculation of the RQ, allowing to control the feed addition regulating the RQ close to the selected set point and below the critical level that trigger the overflow metabolism (RQ≤1). In an initial set of experiment fed-batch was performed cultivating strains SCIGS24 and SCIGS25 using glucose with a concentration of 7.4 Cmol l^{-1} (222 g l^{-1}) as sole carbon source in the feed media. The results of the respiratory quotient control on the feed profile are reported in figure 2. Both

strains display similar characteristics, within the first 15-16 hours of feed the RQ value continue to increase, when the set-point value of 1 was reached the controller respond decreasing the feed addition and the cultivation continued with a decrease exponential rate lower than the theoretically designed with the RQ values oscillate between 1 and 1.2 (Fig. 2). As result the metabolism was maintained fully respirative during the entire feed time and no accumulation of glucose or fermentative products were detected in both strains.

Extractive fermentation was realized using a double phase partitioning set-up, so the product is constantly removed from the cell and trapped into the solvent phase. Organic layer was added before initiating the feed addition corresponding to the start of the production phase. In order to monitor the sesquiterpene production performance during cultivation time, product accumulation in the organic layer was analyzed at regular intervals. Four different sesquiterpene products were detected, with α-santalene as major product followed by *E,E*-farnesol, and minor amount of *trans*-α-bergamotene and *E,E*-farnesene (Fig. 3). Yield and total sesquiterpene concentration were slightly higher in strain SCIGS24 than in strain SCIGS25 (table I). Target compound α-santalene accumulate on strain SCIGS24 and SCIGS25 to final concentration of 11.9 and 11.3 Cmmol l^{-1} respectively, representing 63 Cmol % of the total sesquiterpene produced by these strains. Product to substrate yield of the different compounds are reported in table I and range from 0.0037 for the most abundant sesquiterpene α-santalene to the lowest 0.0003 (Cmmol $Cmmol^{-1}$) for farnesene in strain SCIGS24 and from 0.0033 to 0.0003 (Cmmol $Cmmol^{-1}$) in strain SCIGS25.

Effect of mixed (glucose/ethanol) substrate feed on sesquiterpene production

Under fully oxidative condition *S. cerevisiae* is capable of simultaneous consumption of glucose and ethanol as substrate. The ratio between the two carbon sources determines changes in the metabolic flux in the central carbon metabolism. During growth on glucose alone has carbon source sesquiterpene are obtained directly from the cytosolic acetyl-CoA

produced through pyruvate decarboxylase. Under glucose/ethanol aerobic carbon limited cultures if the ethanol content in the feed is maintained below 0.43 Cmol $Cmol^{-1}$ the ctytosolic acetyl-CoA is derived directly from the ethanol (de-Jong-Gubbels et al., 1995). Here, we investigate the effect of ethanol as alternative substrate for the production of sesquiterpene. To determine if this approach can be applied for improving the production a mixture of 0.57:0.43 glucose:ethanol was used as a feed during the RQ controlled fed-batch process previously described. Oxidation of glucose and ethanol in the selected carbon molar ratio through respiratory metabolism would lead to a respiratory coefficient equal to 0.82. Consistently with the previous experiments the RQ controller was set to maintain an RQ value ($RQ \leq 0.82$), that prevent the occurrence of the fermentative metabolism. The effects of the mixed feed on the fed-batch process are represented in figure 4. Similar to the process on glucose both strains SCIGS24 and SCIGS25 displayed comparable behavior. The RQ value increase until selected set-point during a first phase (15-16 hours) after that was feedback controlled and remains stable around a value of 0.8. When compared to the situation with glucose alone the feed profile of the volume added on time, differs only in minor amount from the theoretical input. However after 16 hours of feed a second phase, were part of the ethanol content in the feed but not glucose was not completely consume occur (Fig. 4). The ethanol fraction that accumulate increase progressively during the cultivation reaching 54%-58% of the ethanol fed and corresponding to 22%- 24% (in Cmol basis) of the total carbon source at the end of the feed for strain SCIGS24 and SCIGS25 respectively. The effects of the mixed substrate on sesquiterpene production are summarized in table I. Consistently with the previous results on glucose α-santalene was the major product (Fig. 5). Ethanol has a positive effect on the sesquiterpene production on both strains. Strains SCIGS24 and SCIGS25 displayed a 14-21% increase α-santalene yield with the best yield of 0.0043 Cmmol $Cmmol^{-1}$ of strain SCIGS24. Interestingly FOH did not change significantly in strain SCIGS24 under

both conditions whereas a 15% improvement was observed on strain SCIGS25 resulting the highest value observed in all process. The yield of the two minor products *trans*-α-bregamotene and *E,E*-farnesene was slightly reduced in both strains under these conditions. When ethanol was used as precursor source for sesquiterpene the total yield was improved for both strains SCIGS24 and SCIGS25 of 4.7 and 13.6 % respectively.

If we compare the production performances of the two process, using mixed substrate was possible to increase the total sesquiterpene productivity for strain SCIGS24 of 49% from 0.038 to 0.076 Cmmol (g biomass)$^{-1}$ h^{-1} (Fig. 6), a minor effect was observed in strain SCIGS25 with 24% increase in productivity from 0.036 to 0.048 Cmmol (g biomass)$^{-1}$ h^{-1}. The best performance was archived using strain SCIGS24 combined with mixed substrate glucose ethanol yielding to a final sesquiterpene concentration of 21.2 Cmol l^{-1} after 28.5 h of feed (table I).

Intracellular sesquiterpene accumulation

Time course analysis of the two process shown that in both cases after c.a. 16 hours of feed time a limitation phase occur leading either a slower consumption during the process with glucose only or incomplete carbon consumption with ethanol accumulation in the process with mixed glucose/ethanol. As direct consequence a decrease in sesquiterpene production in the organic layer was detected (Fig.3 and Fig 5). Several factors could cause the above-described reduction in production performance. In order to investigate if the efflux of sesquiterpene from the cell was related to this effect, intracellular sesquiterpene assay was performed. Only two of the four products detected in the organic layer accumulated intracellularly α-santalene and *trans*-α-bergamotene. Intra and extracellular product accumulation versus feed time for the two different strains under the two different processes are reported in figure 7. Typically the intracellular fraction of α-santalene and *trans*-α-bergamotene measured were between 15-18% and 17-29% of the total amount detected

respectively. It is interesting to notice that the two compound displayed different intracellular accumulation profile. Intracellular *trans*-α-bergamotene amount increase on time and mainly follow the exrtracellular amount accumulated and was retained in the cell in a proportional amount respect to the extracellular concentration. Whereas the intracellular α-santalene concentration reach saturation around a value of 2 Cmmol l^{-1}, corresponding approximately to the time where the entire process became limited. This phenomenon was detected in both strains and seems to be independent of the carbon source utilized as substrate.

Discussion

Develop of an effective bioprocess: impact of the RQ control on the fed-batch process

The developments of an efficient bioprocess for production of sesquiterpene that can compete with the traditional industrial methods require maximization of yield and productivity. Optimization of production performance needs to fast obtain active cell minimizing side product formation. Process design requires detailed knowledge of the product formation in order to achieve optimal balance. In our sesquiterpene engineered strain the extracellular glucose concentration trigger the switch between the flux toward the product formation (Scalcinati et al., 2012a; Scalcinati et al., 2012b) it is therefore critical carefully control the cell metabolism to obtain the best productivity during the entire process. In an initial stage of process optimization we implemented an RQ feedback control method to a programmed limited exponential feed profile of glucose. The RQ control is a physiological type of controller widely applied to improve fed-batch cultivation performances (Kiss et al., 1991; Xiong et al., 2010; Xiao et al., 2006; Bideaux et al., 2006). Here, the feedback control permitted to constantly operate below the critical required value resulting in a robust feed strategy that allow to alleviate glucose repression and Crabtree effect and determine an optimal feed policy. The applied respiratory control systems result in the maximum fed rate

sustainable from the cell without byproduct formation. The fed batch process was integrated with a consolidated *in situ* product removal (ISPR) system (Freeman et al., 1993). Once the target compounds are secreted outside the cell, ISPR allow instant and continuous product recovery, sequestering the products in the organic layer. Extracellular sesquiterpene analysis results in the detection of four different products, beside the target compound α-santalene and the know side product FOH originated from the spontaneous dephosporilation of FPP (Wang et al., 2011), minor product *trans*-α-bergamote and *E,E*-franesene were detected. Both this compounds are secondary product of the santalene syntase (M. Shalk personal communication). Apparently in the tested conditions santalene synthase has a different level of specificity toward α-santalene confirming the high degree of plasticity often reported for this class of enzymes (Yoshikuni et al., 2006).

This methods result in a process capable of producing 12 Cmmol l^{-1} (163 mg l^{-1}) of α-santalene and over 18 Cmmol l^{-1} (261 mg l^{-1}) of total sesquiterpene in 30 h of feed. The minor physiological differences obtained within the two engineered strain tested confirm the applicability and reproducibility of the methods.

Ethanol as alternative C-source for sesquiterpene production, effect of the mixed substrate

In order to obtain a sesquiterpene factory efficient precursors supply is necessary. In yeast sesquiterpene are originated exclusively in the MVA pathway and use cytosolic acetyl-CoA as primary metabolite precursor. Carbon source employed and type of metabolism are known to affect the cellular level of cytosolic acetyl-CoA (Frick et al., 2005; Seker et al., 2005). Previous studies demonstrated that the mevalonate pathway is subject to glucose repression and growth on ethanol lead to an increase in the cytosolyc acetyl-CoA and acetoacetyl-CoA (Quain et al., 1979; Seker et al., 2005). On this bases ethanol represent an attractive carbon source for sesquiterpene production in order to increase the supply of acetyl-CoA for the

mevalonate pathway. In this prospective, ethanol and ethanol/glucose mix has successfully been applied to increase production of sesquiterpene in yeast (Westfall et al. 2011; Tsuruta et al., 2009). However, although market price of ethanol has decrease substantially due the advent of bio-ethanol a mixed cultivation would rather be economically favorable compared to a fully based ethanol feed. Under respiratory conditions the co-consumption of the two sources is highly regulated by the ratio of the two compounds (de Jong-Gubbels et al., 1995). Herewith, it was possible to use ethanol as direct precursor for sesquiterpene formation and glucose for biomass intermediate biosynthesis. Theoretically on Cmol basis α-santalene maximum achievable yield on ethanol (0.83 Cmol $Cmol^{-1}$) is 27% higher than glucose (0.56 Cmol $Cmol^{-1}$). Using mixed substrate we further enhance yield and productivity resulting in a final production of 14 Cmmol l^{-1} (193 mg l^{-1}) of α-santalene and over 21 Cmmol l^{-1} (292 mg l^{-1}) of total sesquiterpene in 28.5 h of feed. The multiple carbon source utilization strategy applied to increase precursor supply, result in a rapid approach to improve of the process performances

Intracellular accumulation and potential derived toxicity

Detailed process characterization show the impossibility to maintain a constant growth rate during the entire process. Independently form the carbon source employed the process was mainly divided into two distinct phase. An initial phase with higher growth rate and a second one with slower growth rate were the process became limited. The previously shown dependency of the productivity on the growth rate was reflected in a reduction of the product formation rate resulting in a suboptimal process. In order to investigate the possible causes of the growth impediment we assay the intracellular content of sesquiterpens. Only two of the four different sesquiterpene produced, α-santalene and *trans*-α-bergamotene were detected intracellularly with α-santalene representing the main intracellular compound. These two compounds differ substantially from the other linear hydrocarbon *E,E*-farnesol and *E,E*-

farnesene because of their multiple cyclic structure. Previous study has proposed that cyclic terpenes can accumulate into the membrane (Sikkema et al., 1994). It is then reasonable to assume that the structural characteristic might be the reason of the difference observed in the capacity to retain these compounds inside the cell. The export of α-santalene from the cell seems to be the main cause of the inhibitory effect detected that limits its productivity. Up to date the mechanisms of secretion of hydrocarbons are not known. In the natural producers organisms terpenes compounds accumulate into a wide variety of specific multicellular secretory structures (Gershenzon et al., 1994 and Besser et al., 2009) but the mechanism of accumulation has not been fully investigated and very few information are available regarding the secretion process. Due to the hydrophobic nature of these compounds, it has been suggested that the excretion can proceed as simple diffusion without require tranporters (Muramatsu et al., 2008). However the saturation kinetics on time observed for santalene might suggest that yeast utilize unspecific transporter to secrete this compound. In yeast strains engineered for production of different sesquiterpene production, transcriptional up regulation of multidrug transporters related genes resulted induced (Verwaal et al., 2010; Ro et al., 2008). Consistently, our transcriptome studies performed in presence of high sesquiterpene concentration were characterized by the overexpression pleoitropic drug resistance (PDR) network genes (unpublished result).

Nowadays, there are only few evidences regarding toxicity of plan terpenes compound on *S. cerevisiae* (Parveen et al., 2004; Sikkema et al., 1995). Indeed, intra-membrane accumulation of cyclic sesquiterpene has been shown to produce toxic effect caused by loss of membrane integrity (Sikkema et al., 1995). The observed physiology obtained can be explained with the excessive intracellular α-santalene accumulation and consequent toxicity related effect. Further investigation would be necessary in order to fully understand and overcome the limits

of the process in order to achieve production rates with commercial potential for a viable biobased production.

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Table I. Titers and yield of target sesquiterpenes obtained during aerobic fed batch cultivation of strains SCIGS24 and SCIGS25. α-santalene yield (Y_{sSan}); *E,E*-farnesol yield (Y_{sFar}); *trans*-α-bergamotene (Y_{sBer}) and *E,E*-Farnesene yield (Y_{Fse}) (Cmmol (Cmmol substrate)$^{-1}$), referred to the feed phase. Total sesquiterpene titer (Tot_{Sesq}) (Cmmol l^{-1}) measured at the end of the feed process. Value represents the mean ±S.D. of three independent cultivations.

Strain	Feed C-source		Sesquiterpene Yield					Titers
	Glucose	EtOH	Y_{sSan}	Y_{sFar}	Y_{sBer}	Y_{sFse}	Y_{sTot}	Tot_{Sesq}
	Cmol l^{-1}		Cmmol (Cmmol substrate)$^{-1}$					Cmmol l^{-1}
SCIGS24	7.40 ±0.11		0.0037 ±0.0005	0.0018 ±0.0004	0.0004±0.0001	0.0003 ±0.0001	0.0061	18.7 ±0.23
	4.15 ±0.04	2.98 ±0.08	0.0043 ±0.0002	0.0018±0.0001	0.0003 ±0.0001	0.0001 ±0.0001	0.0064	21.2±0.11
SCIGS25	7.40 ±0.11		0.0033 ±0.0002	0.0017 ±0.0001	0.0004±0.0001	0.0003±0.0001	0.0057	17.9±0.34
	4.15 ±0.04	2.98 ±0.08	0.0042 ±0.0001	0.0020 ±0.0002	0.0002 ±0.0001	0.0001 ±0.0001	0.0066	19.7±0.27

Figure Legend

Figure 1.

(A) The configuration of the *in situ* product removal (ISPR) fed-batch RQ controlled cultivation process. A stirred tank reactor is operated in fed batch cultivation mode as double phase system adding organic solvent on top of the culture and feeding concentrated culture medium. The product is continuously captured in the organic phase due to its high hydrophobicity. Feed delivery is designed with an exponential policy and it is controlled through a feed-back loop. Fermentation exhaust gas analysis allow the on line determination of the respiratory quotient, the instant RQ measure modulated the fed addition by a PI controller in order to maintain the desired set point. **(B)** Scheme of sesquiterpene (C_{15}) synthesis in yeast. The functional isoprene units isopentenyl diphosphate (3) and its isomers dimethylally diphosphate (4) are synthesized from cytosolic acetyl-CoA (1) through mevalonate (2) intermediate in the mevalonate pathway. The universal sesquiterpene precursor farnesyl diphiosphate (5) is subsequently obtained by the condensation of three units of isopentenyl diphosphate. Sesquiterpenes α-santalene (6); *trans*-α-bergamotene (7); *E,E*-farnesene (8) and *E,E*-farnesol (9) are obtained in one step conversion from farnesyl diphosphate.

Figure 2.

RQ based feed-control development of ISPR aerobic glucose limited fed batch cultivation. Production phase of sesquiterpene producing *S. cerevisiae* strain SCIGS24 **(A)** and SCIGS25 **(B)**. Profiles reported represent the time course of biomass formation (gDCW), RQ measure (CTR/OTR), and comparison between exponential calculated feed profile and experimental value (ml). Data presented are representative of three independent cultures; the error bars represent the standard deviation for three independent cultivations.

Figure 3.

Production phase of aerobic ISPR glucose-limited fed-batch cultivation of *S. cerevisiae* strains SCIGS24 **(A)** and SCIGS25 **(B)**. α-santalene, E,E-farnesol, trans-α-bergamotene and E,E-farnesol (Cmmol l^{-1}) product accumulation as function of feeding time. The error bars represent the standard deviation for three independent cultivations.

Figure4.

RQ based feed-control development of ISPR aerobic glucose/ethanol mixed substrate fed-batch cultivation. Production phase of sesquiterpene producing *S. cerevisiae* strain SCIGS24 **(A)** and SCIGS25 **(B)**. Profiles reported represent the time course of biomass formation

(gDCW), RQ measure (CTR/OTR), ethanol accumulated (Cmmol l^{-1}) and comparison between exponential calculated feed profile and experimental value (ml). Data presented are representative of three independent cultures; the error bars represent the standard deviation for three independent cultivations.

Figure 5.

Production phase of aerobic ISPR glucose/ethanol mixed substrate fed-batch cultivation of *S. cerevisiae* strains SCIGS24 **(A)** and SCIGS25 **(B)**. α-santalene, E,E-farnesol, trans-α-bergamotene and E,E-farnesol (Cmmol l^{-1}) product accumulation as function of feeding time. The error bars represent the standard deviation for three independent cultivations.

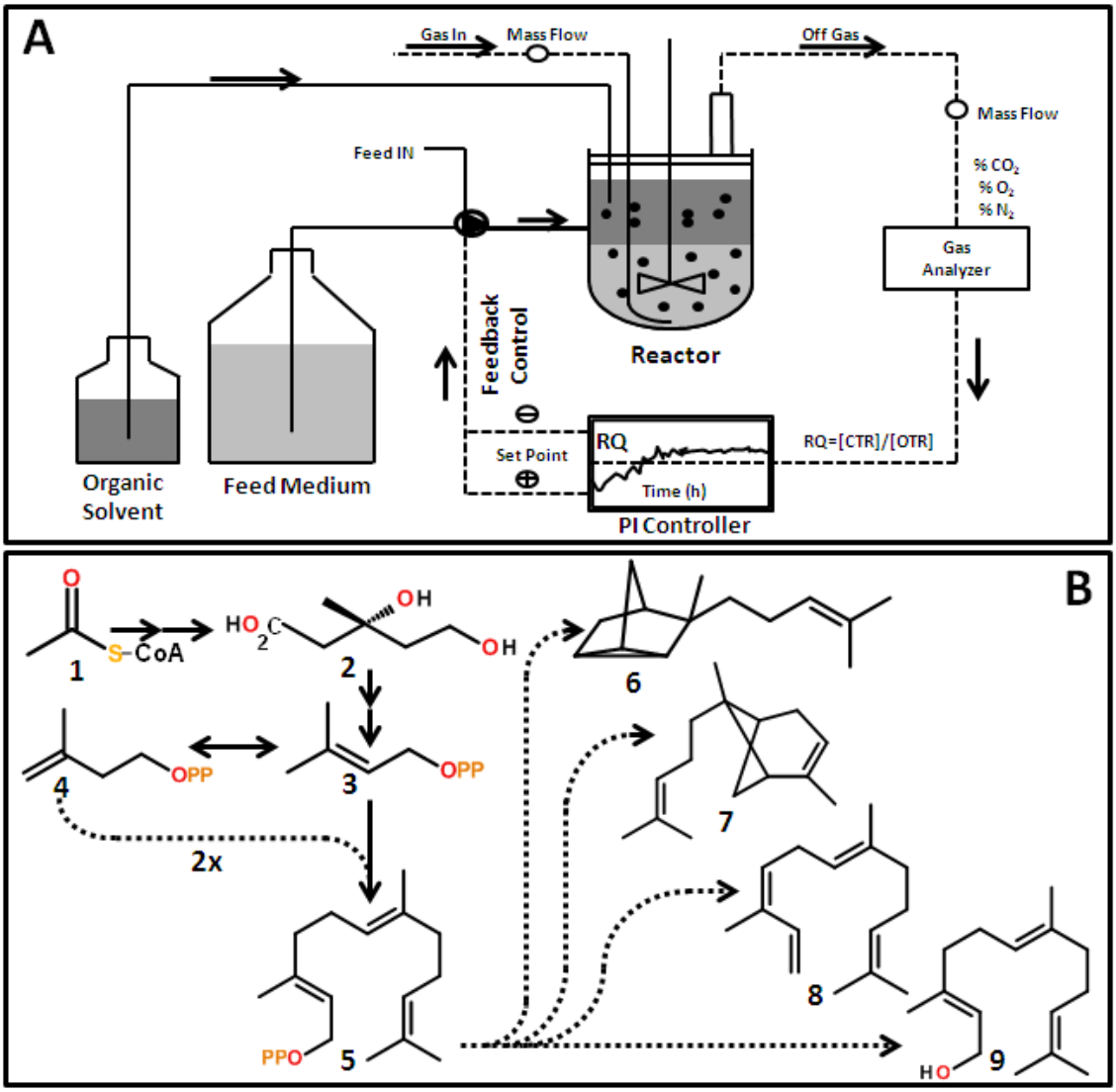
Figure 6.

Sesquiterpene productivity in a two-phase partitioned aerobic fed-batch. α-Santalene and E,E-farnesol, trans-α-bergamotene and E,E-farnesene production rate in Cmmol (g biomass)-1 h-1 (the C-molar weight are 13.62 g Cmol-1 for α-santalene, trans-α-bergamotene, E,E-farnesene and 14.82 g Cmol-1 for E,E-farnesol). Strains SCIGS24 and SCIGS25 are cultivated using glucose or a mixture of glucose and ethanol as carbon source. Error bars represent the standard deviation from three independent cultivations.

Figure 7.

Extracellular (grey area) and intracellular (black area) sesquiterpene α-santalene and trans-α-bergamotene during RQ based double phase aerobic glucose or glucose/ethanol limited fed batch cultivation of strain SCIGS24 and SCIGS25. **(A)** α-santalene, strain SCIGS24 glucose feed; **(B)** *trans*-α-bergamotene, strain SCIGS24 glucose feed; **(C)** α-santalene, strain SCIGS25 glucose feed; **(D)** *trans*-α-bergamotene, strain SCIGS25 glucose feed; **(E)** α-santalene, strain SCIGS24 glucose/ethanol feed; **(F)** *trans*-α-bergamotene, strain SCIGS24 glucose/ethanol feed; **(G)** α-santalene, strain SCIGS25 glucose/ethanol feed; **(H)** *trans*-α-bergamotene, strain SCIGS25 glucose/ethanol feed. Error bars represents the standard deviation from three independent cultivations.

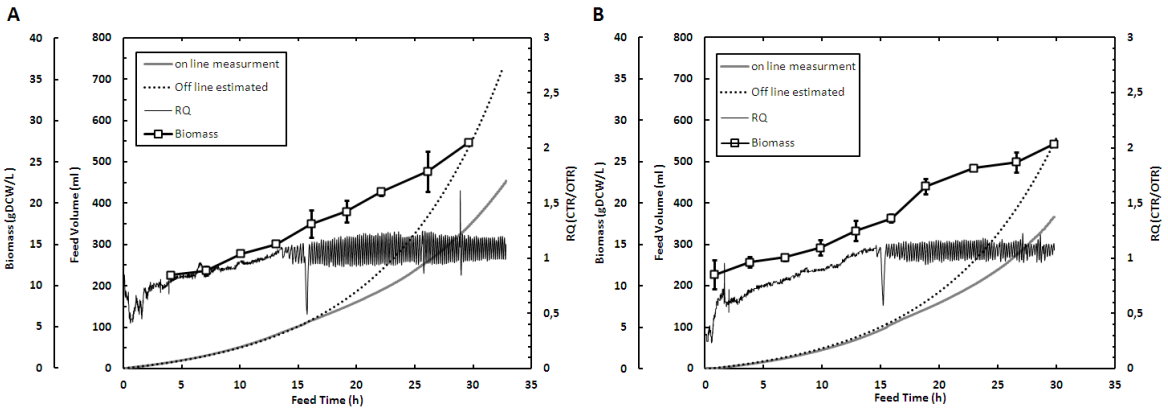
1 Fig.1


A
Gas In
Mass Flow
Off Gas
Mass Flow
Feed IN
% CO_2
% O_2
% N_2
Gas Analyzer
Feedback Control
Reactor
Organic Solvent
Feed Medium
Set Point
RQ
RQ=[CTR]/[OTR]
Time (h)
PI Controller
B
S-CoA
OH
OH
OPP
OPP
2x
PPO
HO
1
2
3
4
5
6
7
8
9

2

3

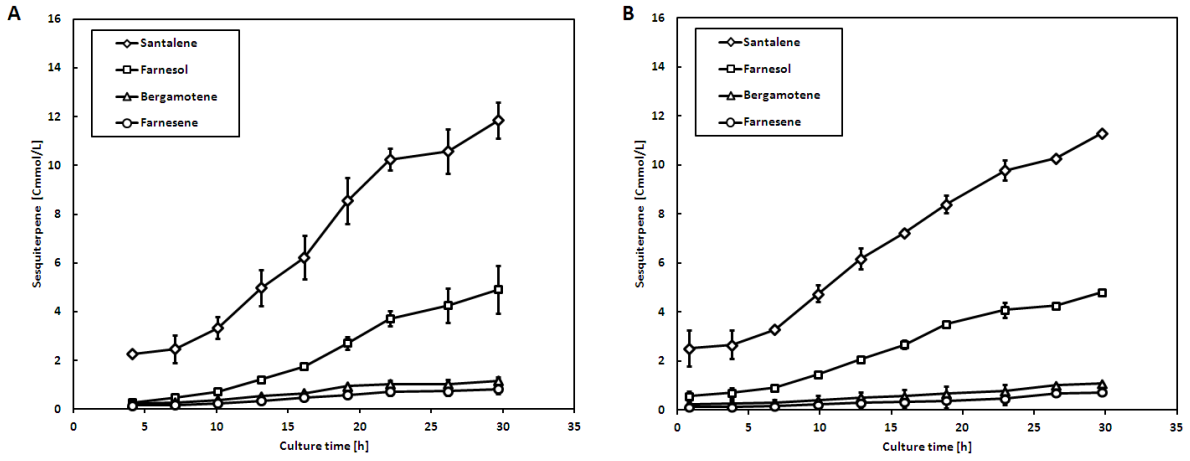
1 Fig.2


A
B
on line measurment
Off line estimated
RQ
Biomass
Biomass (gDCW/L)
Feed Volume (ml)
RQ (CTR/OTR)
Feed Time (h)

2

3

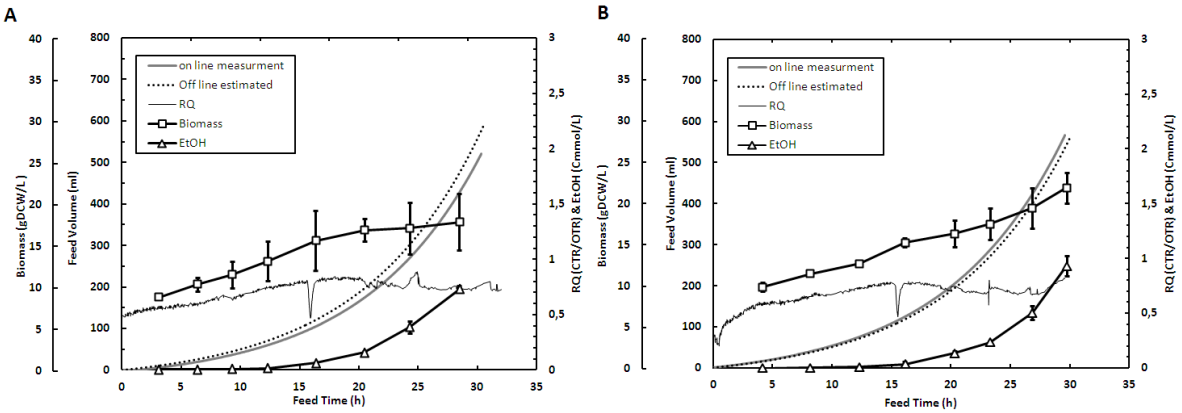
1 Fig.3


A
B
Santalene
Farnesol
Bergamotene
Farnesene
Sesquiterpene [Cmmol/L]
Culture time [h]
0
2
4
6
8
10
12
14
16
0
5
10
15
20
25
30
35

2

3

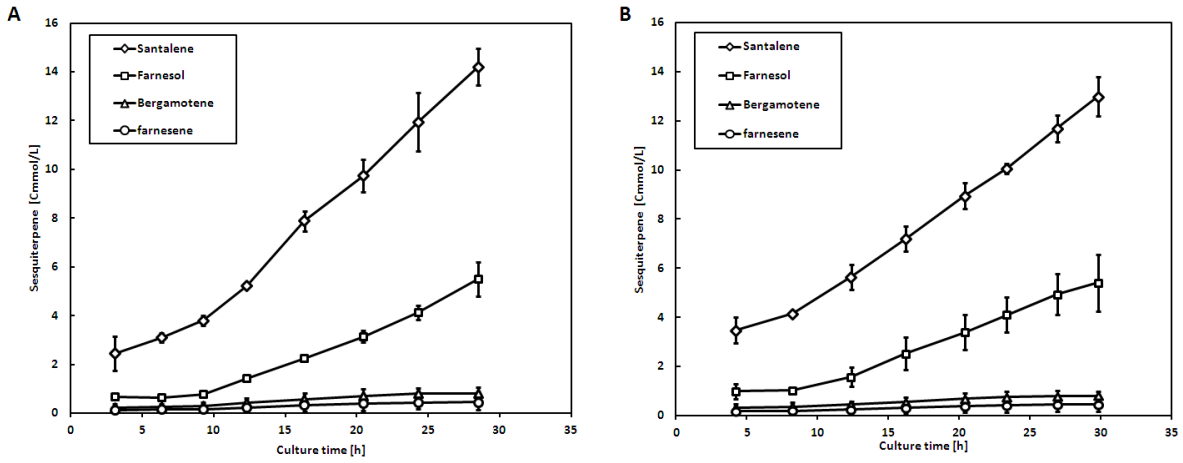
1 Fig. 4


A
B
on line measurment
Off line estimated
RQ
Biomass
EtOH
Biomass (gDCW/L)
Feed Volume (ml)
RQ (CTR/OTR) & EtOH (Cmmol/L)
Feed Time (h)

2

3

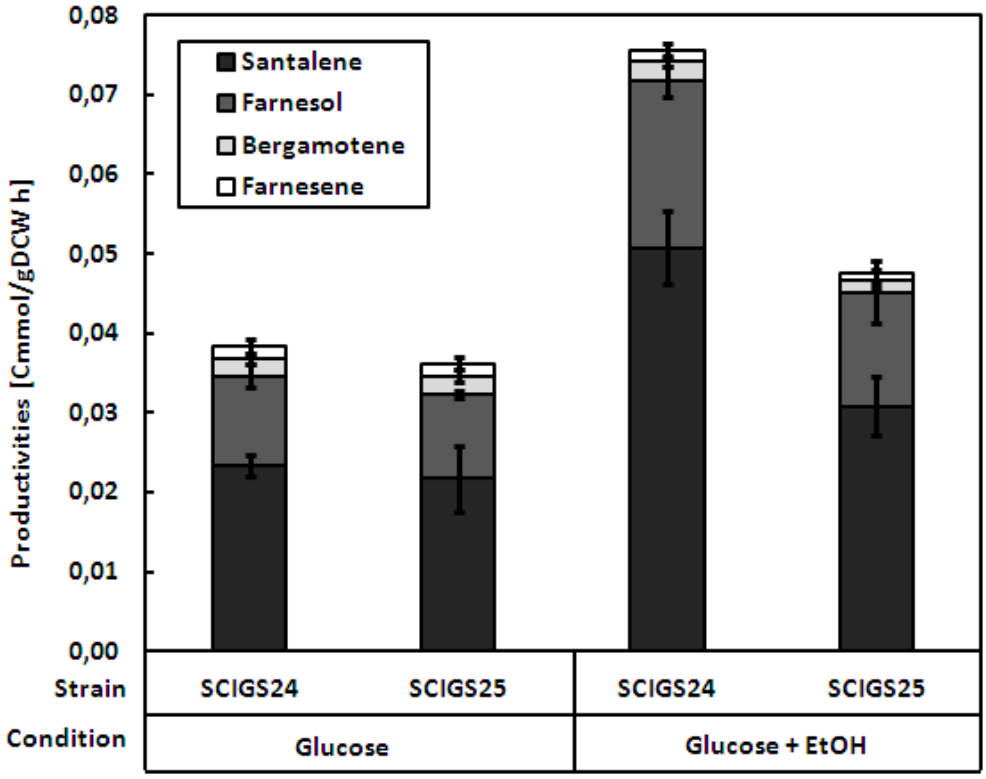
1 Fig.5


A
B
Santalene
Farnesol
Bergamotene
farnesene
Sesquiterpene [Cmmol/L]
Culture time [h]

2

3

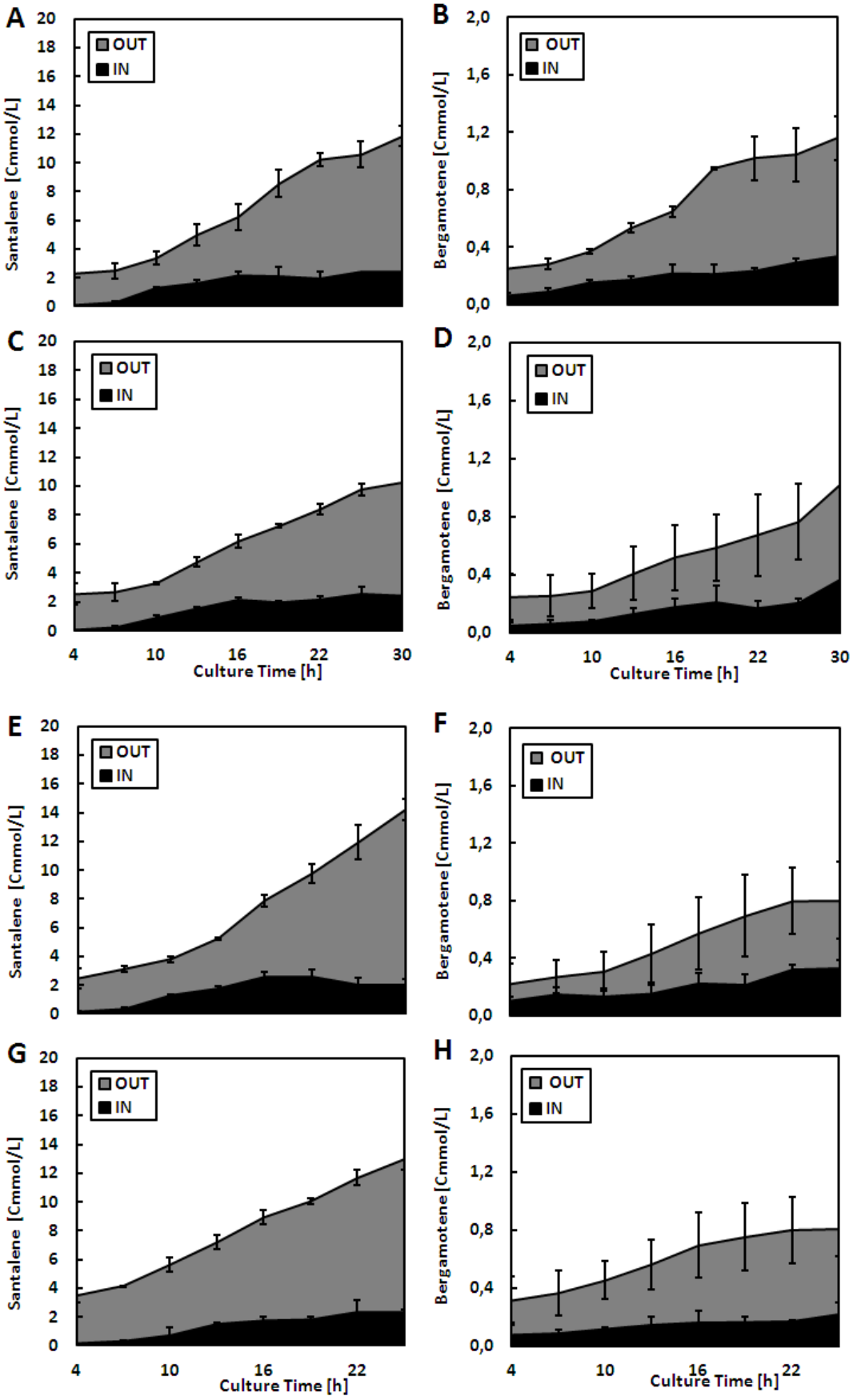
1 Fig.6


0,08
0,07
0,06
0,05
0,04
0,03
0,02
0,01
0,00
Productivities [Cmmol/gDCW h]
Santalene
Farnesol
Bergamotene
Farnesene
Strain
SCIGS24
SCIGS25
SCIGS24
SCIGS25
Condition
Glucose
Glucose + EtOH

2

3

1 Fig.7


A
B
C
D
E
F
G
H
OUT
IN
Santalene [Cmmol/L]
Bergamotene [Cmmol/L]
Culture Time [h]
4
10
16
22
30

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PAPER IV

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RESEARCH ARTICLE

Evolutionary engineering of *Saccharomyces cerevisiae* for efficient aerobic xylose consumption

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Abstract

Industrial biotechnology aims to develop robust microbial cell factories, such as *Saccharomyces cerevisiae*, to produce an array of added value chemicals presently dominated by petrochemical processes. Xylose is the second most abundant monosaccharide after glucose and the most prevalent pentose sugar found in lignocelluloses. Significant research efforts have focused on the metabolic engineering of *S. cerevisiae* for fast and efficient xylose utilization. This study aims to metabolically engineer *S. cerevisiae*, such that it can consume xylose as the exclusive substrate while maximizing carbon flux to biomass production. Such a platform may then be enhanced with complementary metabolic engineering strategies that couple biomass production with high value-added chemical. *Saccharomyces cerevisiae*, expressing xylose reductase, xylitol dehydrogenase and xylulose kinase, from the native xylose-metabolizing yeast *Pichia stipitis*, was constructed, followed by a directed evolution strategy to improve xylose utilization rates. The resulting *S. cerevisiae* strain was capable of rapid growth and fast xylose consumption producing only biomass and negligible amount of byproducts. Transcriptional profiling of this strain was employed to further elucidate the observed physiology confirms a strongly up-regulated glyoxylate pathway enabling respiratory metabolism. The resulting strain is a desirable platform for the industrial production of biomass-related products using xylose as a sole carbon source.

Introduction

Xylose is the most abundant pentose sugar in lignocellulosic feedstocks, including hardwoods and crop residues, and is the second most abundant monosaccharide after glucose (Olsson *et al.*, 2004). The demand for industrial biotechnology processes that leverage sustainable, environmentally favourable and cost-effective raw materials as alternatives to petrochemical feedstocks is receiving unprecedented research focus (Otero *et al.*, 2007; Stephanopoulos, 2010). *Saccharomyces cerevisiae* is a proven, robust, industrial production platform used for the expression of a wide range of therapeutic agents, food and beverage components, added value chemicals (Ostergaard *et al.*, 2000; Chemler *et al.*, 2006; Kim *et al.*, 2012) and commodity chemicals (e.g. bioethanol) across large scales (> 50 000 L) (Olsson *et al.*, 2004; Kumar & Murthy, 2011). Wild-type *S. cerevisiae* is unable to efficiently utilize xylose as a primary substrate. The field has largely focused on metabolic engineering of *S. cerevisiae* for maximizing carbon flux from xylose to bioethanol under anaerobic conditions (Chu & Lee, 2007; Cai *et al.*, 2012); however, the use of *S. cerevisae* is extended as microbial cell factory for a variety of added value chemicals (Chemler *et al.*, 2006). The design of a *S. cerevisiae* platform for broader biomass-coupled production from xylose would be favoured without the loss of carbon to overflow metabolites (ethanol, glycerol, xylitol) particularly in the case of

growth-associated production processes of non-secreted products that require simultaneous formation of biomass and target compound (e.g. poly-3-hydroxybutyrate, ß-carotene and lycopene) (Yamano *et al.*, 1994; Tyo *et al.*, 2010).

Utilization of xylose in yeast and filamentous fungi occurs by a two-step pathway. First, xylose is reduced to xylitol via xylose reductase (XR, primarily NADPH consuming), and then xylitol is oxidized to xylulose via xylitol dehydrogenase (XDH, NADH producing) (Wang *et al.*, 1980). In bacteria, isomerization of xylose to xylulose occurs in a one-step reaction catalysed by xylose isomerase (Misha & Singh, 1993; Harhangi *et al.*, 2003). In yeast, fungi and bacteria, the final conversion of xylulose to xylulose-5P via xylulose kinase (ATP consuming) is conserved.

Recombinant *S. cerevisiae* strains expressing the *Pichia stipitis* xylose reductase (*PsXYL1*), and *P. stipitis* xylitol dehydrogenase (*PsXYL2*) has lead to transformants that can oxidatively and exclusively consume xylose, although resulting in significant xylitol production (Kötter *et al.*, 1990; Kötter & Ciriacy, 1993; Tantirungkij *et al.*, 1993; Walfridsson *et al.*, 1995). While over-expression of the endogenous *XKS1* encoding xylulokinase improved the xylose utilization rate (Ho *et al.*, 1998; Eliasson *et al.*, 2000; Tiovari *et al.*, 2001), xylitol formation persisted. There is a redox imbalance, which results from recombinant co-expression of XR and XDH, and because of the lack of transhydrogenase activity in *S. cerevisiae*, and thereby inability to interconvert NADPH and NADH, there is a surplus formation of NADH and $NADP^+$. Numerous metabolic engineering efforts employed to alleviate the redox imbalance are discussed above and to further improve the xylose consumption rate have been reviewed extensively (Amore *et al.*, 1991; Kuyper *et al.*, 2003, 2004, 2005; Hahn-Hägerdal *et al.*, 2007; van Maris *et al.*, 2007; Matsushika *et al.*, 2009; Van Vleet & Jeffries, 2009).

Among the possible bottlenecks investigated in xylose metabolism, several limiting steps have been identified. The reduced ability of *S. cerevisiae* to grow efficiently on xylose has been attributed to: (1) the inefficient xylose uptake (Amore *et al.*, 1991; Eliasson *et al.*, 2000; Kuyper *et al.*, 2003, 2004, 2005), (2) the insufficient level of expression of xylose transporters to enable significant sugar assimilation (Kötter & Ciriacy, 1993), (3) the redox imbalance generated in the first two steps of xylose metabolism involving the XDH and XR from *P. stipitis* (Kötter & Ciriacy, 1993; Roca *et al.*, 2003), (4) the level of aeration (Skoog & Hahn-Hägerdal, 1990; du Preez, 1994; Walfridsson *et al.*, 1995), (5) insufficient pentose phosphate pathway activity (Kötter & Ciriacy, 1993; Walfridsson *et al.*, 1995) and (6) the inability of pentose sugar metabolism to activate the lower part of glycolysis (Boles *et al.*, 1993; Müller *et al.*, 1995).

Because of the previously described specificity of XR for NADPH and XDH for NAD^+ and the resulting redox imbalance, xylose metabolism is partially regulated by the availability of oxygen in both native and metabolically engineered yeasts (Skoog & Hahn-Hägerdal, 1990; du Preez, 1994; Ho *et al.*, 1998). In the presence of oxygen, excess NADH produced via NAD-dependent XDH can be respired and the NADPH demand for the XR reaction provided by the oxidative part of the pentose phosphate pathway. The level of oxygenation determines the split in carbon flux between biomass and ethanol production under aerobic conditions where xylose is mainly converted into biomass, while ethanol production is favoured under anaerobic conditions (du Preez, 1994). The incomplete respiration of excess NADH under anaerobic conditions leads *S. cerevisiae* to produce and accumulate glycerol followed by xylitol. The xylose consumption rate and the assimilation to biomass increase with increasing aeration level, relieving the accumulation of NADH, yet still resulting in glycerol and xylitol formation (Müller *et al.*, 1995; Jin *et al.*, 2004).

This study aims to metabolically engineer *S. cerevisiae* such that it can consume xylose as the exclusive substrate while maximizing carbon flux to biomass production. Such a platform may then be enhanced with complimentary metabolic engineering strategies that couple biomass production with high value-added chemicals. *Saccharomyces cerevisiae* CEN.PK 113-3C, expressing *PsXYL1* (encoding xylose reductase, XR), *PsXYL2* (encoding xylitol dehydrogenase, XDH) and *PsXYL3* (encoding xylulose kinase, XK) from the native xylose-metabolizing yeast *P. stipitis*, was constructed, followed by a directed evolution strategy to improve xylose utilization rates. The resulting strains were physiologically characterized under aerobic controlled batch fermentations supplemented with glucose and xylose. Transcriptional profiling was employed to further elucidate the strain physiology.

Materials and methods

Saccharomyces cerevisiae strain descriptions

All of the strains constructed in this study were derived from the reference *S. cerevisiae* strain, CEN.PK 113-7D (van Dijken *et al.*, 2000). The strains and plasmids used in this study are listed in Table 1. The strain that was modified using directed evolution is referred to as *evolved*.

Strain CMB.GS001 was derived from the *S. cerevisiae* CEN.PK 113-3C wild-type strain. This strain was transformed with the centromeric plasmid pRS314-X123,

Table 1. *Saccharomyces cerevisiae* strain and plasmid used in this study

Strain or plasmid	Relevant genotype	Origin/reference
CEN.PK 113-7D	*MATa URA3 HIS3 LEU2 TRP1 SUC2 MAL2-8^C*	SRD GmbH*
CEN.PK 113-3C	*MATa URA3 HIS3 LEU2 trp1-289 SUC2 MAL2-8^C*	SRD GmbH*
CMB.GS001	*MATa URA3 HIS3 LEU2 TRP1 SUC2 MAL2-8^C pTDH3-PsXYL1 pTDH3-PsXYL2 pTDH3-PsXYL3*	This study
CMB.GS010†	*MATa URA3 HIS3 LEU2 TRP1 SUC2 MAL2-8^C pTDH3-PsXYL1 pTDH3-PsXYL2 pTDH3-PsXYL3 Evolved*	This study
pRS314-X123	*pTDH3-PsXYL1 pTDH3-PsXYL2 pTDH3-PsXYL3* (TRP1, Centromeric)	Haiying *et al.* (2007)

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†Single colony isolated after repetitive batch evolutionary process. The three final digits of the strain identifier indicate from which cycle in the directed evolution the strain originated, with the starting strain referred to as CMB.GS001.

expressing *TRP1* encoding for N-(5′ phosphoribosyl)-anthranilate isomerase. Into the plasmid pRS314-X123 *PsXYL1* encoding xylose reductase (PsXRp), *PsXYL2* encoding xylitol dehydrogenase (PsXDHp) and *PsXYL3* encoding xylulokinase (PsXKp) all derived from *P. stipitis* were cloned under the glyceraldehyde-3-phosphate dehydrogenase (*TDH3*) constitutive promoter and terminator (Haiying *et al.*, 2007).

Strains CMB.GS010 were evolved from CMB.GS001 after cycles of repetitive culture selection in shake flasks. The three final digits of the strain identifier indicate from which cycle in the repetitive culture the strain originated, with the starting strain referred to as CMB.GS001 (see Directed evolution and selection of strain CMB.GS010, for details).

Yeast strain transformation

Saccharomyces cerevisiae strain CEN.PK113-3C was transformed with plasmid pRS314-X123 (Haiying *et al.*, 2007) using a traditional lithium acetate treatment (Gietz & Woods, 2002). Transformants were selected using synthetic dextrose agar plates without tryptophan (ScD-trp).

Directed evolution and selection of strain CMB.GS010

Mutants of CMB.GS001 with higher specific growth rates on xylose were selected for by serial transfer of cells using repetitive cultures in shake flasks. Specifically, a 500-mL shake flask containing 100 mL of synthetic minimal medium with 20 g L^{-1} xylose was inoculated with CMB.GS001. After 60 h, a new shake flask culture having the same medium composition was inoculated with cells from the preceding shake flask at an initial $OD_{600\ nm}$ of 0.025. This procedure was repeated for four iterations. Thereafter, the culture time was reduced to 48 h. This 48-h cultivation was repeated for six iterations after which strain CMB.GS010 was isolated. Cryovials of stock cultures were prepared following every cycle of repetitive culture. Culture samples were streaked on plates with the same selective condition used throughout the evolution process (minimal media supplemented with 20 g L^{-1} xylose) and growth at 30 °C. Three randomly selected single clones were re-streaked once and thereafter grown in shake flask (see Shake flask cultivation) when the late exponential phase was reached as determined by biomass optical density measurements at 600 nm ($OD_{600\ nm}$), 25% (v/v) sterile glycerol was added, and 1.5 mL sterile cryovials were prepared and stored at −80 °C. From this final evolutionary cycle of the three isolates, the fastest growing strain was designed CMB.GS010 and used for further characterization.

Medium preparation

A previously described synthetic minimal medium containing trace elements and vitamins was used for all shake flasks and stirred tank cultivations (Verduyn *et al.*, 1992). Tryptophan was supplemented for the cultivations of CEN.PK113-3C to satisfy the auxotrophy.

The medium used for stirred tank batch cultivations had the following composition: 5 g L^{-1} $(NH_4)_2SO_4$, 3 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4{\cdot}7H_2O$, 1 mL L^{-1} trace element solution, 1 mL L^{-1} vitamin solution, 0.5 mL L^{-1} antifoam 204 (Sigma A-8311) and 1.25 mL L^{-1} Ergosterol/Tween 80 solution (final concentration 0.01 g L^{-1} Ergosterol and 0.42 g L^{-1} Tween 80). The fermentation medium was pH adjusted to 5.0 with 2 M NaOH and autoclaved. For the cultivations on glucose, the concentration was 20 g L^{-1}, and for the cultivations on xylose, the concentration was 20 g L^{-1}. Both the sugar solutions were added by sterile filtration using a cellulose acetate filter (0.20 μm pore size Minisart®-Plus Satorius AG).

The medium used for shake flask cultivations had the same composition as described above, but the $(NH_4)_2SO_4$ concentration was increased to 7.5 g L^{-1} and the

KH_2PO_4 to 14.4 g L^{-1} together with 20 g L^{-1} of glucose or xylose, and the pH was adjusted to 6.5 prior to autoclaving.

A solid synthetic minimal medium containing 5 g L^{-1} $(NH_4)_2SO_4$, 3 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 1 mL L^{-1} trace element solution, 1 mL L^{-1} vitamin solution, supplemented with 20 g L^{-1} xylose 20 g L^{-1} agarose was used to maintain and isolate the evolved mutant.

A yeast extract peptone dextrose (YPD) complex medium was used for yeast growth prior to transformation with the following composition (g L^{-1}): 10 yeast extract, 20 peptone, 20 glucose and 20 agar.

A synthetic dextrose minus tryptophan medium (ScD-trp) was used as selective media post-transformation with the following composition (g L^{-1}): 7.25 Dropout powder (J.T. Baker), 20 agar and 20 glucose.

Shake flask cultivation

Cultivations were carried out in 500-mL baffled Erlenmeyer flasks with two diametrically opposite baffles and side necks for aseptic sampling by syringe. The flasks were prepared with 100 mL of medium as previously described and cultivated in a rotary shaker at 150 r.p.m. with the temperature controlled at 30 °C. The pH of the medium was adjusted to 6.5 with 2 M NaOH prior to sterilization.

Stirred tank batch fermentations

Stirred tank cultivations were performed in 2.2 L Braun Biotech Biostat B fermentation systems with a working volume of 2 L. The cultivations were operated at aerobic and anaerobic conditions with glucose or xylose as the carbon source. The fermenters were integrated with the Braun Biotech Multi-Fermenter Control System (MFCS) for data acquisition. The temperature was controlled at 30 °C, and agitation was maintained at 600 r.p.m. Dissolved oxygen was monitored using an autoclavable polarographic oxygen electrode. During aerobic fermentations, the sparging flow rate of air was 2 vvm (volume per volume minute). During anaerobic cultivations, nitrogen containing < 5 ppm O_2 was used for sparging at a flow rate of 2 vvm, with $< 1\%$ air-saturated oxygen in the fermenter as confirmed by the dissolved oxygen measurement and the off-gas analyser. The pH was controlled constant at 5.0 by automatic addition of 2 M KOH. Off-gas passed through a condenser cooled to 4 °C to minimize evaporation, and oxygen and carbon dioxide concentrations were determined by the off-gas analyser as previously described (Christensen *et al.*, 1995). Fermentations were inoculated from shake flask precultures to a starting $OD_{600\ nm}$ 0.01.

Analysis

Cell mass determination

The optical density was determined at 600 nm using spectrophotometer (Shimadzu UV mini 1240). Dry cell weight measurements were determined as previously described (Nielsen & Olsson, 1997).

Extracellular metabolite analysis

Extracellular metabolite concentrations were determined by HPLC as previously described (Eliasson *et al.*, 2000).

Transcriptomics

RNA sampling and isolation

Samples for RNA isolation from the late exponential phase of glucose-limited and xylose-limited batch, and continuous cultivations were taken by rapidly sampling 25 mL of culture into a sterile tube with crushed ice. Cells were immediately centrifuged (5000 ***g*** at 0 °C for 2.5 min), the supernatant was discarded and the pellet was frozen in liquid nitrogen. Total RNA was extracted using the RNeasy® Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. RNA sample integrity and quality were determined prior to hybridization with an Agilent 2100 Bioanalyzer and RNA 6000 Nano LabChip kit according to the manufacturer's instruction (Agilent, Santa Clara, CA).

Probe preparation and hybridization to DNA microarrays

Messenger RNA (mRNA) extraction, cDNA synthesis, labelling and array hybridization to Affymetrix Yeast Genome Y2.0 arrays were performed according to the manufacturer's recommendations (Affymetrix GeneChip® Expression Analysis Technical Manual, 2005–2006 Rev. 2.0). Washing and staining of arrays were performed using the GeneChip Fluidics Station 450 and scanning with the Affymetrix GeneArray Scanner (Affymetrix, Santa Clara, CA).

Microarray gene transcription analysis

Affymetrix Microarray Suite v5.0 was used to generate CEL files of the scanned DNA microarrays. These CEL files were then processed using the statistical language and environment R v2.9.1 (R Development Core Team, 2007, www.r-project.org), supplemented with BIOCONDUCTOR v2.3 (Biconductor Development Core Team, 2008,

www.bioconductor.org) packages Biobase, affy, gcrma and limma (Smyth, 2005). The probe intensities were normalized for background using the robust multiarray average (RMA) method only using perfect match (PM) probes after the raw image file of the DNA microarray was visually inspected for acceptable quality. Normalization was performed using the qspline method, and gene expression values were calculated from PM probes with the median polish summary. Statistical analysis was applied to determine differentially expressed genes using the limma statistical package. Moderated *t*-tests between the sets of experiments were used for pair-wise comparisons. Empirical Bayesian statistics were used to moderate the standard errors within each gene, and Benjamini–Hochberg method was used to adjust for multi-testing. A cut-off value of adjusted $P < 0.01$ (referred to as $P_{adjusted}$) was used for statistical significance, unless otherwise specified (Smyth, 2005). Gene ontology process annotation was performed by submitting differentially expressed gene (adjusted $P < 0.01$) lists to the *Saccharomyces* Genome Database GO Term Finder resource and maintaining a cut-off value of $P < 0.01$ for hypergeometric testing of cluster frequency compared to background frequency (Ball *et al.*, 2000). Successively, the reporter feature algorithm (Patil & Nielsen, 2005) has been applied on the dataset to identify transcription factor analysis (TFs) around which the most significant changes occur. Metabolic pathway mapping was performed using Pathway Expression Viewer of the *Saccharomyces* Genome Database, where lists of differentially expressed genes ($P_{adjusted} < 0.01$, |log-fold change| > 1) between two conditions were submitted (Ball *et al.*, 2001).

Results

Physiological characterization of CMB.GS001

Batch cultivations of the xylose-fermenting *S. cerevisiae* strain CMB.GS001 was investigated in synthetic medium supplemented with 20 g L^{-1} xylose. In contrast to the reference strain CEN.PK 113-7D, which cannot grow on xylose, the recombinant strain grew aerobically on xylose with a specific growth rate of 0.02 h^{-1} and a xylose consumption rate of 0.08 g (g dry cell weight)$^{-1}$ h^{-1}, < 2 g L^{-1} xylose was consumed (Fig. 1 and Table 2).

Directed evolution of CMB.GS001

Directed evolution was applied to select a spontaneous mutant with higher specific growth rate on xylose. The constructed xylose-fermenting strain CMB.GS001 was subjected to repetitive serial transfers in batch shake flask cultivations with minimal medium supplemented with 20 g L^{-1} xylose. This approach targeted strain selection based on biomass formation rate, directly coupled to the xylose consumption rate. After four batch cultures, strain CMB.GS001 demonstrated an appreciable improvement in xylose consumption (Fig. 2a). After serial cultivations, over 10 cycles covering a period of 500 h (21 days) the xylose consumption for strain CMB.GS010 increased 15-fold to 20 g L^{-1} and the biomass production increased 52-fold to 9.37 (g dry cell weigh) l^{-1} (Fig. 2a). A total of 74 cell generations were produced across the ten cycles of directed evolution resulting in a doubling time decrease of sixfold from 34.7 to 5.42 h, with the final 50–74 generations not yielding any decrease in the doubling time (Fig. 2b).

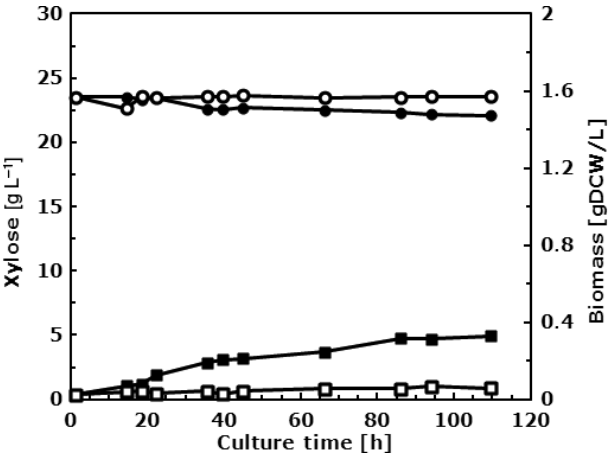

Fig. 1. Time course of aerobic batch culture on defined minimal medium supplemented with 20 g L^{-1} xylose of strain CEN.PK113-7D (empty symbols) and CMB.GS001 (filled symbols). Xylose, (circle) (g L^{-1}) and biomass (square) (g DCW L^{-1}) concentrations are presented as the functions of cultivation time. Data represent the average of three independent cultures.

In order to investigate the possible causes of the dramatic increase in the xylose consumption of CMB.GS010, the plasmid was removed and sequenced, but no mutation was detected compared to the original plasmid pRS314-X123 suggesting that the improved xylose consumption rate is a consequence of mutations in the genome level and not in the plasmid carrying the properties needed for xylose metabolism.

Physiological characterization of CMB.GS010

Batch xylose fermentation

Strain CMB.GS010 was physiologically characterized in aerobic batch fermentations supplemented with 20 g L^{-1} xylose or 20 g L^{-1} glucose. Xylose was completely consumed within 60 h with biomass (62% Cmol C-mol^{-1} xylose) and carbon dioxide (37% Cmol Cmol^{-1} xylose)

Table 2. Physiological parameters obtained during aerobic batch cultivation of strains CMB.GS001, CMB.GS010 and reference strain CEN.PK113-7D. Values represent the mean ± SD of two independent fermentation performed in triplicate (n = 3)

Strain	CMB.GS001	CMB.GS010			CEN.PK113-7D
Carbon source	Xylose	Xylose	Glucose	Glucose/Xylose*	Glucose†
Specific growth rate (h^{-1})	0.02 ± 0.02	0.18 ± 0.01	0.34 ± 0.01	0.32 ± 0.01	0.36
Sugar consumed (Cmol L^{-1})					
Glucose	–	–	0.64 ± 0.01	0.31 ± 0.02	0.66
Xylose	0.07 ± 0.02	0.53 ± 0.04	–	0.05 ± 0.01	–
Sugar consumption rate [g (g dry cell weight)$^{-1}$ h^{-1}]					
Glucose	–	–	2.31 ± 0.06	2.62 ± 0.05	2.36
Xylose	0.08 ± 0.004	0.31 ± 0.02	–	0.21 ± 0.03	–
Biomass yield (Cmol $Cmol^{-1}$)	0.48 ± 0.03	0.62 ± 0.01	0.16 ± 0.02	0.19 ± 0.01	0.15
Carbon recovery (%)	94.9 ± 3.5	100.1 ± 1.1	105.7 ± 2.5	103.6 ± 2.1	103.7
Productivities [g (g dry cell weight)$^{-1}$ h^{-1}]					
CO_2	n.a.	0.21 ± 0.01	0.53 ± 0.001	0.53 ± 0.004	0.49
Ethanol	n.a.	0.02 ± 0.002	0.76 ± 0.002	0.79 ± 0.001	0.96
Xylitol	n.a.	0	0	0	0
Glycerol	n.a.	0.002 ± 0.001	0.10 ± 0.002	0.17 ± 0.001	0.18
Acetate	n.a.	0	0.08 ± 0.003	0.04 ± 0.007	0.02

n.a., not available, because of the relative minimal growth.

*Values relative to the first phase of growth (phase I Fig. 4) when glucose with a small fraction of xylose are used.

†Values from Otero JM, unpublished.

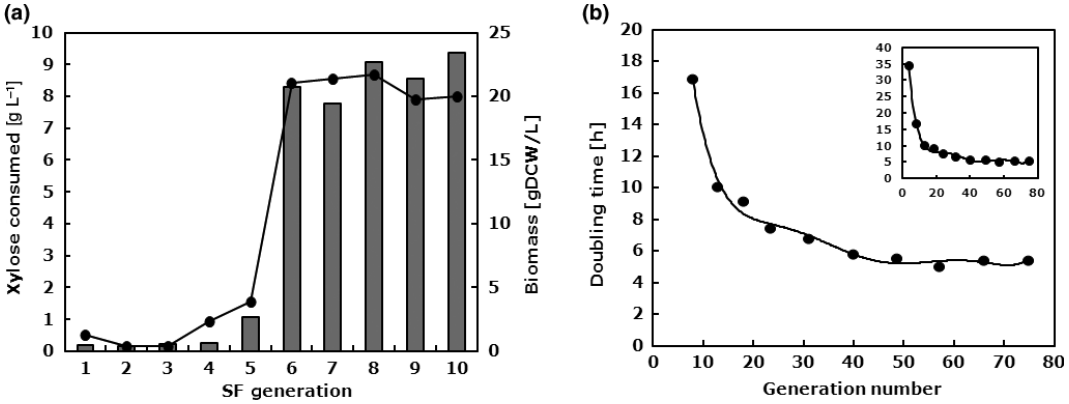

Fig. 2. (a) Comparison in xylose consumption (bars) and biomass production (line) during repetitive growth of *Saccharomyces cerevisiae* CMB.GS001 in shake flask cultures on synthetic medium with 20 g L^{-1} xylose. Shake flask generation represents the number of specific shake flasks in the series of repetitive cultivations performed to select for mutants with higher specific growth rates and xylose utilization rates (for details, see Directed evolution and selection of strain CMB.GS010, Materials and methods). (b) Doubling time during the serial transfers of *S. cerevisiae* in shake flask cultures on synthetic medium with 20 g L^{-1} xylose as a function of the number of cell generations. Each data point represents the doubling time of a single shake flask culture estimated from $OD_{600\ nm}$ measurements. The small plot in the top right represents all 10 cycles, noting the initial doubling time of CMB.GS001 of 35 h, and the rapid decrease to 10 h within < 20 cell generations. For cell generations 50–74, there was no significant improvement in the specific growth rate.

as the major fermentation products, noting the complete absence of xylitol during the culture (Table 2). The xylose consumption rate was highest 0.31 g xylose (g dry cell weight)$^{-1}$ h^{-1} when the extracellular xylose concentration was above 10 g L^{-1}, as demonstrated by the biomass concentration and peak carbon evolution rate (Fig. 3a) subsequently decreasing to 0.08 g xylose (g dry cell weight)$^{-1}$ h^{-1} until xylose exhaustion.

To further investigate whether xylose consumption is sensitive to changes in extracellular xylose concentration, CMB.GS010 was cultivated with synthetic media supplemented with 10 g L^{-1} xylose. Under this condition, the strain exhibits a maximum specific growth rate of 0.11 h^{-1} compared with 0.18 h^{-1} when supplemented with 20 g L^{-1} xylose. The reduced extracellular concentration of xylose to 10 g L^{-1} resulted in an increased lag phase (12–24 h) and maximum specific xylose consumption rate of 0.26 g xylose (g dry cell weight)$^{-1}$ h^{-1}.

Ability of CMB.GS010 to grow under anaerobic condition was tested in batch fermentation conditions with

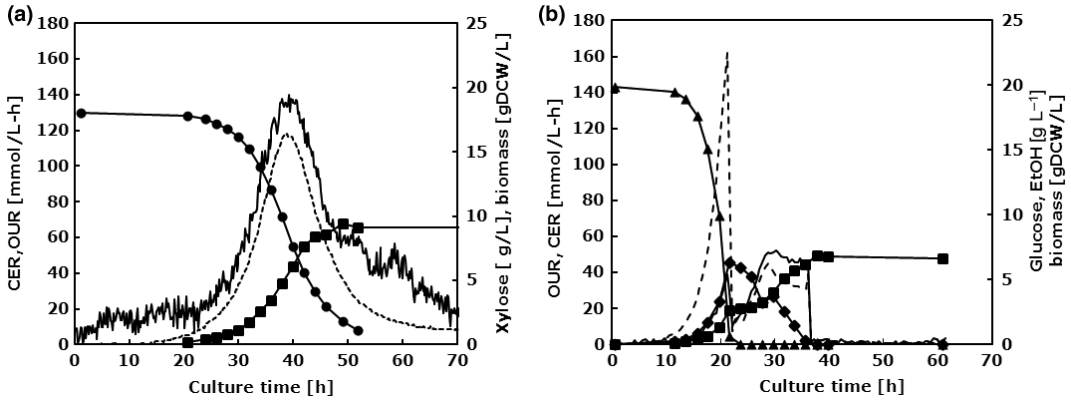

Fig. 3. Time course of aerobic batch culture on defined minimal medium supplemented with 20 g L^{-1} xylose (a) and 20 g L^{-1}glucose (b) of strain CMB.GS010 (evolved strain). For all plots presented, carbon evolution rate (CER) (dashed line), oxygen uptake rate (OUR) (solid line) (mM h^{-1}), and xylose (circle), glucose (triangle), ethanol (diamond) (g L^{-1}); and biomass (square) (g DCW L^{-1}) concentrations as the functions of cultivation time. Data represent the average of three independent cultures.

20 g L^{-1} xylose as the sole carbon source. After 100 h, no growth or xylose consumption was observed. To ensure that the absence of growth was a direct consequence of the anaerobic environment, a recovery experiment was performed, where the culture was aerated quickly from anaerobic to aerobic condition. Growth was immediately restored to the above-described aerobic physiology (data not shown).

Batch glucose fermentation

The aerobic and anaerobic physiology of CMB.GS010 was evaluated in glucose-supplemented batch fermentations to quantify the possible effects of directed evolution on the maximum specific growth rate and the product yields compared to the reference strain CEN.PK113-7D. During aerobic conditions, strain physiology was comparable to previous results with CEN.PK 113-7D (Fig. 3b). The main differences were a small reduction in the maximum specific growth rate and a fourfold higher acetate production, and a small reduction in ethanol production (Table 2). Anaerobic cultivation also showed similar results. Similarly, a reduction in the maximum specific growth rate and in the ethanol yield was observed (data not shown).

Batch mixed substrate fermentation

In order to investigate the proprieties of the strain CMB.GS010 with respect to mixed sugar utilization, the strain was grown aerobically in a mixture containing 10 g L^{-1} glucose and 10 g L^{-1} xylose. The results show that both sugars were completely consumed, however with glucose remaining the preferred substrate. Three different growth phases can be identified (Fig. 4). During the first growth phase (0–21 h), cells consumed 10 g L^{-1} glucose and 1.6 g L^{-1} xylose in the same period (16% more carbon resulting from xylose consumption). The maximum specific growth rate was slightly lower compared with the growth on glucose only (Table 2). Following glucose exhaustion, there was a second growth phase (21–32 h) where the remaining xylose, 8 g L^{-1} (0.27 Cmol L^{-1}), was consumed in conjunction with the re-assimilation of ethanol produced during the glucose consumption phase. During this phase, 0.17 Cmol L^{-1} xylose and 0.15 Cmol L^{-1} ethanol were consumed. In this phase, the maximum specific growth rate decreased 2.5-fold from 0.32 to 0.13 h^{-1}. The maximum xylose consumption rate during the first growth phase on glucose was 0.21 g (g dry cell weight)$^{-1}$ h^{-1}. Once glucose was depleted, the maximum xylose consumption rate was 0.18 g (g dry cell weight)$^{-1}$ h^{-1}. After ethanol re-assimilation, the xylose consumption continued until all the sugar was consumed in the third and final growth phase (> 32 h) with a reduced maximum consumption rate of 0.06 g (g dry cell weight)$^{-1}$ h^{-1}. In contrast to the glucose consumption phase, the xylose–ethanol phase was characterized by a large production of biomass, corresponding to a 28%-increase in biomass yield (Cmol Cmol^{-1}).

The fermentation characteristics of strain CMB.GS010 were also investigated under anaerobic growth on a medium containing 10 g L^{-1} glucose and 10 g L^{-1} xylose. However, only glucose was fully consumed (data not shown).

Transcriptome characterization

Transcriptome characterization was performed with the evolved strain (CMB.GS010) cultivated in batches with xylose and glucose as carbon sources, and the un-evolved

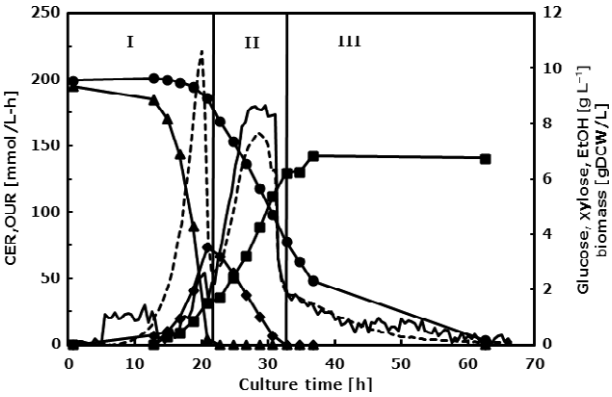

Fig. 4. Time course of aerobic mixed substrate batch cultivation on defined minimal medium supplemented with 10 g L^{-1} xylose and 10 g L^{-1} glucose of strain CMB.GS010 (evolved strain). Carbon evolution rate (CER) (dashed line), oxygen uptake rate (OUR) (solid line) (mM h^{-1}), and xylose (circle), glucose (triangle), ethanol (diamond) (g L^{-1}) and biomass (square) (g DCW L^{-1}) concentrations as functions of cultivation time. Vertical line identifies the three different growth phase (I, II and II). Data represent the average of three independent cultures.

strain (CMB.GS001) with glucose as the sole carbon source in batch cultivations. This different cultivation conditions were selected to elucidate overall carbon flux distributions observed in CMB.GS010 compared to CMB. GS001. The specific comparisons made were focused on identifying fermentative vs. respiro-fermentative metabolism for growth on the different carbon sources. Similar to other studies, the direct comparison with the un-evolved strain on xylose was not possible because of the poor and un-exponential growth on minimal media (Salusjärvi *et al.*, 2006).

Principal component analysis (PCA) of the expression data after normalization showed that the evolved strain grown on xylose in batch conditions clustered with clear separation from the evolved and un-evolved strain grown on glucose (data not shown). Gene ontology (GO) term enrichment analysis from the significant differential gene expressions between the evolved strain (CMB.GS010) grown on xylose or glucose and the un-evolved (CMB. GS001) grown on glucose was performed. A schematic representation of the GO process term identified (*P*-value < 0.01) is represented in Fig. 5a. The terms 'citrate cycle (TCA cycle)', 'glyoxylate and dicarboxylate metabolism', 'peroxisome' and 'oxidative phosphorylation' were among the most represented functional category with respect to metabolism of the evolved strain on xylose compared with the un-evolved or evolved strains grown on glucose. In contrast, processes related to biosynthesis of several amino acids were repressed. TF analysis was used to analyse whether the evolved mutant physiology was affected at a global regulatory level. Figure 5b represents the increase in the expression of genes regulated by different TFs. The main carbon catabolite repressor regulator *SNF1* and several of its known targets *PIP2*, *OAF1*, *CAT8* and *MIG1*, the carbon source responsive *ADR1* and the four subunits of the global respiratory regulator *HAP* complex (*HAP1*, *HAP2*, *HAP3* and *HAP4*) were among the identified over-represented TFs in the evolved strain grown on xylose vs. the un-evolved strain grown on glucose. Interestingly, expression of genes regulated by TFs linked to the glucose sensor *RGT2*, such as *STD1*, and *SNF1* target TFs like *INO2* and *INO4* (involved in the regulation of lipid metabolism) were down-regulated when the evolved and un-evolved strains were compared on glucose. The GO and TF results were consistent with the differences in metabolism expressed by the evolved strain grown on the two sugars. Further, detailed analysis was performed to analyse the change of gene expression at the metabolic pathway level (Fig. 6). The significant mRNA up-regulation of genes encoding enzymes of the central carbon metabolism often correlating with respiration in the TCA cycle and glyoxylate pathways correlates well with the physiological observations that growth on xylose is dominated by respiratory metabolism. The glyoxylate pathway (*ICL1*, *MLS1*, *MDH2*, *AGX1* encoding isocitrate lyase, malate synthase, malate dehydrogenase and glyoxylate aminotransferase) was significantly up-regulated in the evolved strain grown on xylose compared to the evolved strain grown on glucose or the un-evolved strain grown on glucose. This pathway had a significantly higher log-fold change than succinate dehydrogenase, α-ketoglutarate dehydrogenase and succinyl-CoA ligase (*SDH1*, *SDH2*, *SDH3*, *SDH4*, *KGD1*, *KGD2* and *LSC2*, respectively), suggesting that it plays an important role during xylose respiratory metabolism of *S. cerevisiae* as found from studies in chemostat cultures (Regenberg *et al.*, 2006). Finally, *IDP2* and *IDP3* (encoding two isocitrate dehydrogenase) were up-regulated significantly in batch xylose cultivations with the evolved strain (Fig. 6).

The evolved strain, when cultivated on xylose in a batch mode, is able to utilize the glyoxylate bypass to efficiently respire the carbon source. Similar to previous report, the up-regulation of *HXK1* and *GLK1* (encoding hexokinase isoenzyme 1 and glucokinase) supports the hypothesis that xylose is identified as a non-fermentable carbon source and therefore respired (Herrero *et al.*, 1995; Jin *et al.*, 2004; Salusjärvi *et al.*, 2008; Runquist *et al.*, 2009). Furthermore, the expression levels of *MDH2*, *PCK1* and *FBP1* (encoding malate dehydrogenase, phosphoenolpyruvate carboxykinase and fructose-1,6-bisphosphatase, respectively) were up-regulated in the evolved strain cultivated on xylose compared to the evolved or un-evolved strain cultivated on glucose, indicating some glyconeogenic activity. It should be

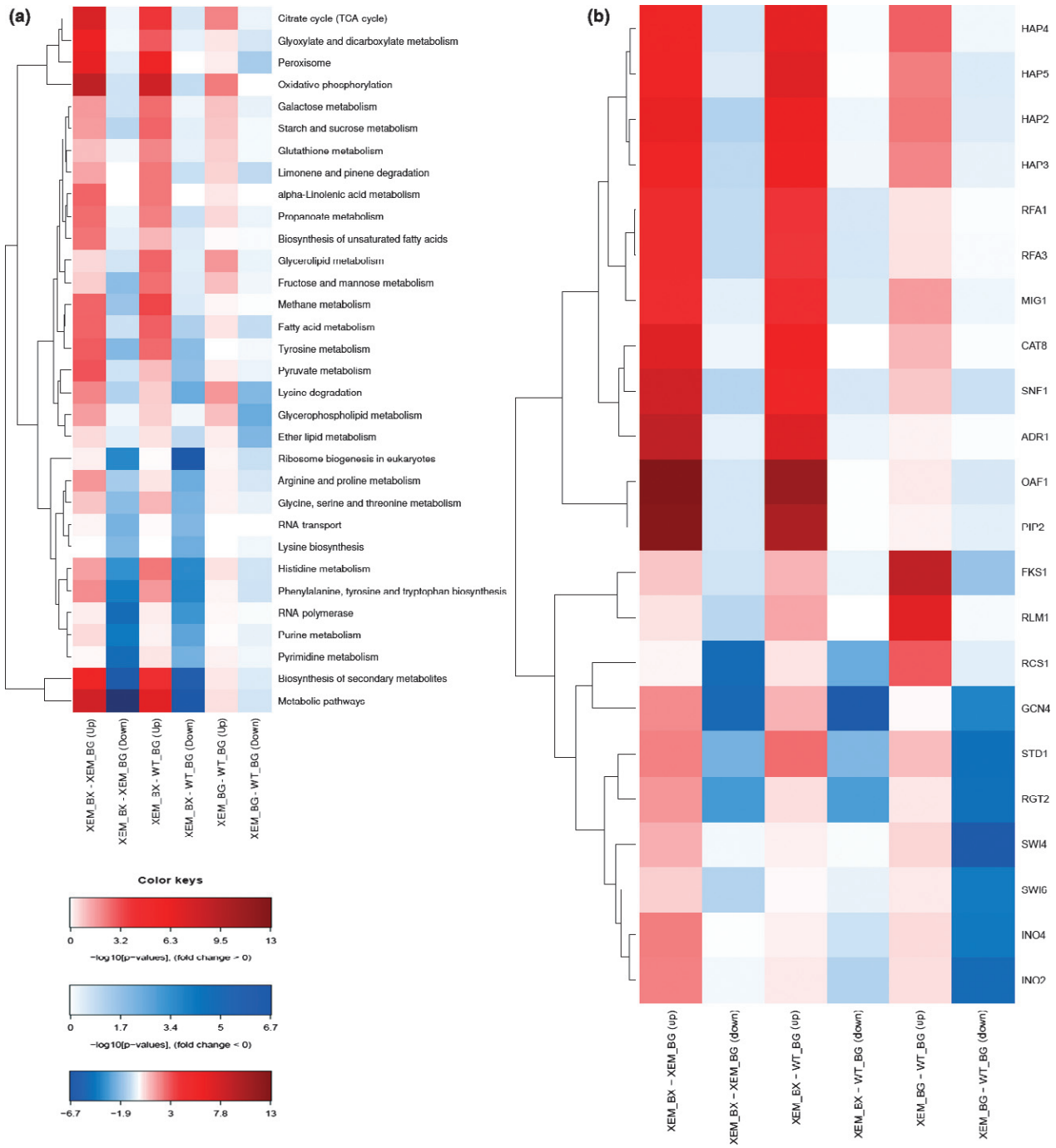

Fig. 5. Integrated analysis of gene ontology (GO) process terms (a) and TFs analysis (b), the cluster frequency is presented on the *y*-axis. Compared conditions are the evolved strain CMB.GS010 strain cultivated on batch xylose (XEM_BX), evolved strain CMB.GS010 cultivated in batch glucose (XEM_BG) and the un-evolved strain CMB.GS001 cultivated on batch glucose (WT_BG). Colour key indicates the different expression in log-fold change ($P_{adjusted} < 0.01$).

mentioned though that these genes are also up-regulated at low dilution rates in glucose-limited chemostat cultures (Regenberg *et al.*, 2006), and expression of these genes may therefore be associated with respiratory metabolism.

The mRNA expression profile of the evolved strain cultivated on xylose suggests a strong flux towards glucose-6-phosphate, requiring inspection of the pentose phosphate pathway (PP pathway). The comparison of differential gene expression in the PP pathway is represented

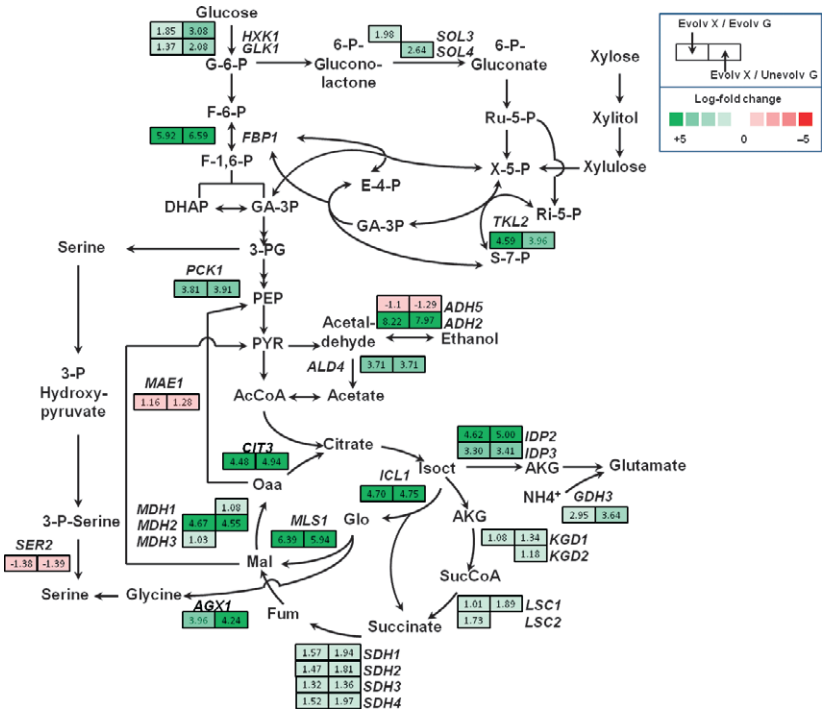

Fig. 6. Gene expression levels of central carbon metabolic pathways: tricarboxylic acid (TCA) cycle, glyoxylate pathway, glutamine/glutamate synthesis and pentose phosphate (PP) pathway are presented. The comparative conditions evaluated include CMB.GS010 cultivated on batch xylose vs. CMB.GS010 cultivated on batch glucose (Evolv X/Evolv G), shown on the left side box; CMB.GS010 cultivated on batch xylose vs. CMB.GS001 cultivated on batch glucose (Evolv X/Unevolv G), shown on the right side box. The log-fold change of significantly differentially expressed genes ($P_{adjusted} < 0.01$, |log-fold change| > 1) is indicated inside each box next to the gene name; boxes are coloured according to log-fold colour scale. If no gene is shown for a given comparative condition, then no significant differential expression changes were detected. The terms evolved and CMB.GS010, and un-evolved and CMB.GS001, are used interchangeably. The pathway intermediate abbreviations are the follows: G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; F-1,6-P, fructose-1,6-biphosphate; GA-3P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetonephosphate; 3-PG, 3-phospho-glycerate; PEP, phosphoenol-pyruvate; PYR, pyruvate; AcCoA, acetyl-CoA; Isoct, isocitrate; AKG, alpha-keto-glutarate; SucCoA, succinyl-CoA; Fum, fumarate; Mal, malate; Oaa, oxaloacetate; Ru-5-P, ribulose-5-phosphate; X-5P, xylulose-5-phosphate; S-7-P, sedoheptulose-7-phosphate; E-4-P, erythrose-4-phosphate.

in Fig. 6. Independent of whether the evolved strain was cultivated on batch xylose or glucose, the *SOL* genes were significantly up-regulated (*SOL3* and *SOL4* encode 6-phosphogluconolactonase) compared to growth on glucose. The evolved strain cultivated on xylose, when compared to the evolved and un-evolved on glucose, exhibited significant up-regulation of transketolase (encoded by*TKL2*, log-fold change 4.59 and 3.96). Transketolase (encoded by major isoform *TKL1* and minor isoform *TKL2*) in combination with transaldolase (encoded by *TAL1*) enables a reversible link between the non-oxidative PP pathway and glycolysis, allowing the cells to adapt their NADPH production and ribose-5-phosphate production to biomass demands (Senac & Hahn-Hägerdal, 1991). The over-expression of *TAL1* and *TKL1* in *S. cerevisiae* over-expressing *PsXYL1* and *PsXYL2* has been previously demonstrated, and there was no influence on growth under either aerobic or anaerobic fermentation conditions in the *TKL1* over-expressed mutant (Walfridsson *et al.*, 1995). *TKL2* over-expression was not considered, and the authors concluded that transaldolase expression in *S. cerevisiae* is insufficient for the effective utilization of PP pathway metabolites (Walfridsson *et al.*, 1995). Furthermore, *TKL2* up-regulation has been correlated with carbon-limited chemostat culture (Boer *et al.*, 2003), but this was not observed here.

Discussion

The strain constructed in this work (CMB.GS010) was obtained through a combination of genetic modification (plasmid introduction) and the application of selective pressure (shake flask repetitive cultivation). Because of the native inability of *S. cerevisiae* to metabolize xylose, three essential genes for xylose uptake from *P. stipitis* were introduced. Using repetitive batch cultivation technique, a strain capable of fast aerobic xylose metabolism was obtained in a relatively short period of time. The

10-fold increase in the specific growth rate on xylose under aerobic growth conditions in only 21 days through repetitive shake flask cultures is the evidence of the efficiency and simplicity of the method. Different evolutionary studies conducted using nutrient limitation as selective pressure has highlighted the high level of adaptability of *S. cerevisiae* (Ferea *et al.*, 1999; Sonderegger & Sauer, 2003; Jansen *et al.*, 2005; Kuyper *et al.*, 2005; Pitkänen *et al.*, 2005). The evolutionary profile observed in this study during the selection period displayed a rapid adaptation to the new fast xylose growing condition rather than a gradual process. Similarly, recent studies reported that when strong selective pressure is applied to yeast cultures in laboratory conditions, adaptation occurs in few steps involving only limited number of mutation (Hong *et al.*, 2011) and the early phase of evolution plays a critical role in the adaptation process (Gresham *et al.*, 2008). Furthermore, plasmid recovery confirms that the genetic modifications during adaptive evolution are present chromosomally in the host rather than any modifications in the plasmid. The *S. cerevisiae* strain CMB.GS010 exhibited a specific growth rate and a xylose consumption rate among the highest reported for *S. cerevisiae* strains metabolically engineered for xylose assimilation with XR, XDH and XK genes and a xylose consumption rate on minimal media under aerobic conditions (Sonderegger & Sauer, 2003; Wahlbom *et al.*, 2003a, b; Jin *et al.*, 2005; Karhumaa *et al.*, 2005; Pitkänen *et al.*, 2005; Parachin *et al.*, 2010). Strain CMB.GS010 clearly exhibited a respiratory metabolism on this sugar. Xylose utilization was almost entirely oxidative as indicated by the respiratory coefficient (RQ = CER/OUR), which remains close to 1 during the entire cultivation time (data not shown), and the high carbon fraction of xylose converted to biomass as compared to glucose metabolism. Furthermore, the physiological observations were supported by transcriptome data analysis at global and metabolic level. The most over-represented gene families in the evolved strain were related to functions or features linked to respiratory process. Consistently, TFs enrichment analysis identified factors primarily involved in carbon catabolite repression response mechanism and regulation of the respiration. Mainly, the significantly enriched TFs in the evolved strain represent transcriptional activator of gene involved in non-fermentative metabolism (Sculler, 2003). Among them is *SNF1* that is a major regulator of carbon metabolism together with several related TFs known to be involved in the generation of precursors of energy and linked to the activation of peroxisomal proteins (*PIP2*, *OAF1*) (Karpichev & Small, 1998) and in the metabolism of non-fermentable carbon sources (*CAT8*, *MIG*) (Usaite *et al.*, 2009). Recent transcriptome studies on recombinant *S. cerevisiae* strain engineered for xylose consumption with the oxoreductive pathway (XR, XDH and XK) indicated the role of partial repression of xylose on TCA and glyoxylate cycle (Salusjärvi *et al.*, 2008), and the physiology of the strain employed during this study differ substantially from the evolved mutant reported here, showing that on batch cultivation, the xylose consumed was partially fermented to ethanol and acetate beside high xylitol production. In contrast, a xylose consuming mutant carrying the oxoreductive pathway with a mutated XR and additionally engineered on the PPP pathway exhibits a full physiological respiratory response without ethanol or xylitol overflow metabolites formation during xylose batch cultivation that correlated with the up-regulation of the TCA cycles at the transcriptional level (Runquist *et al.*, 2009), which is consistent with our findings. Moreover, physiological characterization under continuous cultivation conditions of mutagenesis isolated strains capable of fast growth on xylose shown a clear Crabtree-negative characteristics (Souto-Maior *et al.*, 2009). the observed up-regulation of the glyoxylate pathway in the evolved strain grown on xylose compared to growth on glucose, or the un-evolved strain grown on glucose is in line with observations made at low dilution rates in glucose-limited chemostat cultures in wild-type *S. cerevisiae* (Regenberg *et al.*, 2006). As an extension of the glyoxylate pathway, *IDP2* and *IDP3* were up-regulated significantly in the evolved strain grown on xylose. Xylose metabolism requires the pentose phosphate pathway (PPP), and the first step of the PPP involves the conversion of glucose-6-phosphate to 6-phosphogluconate, catalysed by glucose-6-phosphate dehydrogenase (*ZWF1*). The PPP is essential for the generation of biomass precursors and NADPH cofactor for anabolic reactions (Jeffries, 2006) While the non-oxidative PPP satisfies biomass precursor demands, cytosolic NADPH must still be generated, and the oxidative part of the pathway is bypassed during growth on xylose. Cytosolic isocitrate dehydrogenase (Idp2) catalyses the oxidation of isocitrate to α-ketoglutarate and is $NADP^+$-specific (Cherry *et al.*, 1997). On both fermentable and non-fermentable carbon sources, Zwf1p is constitutively expressed while Idp2p levels are glucose-repressed (Thomas *et al.*, 1991; Minard *et al.*, 1998), whereas Idp2p levels have been demonstrated to be elevated on non-fermentable carbon sources and during the diauxic shift as glucose is depleted (Loftus *et al.*, 1994; DeRisi *et al.*, 1997; Minard *et al.*, 1998). Furthermore, in *Δzwf1 Δadh6 S. cerevisiae* mutants, it was demonstrated that Idp2 is up-regulated and generates enough NADPH to satisfy biomass requirements, noting that the $NADP^+$-specific cytosolic aldehyde dehydrogenase (Adh6p) catalysing acetaldehyde conversion to acetate is the other major cytosolic source of NADPH (Minard & McAlister-Henn, 2005). In the evolved strain, *IDP2* and

IDP3 likely provide a source of NADPH to satisfy biomass requirements.

The native xylose-fermenting strain *P. stipitis*, which is the source of the heterologous expressed enzymes, XR and XDH, does not produce xylitol during xylose fermentations (Skoog & Hahn-Hägerdal, 1990). Extensive xylitol formation has been observed in all the *S. cerevisiae* xylose consuming strains expressing these enzymes (Kötter & Ciriacy, 1993; Tantirungkij *et al.*, 1993; Walfridsson *et al.*, 1995; Ho *et al.*, 1998; Eliasson *et al.*, 2000; Tiovari *et al.*, 2001). The production of xylitol has been shown to be the direct result of a redox imbalance of the NAD(P) cofactors between the XR and XDH (Eliasson *et al.*, 2000; Wahlbom & Hahn-Hägerdal, 2002; Jeppsson *et al.*, 2003; Roca *et al.*, 2003; Träff-Bjerre *et al.*, 2003; Verho *et al.*, 2003; Watanabe *et al.*, 2007). This imbalance has recently been successfully avoided by direct conversion of xylose to xylulose via the introduction of a bacterial isomerase (Kuyper *et al.*, 2003, 2004). Xylitol formation is often described as being the major drawback of the XR-XDH strategy; however, in the engineered strain selected in this study, the formation of xylitol was completely absent during all the xylose fermentations.

The absence of xylitol accumulation under oxidative conditions may be interpreted as a result of complete xylitol oxidation. Consistent with this assumption is that reduction of xylose to xylitol by XR is limited by the availability of NADPH. Perhaps, as the data in this study suggest, up-regulation of IPD2 ensures sufficient NADPH production necessary for the *P. stipitis* NADPH-preferring XR to drive xylose catabolism, whereas the NADH surplus produced is reduced through the respiration eliminating the $NADP^+/NAD^+$ imbalance. Thus, the xylose consumption relies on the external NADH dehydrogenases (Ned1p or Ned2p) or the mitochondrial glycerol-3-phosphate/dihydroxyacetone phosphate shuttle (Gut2p and Gpd1p or Gpd2p) for the re-oxidation of the excess cytosolic NADH (Luttik *et al.*, 1998; Overkamp *et al.*, 2000). This hypothesis is consistent with the previously reported effect of aeration on the reduction of xylitol formation during xylose fermentation (Winkelhausen & Kuzmanova, 1998) and could explain the inability of the evolved strain to consume xylose under anaerobic conditions.

Co-utilization of both sugars, glucose and xylose, is essential for an economically feasible conversion of lignocellulose to industrially relevant bioproducts. Xylose is predominantly consumed after glucose exhaustion. This could be explained a competitive inhibition model for the uptake of the two sugars. Until now, no transporters have been found in *S. cerevisiae* that can exclusively and specifically transport xylose. Nevertheless, it is known that xylose competes with glucose for the same transporters albeit their affinity for xylose is lower (Kötter & Ciriacy, 1993; van Zyl *et al.*, 1999), and so xylose uptake proceeds slower compared to glucose (Leandro *et al.*, 2009). Sugar uptake rate has been related to the carbon catabolite repression and has a role in determining the control the switch between fermentative vs. respirative metabolism (Goffrini *et al.*, 2001; Elbing *et al.*, 2004 Daphne *et al.*, 2012). The evolved mutant displayed sensitivity to different extracellular xylose concentration. In xylose consuming mutant, sugar transport constitutes an important step in determining the fermentation performances (Saloheimo *et al.*, 2007; Jojima *et al.*, 2010). However, the evolved mutant retains a fermentative ability towards glucose comparable to the un-evolved that prevail during mixed sugar cultivation suggesting that the metabolic response of the evolved strain was exclusive to xylose. During glucose–xylose mixed cultivation, the observed poor xylose consumption in the presence of glucose has been attributed to the glucose repression effect (Belinchon & Gancedo, 2003). Recently, several studies propose elegant approaches to overcome the sequential utilization of glucose and xylose, acting on bypass the glucose repression effect on xylose uptake and allowing the co-fermentation of the two sugars (Nakamura *et al.*, 2008; Ha *et al.*, 2010).

Although the efficiency of the evolutionary approach presented in this work is promising, the underlying genetic change that has likely taken place during the direct evolution process remains unclear. Further studies are necessary to gain insight into the possible mutations that contributes to the observed physiology. A detailed genome-wide investigation would offer the opportunity to investigate the genetic basis that result in the ability of the selected strain to consume efficiently xylose as sole carbon source as demonstrated recently in a study on galactose metabolism (Hong *et al.*, 2011).

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Authors' contributions

G.S. and J.M.O. contributed equally to this research.

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