DEVELOPMENT OF YEAST CELL FACTORY FOR IMPROVED PRODUCTION OF ISOPRENOIDS

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By

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DECLARATION

This is to certify that the work presented in the Ph.D. thesis entitled "**Development of Yeast Cell Factory for Improved Production of Isoprenoids**" is a bonafide work done by me under the supervision of **Dr. Sreenivasa Rao Parcha** and was not submitted elsewhere for the award of any degree.

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This thesis is

Dedicated to My Parents

For their endless love, support and encouragement

ABSTRACT

Isoprenoids are most diverse class of natural complex molecules originated through biological systems and diversity of these molecules providing a strong foundation for discovery of bioactive compounds. The superiority of these compounds has expanded their utility from pharmaceutical to fragrances, including biofuel industries. Nevertheless, their structural complication and low abundance in their natural producers, limiting the industrial production of these compounds. However, chemical synthesis is a well-established method for manufacturing of variety of value added chemicals. But, chemical synthesis of natural products is often difficult, expensive and extraction from their natural sources gives low yields and also effects the environment by the extensive usage of solvents. Technical developments over the past century have made microbes as a cell factory for large scale industrial production processes. At present engineering of microbial strains for improved production of desirable end compounds, possible through advancement of synthetic biology and metabolic engineering tools which, enables reconstruction of heterologous metabolic pathways in genetically tractable host organisms. Complex natural products can be produced from inexpensive raw materials through large-scale fermentation processes. In this perspective, investigations aimed towards the production of terpenoids compounds in genetically engineered microbes.

The aim of the present study is to overproduce amorphadiene, an immediate precursor of artemisinic acid (key precursor of artemisinin). Artemisinin is recommended in combinational therapies by the World Health Organization (WHO) to prevent the infection and transmission of multi drug resistant malaria (MDR) malaria. Limited accessibility from their natural producers, environmental concerns raised through contemporary production process restricted the availability of artemisinin to victim in the endemic regions. Metabolic engineering of microbial pathways for production of artemisinic acid, a precursor molecule for the artemisinin, could be a cost effective, environmentally friendly, better quality and reliable source of artemisinin. From the literature, it is clear that considerable efforts made for production of artemisinic acid in engineered microbes. However, *A. annua* cytochrome P450 mono oxygenase (*CYP71AV1*) is unstable and resulting in lower yields of artemisinic

acid, subsequently artemisinic acid led to the induction of pleiotropic drug resistance genes in yeast, which impeding the bulk production of artemisinic acid.

The present study made an attempt to improve the production of amorphadiene, an immediate precursor of artemisinic acid by expressing the Amorphadiene synthase (ADS) in *S. cerevisiae.* Yeast strain capable of producing amorphadiene at better levels was developed by using combinations of metabolic engineering and enzyme fusion technology. Deregulation of the yeast mevalonate pathway was performed through altered expression of two main regulatory genes in the pathway namely *ERG9* and *HMG1*. Attenuated expression of *ERG9* by replacing the native *ERG9* promoter with a regulatable *MET3* promoter in the presence of methionine led to reduced ergosterol content of cells, accumulation of squalene and also formation of farnesol as an FPP derived by-product. Overproduction of the catalytic domain of *HMG-CoA* reductase encoded by *tHMG1* also resulted in accumulation of squalene, farnesol and higher titer of amorphadiene which disclosed the possibility of pooling the available FPP towards amorphadiene. To overcome the natural loss of the metabolic intermediate FPP and its conversion towards farnesol, a chimeric protein was constructed as *ADS-FPPS* and *FPPS-ADS* and strain harboring *FPPS-ADS* protein improved the AD production by 42-folds. Whereas strain harboring *ADS-FPPS* protein not shown any significant effect on AD levels, due to this orientation, active sites were likely far apart and this curtails beneficial proximity effect. In further attempt to take the advantage of statistical techniques to improve the AD production, Response surface methodology (RSM) was applied to optimize the key parameters methionine, KH_2PO_4, pH and temperature and improved AD production additionally by 1.4-folds.

Finally, an *in silico* metabolic engineering approach was used to identify possibilities for integrating native (MEV) and non-native (MEP) pathways, to improve sesquiterpene biosynthesis in yeast by using Petri-Net approach.

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A

AACT Aceto acetyl transferase

Ac-ac-CoA: Acetoacetyl-CoA

Ac-CoA: Acetyl-CoA

ACS: Acetyl-CoA synthetase

AD: Amorpha, 4-11 diene

ADS: Amorphadiene synthase

ADS-FPPS: Amorphadiene synthase -Farnesyl diphosphate synthase-

ALD6: Cytosolic acetaldehyde dehydrogenase

ANOVA: Analysis of variance

AtoB: Acetoacetyl-CoA synthase/thiolase

ATP: Adenosine triphosphate

B

BTS1: Geranylgeranyl diphosphate synthase

C

CCD: Central composite design

CDS: Copalyl diphosphate synthase

ChIp: Chromatin Immuno precipitation

ChIp-seq: Chromatin Immuno-precipitation, sequencing

CIT2: Peroxisomal citrate synthase

CMK: CPP-ME kinase

CMP: Cytidine monophosphate

COX4: Cytochrome C oxidase subunit 4 isoform 1, mitochondrial

CPP: Copalyl diphosphate

CPP-ME 4(cytidine 5′diphospho) 2-C-methyl-D-erythritol

CrtI: Phytoene desaturase

CrtS: Cytochrome-P450 hydroxylase

CrtYB: Lycopene cyclase/phytoene synthase

CUP1: Copper 1

D

DMPP: Dimethylallyl-pyrophosphate

DNA; Deoxy ribonucleic acid

dNTPs; Deoxy nucleotide triphosphate

DPP1: Diacylglycerol pyrophosphatase

DTS: Diterpene synthase

DW: Dry weight

DXP: 1-deoxyxylulose-5-phosphate

DXR: 1-deoxyxylulose-5-phosphate isomer reductase

DXS: 1-Deoxy-d-Xylulose-5-Phosphate Synthase

E

eAS, epi-aristolochene synthase

EDTA: Ethylene di amine tetra acetic acid

ERG10: Acetoacetyl-CoA thiolase

ERG12: mevalonate kinase

ERG13: HMG-CoA synthase

ERG19: Diphospho mevalonate decarboxylase

ERG20: Farnesyl diphosphate synthase

ERG8: Phospho mevalonate kinase

ERG9: Squalene synthase

F

5-FOA: 5-fluoroorotic acid

FOH: (E, E) - farnesol

FPP: (E, E)-farnesyl diphosphate

FPPS-ADS: Farnesyl diphosphate synthase- Amorphadiene synthase

G

G: Gram

G3P: Glyceraldehyde-3-phosphate

GAL1: Galactose 1

GAL10: Galactose 10

GAP: D-glyceraldehyde 3-phosphate

GC/FID: Gas chromatography- flame ionization detector

GC-MS: Gas chromatography –Mass spectrometry

gcpE: MECDP reductase

GGOH: Geranyl geraniol

GGPP: (E, E, E)-geranylgeranyl diphosphate

GYC: Acetyl-CoA in glyoxylate cycle

H

h: Hours

HDR: HMBPP reductase

HDS: HMBPP synthase

HFPNe: Hybrid functional Petri-Net with extension

HMBPP: 1-hydroxy-2-methyl-2-(E)-butenyl 4- diphosphate

HMG1: 3-hydroxy-3-methylglutaryl-CoA reductases

HMG2: 3-hydroxy-3-methylglutaryl-CoA reductases

HMG-CoA: 3-hydroxy-3-methylglutaryl-CoA

HPLC: High performance liquid chromatography

HUM-G6: Engineered version of humulene synthase bearing six such substitutions

I

IDI1: IPP: DMAPP isomerase

IMTECH: Institute of microbial technology

IPP: Isopentenyl-pyrophosphate

IspD: Methylerythritol phosphate cytidyll transferase

ispE: 4- diphosphocytidyl-2-C-methyl-D-erythritol kinase

ispF: 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase

K

kD: Kilo dalton

L

L: Liter

LB: Luria-Bertani

LEU2: Leucine

LPP1: Lipid phosphate phosphatase

lytB: HMBPP reductase

M

MCT: MEP cytidyl transferase

MDS: MEcycPP synthase

MEcycPP: 2-c-methylerythritol-2, 4-cyclodiphosphate

MEP: Methylerythritol phosphate

MET25: Methionine 25

MET3: Methionine 3

MEV: Mevalonate

MEVP: Mevalonate phosphate

MEVPP: Mevalonate diphosphate

mg: Milligram

Min: minute

MK: Mevalonate kinase

mL: Milliliter

MLS1: Cytosolic malate synthase

mM: Mille molar

mRNA: Messenger ribonucleic acid

MTCC: Microbial type culture collection

N

NAD: Nicotinamide adenine dinucleotide

NADP: Nicotinamide adenine dinucleotide phosphate

nm: Nanometer

NOH: (E)-nerolidol

O

OC: Osteocalcin

OD: Optical density

ODEs: Ordinary differential equations

OPN: Osteopontin

ORF: Open reading frame

P

Pck: Phosphoenol pyruvate carboxykinase

pCPPME 4(cytidine 5′diphospho)2-C-methyl-D-erythritol 2-phosphate

PCR: Polymerase chain reaction

PDEs: Partial differential equations

PEP: Phosphoenol pyruvate

PMD: MEVPP decarboxylase

PMK: Phosphomevalonate kinase

Pps: Phosphoenol pyruvate synthase

PTS: Patchoulol synthase

PTS-FPPS: Patchoulol synthase- Farnesyl diphosphate synthase

Pyk-I and Pyk-II: Pyruvate kinase isozymes

R

R: Regression quotient

rDNA; recombinant DNA

RNA: Ribonucleic acid

RPM: Rotations per minute

RSM: Response surface methodology

S

SCPD: *Saccharomyces cerevisiae* Promoter Database

SD: Synthetic defined

SDS-PAGE: Sodium dodecyl phosphate poly acrylamide gel electrophoresis

SeACS (L641P): *Salmonella enterica* acetyl-CoA synthase mutant L641P

SQS: Squalene synthase

STS: Sesquiterpene synthase

T

Taq: Thermus aquaticus

TCA : Tri carboxylic acid

TEMED: N, N, N', N'-tetramethylethylenediamine

tHMGR : truncated 3-hydroxy-3-methylglutaryl-CoA reductases

tHMGR-G9: Engineered tHMGR version with nine mutated residues

U

UBS: Urinary bladder submucosa

UPC: Uptake control

URA3: Uracil

Y

YAC: Yeast artificial chromosomes

YCF: Yeast cell factory

YCp: Yeast centromeric plasmid

Yep: Yeast episomal plasmid

Yip: Yeast integrative plasmid

YPD: Yeast Peptone Dextrose

CHAPTER 1

Introduction

CHAPTER 1

INTRODUCTION

1.1 Bio economy and the microbial cell factories

Since the last two decades the global economy endowed with rapid and exponential increase in the bio-based products (Enriquez, 2009). In current scenario, population growth, environmental concerns, dependence on non-renewable fossil fuels and limitations of synthetic, combinatorial chemistry and high throughput drug discovery, strongly thrusts towards an alternative, potential and sustainable bio-production process (Ganesan, 2004). However, the concept of bio-production was well known since the origin of the fermentation process (Russo et al., 1994) but developments in industrial biotechnology comprehending the bio economy, as it enables the production of a larger spectrum of substances from biomass, rather than non-renewable fossil feedstock (fig. 1.1).

Recent developments in metabolic engineering developed novel strains with improved performance to potentially replace the first generation microbial cell factories (wild type). Yet, limited number of recombinant proteins approved for therapeutic use. Recent development in industrial biotechnology through systems metabolic engineering (integration of metabolic engineering with systems biology and synthetic biology) offers efficient production of chemicals (from pharmaceuticals to bulk chemicals and fuels) and materials (from plastics to high value materials) and sufficiently mature to reach the final stage of full commercialization (Lee et al., 2012). Current industrial biotechnology major market segments are represented by specialty chemicals (28%) base chemicals (24.9%) consumer chemicals (24%) and active pharma ingredients (23%) with EUR 135 billion revenue. According to the forecast by McKinsey & Company the global biotech industry revenue has the potential to produce EUR 340 billion by the year 2017.

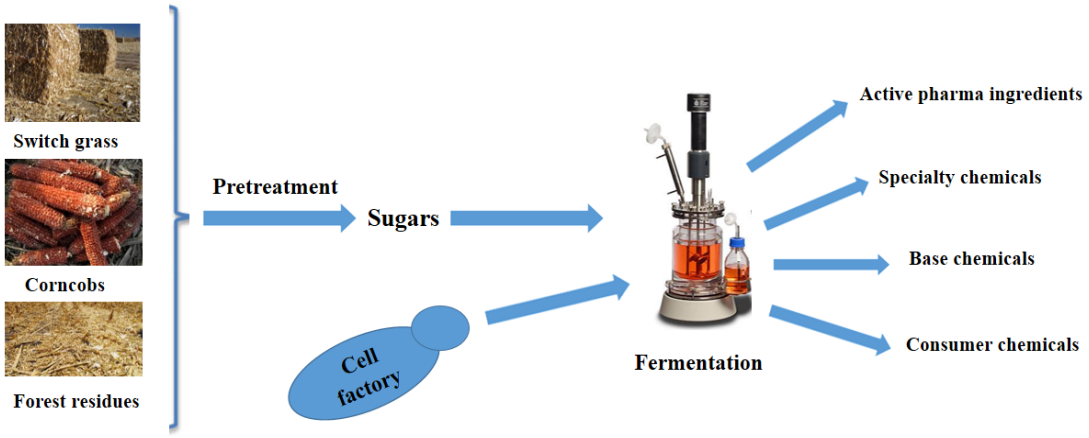

Figure 1.1 Schematic representation of the development of bio-based production (bio refinery) for production of value added chemicals. In a bio refinery, plant-based feed-stocks such as switch grass, corncob and forest residues are converted into sugars that are subsequently converted into a variety of compounds (from active pharma ingredients to consumer chemicals) by microbial cell factories.

1.2 *Saccharomyces cerevisiae* as a cell factory

S. cerevisiae (bakers' yeast), is probably the most studied and characterized yeast, belonging to the class of *Saccharomycetes.* The word *Saccharomyces* comes from the Greek, indicating a fungus that can grow on sugar. *S. cerevisiae* is very well known for making of alcoholic beverages, bread and cake since ancient time earlier the term "biotechnology" has been coined. Due to its interesting physiological features like ability to ferment under fully aerobic conditions (Crabtree effect) made yeast as a unique organism for the production of alcoholic beverages and food since ancient times (Gelinas, 2009). Besides its usage in food and brewing industry (Yeh et al., 2009), the yeast *S. cerevisiae* is commonly used as a model system for the study of numerous cellular processes such as apoptosis, ageing and metabolic disorders (Petranovic and Nielsen, 2008; Klimacek, 2012). Progressive, well established molecular biology techniques and easy cultivation technologies along with its GRAS (Generally Regarded as Safe) status, made yeast as an attractive microbial platform for the production of pharmaceuticals to value added chemicals and fuels (Petranovic and Nielsen, 2008; Klimacek, 2012). Other promising features like low pH tolerance, extended substrate range (Kuyper et al., 2004; Van vleet and Jeffries, 2009; Ha et al., 2011), recovery of the acid in its carboxylated form, low downstream processing costs, moderate post–translational modifications (Buckholz and Gleeson, 1991) made *S. cerevisiae* as tremendous, potential and future

microbial cell factory for production of a sustainable and extended range of value added chemicals (fig.1.2). Presently, *S. cerevisiae* is the preferred cell factory for one of the world largest industrial production of bioethanol (Otero and Nielsen, 2010). Manipulation of S. *cerevisiae* cells are highly relieved by the existence of a wide spectrum of established yeast molecular biology tool kits and availability of the many wild-type and mutant strain collection, plasmid collections and many more, offered by commercial sources such as EUROSCARF (http://web.uni-frankfurt.de), Open bio systems (http://www.openbiosystems.com/Products) or Addgene (http: http://www.addgene.org/. In addition genomic, promoter and biological information about *S. cerevisiae* was available in the *Saccharomyces* Genome Database (SGD, http://www.yeastgenome.org) and the promoter database of *S. cerevisiae* (SCPD http://rulai.cshl.edu/SCPD/). In recent days, the development of "Omics" technologies and next-generation sequencing (NSG) unravel the complexity of omic's information (Nagalakshmi et al., 2008; Daran-Lapujade et al., 2008) and paved the way for *S. cerevisiae* to be exploited in the field of metabolic engineering.

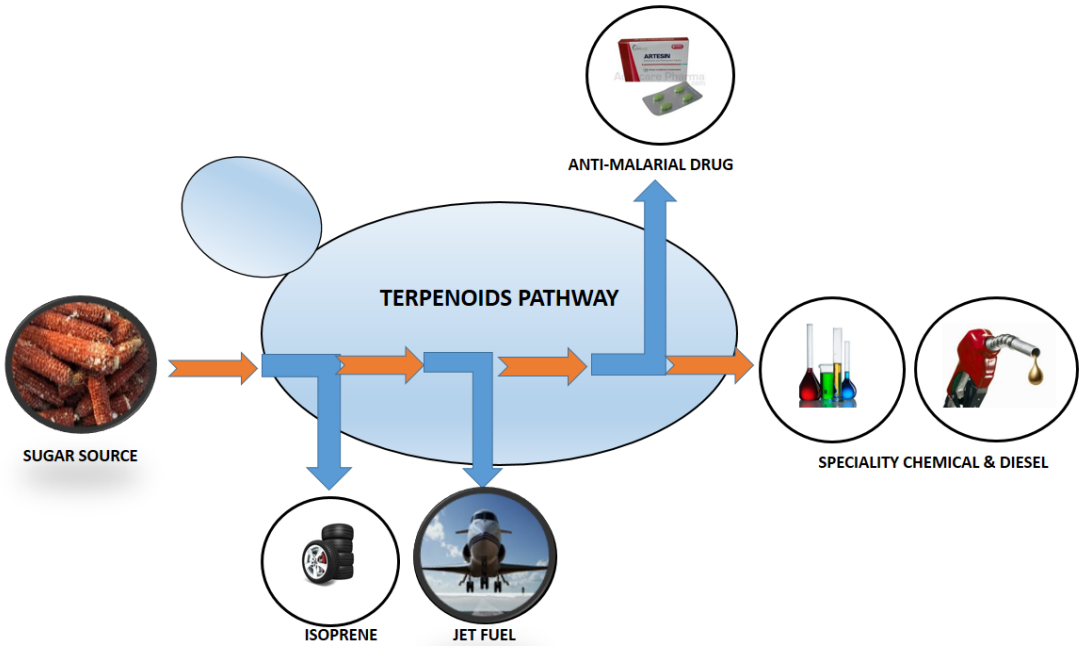

Figure 1.2 Yeast (*Saccharomyces cerevisiae*) cell factory: production of extended range of products from pharmaceutical to jet fuel through engineered isoprenoid pathway.

1.3 Systems Biology and Metabolic Engineering

As *S. cerevisiae* has been used since ancient times for production of goods, there has always been an increasing attention to manipulate the yeast cell in order to improve its performances and to develop strains with new traits and specific characteristics to be utilized in industrial processes. Traditionally, strain improvement was carried out through random mutagenesis followed by a screening of the desired phenotype among a high number of mutants. Despite great success, these methods are time consuming and generate mutation that often are not beneficial and do not contribute to confer the desired characteristics; additionally, even in the successful cases, no understanding of the cellular phenotype obtained can be gained. In respect to that, the development of the science of metabolic engineering provided a valid alternative to traditional mutagenesis for the engineering of the cell factories, allowing the rationale engineering of the desired cellular phenotype(s) through recombinant DNA technologies (Bailey, 1991). Classical metabolic engineering strategies are based on the identification of rate limiting steps in a certain pathway (e.g. pathway for the generation of a desired molecule) and on the attempt to alleviate this regulation by over expressing the genes coding for the responsible enzymes. Through metabolic engineering it is also possible to confer new traits by recruiting heterologous activities and over expressing in the production host. In this respect, the concept of metabolic engineering can be broaden and defined as direct improvement of cellular phenotypes through the introduction, deletion, and modification of metabolic pathways (Ostergaard et al., 2000).

Despite holding great potential, metabolic engineering had to face several challenges related to the engineering of complex phenotypes. This is because the development of efficient strains requires the combination in the production host of different characteristics such as increased production rate of the desired product together with increased growth rates and decreased formation of by-products. These features cannot be easily conferred as the cell machinery is subjected to a tight regulation. The advent of inverse metabolic engineering accounted for an iterative cycle that encompasses 1) the identification, construction or calculation of the desired phenotype 2) the determination of factors that contribute to the desired phenotype and 3) creating that phenotype in another strain through genetic engineering or environmental manipulation (Bailey et al., 2002). Inverse metabolic engineering has developed as a cycle where a previous successful

recombinant host can be further characterized to elucidate its characteristic in order to detect new targets for improved strategies (Otero, 2009). In the last decade, the development of systems biology accounted for improvements in metabolic engineering strategies. Systems biology is a very broad research field and several the definition have been given (Nielsen and Jewett, 2008), however what clearly emerge from the different pioneering works on systems biology is the common aim to furnishing a better understanding of the functioning of the living cell giving by providing a holistic view rather than explaining single, isolated processes and to explain quantitatively biological systems through mathematical modeling (Nielsen and Vidal, 2010). Systems biology is a highly interdisciplinary approach and encompasses multiple techniques, from X-Omic technologies to the application of metabolic mathematical modeling for the prediction of cellular function.

The continuous development in systems biology techniques allowed to better understand the cellular phenotype and contributed to gain insight into the metabolic regulation underlying physiological processes, by integrating experimental analysis and modeling efforts (Heinemann and Sauer, 2010). Hereby systems biology aims at mapping regulatory structure and further de-regulate them (Papini et al., 2010) by combining omics techniques and mathematical modeling on the engineered and wild-type phenotypes in order to identify new, on intuitive targets to ad-hoc design metabolic engineering strategies. A representation of the contribution of systems biology and metabolic engineering to industrial biotechnology can be found in fig.1.3

1.4 Tools of Metabolic engineering

Commercial production of chemicals merely viable with high productivities, titers and yields in microbial hosts. Optimizing/diverting pathway flux, balancing stress on the cell and reducing toxic intermediates are the most important factors vital to reach maximum yields. Consequently, an emphasis on pathway optimization and modulation of enzyme expression is one of the key challenges in metabolic engineering investigations targeting at production of fine chemicals and pharmaceuticals. Present advancements of novel tools targeted the modulation of the central dogma of living cell leading to altered levels of central components, e.g. DNA, RNAs and proteins. Underlying principles of central dogma of cell, guide us to project innovative tools to control the biological processes at a

predictable level. Various biological tools have been developed for optimizing the biochemical pathways with special emphasis of yeast (Siddiqui et al., 2012).

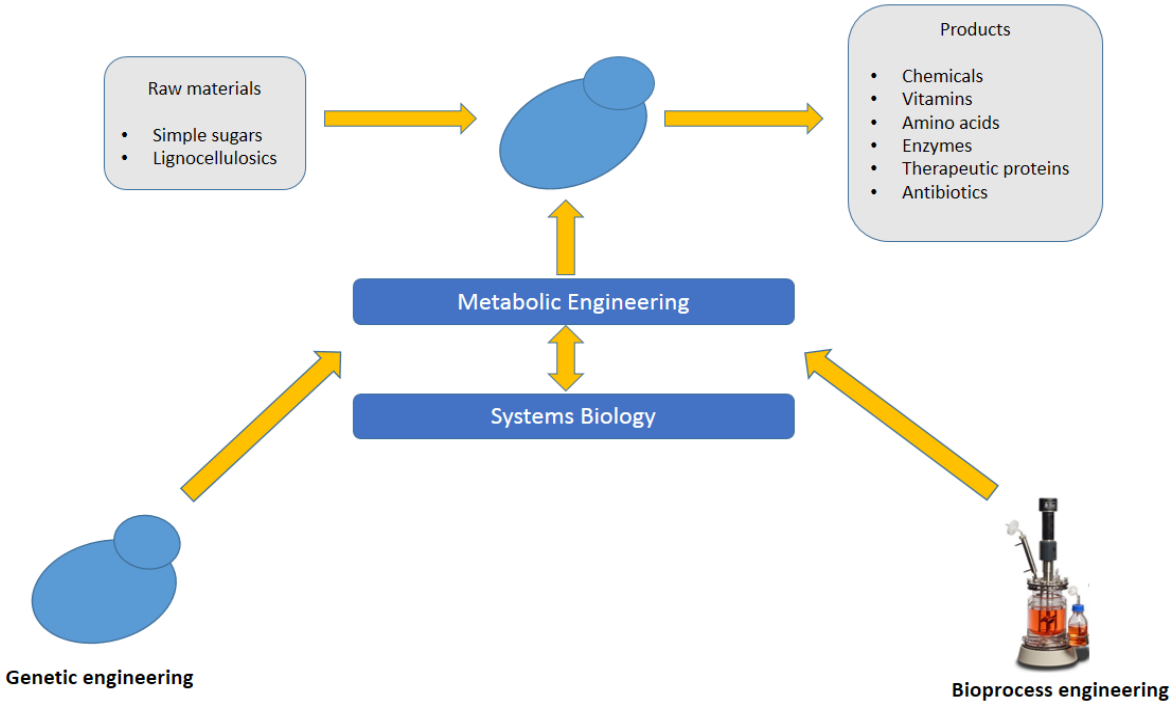

Figure 1.3 Systems biology and metabolic engineering to design the cell factories.

1.4.1 Tools at DNA level

For modulating the expression of DNA, numerous tools have been developed targeting either endogenous or heterologous genes which usually alters the gene copy number or gene dosage (plasmid DNA and yeast artificial chromosomes (YAC)) (Murray, 1983) or offer a precise control over gene copy number and stability (integration of heterologous gene through homologous recombination (fig.1.4) (Shao et al., 2009; Hawkins and Smolke, 2010)

Yeast plasmids are classified into three different classes:1) yeast centromeric plasmid (YCp) 2) yeast episomal plasmid (Yep) and 3) yeast integrative plasmid (Yip). YCp and YEp have been employed for many applications in yeast molecular biology and metabolic engineering (fig.1.5). YCp vectors contain both an origin of replication and a centromere sequence, which gives high segregation stability in selective medium, while maintaining 1-2 copies per cell (Clarke and Carbon, 1980). YEp vectors contain harbors either *S. cerevisiae* native full 2µ sequence or commonly, a 2µ sequence including both the origin and the stability locus (STB), REB3 (Futcher and Cox, 1983; Kikuchi, 1983)

and maintained at more than 10 copies per cell (Romanos et al., 1992). YEp with 2µ sequence with STB are generally more stable in comparison to those which are carrying full 2µ sequence. The third class vectors Yep does not have any origin of replication and need to be integrated into the chromosome through homologous recombination, which happening between complementary target sites on both plasmid and genome, in order to maintain them in the cell. Different target sites have been developed for YIp vector series (Cartwright et al., 1994a; Alberti et al., 2007; Sadowski et al., 2007).

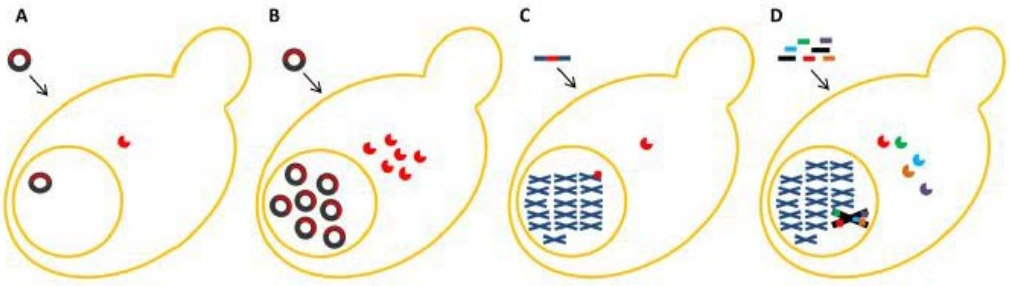

Figure 1.4 Different synthetic biology tools developed at DNA level. A: yeast centromeric plasmid (YCp); B: yeast episomal plasmid (YEp); C: yeast integrative plasmid (YIp); D: yeast artificial chromosome (YAC)

Replacing the native promoter region of plasmid (Auxotrophic markers *URA3* and *LEU2*) with truncated and weak promoters (*URA3-d* and *LEU2-d*) resulted in the enhancement of the plasmid copy number (150 and 111 copies, respectively) (Faulkner et al., 1994). Both YCp and YEp vectors are uncomplicated to use and are ideal for gene overexpression at low or high levels. Though maintenance of two or more YEp (2µ) and/or YCp (CEN/ARS) vectors for stable existence of a single cell can be difficult, in addition these plasmids cannot carry a large size DNA molecule. Which can be evaded by yeast artificial chromosomes (YAC) which offer the possibility to transference large DNA molecule (Murray, 1983; Kouprina and Larionov, 2008).

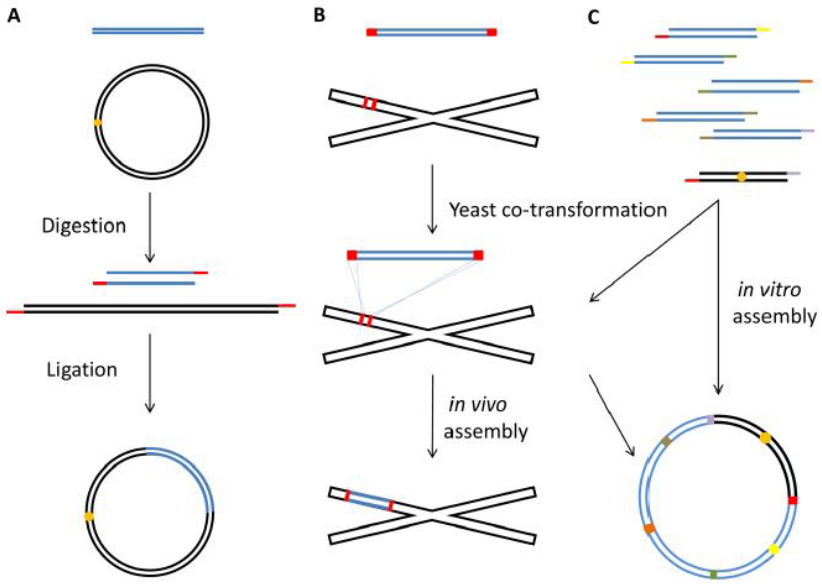

Figure 1.5 Schematic representation of assembly methods for forming DNA constructs; (A) Plasmid-based based on restriction and ligation; (B) chromosomal integration based *in vivo* homologous recombination; (C) YAC based method based on either *in vivo* or *in vitro* homologous recombination.

1.4.2 Tools at transcription level

Most of the biochemical pathways regulated at transcription level during and after the synthesis of RNA. Based upon these particular tools have been developed for modulating RNA levels during the RNA synthesis and controlling the stability of RNA after being synthesized

i) Regulation of transcription process

Promoters are the prime targets for controlling the transcription process by regulating RNA synthesis, consequently, potential targets for synthetic biology and metabolic engineering applications (Ajo-Franklin et al., 2007; Bashor et al., 2008). There are approximately 6000 promoter regions have been found in *S. cerevisiae*, according to SCPD (The Promoter Database of *S. cerevisiae*: http://rulai.cshl.edu/SCPD). These promoters are classified into two categories, constitutive and regulatable (Figure 3), and however, recently different promoter libraries and chimeric promoters have been developed (Jensen, 2003; Alper et al., 2005; Zhang et al., 2012). Promoter with desired constant activity makes them appropriate for the introduction of new pathways in yeast. A few numbers of regulated promoters have been found and employed in yeast. Some of the well-known inducible promoters like *GAL1*and *GAL10*, induced by galactose and repressed by glucose are widely used (Lohr et al., 1995). Another group of promoters

which are known as repressible promoters; *MET25* (Sangsoda et al., 1985), *MET3* (Cherest et al., 1985) and *CUP1* (Etcheverry, 1990) promoters which are retorting to the existence of methionine and copper, respectively.

ii) RNA stability control strategies

RNA molecules plays various important roles in living cells by regulation of gene expression through RNA secondary structure, efficient roles in RNA replication, RNA stability, translation and splicing in both eukaryotes and prokaryotes (Serganov and Patel, 2007). Based on these evidences, diverse synthetic RNA switches to carry regulatory, sensing, information processing and scaffolding activities have been developed to program biological processes. Synthetic RNA switches are generally, comprise of two domains: the sensing domain, which detects signals inside a cell (input); the actuator domain that alters gene expression. Sometimes, a distinct transmitter domain included to the RNA switch, to provide better communication between sensing domain and actuator domain. Based on the type of regulated process, RNA switches are divided into five different categories: transcription-modulation, splicing-modulation, RNA stability-modulation, RNA interference-modulation, translation-modulation and post translation-modulation switches (Chang et al., 2012). From these, different RNA switches for modulating biological systems at transcription, splicing and RNA stability level has been developed in *S. cerevisiae* (Buskirk et al., 2004; Weigand and Suess, 2007; Win and Smolke, 2007; Babiskin and Smolke, 2011a; Babiskin and Smolke, 2011b).

1.4.3. Tools at Protein level

Proteins are multi-functional macromolecules and play a crucial role in moderating biological systems. Diverse control elements based on protein acting through protein degradation have been developed and employed for tuning protein levels (Mateus and Avery, 2000; Hackett et al., 2006; Grilly et al., 2007). These elements usually alter the protein half-lives by introducing a degradation tag signal at the N- or the C- terminus of the target protein (s), leading them into the natural degradation machinery to provide rigid dynamic regulation over biochemical pathways (Mateus and Avery, 2000).

1.4.4. Tools at pathway level

Pathway Engineering is possible through above-mentioned DNA tools by building and optimization of an existing biochemical pathway either in a native or in a heterologous host. Pathway engineering can be designed realistically by mixing and matching well known modular parts (promoters, genes, and proteins) and modulating these parts through

various control mechanisms (Liang, 2011). Failure to control the flux to equilibrium in the synthetic pathway will result in a blockage and the accumulation of intermediates (Pitera et al., 2007). This can be alleviated through transcription optimization (Bennett and Hasty, 2009; Ellis et al., 2009) of the various genes and protein co-localization (Dueber et al., 2009) in the pathway. In addition to above things redesigning and engineering of regulatory parts such as ribosome binding sites (RBSs), ribo switches, and operator–regulator pairs could solve complicated problems in synthetic metabolic pathways (Landrain et al.,2009).

1.4.5. Analysis Tools

Biological engineering have depends on the standard analytical techniques of molecular biology, microbiology, and genetics. However, recent advancements in the 'omic' profiling have improved the analysis in the pathway, whole cell, and multi-cell applications at the genomic, proteomic, and metabolomics levels (Liang, 2011).

i. Genomics tools

Micro array techniques have been widely employed for analysis of simultaneous changes in the expression of large numbers of genes in response to experimental perturbations or environmental variations. (Foret et al., 2007; Weniger et al., 2007). Chromatin Immuno-precipitation (ChIP) method widely used for transcription control at the genomic level (Grandori et al., 1996). Recently, a new method Chromatin Immuno-precipitation followed by sequencing (ChIP-seq) was developed for genome wide profiling of DNA-binding proteins, modifications of his tone or nucleosides, and genome alignment (Trapnell, 2009).

ii. Proteomics tools

The vital analytical technique for proteomics research is mass spectrometry (Domon, 2006) which involves the quantification of all the expressed gene products of a cell type, tissue, or organism. Mass spectrometry-based quantification methods follow two approaches: label-free quantification approaches aim to correlate the number of peptide sequencing events with the relative or absolute protein quantity. Whereas in differential stable isotope labelling specific radio labelled mass tag used which can be recognized by a mass spectrometer and provides the basis for quantification (Mann, 2006)

iii. Metabolomics tools

Metabolomics refers to the analysis metabolic picture of a biological system by measuring the metabolite concentration (Goldsmith et al.,2008) Metabolomics tools have

been used to restore the synthetic metabolism for industrial-scale microbial production of a variety of natural and novel chemicals (Kizer et al., 2008)

1.5. Modelling tools

Metabolic engineering is the manipulation of metabolic pathways in order to improve biological systems for industrial applications, and recombinant DNA technology is the most commonly used tool today for this purpose. However, in order to effectively alter a living system, the first key step should be to understand the inner workings of that system. Especially with the advent of genome projects and new technology for large-scale genomic and proteomic studies, it becomes clear that understanding the function of every component under laboratory conditions will prove technically challenging. Computational modeling tools for the *in silico* study of living systems offer a nice alternative for the identification of key regulatory pathways and genetic circuits that control metabolic pathways, in order to predict potential outcomes of alterations to the organism's genome.

Mathematical modeling is used to describe synthetic constructs and explains the biological phenotypic complexity emerged due to bimolecular interactions (Kaznessis, 2009) mathematical modeling can dramatically increase the speed of the design process as well as reduce the cost of development. Standard Virtual Biological Parts (SVBPs) are an open access repository available on line. Another tool Metabolite to Metabolite (FMM) is available on the web for building of a diversity of possible enzymatic pathways from an input metabolite to an output metabolite, which is useful in pathway engineering (Chou et al., 2009).

Recently a genome-scale metabolic models are most widely used; a network of chemical reactions that take place inside a living organism is primarily reassembled from the information that is present in its genome and the literature and involves steps such as functional annotation of the genome, identification of the associated reactions and determination of their stoichiometry, assignment of localization, determination of the biomass composition, estimation of energy requirements, and definition of model constraints (Baart and Martens, 2012). Some of the widely used and available tools are listed in table 1.1.

DNA Tools	
Biopolymer calculator	Calculate extinction coefficients, Tm's, and base composition for your DNA or RNA; calculate amino acid composition and the extinction coefficient for your protein
Clipboard	Web tool for getting complement, reverse complement, translation and restriction enzyme analysis of a DNA sequence
DNAWorks	A web tool for optimizing melting temperature during gene synthesis.
File format converter	Web tool for converting between sequence file formats
Genome Compiler	It allows you to manipulate genetic information; from genes to plasmids to whole genomes
Gene Design	Collection of online (and some command line) tools for codon optimization and shuffling, restriction site editing, and so on.
Gene Designer	Combine genetic building blocks by drag-and-drop, codon optimize, restriction site editing, sequence oligo design etc.
Geno CAD	An experimental site that relies on formal grammars to design and verify the synthetic DNA sequences.
NEB Cutter	Tool for finding restriction sites, et cetera.
Synthetic Gene Designer	A web platform that allows codon optimization to various extent.
Vector NTI	Free-to-academics tool for sequence analysis and data management.
j5, Device Editor, and Vector Editor	j5: DNA assembly design automation for (combinatorial) flanking homology (e.g., SLIC/Gibson/CPEC/SLiCE/yeast) and type IIs-mediated (e.g., Golden Gate/FX cloning) assembly methods
RNA Tools	
Appendix	Website with many useful nucleic acid parameters.
M Fold	This is the main page with links to sites for predicting RNA and DNA folds, calculating Tm's and free energies.
Protein Tools	
Cn3D	A helper application for your web browser that allows you to view 3-dimensional structures from NCBI's Entrez retrieval service. It doesn't read PDB files but can be more

	straightforward to use than Deep View.
DeepView	Awesome program for viewing and studying protein structure.
ExPASy Proteomics server	Collection of links to many pages to calculate parameters of your favourite proteins
Modeller	For homology or comparative modelling of protein three-dimensional structures.
Zinc Finger Tools	Design Zinc Finger DNA binding proteins
CAD Tools	
Tinker Cell	Construct computational models using biological parts, cells, and modules. Designed to bridge computational and experimental biology
Cell Designer	Modelling and simulation of biochemical and gene regulatory networks
Cell Illustrator	Enables biologists to draw, model, elucidate and simulate complex biological processes and systems.
Metabolic Tinker	Searches for all paths between two compounds.

Table 1.1 Systems metabolic engineering and synthetic biology tools for modeling and simulation of biological pathways (http://syntheticbiology.org/Tools.html)

1.6. Isoprenoids

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 nonvolatile,

cyclic or acyclic, chiral or achiral, reflected in their complexity, due to the multitude of biological activities they fulfill (Bohlmann et al., 2008). They are naturally produced in sub sequential head-tail hetero polymeryzation condensation of isoprene functional units, isopentenyl diphosphate IPP, in all organisms and classified based on the content of isoprene units as: hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), sesterterpenes (C25), triterpenes (C30). The isoprene universal building block IPP is naturally synthesized via two independent pathways: the mevalonate (MEV) pathway and the 1-deoxyxylulose-5-phosphate (DXP) pathway (Kuzuyama et al., 2003). These two biosynthetic pathways are taxonomically distributed, the MEV 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 MEV pathway was recently discovered (Lombard et al., 2011) (fig.1.6). The MEV 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 (fig.1.6). 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).

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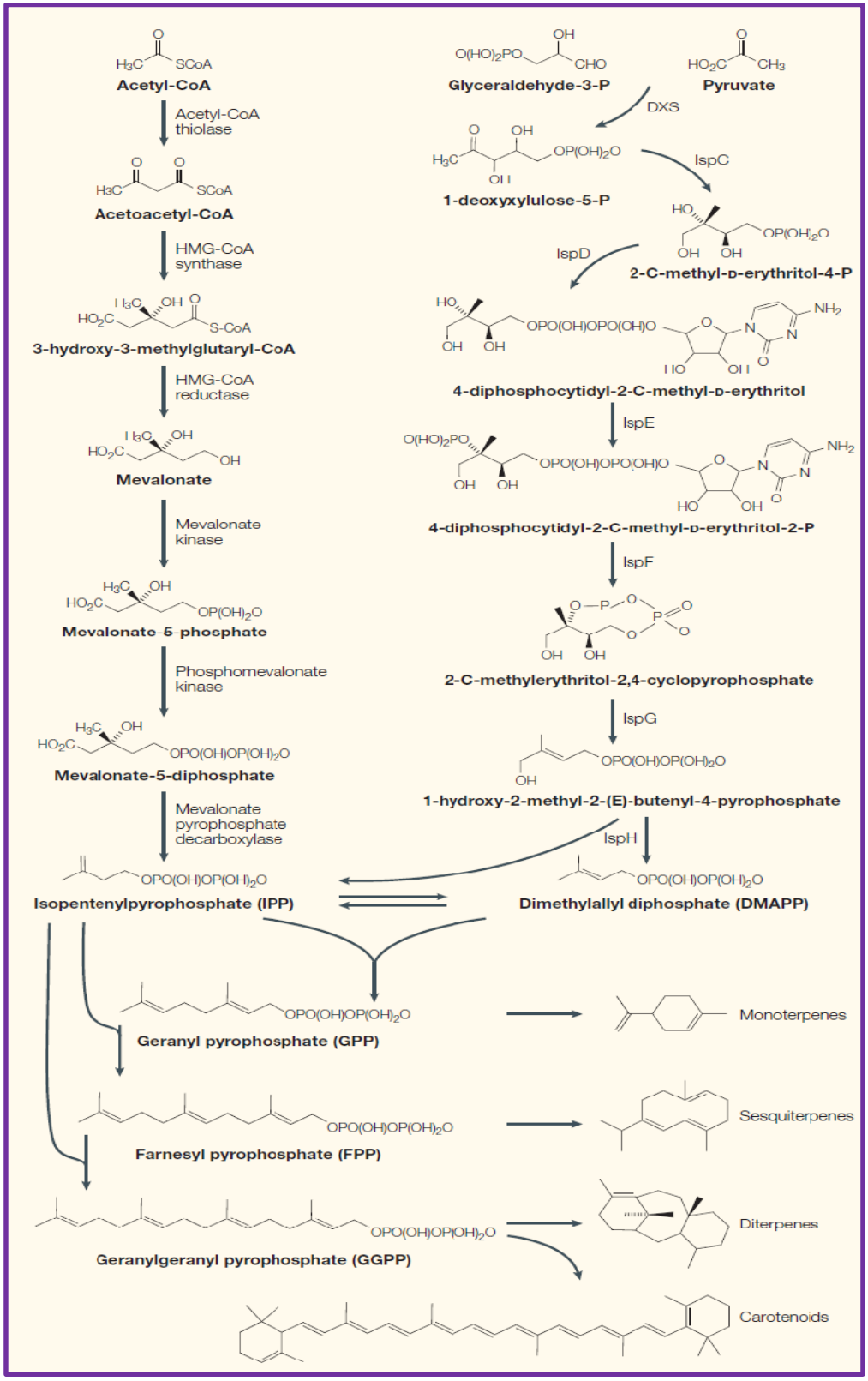


Figure 1.6 Eukaryal mevalonate (MEV) pathway, modified Archeal mevalonate (MEV) pathway and bacterial methylerythritol phosphate (MEP) pathway. (Khosla and Keasling, 2009)

1.6 Market drivers toward microbial production of sesquiterpenes

As mentioned in the introduction, the demand for microbial production of chemicals as an alternative to petrochemical based synthesis is increasing due to economic, 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, 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 (Bohlmann et al., 2008). The complexity of isoprenoid is often the main drawback for the industrial scale production. Now days, most of the isoprene derived compounds are produced via plant extraction and by total or semi synthesis. Extraction of 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 involves 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) increase 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).

CHAPTER 2

Literature Review

CHAPTER 2

LITERATURE REVIEW

2.1 Classification of Isoprenoids

Isoprenoids (terpenoids) are the most structurally diverse class of natural compounds commonly produced in plants (Croteau et al., 2000). Isoprenoids are derived from five-carbon isoprene units (2-methyl-1, 3-butadiene) and the combination of isoprene units leads to the formation of different isoprenoids. Based on the 'isoprene rule' that was first recognized in 1887 by Wallach and later, in 1953, it was extended to the 'biogenetic isoprene rule' by Ruzicka, isoprenoids can be divided into different groups depending on the number of isoprene units in their carbon skeleton as mono terpenoids (C_{10}), sesqui terpenoids (C_{15}), di terpenoids (C_{20}), sesterterpenoids (C_{25}), tri terpenoids (C_{30}), tetra terpenoids (C_{40}) and polyterpenoids (C_n) (Ruzicka, 1953) (table 2.1). More than 55,000 terpenes have been isolated and characterized, consistently doubling in their numbers each decade (Breitmaier, 2006; McGarvey and Croteau 1995). Isoprenoids have diverse functional roles in plants such as growth, defense and development (McGarvey and Croteau 1995). Based on these characteristic features, terpenoids have prominence in pharmaceutical, fragrances and biofuel industries shown in table 2.2 (Breitmaier, 2006; Peralta-Yahya et al., 2012). Terpenoids, being secondary metabolites, produced in very small quantities and scale up with existing plant and microorganism strains (Chang and Keasling, 2006) are not cost effective. With commercial and medicinal uses of plant terpenoids on the rise, there is a need to increase the yield of terpenoid biosynthesis.

2.2 Biosynthesis of Isoprenoids

All isoprenoids originate from the five-carbon basic unit, isopentenyl-pyrophosphate (IPP) and its isomer dimethylallyl-pyrophosphate (DMPP) (fig.2.1). Since 1967, mevalonate pathway (MEV) for cholesterol synthesis was discovered (Katsuki and Bloch, 1967; Lynen, 1967), IPP was assumed to be synthesized through the mevalonate-dependent pathway in all living organisms. However, in recent times, the existence of an alternative pathway, called the 2-methylerythritol 4-phosphate (MEP) pathway, was demonstrated in bacteria, green algae, and higher plants (Rohmer et al., 1993; Rohmer, 1999; Kuzuyama and Seto, 2003; Rohmer et al., 1993) plastids (fig.2.1). The MEP

pathway does not exist in animals or fungi, but both pathways are active in higher-level plants such as *Artemisia annua, Arabidopsis thaliana* and *Helianthus annuus.*

Class	Isoprene units	Carbon atoms	Formula
Monoterpenoids	2	10	$C_{10}H_{16}$
Sesquiterpenoids	3	15	$C_{15}H_{24}$
Diterpenoids	4	20	$C_{20}H_{32}$
Sesterterpenoids	5	25	$C_{25}H_{40}$
Triterpenoids	6	30	$C_{30}H_{48}$
Tetraterpenoids	8	40	$C_{40}H_{64}$
Polyterpenoids	>8	>40	$(C_5H_8)_n$

Table 2.1 Classification of terpenoids based on the isoprene rule

Isoprenoids are functionally important in many different parts of cell metabolism such as photosynthesis (carotenoids, chlorophylls, plastoquinone), respiration (ubiquinone), hormonal regulation of metabolism (sterols), regulation of growth and development (giberellic acid, abscisic acid, brassinosteroids, cytokinins, prenylated proteins), defense against pathogen attack, intracellular signal transduction (Ras proteins), vesicular transport within the cell (Rab proteins) as well as defining membrane structures (sterols, dolichols, carotenoids) (Sacchettini and Poulter, 1997). Many isoprenoids also have considerable medical and commercial interest as flavors, fragrances (e.g. limonene, menthol, camphor), food colorants (carotenoids) or pharmaceuticals (e.g. bisabolol, artemisinin, lycopene, Taxol). Plant tissues are rich source for the isoprenoids extraction. The traditional extraction process neither economical nor environmental friendly for the large-scale production of isoprenoids.

Ttraditional chemical synthesis for the production of value added chemicals and major pharmaceuticals is a well-established field. Chemical synthesis of isoprenoids has also been reported (Mukaiyama et al., 1999; Danishefsky et al., 1996; Miyaoka et al., 2002) and currently most of the industrially interesting carotenoids are produced via chemical synthesis.

Nevertheless, low yields, unwanted side reactions and complex structure of these molecules are some of the major obstacles for chemical synthesis of isoprenoids.

Enzymes established significant expertise in the synthesis of very complex molecules through environmentally friendly methods (Smolke, 2009).

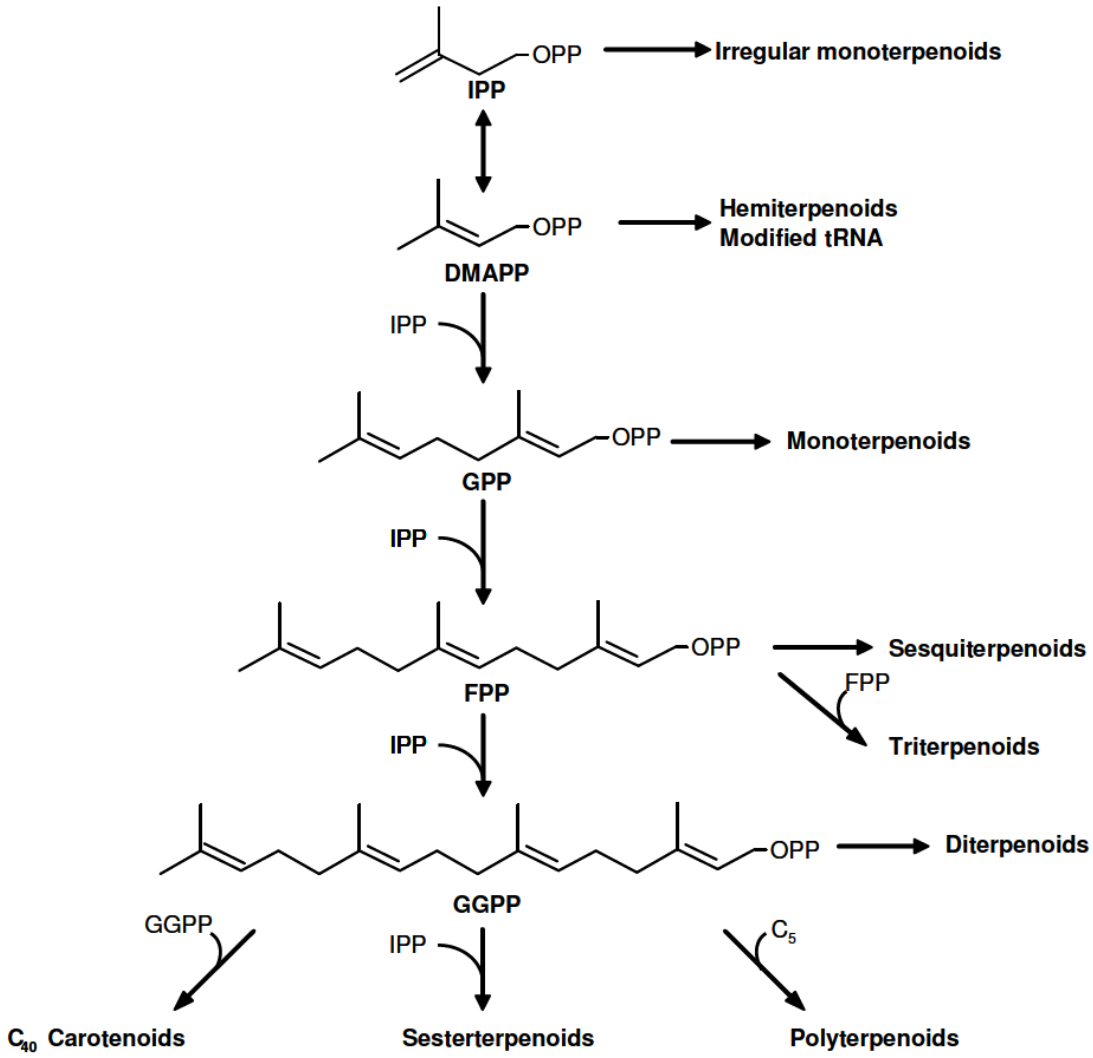


Figure 2.1 The different classes of isoprenoids and their precursors DMAPP: dimethylallyl diphosphate, IPP: isopentenyl diphosphate, GPP: geranyl diphosphate, FPP: farnesyl diphosphate, GGPP: geranylgeranyl diphosphate (Maury et al., 2005)

Approaches which involve biological catalyst, avoids organic solvents, heavy metal catalyzers, and strong acids and bases that are currently employed upon synthetic chemistry based routes. In addition extraction and purification of valuable natural products from native plant sources is difficult as these compounds accumulate in very small amounts, and need many steps and solvents for extractions and purification for example, it would take approximately six, 100-year-old Pacific yew trees to provide enough taxol (paclitaxel) to treat one cancer patient (Horwitz, 1994; Jeandet et al., 2013).

Class	Biological activity	Commercial applications	Examples
Monoterpenoids	Signal molecules, defense agents	Flavors, fragrances, cleaning products, anticancer agents, antimicrobial agents	Limonene, menthol, camphor
Sesquiterpenoids	Antibiotic, antitumor, antiviral, immunosuppressive, and hormonal activities	Flavors, fragrances, potential pharmaceuticals, biofuels	Juvenile hormone, Cubebol, artemisinin, bisabolene α-Santalene, Valencene
Diterpenoids	Hormonal activities, antitumor properties	Anticancer agents	Gibberellins, phytol, Taxol
Sesterterpenoids	Cytostatic activities	None as yet	Haslenes
Triterpenoids	Membrane Components	Biological markers	Sterols, hopanoids
Tetraterpenoids	Antioxidants, photosynthetic components, pigments, and nutritional elements	Food additives (colorants, antioxidants), anticancer agents	β-carotene, Lycopene,

Table 2.2 Biological activities and commercial applications of classical isoprenoids (modified Maury et al., 2005)

Microbial production of chemicals is an accepted environmentally friendly method that may lead to the production of large amounts of high value isoprenoids from simple and cheap carbon sources. Engineered microorganisms would also enable the production of unusual and novel isoprenoids with excellent biological and commercial applications. Development of cell factories using genetic engineering techniques requires comprehensive data about the metabolic pathways and enzymes involved in the biosynthesis of the desired product(s) and also an understanding of the mechanisms by which the flux through the pathway is controlled. One of the major obstacles for the commercial production of isoprenoids by cell factories is the limited supply of precursors. Replenishing the intracellular pool of precursors will need deregulation of the pathways in order to improve the flux towards the biosynthesis of isoprenoid precursors. Hence, for construction of enhanced cell factories proficient with isoprenoid production, the

knowledge of metabolic pathways of isoprenoid synthesis, enzymes and genes involved and also the regulatory network of pathways required.

2.3 The mevalonate pathway of *Saccharomyces cerevisiae*

Involvement of isoprenoids in a variety of physiological and medically important processes, mevalonate pathway has been intensively studied in eukaryotes. Key end products of the mevalonate pathway are sterols, e.g. cholesterol in animal cells and ergosterol in fungi, which are significant regulators of membrane permeability and fluidity (Daum et al., 1998; Veen and Lang, 2004). In addition to sterols, the mevalonate pathway provides intermediates for the synthesis of a number of other essential cellular constituents like hemes, quinones, dolichols or isoprenylated proteins which are all derived from the early part of the pathway (fig.2.2) (Lees et al., 1999). Thus, the mevalonate pathway can be considered to consist of two distinct parts: an early isoprenoid portion of the pathway, common to many branches and ending with the formation of farnesyl diphosphate (FPP), and a late part of the pathway mainly dedicated to ergosterol biosynthesis in *S. cerevisiae* (fig.2.2).

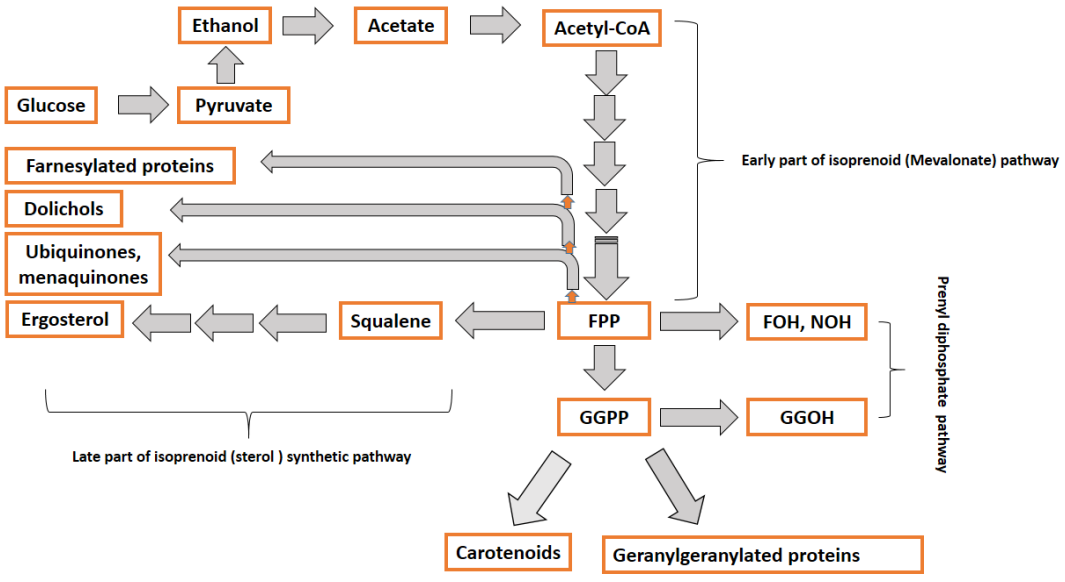


Figure 2.2 Early and late part of the isoprenoids synthesis pathway. FPP, (E, E)-farnesyl diphosphate; GGPP, (E, E, E)-geranylgeranyl diphosphate; FOH, (E, E) -farnesol; NOH, (E)-nerolidol

2.3.1 Regulation of Mevalonate Pathway

The first committed step in the mevalonate pathway is the synthesis of acetoacetyl-CoA from two molecules of acetyl-CoA, catalyzed by the acetoacetyl-CoA thiolase which is encoded by *ERG10* (Figure 2.3). In *S. cerevisiae* this enzyme appear in two forms, with different subcellular locations (the cytosol and the mitochondrion) and this enzymatic reaction is regulated at transcriptional level by late intermediate(s) or product(s) of the pathway (fig.2.3) (Dimster-Denk and Rine, 1996; Dimster-Denk et al., 1999). Though, overexpression studies of *ERG10* did not increased the total sterol content, signifying other enzyme(s) of the sterol biosynthetic pathway as controlling elements (Dimster-Denk and Rine, 1996).

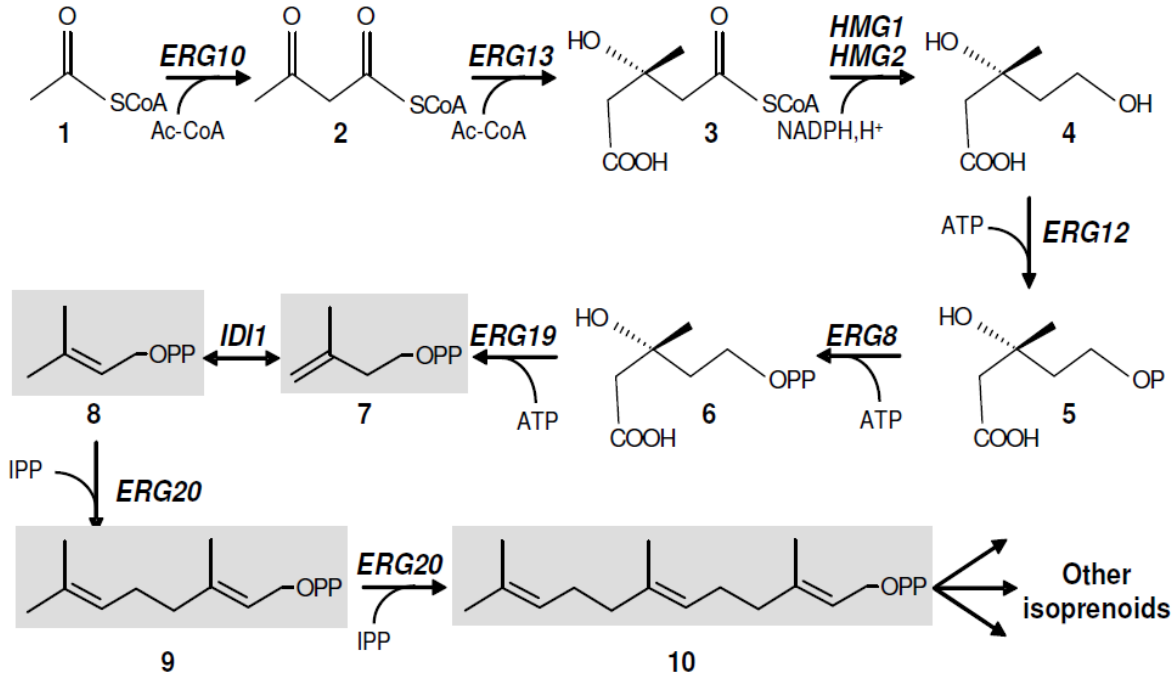


Figure 2.3. The mevalonate pathway in *S. cerevisiae* (Maury et al., 2005)

1: acetyl-CoA, 2: acetoacetyl-CoA, 3: 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), 4: mevalonate, 5: phosphomevalonate, 6: diphosphomevalonate, 7: IPP, 8: DMAPP, 9: GPP, 10: FPP. Gray boxes specify the general precursors for the different classes of isoprenoids. ERG10: acetoacetyl-CoA thiolase, ERG13: HMG-CoA synthase, HMG1, HMG2: HMG-CoA reductases, ERG12: mevalonate kinase, ERG8: phosphomevalonate kinase, ERG19: diphosphomevalonate decarboxylase, IDI1: IPP: DMAPP isomerase, ERG20: FPP synthase.

The second step is catalyzed by the *ERG13* gene product, HMG-CoA synthase involved in the condensation of acetyl-CoA with acetoacetyl-CoA to yield 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). The details of the regulatory mechanism involved remain uncharacterized (Daum et al., 1998). However, the first crystal structure of an HMG-CoA synthase from, *Staphylococcus aureus*, revealed that the amino acid residues involved in the acetylation and condensation reactions are conserved among bacterial and eukaryotic HMG-CoA synthases (Campobasso et al., 2004). The third enzyme in the pathway, HMG-CoA reductase, involved in the conversion of HMG-CoA into mevalonate, which is the most studied enzyme of the mevalonate pathway. In *S. cerevisiae,* the gene encoding HMG-CoA reductase appears in two forms: *HMG1* and *HMG2,* but Hmg1p was major enzyme responsible for more than 83% of the activity in wild type cells (Basson et al., 1986) but disruption of both genes is lethal for cell. This step is extremely regulated at diverse levels and seems to be an important regulatory point in the mevalonate pathway.

In the fourth step phosphorylation of mevalonate at the C-5 position catalyzed by the mevalonate kinase, encoded by *ERG12*. Farnesyl pyrophosphate (FPP) and geranyl diphosphate (GPP) exert an inhibitory effect on the enzyme (fig.2.3). The next step catalyzed by the phosphor mevalonate kinase, the gene product of *ERG8,* is not subject to feedback regulation by ergosterol (Daum et al., 1998). Overexpression studies disclosed that*ERG8*not shown any prominent effect on improving the ergosterol levels, signifying that this enzyme is not a flux controlling for ergosterol production (Lees et al., 1999).

The next two steps of the mevalonate pathway catalysed by mevalonate diphosphate decarboxylase, *ERG19* gene product, which converts mevalonate diphosphate to IPP. Then *IDI1* gene product (isopentenyl diphosphate: dimethylallyl diphosphate isomerase) can convert IPP into dimethylallyl diphosphate (DMAPP). IPP isomerase catalyzes an essential activation step in isoprenoid metabolism by enhancing the electrophilicity of the isoprene unit by at least a billion-fold and converts IPP to DMAPP. Two different classes of IPP isomerases have been reported: the type I enzyme, widely distributed in eukaryota and eubacteria, while the type II enzyme was recently discovered in *Streptomyces* sp. strain CL190 and in the archaeon *Methano thermobacter thermautotrophicus* (Barkley et al., 2004; Kaneda et al., 2001). These two types of isomerases differ in structures and cofactor requirements, hence required different chemical mechanisms for catalysing the isomerization step (Barkley et al., 2004). The properties of mevalonate diphosphate

decarboxylase and of IPP isomerase are mostly uncharacterized. But overexpression studies of *ERG19*, reduced the sterol content which revealed that the accumulation of diphosphate intermediates leading to feedback inhibitions. Hence, *ERG19* could encode a flux controlling step of the mevalonate pathway (Berges et al., 1997). The final step in the early portion of the pathway is the conversion of DMAPP into geranyl and farnesyl diphosphates (GPP and FPP, respectively) which is catalysed by farnesyl (geranyl) diphosphate synthase, the product of the *ERG20* gene. Farnesyl diphosphate synthase (FPPS) is a well characterized prenyl transferase. The enzyme has been purified to homogeneity from several eukaryotic sources including *S. cerevisiae*. FPP is a key molecule positioned at the branch point of several important metabolic pathways leading to sterols, heme, dolichol or quinone biosynthesis and prenylation of proteins and also involved in several key regulations of the mevalonate pathway (fig.2.2). Moreover, overexpression studies improved the ergosterol content in the cell, representing that FPP synthase exerts some flux control in the pathway (Daum et al., 1998).

The regulation of the isoprenoid biosynthetic pathway is known to be complex in all eukaryotic organisms (Hampton et al., 1996). The intervening principle for the regulation of this pathway is several levels of feedback inhibition (fig.2.3). This feedback regulation involves numerous intermediates and appears to act both at different steps of the pathway and at different levels of regulation as it involves changes in gene transcription, mRNA translation, enzyme activity and protein stability. The evolving concept is that the isoprenoid pathway has a number of points of regulation that act to control the overall flux through the pathway as well as the relative flux through the various branches of the pathway (Dimster-Denk et al., 1999). From these complex multilevel regulations, two distinct but interconnected major sites of regulation are evident: one is the HMG-CoA reductase, the other is due to enzymes competing for FPP. The yeast HMG-CoA reductase is subject to complex regulation by a number of factors and conditions, at different levels.

At the transcriptional level, *HMG1* expression is stimulated by heme via the transcriptional regulator Hap1p, while *HMG2* expression is inhibited, indicating a relationship between heme and sterol biosynthesis. Hmg1p was shown to be transcriptionally repressed by a non-sterol product of the pathway (Dimster-Denk et al., 1994). In a different study, the same group reported the induction of *HMG1* reporter gene after inhibition of squalene synthase or lanosterol demethylase suggesting that *HMG1*

responded to the levels of sterol products of the pathway (Dimster-Denk et al., 1999). The two yeast isozymes have also distinctly different posttranslational fates: Hmglp was shown to be extremely stable while Hmg2p was subject to rapid regulated degradation depending on the flux through the mevalonate pathway (Hampton and Rine, 1994). The stability of each isozyme is determined by its non-catalytic amino-terminal domain. Hmg2p was demonstrated to undergo ERAD (endoplasmic reticulum associated degradation), similar to its mammalian ortholog, dependent on ubiquitination (Hampton and Rine, 1994; Shearer and Hampton, 2004). FPP was demonstrated as the source of the regulatory signal controlling and coupling ubiquitination/degradation of Hmg2p with the flux in the mevalonate pathway (Hampton and Rine, 1994; Gardner and Hampton, 1999). In addition to the FPP signal, an oxysterol derived signal positively regulates Hmg2p degradation in yeast, but in contrast with mammals it is not an absolute requirement for degradation in yeast (Gardner et al., 2001).

In summary, the different regulations of HMG-CoA reductase can be grouped as 1) feedback inhibition, i.e. regulation of HMG-CoA reductase activity in response to intermediates or products from the mevalonate pathway, and 2) cross-regulation, i.e. regulation of processes independent of the mevalonate pathway (Hampton et al., 1996). As a consequence, in aerobic conditions Hmg1p is actively synthesized and extremely stable consistent with the constant need of sterols, while in anaerobic conditions the enzyme with a high turnover, i.e. Hmg2p, is dominant to allow rapid adjustment of the balance between cellular demand and the potential accumulation of toxic compounds (Hampton et al., 1996). HMG1 and HMG2 are also expressed differently as a function of the growth phase. FPP, the product of FPP synthase (Erg20p), is a crucial intermediate in the mevalonate pathway leading to the synthesis of several critical end products (Daum et al., 1998). In addition, the farnesyl units and the related geranyl and geranyl geranyl species are important elements for the posttranslational modification of proteins that require hydrophobic membrane anchors for proper placement and function. Furthermore, farnesol, a metabolite causing apoptotic cell death in human acute leukaemia, a molecule involved in quorum sensing in *Candida albicans* (Hornby et al., 2003; Grabinska and Palamarczyk, 2002) and causing growth inhibition in *S. cerevisiae*, is endogenously generated in the cells by enzymatic de phosphorylation of FPP (Machida et al., 1998). To ensure a constant production of the multiple isoprenoid compounds at all stages of growth while preventing accumulation of potentially toxic intermediates, cells must precisely

regulate the level of activity of enzymes of the mevalonate pathway (fig.2.4). A number of experimental data show that biosynthesis of dolichols and ubiquinones, as well as isoprenylated proteins, is being regulated by enzymes distal to HMG-CoA reductase (Szkopinska et al., 2000).

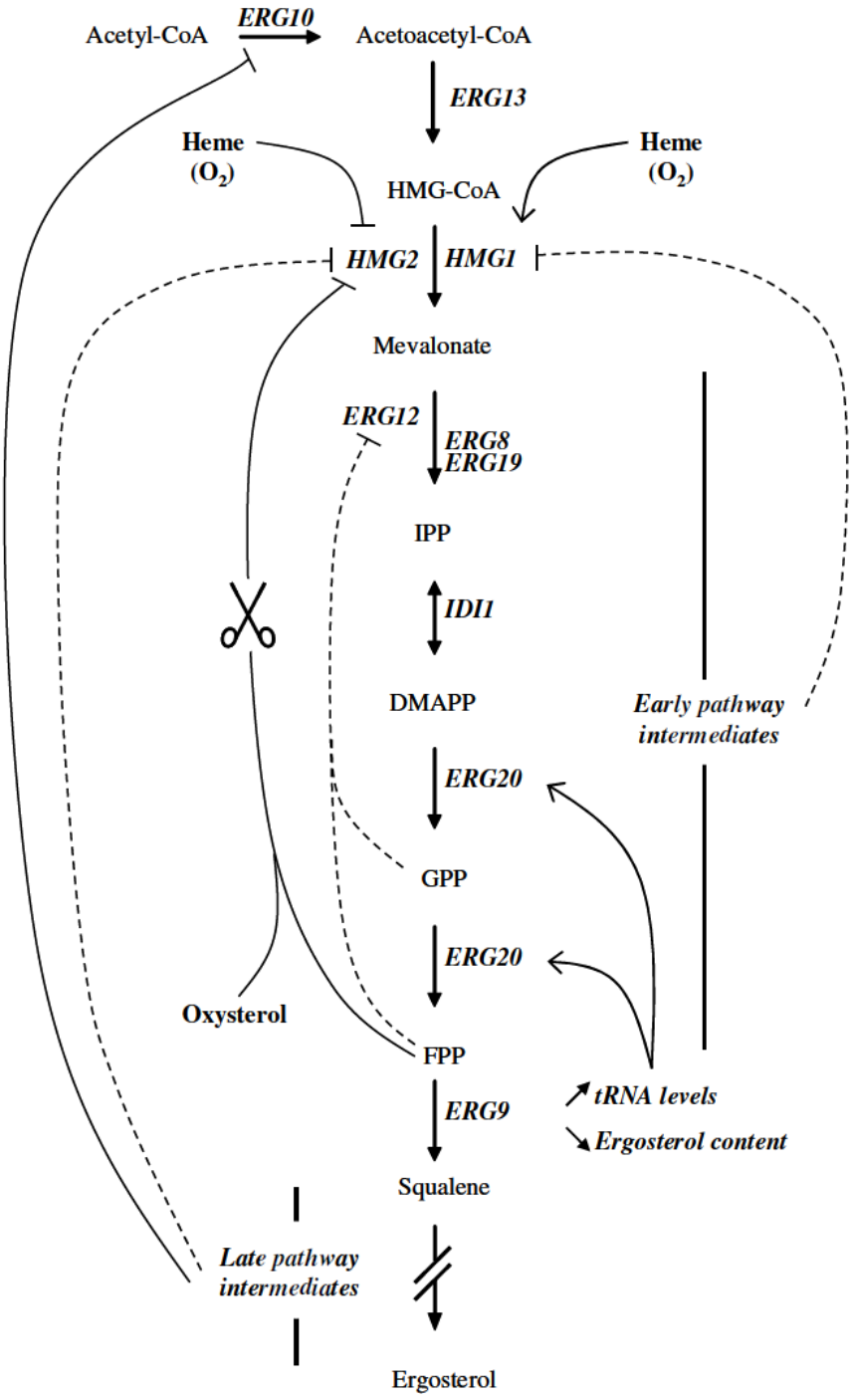


Figure 2.4 Principal regulations of the mevalonate pathway solid lines: regulations at gene expression level, dashed lines: regulations at protein synthesis level, ✂: regulation of protein stability. (Maury et al., 2005)

2.4 The methylerythritol phosphate (MEP) pathway

Until the discovery of the MEP pathway, mevalonate pathway widely accepted as a source for IPP and DMAPP in all living organisms (Zhou and White, 1991) until the discovery of second pathway by the research groups of Rohmer and Arigoni. Experiments conducted with stable isotopes in various eubacteria and plants (Rohmer et al., 1993) revealed that, pyruvate and a triose phosphate could serve as precursors for the formation of IPP and DMAPP (Rohmer et al., 1993). The first enzyme, 1-Deoxy-d-Xylulose-5-Phosphate Synthase (*DXS)* of non-mevalonate pathway was identified and cloned from *E. coli* and the plant *Mentha piperita* (Sprenger et al., 1997; Lange et al., 1998) (fig.2.5). Most of the Gram negative bacteria and *Bacillus subtilis* equipped with MEP pathway for isoprenoid biosynthesis, whereas *staphylococci*, *streptococci*, *enterococci*, fungi and archae use the mevalonate pathway (Hedl et al., 2002). Most of the *Streptomyces* strains possess only MEP pathway, but some strains, in addition to MEP pathway they also have a functional mevalonate pathway to produce terpenoid antibiotics (Takagi et al., 2000a; Hamano et al., 2001). *Listeria monocytogenes* was reported as the only pathogenic bacterium known to contain both pathways concurrently.

In the first step of the MEP pathway, 1-deoxy-D-xylulose 5-phosphate synthase, also named DXP synthase or Dxs, catalyzes the condensation of the two precursors from the central metabolism, D-glyceraldehyde 3-phosphate (GAP) and pyruvate, to form DXP. In addition to IPP and DMAPP, DXP is also a precursor for the biosynthesis of vitamins B1 (thiamine) and B6 (pyridoxal) in *E. coli* (Sprenger et al., 1997). DXP synthase activity requires both thiamine and a divalent cation (Mg^{2+} or Mn^{2+}) as a cofactor (Eisenreich et al., 2004) and its activity relatively high compared to the other enzymes of the pathway. As DXP is the precursor for different kinds of compounds, the committed step of the pathway is catalyzed by DXP isomeroreductase (Dxr) and leads to the formation of 2-C-methyl-D-erythritol 4-phosphate (MEP), hence its name: “MEP pathway”.

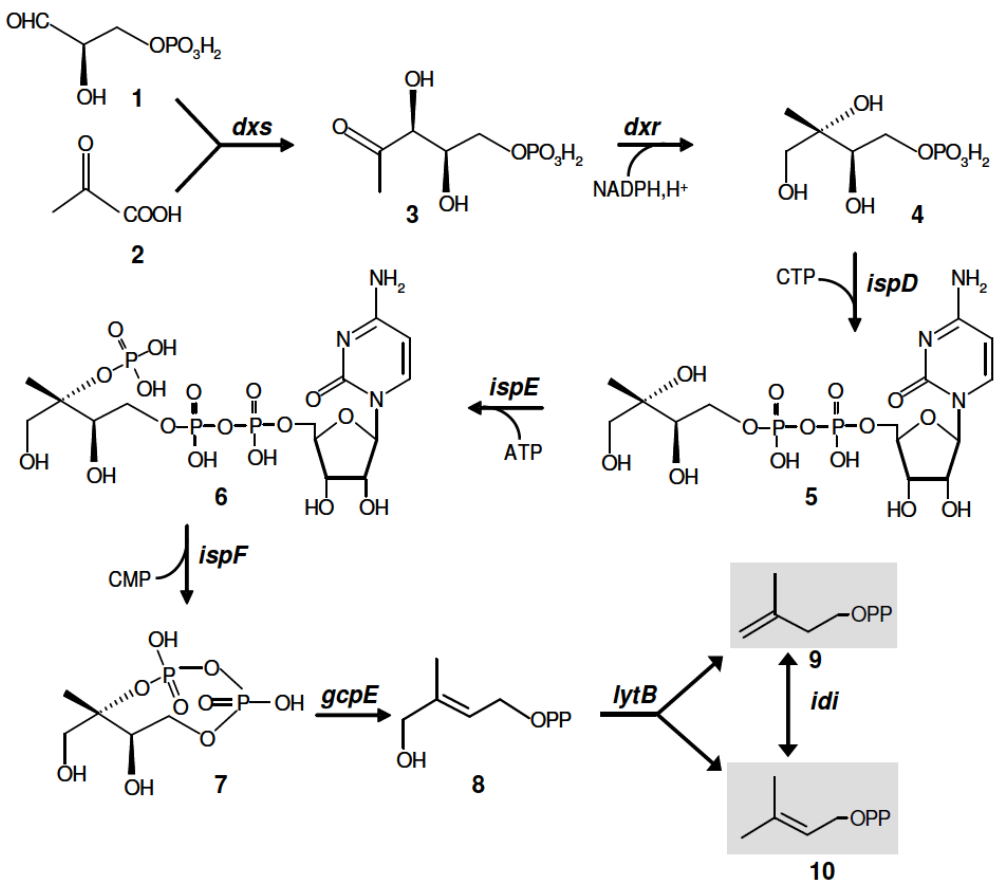


Figure 2.5 The *E. coli* MEP pathway for the synthesis of IPP and DMAPP. (Maury et al., 2005) 1: D-glyceraldehyde 3-phosphate, 2: pyruvate, 3: 1-deoxy-D-xylulose 5-phosphate, 4: 2-Cmethyl- D-erythritol 4-phosphate, 5: 4-diphosphocytidyl-2-C-methyl-D-erythritol, 6: 2-phospho- 4-diphosphocytidyl-2-C-methyl-D-erythritol, 7: 2-C-methyl-D-erythritol 2,4-cyclodiphosphate, 8: 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate, 9: isopentenyl diphosphate, 10: dimethylallyl diphosphate. The enzymes encoded by the different genes are: dxs: DXP synthase, dxr: DXP isomeroreductase, ispD: MEP cytidylyltransferase, ispE: CDP-ME kinase, ispF: MECDP synthase, gcpE: MECDP reductase, lytB: HMBPP reductase.

yaeM gene (dxr enzyme) from *E. coli* was responsible for the rearrangement and the reduction of DXP (Takahashi et al., 1998). The catalytic activity of DXP isomeroreductase (dxr) is substantially lower (12 μmol mg-1 min-1) than DXP synthase (Eisenreich et al., 2004). The reaction catalyzed by DXP isomeroreductase is reversible although the equilibrium is largely displaced in favor of the formation of MEP. Due to the wide distribution of DXP isomeroreductase in plants and many eubacteria, including pathogenic bacteria, and its absence in mammalian cells, this enzyme has been studied as a target for herbicides and antibacterial drugs. Fosmidomycin, an antibacterial agent

active against most Gram negative and some Gram positive bacteria, has been shown to be a strong specific and competitive inhibitor of DXP isomeroreductase activity (Takahashi et al., 1998).

In order to study the MEP pathway, *E. coli* strains were engineered to allow the study of mutations in otherwise essential genes. For this purpose, in addition to the MEP pathway, *E. coli* was transformed with the genes encoding yeast mevalonate kinase, phosphor mevalonate kinase and diphospho mevalonate decarboxylase in one operon. This allowed the study of mutants of the MEP pathway which would have led to the lethality of wild type cells (Campos et al., 2001a). Mutants with a defect in the synthesis of IPP from MEP were isolated and the genes responsible for this defect identified. These genes are ygbP, ychB, ygbB and gcpE. The genes ygbP, ychB, and ygbB are all essential in *E. coli* and the enzymatic steps catalyzed by their gene products belong to the trunk line of the MEP pathway (Campos et al., 2001a). ygbP (ispD) was shown to encode MEP cytidylyl transferase converting MEP into 4- diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME) in the presence of CTP (Kuzuyama et al., 2000a). Its activity is also substantially lower compared to DXP synthase activity. In the presence of ATP, CDP-ME is converted to 2-phospho-4-diphosphocytidyl-2-C-methyl-Derythritol (CDP-ME2P) by the CDP-ME kinase encoded by ispE (Kuzuyama et al., 2000b).

On the basis of sequence comparisons, CDP-ME kinase was recognized as a member of the GHMP kinase family which initially included galactose kinase, homoserine kinase, mevalonate kinase and phosphor mevalonate kinase and more recently mevalonate 5-diphosphate decarboxylase and the archaeal shikimate kinase. 2-C-methyl-D-erythritol 2, 4-cyclodiphosphate (MECDP) synthase, encoded by ygbB (ispF), was demonstrated to catalyze the formation of MECDP from CDP-ME2P with concomitant elimination of cytidine monophosphate (CMP) (Takagi et al., 2000b; Herz et al., 2000). ispF has been shown to be essential (Campos et al., 2001a) and conditional mutation of ispF in *E. coli* or of its ortholog yacN in *B. subtilis* led to a decrease in growth rate and altered cell morphology. In contrast to the dispersed nature of genes belonging to the MEP pathway, ispD and ispF are transcriptionally coupled or, in some cases, fused into one coding region leading to a bifunctional enzyme. IspDF coupling is highly unusual as these enzymes catalyze nonconsecutive steps of the MEP pathway. Interactions have been observed between the bifunctional IspDF and IspE protein. Mono functional IspD, IspF

and IspE proteins have also demonstrated a close interaction suggesting a multi enzymatic complex maybe responsible for the metabolic flux control through the MEP pathway (Gabrielsen et al., 2004).

In contrast to the mevalonate pathway, in which DMAPP is synthesized from IPP by the essential IPP: DMAPP isomerase activity, the finding that IPP: DMAPP isomerase was functional but non-essential for growth of *E. coli* indicated that the MEP pathway was branched i.e. DMAPP and IPP are synthesized by two different routes splitting at late stages of the pathway (Rodriguez-Concepción et al., 2000).The last two steps of the pathway were recently solved by Hintz et al., (2001) who reported the accumulation of the formerly unknown intermediate 1-hydroxy-2-methyl-2-(E)-butenyl 4- diphosphate (HMBPP) in a lytB (ispH) disrupted *E. coli* strain. Several studies aimed at demonstrating the essential nature of gcpE (ispG) and/or lytB (Altincicek et al., 2001a; Altincicek et al., 2001b), their necessity for DXP conversion to IPP and DMAPP (Campos et al., 2001b) and the efficiency of their gene products in converting MECDP into HMBPP (Seemann et al., 2002) and HMBPP into IPP and DMAPP (Altincicek et al., 2002). An important feature of both GcpE and LytB is a [4Fe-4S] cluster as prosthetic group underlying their high sensitivity towards oxygen. This property, common to both enzymes, may explain why the investigations of the terminal reactions of the MEP pathway have been hampered for so long (Seemann et al., 2002).

The finding that a single enzyme is responsible for the formation of both IPP and DMAPP contrasts with the mevalonate pathway where DMAPP is successively formed from IPP by IPP isomerase. As a consequence of these findings, the role of IPP isomerase in microorganisms expressing the MEP pathway comes into question. The non-essential and non-limiting roles of the Idi inter conversion are currently being investigated as on one hand the *E. coli* Idi enzyme was reported to have 20-fold less activity than its yeast counterpart, idi from *E. coli* is dispensable (Rodriguez-Concepción et al., 2000) and *IDI* homologs have not been found in the genomes of many bacteria using the MEP pathway sequenced so far (Cunningham et al., 2000). On the other hand, structurally and mechanistically different IPP isomerases, referred to as class II IPP isomerases, have been identified in Streptomyces sp. strain CL190 and also in a variety of Gram-positive bacteria, cyanobacteria and archaebacteria (Hamano et al., 2001). Furthermore, the overexpression of idi genes from different origins in *E. coli* engineered for the production

of lycopene has always led to carotenoid overproduction (Cunningham et al., 2000; Kajiwara et al., 1997; Wang et al., 1999), these findings keep going the debate about the non-essentiality and non-limiting role of IDI reaction.

2.5 Metabolic Engineering of isoprenoid pathways

At present, *Escherichia coli* and *Saccharomyces cerevisiae* are hired for the microbial synthesis of all possible natural products of interest, though the new platform for microorganism's development (Marienhagen and Bott, 2013). Most of the organisms have the ability to produce isoprenoid precursors and therefore can offer IPP, DMAPP and other subsequent isoprenoid backbones precursors through mevalonate and non-mevalonate pathways as shown in fig.2.6. Due to the established technology developments for analysis and manipulations of the omics data of *E. coli* and *S. cerevisiae*, they have been engineered for the production of plant isoprenoids. In addition, both *E. coli and S. cerevisiae* demonstrated as prominent hosts or "Cell factory" for the creation of isoprenoids and other (plant) natural products (Marienhagen and Bott, 2013).

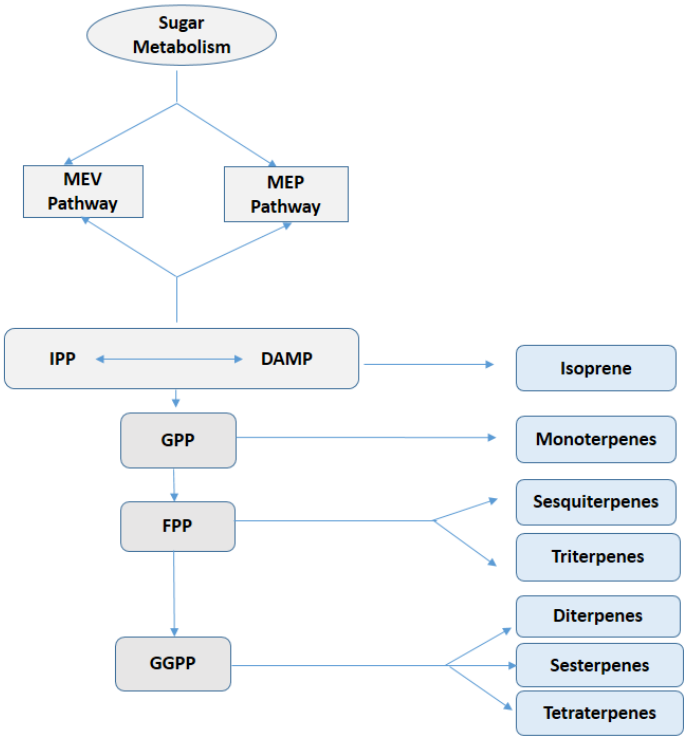


Figure 2.6 Graphical representation of isoprenoid biosynthesis from isopentenyl-pyrophosphate (IPP) and dimethylallyl-pyrophosphate (DMAPP) to important precursors of various isoprenoids. Abbreviations: MEV, Mevalonate pathway; MEP, 2Cmethyl-d-erythritol-4-phosphate; FPP, farnesyl-pyrophosphate; GPP, geranyl-pyrophosphate; GGPP

2.5.1 Metabolic engineering interventions for improved production of isoprenoids through MEV Pathway

The possible intervention in the mevalonate pathway discussed below (fig.2.7)

2.5.1.1 3-hydroxy-3-methyl-glutaryl-CoA reductase *(HMGR)*

The rate limiting step of the pathway catalysed by *HMG-CoA* reductase and regulation takes place at the levels of transcription, translation, post-translational modification and degradation which, generally occurs in two forms: *HMG1* and *HMG2.* Among the two enzymes *HMG1* is considered quite stable whereas *HMG2* undergoes mevalonate products-induced degradation. Truncated and the soluble tHmg1p catalytic domain overexpression causing accumulation of a large amount of squalene (Hampton and Garza 2009). Overexpression of *tHMG1* was applied to improve the amorphadiene and artemisinic acid production in yeast (Ro et al., 2006; Baadhe et al., 2013b). Construction of a point mutation by substituting Lysine 6 by an Arginine (K6R) in *HMG2* signified it's resistant towards ubiquitination (Kampranis and Makris, 2012).

2.5.1.2 Squalene synthase *(ERG9)*

Squalene synthase *(ERG9)* catalyses the first committed step for synthesis of sterols by converting two FPP into squalene. Since majority of FPP involved in the synthesis of sterols, limited substrate available for isoprenoid synthesis, hence suppression of *ERG9* one way to increase the FPP availability, nevertheless complete deletion *ERG9* affects the growth of the cell since sterols involved in the maintenance of cell membrane integrity. Replacement of native promoter *(ERG9)* with repressible promoter *(MET3)* with varying concentrations of methionine repressed the transcription of *ERG9* (Gardner and Hampton, 1999). This approach in combination with over production *tHMG1* of improved the production of major sesquiterpenoids like amorphadiene, cubebol, valencene and patchoulol in various studies (Ro et al., 2006; Asadollahi et al., 2008; Baadhe et al., 2013a, Baadhe et al., 2013b). But in these studies accumulation of farnesol as by-product, was observed, thus indicates destitute regulation of the promoter.

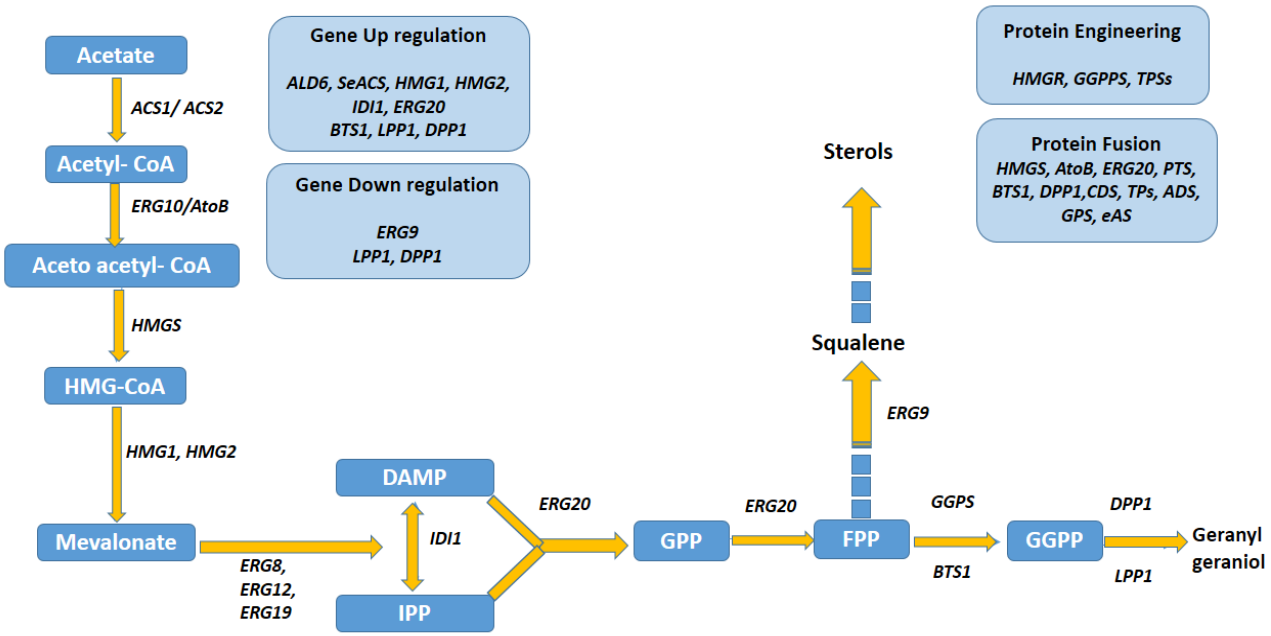


Figure 2.7. Outline of terpene biosynthesis in yeast indicating the genes involved and the metabolic engineering contributions. (CPP, copalyl diphosphate; CDS, copalyl diphosphate synthase; DTS, diterpene synthase; SeACS(L641P), *Salmonella enterica* acetyl-CoA synthase mutant L641P; AtoB, acetoacetyl-CoA synthase/thiolase; PTS, patchoulol synthase; eAS, epi-aristolochene synthase).

Consequently to provide a tighter control of *ERG9* expression, the glucose regulated promoters *PHTX1* and *PHTX2* were tested and PHTX1 was shown to be efficient in down regulating *ERG9* expression under glucose limiting conditions. In addition, regulating the transcript stability and heterozygous deletion of the gene in a diploid strain increased the monoterpenes and sesquiterpenes (Ignea et al., 2011).

2.5.1.3 Farnesyl diphosphate synthase *(ERG20)*

The *ERG20* enzyme catalyses the formation of GPP and later FPP, by the condensation of IPP and DMAP. The overexpression of the *ERG20* not improved the amorphadiene production (Ro et al., 2006). But under control strong promoter (P_{GAL1}) it improved the production of some terpenoids (Ignea et al., 2011). Mutations in the lysine 197 of *ERG20* (K197E) increased the formation of GPP and consequently, geraniol productivity by the expression of geraniol synthase, thus can be used for improved production of monoterpenes (Blanchard and Karst, 1993; Fischer et al., 2011)

2.5.1.4 Acetyl-CoA Metabolism

Apart from contribution in energy generation through TCA cycle, Acetyl-CoA also involved in the initiation of the MEV pathway (Figure 3). The pyruvate dehydrogenase bypass converts pyruvate into acetyl-CoA by the action of pyruvate decarboxylase, cytosolic acetaldehyde dehydrogenase (*ALD6)*, and acetyl-CoA synthetase *(ACS).* ThusOver production of heterologous, acetaldehyde dehydrogenase (*ALD6)* from *Salmonella enterica* (L641P) together with *tHMG1* expression attained considerable enhancements in amorphadiene production (Shiba et al., 2007). Later push-pull back approach (Chen et al., 2013) improved the Acetyl-CoA supply towards MEV pathway (fig.2.8). This strategy involved a push of carbon from ethanol via acetaldehyde to cytosolic acetyl-CoA and a block of further conversion of acetyl- CoA by competing pathways. The push part of the strategy involved over-expression of the endogenous *ADH2* gene, encoding alcohol dehydrogenase, by using the glucose-regulatable *HXT7* promoter, as well as constitutive over-expression of *ALD6*, encoding NADP-dependent aldehyde dehydrogenase, and a codon- optimized ACS variant (L641P) from *S. enterica* (acsSE L641P), encoding acetyl-CoA synthetase. Acs $_{SE}{}^{L641P}$contains a point mutation that prevents the enzyme from being inhibited by acetylation (Starai et al., 2005).

Over-expression of *ADH2* ensures that ethanol produced byAdh1p during growth on glucose can be converted back to acetaldehyde and from here further to acetyl-CoA. To ensure pulling of acetyl-CoA towards the products of interest *ERG10* over-expressed which encodes acetyl- CoA C-acetyl transferase that catalyses the conversion of acetyl-CoA to aceto acetyl-CoA (AcAcCoA), . Finally, the block part involves the reduction precursor acetyl-CoA loss by avoiding the involvement and consumption of acetyl-CoA in glyoxylate cycle (GYC), by inhibiting the key enzymes peroxisomal citrate synthase, encoded by CIT2, and cytosolic malate synthase, encoded by MLS1.

2.5.1.5 Uptake Control *(UPC2)*

Upc2p and *Ecm22p* are two highly homologous zinc cluster proteins which regulates a number of *ERG* genes in the yeast ergosterol biosynthetic pathway and of DAN/TIR gene products. They relocates from intracellular membranes to perinuclear foci on sterol depletion; they positively regulate transcription by binding to sterol response elements in the promoters of the target genes. The *upc2-1* mutant contains a single amino acid change

(G888D) within the activation domain of the protein. Overexpression of *upc2-1* in combination with *tHMG1* and P_{MET3}-*ERG9* had a prominent effect on the synthesis of amorphadiene. (Ro et al., 2006; Peralta et al., 2011; Westfall et al., 2012)

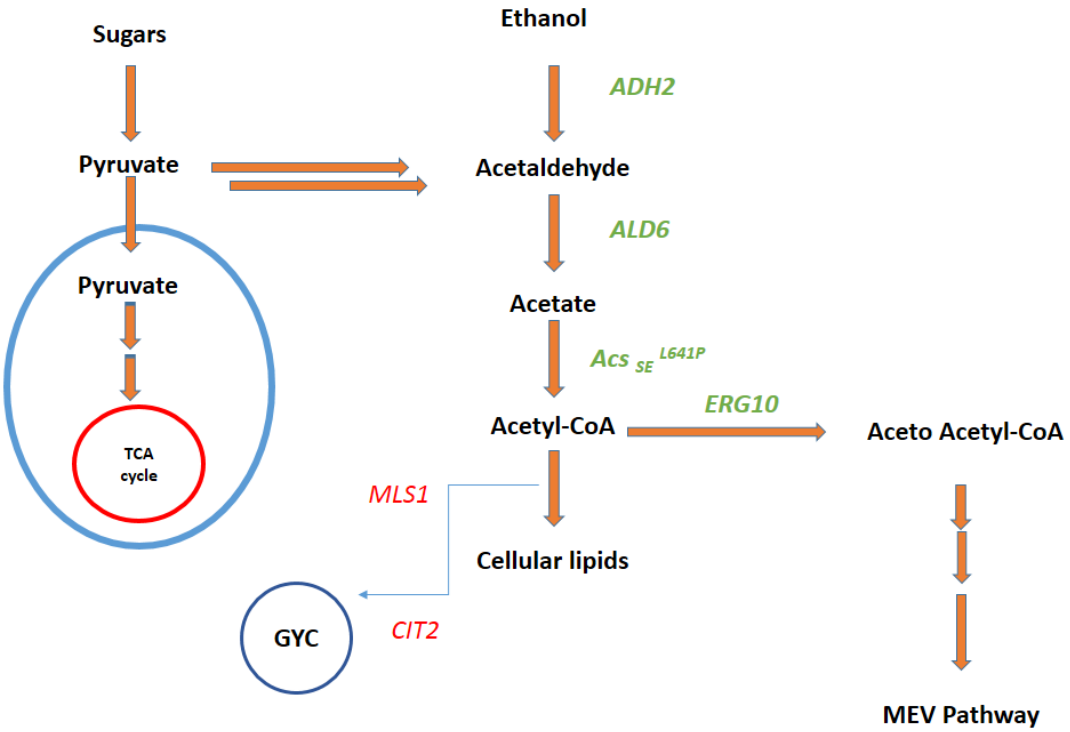


Figure 2.8. Illustration of the push-pull-block strategy for improving the production of Acetyl-CoA metabolism by acetyl-CoA in the cytosol.

2.5.1.6 Geranylgeranyl diphosphate synthase (*BTS1)*

The enzyme catalyses the synthesis of GGPP by using FPP and IPP as substrate, for ubiquinone biosynthesis and geranylgeranylation of proteins for membrane attachment. GGPP is a precursor for diterpene and carotenoid biosynthesis (Figure 1). Overexpression of *BTS1* has been combined with lycopene cyclase/phytoene synthase (*CrtYB)* and phytoene desaturase (*CrtI)* from *Xanthophyllomyces dendrorhous* to generate β-carotene and with cytochrome-P450 hydroxylase (*CrtS)* to produce astaxanthin (Verwaal et al., 2007; Ukibe et al., 2009).

2.5.1.7 Lipid phosphate phosphatase *(LPP1)* and Diacylglycerol pyrophosphatase *(DPP1)*

Down regulation of *ERG9* in mammalian species was shown to lead to conversion of FPP to farnesol which was also understood in yeast strains (Kuranda et al., 2010). It has been

hypothesised that dephosphorylation of FPP and GPP may be a mechanism to alleviate the possibly toxic effects of substrate accumulation. *LPP1* and *DPP1*, two enzymes initially recognised as phosphatidic acid hydrolases later as dephosphorylate isoprenoid phosphates (Faulkner et al., 1999). A modest increase in the production of the sesquiterpene α-santalene and a 24% drop in farnesol accumulation was observed with the deletion of *DPP1* (Scalcinati et al. 2012). However, other studies targeting at high sesquiterpene production did not perceive noteworthy improvements (Takahashi et al., 2007; Albertsen et al., 2011). Still, when *LPP1* and *DPP1* were overexpressed fused to *BTS1*, they employed a strong positive effect on geranylgeraniol production, with *DPP1* exerting the strongest effect, yielding 2.9-fold higher levels of geranylgeraniol (GGOH) than simple co-expression of the genes (Tokuhiro et al., 2009).

2.5.1.8 Protein engineering interventions for improved production of isoprenoids

Protein engineering strategies includes, the increase of product yield and to interfere with the cyclization chemistry of the terpene synthases, which improves the enzyme specificity or alter the products out of a specific enzyme. Extensive studies on *E. coli*, metabolic enzymes sequences noticed that Gly and Pro were significantly less frequently mutated than other amino acids enzymes. To know their effects on the catalytic activity, engineered version of *tHMGR (tHMGR-G9),* with nine mutated residues, revealed a 2.5- to 3-fold increase in the production of mevalonate compared to the wild-type *tHMGR.* Similarly, HUM-G6 (an engineered version of humulene synthase bearing six such substitutions) improved sesquiterpene production by 80 folds. Further Integration of the *tHMGR-G9* and *HUM-G6* mutants into the same host resulted into a three- to four-fold improvement in growth and nearly 1000-fold overall improvement in sesquiterpene production.

2.5.1.9 Metabolic channelling by expression of chimeric enzymes

Heterologous expression of pathways/enzymes in microbial hosts is complex due to the presence of numerous native host enzymes and tight regulation of the host machinery. Heterologous product formation is not only affected by the host environment, but also by the loss of intermediate metabolites through diffusion, degradation, or by competitive pathways In order to avoid such intermediates loss and make heterologous expression more efficient, enzymes catalysing successive reactions are often fused in close proximity

to each other by using linkers. Linkers are the sequence coding for few amino acids, which separates the two proteins in space with a small distance allowing them to fold properly without restraints from each other. Consequently, the substrate was channelled between active sites of two or more sequential enzymes of a pathway, without allowing free diffusion of the intermediates. Subsequently it reduces the transit time required for the intermediates to spread the enzyme that catalyses the next step in the reaction. (Baadhe et al., 2013b).

Fusions of *ERG20* to PTS (*FPPS-PTS*), and amorphadiene synthase (*ADS) (FPPS-ADS)* were tested for the production of patchoulol and unveiled higher yields than the individual enzymes. Sometimes orientation enzymes during the fusion also affect their catalysis. C-terminal fusion of EYFP to a sesquiterpene synthase (STS) caused a significant reduction in enzymatic activity. Similar effect was observed with ADS-FPPS and PTS-FPPS (Albertsen et al., 2011; Baadhe et al., 2013b). In another approach, the fusion of STS or FPPS to targeting proteins of organelle improved the isoprenoids. The Cytochrome C oxidase subunit 4 isoform 1, mitochondrial (COX4), a mitochondrial targeting sequence, diverts pathway to the organelles where FPP pools are naturally present for the synthesis of ubiquinone, heme A etc. The fusion FPPS or STS to COX4 and combined with cytosolic *tHMG1* overexpression, had a significant improvement in the sesquiterpene production (Farhi et al., 2011).

2.5.2 Metabolic engineering of MEP Pathway for Isoprenoids Production

Numerous studies have reported the engineering of the MEP pathway to increase the supply of isoprenoid precursors in *E. coli*. Balancing the pool of glyceraldehyde-3-phosphate and pyruvate, or overexpression of 1-deoxy-D-xylulose 5-phosphate synthase (DXS; encoded by the gene dxs) and IPP isomerase (encoded by idi), driven the increased carotenoid build-up in the cell. Nevertheless enhancements in isoprenoid production were noted, some limitations rose by the native host by controlling the MEP pathway in *E. coli.* To circumvent this pathway by expressing the *S. cerevisiae* mevalonate-dependent pathway in *E. coli* abundant isoprenoid precursors observed, but that some time inhibited the growth. The synchronised expression of a synthetic ADS resulted in high-level production of amorphadiene and circumvented growth inhibition (Martin et al., 2003)

E. coli MEP pathway was reconstructed in *S. cerevisiae* by cloning major seven genes of the pathway for isoprenoids production and the pathway was shown able to ensure growth of yeast, under inhibition of it native MEV pathway. The resulting strain harbouring valencene synthase produced up to 1 mg/L of valencene but it is very less compared to other reported studies; however optimization of expression of the MEP pathway in yeast may improve the isoprenoids production (Maury et al., 2008). But non-functionality of the MEP pathway in *S. cerevisiae* is revealed by the lack of the enzyme activity of IspG and/or IspH, which catalyze the last two reactions of the MEP pathway.

2.5.3 Metabolic engineering of MEP Pathway

Amongst the different enzymes in the MEP pathway, DXP synthase (encoded by *dxs*), IPP isomerase (encoded by *idi*) and DXP isomeroreductase (encoded by *dxr*) have been the main targets for the metabolic engineering investigations.

2.5.3.1DXP synthase

Overexpression of *dxs* in *E. coli* strains harboring the carotenogenic genes resulted in up to 10.8- and 3.9-fold increase in the accumulated levels of lycopene and zeaxanthin, respectively. Overproduction of DXP synthase also had a great impact on the biosynthesis of taxadiene (Huang et al., 2001) as the required intermediate for the synthesis of paclitaxel (Taxol), and lycopene in *E. coli* (Harker and Bramley, 1999). Chromosomal replacement of the native *dxs* promoter with a strong bacteriophage T5 promoter improved _-carotene production 2- to 3.3-fold in *E. coli* (Yuan et al., 2006). The strength of promoter and plasmid copy number are important in balancing expression of *dxs* with overall metabolism (Kim and Keasling, 2001).

2.5.3.2 DXP isomeroreductase

DXP isomeroreductase, has been shown to control the flux to isoprenoid precursors in *E. coli* (Kim and Keasling, 2001; Albrecht et al., 1999). Co-overexpression of *dxr* and *dxs* was concomitant with a 1.4- to 2-fold increase in lycopene level compared to the strains overexpressing only *dxs* (Kim and Keasling, 2001). However, overexpression of *dxs* had a greater impact on lycopene production than overexpression of *dxr*. In another study (Albrecht et al., 1999), simultaneous overexpression of *dxs* and *dxr* in the ß-carotene and zeaxanthin producing *E. coli* strains were lethal for the cells, probably due to restricted

storage capacity for lipophilic carotenoids, which causes membrane overload and loss of its functionality. This problem implies the need for host microorganisms with higher storage capacity for heterologous production of carotenoids (Sandmann et al., 1999; Sandmann, 2001). However, in contrast to the previous study (Albrecht et al., 1999) that showed 1.6 mg/g DW as the maximum membrane capacity of *E. coli* for ß-carotene and zeaxanthin storage, Yoon et al., (2006) did not observe any toxicity effect in the *E. coli* strains accumulating 49.3 mg ß-carotene/g DW.

2.5.3.3 IPP isomerase

Isomerization of IPP to DMAPP has been another target for improving isoprenoid biosynthesis in the MEP pathway, and several studies have shown the enhancing effect of IPP isomerase overproduction (Kajiwara et al., 1997; Wang et al., 1999; Huang et al., 2001; Reiling et al., 2004; Martin et al., 2003; Albrecht et al., 1999; Yuan et al., 2006). Overexpression of *idi* genes from different organisms in the recombinant *E. coli* showed 1.5- to 4.5-fold increase in the lycopene, ß-carotene, and phytoene levels compared to the control strains (Kajiwara et al., 1997). Positive effects of *idi* or *dxs* overexpression on ß-carotene and zeaxanthin accumulation in *E. coli* have also been shown. Amplification of *idi* or/and *dxs* resulted in approximately 2- to 3.5-fold more carotenoid accumulation in the recombinant strains than the control (Albrecht et al., 1999; Yuan et al., 2006). Engineered lycopene producing *E. coli* overexpressing *dxs, idi,* and *ispA* (responsible for FPP synthase activity in *E. coli*) produced 6-fold more lycopene than the control strain (Reiling et al., 2004). Simultaneous amplification of *idi* and GGPP synthase gene (*gps*) in astaxanthin producing *E. coli* strains increased the astaxanthin level from 33 µg/g dry weight in the control strain to 1419 µg/g dry weight in the recombinant strain (Wang et al., 1999). In the same laboratory, subjecting *gps* gene to direct evolution resulted in 2-fold increase in the lycopene level and subsequent co-overexpression of *dxs* gene further enhanced the lycopene accumulation (Wang et al., 2000).

2.5.3.4 Pyruvate and glyceraldehyde -3 -phosphate (GAP)

The MEP pathway is initiated with combining pyruvate and glyceraldehyde -3 -phosphate (GAP) in equal amounts catalyzed by DXP synthase. Hence, balanced pools of pyruvate and GAP would be an important factor in efficient directing of central carbon metabolism to the isoprenoid pathway. Pyruvate is required as a precursor in many cellular pathways

and presumably it is more available than GAP for isoprenoid biosynthesis. It was shown that overproduction or inactivation of enzymes that leads to redirection of flux from pyruvate to GAP results in higher lycopene production in *E. coli* (Farmer and Liao, 2001). Thus, overproduction of phosphoenolpyruvate (PEP) synthase (Pps) and PEP carboxy kinase (Pck) or inactivation of pyruvate kinase isozymes (Pyk-I and Pyk-II) were shown to enhance lycopene production in *E. coli*.

2.5.3.5 Central carbon metabolism

Effect of manipulating central carbon metabolism on lycopene production in *E. coli* by inactivating the competing pathways at acetyl-CoA and pyruvate nodes has been studied (Vadali et al., 2005). Although strong competition exists for these two substrates which are central metabolites involved in several pathways, such as the tri carboxylic acid cycle, glycolysis and gluconeogenesis and the pentose phosphate pathway, they have shown that lycopene accumulation is controlled by the G3P/pyruvate ratio and not by substrate availability (Farmer and Liao, 2001).

In brief the contemporary developments indicate the great ability for the production of isoprenoid compounds in either *E. coli* or *S. cerevisiae* host platforms. Though many attempt made for increasing flux towards isoprenoid precursors by overexpressing, up regulating, and down regulating the endogenous genes. Still much effort continuing for improving the intermediate fluxes. Some successful attempt made, still the thrust driving towards improving the acetyl- CoA precursor flux. With the application and combination of the latest tools and strategies in systems biology, synthetic biology, protein engineering, process engineering, high-throughput technologies for cultivation, screening and analysis, metabolic engineering of microorganisms for isoprenoids, significantly enlarge the number as well as commercialization of isoprenoids.

2.5.4 Modelling and simulation approaches

Advancements in analytical technologies for analysis of omic's data and modelling tools enabled the construction of metabolic models of the host organisms. Minimization of metabolic adjustments algorithm (MOMA) recognised *GDH1* as a conceivable target which could swing the metabolic flux towards the ergosterol pathway. The gene encodes a glutamate dehydrogenase involved in ammonium metabolism in yeast and requires NADPH for its function. The conversion of *HMG-CoA* to mevalonate is an NADPH

requiring step, thus deletion of *GDH1* was assumed to be beneficial for carbon flux through the mevalonate pathway by increasing the pool of available NADPH for *HMGR*. In yeast there are two other glutamate dehydrogenase enzymes encoded by *GDH2* and *GDH3*. *GDH3* appears to have arisen from genome duplication of *GDH1*, while *GDH2*, unlike the other two, is an NADH-dependent enzyme. Deletion of *GDH1* in cells expressing cubebol synthase led to approximately 85% increase in the final titer (Asadollahi et al., 2009).

Redox cofactors NAD (H) and NADP (H) are essential for the transfer of reducing equivalents in many enzymatic reactions and availability of these intracellular pools very important for metabolic engineering. Though, these cofactors species activities and specificities manipulated by using genetic, metabolic and protein engineering techniques. Identifying a potential target among the hundreds of reactions is tedious. A novel cofactor modification analysis (CMA) within the constraints-based flux analysis framework was a good approach to identify the target species. Analysis of isoprenoid synthesise pathway revealed that the IPP yield can be improved by either by decreasing NADPH consumption or by increasing NADPH regeneration through modification of substrate specificity either from NADPH to NADH or from NAD to NADP, respectively. Between the enzyme targets identified by CMA, the alteration of cofactor specificity of glyceraldehyde-3- phosphate dehydrogenase from NAD to NADP was found to cause the best IPP yield improvement in both *E. coli* and *S. cerevisiae* (Chung et al., 2013).

OBJECTIVES

OBJECTIVES OF THE PRESENT STUDY

Malaria is a most overwhelming human disease in the tropical areas, which threatens 300-500 million people and kills approximately two million people annually and ranked in the top three of communicable diseases it terms of deaths. These deaths are primarily among the children and pregnant women. It is estimated that a child dies from malaria every 40 seconds, resulting in the loss of thousands of adolescents every day. The disease occurs due to the mosquito-mediated infection of erythrocytes mainly by the malaria parasite *Plasmodium falciparum*. Presently major classes of anti-malarial are quinolines have efficiently controlled malaria for decades, multi-drug resistant (MDR) malarial strains have appeared in recent years which have led to a substantial increase of mortality in malaria patients.

Fortunately, Artemisinin Combinational Therapies (ACTs), recommended by the World Health Organization (WHO) was proved to be successful in preventing the infection and transmission of MDR malaria. However, ACTs are not always available for every malaria victim in the endemic regions because naturally artemisinin is produced in *Artemisia annua L*. (sweet wormwood), which will give low yield and time consuming process. Traditional chemical synthesis procedures were also not economical due to their complex structure. The contemporary production process involves, consumption of organic solvents for extraction artemisinin from plant sources, which again connected with environmental issues. All the above consequence limiting the accessibility of artemisinin to the endemic areas. Metabolic Engineering of microbial pathways for production of artemisinic acid, a precursor molecule for the artemisinin, could be a cost-effective, environmentally friendly, high-quality and reliable source of artemisinin. For this purpose, practical approaches have been developed to transplant plant biosynthetic genes into genetically tractable microbial hosts. Many groups engineered Mevalonate pathway for artemisinic acid. From the literature, it is clear that considerable efforts made for production of artemisinic acid in engineered microbes. However, *A. annua* cytochrome P450 monooxygenase (*CYP71AV1*) instable and resulting lower yields of artemisinic acid, subsequently artemisinic acid led to the induction of pleiotropic drug resistance genes in yeast, which impeding the bulk production of artemisinic acid. In this study an attempt made to improve the production of amorphadiene, the immediate precursor of artemisinic acid in *S. cerevisiae*.

In the present study we developed a yeast strain capable of producing amorphadiene at better levels with the combination of metabolic engineering and enzyme fusion technology, which comprise following specific objectives

Specific Objectives:

1. Replacement of *ERG9* promoter with *MET3* promoter for down regulation of squalene/ergosterol synthesis in *S. cerevisiae* MTCC 3157.
2. Overexpression of *tHMG1* for enhanced production of AD in *S. cerevisiae*.
3. Combination of metabolic engineering and enzyme fusion technology for improved production of AD in *S. cerevisiae*.
4. Optimization of process conditions for enhanced production of AD by Response Surface Methodology.
5. Development of *in silico* model for improved production of FFP.

CHAPTER 3

Effect of *SQS* Repression and *tHMG1* over Expression on Amorphadiene synthesis in *S. Cerevisiae*

CHAPTER 3

EFFECT OF *SQS* REPRESSION AND *tHMG1* OVER EXPRESSION ON AMORPHADIENE SYNTHESIS IN *S. cerevisiae*

3.1 Introduction

Plants are natural producers of diverse, complex metabolites including isoprenoids, alkaloids, phenyl propanoids and all allied phenylic compounds (Croteau et al., 2000). Isoprenoids are the largest group of compounds with diverse structures (Withers and Keasling, 2007). Sesquiterpenes are the most diverse class of isoprenoids consisting of 15 carbon units, which are derived from three isoprenoid (C_5) units and distributed mainly in higher plants as a constituents of essential oils as volatile compounds. More than 7000 natural compounds are characterized (Connolly and Hill, 1991) which can act as pheromones, juvenile hormones, phytoalexins and antifeedants etc. in plants (Harborne, 1991). Based on these superior characteristics, sesquiterpenes have prominence in chemical, pharmaceutical, fragrances and biofuel industries (Breitmaier 2006; Peralta-Yahya et al. 2012). Industrial production of sesquiterpenes through plants is not economical due difficulties in extraction, low growth, varying composition and concentration depending on the topographical location and environment circumstances. Therefore microbial hosts is best possible alternative for commercial terpenoids production (Chang and Keasling 2006). All sesquiterpenes are derived from intermediate compound FDP/FPP (farnesyl di (or) pyro phosphate of mevalonate (MEV) pathway. But the intracellular levels of this intermediates not adequate for high level production. Thus deregulation of the biosynthetic isoprenoid pathway improves the isoprenoid production (Martin et al., 2001; Martin et al., 2003; Ro et al., 2006; Takahashi et al., 2007). Several studies used *E.coli* and yeast as microbial host for metabolic engineering of isoprenoid production (Alper et al., 2005; Carter et al., 2003; Martin et al., 2001, DeJong et al., 2006; Jackson et al., 2003; Miura et al., 1998a; Miura et al., 1998b; Shimada et al., 1998; Verwaal et al., 2007; Takahashi et al., 2007; Ro et al., 2006). The vital role of MEV pathway and eventual product (ergosterol) proportion from this pathway increased the interest for the engineering of isoprenoid pathway in yeast for the production of heterologous isoprenoids (Lamacka and

Sajbidor, 1997). This distinctive ability of yeast enabled to divert the isoprenoids precursors towards the production of heterologous compounds. In yeast, for ergosterol production, FPP is converted to squalene by squalene synthase (*SQS*), which is encoded by the *ERG9* gene located on Chromosome VIII. Escalation of FPP levels and diversion of the FPP flux towards heterologous compounds may be possible by limiting the *SQS* synthesis. But the essence of ergosterol for yeast growth and inability of external ergosterol assimilation by yeast during aerobic growth circumvented the deletion of the *ERG9* gene. Regulation of the mevalonate pathway in eukaryotes is very complex, but *HMG-CoA* reductase and FPP branch-point represent two key regulation sites in the pathway (Maury et al., 2005). *HMG-CoA* reductase, which catalyzes the conversion of *HMG-CoA* to mevalonate, is a highly regulated enzyme that is generally considered to represent the main flux controlling step in the pathway. *S. cerevisiae* possesses two isozymes of *HMG-CoA* reductase, Hmg1p and Hmg2p, encoded by the *HMG1* and *HMG2* genes, respectively. At aerobic conditions Hmg1p has the highest activity (Hampton et al., 1996). Both the yeast isozymes have a C-terminal catalytic domain and an N-terminal domain which consists of several transmembrane spans to anchor the protein in the endoplasmic reticulum (Hampton and Rine, 1994). Several studies have shown that overexpression of the region of gene encoding the catalytic domain of Hmg1p leads to enhanced isoprenoid production in yeast (Jackson et al., 2003; Ro et al., 2006; Verwaal et al., 2007). The FPP branch-point represents another regulatory site in the mevalonate pathway where several enzymes compete for FPP as substrate. However, most of the FPP is required for the sterol biosynthesis and hence minimizing the flux towards sterols would provide more FPP for the other enzymes of the branch-point including sesquiterpene synthases.

In the present study *S. cerevisiae* MTCC 3157 was used for the production of amorphadiene (AD) and *ERG9* expression was down regulated by replacing the native *ERG9* promoter with repressible *MET3* promoter (Mountain et al., 1991; Asadollahi et al., 2008). The mevalonate pathway yeast strain was deregulated by overexpression of the truncated *HMG1* (*tHMG1*) both from the genome and using plasmids. The overexpression of *tHMG1* was combined with the down-regulation of *ERG9* in order to further improve AD production. The constructed yeast strains were characterized in batch fermenters and the effect of the modifications were studied on AD production as well as accumulation of ergosterol and squalene.

3.2 Materials and methods

3.2.1. Strains, media and reagents

All the reagents and media used in this study are analytical grade and procured either from Himedia (India) or Merck (India) or Sigma (India). Microbial strains used in this study, *Saccharomysces cerevisiae* MTCC 3157 and *E.coli* DH5α MTCC 1652 procured from microbial type culture collection and gene bank (MTTC), Chandigarh, India.

3.2.2. *ERG9* promoter replacement with *MET3* promoter

The *MET3* promoter was amplified from genomic DNA of *S. cerevisiae* (MTCC 3157) using the primer pair 1 and 2 (Refer table. 1) containing restriction sites BcuI and Cfr42I. PCR was carried out in a total volume of 50 µL with the following reagents: 1x Ex Taq DNA Polymerase buffer (Stratagene), 0.2 mM dNTPs (Pharmacia), 20 pmol of each primer (appendix section) and 1U Ex Taq DNA Polymerase (Takara, Japan). This PCR includes: two cycles of 94 ^{o}C (0.5 min), 50 ^{o}C (1.0 min), 72 ^{o}C (1.5 min); 29 cycles of 94 ^{o}C (0.5 min), 56 ^{o}C (1 min), 72 ^{o}C (1.5 min); 72 ^{o}C (5 min). Resultant PCR fragment and pUG6 plasmid (Euroscarf, Germany) are digested with BcuI-Cfr42I restriction enzymes. The vector and PCR fragments were purified with NucleoSpin® gel and PCR clean-up kit (Macherey-Nagel, Germany) and separated on 1% agarose gel (Merck Biosciences, India) and gel was further purified using NucleoSpin® gel and PCR clean-up kit (Macherey-Nagel, Germany). Ligation of the vector and PCR products carried as per the standard protocol given for T_4 DNA ligase (Merck Biosciences, India). The ligated plasmid mix was transformed into competent *E. coli* (DH5α) MTCC 1652 and transformants were selected on LB medium supplemented with ampicillin (50mg/L) and the plasmid obtained was named as pUG6MET3. *ERG9* promoter was replaced with methionine promoter by using fusion PCR, briefly four fragments were separately amplified before fusing them together in pairs by using fusion PCR and a bipartite gene targeting method (fig.3.1) (Erdeni et al., 1997; Asadollahi, et al., 2008). Two fragments containing the *MET3* promoter and the KanMX selection cassette were amplified from plasmid pUG6MET3 in two separate, but overlapping, fragments using the two pairs of primers 3a, b and 4 a, b (table.1.). Also 500bp upstream region of the *ERG9* promoter in the genome of *S. cerevisiae* were amplified using primers 5 and 6. Subsequently the first 500bp of the *ERG9* open reading frame (ORF) region was amplified using primer pairs 7 and 8 (table 3.1). Finally PCR fragments

were gel purified using the NucleoSpin® gel and PCR clean-up kit (Macherey-Nagel, Germany) and subsequently fused together in pairs using fusion PCR to obtain two fragments, first one with *MET3* promoter and 500bp ORF region of *ERG9* using primers 9 and 10 and a second fragments containing *KanMX* and first 500bp upstream region of ERG9 using primers 11and 12 (table 3.1). Subsequently fusion PCR fragments were gel purified with the NucleoSpin® gel and PCR cleanup kit (Macherey-Nagel, Germany).

List of primers
1. GGGACTAGTGTTTAATTTAGTACTAACAGAGACTT
2. CCCCCGCGGCATGTTAATTATACTTTATTCTTGTT
3. a)GATCCCCGGGAATTGCCATG/3b)ACGCTGCAGGTCGACAACCC
4. a)CCATGAGTGACGACTGAATCCGG/ b)CTATCGATTGTATGGGAAGCCCG
5. CTATCGATTGTATGGGAAGCCCG
6. CAATGCCAATTGTAATAGCTTCCCAT
7. GTTAATTATACTTTATTCTTGTTATTATTATAC
8. AGCCTCAGTACGCTGGTACCCG
9. CATGGCAATTCCCGGGGATCTGGGCTATGAAATGTACTGAGTCAG
10. ATGGGAAAGCTATTACAATTGGCATTG
11. GTCGTA-GTCGTGGACGGTTTGC
12. AGCCTCAGTACGCTGGTACCCG

Table. 3.1 List of the primers used in the promoter replacement

3.2.3. Integration of *tHMG1* into genome of yeast

For genomic integration of *tHMG1* under control of *TPI1* promoter a plasmid bearing a truncated version of *HMG1*, associated with a selective marker, *Kl LEU2*, was constructed. *tHMG1* was PCR amplified from pBAD18HMGR (#Addgene 19041) MTCC 3157 genomic DNA using primers containing overhangs with *Eco*RI restriction site on one side (5'accggaattcACT***ATG***GACCAATTGGTGAAAACTG3') and *Nhe*I restriction site on the other side (5'tctagcttagcCACATGGTGCTGTTGTGCTT3'). After digestion with *Eco*RI and *Nhe*I, the PCR fragments were ligated into pYX212 plasmid, in between the two restriction sites *Eco*RI and *Nhe*I. *Kl LEU2* was further cloned into pYX212 bearing *tHMG1* at the *Nhe*I restriction site. *Kl LEU2* flanked by two direct repeats was first PCR amplified from the plasmid pUG73 (Hegemann and Heick, 2011) using the couple of primers OL5' (5' CAGCTGAAGCTTCGTACGC 3' and

OL3’ (5’ GCATAGGCCACTAGTGGATCTG 3’. After digestion with *Nhe*I, *Kl LEU2* flanked by the two direct repeats was ligated into the *Nhe*I restriction site of pYX212 bearing *tHMG1*. This plasmid was named pTTU. In order to integrate the *TPIp-tHMG1-Kl LEU2* construct into the *LEU2* locus, fusion PCR and the bipartite gene targeting method (Erdeniz et al., 1997) was used. *TPIp-tHMG1-Kl LEU2* was amplified from the plasmid pTTU in two separate, but overlapping, fragments using the primers listed in table 3.2. Furthermore, 500 bp upstream and downstream of *LEU2* on the genome of *S. cerevisiae* were amplified. The four resulting PCR fragments were gel-purified and fused together in pairs by fusion PCR. The primers *LEU2*sl500 and 3’int KL *LEU2* were used for fusion of the “left fragment”; primers 5’int KL *LEU2* and *LEU2*3rr500 were used for fusion of the “right fragment”. The resulting two fusion PCR fragments were purified after gel electrophoresis.

Primer	Sequence
LEU2-tHMG1s	atgtcgaaagctacatataaggaacgtgctgctactcatcctagtcctgtGAAAAGT GCCACCTGACGTC
LEU2-tHMG1r t	ttgctggccgcatcttctcaaatatgcttcccagcctgcttttctgtaacgTGTAAAAC GACGGCCAGTGAG
5’int KL *LEU2*	CTTGACGTTCGTTCGACTGATGAGC
3’int KL *LEU2*	3’int KL URA3 GAGCAATGAACCCAATAACGAAATC
*LEU2*3sl500	AAACGACGTTGAAATTGAGGCTACTGCG
LEU2 rl500	GGACTAGGATGAGTAGCAGCACGTTCC
*LEU2*3 sr500	GGGAAGCATATTTGAGAAGATGCGGC
LEU2 rr500	URA3rr500 GGAAACGCTGCCCTACACGTTCGC

Table. 3.2 List of the primers used in the *tHMG1*genome integration

3.2.4. Plasmid construction

For construction of plasmid pESC-URA-ADS, first *ADS* sequence was amplified form pRS425ADS (#20119, Addgene, USA) (Ro et al., 2006) using primer pair 13 and 14 and cleaved with BamHI and SrfI restriction enzymes and inserted in to pESC-URA plasmid digested with same restriction enzymes. Ligation of the vector and PCR products carried as per the standard protocol given for T_4 DNA ligase (Merck Biosciences, India).

The plasmid p101 was constructed by cloning HMGR from pBAD18HMGR in to pESC-URA-ADS using primers pair 5'TTAATGCAGGTGACGGACCC3' and 5'GTGGTAAGAGTGTCGTCGCA3' vector under control of the *GAL1* promoter using *BamH*I and *Xho*I restriction sites.

3.2.5. Strain construction

Strain YCF-ADS was constructed by the transformation of pESC-URA-ADS plasmid in to MTCC 3157. Strain YCF-002 was generated by transforming the fusion PCR fragments in to strain YCF-ADS and selected on Kanamycin and synthetic defined (SD)-URA drop out plates. Finally strain YCF-001 obtained by exclusion of plasmid pESC-URA-ADS from strain YCF-002 by selection on plates containing 5-fluoroorotic acid (5-FOA). YCF-00b was generated by transforming the fusion PCR fragments (section 3.2.3) in to MTCC 3157. Transformants were selected on SC-LEU medium after incubation at 30 °C for 2-4 days. Several transformants were selected and their genomic DNA was isolated. Correct integrants were identified by PCR using both external primers (*LEU2* sl500 and *LEU2* rr500) and a combination of external and internal primers (5'int KL *LEU2* and *LEU2* rr500). Strain YCF-2 and YCF-3 generated by transforming the p101 into YCF-00b and YCF-001 (table 3.3) and selected on corresponding drop-out media.

Strain	Genotype	Plasmid	Plasmid description
MTCC 3157	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	None	
YCF-ADS	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pRS425 ADS*	pESC-URA-ADS	pESC-URA 2μ *URA3* P_{GAL1}- ADS
YCF-001	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0erg9:: PMET3-ERG9*	None	
YCF-002	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0erg9:: PMET3-ERG9*	pESC-URA-ADS	pESC-URA 2μ *URA3* P_{GAL1}- ADS
YCF-00b	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ ::PTPI1-tHMG1*	None	
YCF-2	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ ::PTPI1-tHMG1*	p101	pESC-URA 2μ *URA3* P_{GAL1}- ADS, 18HMGR
YCF-3	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0erg9:: PMET3-ERG9 ::PTPI1-tHMG1*	p101	pESC-URA 2μ *URA3* P_{GAL1}- ADS, 18HMGR

Table. 3.3 Strains and plasmids used in this study

3.2.6. Media for shake flasks

Hundred milliliter medium was prepared with the following composition (g/L): Galactose, 20; KH_2PO_4, 14.4; $(NH_4)_2\ SO_4$, 7.5; $MgSO_4\ 7H_2O$, 0.5; trace metal solution, 2; vitamin solution, 1 mL and 50 µl/L silicone anti-foam. The pH was adjusted to 6.20 using 1 M NaOH and autoclaved separately from the carbon source solution. Vitamin solution was filter sterilized and aseptically added to the medium after autoclaving. Varied concentrations of methionine (0-3 mM) used to know the minimal methionine concentration for the repression of *ERG9* expression. Flasks were further incubated in a shaking incubator (Remi, India) at 30 ºC with 150 rpm.

3.2.7. Batch fermentation

Batch fermentation were carried out in a controlled bioreactor (Spectrochem, India) containing 2 L mineral medium consists of (g/L): Glucose (or) Galactose 20; $(NH_4)2\ SO_4$, 5; KH_2PO_4, 3; $MgSO_47H_2O$, 0.5; EDTA, 0.015; $ZnSO_4.7H_2O$, 0.0045; $CoC1_2.6H_2O$, 0.0003; $MnC1_2\ 4H_2O$, 0.001; $CuSO_4\ 5H_2O$, 0.0003; $CaC1_2.2H_2O$, 0.0000045; $FeSO_4.7H_2O$, 0.0003; $NaMoO_4$,.$2H_2O$, 0.0004; H_3BO_3, 0.001; KI, 0.0001; and 0.025 mL silicone anti-foam (Merck). This medium further autoclaved at 121 ºC for 20 min. Then filter sterilized vitamin solution containing (mg/L): biotin, 0.05; calcium pantothenate, 1; nicotinic acid, 1; inositol, 25; thiamine HCl, 1; pyridoxine HCl, 1; and para-amino benzoic acid, 0.2 was added to the media. Finally media were supplemented with 2 mM filter sterilized methionine. During the fermentation process the temperature was kept constant at 30 ±2 ºC and dissolved oxygen tension (50%) was maintained with sterilized air (0.2 µ filter) with airflow 1 L/min , and with 250 rpm agitation. The off-gas passed through an outlet port. pH was controlled between 6.20 ± 0.5 by automatic addition of 1 M NaOH and 1 M HCl. Seed culture with OD_{600} of 0.02 from shake flask inoculated in to batch fermentor. After cells reaching 1 at OD_{600} 20% (vol/vol) isopropyl myristate (Merck Millipore, Germany) was added aseptically to the media.

3.2.8. OD measurement and dry weight analysis

All optical density values of samples in triplicates were measured at 600nm (OD_{600}) by using UV-Spectrophotometer (Thermo Scientific, USA). Cultures were adjusted by dilution to get the OD_{600} as <1.0 (Lamacka and Sajbidor, 1997). The dry weight was analyzed by using

nitrocellulose filter papers (pore size 0.45 µm, Whatman). The filters were pre-dried in a microwave oven at 60 ^{o}C for 10 min. A known volume of the cell culture was filtered and the residue was washed with distilled water and dried on the filter for 60 ^{o}C in a microwave oven at 60 ^{o}C (Dynesen et al., 1998).

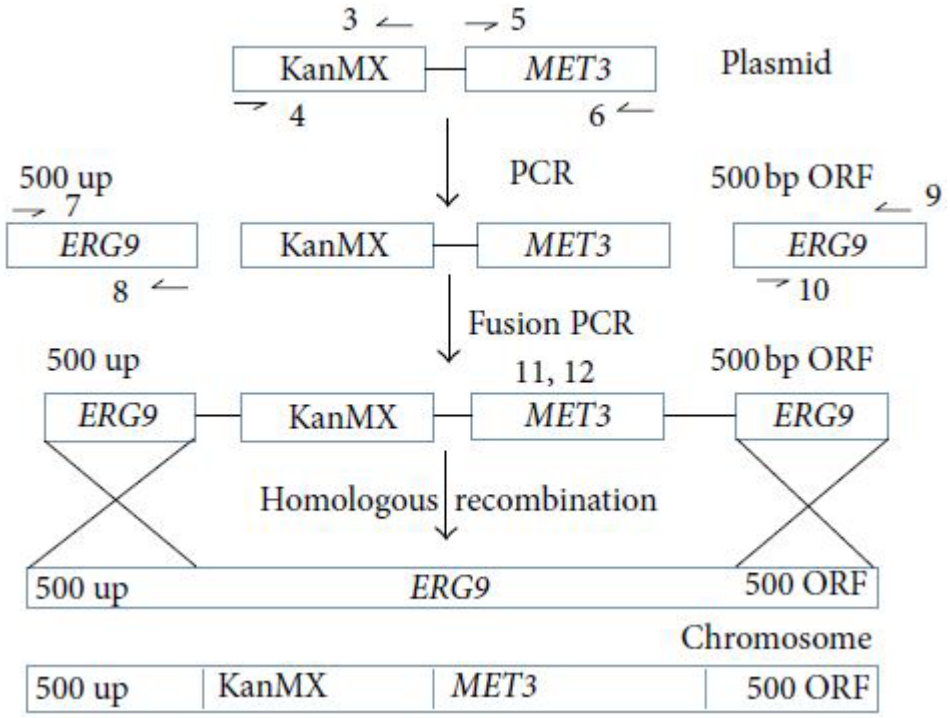


Figure 3.1 Promoter replacement of *ERG9* gene by using bipartite gene fusion method in *S. cerevisiae*. The KanMX3 and MET3 construct is amplified from pUG6MET3 in two separate, but overlapping, reactions using primers at the extremities with adaptamers homologous to the insertion site. 500 bp up and downstream from the insertion site are amplified as well. After a second round of PCR where the four resulting fragments are fused together in pairs the two fusion fragments are transformed into *S. cerevisiae*.

3.2.9. Ergosterol extraction and analysis

An overnight culture grown in minimal medium supplemented with 1.5 mM methionine (glucose 20 g/L) was centrifuged at 5,000 rpm for 5 min to get approximately 3 g of dry cells. The cell pellet was washed with distilled water and the cell suspension was centrifuged for another 5 min at 5,000 rpm. Further cell pellet was mixed with 300 mL of 25% alcoholic KOH

solution and vortexed for 1 min and the suspension was saponified for 3 h at 90 ºC in a reflux. After cooling them to room temperature, non-saponified sterols were extracted by adding 300 mL heptane followed by vortexing for 2 min. A vortex mixture of 10 mL heptane and 10 mL of alcoholic KOH solution used as blank. After clarification of heptane layer 0.5 mL of heptane from both sample and blank was diluted ten-folds with 4.5 mL absolute ethanol. The absorbance of all samples was read against blank at 230 and 281.5 nm respectively (Owades, 1957; Lamacka and Sajbidor, 1997)

The ergosterol content was calculated as milligram ergosterol per gram dry weight using the following equation:

Ergosterol = % ergosterol -%24 (28) –dehydroergosterol

$$Ergosterol(\frac{mg}{gDW}) = (\frac{OD281.5}{290} - \frac{OD230}{580})\, XF$$

Where F is a correction factor for dilutions and sample sizes and 290 and 518 are E (1%, 1cm) of crystalline ergosterol and 24 (28) -dehydroergosterol, respectively.

3.2.10. Analysis of sesquiterpenes

This isopropyl myristate layer was sampled and centrifuged at 5000 rpm for 5 min, and diluted directly into ethyl acetate and mixed for 30 min on a vortex mixer. After phase separation 0.6 mL of the ethyl acetate layer was transferred to a capped vial for analysis. The ethyl acetate-extracted samples were analyzed using the GC/FID. A 1 µL sample was split 1:20 and separated using a DB-WAX column (50 m × 200 µm × 0.2 µm) with hydrogen as the carrier gas at flow rate at 1.57 mL/min. The temperature program for the analysis was as follows: the column was initially held at 150 °C for 3.0 min, followed by a temperature gradient of 5 °C per min to a temperature of 250 °C. Amorpha- 4, 11-diene and farnesol peak areas were converted to concentration values from external standard calibrations using authentic compounds (Westfall et al., 2012)

3.2.11. Analysis of sugars and extracellular metabolites

To determine the concentration of sugars and extracellular metabolites in the culture media, 2 ml samples were withdrawn from the fermenter and immediately filtered through a 0.45 µm pore size cellulose acetate filter (Sartorius). The filtrate was stored at -20 ºC until HPLC

analysis. Glucose or galactose, concentrations were determined in a Waters 717 plus Auto sampler HPLC system equipped with a Bio-Rad Aminex HPX-87H reverse phase column (Biorad) at 60 ºC using 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 ml/min. Glucose or galactose, were detected refractometrically (Waters 410).

3.2.12. Analysis of methionine

The concentration of methionine in the media was measured by HPLC by the method of Barkholt and Jensen (1989).

3.2.13. Analysis of squalene

Samples from sterol extraction used for analyzing squalene by GC-MS. Sterols were separated on a SLB-5ms capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) using helium as carrier gas at the flow rate of 1.2 ml/min. A split/splitless injector was used in the splitless mode. The initial oven temperature was 80 ºC and injector temperature was 250 ºC. After 1 min the oven temperature was increased to 270 ºC.

3.2.14. *HMG-CoA* reductase activity

Baffled, cotton-stopped, shake flasks containing 100 ml minimal medium and 20 g/L of glucose as carbon source were inoculated with overnight precultures to an initial OD_{600} of 0.02 and incubated for 24 h in a shaking incubator at 30 ºC and 150 rpm. Fifty milliliters of culture medium was harvested by centrifuging at 5000 rpm for 10 min. The cell pellets were washed twice with 25 ml of 50 mM phosphate buffer (pH 7.0). After removing the supernatant, cells were resuspended in 300 μl of lysis buffer (100 mM phosphate buffer, pH 7.0; 1 mM Na_2EDTA, pH 8.0; 5mM DTT). The suspension was then transferred to a 2 ml FastPrep® tube and precooled glass beads (0.25-0.50 mm) were added to reach the meniscus. The cells were broken by vortexing at the speed set to 5.0 m/sec for 3×20 sec separated by periods of cooling on ice. An additional 300 μl of lysis buffer was added to assist the recovery of the extract. The extract was transferred to a new Eppendorf tube and Nonidet P-40 was added to 0.5% (w/v) and incubated on ice for 1 h. The extract was centrifuged at 10000 g for 2 min at 4 ºC. Activity assays were modified from the procedure of Quain and Haslam (1979). For the assay a reaction buffer containing 100 mM KPO4 buffer, pH 7.0, 0.5% (w/v) Nonidet P-40, 5 mM DTT was

prepared. Stock solutions of NADPH and HMG-CoA in KPO_4 buffer, 3 mM, pH 7.0, were prepared in separate tubes. Assays were done in 1 ml (1 cm path length) quartz cuvettes by adding reaction buffer and 150 μM NADPH. The cuvette temperature was kept constant at 30 ºC by circulating water. The cuvette was put in the place at 30 ºC for 10 min to equilibrate. Then different amounts of cell extract (50, 100, and 150 μl) were added to the cuvette. After 5 min of incubation at 30 °C to stabilize the endogenous oxidation of NADPH, 150 μM HMG-CoA was added to the cuvette as substrate. The initial change in absorbance at 340 nm due to oxidation of NADPH was recorded. The protein content of cell extract was determined using the Lowry method (Lowry et al., 1951) using BSA as standard.

3.3. Results and Discussion

3.3.1. Effect of methionine concentration on *ERG9* repression

The minimum concentration of methionine for expression of *ERG9* was determined by growing the *ERG9* repressed yeast cells in shake flasks supplemented with varying quantities of methionine (fig.3.2). In the absence of methionine yeast cells not resided in lag phase and there is a steep elongation in the log phase up to 10 h and there is a sudden drop at this point and a slight rise at 12 h of growth which continued up to 20 h. The methionine concentration at 1 mM elongated the lag phase and a lower final biomass concentration observed. There was a drastic decrease in the growth of the yeast beyond 2.5 mM methionine concentration and the consequences were observed by measuring the ergosterol content. The final ergosterol content of control yeast strain (MTCC 3157) observed as 19.25mg /g DW and strain YCF-002 given varied quantities of ergosterol at different methionine concentrations (0, 1, 2, 2.5 and 3 mM) as 19.25, 10.95, 4.52 and 3.75 and 2.31 mg /g DW respectively.

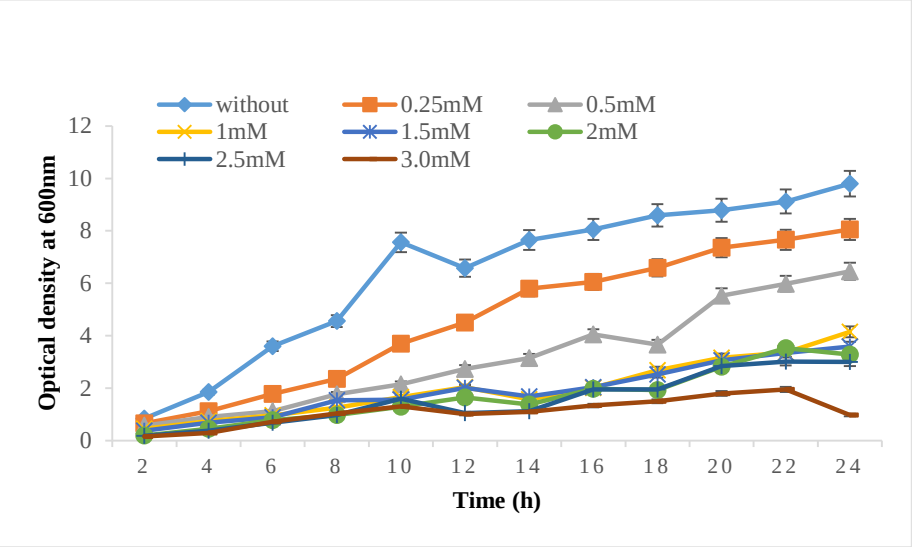


Figure 3.2 Effect of methionine on growth pattern of *ERG9* repressed yeast strain (YCF-002) cultivated in shake flasks containing minimal medium and 20 g/L glucose.

3.3.2. *ERG9* repression diverts FPP towards farnesol

Analysis of ethyl acetate layer from batch fermentation of the *ERG9* repressed strain without any amorphadiene synthase (YCF-002) revealed formation of farnesol as an FPP derived compound during fermentation. The yield of farnesol when cells were assimilating galactose as carbon source was 10.90 mg/g DW and the final titer was 12.25 mg/L. The concentration and yield of the compounds directly derived from FPP are higher when *ERG9* is repressed. Taken together with the reduced ergosterol content of the *ERG9* repressed strains, this confirms the possibility of using this strategy for enhancing FPP availability for the sesquiterpene synthases.

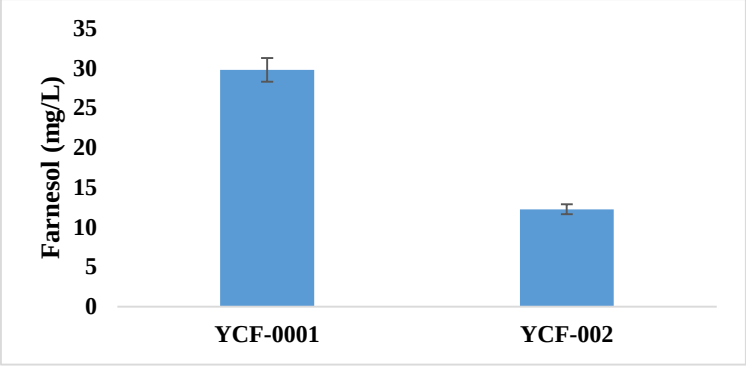


Figure 3.3 Effect of *ERG9* repression leads to the synthesis of farnesol in different yeast strains.

3.3.3. Combination of *ERG9* repression and amorphadiene synthase expression

Repression of *ERG9* gene (YCF-001) resulted in accumulation of FPP derived farnesol (fig. 3.3) and revealed that feasible amounts of FPP is available and that can be converted to AD. Further it was confirmed by combining the *ERG9* repression and amorphadiene synthase expression. It was expected to give rise to higher accumulation of amorphadiene. Strains YCF-ADS and YCF-002 were cultivated in 3 L batch fermenter and farnesol was observed as FPP derived by-product (fig.3.4). Comparison of final concentrations of amorphadiene and farnesol produced by strains YCF-ADS (control) and YCF-002 (repressed) (table 3.4) and it was observed that repression of *ERG9* improved the AD production by 2-folds but, could not effectively diverts total FPP towards AD, which was accumulated in significant amounts as farnesol and its by- products.

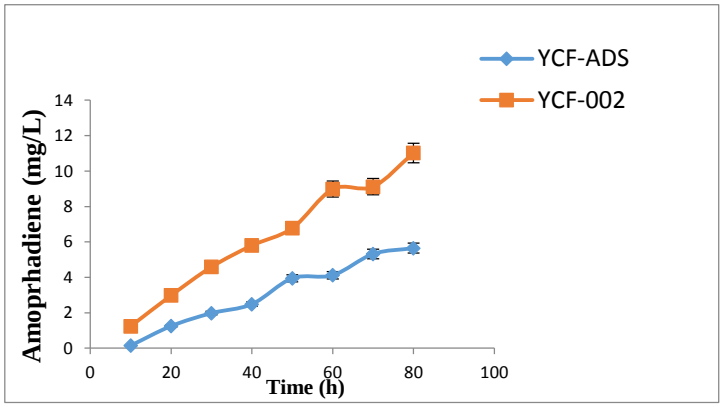


Figure 3.4 Amorphadiene concentrations as a function of time for different yeast strains

3.3.4. Regulation of methionine concentration alters the flux towards amorphadiene

ERG9 down-regulation tightly controlled the ergosterol synthesis and yeast growth. Consequently it increased the yield of amorphadiene approximately 2-folds when grown on media containing 2 mM methionine. Further to know the effect of methionine concentration on the level of amorphadiene synthesis fermentations performed with the YCF-002 strain using higher amounts of methionine (3 mM) added to the medium at different times during fermentation. Maintaining the methionine concentration at higher level during the fermentation resulted in a 1.5-fold increase in the final amorphadiene titer and a 1.64-fold increase in the final farnesol titer. Thus, the final concentrations of amorphadiene and farnesol reached to 17.1

and 20.2 mg/L, respectively. It was speculated that methionine was metabolized by the cells and the subsequent reduced levels of methionine would relieve a true repression of the *ERG9* gene and consequently lowered the accumulation rate of amorphadiene. Analyzing the methionine concentration in the culture media confirmed that almost total methionine consumed (fig.3.5).

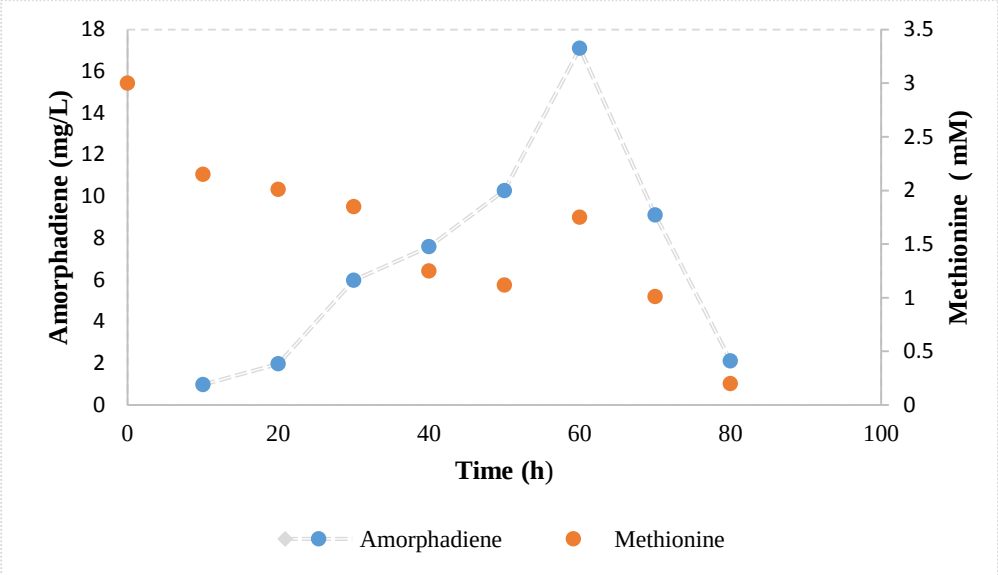


Figure 3.5 Concentrations of amorphadiene and methionine as a function of time with YCF-002 strain

3.3.5. Overexpression of *tHMG1* by integrating it into genome

Enzymatic activity of HMG-CoA reductase was assayed for MTCC 3157 and YCF-00b. Since HMG-CoA reductase consumes NADPH as a cofactor for reduction of HMG-CoA to mevalonate, the enzyme activities were tested based on the ability of cell extracts to oxidize NADPH in the presence of HMG-CoA as substrate. Overexpression of *tHMG1* increased the activity of this enzyme from 4.26 ± 0.52 in MTCC 3157 to 6.22 ± 0.52 U/ (mg protein) in the YCF-00b strain where U is defined as the nmol NADPH oxidized min^{-1}.

The effect of *tHMG1* overexpression on AD biosynthesis was examined by measuring sesquiterpenes produced by the strains YCF-00b and this strain produced approximately 5-and 2.5-fold more sesquiterpene compared to their corresponding control strain, YCF-ADS and repressed strain YCF-002, respectively (table 3.4 and fig.3.6).

Yeast Strain	AD (mg/L)	Farnesol (mg/L)	Yield of AD biomass (mg/g DW)	Yield of Farnesol on biomass (mg/g DW)	Yield of AD on galactose (mg/g galactose)
YCF-ADS	5.65		0.565		0.31
YCF-001		29.8		2.98	
YCF-002	11.02	12.25	1.37	1.53	0.61
YCF-0b	28.14				
YCF-2	47.25				
YCF-3	70.25	30.15		3.35	

Table.3.4 Final concentrations and yields of amorphadiene for the *ERG9* repressed yeast strains.

3.3.6. Overexpression of *tHMG1* from plasmid

Since single copy integration of *tHMG1* in the genome led to only 1.4-fold increase in the HMG-CoA reductase activity, which further enhance the enzyme activity by expressing the *tHMG1* gene under control of a strong promoter in a high copy number plasmid. Therefore *tHMG1* was cloned together with ADS into a plasmid under control of the strong inducible *GAL* promoters to make the plasmid p101. The strains overexpressing *tHMG*1 were characterized in fermentation for both growth and AD production (fig. 3.6 and 3.7). For all the strains overexpressing *tHMG1* resulted in a decrease in the specific growth rate (fig.3.6). This could be due to accumulation of squalene and its cytotoxic effects at high concentrations (Donald et al., 1997). Treatment of fungi with squalene epoxidase inhibitors such as terbinafine results in ergosterol depletion of fungal cell membrane and also high intracellular concentration of squalene which is believed to interfere with normal fungal membrane function and cell wall synthesis (Mukherjee et al., 2003; Liu et al., 2004). In contrast to this Donald et al. (1997), Veen et al. (2003) did not observe any adverse effect of squalene accumulation on cell growth.

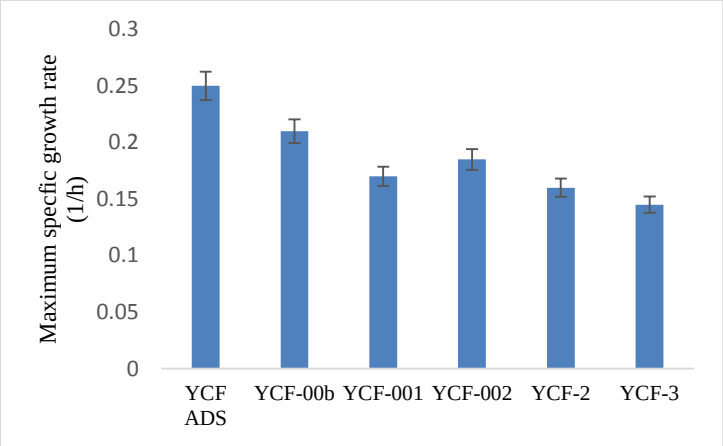


Figure 3.6 Effect of *tHMG1* overexpression on the maximum specific growth rate of yeast strains

Overexpression of *tHMG1* led to increased final titers and yields of AD compared to the control strain, YCF-ADS (table 3.4 and figure 3.6). Overexpression of *tHMG1* from the genome or plasmid enhanced the AD production, but plasmid overexpression had a greater impact. However, simultaneous overexpression of *tHMG1* both from the plasmid and the genome in a single strain further improved AD production 2-folds (Figure 3.6).

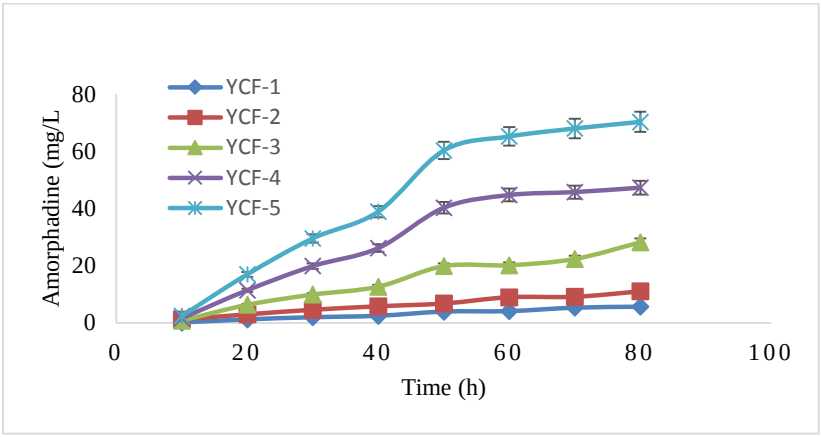


Figure 3.7 Concentrations of amorphadiene and methionine as a function of time in different yeast strains.

3.3.7. Combining *tHMG1* overexpression and *ERG9* down-regulation

It was found that repression of *ERG9* in AD producing yeast strains led to accumulation of the FPP derived compound, farnesol, indicating an increase in the intracellular pool of FPP. Here down-regulation of *ERG9* and overexpression of *tHMG1* combined in AD producing yeast strain. Down-regulation of *ERG9* alone produced 11.02 mg/L of AD, but combination of *ERG9* down-regulation and *tHMG1* overexpression improved the AD production by 7-folds (table 3.2). FPP is a branch-point intermediate of the mevalonate pathway that serves as a substrate for several important enzymes. However most of the FPP is used for the biosynthesis of sterols and therefore minimizing the flux of FPP towards sterols would enhance available FPP for other enzymes of the branch-point including all sesquiterpene synthases. Replacement of the endogenous *ERG9* promoter with a repressible *MET3* promoter led to reduced ergosterol content of cells and enhanced AD biosynthesis, but it was observed that there was accumulation of farnesol as a major FPP derived by-product (table 3.4). In contrast *tHMG1* over expressed strains not shown any accumulation of farnesol (table 3.6)

3.3.8. Effect of *tHMG1* overexpression on ergosterol and squalene content of the cells

To assess the effect of *tHMG1* overexpression on ergosterol which is the end product of the pathway, the ergosterol and squalene content of *tHMG1* overexpressed strains were determined and compared with the control (YCF-ADS) and wild type MTCC 3157 strains (fig 3.7). The increase in the ergosterol content of cells is not proportionally correlated with the increase in the HMG-CoA reductase activity and increase in the AD titer meaning that conversion of HMG-CoA to mevalonate is not the only rate controlling step in the complete ergosterol pathway and there are other regulatory sites downstream in the pathway towards ergosterol (Polakowski et al., 1998). Extraction and measurement of squalene in the *tHMG1* overexpressed strains also revealed a dramatic change in squalene concentration from trace amounts in the MTCC 3157 and control strains to several milligrams per gram of biomass for the mutants (fig 3.8).

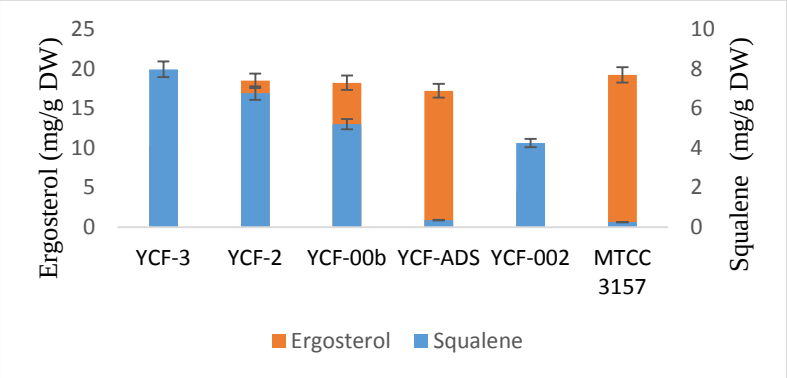


Figure 3.8 Influence of *tHMG1* overexpression on squalene accumulation in the yeast strains. Cells were grown in shake flasks containing 100 ml medium and 20 g/L galactose

Squalene accumulating organisms are scarce in the nature and the liver oil of deep-sea shark, which is traditionally used for squalene production (Donald et al., 1997), is one of the few exceptions with up to 60% squalene (Pietsch and Jaeger, 2007). Although the wild type strain contained trace amounts of squalene, the *tHMG1* overexpressed strains were able to accumulate up to 9 mg squalene/g DW. Accumulation of squalene as a result of *tHMG1* overexpression has been reported by other investigators as well (Donald et al., 1997; Polakowski et al., 1998) and proposes the reaction catalyzed by squalene epoxidase as a flux controlling step in the ergosterol biosynthetic pathway (Polakowski et al., 1998; Veen et al., 2003). Comparison of kinetic parameters of the enzymes squalene synthase and squalene epoxidase may explain why squalene expoxidase is a flux controlling step in the ergosterol pathway. The yeast squalene synthase has a Km value of 2.5 µM for its substrate FPP (LoGrasso et al., 1993) and the wild type specific activity for this enzyme was found to be 460 pmol min^{-1} mg^{-1} (Jennings et al., 1991) whereas the reported numbers for the yeast squalene epoxidase are 13.5 µM and 32.1 pmol min^{-1} mg^{-1}, respectively (Satoh et al., 1993). The higher K_m value of squalene epoxidase for its substrate and lower specific activity of this enzyme suggest that squalene epoxidase has lower capacity than squalene synthase and this could lead to accumulation of squalene if the flux through the pathway exceeds a certain limit e.g. when HMG-CoA reductase is overproduced. Typical K_m values of sesquiterpene synthases for FPP are in the range of 0.4-10 µM (Picaud et al., 2005) which is comparable with that of yeast squalene synthase. The kcat values for amorpha-4, 11-diene synthase from *Artemisia annua* L. vary from 3.4 × 10-4 to 15.4 × 10-3 s^{-1} depending on pH and the divalent metal ions (Picaud

et al., 2005) and germacrene D synthase from *Zingiber officinale* has a kcat value of 3.34 × 10-3 s^{-1} (Picaud et al., 2006) whereas squalene synthase has a k_{cat} value of 0.53 s^{-1} (LoGrasso et al., 1993). These low values indicate that the conversion of FPP to sesquiterpenes by sesquiterpene synthases is less efficient than the condensation of two FPP molecules to squalene by squalene synthase. But some of the previous studies reported that accumulation of squalene implies high ergosterol content and squalene epoxidase is inhibited by feedback regulation triggered by ergosterol. This feedback regulation leads to the inhibition of HMG-CoA reductase and thus increased expression of *tHMG1* cannot enhance the carbon flux towards FPP (Asadollahi et al., 2008).

3.4. Conclusions

The corresponding amorphadiene synthase expressed in yeast and was shown to be functional. The availability of FPP which is the precursor for all sesquiterpene synthases was enhanced by replacement of the native *ERG9* promoter with a regulatable *MET3* promoter and repressing the promoter with the presence of 2 mM methionine. The ergosterol content was drastically reduced as a consequence of this repression and improved the amorphadiene production by approximately 2-folds. Metabolism of methionine during the growth phase relieved the repression of *ERG9*, which was accompanied by a reduction in the rate of amorphadiene synthesis. Further experiments showed that it was possible to improve the amorphadiene production by supplying more methionine during fermentation. Under these conditions the final titer of amorphadiene and farnesol reached to 17.1 and 20.2 mg/L, respectively. More precise regulation of methionine should improve further the efficiency of sesquiterpene biosynthesis. Considering that the reported titers and yields have been achieved only by down-regulating one gene in the pathway. Further overexpression of the truncated *HMG1*encoding the catalytic domain of HMG-CoA reductase was first done by integrating *tHMG1*into the genome under control of the strong constitutive *TPI1* promoter. This modification enhanced the activity of the enzyme by approximately 1.5-fold and increased the biosynthesis of AD by roughly 2-fold in shake flask experiments. The *tHMG1* gene was further overexpressed by cloning it into a high copy number plasmid under control of the strong inducible *GAL10* promoter. Overexpression of *tHMG1* from plasmid was more efficient and the final concentration of AD in batch fermenter reached to 47.25 mg/L whereas the AD concentration

was 28.14 mg/L when *tHMG1* was overexpressed by genomic integration. Combining the repression and overexpression of *tHMG1*improved the production by 7-folds. This work demonstrates the capacity of yeast as a cell factory for the production of sesquiterpenes. In addition to enhancing sesquiterpene production, overexpression of *tHMG1* resulted in accumulation of squalene in the cells, indicating the accessible carbon flux for sesquiterpenes production. This can be possible by limiting the loss of intermediate FPP towards other unwanted side reactions. This can be achieved by applying the enzyme fusion technology to the tradition metabolic engineering techniques and same was carried out in present study (chapter 4).

CHAPTER 4

Combination of Metabolic Engineering and Enzyme Fusion Technology for Improved Production of Amorphadiene in *S. Cerevisiae*

CHAPTER 4

COMBINATION OF METABOLIC ENGINEERING AND ENZYME FUSION TECHNOLOGY FOR IMPROVED PRODUCTION OF AMORPHADIENE IN *Saccharomyces cerevisiae*

4.1. Introduction

Microbial fermentation ensures production of industrially important metabolites in large quantities. Implication of r-DNA technology in microbial fermentations offers production of desired heterologous proteins at large scale. Apart from the proteins, nature offers diverse classes of complex metabolites (isoprenoids) that are utilized in the food, cosmetic, and pharmaceutical industries and so forth (Chang and J. D. Keasling, 2006) Many of these complex metabolites are produced naturally in low quantities in plants that are difficult or expensive to cultivate. Metabolic engineering, systems, and synthetic biology principles and methods allowed easy transfer of heterologous pathways from natural plant producer to a suitable microbial host such as yeast and *E. coli* (Ro et al., 2006). *E. coli* was the most studied host for metabolic engineering of isoprenoid by modulating 1-deoxyxylulose-5-phosphate (DXP) and mevalonate (MVA) pathways (Alper at al., 2005; Carter et al., 2003; Jackson et al., 2003). Other studies on MVA pathway deregulation in yeasts have improved the biosynthesis of different isoprenoids (Shimada et al., 1998; Albertsen et al., 2011). The vital role of MVA pathway and eventual product (ergosterol) proportion from this pathway increased the interest for the engineering of isoprenoid pathway in yeast for the production of heterologous compounds (Lamacka and J. Sajbidor 1997). Ergosterol synthesis was carried out by squalene synthase (*ERG9*), and conversion of farnesyl pyrophosphate (FPP) to squalene was successfully regulated by replacing the native promoter with repressible promoters (Ro et al., 2006; Asadollahi et al., 2008). ATP sulfurylase (*MET3*) catalyzes the reduction of sulfate to sulfide, involved in methionine metabolism. Addition of methionine had the ability to repress the *MET3* promoter in *S. cerevisiae* and *Ashbya gossypii* (Mao et al 2002), and *MET3* promoter was widely used as a molecular tool for yeast genetics. Further *tHMG1* over expression had significant effect on the final tires of targeted sesquiterpenes. However Heterologous

expression of pathways/enzymes in *S. cerevisiae* is complex due to the presence of numerous native host enzymes and tight regulation of the intermediate metabolites generated by the host machinery. The non-native product formation is not only affected by the host environment but also by the loss of intermediate metabolites through diffusion, degradation, or converted by competitive enzymes/pathways (Conrado et al., 2008; Jorgensen et al., 2005). From our previous experiments, FPP derived farnesol and squalene accumulation observed, which indicating, some of the FPP flux diverting towards other compounds. In order to avoid such intermediates loss and make heterologous expression more efficient, enzymes catalyzing successive reactions are often fused in close proximity to each other by using small linkers. Small linkers are the sequence coding for few amino acids, which separates the two proteins in space with a small distance allowing them to fold properly without constraints from each other. Consequently, the substrate was channeled between active sites of two or more sequential enzymes of a pathway, without allowing free diffusion of the intermediates. Subsequently it reducing the transit time required for the intermediates to spread the enzyme that catalyzes the next step in the reaction. Several elucidations *in vitro* and *in vivo* recommended that this strategy can be used to improve the flux through a metabolic pathway (Bulow 1987; Orita 2007; Seo 2000)

In the present study *S. cerevisiae* (MTCC 3157) used for heterologous expression of amorphadiene synthase (*ADS*) for one-step conversion of FPP to amorphadiene. In order to increase the concentration and flux of FPP towards heterologous product, squalene synthase (*ERG9*) a key enzyme in ergosterol synthesis was repressed and another regulatory enzyme in the pathway *tHMG1* over expressed. Further to avoid FPP loss through competitive pathways, enzyme fusion strategy was applied and composed a chimeric fusion protein between farnesyl diphosphate synthase (*FPPS*) of yeast and amorphadiene synthase (*ADS*) of *Artemisia annua* L. and was expressed in improved yeast strains.

4.2. Materials and methods

4.2.1. Microbial strains, media, and reagents

All the reagents and media used in this study were of, analytical grade and procured either from Himedia (India) or Merck (India) or Sigma (India). The strains used in this study were *Saccharomyces cerevisiae* (MTCC 3157), and *E. coli* DH5α (MTCC 1652) procured from

MTCC, Chandigarh and strains YCF-ADS, YCF-001 and YCF-002 constructed in previous studies (chapter 3).

4.2.2. *ERG9* Promoter replacement and *tHMG1* over expression

YCF-001 was generated by replacing the native *ERG9* promoter with *MET3* by using fusion PCR and a bipartite gene targeting method and YCF-ADS strain generated by using gap repair technique with high efficiency homologous recombination in previous studies (chapter 3)

4.2.3. Construction of fusion proteins

An FPPS (*ERG 20*) gene fragment was obtained by PCR amplification as mentioned above using genomic DNA of *S. cerevisiae* MTCC 3157 with the primer pairs (13) and (14). The fragments were digested with EcoRI and ClaI and inserted into an EcoRI-ClaI vector fragment 2μ based pY01URA plasmid (Genecopeia USA) and the resulting plasmid is designated as pY01*FPPS*. For construction of plasmid expressing fusion protein *FPPS-ADS*, first *FPPS* and *ADS* were amplified separately using primer pairs (table 4.2) (13), (14) and (15), (16) using pY01*FPPS* and pRS425ADS (Addgene#20119) as templates (Ro et al., 2006). The two resulting PCR fragments were fused in the second round of PCR using primers (17) and (18). Similarly, *ADS-FPPS* fragments were generated in the first round using the primer pairs (19), (20) and (21), (22) and the resulting fragments fused together in second round of PCR using primer pair (23) and (24). The resulting two PCR fragments were cut with EcoRI-ClaI and individually cloned into EcoRI-ClaI plasmid 2μ-based pY01URA plasmid (Genecopeia, USA) and the resulting plasmids were named as pY01*FPPS-ADS* and pY01*ADS-FPPS*, respectively.

4.2.4. Strain Construction

Transformation of all strains of *S. cerevisiae* was performed by lithium acetate and PEG mediated transformation by using Yeast maker transformation system 2 kit (Clontech, USA). *S. cerevisiae* strain (MTCC 3157), was used as the parent strain for all *S. cerevisiae* strains used in this study. Strain YCF-ADS was constructed by the transformation of pESC-URA-ADS plasmid (constructed by using pESC-URA and Addgene#20119) in to MTCC 3157. Strain YCF-002 was generated by transforming the fusion PCR fragments in to strain YCF-ADS and selected on Kanamycin and synthetic defined (SD)-URA drop out plates. Finally

strain YCF-001 obtained by exclusion of plasmid pESCURA- ADS from strain YCF-002 by selection on plates containing 5-fluoroorotic acid (5-FOA). Strain YCF-004, YCF- 005, YCF-006, YCF-00c, YCF-00d, YCF-001a, YCF-002b, YCF-00C_a,YCF-3, YCF-4 and YCF-5 constructed by transforming the corresponding plasmids (table 4.1) and the transformants were selected on SD-URA drop out plates.

Strain	Genotype	Plasmid	Plasmid description
YCF-ADS	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pRS425 ADS	pESC-*URA-ADS*	pESC-URA 2μ *URA3* P_{GAL1}- ADS
YCF-001	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0erg9::MET3	None	
YCF-002	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0erg9::ADS	pESC-URA-*ADS*	pESC-URA 2μ URA3 PGAL1- ADS
YCF-00b	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ *::PTPI1-tHMG1*	None	
YCF-004	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0FPPS	pY01*FPPS*	2μ URA3 PGAL1- FPPS 6x-His
YCF-005	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0erg9::FPPS-ADS	pY01*FPPS-ADS*	2μ URA3 PGAL1- *FPPS-ADS* 6x-His
YCF-006	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0erg9:: ADS-FPPS	pY01*ADS-FPPS*	2μ URA3 PGAL1- *ADS-FPPS* 6x-His
YCF-00C_a	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0ADS	pY01*ADS*	2μ URA3 PGAL1- *ADS* 6x-His
YCF-3	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0erg9:tHMG ADS, tHMG	pBAD thmg1	2μ URA3 PGAL1- Thmg1
YCF-4	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0erg9:tHMG, tHMG, FPPS-ADS	pY01*ADS*	2μ URA3 PGAL1- *ADS* 6x-His
YCF-5	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0erg9:tHMG ADS, tHMG, ADS-FPPS	pY01*ADS*	2μ URA3 PGAL1- *ADS* 6x-His

Table 4.1 Strains and plasmid used in this study

4.2.5. OD measurement and dry weight analysis

Optical density values of samples in triplicates were measured at 600 nm (OD_{600}) by using UV-Spectrophotometer (Thermo Scientific, USA). Cultures were further diluted until the OD_{600} value as <1.0 (Lamacka and Sajbidor, 1997). The dry weight was analyzed by using nitrocellulose filter papers (pore size 0.45 μm, Whatman).The filter papers were predried in a microwave oven at 60 ^oC for 10min. A known volume of the cell culture was filtered, and the residue was washed with distilled water and dried in an oven at 60 ^oC (Dynesen 1998)

(13) ATGGCTTCAGAAAAAGAAATTAGGAGAGAGAGA
(14) TTCAGTCAAGGCCACTATTTGCTTCTCTTGTAAACTTTGTTCAAGAAC
(15) GAGAAGCAAATAGTGGCCTTGACTGAAGAGAAACCTATAAGGC
(16) TTAGATAGACATAGGGTAAACTAGCAATGATTTGATCAA
(17) ATGGCTTCAGAAAAAGAAATTAGGAGAGAGAGA
(18) TTAGATAGACATAGGGTAAACTAGCAATGATTTGATCAA
(19) ATGGCCTTGACTGAAGAGAAACCT
(20) ATTTCTTTTTCTGAAGCCATTTAGATAGACATAGGGTAAACTAGCAATGATTTG ATCAA
(21) TTTACCCTATGTCTATCTAAATGGCTTCAGAAAAAGAAATTAGGAGAGAGAG
(22) CTATTTGCTTCTCTTGTAAACTTTGTTCAAGAACG
(23) ATGGCCTTGACTGAAGAGAAACCT
(24) CTATTTGCTTCTCTTGTAAACTTTGTTCAAGAACG

Table 4.2. List of primers used in this study

4.2.6. Ergosterol extraction and analysis

An overnight culture grown in minimal medium supplemented with 1.5 mM methionine (glucose 20 g/L) was centrifuged at 5,000 rpm for 5min to get approximately 3 g of dry cells. The cell pellet was washed with distilled water, and the cell suspension was centrifuged for another 5min at 5,000 rpm. Further cell pellet was mixed with 300 mL of 25% alcoholic KOH solution and vortexed for 1min, and the suspension was saponified for 3 h at 90 ^{o}C in a reflux. After cooling them to room temperature, non saponified sterols were extracted by adding 300 mL heptane followed by vortexing for 2 min. A vortex mixture of 10mL heptane and 10 mL of alcoholic KOH solution is used as blank. After clarification of heptane layer 0.5 mL of heptane from both sample and blank was diluted ten-folds with 4.5 mL absolute ethanol. The absorbance of all samples were read against blank at 230 and 281.5 nm, respectively (Lamacka and Sajbidor 1997). The ergosterol content was calculated as milligram ergosterol per gram dry weight using the following equation (Breivik and Owades 1957)

$$\text{Ergosterol} = \%\ \text{ergosterols} - \%24\ (28)\text{-dehydroergosterol}$$

$$Ergosterol(\frac{mg}{gDW}) = (\frac{OD281.5}{290} - \frac{OD230}{580})\ XF$$

Where F is a correction factor for dilutions and sample sizes, and 290 and 580 are E (1%, 1 cm) of crystalline ergosterol and 24 (28)-dehydroergosterol, respectively.

4.2.7. Batch Fermentation

Batch fermentation was carried out in a controlled bioreactor (Spectrochem, India) containing 2 L mineral medium that consists of (g/L) galactose 20; $(NH_4)_2SO_4$, 5; KH_2PO_4, 3; $MgSO_4 7H_2O$, 0.5; EDTA, 0.015; $ZnSO_4 \cdot 7H_2O$, 0.0045; $CoC1_2 \cdot 6H_2O$, 0.0003; $MnC1_2$ $4H_2O$, 0.001; $CuSO_4$ $5H_2O$, 0.0003; $CaC1_2 \cdot 2H_2O$, 0.0000045; $FeSO_4 \cdot 7H_2O$, 0.0003; $NaMoO_4, \cdot 2H_2O$, 0.0004; H_3BO_3, 0.001; KI, 0.0001; and 0.025mL silicone antifoam (Merck). This medium was further autoclaved at 121°C for 20 min. Further filter sterilized vitamin solution containing (mg/L): biotin, 0.05; calcium pantothenate, 1; nicotinic acid, 1; inositol, 25; thiamine HCl, 1; pyridoxine HCl, 1; and para-aminobenzoic acid, 0.2 was added to the autoclaved mineral medium. Finally, media were supplemented with 2 mM filter sterilized methionine. During the fermentation process, the temperature was kept constant at 30 ± 2 °C, and dissolved oxygen tension (50%) was maintained with sterilized air (0.2 μ filter) with airflow 1 L/min and with 250 rpm agitation and the off gas passed through an outlet port. pH was controlled between 6.20 ± 0.5 by automatic addition of 1M NaOH and 1M HCl. Seed culture with OD_{600} of 0.02 from shake flask was inoculated into batch fermentor. After cells reaching 1 at OD_{600} 20% (vol/vol) isopropyl myristate (Merck Millipore, Germany) was added aseptically to the media. This isopropyl myristate layer was sampled and diluted with ethyl acetate for determination of amorphadiene concentration by gas chromatography coupled mass spectrometry (GC-MS) (Agilent Technologies, USA).

4.2.8. Analysis of sesquiterpenes

Amorpha-4, 11-diene and farnesol were analyzed by gas chromatography with flame ionization detector (GC/FID). Samples from fermentor were centrifuged at 5000 rpm for 5 min, diluted directly into ethyl acetate, and mixed for 30min on a vortex mixer. After phase separation 0.6mL of the ethyl acetate, layer was transferred to a capped vial for analysis. The ethyl acetate-extracted samples were analyzed using the GC/FID. A1 μL sample was split 1: 20 and separated using a DB-WAX column (50m. 200 μm. 0.2 μm) with hydrogen as the carrier gas with flow rate of 1.57 mL/min. The temperature program for the analysis was as follows. The column was initially held at 150 °C for 3.0 min, followed by a temperature gradient of 5 °C per min to a temperature of 250 °C. Amorpha-4, 11-diene and farnesol peak

areas were converted to concentration values from external standard calibrations using authentic compounds (Westfall et al., 2012)

4. 2.9. Expression, purification, and SDS-page analysis of the proteins.

Different yeast strains carrying the plasmids pY01*ADS*, pY01*FPPS*, pY01*FPPS-ADS*, and *pY01ADS-FPPS* were grown in appropriate SD drop out media at 37 ^{o}C. After cells optical density values were reaching 2.5 at OD_{600}, they were harvested and centrifuged at 5000 rpm for 5min, and cell pellet was re suspended in 10mL distilled water, and proteins were extracted according to CelLytic Y plus Kit (Sigma, USA). Purification was carried out in a single step using immobilized metal affinity chromatography (IMAC). The supernatant was applied to a 5mL Nickel CL-Agarose Column (Merck Biosciences, India), loaded with nickel. Further non bound proteins were removed with washing buffer. Elution of adsorbed recombinant proteins was achieved with elution buffer (Merck Biosciences, India). Fractions were collected either used or stored at 4 ^{o}C. SDS/PAGE was carried out in 10% resolving and 5% stacking gels according to instructions given by the manufacturer (Merck Biosciences, India) and the samples along with protein marker were loaded on Mini- PROTEAN Tetra cell (Bio-Rad, USA). After electrophoresis, the gel was stained with coomassie blue. Gel, visualized and analyzed in Gel Doc Fire.

4.3. Results and Discussion

4.3.1. Combined effect of *ERG9* repression and chimeric protein expression

Transformed yeast strains harboring amorphadiene synthase (ADS) was able to produce amorpha, 4-11- diene (Figure 3). Strain expressing ADS analyzed for amorphadiene production by GC-MS as mentioned in materials and methods. Analysis of GC-MS for ethyl acetate extracts revealed the presence of amorphadiene major peak and other sesquiterpene as a minor peak (Figure 4.1(b)). Retention time, mass spectrum of main component (19.75 and 14.21 min) were almost matching with standard (19.70 and 14.18 min) (Figures 4.1(a), 4.1(c), and 4.1(d)). When AD producing strains analyzed for amorphadiene, strain YCF-002 produced 11.02 mg/L of amorphadiene, which was approximately 2-fold higher than AD (5.65 mg/L) produced by YCF-ADS respectively (fig.4.2). FPP conversion to farnesol in the strain YCF-004 was limited by expressing the fusion protein in yeast and to probe the effect of enzyme

fusion on diversion of the flux more efficiently towards amorphadiene production. Different strains expressing the native proteins and chimeric proteins *ADS*, *FPPS-ADS*, and *ADS-FPPS* were analyzed. The strain transformed with the plasmid expressing ADS-FPPS produced amorphadiene (12.08 mg/L) at the same level as that for the strain expressing free *ADS* (Figure 4). More interestingly, the strain transformed with the plasmid expressing *FPPS-ADS* produced amorphadiene at an almost 2-fold higher level (25.06 mg/L) than that for the strain expressing free *FPPS* and *ADS* (fig.4.2).

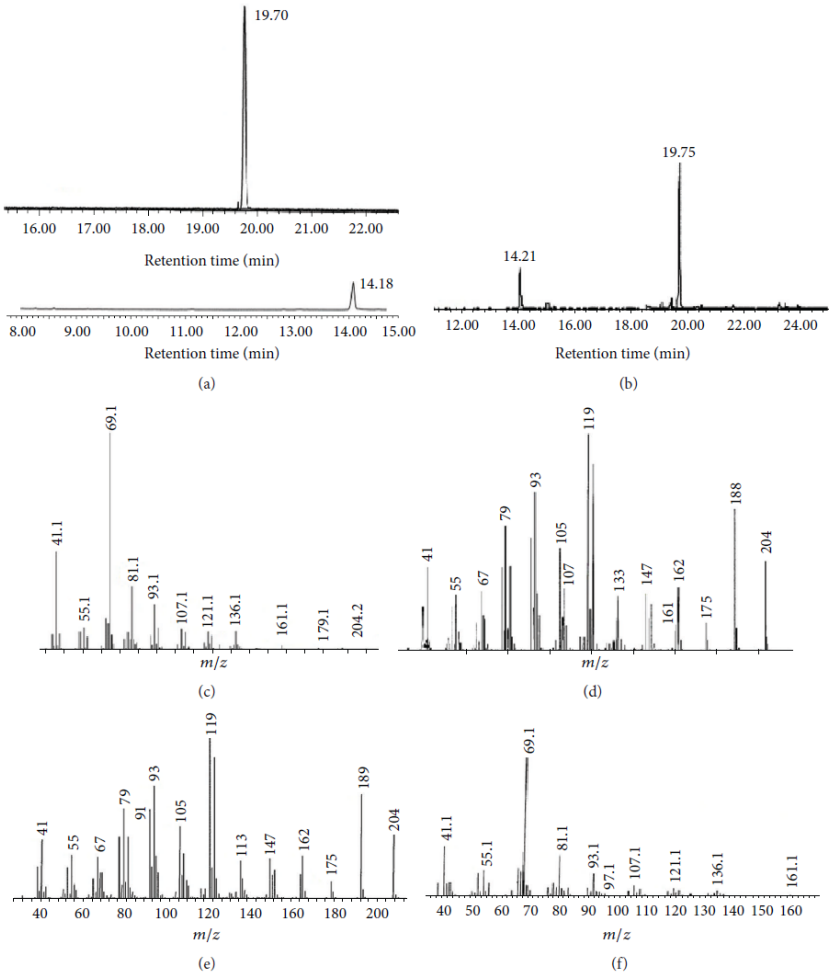


Figure 4.1 GC-MS profile of the amorphadiene and farnesol produced by YCF-005 strain. (a) Standard chromatogram of amorphadiene and farnesol, (b) ethyl extract sample chromatogram, (c) farnesol authentic standard mass spectrum, (d) amorphadiene authentic standard mass spectrum, (e) amorphadiene, and (f) farnesol sample mass spectrum generated from sample chromatogram (b).

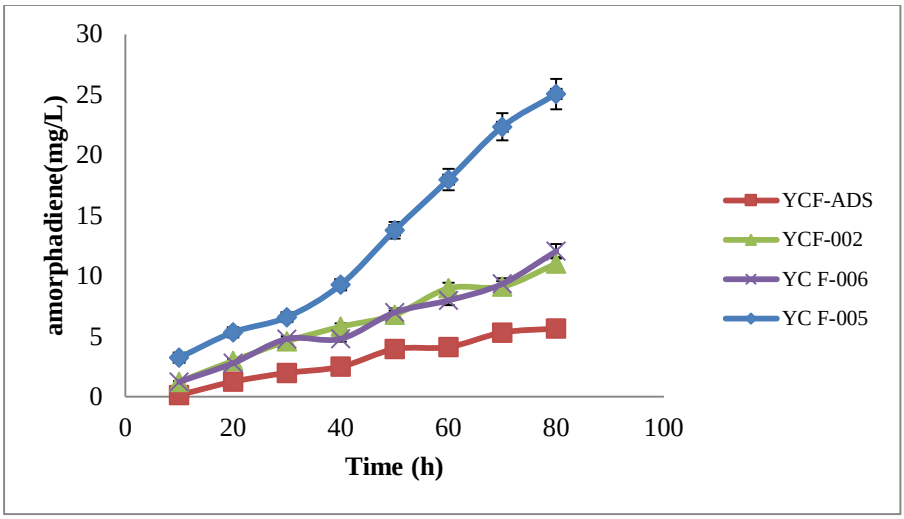


Figure 4.2 Amorphadiene concentration as a function of time in repressed (YCF-002, YCF-005, YCF-006) and non-repressed (YCF-ADS) yeast strains expression free enzymes (*ADS*) and chimera enzymes (*FPPS-ADS* and *ADS-FPPS*) (average data obtained from triplicate of the experiments were represented).

4.3.2. Chimeric protein expression in *tHMG1* over expressed and repressed (*ERG9*) strains

Further to know the combined effect of the *tHMG1* over expression and repression on fusion protein expression and final product concentration, yeast strains YCF-3, YCF-4 and YCF-5 analyzed for amorphadiene synthesis. As expected the final amorphadiene synthesis was improved by approximately 10-folds in YCF-4 compared to strain YCF-005. *tHMG1* overexpression (YCF-00b) alone improved the amorphadiene synthesis by 2.5- folds, further collective consequence of *ERG9* repression and *tHMG1* over expression with an additional copy improved the AD synthesis by 2.5- folds in strain YCF-3. But expression of fusion proteins (*FPPS-ADS*) in the YCF-3 strain increased the final titer of AD by 2.5-folds, cumulative improvement about 21.7-folds compared to control strain (fig.4.3). Likewise in YCF-002, orientation of fused genes (FPPS-ADS) had a significant effect on the final titers of AD, which was further confirmed by analyzing the AD synthesis levels in YCF-5 (fig.4.3), AD synthesis level of this strain almost equal to the strain YCF-3. In the reverse orientation of

the two genes (*ADS-FPPS*) the active sites were likely far apart to produce a beneficial proximity effect, which was also confirmed form previous studies (Albertsen et al., 2011).

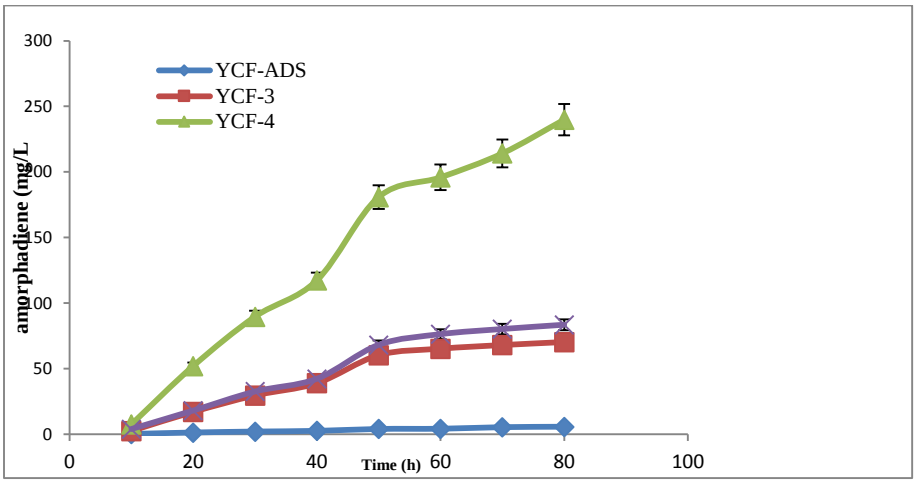


Figure 4.3 Amorphadiene concentration as a function of time in repressed (YCF-3 YCF-4, YCF-5) and non-repressed (YCF-ADS) yeast strains expression free enzymes (*ADS*) and chimera enzymes (*FPPS-ADS* and *ADS-FPPS*) (average data obtained from triplicate of the experiments were represented).

Further, analysis of squalene levels in the final strains (fig.4.4), revealed that FPP conversion to farnesol was limited by the expression of fused proteins. Almost 40-fold reduction in the synthesis of squalene observed, but strain harboring (*ADS-FPPS*) plasmid not have any significant effect on the squalene levels reduction.

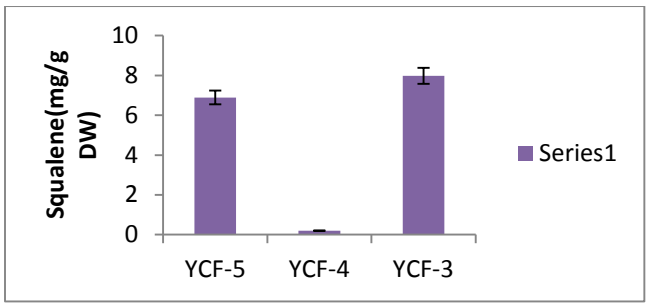


Figure 4.4 Influence of combination of *ERG9* down-regulation, *tHMG1* overexpression and fusion protein expression (*FPPS-ADS* and *ADS-FPPS*) on squalene accumulation in the different yeast strains.

Table 4.3 Final concentrations and yields of AD for the different yeast strains

Yeast Strain	Amorphadiene (mg/L)	Farnesol (mg/L)	Yield of Amorphadiene on biomass (mg/g DW)	Yield of Farnesol on biomass (mg/g DW)	Yield of AD on galactose (mg/g galactose)	Yield of Farnesol on galactose (mg/g galactose)
YCF-ADS	5.65		0.56		0.31	
YCF-3	70.25	30.15	7.80	3.35	3.90	1.67
YCF-4	239.85	6.29	28.12	7.3	13.25	0.34
YCF-5	83.45	28.18	9.17	3.09	4.63	1.56

Further analysis of ergosterol content, confirmed the tight regulation of the promoter with highest methionine (3 mM) concentration. Probably down regulation of ergosterol synthesis not contributed towards improved production of AD in yeast strains (fig. 4.5) harboring fused proteins.

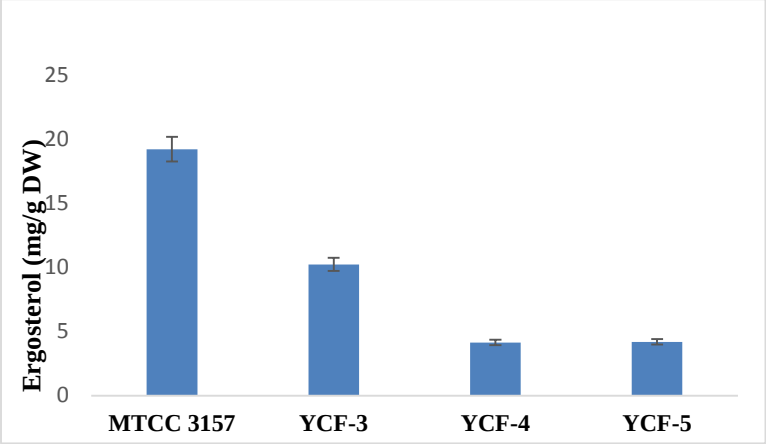


Figure 4.5 Influence of combination of *ERG9* down-regulation, *tHMG1* overexpression and fusion protein expression (*FPPS-ADS* and *ADS-FPPS*) on ergosterol synthesis in different yeast strains.

Which was confirmed by observing ergosterol levels in the final strains (fig.4.5), *ERG9* repression and *tHMG1* over expression (YCF-3) reduced the ergosterol content of the cell and

almost same levels of ergosterol synthesized in remaining two strains (YCF-4 and YCF-5). However it leads to the accumulation of farnesol (Buurman et al., 2004; Hornby et al., 2003) and also at high concentration it inhibits the growth of the cells by cell cycle arrest in some yeast cells (Machida et al., 1999). Nevertheless, not any effect observed on the growth of the strains used in this study. After analyzing the farnesol levels, there was much difference observed between strains YCF-4 and YCF-5, from this it was hypnotized that, orientation of fused proteins is very important, and correct orientation (*FPPS-ADS*) significantly improved the AD titres, compared other orientation (*ADS-FPPS*) (table 4.1). Active site access was limited in other orientation, which leads to the accumulation of farnesol in yeast cells (table 4.1).

4.3.3. SDS-PAGE analysis of proteins.

The four recombinant proteins were produced using the conditions reported as above. On SDS-PAGE the four proteins were compared across the protein molecular marker (fig.4.6). Both fused proteins *FPPS-ADS* and *ADS-FPPS* molecular weights were approximately 105 kD which was almost equal to the sum of two individual proteins (FPPS 40 kD and 63 kD). This indicates the efficacious fusion of the two proteins to form a chimeric protein.

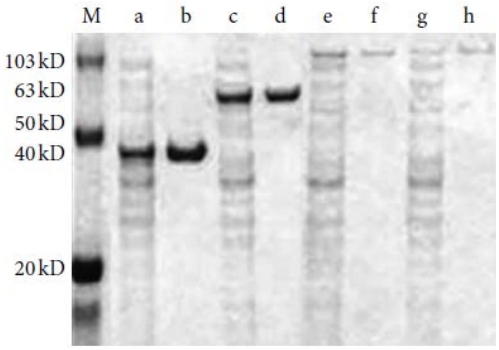


Figure 4.6 SDS/PAGE of recombinant enzymes produced in different yeast strains. Lane M: molecular mass standards; lane a, crude extract FPPS; lane b: purified FPPS; lane c: crude extract ADS; lane d: purified ADS; lane e: crude extract FPPS-ADS; lane f: purified FPPSADS lane g: crude extract ADS-FPPS; lane h: purified ADS-FPPS. The calculated molecular weights of FPPS, ADS, and the fusion enzymes were 40, 63, and 103 kDa, respectively.

4.3.4. Conclusions

In the present study, amorphadiene production improved by 42-fold in the yeast *S. cerevisiae* (MTCC 3157) compared to the control strain. In order to decrease the FPP flux towards ergosterol and competitive pathways, *ERG9* promoter was replaced with repressible methionine (*MET3*) promoter. From previous studies high methionine (3 mM) was selected for the regulation of the *MET3* promoter. The ergosterol content of the *ERG9* repressed strain was quite low compared with control strain (MTCC 3157). Subsequent repression of *ERG9* leads to the accumulation of FPP derived farnesol. Strain YCF-4 produced only 30.15 mg/L of farnesol and it disclosed the possibility of pooling the available FPP towards amorphadiene by enzyme fusion technology. To overcome the natural loss of the metabolic intermediate FPP and its conversion towards farnesol, a chimeric protein with small Gly-Ser-Gly linker was constructed as *ADS-FPPS* and *FPPS-ADS*. Strain YCF-4 expressing *ADS-FPPS* produced 238.85 mg/L whereas YCF-5 produced 83.45 mg/L due to the limited access of active site to the substrate. The fusion phenomenon of the enzyme further confirmed by isolation and purification of *FPPS*, *ADS*, *ADS-FPPS*, and *FPPS-ADS* enzymes with SDS-PAGE and fused proteins molecular weights (105 kD) were almost equal to the sum of the individual enzymes ADS (63 kD) and FPPS (40 kD). In the summary a strain was developed which has capable producing 239.85mg/L of AD by using combination metabolic engineering and enzyme fusion technology. Apart from the strain improvement, process optimization plays a major role for enhancing the production levels of microbial products. With that emphasis an attempt to made optimize the key process parameters for production AD, which was carried in the next section (chapter 5)

CHAPTER 5

Optimization of Amorphadiene Production in Engineered Yeast by Response Surface Methodology

CHAPTER 5

OPTIMIZATION OF AMORPHADIENE PRODUCTION IN ENGINEERED YEAST BY RESPONSE SURFACE METHODOLOGY

5.1. Introduction

Isoprenoids (terpenoids) are the most structurally diverse class of natural compounds commonly produced in plants (Croteau et al., 2000). Terpenoids are classified according to their carbon number (basic isoprene (C5) unit) as mono (C10), sesqui (C15), di (C20), sester (C25), tri (C30), tetra (C40) and polyterpenoids (Cn) (Ruzicka, 1959). More than 55,000 terpenes have been isolated and characterized, consistently doubling in their numbers each decade (Breitmaier, 2006; McGarvey and Croteau, 1995). Isoprenoids have diverse functional roles in plants such as growth, defense and development (McGarvey and Croteau, 1995). Based on these characteristic features, terpenoids have prominence in pharmaceutical, fragrances and biofuel industries (for e.g. bisabolene is an alternative source for jet fuel (Breitmaier, 2006; Peralta-Yahya et al., 2012). Artemisinin is well-known sesquiterpene lactone peroxide, extracted from the shrub *Artemisia annua*. 'Artemisininins' (artemisinin and its derivatives) are recommended by the World Health Organization (WHO) in combination with other effective anti-malarial drugs, known as artemisinin-based combination therapy (ACT) for malarial treatment (Bloland, 2001). Since then, the incompetence in large-scale chemical synthesis of artemisinin and enormous demand and price directed the scientific world towards the semi-synthesis of artemisinin followed by microbial production of the precursor amorpha- 4, 11-diene. Heterologous production of amorpha-4, 11-diene was first established in *Escherichia coli* by the expression of the mevalonate pathway from yeast and amorpha-4, 11-diene synthase (*ADS*) from *A. annua* (Martin et al., 2003). The production of amorpha-4, 11-diene from *Saccharomyces cerevisiae* revealed that cytochrome P450 enzyme was responsible for the production of artemisinic acid (Mercke et al., 2000; Martin et al., 2003; Ro et al., 2006). Artemisinic acid was produced from yeast by a series of alterations and adjustments to the endogenous mevalonate

pathway, such as high-level expression of ADS, over expression of farnesyl diphosphate synthase (*FPPS*), expression of the catalytic domain of HMG-CoA reductase (*HMGR*), reduced expression of squalene synthase (SQS) and increased expression of *UPC2* allele transcription factor (Ro et al., 2006). Artemisinic acid was produced by a three-step oxidation of amorphadiene, by cytochrome P450 reductase (*A. annua*) (Ro et al., 2006). However, cytochrome P450 reductase instability and lower yields of artemisinic acid compared to amorphadiene drew attention towards improving the production of amorphadiene, the precursor of artemisinic acid in *S. cerevisiae*. (Westfall et al., 2012). In combination with traditional metabolic engineering, enzyme fusion technology was applied for improving the production of amorphadiene in *S. cerevisiae* (YCF-3 (renamed as YCF-AD-1) and YCF-4 (renamed as YCF-AD-2)). From previous studies it is observed that, engineered yeast mevalonate pathway is tightly regulated by methionine and phosphate levels along with other physical parameters such as pH and temperature. Optimization of these parameters by classical experimental optimization is difficult because it involves changing one variable at a time while keeping the others constant. In addition, it is not practical to carry out experiments with every possible factorial combination of the test variables, because of the large number of experiments required to be done and/or evaluated (Akhnazarova and Kafarov, 1982; Myers and Montgomery, 1995) which does not emphasize the effect of interactions among various parameter. Besides this, it will be a tedious and time-consuming process, especially when there are a large number of parameters to take into consideration. An alternative and more efficient approach is the use of the statistical method to resolve this kind of practical hurdles. Response surface methodology (RSM) has been widely used to evaluate and understand the interactions between different process parameters (Khuri and cornell, 1996). RSM was applied successfully for optimizing process parameters for various processes in biotechnology, from biological treatment of toxic wastes to enzyme production (Doddapaneni et al. 2007; Tatineni et al. 2007; Ravichandra et al., 2008a, b; Chennupati et al., 2009) including recombinant products (Vellanki et al., 2009; Farhat-Khemakhem et al., 2012). Till date, studies with statistical optimization of parameters for production of amorphadiene have not been reported elsewhere. The present work emphasizes the key parameters (KH_2PO_4, methionine, pH and temperature) affecting amorpha-4, 11-diene

production in engineered *S. cerevisiae* strains (YCF-AD-11and YCF-AD-2), optimized using RSM.

5.2. Materials and methods

5.2.1. Microbial strain and inoculum preparation

The yeast strain *S. cerevisiae* (YCF-AD-1and YCF-AD-2) used in this study was developed in previous studies (chapter 3 and 4) and originated from *S. cerevisiae* MTCC 3157. The strain was cultured in 250 mL Erlenmeyer flasks containing 100 mL medium with the following composition (g/L): galactose, 20; $(NH_4)_2.SO_4$, 7.5; $MgSO4.7H_2O$, 0.5; trace metals solution, 2 mL; vitamins solution, 1 mL and 50 µ/L silicone anti-foam. The pH of the media was adjusted to 5.0 using 1 M NaOH and further autoclaved. Filter-sterilized vitamin solution and galactose solution were aseptically added to the sterile medium. The flasks were incubated for 24 h at 28 ± 2 ^oC at 150 rpm.

5.2.2. Amorphadiene production

The media components KH_2PO_4 and methionine were added according to experimental designs (table 5.2) to the minimal medium (Verduyn et al. 1992) which consisted of (g/L): galactose, 20; $(NH4)_2SO_4$, 5; $MgSO_4.7H_2O$, 0.5; EDTA, 0.015; $ZnSO_4.7H_2O$, 0.0045; $CoC1_2.6H_2O$, 0.0003; $MnC1_2.\ 4H_2O$, 0.001; $CuSO_4.5H_2O$, 0.0003; $CaC1_2.2H_2O$, 0.0000045; $FeSO_4.7H_2O$, 0.0003; $NaMoO_4.2H_2O$, 0.0004; $H3BO_3$, 0.001; KI, 0.0001; 25 ml/L silicone anti-foam (Merck). It was autoclaved and cooled to room temperature. The filter solution was added to this sterile medium (Dynesen et al., 1998). The pH was adjusted according to the experimental design (table 5.2 and 5.3). Aseptically, 1 % of inoculum was added to the flask, mixed thoroughly and incubated at the temperature specified in the experimental designs (table 5.1) for 80 h with 150 rpm. After cells reached OD_{600} value of 1.0, 20 % (v/v) of isopropyl myristate (Merck Millipore, Germany) was added aseptically to the media. This isopropyl myristate layer was sampled and diluted with ethyl acetate for determination of amorphadiene by gas chromatography coupled with mass spectrometry GC-MS (Agilent Technologies, USA).

5.3. Analytical methods

5.3.1. Amorpha-4, 11-diene analysis

Amorpha-4, 11-diene was analyzed by gas chromatography with flame-ionization detection (GC-FID). Samples from flasks were centrifuged at 5,000 rpm for 5 min and diluted directly into ethyl acetate and mixed for 30 min on a vortex mixer. After phase separation, 0.6 mL of the ethyl acetate layer was transferred to a capped vial for analysis. The ethyl acetate-extracted samples were analyzed using the GC-FID with a split ratio of 1:20 and separated using a DB-WAX column (50 m x 200 lm x 0.2 lm) with hydrogen as carrier gas with a flow rate of 1.57 mL/min. The temperature program for the analysis was as follows: the column was initially held at 150 ^{o}C for 3 min, followed by a temperature gradient of 5 ^{o}C per min to a temperature of 250 ^{o}C. Amorpha- 4, 11-diene peak areas were converted to concentration values from external standard calibrations using trans-caryophyllene standard (Westfall et al., 2012).

5.3.2. Experimental design and response optimization

Response optimization method was used to increase the yield of amorphadiene by using RSM. On the basis of previous experience (unpublished data), four critical parameters for amorphadiene production were selected and further evaluated for their interactive behavior by using statistical approach. The levels of the four medium variables, KH_2PO_4, 8 (x1); methionine, 2 (x2); pH, 5.0 (x3); and temperature, 31^{o}C (x4), were selected as central points, and each variable was coded at five levels, -2, -1, 0, +1 and +2, using Eq. (1). For statistical calculations, the center variable Xi was coded as xi according to the following transformation. The range and levels of the variables in coded units for RSM studies are given in table 5.1.

$$x_{i} = X_i - X_0 / \Delta X \qquad (1)$$

Where xi is the dimensionless coded value of the variable X_i, X_0 represents the value of X_i at the center point and ΔX the step change.

The behavior of the system is explained by the following quadratic model [Eq. (2)].

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \qquad (2)$$

Where Y is the predicted response, b_0 is the intercept term, bi the linear effect, b_{ii} the squared effect and b_{ij} the interaction effect.

Variables	-2	-1	0	+1	+2	ΔX
KH_2PO_4 (x_1)	0	4	8	12	16	4
Methionine, (x_2)	0	1	2	3	4	1
pH, (x_3)	4.0	4.5	5.0	5.5	6.0	0.5
Temperature, ^oC (x_4)	27	29	31	33	35	2

Table 5.1 Range and levels of the variables in coded units for Response Surface Methodology studies.

The full quadratic equation for four factors is given by the following model [Eq. (3)].

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4 \quad (3)$$

Previous experimental studies have considered such models using central composite design (CCD) (Cochran and CoxIn 1957; Montgomery 2001). In this study, a 2^4 full-factorial design with eight star points and six replicates at the central points were employed to fit the second-order polynomial model, where we carried out a set of 30 experiments. Data obtained in the above experiments were analyzed for regression, and graphical analysis using Design Expert software (Stat-Ease Inc, USA) was used for regression and graphical analysis of the data obtained. The optimal combination of variables for the amorphadiene production was analyzed using CCD experiments and was tabulated in table 5.2. Table 5.3 shows the results of CCD experiments used for studying the effect of four independent variables along with the mean predicted and experimental responses. Each response was analyzed, and a second-order regression model was developed. The model was validated in each case, and a set of optimal values were calculated.

5.4. Results and discussion

5.4.1. Multiple responses optimization and building model

RSM is a sequential and effective procedure where the primary objective of the methodology is to run rapidly and efficiently along the path of enhancement towards the general vicinity of

the optimum, identifying the optimal region for running the process (Mekala et al., 2008; Chennupati et al., 2009; Potumarthi et al., 2012). The four independent variables such as KH_2PO_4, methionine, pH and temperature were chosen for optimized production of amorphadiene and experiments were performed according to the given CCD experimental design (table 5.2 and 5.3), to obtain optimal combination of variables for the process. For each strain thirty experimental runs with different combinations of four factors were carried out. For each run, the experimental responses along with the predicted response were calculated from the regression Eq. (4) for (YCF-AD-1) and (5) for (YCF-AD-2).

$$Y = 190.777 - 2.867\,X_1 - 1.756\,X_2 - 0.123\,X_3 + 6.121\,X_4 - 0.0719\,X_1X_2 + 1.4744\,X_1X_3 - 1.1194X_1X_4 - 0.3944X_2X_3 - 2.243\,X_2X_4 + 0.0956\,X_3X_4 - 3.481\,X_1^2 - 111.521\,X_2^2 - 13.075X_3^2 - 14.7455X_4^2 \quad (4)$$

$$Y = 322.5 + 7.66X_1 + 30.74X_2 + 11.90X_3 + 27.15X_4 + 4.43X_1X_2 + 2.97\,X_1X_3 + 2.63X_1X_4 + 9.98X_2X_3 13.88\,X_2X_4 + 13.72\,X_3X_4 - 92.26\,X_1^2 - 38.70\,X_2^2 - 99.75X_3^2 - 31.73X_4^2 \quad (5)$$

Where, Y is the predicted response, and X_1, X_2, X_3andX_4 are coded values of KH_2PO_4, methionine, pH and temperature, respectively. The regression equation was used to calculate the predicted responses given in Table 5.2 and 5.3, and assessment of the predicted values with the experimental values indicated that these data were in reasonable agreement. The maximum response (205.34 mg/L (YCF-AD-1) was obtained in run number 7, and in general all the runs with middle levels of parameters gave higher yields compared to other combinations. In contrast to this YCF-AD-2 gave maximum response at duplicate experiments as 325.12 mg/L. The data were analyzed by regression analysis, and the optimized values to maximize the responses were observed withX_1, 4, X_2, 1.49, $X_3$5.47 and $X_4$33.13 for strain YCF-AD-1 and X_1, 7.5, X_2, 2.3, X_3 5.6 and $X_4$34forstrain YCF-AD-2.Suitability of the model was confirmed by the analysis of variance (ANOVA) using Design Expert software and the results are shown in Table 5.4 and 5.5.

Std. order	Run Order	x_1	x_2	x_3	x_4	AD (mg/L) Experimental	AD (mg/L) Predicted
1	14	-1	-1	-1	-1	41.98	44.31
2	10	1	-1	-1	-1	40.12	38.01
3	22	-1	1	-1	-1	46.24	46.22
4	8	1	1	-1	-1	42.37	39.63
5	30	-1	-1	1	-1	48.24	41.71
6	2	1	-1	1	-1	39.21	41.31
7	29	-1	1	1	-1	46.21	42.04
8	9	1	1	1	-1	40.35	41.35
9	26	-1	-1	-1	1	68.24	63.09
10	1	1	-1	-1	1	48.25	52.31
11	18	-1	1	-1	1	58.23	56.02
12	3	1	1	-1	1	42.58	44.96
13	21	-1	-1	1	1	58.24	60.87
14	11	1	-1	1	1	60.12	55.99
15	15	-1	1	1	1	54.27	52.23
16	25	1	1	1	1	49.5	47.06
17	4	-2	0	0	0	175	190.15
18	17	2	0	0	0	182.54	184.42
19	27	0	-2	0	0	74.21	81.00
20	20	0	2	0	0	67.25	77.49
21	19	0	0	-2	0	174.35	177.81
22	16	0	0	2	0	164	177.57
23	24	0	0	0	-2	159.77	169.90
24	28	0	0	0	2	175.24	182.14
25	7	0	0	0	0	205.34	190.77
26	23	0	0	0	0	201.27	190.77
27	12	0	0	0	0	198.24	190.77
28	6	0	0	0	0	195.28	190.77
29	13	0	0	0	0	197.32	190.77
30	5	0	0	0	0	198.25	190.77

Table 5.2 Design of experiments by Central Composite Design for Response Surface Methodology studies for strain YCF-AD-1.

Std. order	Run Order	x_1	x_2	x_3	x_4	AD (mg/l) Experimental	AD (mg/l) Predicted
1	21	-1	-1	-1	-1	26.98	30.23143
2	25	1	-1	-1	-1	24.12	25.48184
3	29	-1	1	-1	-1	38.24	35.12129
4	13	1	1	-1	-1	50.37	48.0842
5	2	-1	-1	1	-1	5.24	0.67462
6	27	1	-1	1	-1	10.21	7.822537
7	7	-1	1	1	-1	42.21	45.48698
8	18	1	1	1	-1	68.35	70.3474
9	4	-1	-1	-1	1	26.24	24.06629
10	30	1	-1	-1	1	34.25	29.8392
11	1	-1	1	-1	1	83.23	84.48365
12	17	1	1	-1	1	103.58	107.9691
13	3	-1	-1	1	1	48.24	49.39198
14	22	1	-1	1	1	64.12	67.0624
15	28	-1	1	1	1	151.27	149.7318
16	9	1	1	1	1	189.5	185.1148
17	5	-2	0	0	0	220.12	222.5819
18	24	2	0	0	0	235.12	237.8986
19	8	0	-2	0	0	248.23	253.0597
20	23	0	2	0	0	314.12	314.5308
21	14	0	0	-2	0	209.12	210.853
22	19	0	0	2	0	231.14	234.6475
23	10	0	0	0	-2	261.15	263.6197
24	12	0	0	0	2	315.15	317.9208
25	16	0	0	0	0	325.12	322.4997
26	26	0	0	0	0	325.12	322.4997
27	15	0	0	0	0	325.12	322.4997
28	11	0	0	0	0	325.12	322.4997
29	20	0	0	0	0	325.12	322.4997
30	6	0	0	0	0	325.12	322.4997

Table 5.3 Design of experiments by Central Composite Design for Response Surface Methodology studies for strain YCF-AD-2

ANOVA of the quadratic regression model suggests that the both model were significant with a computed F values of 101.6917, 1874. 25 strain YCF-AD-1 and AD-2 respectively

and a P<F value less than 0.05 in case of both strains. A lower value for the coefficient of variation suggests higher consistency of the experiment and in the both cases we obtained CV values of 9.19 % and 2.49% demonstrates a greater reliability of the trials. R^2 is the coefficient of variance of response under test and whose values are always between 0 and 1; closer the value of R^2 to 1, the stronger is the statistical model and better is the prediction of response (Myers and Montgomery 1995). The coefficient of determination (R^2) for response of amorphadiene in both cases 0.9896 and 0.9994 respectively (Table 5.4 and 5.5), indicating that the both statistical model can explain 98.96 % (YCF-AD-1) and 99.99% (YCF-AD-2) of variability in the response and only 1.04 %, 0.11% of the variations for amorphadiene not explained by the models. The adjusted R^2 value corrects the R^2 value for the sample size and for the number of terms in the model.

Table 5.4 Model summary and analysis of variance for the quadratic model for YCF-AD-1

Source of variations	Sum of squares	Degree of freedom	Mean square	*F* value	Probability (*p*)
Regression	132761.320	14	9482.95	101.69	< 0.0001
Residual	1398.780	15	93.25		
Total	134160.099	29			

R=0.9947, R^2 =0.9896, Adjusted R^2 =0.9798, CV=9.19%

Table 5.5 Model summary and analysis of variance for the quadratic model.

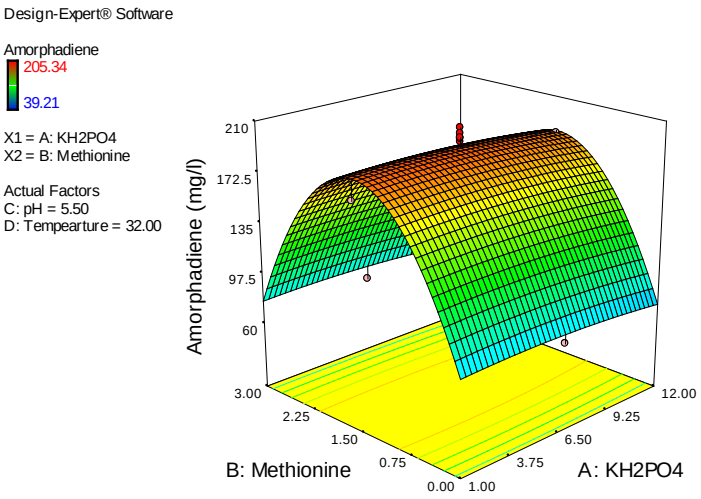
Source of variations	Sum of squares	Degree of freedom	Mean square	*F* value	Probability (*p*)
Regression	442221.3295	14	31587.24	1874.25	< 0.0001
Residual	252.80	15	16.85		
Total	442474.1295	29			

R=0.9996, R^2 =0.9994, Adjusted R^2 =0.9989, CV=2.49%

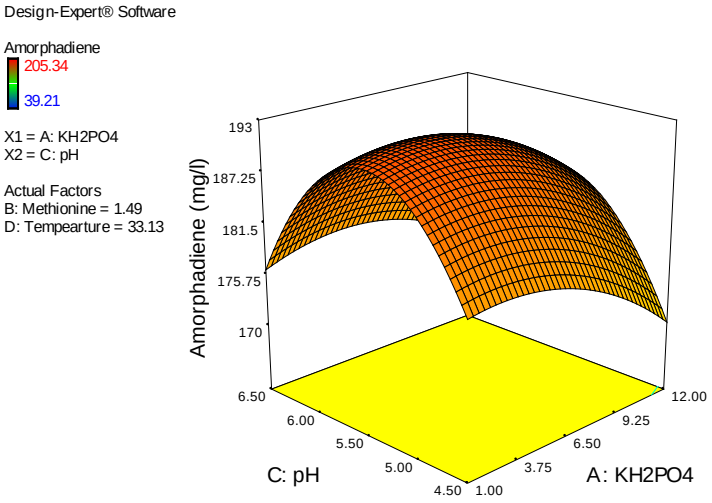
The value of the adjusted determination coefficient (Adj R^2) for amorphadiene in both cases good, supporting the significance of these developed models (Cochran and CoxIn 1957). The significance of individual variables can be evaluated from their P values, with the more significant terms having a lower P value (table 5.4 and 5.5). The values of P>F less than 0.05 indicate that the model terms are significant and in YCF-AD-1 case X_4, X_2^2 ,X_3^2 and X_4^2

were found to be significant model terms and there were no significant interactions between the parameters. In contrast to this in case of YCF-AD-2, all four variable shown significant effect on the response and P>F less than 0.05 for X_2, X_3, X_4, indicating the interactions between the parameters.

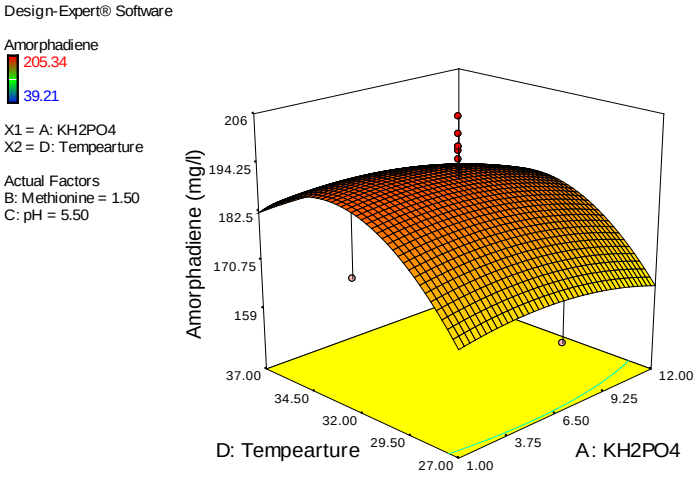
Surface plots are generally the graphical representation of the regression equation for identifying the optimal levels of each parameter for attaining the maximum response (amorphadiene) production. fig. 5.1 and 5.2 a-f shows the response surfaces obtained for the interaction effects of tested variables. In each response graph, the effect of the two variables on amorphadiene production was shown when the other two variables were kept constant. Fig. 5.1 and 5.2 shows the interaction relationship between the two independent variables, namely, KH_2PO_4/methionine and their effects on amorphadiene production. It was observed from fig.5.1a that amorphadiene synthesis was significantly affected by methionine concentration. Amorphadiene synthesis was increased with increase in methionine concentration up to 1.5 mM and further increase in methionine concentration did not show any influence on amorphadiene production, whereas the further addition resulted in decreased production. The same pattern was observed in other graphs (fig. 5.1d, e). This indicates that the increase in the methionine concentration tightly regulates the engineered repressible methionine promoter in *S. cerevisiae* by limiting the conversion of farnesyl pyrophosphate into squalene (Asadollahi et al. 2008). Studies on the effect of varied methionine concentration (0-2 mM) with engineered yeast reported approximately 125 mg/L of amorphadiene with 0.2 mM methionine concentration. In previous studies, 1.5 and 2 mM concentrations of methionine were considered for the production of plant sesquiterpenes in yeast during batch and fed-batch operations, respectively (Asadollahi et al. 2008; Paradise et al. 2008). But these reported studies were not statistically optimized for methionine concentration; in the present work, it was observed that 1.49 mM of methionine was the optimum concentration with combinations of other optimum variables leading to synthesis of 191.5 mg/L of amorphadiene. The effect of KH_2PO_4 did not have significant effect in combination with methionine concentration, but there was significant effect observed in combination with the other two variables, temperature and pH (fig. 5.1a, b and c).


Design-Expert® Software
Amorphadiene
205.34
39.21
X1 = A: KH2PO4
X2 = B: Methionine
Actual Factors
C: pH = 5.50
D: Tempearture = 32.00
Amorphadiene (mg/l)
210
172.5
135
97.5
60
3.00
2.25
1.50
0.75
0.00
B: Methionine
1.00
3.75
6.50
9.25
12.00
A: KH2PO4

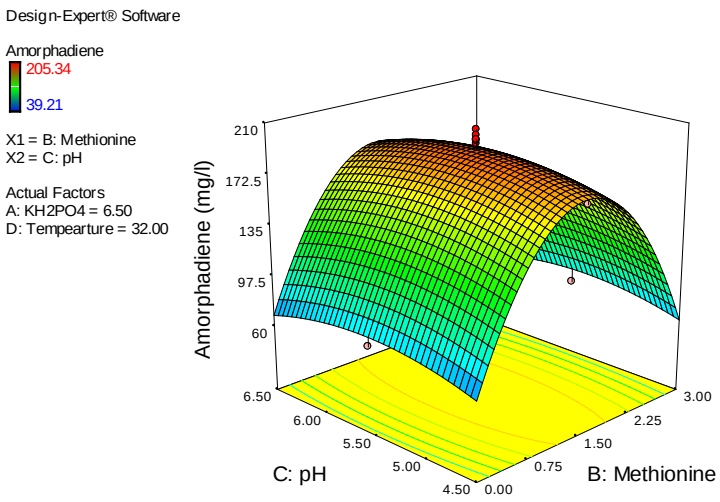
a)


Design-Expert® Software
Amorphadiene
205.34
39.21
X1 = A: KH2PO4
X2 = C: pH
Actual Factors
B: Methionine = 1.49
D: Tempearture = 33.13
Amorphadiene (mg/l)
193
187.25
181.5
175.75
170
6.50
6.00
5.50
5.00
4.50
C: pH
1.00
3.75
6.50
9.25
12.00
A: KH2PO4

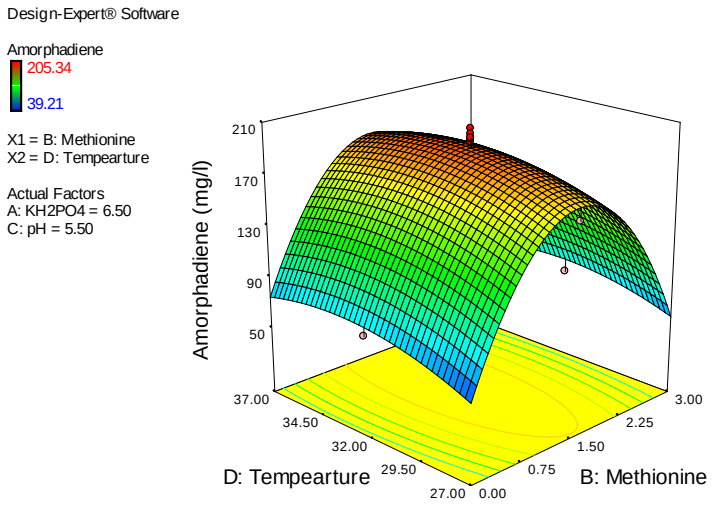
b)


Design-Expert® Software
Amorphadiene
205.34
39.21
X1 = A: KH2PO4
X2 = D: Tempearture
Actual Factors
B: Methionine = 1.50
C: pH = 5.50
Amorphadiene (mg/l)
206
194.25
182.5
170.75
159
37.00
34.50
32.00
29.50
27.00
D: Tempearture
1.00
3.75
6.50
9.25
12.00
A: KH2PO4

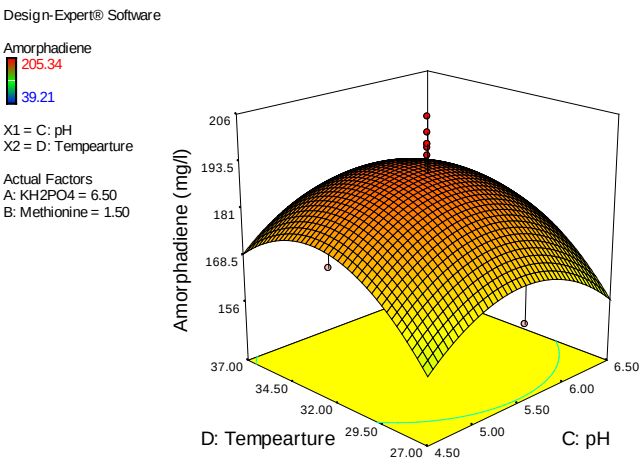
c)



d)

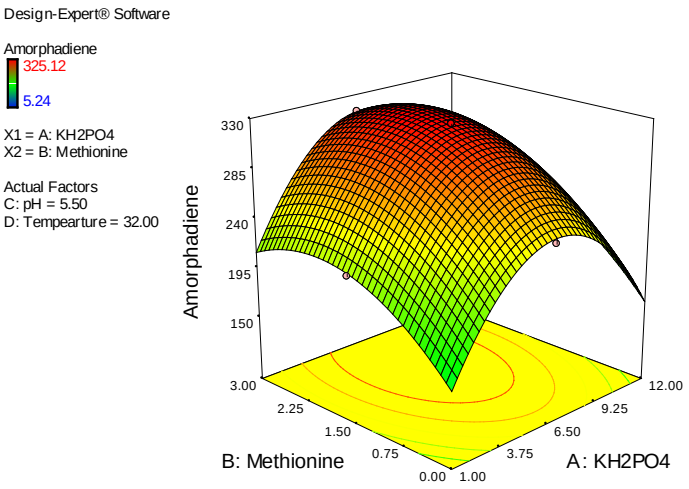


e)

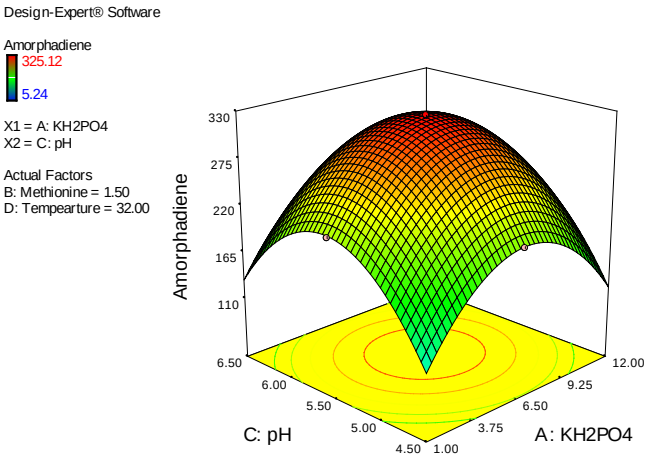


f)

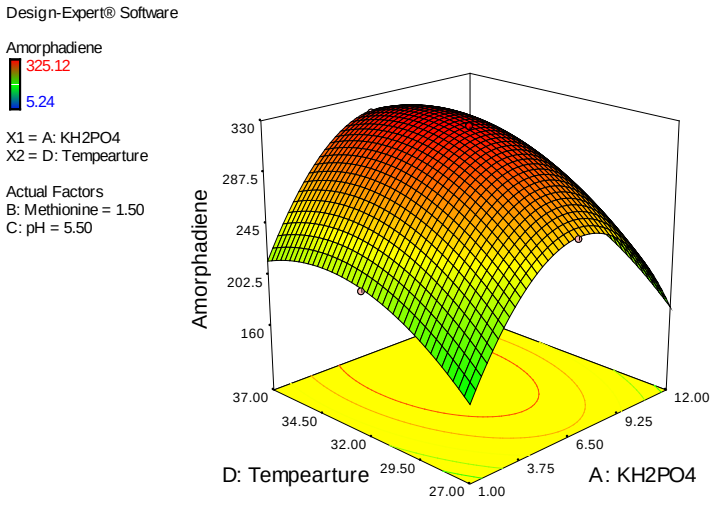
Figure. 5.1 a–f 3-D surface and contour plot of amorphadiene production by *S. cerevisiae* (YCF-AD-1) (mg/L): the effect of two variables while the other two were held at 0 level


Design-Expert® Software
Amorphadiene
325.12
5.24
X1 = A: KH2PO4
X2 = B: Methionine
Actual Factors
C: pH = 5.50
D: Tempearture = 32.00
Amorphadiene
330
285
240
195
150
3.00
2.25
1.50
0.75
0.00
B: Methionine
1.00
3.75
6.50
9.25
12.00
A: KH2PO4

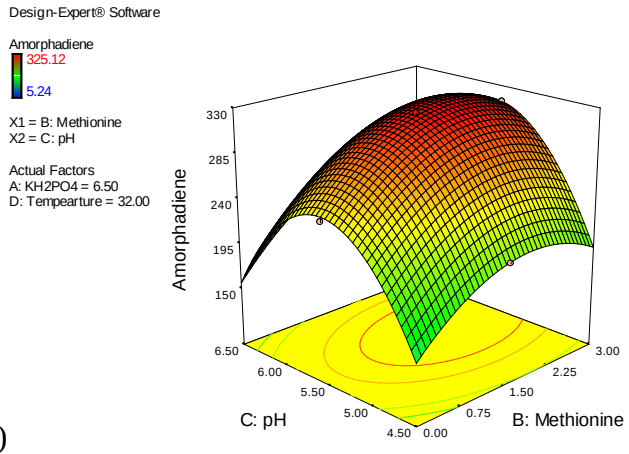
a)


Design-Expert® Software
Amorphadiene
325.12
5.24
X1 = A: KH2PO4
X2 = C: pH
Actual Factors
B: Methionine = 1.50
D: Tempearture = 32.00
Amorphadiene
330
275
220
165
110
6.50
6.00
5.50
5.00
4.50
C: pH
1.00
3.75
6.50
9.25
12.00
A: KH2PO4

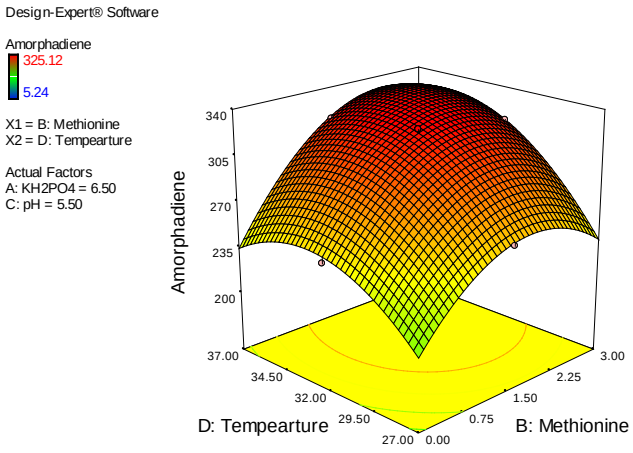
b)


Design-Expert® Software
Amorphadiene
325.12
5.24
X1 = A: KH2PO4
X2 = D: Tempearture
Actual Factors
B: Methionine = 1.50
C: pH = 5.50
Amorphadiene
330
287.5
245
202.5
160
37.00
34.50
32.00
29.50
27.00
D: Tempearture
1.00
3.75
6.50
9.25
12.00
A: KH2PO4

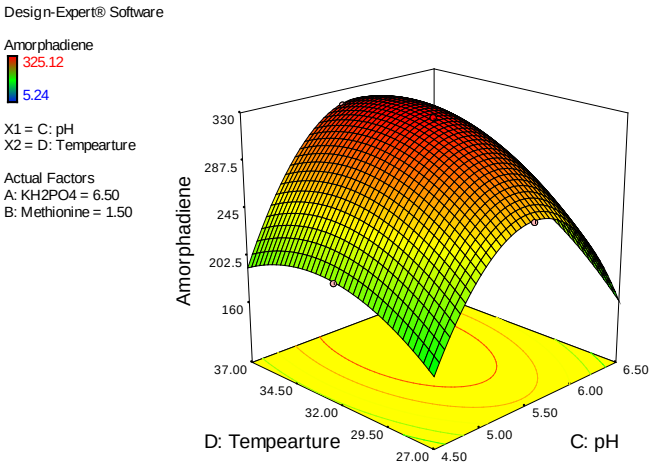
c)



d)



e)



f)

Figure. 5.2 a–f 3-D surface and contour plot of amorphadiene production by *S. cerevisiae* (YCF-AD-2) (mg/L): the effect of two variables while the other two were held at 0 level

There was a significant increase in amorphadiene production with increase in KH_2PO_4 concentration up to 6.5 g/L and further increase in its concentration did not show any significant improvement in amorphadiene production. But in case of YCF-AD-2 increase methionine concentration (fig 5.2 a, d, e) improved AD production and change in pH and KH_2PO_4 also had a significant effect on the response. Previous studies reported that low phosphate concentration improved amorphadiene production, which may be by limiting the growth and channeling the carbon flux towards amorphadiene production (Westfall et al. 2012). In this study, 4.01 (YCF-AD-1) 7.35 g/L (YCF-AD-1) of KH_2PO_4 was the recommended concentration for the optimized production of amorphadiene in combination with other optimized parameters. fig. 5.1 b, d, f and 5.2 a, d, f shows the effect of pH on amorphadiene production in combination with KH_2PO_4 and temperature. There is increase in amorphadiene production with increase in pH and the maximum production was at pH 5.5, 5.9 in cases of two strains. In previous studies, the production of plant sesquiterpenes in yeast was carried out at pH 6.50, 5 ± 0.5, 5.0 for shake flasks, batch and fed-batch cultivation, respectively (Asadollahi et al., 2008), whereas the enzyme responsible for amorphadiene production (amorphadiene synthase) showed optimum activity at varied pH 6.5-7.5 levels in *Artemisia annua* (Bouwmeester et al., 1999; Mercke et al., 2000; Picaud et al., 2005; Picaud et al., 2007). In this study, *S. cerevisiae* it was observed 5.5 and 5.9 as optimum pH and the present model gave 5.47 and 5.96 as an optimum value along with other optimal parameters. The effects of temperature in response to combination with other variables, KH_2PO_4, methionine and pH, are shown in fig. 5.1 c, e, f. and 5.2 c, e, f. At low temperature (27 ^oC), amorphadiene synthesis was very less and increased with increment in temperature up to 33 ^oC in both cases. There was a rapid increase in amorphadiene production in combination with KH_2PO_4 and pH, whereas in combination with methionine the effect of temperature was not significant. Based on these models, the optimal combination of all parameters for YCF-AD-1 and YCF-AD-2 are KH_2PO_4, 4.01, 7.35; methionine, 1.49, 2.31; pH, 5.47, 5.96; and temperature 33.13 ^0C, 35.76 ^0C with a predicted response value of 192.119 mg/L and 327.56 mg/L respectively.

Experiments conducted with the same optimal conditions, such as KH_2PO_4, 4.0, 7.3; methionine, 1.49, 2.3; pH, 5.4, 5.9; and temperature 33 ^oC, 35 ^oC, yielded 191.5 mg/L and 317.5 mg/L of amorphadiene, which is very close the predicted response. Finally, these

results suggest all four variables had significant effect on amorphadiene production in YCF-AD-2. But in case of YCF-AD-1 only methionine had a significant impact on AD production. This could be due to the accumulation of farnesol and squalene in YCF-AD-1, whereas in case of YCF-AD-2 increase in methionine (2.3 mM), not shown any adverse effect, this effect may be due to the immediate conversion of FPP in to AD, which avoids the loss of FPP towards other intermediates. Hence, the maximum amorphadiene production can be achieved with a relatively limited number of experimental runs using the appropriate statistical design and optimization technique.

5.5. Conclusion

The use of RSM with a full-factorial rotatable CCD for determination of optimal medium and physical parameters for amorphadiene production was demonstrated using the essential parameters. The use of this methodology will be successful for any combinational analysis, in which an analysis of the effects and interactions of many experimental factors are required. Rotatable central composite experimental design maximizes the amount of information that can be obtained while limiting the number of individual experiments. Thus, smaller and less time-consuming experimental designs could generally be sufficient for optimization of many such fermentation processes (Tatineni et al., 2007). The superiority of terpenoids has expanded their utility from pharmaceutical to fragrances, including biofuel industries. Significant efforts have been made for establishing microbial cell factories for the production of a wide variety of high value-added chemicals. However, there are some difficulties for the large-scale production of these chemicals. In addition to the synthetic biology and metabolic engineering approaches, statistical optimization methods will provide insights into the production of high value-added chemicals. In the present study, the overall view on the optimization of the process using essential parameters for amorphadiene production provides insights into the process development and further scaling-up process. The results of ANOVA and regression of the second order model showed that the linear effects of temperature and the interactive effects of the three variables, methionine, pH and temperature, were significant for amorphadiene production. Among these three variables, methionine has a more significant interactive effect. Finally, this study concludes by stating that the optimization of amorphadiene production was by the second-order model, and

ANOVA requires optimal conditions of KH_2PO_4, 4.01, 7.35; methionine, 1.49, 2.31; pH, 5.47, 5.96; and temperature 33.13 ^{0}C, 35.76 ^{0}C for YCF-AD-1 and YCF-AD-2 respectively.

CHAPTER 6

Development of Petri-Net Based *In Silico* Model for Improved Production of FPP

CHAPTER 6

DEVELOPMENT OF PETRI-NET BASED *IN SILICO* MODEL FOR IMPROVED PRODUCTION OF FPP

6.1. Introduction

Over the last few years, usage of terpenoids has increased exponentially in medicine and aromatics. They offer a viable commercial alternative to chemically synthesized products of similar applications. Terpenoids are typically extracted from plants (Dubey et al., 2003) and microorganisms. Terpenoids, being secondary metabolites, produced in very small quantities and scale up with existing plant and microorganism strains (Chang and Keasling, 2006) are not cost effective. With commercial and medicinal uses of plant terpenoids on the rise, there is a need to increase the yield of terpenoids biosynthesis. Development of simulated models will diminish the troubleshooting and points the bottlenecks during the experimentation by understanding the relation between the complex biological pathway structures and dynamics of the system (Kitano, 2002).Various computational, mathematical, and Perti-Net models are developed in order to understand the relation between numerous pathways. This gives thorough observation of troubleshoots, which solved easily or should find the alternatives (Sweetlove et al., 2003; Sweetlove and Fernie, 2005; Hawari and Mohamed-Hussein, 2010). In addition to this, flux analysis enables us to estimate and enumerate the flow of carbon and energy within a given system of bioprocesses. Flux analysis helps not only to build a better target model but also to eliminate unwanted side effects that might potentially be encountered in engineered organisms.

Biological pathways fall under three categories: gene regulatory networks, metabolic pathways, and signaling pathways (Koch and Heiner, 2008) whose behavior widely described using methods like ordinary differential equations (ODEs), partial differential equations (PDEs) and non-ODE approaches (Gilbert et al., 2006; Goryanin et al., 1999). The present model deals with metabolic pathways and explains the improved production of farnesyl pyrophosphate (FPP), an intermediate for all major isoprenoids or terpenoids

synthesis through two independent pathways (mevalonate (MEV) and non mevalonate (MEP)) in yeast. The MEV pathway is frequently found in the eukaryotic cytoplasm, while the MEP pathway is observed in the eubacteria such as *Escherichia coli* and *Streptomyces species* (Rohdich et al., 2002; Voynova et al., 2004) as well as in plant plastids. The MEP pathway is not found in animals or fungi, but both pathways are operational in higher-level plants such as *Arabidopsis thaliana* and *Helianthus annuus* and *Artemisia annua* (Schulte et al., 2000)

In this case study, FPP biosynthetic network designed using hybrid functional Petri-Net with extension (HFPNe) and it is derived from traditional Petri-Net theory (Petri, 1962) and allows easy modeling with graphical approach of various types of entities in the networks together. A Petri-Net is a graphical diagram consisting of circles and lines representing the current status of a rule-based state-dependent procedural system. For this reason, a Petri-Net is also called a place transition network. To support more complicated networks with varying degrees and kinds of control structures, HFPNe are used (Matsuno et al., 2003).These networks support concepts essential to pathway design like quantitative (equation or value based) induction, inhibition, and repression. Petri-Net offers a versatile graphical language to design, integrate, and simulate multiple pathway networks (Koch et al., 2005). Petri-Nets are suitable because of their intuitive graphical representation and their capabilities for mathematical analyses. Prospect to find out new option for amplifying the production of FPP was our major objective, which can be further utilized to synthesize variety of isoprenoids having medical as well as industrial importance.

6. 2. Resources and Methods

6.2.1. Pathway databases

The information and data desirable to build this model was obtained from biological databases: KEGG, BRENDA, ENZYME, IUBMB, MetaCyc, and PATHWAYDATABASE (Kanehisa and Goto, 2000; Kanehisa et al., 2006; Kanehisa et al., 2008; Chang et al., 2009). These databases include information on the substrates, products, enzyme consumption, and production rates involved in the MEV and MEP pathways. Along with the above data, stoichiometric and enzyme mechanisms are also taken into consideration in developing the

dynamic model. This information was used to design the model layout and parameter assignments for each of the HFPNe elements.

6.2.2. Model pattern design

The HFPNe elements (place, transition, and arc (fig.6.1) represent the metabolites and processes that comprise the MEV and MEP biosynthetic pathways. Continuous places correspond to the substrates, products, inhibitors, and enzymes in both the pathways. While continuous transitions correspond to biological processes such as synthesis, catalysis, and glycosylation, generic places represent the on/off switches and parameter modulators. The normal arc connects places (enzyme, substrate, and product) to transitions while inhibitory arcs connect inhibitors to their potential inhibited processes. All these entities were arranged according to the reaction stoichiometry and enzymatic mechanism in their order of incidence in the respective pathways. Each of the HFPNe elements possesses diverse features that characterize its function in the model. Parameter values are assigned to each of these attributes to control the behavior of the model during simulation. These values are generated based on the biological data obtained from databases.

6.2.3. Model development

Developmental stages as well as the simulation and validation processes of the model were carried out using Cell Illustrator 5.0 www.cellillustrator.com (developed by Human genome center, Institute of medical science. The University of Tokyo, Japan).

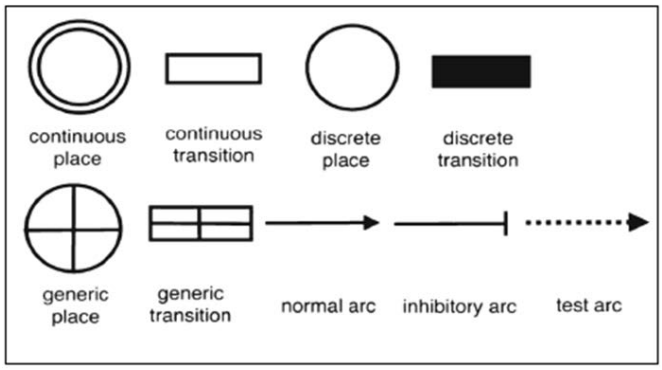


Figure 6.1 Basic entities of HFPNe method

6.2.4. Simulation and validation

Simulations were carried out with four different conditions:

1. Conventional production of FPP through the MEV pathway.

2. Conventional production of GPP2 through the MEP pathway. 3. Combined production of FPP through integrated MEV and MEP pathways.

4. Production of amorphadiene through integrated MEV and MEP pathways.

Simulation results were calculated as concentration (unit) versus time (pt) graphs. Petri net time (pt) indicates virtual Petri net time that do not match to real time; concentration also is given in general concentration units (unit) that do not specifically correspond to standard concentration units such as mM and μM. The changes in metabolite concentrations (unit), over time predicted by each simulation were confirmed against known biological data to identify breaks and variations. Unmatched biological processes were re-examined and previous developmental steps (pattern design and parameter assignment) were repeated. The simulation process was then executed and revalidated using cell illustrator. The entire process was carried out repetitively in order to rule out contradictions and obtain a best possible system.

6.3. Results and Discussion

6.3.1. Outlines of the Model

All of the metabolites involved in the pathways are represented using HFPNe elements and are interconnected to each other based on their reaction stoichiometry and enzymatic mechanisms. The network consists of 84 continuous transitions representing various reactions and production/degradation processes; 61 places representing 56 metabolites; three on/off switches; and two parameter modulators (fig. 6.2). Eighteen main enzymatic reactions are involved in this pathway network. MEV pathway network consists of ten main enzymatic reactions whereas MEP pathway network contains eight main enzymatic reactions. Conventional Production of FPP through the MEV Pathway the MEV pathway consists of ten main enzymatic reactions (fig.6.3). In the mevalonate pathway, three molecules of acetyl-coenzyme-A (CoA) couple to yield 3- hydroxy-3-mehtylglutarylCoA (HMG-CoA),which is

subsequently reduced by the enzyme HMG-CoA reductase to yield mevalonicacid (MVA). In the next two steps, mevalonate kinase and mevalonate5-phosphatekinase catalyze MVA to form mevalonate5-diphosphate, which is subsequently de carboxylated to yield isopentenyl pyrophosphate (IPP) (Miyano, 2004; Newman and Chappell, 1999). The mevalonate pathway provides IPP for the synthesis of some sesquiterpenes, sterols and triterpenes and is localized in the cytosol .The behavior of each reaction in the pathway is described using HFPNe element (Gilbert et al., 2006; Nagasaki, 2004). All simulations carried out in this study are based on known information retrieved from literature and database like KEGG, BRENDA and MetaCyc. Usually, in yeast, FPP produced through the MEV pathway can be assumed as test or control for our experiment. Most important step of this pathway is synthesis of mevalonate catalyzed by HMGR. This situation yields 259.91U (fig. 6.4) of FPP per 100 pt (sec). At the end of the simulation, the universal precursors IPP and DAMP were accumulated in low concentrations, 3.21U and 0.023U, respectively

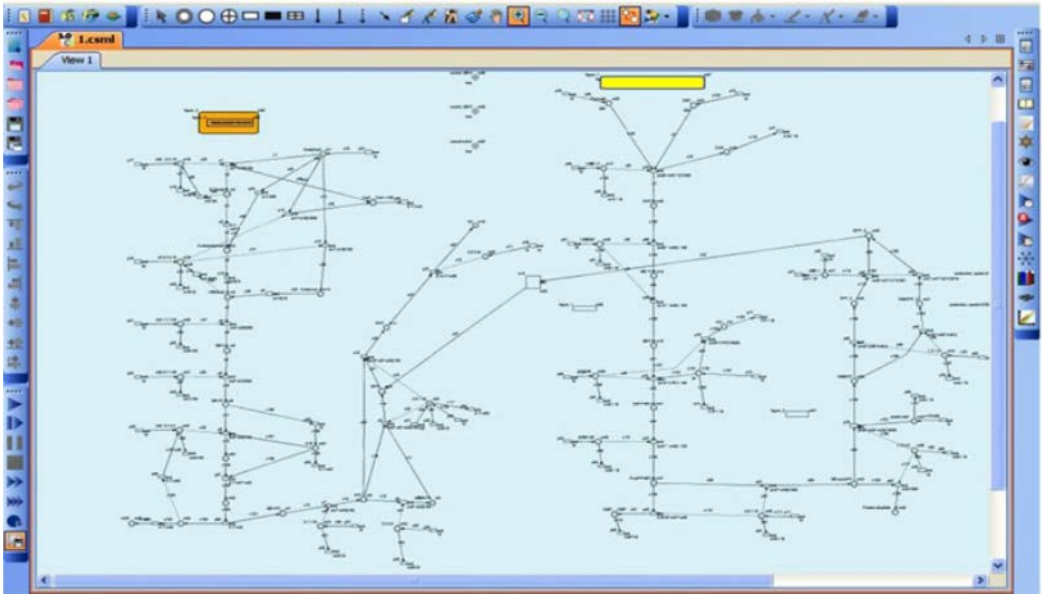


Figure 6.2 Outline of the integrated MEV and MEP pathway. The MEV pathway is on the left and the MEP pathway on right and both are connected through GPP2 flux (MEP) directed towards GPP (MEV). All metabolites are symbolized using continuous places. Each transition entity represents a biological process.

6.3.2. Conventional Production of GPP2 through the MEP Pathway

MEP pathway usually presents in higher plant plastids and eubacteria are involved in the production of mono- and diterpenes. MEP pathway starts from transketolase type condensation of pyruvate and glyceraldehyde-3-phosphate to form Deoxylulose5-phosphate (DXP), followed by the rearrangement and reduction of DXP to MEP. MEP transforms to the cytidine 5-diphosphate derivative and sequential phosphorylation at C,and cyclization leads to 2-C-methylerythritol-2,4-cyclodiphosphate. Loss of CMP forms MEcycPP which is further converted to 1-hydroxy-2methyl-2-(E)-Butenyl 4-diphosphate. IPP and DMAPP are produced as final products. DXP synthase is the first enzyme and rate limiting enzyme in this pathway which leads to the production of DXP. The constructed MEP Perti net model produced 229.0U of the GPP2 per 100 pt (fig. 6.5). At the end of the simulation, precursors accumulated. Unconverted or accumulated IPP2 is more (99.0U/100 pt) than DAMP2 concentrations (30U/100 pt).

6.3.3. Combined Production of FPP through Integrated MEV and MEP Pathways

MEV and MEP pathways are integrated by routing the GPP2 flux towards GPP which finally leads to the improved production of FPP. The fifth reaction of MEV pathway acts in two-substrate/two-product sequential enzymatic mechanism. Studies have shown that high concentration of ATP halts the production of MEVPP and thus blocks the entire pathway from continuing to synthesize other intermediates.

$$\boldsymbol{MEVP + ATP} \; \boldsymbol{---} \; \boldsymbol{MEK}(\boldsymbol{EC\ 2.7.4.2}) \; \boldsymbol{----\rightarrow} \; \boldsymbol{MEVPP + ADP}$$

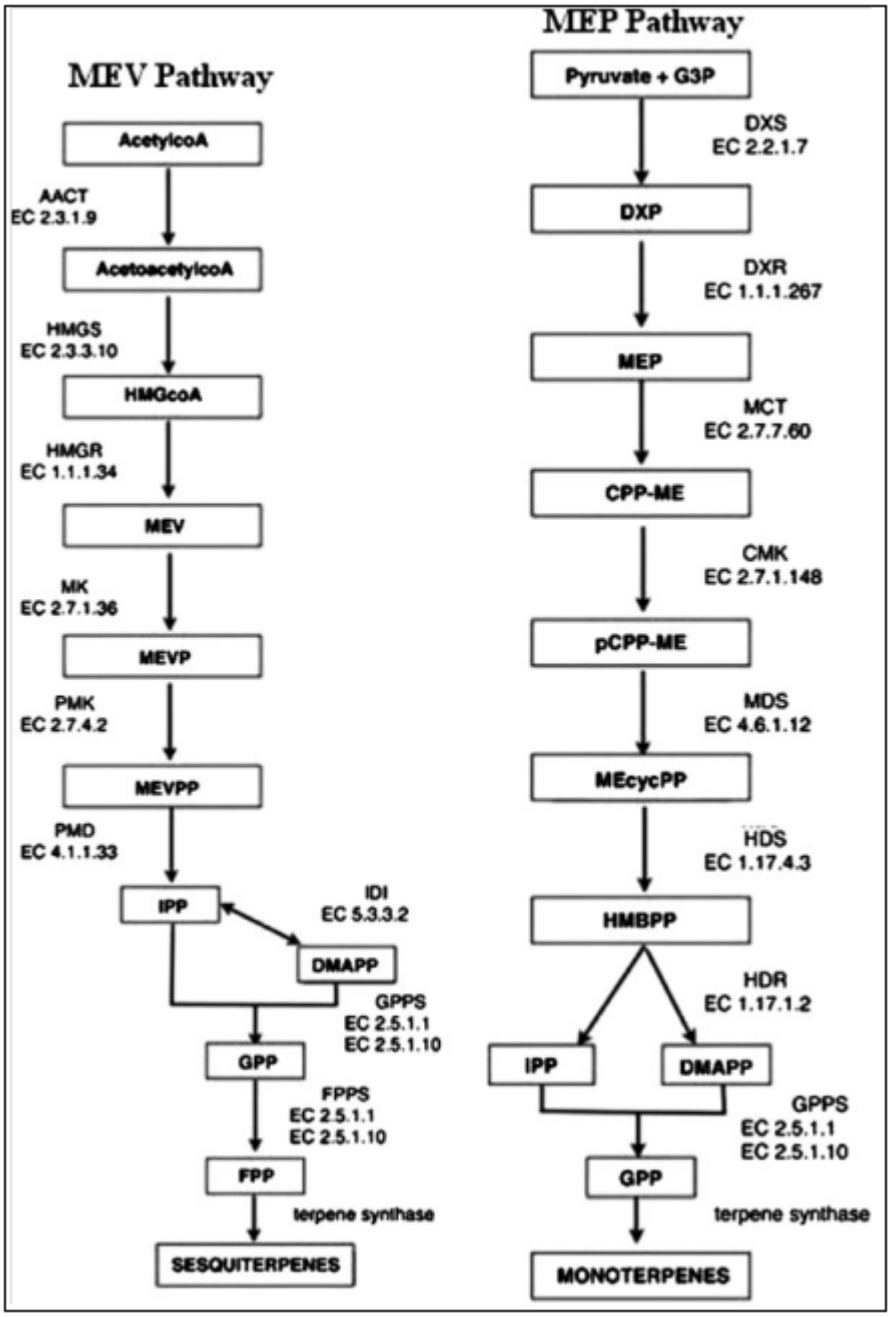


Figure 6.3 Sequential enzymatic steps involved in synthesis of sesqui- and monoterpenoids independently by MEV and MEP pathways. HMG-CoA Hydroxymethyl glutaryl-CoA, MEV Mevalonate, MEVP Mevalonate phosphate, MEVPP Mevalonate diphosphate, G3P Glyceraldehyde-3-phosphate, DXP Deoxylulose-5-phosphate, MEP Methylerythritol 4-phosphate, CPP-ME 4(cytidine 5′diphospho)2-C-methyl-D-erythritol, pCPPME 4(cytidine 5′diphospho)2-C-methyl-D-erythritol 2-phosphate, MEcycPP 2-c-methylerythritol-2,4-cyclodiphosphate, HMBPP 1-hydoxy-2-methyl-2-(E)-butenyl-4-diphosphate, IPP Isopentenyl pyrophosphate, DMAPP Dimethylallyl pyrophosphate, GPP

Geranyl pyrophosphate, FPP Farnesyl Pyrophosphate, AACT Aceto acetyl transferase, HMGS HMG-CoA synthase, HMGR HMG-CoA reductase, MK Mevalonate kinase, PMK Phosphomevalonate kinase, PMD MEVPP decarboxylase, IDI Isopentenyl diphosphate isomerase, GPPS GPP synthase, FPPS FPP synthase, DXS DXP synthase, DXR deoxyxylulose phosphate reductoisomerase, MCT MEP cytidyltransferase, CMK CPP-ME kinase, MDS MEcycPP synthase, HDS HMBPP synthase, HDR HMBPP reductase

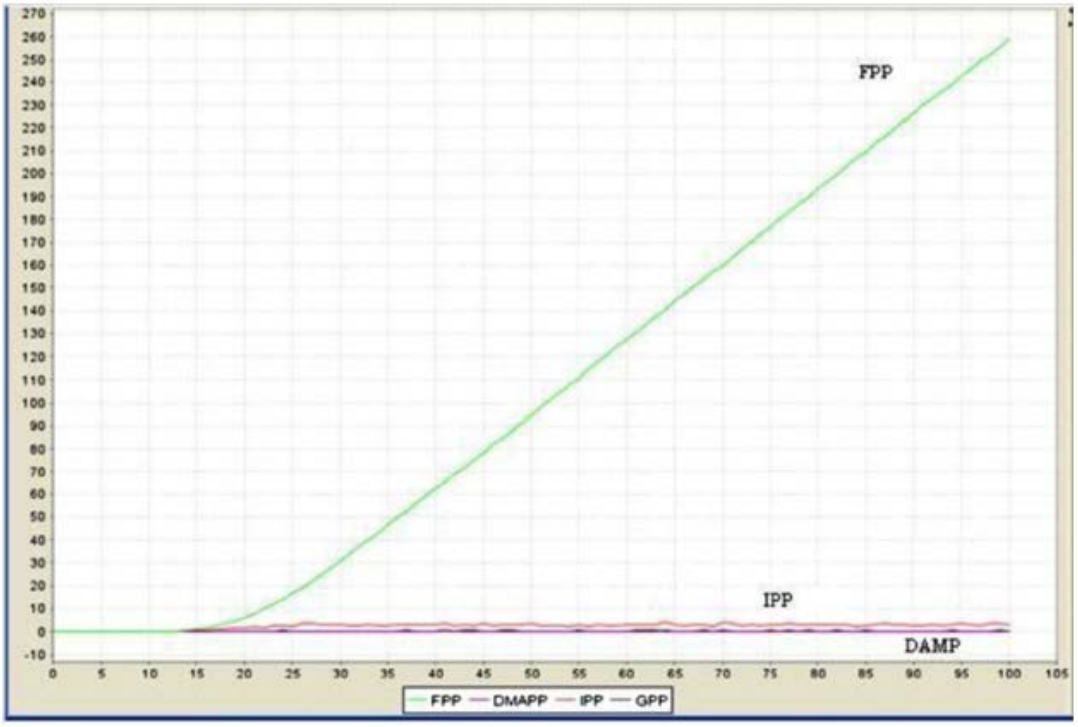


Figure 6.4 Concentration changes of main precursors in MEV pathway over time (pt).Universal precursors DAMP and IPP concentrations are completely converted to GPP and further to FPP(concentration in Unit (U) on Y-axis whereas time (pt) is on X-axis

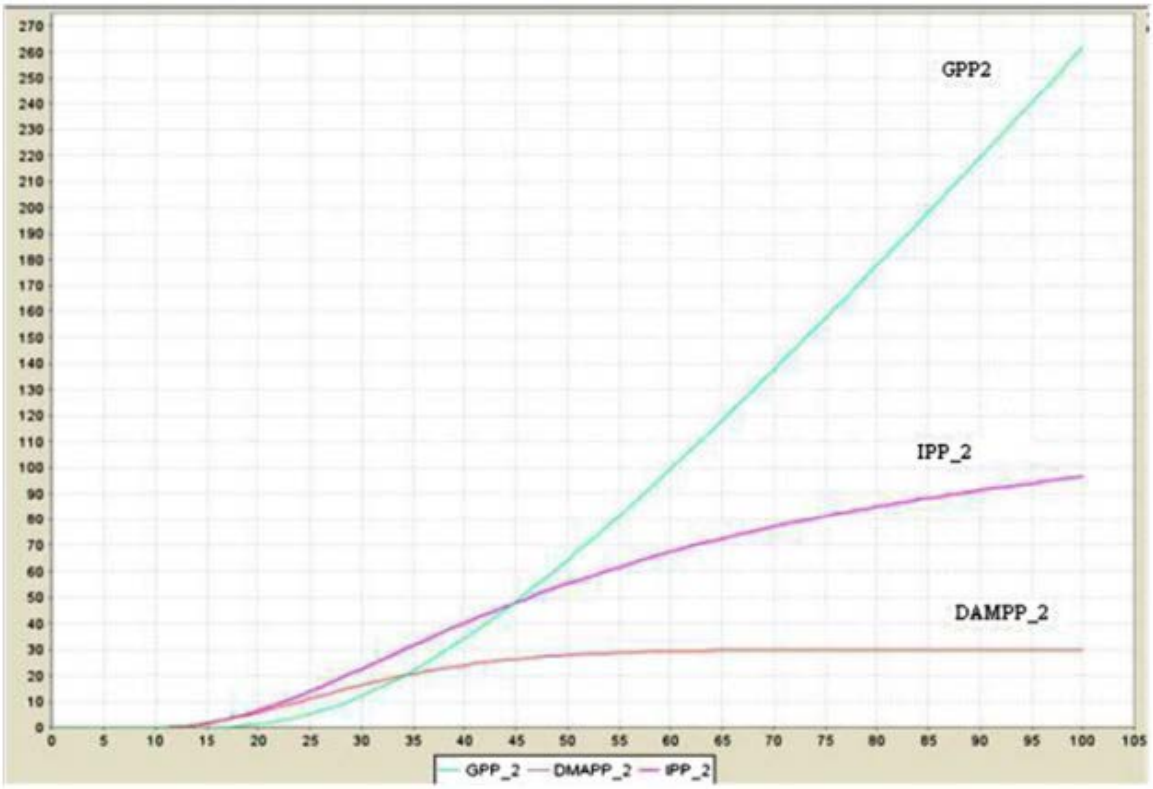


Figure 6.5 Concentration changes of metabolites in MEP pathway over time. Concentration in Unit (U) on Y-axis whereas time (pt) is on X-axis

ATP acts as a competitive inhibitor; this obstructs the downstream reactions in the pathway. However, the increase in the concentration of MEVP shows that the inhibitory effects of ATP could be reversed. Higher concentrations of MEVP inhibit the synthesis of ATP, lowering the concentration of ATP to a manageable level allows the synthesis of MEVPP well as downstream reactions. This model serves as a tool for better understanding of the reactions involved in both pathways and how they affect each other. Diversion of GPP2 flux (MEP pathway) towards MEV pathway increased FPP production approximately two fold, 259.91u/100 pt (sec) to 431.168 u/ 100 pt which will increase the sesquiterpene production and concentration of GPP and GPP2, 0 and 4.11U/pt, respectively (fig.6.5 and fig. 6.6).

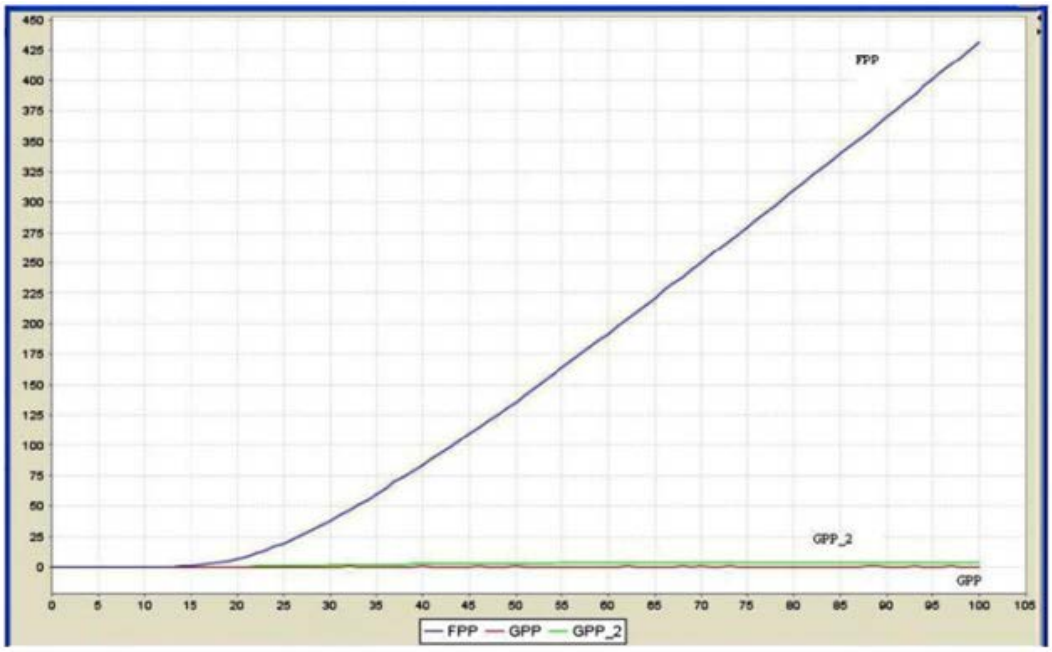


Figure 6.6 Concentrations of FPP, GPP and GPP2 after the integration of MEV and MEP pathways. After the integration of two pathways, FPP concentration was increased, whereas GPP, GPP2 converted to FPP

6.3.4 Production of Amorphadiene through Integrated MEV and MEP Pathways

FPP is a common precursor for sesquiterpenoids like artemisinin which is well-known for its antimalarial, anti-cancer, and anti-viral activities. FPP is converted to amorphadiene (AD) (fig.6.7) by amorphadiene synthase (EC 4.3.2.24), which is an immediate precursor of artemisinin. Kinetic data of the enzyme obtained from the biological databases and this additional reaction was added to this model yielding 436.5U/pt of AD which is approximately two folds greater than that of 258.5U/pt AD given exclusively by MEV pathway (fig. 6.8 and 6.9). Improved production of AD will have great impact on artemisinin productivity, which is not affordable to most of the malarial patients. No kinetic data is available for cytochrome P450 mono oxygenase (CYP71AV1) which converts AD to artemisinin in order to design the model for enhanced production of artemisinin.

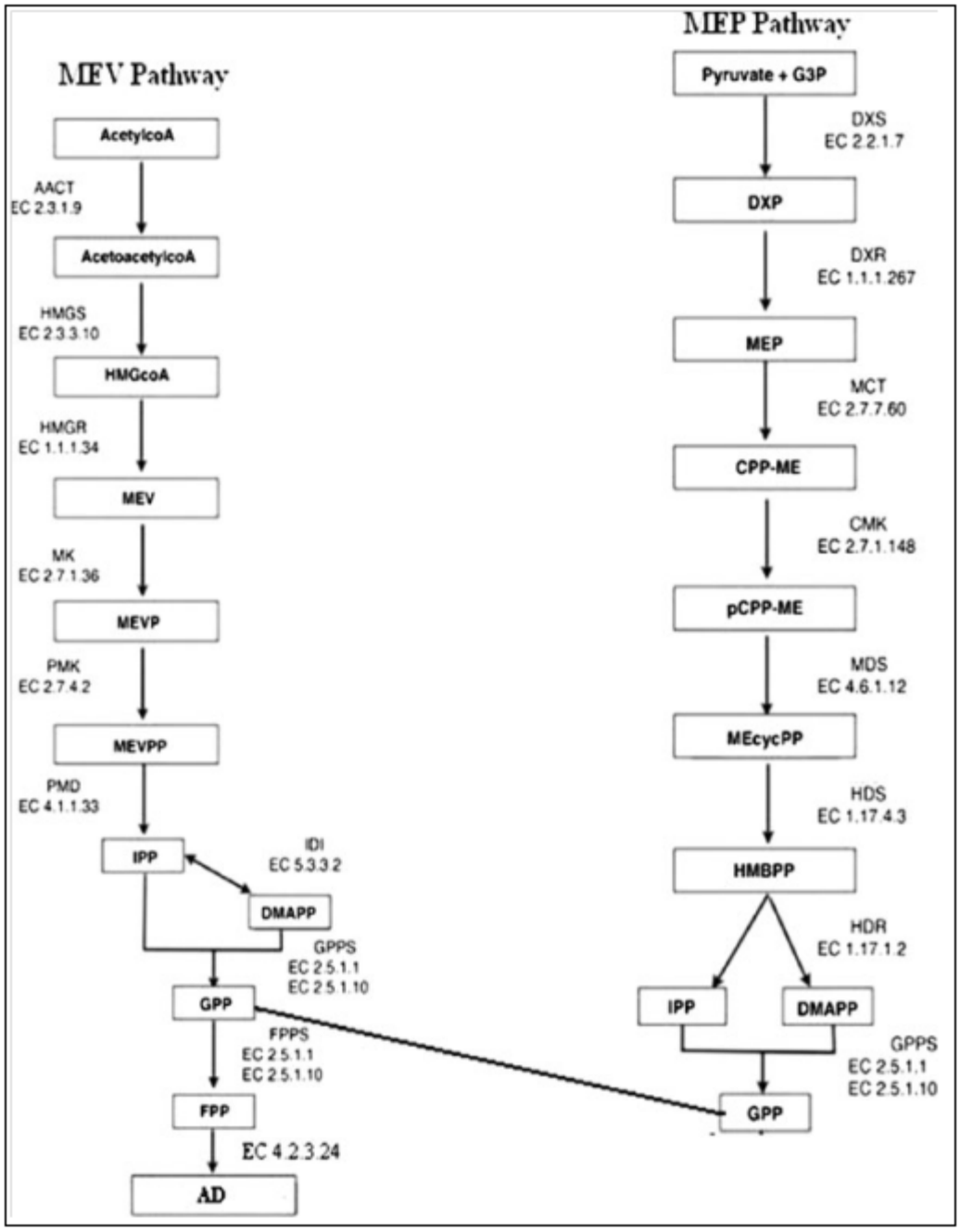


Figure 6.7 Integrated MEV and MEP pathways for synthesis of AD. GPP flux from MEP pathway is directed towards MEV pathway which will successfully increase the FPP synthesis further it was converted to AD, an immediate precursor of artemisinin (Abbreviations are same as in Fig. 3)

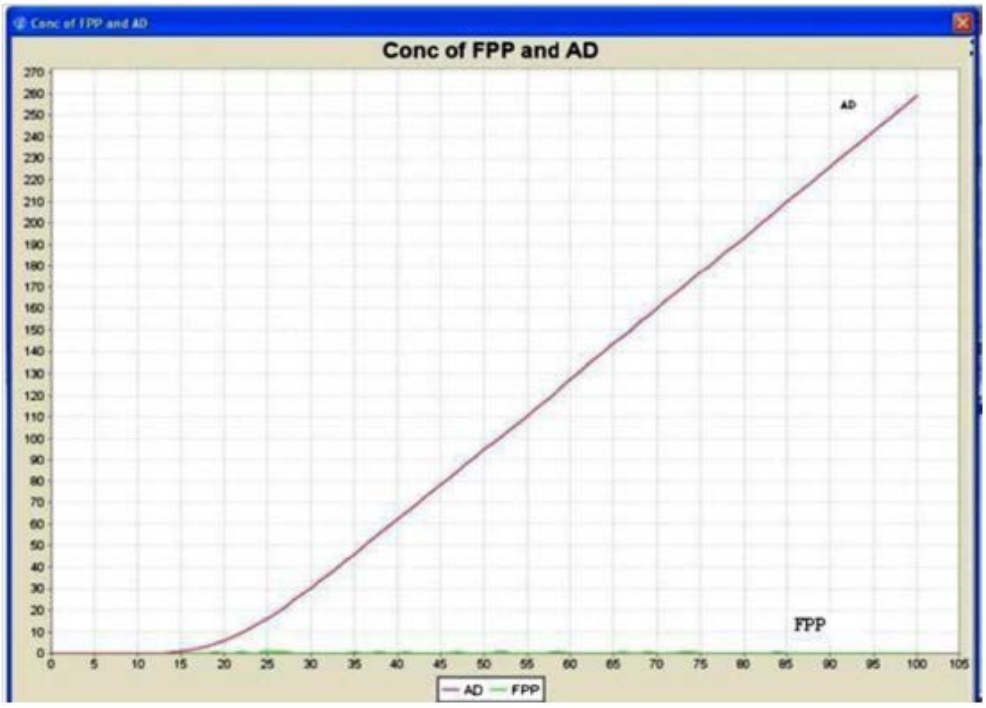


Figure 6.8 Concentrations of FPP and AD in MEV pathway .Total FPP converted to AD, no accumulation FPP observed

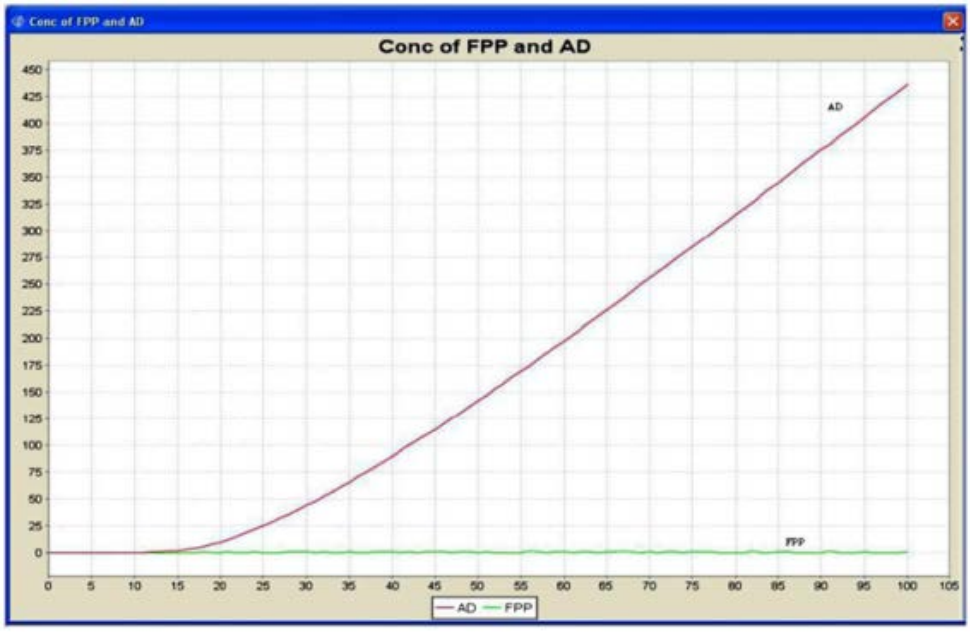


Figure 6.9 Concentration of FPP and AD in integrated MEVand MEP pathways. Total available FPP is converted to AD and its concentration increased by two folds

6.4. Conclusions

HFPNe technique enables complicated modeling tasks to be viewed and solved in a graphical manner. The model serves as a tool to better understand the reactions involved in combinatorial FPP synthesis and how they interact to each other. This gives needful information for finding the alternatives for production of isoprenoids. Apart from this, most of the acetyl-CoA in the MEV pathway is either transported from the cytosol into the mitochondrion for oxidization by TCA cycle or utilized in fatty acid and ergosterol synthesis. Channeling more acetyl-CoA into the mevalonate pathway by limiting acetyl-CoA transport to the mitochondrion or inhibiting ethanol and fatty acid synthesis, it can be possible to further increase the FPP production which is having tremendous significance in chemical industry and medicine.

CONCLUSIONS

CONCLUSIONS

In this study improved production of amorphadiene in the yeast *S. cerevisiae* reported by using combination of metabolic engineering and enzyme fusion technology. The corresponding amorphadiene synthase expressed in yeast and was shown to be functional. The availability of FPP which is the precursor for all sesquiterpene synthases was enhanced by replacement of the native *ERG9* promoter with a regulatable *MET3* promoter and repressing the promoter with the presence of 2 mM methionine. The ergosterol content was drastically reduced as a consequence of this repression and improved the amorphadiene production by approximately 2-folds. Metabolism of methionine during the growth phase relieved the repression of *ERG9*, which was accompanied by a reduction in the rate of amorphadiene synthesis. Further experiments showed that it was possible to improve the amorphadiene production by supplying more methionine during fermentation. Under these conditions the final titer of amorphadiene and farnesol reached to 17.1 and 20.2 mg/L, respectively. Further over expression of *tHMG1* improved AD production by 2-folds. In addition to this, expression of additional copy of *tHMG1* added 2-folds improvement in AD synthesis. It was observed that subsequent repression of *ERG9* leads to the accumulation of FPP derived farnesol and strain YCF-4 produced 30.15 mg/L of farnesol and disclosed the possibility of pooling the available FPP towards amorphadiene. To overcome the natural loss of the metabolic intermediate FPP and its conversion towards farnesol, a chimeric protein with small Gly-Ser-Gly linker was constructed as *ADS-FPPS* and *FPPS-ADS*. Strain YCF-4 expressing *ADS-FPPS* protein produced 238.85 mg/L whereas YCF-5 produced only 83.45 mg/L due to the limited access of active site to the substrate. The fusion phenomenon of the enzyme further confirmed by isolation and purification of FPPS, ADS, ADS-FPPS, and FPPS-ADS proteins. Analysis of SDS-PAGE revealed that molecular weights (105 kD) of fused proteins were almost equal to the sum of the individual enzymes ADS (63 kD) and FPPS (40 kD). The final strain constructed through combination of metabolic engineering and enzyme fusion technology improved the improved the AD production by 42-fold in the yeast *S. cerevisiae* compared to the control strain.

From the previous observations noticed, that engineered yeast, mevalonate pathway is tightly regulated by methionine and phosphate levels along with other physical parameters such as pH and temperature. Till date, studies with statistical optimization of parameters for production of amorphadiene have not been reported elsewhere. To take the advantage of statistical techniques to improve the AD production we used to improved strains for optimization (YCF-3 (renamed as YCF-AD-1) and YCF-4 (renamed as YCF-AD-2)). We emphasizes on the key parameters (KH_2PO_4, methionine, pH and temperature) affecting amorpha-4, 11-diene production in engineered *S. cerevisiae* strain (YCF-AD-1 and AD-2) and optimized using Response Surface Methodology. The results of ANOVA and regression of the second order model showed that the linear effects of temperature and the interactive effects of the three variables, methionine, pH and temperature, were significant for amorphadiene production. Among these three variables, methionine has a more significant interactive effect. The optimization of amorphadiene production was by the second-order model, and ANOVA requires optimal conditions of: KH_2PO_4, 4.0; methionine, 1.49; pH, 5.4; temperature 33 ^{0}C for strain YCF-AD1 and produced 191.5 mg/L of AD whereas finally improved strain (YCF-AD-2) synthesized 317.5 mg/L of AD at KH_2PO_4, 7.3; methionine, 2.3; pH, 5.9; temperature 35 ^{o}C.

Further to take the advantage of FPP flux, we hypnotized to integrate the native MEV and non- native MEP pathways in yeast. Since both pathways have common intermediate IPP and DAMP, FPP levels significantly improved, which can directed towards improved production of AD. Since integration of pathways and expression of MEP pathway genes in yeast complex and time consuming. We concentrated on development of simulated models, which will diminish the troubleshooting and bottlenecks during the experimentation by understanding the relation between the complex biological pathway structures and dynamics of the system. In this study a model of farnesyl pyrophosphate (FPP) biosynthetic network developed using hybrid functional Petri net with extension (HFPNe) which is derived from traditional Petri-net theory and allows easy modeling with graphical approach of various types of entities in the networks together. By integrating these two pathways in yeast, it augmented the FPP synthesis approximately two folds higher (431.16 U/pt) than in MEV pathway alone (259.91 U/pt) by using HFPNe technique. Further enhanced FPP levels converted to AD by amorphadiene synthase gene yielding 436.5 U/pt of AD which

approximately two-folds higher compared to the AD (258.5 U/pt) synthesized by MEV pathway exclusively. Simulation and validation processes performed using these models were reliable with identified biological information and data.

Finally we conclude that enzyme fusion technology will be an additive step to the metabolic engineering, and combination of metabolic engineering and enzyme fusion technology improves the flux of the intermediate metabolites in order to expand the heterologous components production.

FUTURE PROSPECTS

FUTURE PROSPECTS

Terpene biosynthesis is a complicated process associated with two independent biosynthetic pathways, the mevalonate (MEV) pathway and the methyl erythritolphosphate (MEP) pathway. These two pathways are distinct in terms of organism, cellular localization and products as well as in the metabolites and precursors involved. The classical pathway, the MEV pathway, was thought to be the only pathway involved in terpenoid biosynthesis until the MEP pathway was discovered in the early 1990s (Lange et al. 1998; Hahn et al. 2001).

The MEV pathway usually operates in the cytoplasm of eukaryotic organisms, whereas the MEP pathway takes place in the plastids of eubacteria such as *Escherichia coli* and *Streptomyces species*, as well as in the plastids of plants. However, both pathways are operational in higher-level plants such as *Arabidopsis thaliana* and *Helianthus annuus* and there is cross-talk mechanism between the two pathways for exchange of precursor molecules (Hoeffler et al. 2002; Rohdich et al. 2002; Voynova et al 2004). The type of terpene produced also depends on the route of biosynthesis. The MEV pathway is more likely to produce sesqui and triterpenes, whereas the MEP pathway produces mono- and diterpenes (Eisenrich et al. 2001; Rohdich et al. 2001; Dubey et al. 2003).

Based on these native pathways in higher plants we are proposing a new method for enhanced production of terpenoid intermediate FPP by combinatorial engineering of these two pathways in microorganisms like yeast. Further combinatorial expression of *tHMGR* and up regulation of *UPC2 alleles* with fusion technology accelerate the terpenoid production. Further channeling acetyl-CoA into the mevalonate pathway will make it possible to further increase the FPP production which is having tremendous significance in chemical industry as well as in medicine. Some our future forecasts includes:

1. Carbon flux analysis of MEV and MEP pathways after their integration in yeast.
2. Effect of MEP pathway influence on the yield of FPP

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APPENDIX

APPENDIX

1. Revival of freeze - dried cultures

Cultures were revived as per the instruction given by the suppliers (MTCC, IMTECH); briefly the ampoules containing freeze dried cultures were surface sterilized with alcohol (70%) around the marked and wrapped with sterilized cotton and broken at marked area. Cotton plug inside the ampoule gently removed and 0.4ml of malt yeast broth (*S. cerevisiae* MTCC 3157) and LB Medium supplemented with 50.0 µg/ml of Naladixic acid (E. coli MTCC 1652) were added to make a suspension culture. Further it was used for streaking in respective agar plates or suspension culture in the respective broth and incubated at 30^0C (*S. cerevisiae*) or 37^0C (*E. coli*), respectively.

2. Revival of stab cultures

Plasmid pRS425ADS (Addgene #20119) was received as a bacterial stab culture and stored at 4°C upon receipt. Culture was revived as per the instructions given by the suppliers (Addgene, USA) on LB agar plates (5g NaCl; 5g tryptone; 2.5g yeast extract; 7.5g agar; 500mL dH2O and 100 µg/mL Ampicillin) and incubated at 37^0C for overnight (12-18h) to get the single colonies. Using sterile pipette or tooth pick single colony from the LB plates was inoculated in to LB broth (4g NaCl; 4g Tryptone; 2g Yeast Extract; 400mL dH_2O and 100 µg/mL Ampicillin) and incubated at 37^0C for 12-18h in a shaking incubator.

3. Preservation of cultures for long term storage

Yeast cultures were grown in normal YPD medium for 28-48 h and mixed with 50% glycerol (w/v) equally mixed thoroughly and stored at $–80^0$C in 2 mL screw top tube (Open Wet Ware). *E. coli* carrying the plasmid pRS425ADS (Addgene #20119) was grown on LB broth for 12-18h and 500 µL of the overnight culture mixed with 500µL of 50% glycerol in a 2 mL screw top tube and gently mixed and stored at -80^0C (Addgene).

4. PCR amplification kit composition

Kit Components

1.	*TaKaRaTaq*DNA Polymerase*[1](5 units/μl)	250 units
2.	dNTP Mixture*[2] (2.5 mM each)	1.28 mL
	10X PCR	
3.	Buffer	1 mL
	100 mM Tris-HCl (pH8.3 at 25^0C) 500 mM KCl 15 mM $MgCl_2$	
4.	10X PCR Buffer (−) (Mg^{2+} Free)	1 mL
	100 mM Tris-HCl (pH8.3 at 25^0C) 500 mM KCl	
5.	MgCl2(25mM)	1 mL
6.	Control Template (1 μg/mL λDNA)	100 μL
7.	Control Primer 1*[3] (20 pmol/μL)	50 μL
8.	Control Primer 2*[3] (20 pmol/ μL)	50 μL
9.	Control Primer 3*[3] (20 pmol/ μL)	50 μL
10.	λ*Eco*T14 I Marker (100 ng/ μL)*[4]	40 μL
11.	6X Loading Buffer*[5]	1 μL

∗ 1 : *TaKaRaTaq* (5 units/μL)

The sequence of Control Primer

Control Primer 1: 5'-GATGAGTTCGTGTCCGTACAACT-3'

Control Primer 2: 5'-CCACATCCATACCGGGTTTCAC-3'

Control Primer 3: 5'-GGTTATCGAAATCAGCCACAGCGCC-3'

5. SDS-PAGEReagents

30% acrylamide/bisacrylamide (37.5:1) aqueous solution (stored in the dark)

1.5 M Tris-HCl buffer (pH 8.8)

0.5 M Tris-HCl buffer (pH 6.8)

10% ammonium persulfate (APS) solution (always should be prepared freshly)

TEMED

1X Tris-glycine-SDS Buffer

(10X buffer diluted to 1X concentration prior use

I. Resolving Gel Preparation

Component	Volume:10 mL resolving gel 10%
Deionized water	4.13 mL
30% acrylamide/ bisacrylamide	3.3 mL
1.5 M Tris-HCl containing 0.4% SDS, pH 8.8	2.3 mL
10% APS	60µL
TEMED	15 µL

II. Stocking Gel Preparation

Component	Volume:5 mL stacking gel 5%
Deionized water	2.1 mL
30% acrylamide/ bisacrylamide	2.5 mL
1.5 M Tris-HCl containing 0.4% SDS, pH 8.8	1.56 mL
10% APS	31.25µL
TEMED	18.75 µL

Sample buffer:

100 mM Tris, pH 6.8,

2% SDS,

5% ß- mercaptoethanol,

15% glycerol

6. Yeast Transformation

Components

- 2 x 1 mL 10 mg/mL Yeastmaker Carrier DNA, denatured

- 20 µL pGBT9 (positive control plasmid), 0.1 µg/µ
- 2 x 50 mL 50% PEG 3350 (Sigma, Cat. No. P4338)
- 50 mL 1 M LiAc (10X)
- 50 mL 10X TE Buffer
- 50 mL YPD Plus Liquid Medium

1.1X TE/LiAc Solution

- Prepare fresh just prior to transformation using the stock solutions provided. Combine 1.1 mL of 10X TE Buffer with 1.1 ml of 1 M LiAc (10X). Bring the total volume to 10 mL using sterile, deionized H_2O.

PEG/LiAc Solution (polyethylene glycol 3350/lithium acetate)

- Prepare fresh just prior to transformation using the stock solutions provided.

	Final Conc.	To prepare 10 mL of solution
PEG 3350	40%	8 ml of 50% PEG 3350
TE buffer	1X	1 ml of 10X TE Buffer
LiAC	1X	1 ml of 1 M LiAc(10X)

- 0.9% (w/v) NaCl Solution Dissolve 0.9 g of NaCl in 100 mL of deionized H_2O and filter-sterilize the solution.

A. Protocol: Preparation of Competent Yeast Cells

1. Materials:

Yeastmaker Yeast Transformation System 2 [provided with the Two-Hybrid Kit or available separately (Cat. No. 630439)]

1x TE/LiAc (Section IV)

YPDA agar plates

YPDA liquid medium

Appropriate SD selective medium

Frozen stock of yeast cells (S. cerevisiae)

Sterile, deionized water

- Streak a YPDA agar plate with your chosen yeast cells from a frozen yeast stock. Incubate the plate upside down at 30^0C until colonies appear (~3 days).

- Inoculate one colony (diameter 2-3 mm, < 4 weeks old) into 3 ml YPDA medium in a sterile 15 mL culture tube.
- Incubate at 30^0C with shaking at 250 rpm for 8-12 h.
- Transfer 5 µl of the culture to 50 ml of YPDA in a 250 mL flask.
- Incubate shaking until the OD 600 reaches 0.15–0.3 (16–20 h).
- Centrifuge the cells at 700 g for 5 min at room temperature. Discard the supernatant and resuspend the pellet in 100 mL of fresh YPDA.
- Incubate at 30^0C until the OD600 reaches 0.4-0.5 (3-5 h).
- Divide the culture into two 50 mL sterile Falcon conical tubes. Centrifuge the cells at 700 g for 5 min at room temperature. Discard the supernatant and resuspend each pellet in 30 mL sterile, deionized H_20.
- Centrifuge the cells at 700 g for 5 min at room temperature. Discard the supernatant and resuspend each pellet in 1.5 mL of 1.1xTE/LiAc.
- Transfer the cell suspensions to two respective 1.5 mL microcentrifuge tubes; centrifuge at high speed for 15 sec.
- Discard the supernatant and resuspend each pellet in 600 µL of 1.1xTE/LiAc. The cells are now ready to be transformed with plasmid DNA.

B Protocol: Transformation of Competent Yeast Cells

Materials:

- Yeastmaker Yeast Transformation System 2
- Competent Yeast Cells (Section VI.A)
- PEG/LiAc (Section IV)
- 0.9% (w/v) NaCl
- DMSO

Combine the following in a pre-chilled, sterile tube:

Plasmid (100 ng) and Yeastmaker Carrier DNA (5 µL) (denatured**; 10 µg/µL)

Add **competent cells** and gently mix.	50 µL
Add **PEG/LiAc** and gently mix.	500 µL

Incubate at **30° C**.	30 min
Add **DMSO** and mix.	20 µL
Place the tube in a **42° C** water bath.	15 min
Centrifuge to pellet yeast cells.	high speed
Remove the supernatant and resuspend in YPD Plus Medium.	1 mL
Incubate at **30° C with shaking**	90 min
Centrifuge to pellet yeast cells. For speeds and times,	high speed
Discard the supernatant and resuspend in 0.9% (w/v) NaCl	1 mL

Media Composition

Luria Broth (LB) (500 mL):

5 g Tryptone

2.5 g Yeast extract

5 g NaCl

500 mL H_2O

YPD Media

20 g/L Difco peptone

10 g/L Yeast extract

20 g/L Agar (for plates only)

SD medium

6.7 g Yeast nitrogen base without amino acids

20 g Agar (for plates only)

850 ml H_2O

100 ml of the appropriate sterile 10X Dropout Solution

LIST OF ACCOMPLISHMENTS BASED ON THE THESIS

Research Papers:

1. **Rama Raju Baadhe**, Naveen Kumar Mekala, Satwik Reddy Palagiri and Sreenivasa Rao Parcha. Development of petri net-based dynamic model for improved production of farnesyl pyrophosphate by integrating mevalonate and methylerythritol phosphate pathways in yeast, ***Appl. Biochem. Biotechnol***, 167:1172–1182 2012.

2. **Rama Raju Baadhe**, Naveen Kumar Mekala, Sreenivasa Rao Parcha and Prameela Devi. Combination of ERG9 repression and enzyme fusion technology for improved production of amorphadiene in yeast. ***Journal of Analytical Methods in Chemistry*** **http://dx.doi.org/10.1155/2013/140469** Volume 2013, Article ID 140469, 8 pages.

3. **Rama Raju Baadhe**, Naveen Kumar Mekala, Sreenivasa Rao Parcha and Prameela Devi. Optimization of sesquiterpene production in engineered yeast by response surface methodology. **3 Biotech DOI 10.1007/s13205-013-0156-y**

4. **Rama Raju Baadhe**, Naveen Kumar Mekala, Sreenivasa Rao Parcha and Prameela Devi. Combination of metabolic engineering and enzyme fusion technology for improved production of sesquiterpens in yeast. (Under preparation)

Review Papers:

1. **Rama Raju Baadhe**, Naveen Kumar Mekala, Sreenivasa Rao Parcha and Prameela Devi. Genetic engineering to metabolic engineering-New trends in production of natural complex molecules. (Under preparation)

Book chapters:

1. **RR Baadhe**, NK Mekala, SR Parcha, Principles of Systems and synthetic biology In ***Recent Developments in Biotechnology*** by Eds: J. N. Govil, Studium Press LLC, Houston, USA 2013 (In press).

International conferences

1. **Rama Raju Baadhe**, Naveen Kumar Mekala, Sreenivasa Rao Parcha. "Genetic Engineering to Metabolic Engineering –New Trends in Production of Natural Complex Molecules". ***International Conference on Biotechnology: A global Scenario*** organized by Kakatiya University Warangal, during 2-4 November 2010.

2. **Rama Raju Baadhe**, Naveen Kumar Mekala, Sreenivasa Rao Parcha. "Development of petri net-based dynamic model for improved production of farnesyl pyrophosphate by integrating mevalonate and methylerythritol phosphate pathways in yeast" ***New Horizons in Biotechnology 2012***, Trivandrum during November 21-24, 2011.

3. **Rama Raju Baadhe**, Naveen Kumar Mekala, Sreenivasa Rao Parcha "Combination of Metabolic engineering and Enzyme fusion technology for improved production of Amorphadiene in Yeast***" Metabolic Engineering-IX***, ***Biarritz, France*** during 3-7 June, 2012.

4. **Rama Raju Baadhe**, Naveen Kumar Mekala, Sreenivasa Rao Parcha "Metabolic engineering and enzyme fusion technology for improved production of amorphadiene in yeast" International Conference on Industrial Biotechnology -2012, Punjab University, Patiala, during 21-23 November, 2012.

Awards and Grants

1. **Young Scientist Award**

   Young Scientist Award-2013 (Biology) received from **K. V. Rao Scientific Society** Hyderabad.

2. **International Travel Grant for Young Scientist-2012.**

   From DST and DBT to present a paper in "Metabolic Engineering-IX" conference at Biarritz, France.

BIO-DATA

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