Metabolic Engineering of *Saccharomyces cerevisiae* for the Production of Isopentenol, a Valuable Biochemical and Potential Biofuel

by

Diana Phuong Hong Pham

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

Department of Biochemistry
University of Alberta

Abstract

The rising concern about limited fossil fuel resources and the environmental factors associated with it has motivated the development of alternative fuels. Ethanol is currently the primary substitute to gasoline, however the intrinsic properties of ethanol have limited its ability to fully replace petroleum based gasoline. Microbial production of higher chain alcohols is currently being explored to circumvent the problems associated with ethanol. Isoprenol is a 5-carbon alcohol that has a higher energy density and a lower water affinity when compared to ethanol. These characteristics allow isoprenol to fit well with existing fuel infrastructure. However, reported yields of isoprenol remain low and the process thus requires further optimization to increase production. Microbial production of isoprenol has been limited to synthetic pathways introduced into *Escherichia coli*. The approach in this study is to utilize the endogenous, *Saccharomyces cerevisiae*'s isoprenoid pathway and apply metabolic engineering approaches to manipulate gene expression and protein stability to increase flux through the pathway, maximizing isopentenyl pyrophosphate production. Isopentenyl pyrophosphate is an intermediate in the isoprenoid pathway that can be dephosphorylated to produce isoprenol. NudF, an exogenous phosphatase, with potential activity towards isopentenyl pyrophosphate, was expressed in *Saccharomyces cerevisiae* to produce isoprenol. Optimization of the isoprenoid pathway in *Saccharomyces cerevisiae* and expression of NudF did not lead to isoprenol production but did yield production of isopentyl alcohol. This work emphasizes the existence of inherent limitations when introducing novel processes into the cell.

Acknowledgments

This thesis would not be possible without the support and guidance from my supervisor, Dr. David Stuart. I would like to thank him for generously accepting me into his research laboratory. He has provided an unprecedented research and learning environment that I'm immensely grateful for. An undertaking of this magnitude would not be possible without his encouragement and optimism.

I would also like to thank my supervisory committee, Dr. Joel Weiner and Dr. David Bressler for guiding me through my studies. Their expertise and direction has been greatly appreciated.

Special thanks to both current and prior lab members, Dr. Isabella Wong, Dr. Carlos Fernandez, Jingui Lan, Audric Moses, and Jack Moore for their invaluable contribution to my studies.

At last, but not least, I would like to thank my family for their unconditional love and support.

Table of Contents

List of Tables

List of Figures

List of Abbreviations and Symbols

α - alpha or anti

β - beta

μ - micro

a.a. – amino acid

Abs - absorbance

Amp - ampicillin

Bb – biobrick plasmid

bp – base pairs

BME - β - mercaptoethanol

BSA – bovine serum albumin

CIP – calf intestinal phosphatase

CoA – coenzyme A

CV – column volume

ddH_2O – double distilled water

dex - dextrose

DMAPP – dimethylallyl pyrophosphate

DNA – deoxyribonucleic acid

dNTP - deoxyribonucleotide triphosphate

DMAPP – dimethylallyl pyrophosphate

DMSO – dimethylsulfoxide

DTT – dithiothreitol

E. coli – *Escherichia coli*

EDTA – ethylenediaminetetraacetic acid

Δ*erg20* – truncated *ERG20*

ERG20 – *Saccharomyces cerevisiae* gene encoding farnesyl-pyrophosphate synthetase

FPP – farnesyl pyrophosphate

g – gravitational force

g - grams

GC – gas chromatography

GC-FID – gas chromatography-flame ionization detector

GST – Glutathione S-Transferase

GST-NudF – GST tagged NudF

H_2O_2 –hydrogen peroxide

HA – haemagglutinin epitope tag

HCl – hydrochloric acid

His - histidine

HMG1 – *Saccharomyces cerevisiae* gene encoding HMG-CoA reductase

t*HMG1* – truncated *HMG1* – hyperactive mutation

t*hmg1MYC* – Myc tagged truncated *HMG1*

HMG-CoA – 3-hydroxy-3-methyl-glutaryl-CoA

HMGCR – HMG-CoA reductase

HMGCRMyc – Myc tagged HMGCR

IPTG – isopropyl β-D-1 thiogalactopyranoside

IPP – isopentenyl pyrophosphate

kb – kilo base pair

KPO_4 – potassium phosphate

L – liters

LacI – Lac repressor protein

LB – luria-bertani

LPP - lipid phosphate phosphatase

LPP1 - *Saccharomyces cerevisiae* gene encoding lipid phosphate phosphatase

leu – leucine

LEU2 - *Saccharomyces cerevisiae* gene encoding for β-isopropylmalate dehydrogenase

M – molar

m – milli

mA – milli amps

met – methionine

MET3 - *Saccharomyces cerevisiae* gene encoding ATP sulfurylase

$MgCl_2$ - magnesium chloride

mins – minutes

$MnCl_2$ – manganese chloride

Myc- c-myc epitope tag

$NaCl_2$ - sodium chloride

NADP+ - Nicotinamide adenine dinucleotide phosphate

NADPH - reduced form of NADP+

NaOH – sodium hydroxide

nm - nanometers

nudF – *Bacillus subtilis* gene encoding NudF protein

NudF – NudF protein

NudFHA-His – HA and His tagged NudF

OD_{600} – optimal density at 600nm

P_i : free inorganic phosphate

PCR – polymerase chain reaction

PDC1 – *Saccharomyces cerevisiae* gene encoding pyruvate decarboxylase

PMSF – phenylmethanesulfonylfluoride

PVDF – polyvinylidine fluoride

PYK1 – *Saccharomyces cerevisiae* gene encoding pyruvate kinase

rNudFHA-His – recombinant NudFHA-His

rpm – revolutions per minute

S. cerevisiae – *Saccharomyces cerevisiae*

SDS - sodium dodecyl sulfate

SDS PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

secs - seconds

trp – tryptophan

U - Units

URA3 - *Saccharomyces cerevisiae* gene encoding for Orotidine-5'-phosphate decarboxylase

ura – uracil

V – volts

W303 – a Lab strain of *Saccharomyces cerevisiae*

YEPD - yeast extract peptone dextrose

CHAPTER 1

Introduction

1.1 Metabolic Engineering: A Platform for Biofuel Production

The development of metabolic engineering has given rise to the expansion of industrial biotechnology[24]. Metabolic engineering can be defined as the manipulation of genetic and or regulatory properties of cells to enhance production of a desired compound[24]. In other words, metabolic engineering imports or replaces existing metabolic pathways to improve biological production of chemical compounds of interest[24]. A key advantage of metabolic engineering is that many compounds of interest can be mass-produced in a sustainable and environmentally friendly manner. For example, numerous microorganisms have been found to produce compounds that have medicinal properties, but only in minute amounts. However, the use of metabolic engineering techniques could potentially optimize the production of these compounds in the natural producers or, alternatively, the pathways involved could be imported and expressed in a suitable non-native host.

For many years, metabolic engineering approaches have been applied to microorganisms for fermentation practices and drug synthesis[1]. These biotechnology applications have led to significant advancements by improving existing applications and also by opening up new possibilities. The ability to modify organisms has led to the microbial production of biofuels. There is a growing interest in this area because of the rising concern about the sustainability and the environmental impact of using fossil fuels[12].

Modern society relies heavily on fossil fuels as a source of energy and therefore increasing efforts have been implemented towards producing fuel alternatives from renewable resources[9]. The production of advanced fuel substitutes that are compatible with existing infrastructure cannot be achieved economically using natural microorganisms. Therefore, metabolic engineering techniques offer an approach to engineering microorganisms for the mass production of biofuels.

1.2 Ideal Host Organism

In order to become cost and energy effective, the production of alternative biofuels must utilize organisms that can be easily manipulated. Host organisms can be native producers or non-native hosts. The use of native producers allow for higher production efficiency and tolerance to product toxicity[25]. Unfortunately, genetic engineering cannot be applied to many native producers, as they are poorly characterized or resistant to manipulation. However, well-characterized microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae* are promising host organisms as they can easily be manipulated to maximize the production of candidate biofuels. The vast physiological knowledge available on these hosts along with the accessibility to various genetic tools has allowed for efficient engineering of these microorganisms[9].

1.3 Ideal Biofuel

Ideal biofuels are designed to replace current fuel in internal combustion engines, diesel engines, and jet engines[37]. A biofuel should be structurally identical to petroleum-derived transportation fuel and cannot be corrosive or hygroscopic in nature. For gasoline replacement, the biofuel should have an energy density similar to gasoline and a high octane number. An octane number is essentially a measure of the fuel's ability to resist compression. A high octane number in a fuel is preferred as it effectively reduces harmful atmospheric emissions. Biofuels possessing these properties are compatible with existing infrastructure for fuel transportation, storage, and use. Lastly, production of these biofuels should use minimal energy, be non-toxic to host microorganisms and be efficiently produced from a variety of feedstock[40].

1.4 Recent Advances in Biofuel Production

1.4.1 Bioethanol

Ethanol is commonly produced by fermentation of crops high in starch and sugar such as corn and sugarcane. *Saccharomyces cerevisiae* and *Zymomonas mobilis* can naturally covert C_6 sugars into ethanol and are the most widely used microbes to produce ethanol[27]. The metabolic production of ethanol in these microbes is very efficient therefore further improvement has been focused on enhancing ethanol tolerance and expanding the substrate range[25]. Currently, ethanol is being produced from food crops but the use of edible feedstock is not economically sustainable[9]. Effort has been focused on

developing native ethanol producers to efficiently metabolize lignocellulosic biomass[10].

Ethanol is the major biofuel in commercial production. However, ethanol is not an ideal biofuel. Ethanol contains approximately 70% of the energy content of gasoline (21MJ/L versus 32 MJ/L for gasoline)[30]. The majority of ethanol produced is currently being blended in petrol to improve fuel combustion in vehicles[19]. Ethanol's inherent hygroscopic and corrosive characteristics make it incompatible with current fuel infrastructure, limiting its economic use. For this reason new approaches have been implemented to enable production of advanced fuels that have a higher energy content and that are more compatible with existing fuel infrastructure. Although fuel quality compounds are not commonly produced biologically, the escalating genomic information, molecular biology techniques, and metabolic engineering tools, have enabled significant advancements in developing microorganisms to produce fuel grade compounds. These compounds include higher chain alcohols, fatty acid derived biofuels, and isoprenoids derived biofuels.

1.4.2 Higher Chain Alcohols

The production of higher chain alcohols (C3-C5) such as butanol, isopropanol, isobutanol, and pentanol isomers have been examined as alternative fuels. In comparison to ethanol, higher chain alcohols have a higher energy density and are less hygroscopic which makes these alcohols

more compatible with the current fuel infrastructure. These alcohols have the potential to meet fuel standards and are currently being explored.

Traditionally, butanol is produced via fermentative processes in *Clostridium acetobuylicum* with a maximum titer of 19.6 g/L[50]. This process consists of condensing two acetyl-CoA molecules and then reducing the product to butanol, which requires six enzymatic reactions[35]. Butanol has about 90% of the energy density of gasoline, and boasts a high octane number. Another advantage of butanol is its low vapor pressure, which allows it to be mixed with gasoline at any ratio[20]. Despite these virtues, the production of butanol in *Clostridium* has its drawbacks. The complex physiology and lack of regulatory understanding on *Clostridium* makes it a difficult model for maximizing the organism's efficiency. To overcome this limitation, the pathway responsible for butanol production in *Clostridium* was constructed and expressed in user-friendly organisms. For example, the *Clostridium acetobutylicum* butanol biosynthetic pathway was transferred to and expressed in *E. coli*[2]. The expression of this pathway, the over expression of *atoB* gene from *E. coli*, and the removal of competing pathways for 1-butanol synthesis lead to a maximum butanol titer of 550 mg/L[2]. A similar platform was introduced into *S. cerevisiae,* resulting in the production of 2.5 mg/L of butanol[43]. Additionally, it has been reported that 30 g/L of butanol was produced in *E. coli* when flux and balancing redox cofactors were taken into consideration[40]. This work demonstrates the feasibility of butanol production, however, further research is required for commercialization.

Isopropanol is also naturally produced in *Clostridium acetobutylicum* via the acetone pathway with a maximum titer of 2 g/L[50]. Similar to butanol, isopropanol synthesis was accomplished by transferring the native *Clostridium acetobutylicum* acetone production pathway and expressing it in *E. coli*[17]. A secondary alcohol dehydrogenase from *E. coli* was also expressed in order to convert acetone to isopropanol[17]. This platform led to a maximum production of 4.9 g/L of isopropanol[17]. However, using a similar scheme combined with the expression of an alcohol dehydrogenase gene from *Clostridium bijerinkckii* resulted in 13.6 g/L of isopropanol[23]. Furthermore, a gas stripping method has been used to alleviate isopropanol toxicity resulting in a titer of 143 g/L over a 240-hour fermentation period[21].

Isobutanol, an isomer of butanol, has a high octane number and its production has been engineered in *E. coli* using the acetohydroxy acid synthase chain elongation pathway[3]. The keto acid elongation scheme produces 2-ketoisovalerate from pyruvate. The 2-ketoisovalerate can be converted into an aldehyde, which then can be reduced to isobutanol through the expression of a decarboxylase from *Lactococcus lactis* (2-ketoiosvalerate decarboxylase) and an alcohol dehydrogenase such as YqhD from *E. coli*[3]. This platform is very efficient as it produces more than 20 g/L of isobutanol[3]. These results illustrate that commercialization of isobutanol is promising.

Pentanol isomers have a low affinity for water and a high energy density of 28 MJ/L[5]. Pentanol isomers such as 2-methyl-1-butanol and 3-methyl-1-

butanol are chemicals that have potential as biofuels. These isomers are found as natural byproducts of microbial fermentations from amino acid substrates[5]. While natural production of these isomers is too low for commercial production, metabolic engineering has made the synthesis of these fuel alternatives possible. It has been shown in *E. coli* that 2-methyl-1-butanol and 3-methyl-1-butanol can be produced from the amino acid biosynthetic pathways of isoleucine and leucine, respectively[5]. The biosynthesis of 2-methyl-1-butanol first requires the condensation of pyruvate and 2-ketobutyrate to form 2-keto-3-methylvalerate. This 2-ketoacid can be converted into an aldehyde through expression of the KDC (2-ketoacid decarboxylase) enzyme from *Lactococcus lactis* gene, *kivd*. The aldehyde can be reduced to an alcohol by an alcohol dehydrogenase. KDC combined with alcohol dehydrogenase (encoded by the *ADH2* gene from *S. cerevisiae*) activity facilitates production of 2-methyl-1-butanol in *E. coli*[3]. The production of 3-methyl-1-butanol (isopentyl alcohol) in *E. coli* also requires the expression of both the *kivd* gene from *L. lactis* and *ADH2* gene from *S. cerevisiae*. The decarboxylase and alcohol dehydrogenase facilitate the conversion of 2-ketoisocaproate to the pentanol isomer[3]. Current production of these pentanol isomers is very low as their titers fall just above 1.0 g/L[11]. These levels are too low for any industrial applications but more research on this area could lead to significant improvements in production efficiency.

1.4.3 Fatty Acid Derived Biofuels

Fatty acids have a high energy density; the wide range of chain length and degree of saturation allow fatty acids to offer the possibility for the production of various biofuel candidates that are comparable to molecules found in petroleum based fuels[9]. Free fatty acids cannot be used as fuel directly, but their derivatives such as fatty alcohols, fatty acid alkyl esters, fatty-acid derived alkanes and alkenes, are potential biofuel alternatives[50]. These derivatives have a high energy density, low water solubility, and low toxicity to the host[50].

Biodiesel is currently a substitute for petroleum based diesel fuel; it can be mixed at any ratio with pure diesel. Biodiesel is made from plant oil or animal fat through chemical transesterification of triacylglycerols with methanol or ethanol to produce fatty acid methyl esters (FAME) or fatty acid ethyl esters (FAEE)[48]. The use of plant oils and animal fat for biodiesel production has its disadvantages. Plant oil is highly viscous and not very volatile which causes serious engine problems[34]. Triacylglyercols from animal fat have a high degree of saturation and thus exhibit poor cold flow properties[34]. These limitations have stimulated finding alternative oil sources to optimize the production of biodiesel.

To produce more economical biodiesel fuel *E. coli* has been engineered to produce FAEE. For example, ethanol production was combined with esterification of the ethanol with the acyl moieties of coenzyme A thioesters

of fatty acids by over expressing an acyltransferase from *Acinetobacter baylyi*[45]. This engineered *E. coli* produced 1.3 g/L of FAEE[45]. Other efforts have been made to engineer microorganisms to produce alkanes and alkenes, which are predominant components of diesel fuel[50]. This new approach for biodiesel production may increase industrial efforts in utilizing microorganisms for the production of various types of diesel fuels.

1.4.4 Isoprenoid Derived Biofuels

Isoprenoids are the largest class of natural products[6]. Isoprenoids are hydrocarbons synthesized in a wide range of organisms. They are valued for their fragrance and flavors as they are primary constituents of the essential oils of plants and flowers[6]. The intermediates and products of the isoprenoid pathway are attractive targets as pharmaceuticals[25]. Isoprenoids are also known for their diverse biological roles. Their functions range from hormones to their roles in membrane fluidity[6]. Synthesis of isoprenoids can be divided into four basic steps (Figure 1.1). The first step is the production of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) within the mevalonate pathway or the methylerythritol phosphate pathway. The second step is the condensation of multiple IPP with DMAPP by FPP (farnesyl pyrophosphate) synthase to form geranyl pyrophosphate (GPP) and subsequently FPP, and geranylgeranyl pyrophosphate (GGPP). These long chair pyrophosphate units are then rearranged by terpene synthases to form branched chain or cyclic terpene backbones. Lastly, modifications by tailoring enzymes generate the final isoprenoid products.

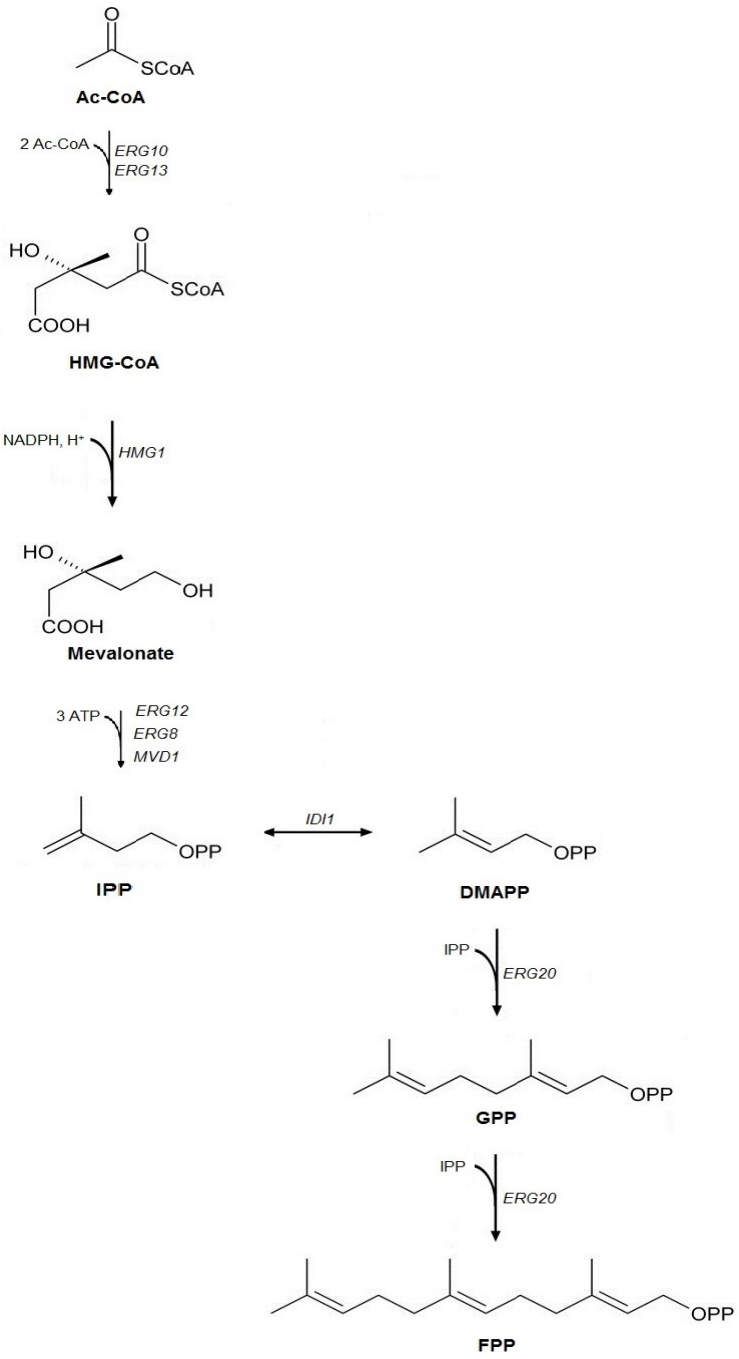


Figure 1.1 Simplified isoprenoid pathway in *S. cerevisiae* Isoprenoid biosynthesis in *S. cerevisiae* via the mevalonate pathway. Figure adapted from Chemler *et al.*. Ac-CoA = Acetyl coenzyme A, *ERG10* = thiolase gene; *ERG13* = HMG-CoA synthase gene; HMG-CoA = 3-hydroxy-3-methyl-glutaryl-coenzyme A; *ERG12* = mevalonate kinase gene; *ERG*8 = phosphomevalonate kinase gene; *MVD1* = mevalonate-5-pyrophosphate decarboxylase gene; IPP = isopentenyl pyrophosphate; *IDI1*= isopentenyl pyrophosphate isomerase gene; DMAPP = dimethylallyl pyrophosphate; *ERG20* = FPP Synthase gene; GPP = geranyl pyrophosphate; FPP = farnesyl pyrophosphate.

The isoprenoid biosynthetic pathway generates a diverse array of branched chain and cyclic hydrocarbon intermediates that may have the properties favorable for alternative biofuel (Figure 1.2)[5]. The most relevant intermediates are IPP, DMAPP, GPP and FPP. With modifications, these intermediates can lead to alcohol production that may substitute gasoline, whereas the production of longer cyclic or branched chain terpenes may act as alternatives to petroleum based diesel or jet fuel[30]. For example, it has been shown that engineered *E. coli* expressing a heterologous mevalonate pathway and over expressing FPP synthase can produce 135 mg/L of farnesol, a proposed diesel substitute[46]. Terpenes including pinene and limonene are currently being explored as potential jet fuel alternatives[50].

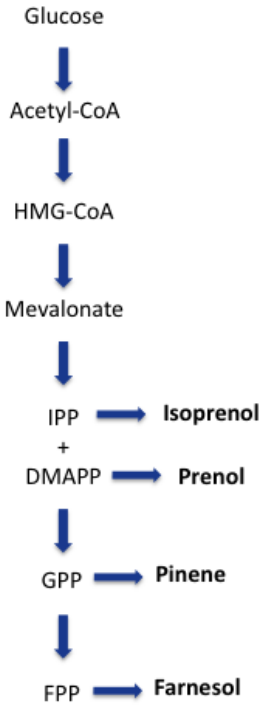


Figure 1.2 Isoprenoid derived fuels Conversion of isoprenoid intermediates into potential fuel alternatives. HMG-CoA = 3-hydroxy-3-methyl-glutaryl-coenzyme A; IPP = isopentenyl pyrophosphate; DMAPP = dimethylallyl pyrophosphate; GPP = geranyl pyrophosphate; FPP = farnesyl pyrophosphate. Potential fuel alternatives are bolded.

Isoprenol, an attractive fuel alternative, has been found in trace amounts in the resin of some tree species and has also been detected at very low concentrations in *E. coli* strains engineered to produce terpenoids[5]. Withers *et al.* carried out a screen on a *Bacillus subtilis* cDNA library for enzymes that were able to relieve toxicity of accumulated prenyl diphosphates in *E. coli* Initially, they wanted to find an enzyme capable of converting DMAPP to isoprene however, they discovered a pyrophosphatase-like enzyme called NudF from *Bacillus subtilis* that was able to convert IPP into isoprenol[47]. Expression of the *nudF* gene and the mevalonate pathway (from acetyl-CoA

to IPP and DMAPP) in *E. coli* produced 112 mg/L of isopentenol (isoprenol and prenol)[47]. More recently, Zheng *et al.* enhanced mevalonate synthesis and investigated the activity of different phosphatases in their engineered *E. coli* strain. Their work resulted in high-specificity production of isoprenol (1.3 g/L) and prenol (0.2 g/L). Liu *et al.* used a different approach and optimized the native *E. coli* methylerythritol phosphate pathway for the production of isoprenol and prenol. The methylerythritol phosphate pathway is able to convert glyceraldehyde-3-phosphate and pyruvate into IPP and subsequently DMAPP. Liu *et al.* believed this pathway was more energetically balanced and efficient, however their engineered strain was only able to produce 61.9 mg/L of isopentenol.

1.5 Research Hypothesis and Objectives

Microbial production of isoprenol has only been examined in *E. coli*. The mevalonate pathway exists in *S. cerevisiae* and therefore it was hypothesized that if metabolic engineering strategies were employed to increase IPP production and accumulation, and NudF was expressed, an isoprenol producing *S. cerevisiae* strain could be developed.

The goal of this research is to apply metabolic engineering techniques in *S. cerevisiae* to manipulate gene expression and protein stability to increase flux through the isoprenoid pathway, maximizing IPP production. Expression of *nudF* will promote the conversion of IPP into its alcohol derivative, isoprenol.

This research is comprised of several elements that will allow for the assembly of an isoprenol producing *S. cerevisiae* strain:

1. Expression of an active NudF in *S. cerevisiae* – NudF will dephosphorylate endogenous IPP to produce isoprenol.

2. Expression of a hyperactive HMG-CoA Reductase in *S. cerevisiae* – A hyperactive HMG-CoA Reductase will circumvent the rate-limiting step in the mevalonate pathway and increase production of IPP.

3. Conditional regulation of *ERG20* in *S. cerevisiae* – Shutting down *ERG20* expression will allow IPP to accumulate.

4. Evaluation of the *S. cerevisiae* isoprenol producing strain.

CHAPTER 2

Materials and Methods

2.1 List of Materials

2.1.1 Chemicals, Reagents and other Materials

Acetic Acid	Sigma-Aldrich
Acetonitrile	Fisher Scientific
Agarose	Fisher Scientific
Ammonium bicarbonate (NH_4HCO_3)	Fisher Scientific
Ampicillin Sulfate	Sigma-Aldrich
Aproptinin	Fisher Scientific
Ammonium Persulfate	BioRad
Bradford dye reagent	BioRad
BSA	NE Bio Labs
BME	Sigma-Aldrich
Chelating sepharose	GE Healthcare
Coomassie Brilliant Blue R	Sigma-Aldrich
p-Cumeric acid	Sigma-Aldrich
Diethanolamine	Sigma-Aldrich
DMAPP	Sigma-Aldrich
DMSO	Fisher Scientific
DTT	Fisher Scientific
EDTA	Fisher Scientific
Ethanol	Fisher Scientific
Eppendorf microfuge tube	Fisher Scientific
Glycerol	Fisher Scientific
Glycine	Fisher Scientific
HCl	Fisher Scientific
Hexane	Sigma-Aldrich

Imidazole	Fisher Scientific
IPTG	Fisher Scientific
IPP	Sigma-Aldrich
Isobutanol	Fisher Scientific
Isoprenol	Sigma-Aldrich
Leupeptin	Fisher Scientific
$MgCl_2$	Fisher Scientific
$MnCl_2$	Fisher Scientific
Methanol	Fisher Scientific
$NaCl_2$	Fisher Scientific
Nickel Sulfate ($NiSO_4$)	Fisher Scientific
Pepstatin	Sigma-Aldrich
PMSF	BioShop Canada Inc
Prenol	Sigma-Aldrich
Salmon sperm DNA	Sigma-Aldrich
Screw cap glass vial	Fisher Scientific
SDS	Fisher Scientific
Tergitol	Sigma-Aldrich
Tris Base	Fisher Scientific
Triton X – 100	VWR

2.1.2 Media and Plates

Luria Bertani media

1 % Tryptone (Fisher Scientific), 0.5 % Yeast Extract (Fisher Scientific), and 1 % NaCl.

LB + Amp media

1 % Tryptone, 0.5 % Yeast extract, 1 % NaCl, and 100 μg/mL Ampicillin.

LB plates

1 % Tryptone, 0.5 % Yeast extract, 1 % NaCl and 1.5 % Agar.

LB + Amp plates

1 % Tryptone, 0.5 % Yeast extract, 1 % NaCl, 100 μg/mL Ampicillin, and 1.5 % Agar.

YEPD media

1 % Yeast extract, 2 % Peptone (Fisher Scientific), 30 mg/L Adenine Sulfate (Fisher Scientific), 30 mg/mL tryptophan (Sigma), and 2 % Dextrose (Fisher Scientific).

YEPD plates

1 % Yeast extract, 2 % Peptone, 30 mg/L Adenine Sulfate and 30 mg/mL tryptophan, 2 % Dextrose, and 1.5 % Agar.

Yeast Selective media

1.6 g/L Difco Yeast Nitrogen Base (Becton, Dickson and Co.), 5 g/L ammonium sulfate (Fisher), 2 % Dextrose, and 2 g/L amino acid dropout mix. Dropout mixtures contain 0.5 g Adenine Sulphate, 2 g Uracil (Sigma) if needed, and 2 g of the required essential amino acids (Fisher, Sigma, MP Biomedicals, and Acros Organics) with the exception of Leucine (Fisher Scientific), which requires 4 g if needed.

Selective plates

1.6 g/L Difco Yeast Nitrogen Base, 5 g/L Ammonium Sulfate, 2 % Dextrose, 2 g/L amino acid dropout mixtures, and 15 g/L Agar.

2.1.3 Buffers

2X Sample buffer

100 mM Tris-HCl pH 6.8, 4 % SDS, 20 % Glycerol, 0.2 % Bromophenol blue, and 200 mM DTT.

TE Buffer

10 mM Tris-HCl pH 8.0 and 1 mM EDTA.

TB Buffer

10 mM Pipes (Fisher Scientific), 55 mM $MnCl_2$, and 15 mM $CaCl_2$ (Fisher Scientific) pH to 6.7 with KOH.

2.2 Methods

2.2.1 DNA Cloning Methodology

2.2.1.1 Primer design and synthesis

Primers for DNA synthesis were designed with the help of the DNA strider 1.2.1 software program and synthesized by Integrated DNA Technologies. All oligonucleotides synthesized are listed in Table 2.1.

Table 2.1 Oligonucleotides used for cloning

Oligonucleotide	Sequence (5'→3')	Sense*	Source
DPP FWD	GCAAGATCTCCTAGGAT GAACAGAGTTTCGTTTA TTAAA	+	IDT
DPP REV	GCACTGCAGGCGGCCGCA CTAGTCTACTCAACTGCT TCCTAAGA	-	IDT
Δerg20 FWD	GGCTCCATGGCTTCAGAA AAAGAAATTAGG	+	IDT
Δerg20 REV	ACTAGTCTTTTCATCCGT GATACCGGCAAC	-	IDT
thmg1 FWD	GGACCTAGGATGGTTTT AACCAATAAAACAGTC	+	IDT
thmg1 REV	GGACTGCAGGCGGCGCG ACTAGTGTTGAATTTTC AGGTAAAGCTC	-	IDT
thmg1MYC FWD	GGATCTAGAATGGTTTT AACCAATAAAACAGTC	+	IDT
thmg1MYC REV	GGACTGCAGGCGGCGCG ACTAGTGTTGAATTTTC AGGTAAAGCTC	-	IDT
LPP FWD	GCAGGATCCTCTAGAAT GATCTCTGTCATCGCGGA T	+	IDT
LPP REV	GCACTGCAGGCGGCCCTA GTGAAATATTACACTGC ACCGCA	-	IDT
NudF-pGEX-6P-1 FWD	CCGGGATCCATGAAATCC TTGGAAGAAAAAAC	+	IDT
NudF-pGEX-6P-1 REV	CGGGAATTCCTACTATTT TTGAGCTTGTAAGGCTTC C	-	IDT
NudF FWD	CCGTCTAGAAAAATGAA ATCCTTGGAAGAAAAAA C	+	IDT
NudF REV	CGGCTGCAGTCGACTAGT TTTCTATTTTTGAGCTTG TAAGGCTTCC	-	IDT
NudFHA FWD	CCGTCTAGAAAAATGAA ATCCTTGGAAGAAAAAA C	+	IDT
NudFHA REV	CGGCTGCAGTCGACTAGT TTTCTATTTTTGAGCTTG	-	IDT

	TAAGGCTTCC		
NudFHAHis FWD	CCACATATGAAATCCTTG GAAGAAAAAACC	+	IDT
NudFHAHis REV	ACTGGATCCTAGTGATG GTGATGGTGATGGGCAT AATCTGGGACATCGTAT GG	-	IDT
pho8Nt FWD	GCACCTAGGATGCACAA GAAGAAGAATGTCATA	+	IDT
pho8Nt REV	GGACTGCAGGCGGCCGCT CTAGATCAGTTGGTCAA CTCATGGTA	-	IDT
pho8NCt FWD	GCACCTAGGATGCACAA GAAGAAGAATGTCATA	+	IDT
pho8NCt REV	GGACTGCAGGCGGCCGCT CTAGATCAATCTGATGT GTGTTGGT	-	IDT
pho8Nt-pYES2 FWD	GCAGGATCCATGCACAA GAAGAAGAATGTCATA	+	IDT
pho8Nt-pYES2 REV	GGACTGCAGGCGGCCGCT CTAGATCAGTTGGTCAA CTCATGGTA	-	IDT
pho8NCt-pYES2 FWD	GCAGGATCCATGCACAA GAAGAAGAATGTCATA	+	IDT
pho8NCt-pYES2 REV	GGACTGCAGGCGGCCGCT CTAGATCAATCTGATGT GTGTIDTGGT	-	IDT

2.2.1.2 Polymerase chain reaction

Prepared *Saccharomyces cerevisiae* genomic DNA was generally used as a template in the polymerase chain reaction (PCR). PCR was normally carried out in a 25 μL reaction containing 1 μL of prepared *S. cerevisiae* genomic DNA, 100 nM of each primer, 100 μM dNTPs (Invitrogen), 1 U of Taq DNA polymerase (Invitrogen) or 1 U of Vent DNA polymerase (NE Bio Labs), and 1X Taq thermo buffer (Invitrogen). PCR reactions were performed in a Techne Genius PCR thermal cycler. Typical PCR reaction conditions are an

initial hot start, 5 mins at 95°C followed by 30 cycles of 1 min at 95°C for denaturation, 1 min at variant annealing temperatures, and 72°C for elongation, the time is determined by the length of the PCR product (1 min per kb). A final hold at 4°C completes the reaction. Samples were stored at -20°C.

2.2.1.3 Agarose gel electrophoresis

Agarose gel electrophoresis was commonly carried out to aid in the purification of PCR products and plasmid DNA as well as confirmation of correct DNA ligations. DNA samples were mixed with 10X Loading Buffer (Invitrogen) and were separated by electrophoresis on a 0.8 % w/v agarose gel (2.4 g agarose in 300 mL 1X TAE and 15 μL of a 500 mg/mL stock of EthBr (Sigma)). Electrophoresis was carried out in 1X TAE buffer (50X stock: 242 g Tris base, 57.1 mL Glacial acetic acid, and 100 mL 0.5M EDTA pH 8.0 in a final volume of 1L) at 100V. The agarose gel was visualized using the BioRad Gel Doc XR+ imaging system and the Invitrogen 1-Kb Plus DNA Ladder (20% 1 Kb Plus DNA Ladder and 1X Loading Buffer) was used as a reference.

2.2.1.4 Gel extraction and purification

A Qiagen QIAquick Gel Extraction Kit was used to extract and purify the DNA. Briefly, correct bands were excised and dissolved in the Qiagen QG Buffer at 50°C. The samples were transferred to a QIAquick DNA column, washed with Qiagen PE Buffer, and the DNA was subsequently eluted with 25 μL of sterile

ddH_2O by centrifugation at 13,000 rpm for 1 min.

2.2.1.5 DNA ligation of PCR products

Taq polymerase frequently generates 3'A overhangs allowing cloning to a vector with 3' T overhangs. PCR products generated from Taq Polymerase were normally ligated into the TA cloning vector, pGEM-T. A pGEM-T Easy Kit from Promega was used for this experiment. Ligations were carried out according to manufactures instructions. In brief, 2 ng of pGEMT, 1.5 U of T4 DNA ligase, 2.5 µl of 2X rapid ligation buffer and 1.5 µl of the PCR product were mixed together and incubated at room temperature for 1 hour or at 4°C overnight.

2.2.1.6 Bacterial transformation

All bacterial strains and plasmids used for transformation are listed and described in Table 2.2 and 2.3, respectively.

Table 2.2 *E. coli* strains used for transformation

Genotype	Description/Properties	Growth Medium
DH5α	*fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17* - contains multiple mutations that allow for high-efficiency transformations	LB
BL-21(DE3) Rosetta	*E. coli F- ompT hsdS$_B$(R$_B$- m$_B$-) gal dcm λ(DE3 [lacI lacUV5-T7* gene 1 *ind1 sam7 nin5*]) pLysSRARE (Cam^R) Enhances the expression of eukaryotic proteins that contain codons rarely used in *E. coli*. - supplies tRNAs for 7 rare codons on a compatible chloramphenicol-resistant plasmid	LB

Table 2.3 Bacterial plasmids used for cloning

Plasmids	Description	Growth Medium	Source
ScNUD_pMA-T	*Saccharomyces cerevisiae* replicating plasmid containing *nudF* gene	LB + Amp	This study
pGEM-T	TA cloning Vector	LB + Amp + IPTG + X-gal	Promega
pET3a	*E. coli* expression plasmid containing a bacteriophage T7 promoter and *AmpR* gene	LB + Amp	Novagen
pGEX-6P-1	*E. coli* GST gene fusion plasmid	LB + Amp	GE Healthcare

2.2.1.6.1 Preparation of DH5α competent cells

Competent DH5α cells were prepared as described by Inoue *et al.*. Briefly a frozen stock of DH5α cells were thawed, streaked on an LB agar plate, and cultured overnight at 37°C. Approximately 10-12 large colonies were selected and inoculated to 250 mL of SOB medium (2 % w/v Tryptone, 0.5 % w/v Yeast extract, 10 mM NaCl, and 2.5 mM KCl) in a 2 L Erlenmeyer flask. The culture was grown to an OD_{600} of 0.6 at room temperature, shaking at 200 rpm. Once the appropriate OD_{600} reading was reached the flask was placed on ice for 10 mins. The culture was then transferred to a centrifuge bottle and centrifuged at 3,000 rpm in Beckman Avanti J25 for 10 mins at 4°C. The cell pellet was resuspended in 80 mL of ice-cold TB buffer and incubated in an ice bath for 10 mins and subsequently centrifuged at 3,000 rpm. The cell pellet was gently resuspended in 20 mL of TB buffer and DMSO was added with gentle swirling to a final concentration of 7 %. The cell suspension was incubated in an ice bath again for 10 mins. Following incubation the suspension was dispensed into cell-freezing tubes and immediately immersed into liquid nitrogen. The frozen competent cells were stored at -80°C.

2.2.1.6.2 Plasmid transformation

Prepared competent *E. coli* strain DH5α was normally used for plasmid transformation. Transformations were carried out using a heat shock procedure. Briefly, 50 μL of competent cells were incubated with DNA for 20

mins on ice followed by 45 secs incubation at 42°C. The cells were then incubated on ice for 2 mins. The cells were subsequently supplemented with 1mL of LB media and incubated at 37°C for 1 hour. Following the incubation, cells were plated on LB agar plates containing 100 μg/μL Ampicillin, 20 mg/mL X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranosid) and 0.1 mM IPTG to allow for blue/white screening of recombinants. Plates were incubated at 37°C overnight. The white colonies harbouring the pGEM-T plasmid carrying an insert were selected for further analysis. DH5α strains transformed with other plasmids were plated onto LB agar plates containing 100 μg/μL ampicillin and incubated overnight at 37°C. Successful transformants were selected for further analysis.

2.2.1.7 Plasmid preparation

Selected transformed *E. coli* were cultured overnight in 3 mL of LB + Amp. The cultures were collected by centrifugation at 8,000 rpm for 3 mins to obtain the cell pellet. High copy plasmid DNA was purified using the Qiagen QIAprep Spin Miniprep Kit. Briefly, cell pellets were lysed and cell membrane, proteins, and high molecular weight DNA were precipitated out using Qiagen buffers P1, P2 and N3. Samples were transferred to a QIAquick DNA column to separate the plasmid DNA from the supernatant. The samples were washed with Qiagen PE buffer and subsequently eluted with 50 μL sterile ddH_20.

2.2.1.8 Restriction endonuclease digestion

All restriction enzymes used for restriction endonuclease digestion are listed in Table 2.4.

Table 2.4 Restriction endonuclease digestion enzymes used

Enzyme	Source
Avr II	NE Bio Labs
Bam HI	Invitrogen
Bgl II	Invitrogen
Eco RI	NE Bio Labs
Hind III	Invitrogen
Not I	Invitrogen
Pst I	Invitrogen
Spe I	Invitrogen
Xba I	Invitrogen

Restriction endonuclease digestions were generally carried out to produce suitable ends for further cloning or to determine whether or not DNA ligation was successfully completed. Briefly, restriction endonuclease digestions were carried out in 20 μL reaction volume containing 10-15 U of specific restriction enzyme and the appropriate reaction buffer. Digestions were allowed to proceed at 37°C for 1 hour.

2.2.1.9 DNA ligation

DNA fragments generated from restriction endonuclease digestions for further cloning were extracted and purified as described in section 2.2.1.4. Correct purified fragments and recipient vectors were mixed together with 1

U T4 DNA ligase (NE Bio Labs) and 1X T4 ligase buffer (NE Bio Labs) in a total volume of 20 μL. This reaction was incubated at room temperature for 1 hour. This reaction mixture was further processed for transformation.

2.2.1.10 DNA sequencing

The DNA sequences of all plasmid constructs were verified by sequencing. The Applied Genomics Centre at the University of Alberta completed all the DNA sequencing. The Basic Local Alignment Search Tool (BLAST) program provided by the National Center for Biotechnology Information was used to analyze the DNA sequences.

2.2.2 DMSO Assisted Transformation of *Saccharomyces cerevisiae*

All *S. cerevisiae* strains and plasmids used for transformation are listed and described in Table 2.4 and 2.5, respectively.

Table 2.5 *S. cerevisiae* strain used for transformation

Genotype	Description/Properties	Medium
W303	*MAT*a {*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*}	YEPD

Table 2.6 *S. cerevisiae* plasmids used

Plasmids	Description/Properties	Medium
P_{PDC}Bb	Episomal plasmid – *PDC1* promoter, *URA3* marker, and *AmpR* gene	-ura + dex
pRS405-*MET3*	Integrating plasmid - *MET3* promoter, *LEU2* marker and *AmpR* gene	-met + dex
P_{PYK}Bb	Episomal plasmid – *PYK1* promoter, *URA3* marker, and *AmpR* gene	-ura + dex
pYES2	Episomal plasmid – *GAL1* promoter, *URA3* marker and *AmpR* gene	-ura + dex
YEplac195	Episomal Biobrick plasmid - uracil marker and *AmpR* gene	-ura + dex
YCplac22	Centromeric plasmid – *TRP1* marker and *AmpR* gene	-trp + dex
YCplac33	Centromeric plasmid – *URA3* marker and *AmpR* gene	-ura + dex
pESC-URA	Episomal plasmid – *GAL1* and *GAL 10* promoter, URA3 marker and *AmpR* gene	-ura + dex

A 20 mL W303 *S. cerevisiae* culture was grown overnight at 30°C. On the following day the culture was diluted in 50 mL of YEPD to an OD_{600} reading of approximately 0.2-0.3. The cultures were incubated at 30°C until the culture reached an OD_{600} reading of approximately 0.5-0.7. The cell pellet was collected from the culture and subsequently was washed with sterile ddH_2O. The cell pellet was re-suspended in 1mL of 100 mM LiAc (100 mM LiAc (Sigma-Aldrich) in 10 mM Tris, pH 8.0) and incubated with gentle agitation at 30°C for 15 mins. 100 μL of the cell suspension was incubated for 5 mins at room temperature with 10 μg salmon sperm DNA and the specific *S.*

cerevisiae plasmid generated from cloning. 300 μL of PEG solution (50% of PEG 600 (Fisher Scientific) in 100 mM LiAc) was added to the cell suspension and incubated at 30°C for 1 hour. After incubation, 43 μL of DMSO was added followed by incubation at 42°C for 5 mins. Following incubation, the cell pellet was collected and washed with 1 mL ddH_2O and subsequently resuspended with 60 μL of sterile ddH_2O. The cells were plated onto appropriate selective plates and incubated at 30°C for 2-3 days. Once colonies became visible, a few colonies were selected and patched on appropriate selective plates.

2.2.3 Protein Methodology

2.2.3.1 Trichloroacetic acid (TCA) total protein extraction

Total cellular protein was prepared from *S. cerevisiae* using a TCA extraction protocol as described by Foiani *et al.*. A 10 mL *S. cerevisiae* culture was grown overnight at 30°C. On the following day the culture was diluted in 15 mL of appropriate media to an OD_{600} reading of approximately 0.2-0.3. The cultures were incubated at 30°C until the culture reached an OD_{600} reading of approximately 0.5. The cell pellet was collected and re-suspended in 500 μL of 20 % TCA solution. A 100 % TCA (EM Science) stock solution was prepared from 500 g of pure crystal in 227 mL of ddH_2O. The cell suspension was transferred to an eppendorf microfuge tube. The suspension was centrifuged and the supernatant was discarded. The pellet was then re-suspended in 200 μL of 20 % TCA solution. 0.5 mm glass beads (Biospec

Products Inc.) were added to the meniscus and vortexed at max speed 3 times for 5 mins with a 5 min incubation on ice in between each vortex. 400 μL of 5 % TCA solution was added and the aqueous extracts were transferred to new tubes. The suspension was centrifuged at 14,000 rpm for 10 mins to pellet the protein. The supernatant was discarded and the pellets were washed with 500 μL of ice-cold acetone (Fischer Scientific) to remove residual TCA. The suspension was then centrifuged at 10,000 rpm for 10 mins. The supernatant was discarded and pellets were left to dry on the bench to removal residual acetone. The protein pellet was re-suspended in 200 μL of 2X Sample Buffer and 200 μL of 1 M Tris-HCL pH 8.5. Protein samples were incubated at 100°C for 5 mins. The samples were then centrifuged at 14,000 rpm for 10 mins and the supernatant was collected and stored at -20°C.

2.2.3.2 SDS poly-acrylamide gel electrophoresis (SDS-PAGE)

All protein samples were resolved on a 12 % poly-acrylamide gel (10 mL of 12 % Separating buffer (3.33 mL ddH_20, 2.5 mL 1.5M Tris-HCl pH 8.8, 4 mL 30 % Acrylamide (Bio Rad), 100 μL 10 % SDS, 100 μL 10 % APS (Bio Rad), and 10 μL TEMED (Fisher Scientific)) and 5 mL of 4 % Stacking buffer (2.6 mL ddH_20, 525 μL 1 M Tris-HCl pH 6.8, 670 μL 30 % Acrylamide 50 μL 10 % SDS, 50 μL 10 % APS, and 5 μL TEMED)). The PageRuler protein ladder (Fermentes) was used as the prestained molecular weight ladder. SDS-PAGE

was carried out in 1X SDS running buffer (10X Stock: 250 mM Tris, 2.5 M Glycine, and1 % SDS, pH 8.3) at 100-150 V.

2.2.3.3 Semi-dry transfer

Proteins resolved by SDS-PAGE were transferred from polyacrylamide gels to PVDF (polyvinylidine fluoride) membranes (Millipore). The transfer was carried out using a semi-dry transfer apparatus (Tyler Research Instruments), Whatman chromatography paper (Fischer Scientific), and 1X semi-dry transfer buffer (2.9 g Glycine, 5.8 g Tris base, 0.37 g SDS, 200 mL methanol in a final volume of 1 L pH 8.3). Prior to transfer, the PVDF membrane was activated with methanol. The semi-dry transfer was carried out at 200 mA for 45 mins. Following the transfer the membrane was blocked with 10 % skim milk powder diluted in 1X TBS-Tween buffer (50 mM Tris, 150 mM NaCl, and 0.05 % Tween 20 (Fisher Scientific) pH 7.6) for 1 hour at room temperature.

2.2.3.4 Western blotting

All antibodies and concentrations used for western blot analysis are listed in Table 2.6.

Table 2.7 Antibodies and concentrations used

Antibody	Concentration
Mouse α HA ascites fluids	1:10,000
Mouse α Myc ascites fluids	1:10,000
Rabbit α mouse-HRP	1:5,000

Following membrane blocking, the PVDF membrane was immunoblotted with a specific primary antibody. The antibody was diluted in 5 % skim milk powder in 1X TBS-Tween buffer. The PVDF membrane was incubated in the prepared antibody solution overnight on a shaker at 4°C. On the following day the PVDF membrane was washed twice for 15 mins with 1X TBS-Tween buffer while shaking at room temperature to remove unbound primary antibody. Subsequently, the PVDF membrane was incubated at room temperature for 2 hours with the appropriate secondary HRP (Horse Radish Peroxidase)-conjugated antibody that was prepared in 5 % skim milk powder in 1X TBS-Tween buffer. Finally, the PVDF membrane was washed again twice for 15 mins with 1X TBS-Tween buffer. The PVDF membrane was then incubated in prepared enhanced chemiluminescence solution (Solution 1: 17.71 mL ddH_2O, 2 mL Tris-HCl pH 8.5, 200 for 200 µL Luminol (250 mM Stock: 0.44 g Luminol (Sigma-Aldrich) in 10 mL DMSO), and 88 µL p-Cumeric Acid (90 mM Stock: 0.15 g of p-Cumeric Acid (Sigma-Aldrich) in 10 mL DMSO). Solution 2: 17.87 mL ddH_2O, 2 mL Tris-HCl pH 8.5, and 13 µL 30 % H_2O_2 (Fisher Scientific) for 2 mins and the western blot was visualized by exposing the treated PVDF membrane to Fuji SuperRX X-ray film.

2.2.3.5 Bradford protein assay

BSA protein standards between 0 µg/mL and 10 µg/mL were prepared via serial dilutions from a stock concentration of 10 µg/µL. 800 µL of each standard was mixed with 200 µL of the Bradford dye (Bio Rad) reagent and

vortexed. The protein-dye suspension was incubated at room temperature for 5 mins. The absorbance at 595 nm was measured with the Biochrom Ultraspec 3000 spectrometer. A protein standard curve was generated from the data and used to determine concentrations of protein samples. Typically, protein samples were diluted 1000X in order to achieve an absorbance reading within the linear range.

2.2.4 Expression of *nudFHA* in *S. cerevisiae*

A *S. cerevisiae* replicating plasmid, expressing a codon optimized *nudF gene* was created. The *nudF* gene was amplified via PCR to contain an HA tag at the 3' end. The PCR product was inserted into pGEM-T and transformed into DH5α. Appropriate clones were selected and the *nudFHA* insert was confirmed via restriction endonuclease digest. The *nudFHA* fragment was excised, purified, and inserted into episomal P_{PYK}Bb plasmid. The P_{PYK}Bb*nudFHA* plasmid was transformed into DH5α and the appropriate clones were selected. The *nudFHA* insert was confirmed via restriction endonuclease digest. The P_{PYK}Bb*nudFHA* plasmid was transformed into the W303 *S. cerevisiae* strain. Transformants were plated and selected on -ura + dex plates.

2.2.5 Expression of *nudFHAHis* in BL-21(DE3) Rosetta

The *nudF* gene containing an HA tag and six histidine sequence at the 3' end was amplified via PCR, inserted into pGEM-T, and cloned into DH5α. Appropriate clones were selected and the *nudFHAHis* insert was confirmed

via restriction endonuclease digest. The *nudFHAHis* fragment was then excised, purified, and inserted into pET3a, a bacterial expression vector with a phage T7 promoter and transcriptional terminator. The pET3a containing *nudFHAHis* plasmid was transformed into DH5α and the appropriate clones were selected. The *nudFHAHis* insert was confirmed and pET3a containing *nudFHAHis* was transformed into competent BL-21(DE3) Rosetta and plated and selected on LB + Amp plates.

2.2.6. Testing Protein Production in Recombinant NudFHAHis

A 3 mL LB + Amp culture of BL-21(DE3) Rosetta containing pET3a-*nudFHAHis* was grown overnight at 37°C. On the following day the culture was diluted to an OD_{600} reading of 0.1 in 25 mL of LB + Amp. When the OD_{600} reading reached approximately 0.5 the culture was induced with 1 mM IPTG for 4 hours at 37°C. The culture was then centrifuged at 3,000 rpm for 5 mins and subsequently the supernatant was discarded and the cell pellet was washed twice with sterile ddH_2O. The cell pellet was resuspended in 300 μL of TE buffer. The pellet was sonicated 4 times for 30 secs with incubation on ice between each sonication. After sonication, the lysate was clarified by centrifugation at 14,000 rpm for 10 mins at 4°C. 1X Sample Buffer and 50 mM DTT was added to 10 μL of the clarified lysate. Prepared samples were heated at 100°C for 10 mins. NudFHAHis protein was resolved via SDS-PAGE. The 12 % gel was stained with Coomassie Brilliant Blue for 30 mins and destained with a solution containing 30 % methanol and 10 % acetic acid.

The gel was subsequently visualized with the BioRad Gel Doc XR+ imaging system.

2.2.7 Purification of NudFHAHis via Immobilized Metal Ion Affinity Chromatography Using a Phosphate Buffer

A 50 mL culture of the BL-21(DE3) Rosetta strain containing pET3a-*nudFHAHis* was grown in LB + Amp overnight at 37°C. On the following day the culture was diluted to an OD_{600} reading of approximately 0.2 in 200 mL of appropriate media. When the culture reached an OD_{600} reading of approximately 0.5 the culture was induced with 1 mM IPTG for 4 hours. Afterwards the culture was collected and centrifuged at 3,000 rpm for 5 mins. The supernatant was discarded and the cell pellet was washed twice with sterile ddH_2O and stored at -80°C. When required the cell pellet was resuspended in 2 mL of Lysis buffer (50 mM NaH_2PO_4/Na_2HPO_4 pH 7.6, 500 mM NaCl, 10 mM Imidazole, 1% Triton X-100, 1 mM PMSF, 1 µg/mL Aproptinin, 0.5 µg/mL Leupeptin, and 0.5 µL/mL Pepstatin). The sample was then sonicated 4 times for 30 secs with incubation on ice between each sonication. The lysate was clarified by centrifugation at 14, 000 rpm for 10 mins at 4°C. The immobilized metal ion affinity chromatography experiment was carried out following GE Healthcare's Chelating Sepharose Fast Flow procedure. The chromatography experiment was carried out using gravity flow at 4°C. Briefly, pre-swollen chelating sepharose fast flow was decanted and washed and resuspended with sterile ddH_2O. The BioRad poly-prep

chromatography column was assembled, packed with 0.5 mL of chelating sepharose, and washed with 2 column volumes (CV) of sterile ddH_2O. 0.2 CV of 100 mM $NiSO_4$ solution was applied to the column and subsequently the column was washed with 5 CV of sterile ddH_2O, and 5 CV of Acidic buffer (0.02 M Sodium Acetate and 0.75 M NaCl pH 4.0). The column is then equilibrated with 2 CV of the Lysis buffer (50 mM NaH_2PO_4/Na_2HPO_4 pH 7.6, 500 mM NaCl, 1 % Triton X-100, and 10 mM Imidazole). The clarified cell extract was applied to the column and the flow through was collected in 200 μL fractions. Once the extract had made its way down the column, the column was washed with 5 CV of Wash buffer (50 mM NaH_2PO_4/Na_2HPO_4 pH 7.6, 500 mM NaCl, 1 % Triton X-100, and 20 mM Imidazole). The wash was collected in a single batch. Bound proteins were then eluted with 2 CV of Elution buffer (50 mM NaH_2PO_4/Na_2HPO_4 pH 7.6, 500 mM NaCl, 1 % Triton X-100, and 250 mM Imidazole). The elution of bound proteins was achieved by competitive elution with a higher concentration of imidazole. The eluents were collected in 200 μL fractions. SDS-PAGE was carried out on 20 μL of each fraction collected. The 12 % gel was subsequently stained with Coomassie Brilliant Blue R250 for 30 mins, destained with a 30 % methanol and 10 % acetic acid solution until bands were visible and analyzed using the BioRad Gel Doc XR^+ imaging system. A western blot was also carried out on these samples. An αHA antibody was used for detection. The eluent fractions containing the protein band of interest were pooled and dialyzed against a dialysis buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, and 1 mM DTT) for 2 hours at 4°C.

The Spectra/Por molecular porous membrane tubing was used for dialysis. Following dialysis, the tubing containing the purified sample was immersed in PEG 8000 powder for 1 hour to concentrate the sample. The purified sample was stored at -20°C. .

2.2.8 NudFHAHis Activity Assay via Enzymatic Dephosphorylation

To test purified recombinant NudFHAHis activity a reaction mixture containing 0 µL or 5 µL of purified protein sample, 25 mM $MgCl_2$, 25 mM $MnCl_2$ and 2 µg of IPP or DMAPP was assembled. A set of positive control samples was prepared with 8 µL of elution buffer, 2 U of CIP (calf intestinal phosphatase (CIP), and 2 µg of IPP or DMAPP. The reactions were incubated at 37°C for 45 mins. The samples were then analyzed via GC-FID for isoprenol and prenol production (2.2.27).

2.2.9 Purification of NudFHAHis via Immobilized Metal Ion Affinity Chromatography Using a Tris Buffer

A 20 mL culture of the BL-21(DE3) Rosetta strain containing pET3a-*nudFHAHis* was grown in LB + Amp overnight at 37°C. On the subsequent day the culture was diluted to an OD_{600} reading of approximately 0.2 in 200 mL of appropriate media. When the culture reached an OD_{600} reading of approximately 0.5 the culture was induced with 1 mM IPTG for 4 hours. Afterwards the culture was collected and centrifuged at 3,000 rpm for 5 mins. The supernatant was discarded and the cell pellet was washed twice with sterile ddH_2O and stored at -80°C. When necessary the cell pellet was

resuspended in 2 mL of Lysis buffer (20 mM Tris-HCl pH 7.5, 250 mM NaCl, 0.5 % Terigtol, 5 % Glycerol, 1 mM PMSF, 1 µg/mL Aproptinin, 0.5 µg/mL Leupeptin, and 0.5 µL/mL Pepstatin). The sample was then sonicated 4 times for 30 secs, with incubation on ice between each sonication. The lysate was clarified by centrifugation at 14, 000 rpm for 10 mins at 4°C. The immobilized metal ion affinity chromatography experiment was carried out following GE Healthcare's Chelating Sepharose Fast Flow procedure. The chromatography experiment was performed using gravity flow at 4°C. Briefly, pre-swollen chelating sepharose fast flow was decanted and washed and resuspended in sterile ddH_2O. The BioRad poly-prep chromatography column was assembled, packed with 2 mL of chelating sepharose, and washed with 5 CV of sterile ddH_2O. 1 CV of 100 mM $NiSO_4$ solution was applied to the column and subsequently the column was washed with 5 CV of sterile ddH_2O. The column was then equilibrated with 5 CV of the Equilibration buffer (20 mM Tris-HCl pH7.5, 250 mM NaCl, 0.5 % Tergitol, and 5 % Glycerol). The clarified cell extract was applied to the column and the flow through was collected in several fractions. Once the extract had made its way through the column, the column was washed with 10 CV of Wash buffer (20 mM Tris-HCl pH 7.5, 0.5 M NaCl, 0.5 % Tergitol, 5 % Glycerol, and 10 mM Imidazole). The wash flow through was collected in a single batch. Competitive elution with a higher concentration of imidazole was carried out to elute bound proteins from the column. 5 CV of Elution buffer (20 mM Tris-HCl pH 7.5, 0.5 M NaCl, 0.5 % Tergitol, 5 % Glycerol, and

250 mM Imidazole) was used. The eluents were collected in 1 mL fractions. 10 µL of the collected flow through, wash, and eluents were ran on a 12 % mini gel stained with Coomassie Brilliant Blue R250 for 30 mins, destained with a 30 % Methanol and 10 % Acetic acid solution and visualized using the BioRad Gel Doc XR+ imaging system. The eluent fractions containing the protein band of interest were pooled. Finally, the pooled eluent sample was dialyzed against a buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl and 1 mM EDTA, 0.1 % BME, and 50 % Glycerol) for 2 hours at 4°C. The Spectra/Por molecularporous membrane tubing was used for dialysis. The purified sample was stored at -20°C.

2.2.10 NudFHAHis Activity Assay via P_iColorLock Gold

Innova Biosciences's P_iColorLock Gold kit (Innova Biosciences) was used to test the activity of the purified recombinant NudFHA-His. P_iColorLock Gold kit measures P_i based on the change in absorbance of malachite green. A 100 µL reaction mixture containing varying volumes of purified protein and control (0 µL, 1 µL, 5 µL, and 10 µL), 25 mM $MgCl_2$, 25 mM $MnCl_2$, and 2 µg of IPP or DMAPP was prepared. 100 µL reaction mixtures were also prepared for the positive control samples containing 2 U of CIP, 25 mM $MgCl_2$, 25 mM $MnCl_2$, and 2 µg of IPP or DMAPP. The manufacture's protocol was followed to carry out the reaction. Briefly, the phosphatase assay was performed in 96 well plates. All reactions were performed in triplicates. 25 µL of the prepared Gold mix was added to 100 µL reaction samples and incubated at room temperature for 5 mins. Following incubation, 10 µL of the Stabiliser was

added to the reaction samples. The reaction was carried out for an additional 30 mins at room temperature to allow for colour development. The plate was then measured using a plate reader (ThermoLabsytems Multiskan Ascent) at 600 nm. The amount of P_i present was determined by preparing a standard curve with the standard provided in the kit.

2.2.11 Recombinant NudFHAHis Protein Concentration

A series of BSA concentrations were prepared (0.05, 0.1, 0.2,0.4,0.6, and 1.0 mg/mL). These standards and NudFHAHis were mixed with 2X Sample buffer, boiled at 95°C and electrophoresed on a 12 % SDS-PAGE along with a MW protein ladder. The gel was stained with Coomassie Brilliant Blue R250 for 30 mins and destained with a solution containing 30 % Methanol and 10 % acetic acid. The gel was then visualized and analyzed with the BioRad Gel Doc XR^+ imaging system. The band intensity of each BSA standard was obtained and used to produce a standard curve. The standard curve was used to determine the concentration of NudFHAHis protein band.

2.2.12 Expression of *nudF* in *S. cerevisiae*

The *GAL1* promoter sequence was excised from the pESC-URA plasmid. The promoter sequence was then purified, ligated into YCplac33, and transformed into DH5α. Appropriate clones containing the YCplac33*GAL1* vector was confirmed via restriction endonuclease digest. The YCplac33*GAL1* vector was then digested and purified for further cloning. The *nudF* open reading frame with no epitope tag was amplified via PCR. The PCR product

was inserted into pGEM-T and transformed into DH5α. Appropriate clones were selected and the *nudF* insert was confirmed via restriction endonuclease digest. The *nudF* fragment was excised, purified, and inserted into the digested YCplac33*GAL1* vector. The YCplac33*GAL1nudF* plasmid was transformed into DH5α and the appropriate clones were selected. The *nudF* insert was confirmed via restriction endonuclease digest. The YCplac33*GAL1nudF* plasmid was transformed into *S. cerevisiae*. Transformants were plated and selected on appropriate plates.

2.2.13 GST-NudF Fusion Protein Production

The *nudF* open reading frame was amplified via PCR. 5 µL of the *nudF* PCR product was electrophoresed on a 0.8 % Agarose gel to confirm synthesis of the product. The PCR product was purified using a PCR clean up column (Qiagen) and subsequently digested. The cut product was then electrophoresed on a gel and purified. The purified cut product was ligated into pGEX-6p1 and transformed into DH5α. The *nudF* insert was confirmed and pGEX-6p1 containing *nudF* was transformed into competent BL-21(DE3) Rosetta and plated and selected on LB + Amp plates.

2.2.14 Purification of Recombinant GST-NudF Fusion Protein

A 3 mL LB + Amp culture of BL-21(DE3) Rosetta containing pGEX-6p1*nudF* was grown overnight at 37°C. On the following day, the culture was diluted into 100 mL of LB + Amp in a 500 mL flask. The culture was grown at 30°C until the final OD_{600} reading was between 0.5-1.0. The culture was then

induced with 1 mM IPTG for 3 hours. Afterwards, the culture was collected and centrifuged at 2500 rpm for 10 mins at 4°C. The media was discarded and the cell pellet was stored at -20°C. When required, the cell pellet was resuspended in 10 mL of GST binding buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 1mM PMSF, 1 µg/mL Aproptinin, 0.5 µg/mL Leupeptin, and 0.5 µL/mL Pepstatin). 10 % Triton X-100 was added to a final concentration of 0.5 % and the sample was vortexed at a low speed for 1 min. The sample was then incubated at -80°C for 30 mins. Following incubation the sample was thawed and subsequently sonicated for 10 secs and incubated on ice for 1 min. This cycle was repeated two more times. The lysate was centrifuged at 10, 000 rpm for 10 mins at 4°C. The supernatant was collected. 1 mL of glutathione beads was added to the cell extract and the suspension was incubated on a rocker at 4°C for 1 hour. The mixture was poured into a disposable column (Bio Rad). The flow through was collected. The column was then washed with 10 mL of wash buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 µg/mL Aproptinin, 0.5 µg/mL Leupeptin, and 0.5 µL/mL Pepstatin). The wash flow through was also collected. Next, the column was eluted with 5 mL of Elution buffer (10 mM reduced glutathione, 50 mM Tris-HCl pH 8.0, 5 % glycerol, and 1 mM DTT). The eluent was collected in fractions and all samples collected were stored at -20°C for further analysis. Fusion protein production was determined by analyzing the fractions via SDS-PAGE and Coomassie Brilliant Blue staining.

2.2.15 GST-NudF Activity Assay via pNPP Phosphatase Assay

A series of CIP controls were prepared (0.001 U, 0.002 U, 0.003 U, 0.004 U, and 0.005 U). Briefly, the phosphatase assay was carried out in 100 µL reactions in 96 well plates. 10 µL of each CIP standards and GST-NudF fusion protein were mixed with 50 µL of pNPP buffer (50 mM Tris-HCl pH 8.3, 1.0 mM EGTA, 30 mM $MgCl_2$, 1 mg/mL BSA, and fresh 0.5 mM $MnCl_2$ and 0.2 % BME). The reactions were incubated at 37°C for 10 min and subsequently 10 µL of 30 mM pNPP was added to each well. The reactions were then incubated at 37°C for 2 hours for absorbance to reach approximately 0.5 at 405 nm.

2.2.16 Expression of t*hmg1MYC* in *S. cerevisiae*

The *HMG1* gene sequence containing 1596 bp deletion from the 5' end and a Myc tag at the 3' end was amplified via PCR, inserted into pGEM-T, and cloned into DH5α. Clones were selected and the t*hmg1MYC* insert was confirmed via restriction endonuclease digest. The t*hmg1MYC* fragment was then excised, purified, and inserted into *S. cerevisiae* episomal plasmid, P_{PDC}Bb. The t*hmg1MYC* containing plasmids were transformed into DH5α and the appropriate clones were selected. The t*hmg1MYC* insert was confirmed by restriction endonuclease digest and subsequently the P_{PDC}Bbt*hmg1MYC* plasmid was transformed into a *S. cerevisiae* strain. Transformants were plated and selected on appropriate plates.

2.2.17 HMG-CoA Reductase Enzyme Assay

The HMG-CoA activity assay was adapted from Qureshi *et al.*. A 25 mL *S. cerevisiae* culture expressing a hyperactive HMG-CoA reductase was grown overnight at 30°C. On the next day the culture was diluted in 25 mL of appropriate media to an OD_{600} reading of approximately 0.2. The cultures were incubated at 30°C until the culture reached an OD_{600} reading of approximately 0.5-0.7. The cell pellet was collected and subsequently was washed twice with sterile ddH_2O. The cell pellet was resuspended in 300 μL of Lysis buffer (50 mM Tris-HCL pH 7.5, 100 nM NaCl, 1 mM EDTA and 0.1 % Tergitol). Glass beads were added to the meniscus and the cell suspension was vortexed at max speed 3 times for 5 mins with a 5 min incubation between each vortex. An additional 300 μL of Lysis buffer (100 mM Phosphate buffer pH 7.0, 1mM EDTA pH 8.0, and 5 mM DTT) was added and the cell suspension were transferred into new tubes and incubated on ice for approximately 1 hour. The cell suspension was centrifuged at 14,000 rpm for 2 mins at 4°C. The supernatant was collected and the cell extract was incubated on ice until needed. 1 mL reactions were carried out to determine activity of HMGCR. A reaction buffer containing 100 mM KPO_4 pH 7.0, 0.5 % w/v Tergitol, and 5 mM DTT was prepared. The reaction buffer was incubated at 30°C with 150 μM of NADPH (Sigma) diluted in 3 mM KPO_4 pH 7.0 for a minute. The cell extract was then added to this mixture and incubated again at 30°C for 5 mins. This step stabilizes endogenous oxidation of NADPH. An initial absorbance reading at 340 nm was taken with the

Biocrom Ultraspec 3000, an UV/Visible spectrometer, this reading was considered to be time zero. After the addition of 150 μM of HMG-CoA (Sigma) diluted in 3 mM KPO_4 pH 7.0 the absorbance at 340 nm was measured at specific time intervals, 15, 30, 45, 60, 90, 120, 180 and 240 secs. The total protein amount was quantified by carrying out the Bradford protein assay, as described previously (2.2.3.5). The initial rate of the reaction was determined by concentrating on the linear section of the assay. In this case, the initial rate was the change in absorbance between 0 and 60 secs. Lastly, the specific activity of HMGCR was calculated by dividing the initial rate with the total protein concentration.

2.2.18 Expression of t*HMG1* in *S. cerevisiae*

The *HMG1* gene sequence containing 1596 bp deletion at the 5' end and no epitope tag was amplified via PCR, inserted into pGEM-T, and cloned into DH5α. Clones were selected and the t*HMG1* insert was confirmed via restriction endonuclease digest. The t*HMG1* fragment was then excised, purified, and inserted into *S. cerevisiae* episomal plasmid, P_{PDC}Bb and centromeric plasmid, YCplac22. The t*HMG1* containing plasmids were transformed into DH5α and the appropriate clones were selected. The t*HMG1* insert was confirmed by restriction endonuclease digest and subsequently the P_{PDC}Bbt*HMG1 and* YCplac22t*HMG1* plasmids were independently transformed into a *S. cerevisiae* strain. Transformants were plated and selected on -ura + dex or -trp + dex plates.

2.2.19 Construction of a Methionine Regulated *ERG20 S. cerevisiae* Strain

A truncated *ERG20* fragment containing the first 660 bp of the open reading frame was generated via PCR. This fragment was cloned into a pGEM-T and the DNA was purified. The Δ*erg20* fragment was excised from the pGEM-T via restriction endonuclease digestion with BamH1 and Spe1 and subsequently gel purified. This fragment was inserted into the *S. cerevisiae* integrating pRS405-*MET3* plasmid and transformed into *E. coli*. Transformants were grown on LB + Amp plates, colonies were selected and the plasmid was purified. The plasmid was subsequently digested with HinD III to linearize the plasmid. A control restriction endonuclease digest with EcoRI was also carried out independently. After the restriction endonuclease digestion 5 μL of salmon sperm DNA was added and the linearized DNA was subsequently precipitated with 1/10 volume of 3M NaOAC and 2 volumes of 95 % ethanol. The suspension was incubated at -20°C for 1 hour. Following incubation the DNA was collected via centrifugation at 14,000 rpm for 20 mins. The DNA precipitate was washed with 70 % ethanol, dried, and resuspended in 1X TE buffer. The precipitated DNA was then used for *S. cerevisiae* transformation. Control and experimental transformations were plated on -met -leu + dex plates. Once colonies appeared a few single colonies were split and patched onto two different plates, -met -leu + dex plates and -leu + dex plates. The strains harbouring the correct integration grew well on -met -leu + dex plates and poorly on -leu + dex plates.

2.2.20 Analysis of DMAPP via Reverse-Phase HPLC

Reverse-phase HPLC analysis was adapted from Zhang and Poulter, and Henneman *et al.*. The HPLC system consisted of a ABI 140D Microgradient Delivery System, a ABI 785A UV/VIS Detector, and a Kipp & Zonen CD41 chart recorder (Life Technologies). Using a syringe, 10 µL of the DMAPP standard was injected onto a C18 column (Thermo Scientific). The standard was separated by a linear gradient between solution A (25 mM NH_4HCO_3 pH 8.0) and solution B (100 % Acetonitrile). The gradient started at 1 % solution B and increased to 65 % solution B and the flow rate was 50 µL/min. When peaks were detected on the chromatogram the eluent corresponding to each peak was manually collected and analyzed via electrospray ionization mass spectrometry (EIS-MS)(2.2.23).

2.2.21 Extraction of IPP and DMAPP for Mass Spectrometry (MS) Analysis

The method described by Tamaki *et al.* was adapted to extract IPP and DMAPP from the *ERG20* conditional strain over expressing a hyperactive HMG-CoA reductase. A 50 mL culture was grown overnight at 30°C and on the following day the culture was diluted to an approximate OD_{600} of 0.2. The culture was the grown at 30°C until it reached an approximate OD_{600} reading of 0.5. The culture was then split into two separate flasks containing 20 mL of culture in each flask. One culture was treated with 2 mM methionine and the other was treated with ddH_20. The cultures were incubated for 4 hours or overnight at 30°C. After treatment the culture was centrifuged for 5 mins at

3000 rpm at room temperature. The supernatant was discarded and the cell pellet was washed once with 100 mM NH_4HCO_3 and then resuspended in 300 μL of extraction solution (2-propanol:100 mM NH_4HCO_3 (1:1 v/v)). The sample was then incubated at 70°C for 5 mins. The sample was vortexed for 1 min and subsequently sonicated three times for 1 min with incubation on ice between each sonication. After sonication 500 μL of extraction solution and 800 μL of acetonitrile was added. The mixture was vortexed for 10 secs and then incubated on ice for 10 mins. Following incubation the sample was centrifuged at max speed for 5 mins. The supernatant was then evaporated using a centrifugal vacuum evaporator over night. The residue was re-dissolved in 100 μL of 25 mM NH_4HCO_3. The extract was analyzed via electrospray ionization mass spectrometry (EIS-MS).

2.2.22 Electrospray Ionization Mass Spectrometry (ESI-MS)

Mass spectrometry was performed using an LTQ Orbitrap XL mass spectrometer (Thermo Scientific) equipped with an electrospray ionization source. Samples were introduced into the instrument via direct infusion with a glass syringe connected to a syringe pump at a flow rate of 5 μL/min. The mass spectrometer was operated in negative mode with a spray voltage of -5 kV, a sheath gas rate of 20, a capillary temperature of 200˚C, and a tube lens voltage of 100.

2.2.23 Enzymatic Dephosphorylation to determine endogenous concentrations of IPP and DMAPP

The method developed by Vallon *et al.* was adapted for the enzymatic dephosphorylation of IPP and DMAPP. A 50 mL culture of the *ERG20* conditional strain expressing a hyperactive HMG-CoA reductase was grown overnight at 30°C and on the next day the culture was diluted to an approximate OD_{600} reading of 0.2. The culture was grown at 30°C until it reached an approximate OD_{600} reading of 0.5. The culture was then split into two separate flasks each containing 20 mL of culture. One culture was treated with 2 mM methionine to shut off expression of *MET3-ERG20* and the other was treated with the same volume of ddH_20. The cultures were incubated for 4 hours or overnight at 30°C. After treatment the culture was centrifuged for 5 mins at 3000 rpm at room temperature. The supernatant was discarded and the cell pellet was washed with ddH_2O. The cell pellet was resuspended in 2 mL of Hydrolysis buffer (1 M Diethanolamine (Sigma) pH 9.8, 0.5 mM $MgCl_2$) and vortexed for 1 min. Cell lysis was performed by sonication of the cell pellet for 20 mins. The suspension was vortexed for 1 min. Dephosphorylation was carried out with 3 U of CIP. Hydrolysis was performed for 45 mins at 65°C. The suspension was vortexed again for 1 min and subsequently extracted with 2 mL of hexane. The mixture was vortexed for 3 mins and centrifuged at 12000 x*g* for 15 mins. The hexane phase was collected for GC-FID analysis (2.2.27).

2.2.24 Labeled Thin Layer Chromatography to Measure Endogenous IPP

The method described by Beyer *et al.* was adapted to measure endogenous IPP. The *S. cerevisiae* strain containing the YCplac22t*HMG1* plasmid was grown overnight at 30°C. When the culture reached an OD_{600} reading of approximately 1.0, 5 mL of culture was treated with 2 mM methionine to repress expression of *MET3-ERG20* and another 5 mL was treated with ddH_2O. The cultures were incubated for 4 hours at 30°C. After stimulation the cultures were labeled with 10μCi C^{14}-Acetate (Perkin-Elmer) for 45 mins. 20 μL of 20 mM Sodium Azide was added to stop the labeling. The cultures were centrifuged at 3000 rpm for 5 mins. The supernatant was discarded and the cell pellet was resuspended and washed with 1 mL ice-cold water. 50 μL of each sample was collected in order to determine total counts incorporated. The remaining sample was centrifuged again at 3000 rpm for 5 mins. The pellets were stored at -20°C. On the following day, whole cell lipid extraction was carried out on the pellets collected. The extraction method was adapted from the protocol described by Schneiter and Daum. The pellets were resuspended in 1 mL methanol and transferred to a 15 mL glass vial. Glass beads were added to the top of the meniscus and the suspension was vortexed for 30 secs with 30 secs cooling intervals. 2 mL of chloroform was added to the samples and the suspension was stirred for 1 hour on a stirrer at room temperature. 1 mL of ddH_2O was added to the suspension and subsequently vortexed for 1 min. The extract was centrifuged at 3000 rpm for 5 mins. The upper aqueous layer was collected and discarded and the

organic phase was collected into glass vials (Fisher Scientific). The organic phase was evaporated with N_2 gas and the lipid film was dissolved with 200 μL of methanol. 20 μL of the treated and untreated sample was applied to a precoated cellulose sheet (Eastman Kodak Company) six times. Three of the spots from each sample were spiked with 5 μg of IPP and the other three spots were spiked with 4 μg of FPP. The TLC sheets were developed with 1-propanol/NH_3 (25 % aqueous solution)/H_2O (6:3:1). Once the solvent front reached about an inch from the top of the sheet, the separation process was halted and the TLC sheet was air-dried. The sheet was then stained with iodine and the spots corresponding to IPP and FPP were collected and suspended in scintillation vials (Fisher Scientific) containing 10 mL of scintillation fluid (Fisher Scientific). A liquid scintillation counter (Packard Tricarb 2100 TR – A210001) was used to determine C^{14} counts.

2.2.25 Construction of an Isoprenol Producing *S. cerevisiae* Strain

An *S. cerevisiae* strain was engineered to produce isoprenol. The methionine regulated *ERG20* strain, *erg20*::P_{MET3}*ERG20* was further manipulated by methods described previously to express a hyperactive HMG-CoA reductase and an active NudF. Briefly, the *nudF* containing vector, P_{PYK}*nudFHA* and the highly expressed truncated *HMG1* containing vector P_{PDC}Bb*thmg1* were integrated together into a single plasmid and transformed into e*rg20*::P_{MET3}*ERG20*. The *nudF* containing vector YCplac33*GAL1nudF* and the single copy truncated *HMG1* containing vector YCplac22*tHMG1* were also transformed into e*rg20*::P_{MET3}*ERG20*. The correct transformants were

selected by plating the same colony onto both – met + dex and + met + dex plates. Colonies that did not grown on + met plates were used for isoprenol analysis. Table 2.8 outlines all the *S. cerevisiae* strain created.

Table 2.8 *Saccharomyces cerevisiae* strains developed

Strain	Description/Properties	Medium
erg20::P$_{MET3}$*ERG20* P$_{PDC}$Bb	Empty vector control	-met -ura + dex
erg20::P$_{MET3}$*ERG20* P$_{PDC}$Bbt*HMG1*	Over expresses the hyperactive *HMG1* gene	-met -ura + dex
erg20::P$_{MET3}$*ERG20* P$_{PDC}$Bbt*HMG1MYC*	Over expresses the truncated *HMG1* gene	-met -ura + dex
erg20::P$_{MET3}$*ERG20* P$_{PDC}$Bbt*HMG1*P$_{PYK}$Bb*nudFHA*	Over expresses the hyperactive *HMG1* gene and over expresses *nudF*	-met -ura + dex
erg20::P$_{MET3}$*ERG20* P$_{PDC}$Bbt*hmg1MYC*P$_{PYK}$Bb*nudFHA*	Over expresses the truncated *HMG1*gene and over expresses *nudF*	-met -ura + dex
erg20::P$_{MET3}$*ERG20* YCplac22	Empty vector control	-met -trp + dex
erg20::P$_{MET3}$*ERG20* YCplac22t*HMG1*	Expresses the hyperactive *HMG1* gene in single copy	-met -trp + dex
erg20::P$_{MET3}$*ERG20* YCplac22YEplac195	Empty vector control	-met -ura -trp + dex
erg20::P$_{MET3}$*ERG20* YCplac22t*HMG1*P$_{PYK}$Bb*nudFHA*	Expresses the hyperactive *HMG1* in single copy and over expresses *nudF*	-met –ura -trp + dex
erg20::P$_{MET3}$*ERG20* YCplac22YCplac33*GAL1*	Empty vector control	-met -ura -trp + dex
erg20::P$_{MET3}$*ERG20* YCplac22t*HMG1*YCplac33*GAL1nudF*	Expresses the hyperactive *HMG1 gene* in single copy and over expresses an active *nudF* gene	-met -ura -trp + dex

2.2.26 *S. cerevisiae* Culture Preparation for Qualitative Analysis of Isoprenol (1)

A 50 mL culture was grown over night at 30°C and on the following day diluted to an approximate $0.D._{600}$ of 0.2 and grown at 30°C until it reached an approximate OD_{600} reading of 0.5. The culture was then split into two separate flasks containing 20 mL of culture. One culture was treated with 2 mM methionine and the other was treated with the same volume of ddH_20. These cultures were then incubated at 30°C for 4 hours or overnight. After incubation the cultures were centrifuged for 5 mins at 3000 rpm. 10 mL of the supernatant was collected and set aside. The cell pellet was then washed twice with ddH_20 and subsequently resuspended in 2 mL of Lysis buffer (10 mM Tris, 150 mM NaCl pH 8.0). Next, glass beads were added to the cell lysis suspension and vortexed for 1 min. The cell lysis suspensions and supernatants collected were then extracted with 2 mL of Hexane. The sample was vortexed 3 times for 1 min and centrifuged for 15 mins at 3000 rpm. The hexane layer was collected, stored in a glass vials with Teflon lined screw caps, and analyzed via GC-FID (2.2.27).

2.2.27 Gas Chromatography (1)

Gas chromatography methodology was adapted from Withers *et al.*. 0.8 mL of the hexane sample was transferred into a GC vial (Fisher) containing 0.2 mL of commercial internal standard. Water (blank) and 0.5 % Isobutanol (internal standard) were used for polar samples and hexane (blank) and 0.5 % Hexanol (internal standard) were used for non-polar samples (samples

extracted with hexane). Standard samples were prepared by mixing 0.2 mL of each commercial standard (0.5 % Isoprenol, 0.5 % Prenol and 0.5 % Isobutanol or 0.5 % Hexanol) with 0.6 mL of water (polar samples) or hexane (non polar samples). Analysis for the polar samples was carried out using the Agilent Technologies 7890A GC System with the Agilent Technologies 7693 Autosampler and the Restek Stabilwax-DA column (30 m by 0.53 mm inner diameter, 0.5 μm film thickness). A 1 μL injection in split mode (10:1 split ratio) was used. The injector temperature was 170°C and the FID temperature of 200°C. The helium carrier gas was at constant pressure mode (7.5 lb/psi). The initial oven temperature was at 35°C held for 3 mins, followed by 20°C/min ramp to 190°C with a final hold of 5 mins. Analysis for non-polar samples was carried out using an Agilent Technologies 6890N GC System with an Agilent 7683B Autosampler and an HP-5 column. A 1 μL injection in split mode (20:1 split ratio) was used. The injector temperature was 60°C and the FID temperature of 320°C. The helium carrier gas was at constant pressure mode (12.48 lb/psi). The initial temperature was at 60°C for 3 mins followed by a 15°C/min ramp to 300°C with a final hold for 1 minute. The data obtained from each sample's chromatogram was compared to the chromatograms of the commercial standards.

2.2.28 *S. cerevisiae* Culture Preparation for Quantitative Analysis of Isoprenol (2)

A 50 mL culture was grown over night at 30°C and on the following day diluted to an approximate $0.D._{600}$ of 0.5 and grown at 30°C until it reached an

approximate OD_{600} reading of 0.9. The culture was then split into two separate flasks containing 20 mL of culture. One culture was treated with 2 mM methionine and the other was treated with the same volume of ddH_20. Both cultures were treated with 2 % galactose. These cultures were then incubated at 30°C for 4 hours or overnight. After incubation the cultures were centrifuged for 5 mins at 3000 rpm. 10 mL of the media was collected and spiked with 40 µg of the internal standard, 1-hexanol. The collected media was initially extracted with 2 mL of ethyl acetate. The samples were vortexed 3 times for 30 secs and centrifuged for 5 mins at 3000 rpm. 1 mL of ethyl acetate layer was recovered and collected. An additional 1 mL of ethyl acetate was added to the samples. Samples were vortexed and recovered as described earlier. This was carried out twice, resulting in three 1 mL extracts for each sample. Extracts were stored at -20°C in a glass vials with Teflon lined screw caps, and analyzed via GC-FID (2.2.29).

2.2.29 Gas Chromatography (2)

Gas chromatography methodology was adapted from Withers *et al.*. 200 µL of the ethyl acetate extract sample was transferred into a GC vial (Agilent Technologies). Standard samples were prepared by mixing each commercial standard (0.5 % Isoprenol, 0.5 % Prenol, and 0.5 % Isopentyl alcohol) to varying final concentrations (75 µg/mL, 50 µg/mL, 30 µg/mL, 20 µg/mL, and 10 µg/mL). Analysis was carried out using the Thermo Scientific TRACE GC Ultra System with the Thermo Scientific Triplus Autosampler and the HP Innowax column (30 m by 0.53 mm inner diameter, 0.5-µm film thickness). A

1 µL injection in split mode (6:1 split ratio) was used. The injector temperature was 250°C and the FID temperature of 225°C. The helium carrier gas was at constant pressure mode. The initial oven temperature was at 50°C, held for 1 min, followed by an initial 10°C/min ramp to 150°C, held for 2 mins, and finally a 40°C/min ramp to 230°C, held for 3 mins. The data obtained from each standard mixture and sample was analyzed using the Thermo Xcalibur program. A standard curve was generated and used to determine the presence and concentration of each alcohol in the samples. Intrinsic properties of the alcohols prevent them from extracting in the same manner as one another. A set of commercial standards were extracted with ethyl acetate and analyzed via GC to determine percent recovery. The percent recovery was used to determine actual concentration of alcohol in the initial sample.

2.2.30 Isoprenol Toxicity Assay

A 10 mL culture of the W303 *S. cerevisiae* strain was grown overnight at 30°C in YEPD media. On the following day the culture was diluted to an OD_{600} reading of 0.05 in 50 mL of YEPD media containing isoprenol. Individual cultures were grown in various concentrations of isoprenol (0.00 %, 0.25 %, 0.50 %, 0.75 %, 1.00 %, 1.50 %, and 2.00 %) for 24 hours. The OD_{600} reading was measured at 2, 4, 6, and 24 hours.

2.2.31 Changes in Isoprenol Concentration in Growing *S. cerevisiae* Cultures

A 50 mL culture the W303 *S. cerevisiae* strain was grown overnight at 30°C in YEPD media. On the following day the culture was diluted to an OD_{600} reading of 0.05 in 50 mL of YEPD media containing 0.5 % isoprenol. The culture was grown for 24 hours and at 2, 4, 6, and 24 hours an OD_{600} reading was measured and a 1 mL sample was collected for GC-FID analysis (2.2.27).

2.2.32 Expression of other Phosphatases in *S. cerevisiae*

The *PHO8* gene sequence containing 189 bp deletion from the 5' end and the *PHO8* gene sequence containing the same 5' end deletion and a 69 bp deletion from the 3' end were amplified via PCR, inserted into pGEM-T, and cloned into DH5α. Clones were selected and the *pho8*Nt and *pho8*NCt insert was confirmed via restriction endonuclease digest. The truncated *PHO8* fragments was then excised, purified, and inserted into *S. cerevisiae* episomal plasmid, P_{PYK}Bb. The *PHO8* containing plasmids were transformed into DH5α and the appropriate clones were selected. The inserts were confirmed by restriction endonuclease digest and subsequently P_{PYK}Bb*pho8*Nt and P_{PYK}Bb*pho8*NC*t* plasmids were transformed into a *S. cerevisiae* stain.

The same truncated *PHO8* genes described above along with *LPP1* and *DPP1* were amplified via PCR, inserted into pGEM-T, and cloned into DH5α. Clones were selected and the inserts were confirmed via restriction endonuclease digest. The inserts were then excised, purified, and inserted into *S. cerevisiae*

episomal plasmid, pYES2. The phosphatase containing plasmids were transformed into DH5α and the appropriate clones were selected. The inserts were confirmed by restriction endonuclease digest and subsequently the phosphatase containing plasmids were transformed into a *S. cerevisiae* strain.

CHAPTER 3

Expression of *nudF* in *S. cerevisiae*

3.1 *nudF*

The *Bacillus subtilis nudF* gene encodes a protein called NudF, composed of 188 a.a. that belongs to the Nudix hydrolase superfamily of enzymes; this superfamily of enzymes catalyzes reactions on nucleoside diphosphate groups. More specifically, NudF belongs to the ADP-ribose pyrophosphatase subfamily. NudF contains a Nudix hydrolase domain, which inherently leads to its classification as a hydrolase.

E. coli harbouring a synthetic mevalonate pathway suffers from IPP toxicity[16]. Toxicity screens carried out by Withers *et al.* led to the discovery of genes that encoded for proteins capable of rescuing *E. coli* from IPP toxicity by converting IPP into its alcohol product[47]. Withers *et al.* demonstrated that *nudF*'s protein product, NudF had an affinity for IPP and that, *nudF* expression in *E. coli* harbouring a synthetic mevalonate pathway resulted in the production of isoprenol.

Given that the mevalonate pathway exists endogenously in *S. cerevisiae* it would be an excellent framework to manipulate to produce isoprenol. In order to convert endogenous IPP into its alcohol product, *nudF* was expressed in *S. cerevisiae*.

3.2 Expression of *nudFHA* in *S. cerevisiae*

Since *nudF* is a gene from *Bacillus subtilis*, a *nudF* open reading frame with an HA epitope tag, codon optimized for expression in *S. cerevisiae* was synthesized (ScNUD_pMA-T). The *nudF* open reading frame was introduced into an expression vector for protein production in *S. cerevisiae*. *nudFHA*'s expression is under the regulation of a *PYK1* promoter (2.2.4). The *PYK1* promoter is a constitutively active promoter which allows for the over expression of *nudFHA*. Figure 3.1 illustrates that *nudFHA* is expressed in *S. cerevisiae* since NudFHA production can be detected via western blot analysis of crude extracts. An HA antibody was able to detect the HA epitope tag on NudF at the expected molecular weight (lanes 5-8). In contrast, cells harbouring the expression vector alone displayed no signal on the western blot (lanes 3-4). These results confirm that *nudF* from *Bacillus subtilis* can be expressed in *S. cerevisiae* since protein production of NudFHA can be visualized.

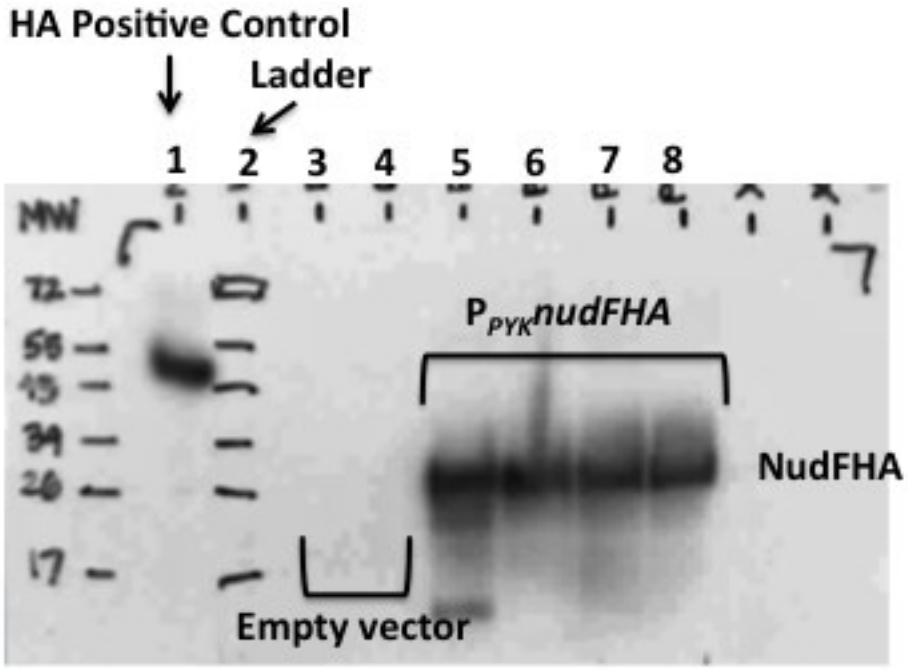


Figure 3.1 Expression of *nudFHA* in *S. cerevisiae*. Western blot was carried out on protein extractions from independent *S. cerevisiae* strains harbouring P*PYK*nudFHA *and* P*PYK*Bb. An HA antibody was used for detection. Lane 1: HA positive control, Lane 2: MW ladder, Lane 3-4: P*PYK*Bb, and Lanes 5-8: P*PYK*nudFHA containing strains.

3.3 Expression of *nudFHA-His* in *E. coli* BL-21(DE3) Rosetta

Since *S. cerevisiae* was able to express NudFHA it would be essential to determine if the NudFHA enzyme displays activity towards IPP and DMAPP. NudF has been shown to have affinity for IPP *in vivo* in *E. coli*, based upon the ability of *nudF* expression to produce isoprenol in *E. coli*[47]. The activity of NudFHA was assayed to investigate whether or not NudFHA would act directly on IPP and DMAPP and convert them into their respective alcohol products. To do this *nudF* was expressed in *E. coli* and subsequently purified to determine its activity towards IPP and DMAPP. The *nudF* opening reading

frame containing an HA epitope and a six histidine sequence at the 3' end was inserted into a bacterial replicating plasmid called pET3a where the expression of *nudF* is controlled by a bacteriophage T7 promoter (2.2.5.2). To induce expression of *nudFHA-His* the transformants were induced with IPTG. IPTG induces T7 RNA polymerase expression and subsequently induces the expression of *nudFHA-His*. SDS-PAGE was carried out on extracts prepared from stimulated and non-stimulated BL-21(DE3) Rosetta strains harbouring pET3a empty vector and pET3a containing *nudFHA-His* (2.2.5.3) Figure 3.3 verifies NudFHA-His protein production upon a 4 hour stimulation with 1 mM IPTG.

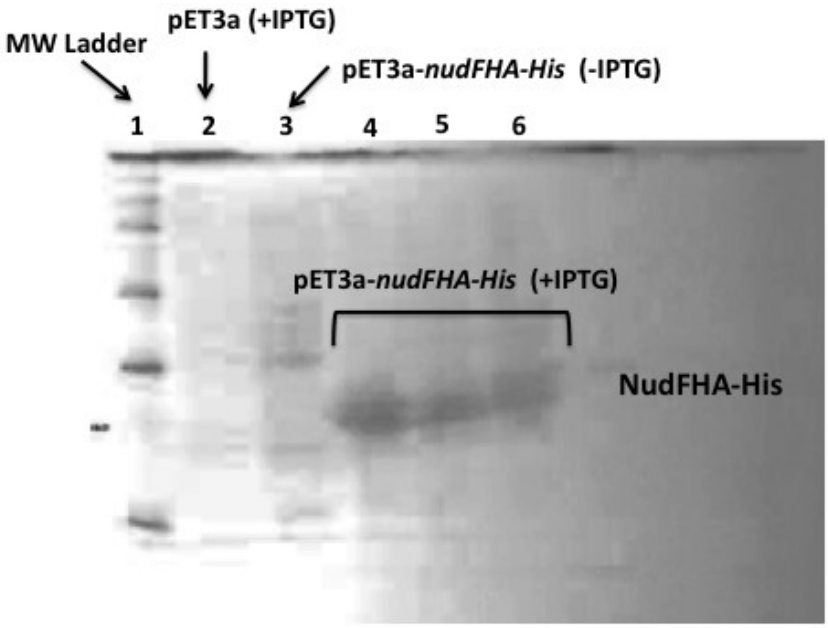
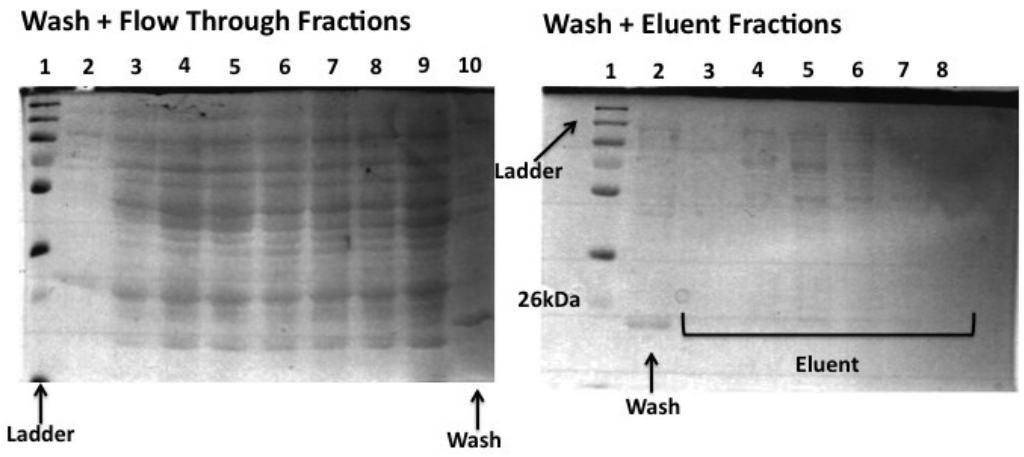


Figure 3.2 Recombinant NudFHA-His protein production. SDS-PAGE was carried out on cell extracts obtained from IPTG induced and non induced BL-21 Rosetta strains harbouring empty vector pET3a and pET3a containing *nudFHA-His*. The mini gel was stained with Coomassie Brilliant Blue. Lane 1: MW ladder, Lane 2: Empty vector stimulated with 1 mM IPTG. Lane 3: pET3a containing *nudFHA-His* non-stimulated, and Lane 4-6: pET3a containing *nudFHA-His* stimulated with 1 mM IPTG.

3.4 Recombinant NudFHA-His Purification

After verification of protein production, NudFHA-His was subsequently purified using a nickel column for functional analysis (section 2.2.5.4). A nickel column was used because the histidine sequence at the carboxyl terminus of the recombinant NudFHA-His can form complexes and bind with the transition metal Ni^{2+}. Figure 3.3a is a Coomassie Brilliant Blue stained mini gel of the flow through, wash and eluent samples collected from the chromatography experiment. Figure 3.3b is a western blot of the same samples using an HA antibody for detection of NudFHA-His. A western blot analysis was carried out because Coomassie Brilliant Blue staining was unable to detect NudFHA-His in the eluent fractions (Figure 3.3a). Western blot analysis confirms partial purification of NudFHA-His since the HA antibody was able to detect NudFHA-His at 26kD in the flow through fractions (Figure 3.3b). Eluent fractions 2 to 5 were pooled for further analysis.

a)



b)

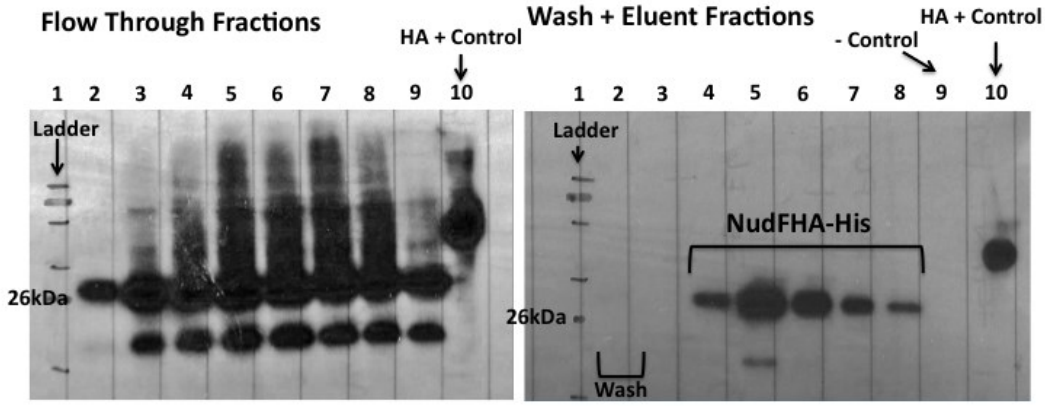


Figure 3.3 Recombinant NudFHA-His purification with phosphate buffer **a)** SDS-PAGE was carried out on flow through, wash and eluent samples. The mini gels were stained with Coomassie Brilliant Blue. Left, Lane 1: MW ladder, Lanes 2-9: flow through fractions, and Lane 10: pooled wash fractions. Right, Lane 1: ladder, Lane 2: pooled wash fractions, and Lanes 3-8: eluent fractions. **b)** Western blot of flow through, wash and eluent samples using an HA antibody. Left, Lane 1: MW ladder, Lanes 2-9: flow through fractions, and Lane 10: HA positive control. Right, Lane 1: ladder, Lane 2: pooled wash fractions, and Lanes 3-8: eluent fractions, Lane 9: negative control, and Lane 10: HA positive control.

3.5 Qualitative Analysis of NudFHA-His Activity

Once recombinant NudFHA-His was purified a functional enzyme assay was carried out to determine NudF's affinity for IPP and DMAPP and its ability to convert these intermediates into their alcohol products (2.2.8). Gas chromatography was performed to screen the reactions for isoprenol and prenol in the reaction mixtures. Figure 3.4 illustrates the chromatogram generated from a mixture of commercial standards containing isoprenol, prenol, and the internal standard, isobutanol. This chromatogram was used to deduce the presence and production of isoprenol and prenol in the experimental reactions.

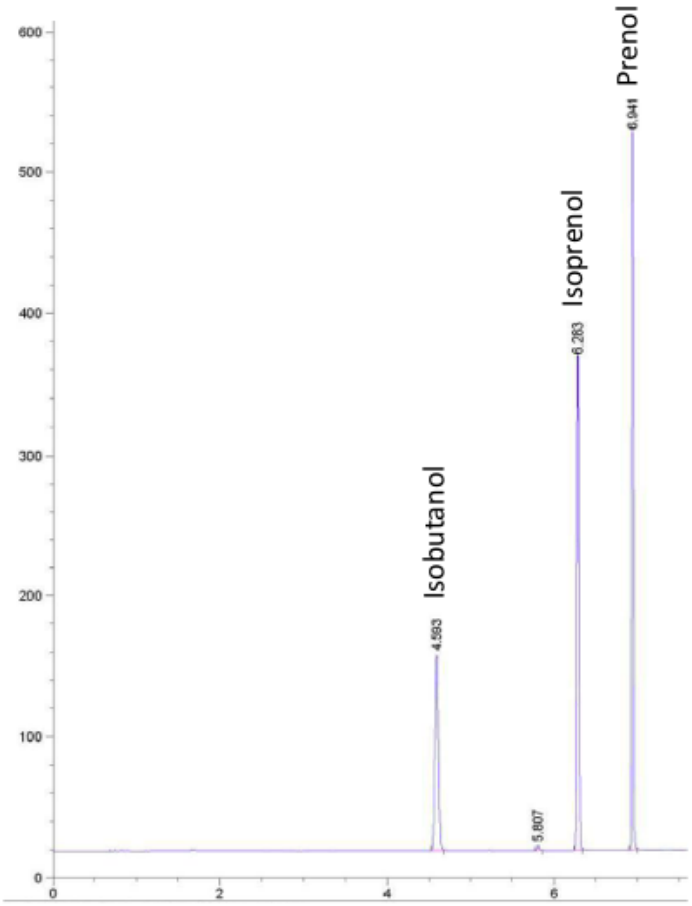


Figure 3.4 Chromatogram of standards Gas chromatography was performed on commercial standards. From left to right, the first peak at a retention time of 4.593 mins is the internal standard isobutanol. The peak at 6.283 mins is isoprenol and the last peak at 6.941mins is prenol.

Figures 3.5 and 3.6 are chromatograms of reactions mixtures containing commercial calf intestinal alkaline phosphatase (CIP) and IPP (Figure 3.5) or DMAPP (Figure 3.6). CIP can efficiently remove phosphate groups and was therefore used as a positive control. Figure 3.5 and 3.6 illustrate that CIP can effectively remove the phosphate groups from IPP and DMAPP to produce their alcohol derivatives, isoprenol and prenol respectively.

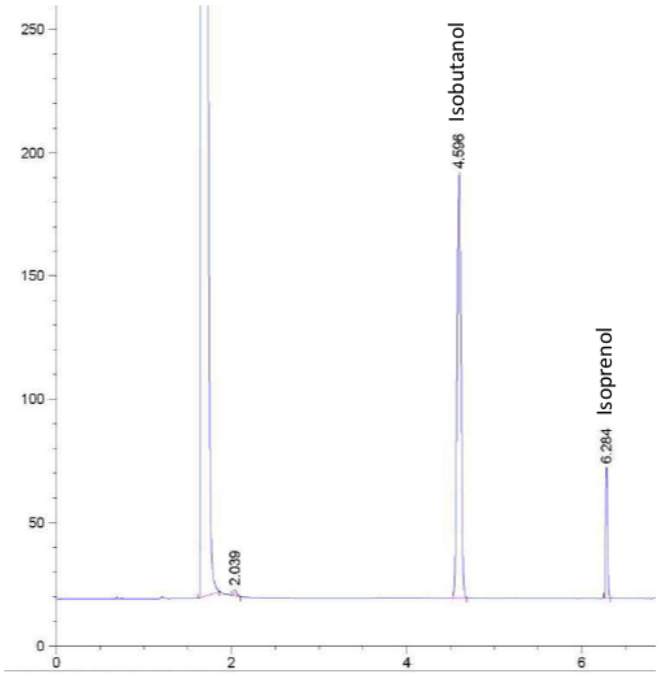


Figure 3.5 Chromatogram of CIP and IPP reaction mixture Gas chromatography was performed on a reaction mixture containing 8 µL elution buffer, 2 U CIP, and 2 µg IPP. The peak at the retention time of 4.596 mins is the internal standard isobutanol and the peak at 6.284 is isoprenol.

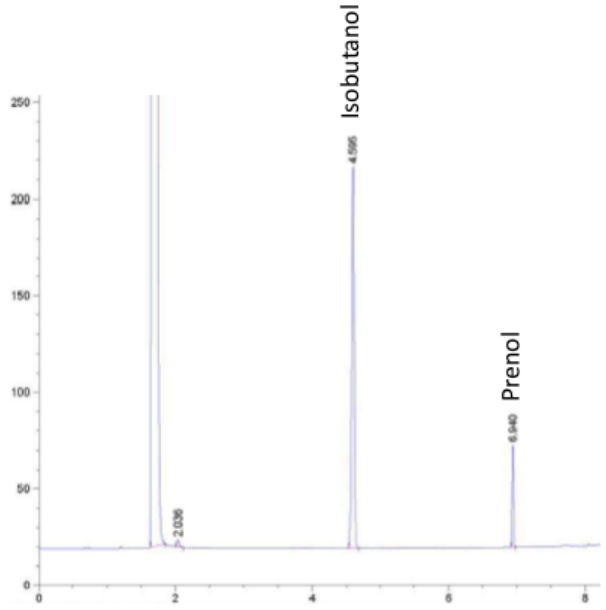


Figure 3.6 Chromatogram of CIP and DMAPP reaction mixture Gas chromatography was performed on a reaction mixture containing 8 µL elution buffer, 2 U CIP, and 2 µg DMAPP. The peak at the retention time of 4.595 mins is the internal standard isobutanol and the peak at 6.940 is prenol.

Figures 3.7 and 3.8 are chromatograms from reaction mixtures containing the purified recombinant NudFHA-His sample and IPP (Figure 3.7) or DMAPP (Figure 3.8). Samples containing the purified recombinant NudFHA clearly show a product peak for isoprenol (Figure 3.7) and prenol (Figure 3.8).

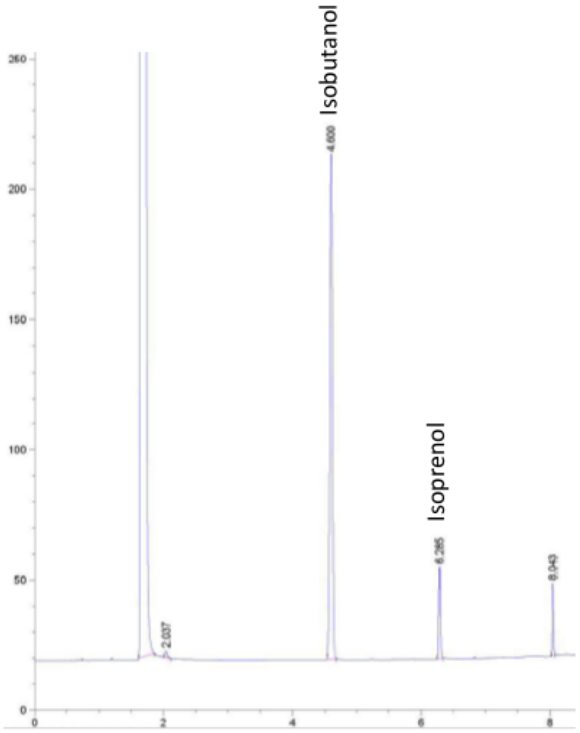


Figure 3.7 Chromatogram of recombinant NudFHA-His and IPP reaction mixture Gas chromatography was performed on a reaction mixture containing 5 µL recombinant NudF and 2 µg IPP. The peak at the retention time of 4.600 mins is the internal standard and the peak at 6.285 is isoprenol.

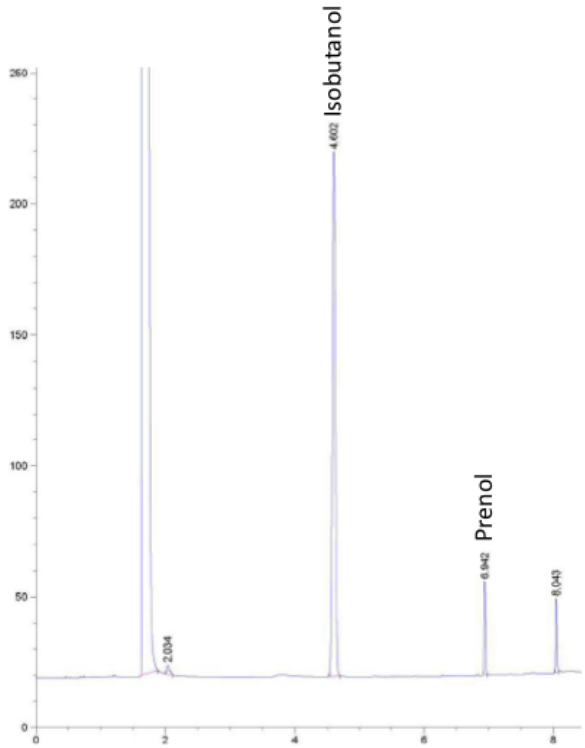


Figure 3.8 Chromatogram of recombinant NudFHA-His and DMAPP reaction mixture A reaction mixture containing 5 µL recombinant NudF and 2 µg DMAPP was prepared. The peak at the retention time of 4.602 mins is the internal standard and the peak at 6.9425 is prenol.

3.6 Quantitative Analysis of NudFHA-His Activity

Since the purified recombinant NudFHA-His sample was able to convert IPP and DMAPP into their alcohol products it would be informative to clarify that NudFHA-His was in fact involved in these dephosphorylation reactions. Histidine rich proteins present BL-21(DE3) Rosetta can be purified along with NudFHA-His, therefore it would be important to demonstrate that these co-purified proteins were not involved in the dephosphorylation reactions illustrated previously. Also, it would be informative to determine the activity of NudFHA-His. Given this, the BL-21(DE3) Rosetta strain harbouring the

empty vector, pET3a and vector containing *nudF* (pET3a-*nudFHA*-His) were grown and purified via chromatography (2.2.9). Figure 3.9 is Coomassie Brilliant Blue stained mini gels of the flow through, wash, and eluent samples collected from the empty vector strain. Figure 3.10 is Coomassie Brilliant Blue stained mini gels of the fractions collected from the strain harbouring the *nudF* gene. Figure 3.10 illustrates a high production of a protein around 26 kD in the *nudF* containing strain when compared to the empty vector. This observation suggests that NudFHA-His was enriched in this chromatography experiment. Eluent fractions 2 to 4 were pooled from the empty vector purification and eluent fractions 1 to 3 were pooled from the NudF purification for further analysis.

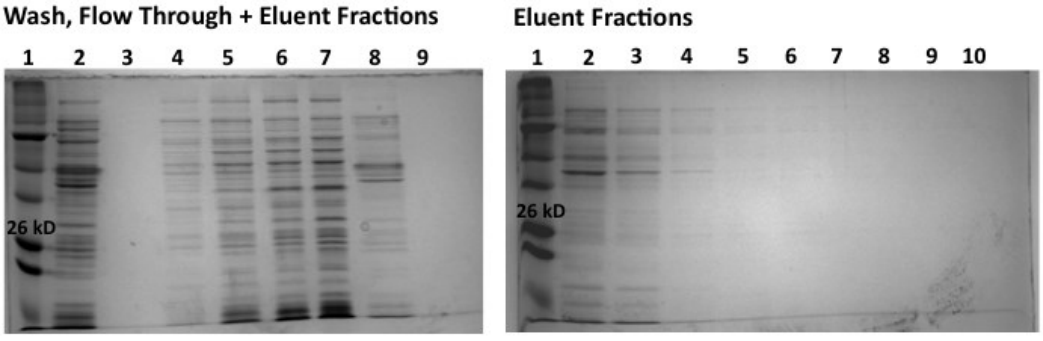


Figure 3.9 Recombinant control purification with Tris buffer SDS-PAGE was carried out on flow through, wash and eluent samples. The mini gels were stained with Coomassie Brilliant Blue. Left Gel, Lane 1: MW ladder, Lane 2: pooled wash fraction, Lanes 3-8: flow through fractions, and Lane 9: eluent fraction 1. Right Gel, Lane 1: ladder, Lane 2-10: eluent fractions 2-10.

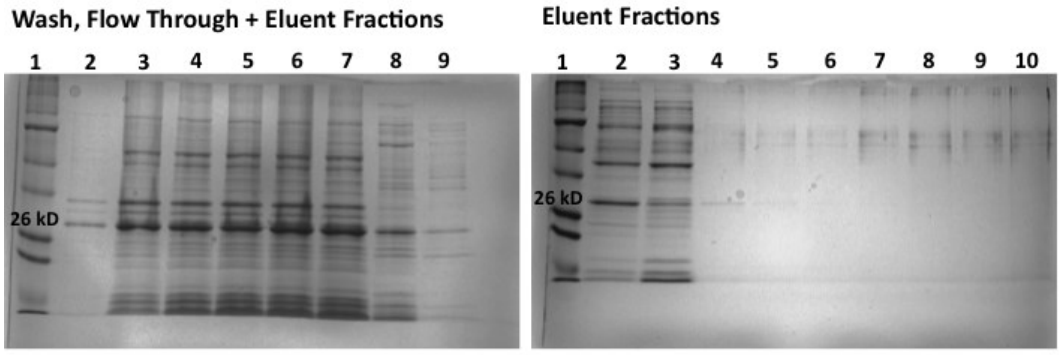


Figure 3.10 Recombinant NudFHA-His purification with Tris buffer SDS-PAGE was carried out on flow through, wash and eluent samples. The mini gels were stained with Coomassie Brilliant Blue. Left Gel, Lane 1: MW ladder, Lane 2: pooled wash fraction, Lanes 3-8: flow through fractions, and Lane 9: eluent fraction 1. Right Gel, Lane 1: ladder, Lane 2-10: eluent fractions 2-10.

Once recombinant NudFHA-His and the co-purified proteins were assessed via SDS-PAGE a phosphatase assay was carried out to determine NudF's and the eluent fractions from empty vector extract's ability to dephosphorylate IPP and DMAPP. Dephosphorylation of these substrates release inorganic phosphate (P_i). The P_iColorLock Gold kit from Innova Biosciences was used to measure P_i in the reaction mixtures (2.2.10). Table 3.1 represents the data obtained from the phosphatase assay. The eluent fractions from the control strain and the NudF expressing strain show similar specific activities for the substrate DMAPP, $2.3x10^{-14}$ and $2.2x10^{-14}$, respectively. These results suggest that rNudFHA-His has no activity against DMAPP. Furthermore, there is a small difference between specific activities of the control and recombinant NudF samples for IPP, $1.6x10^{-14}$ and $2.0x10^{-14}$, respectively. When these values are compared they suggest that the presence of NudF results in a

higher IPP dephosphorylation rate. However these findings do not prove that NudF is responsible for the increase in specific activity alone.

Table 3.1 Specific activities of recombinant proteins

Sample	P_i (μM)	Enzyme Activity (moles/min)	Total Protein (μg)	Specific Activity
Co-Purified Proteins	IPP: 4.1	$13.7x10^{-12}$	842	$1.6x10^{-14}$
	DMAPP: 5.7	$19.0x10^{-12}$		$2.3x10^{-14}$
Purified rNudFHA-His Sample	IPP: 7.4	$24.7x10^{-12}$	1264	$2.0x10^{-14}$
	DMAPP: 8.4	$28.0x10^{-12}$		$2.2x10^{-14}$

*10 μL of each purified sample was used. The enzyme activity was calculated by dividing moles of P_i by 30 mins. Total protein was determined by the Bradford method. Specific activity was calculated by dividing the enzyme activity by the total protein amount.

In order to get a clear understanding on NudF's activity for IPP the amount of rNudFHA-His protein was determined. A series of known BSA standards were electrophoresed on SDS-PAGE and subsequently used to determine the relative amount of rNudFHA-His protein in the reaction sample (2.2.1). Table 3.2 illustrates that the total amount of co-purified proteins in each sample are very similar and suggests that the increase in specific activity for IPP in the samples containing rNudFHA-His is the result of more co-purified proteins present. These results show that the co-purified proteins are able to dephosphorylate IPP and DMAPP and release P_i.

Table 3.2 Specific amounts of recombinant proteins

Sample	Amount of NudFHA-His (μg)	Amount of co-purified proteins (μg)	Enzyme Activity for IPP (moles/min)
Co-Purified Proteins	0	842	$13.7x10^{-12}$
Purified rNudFHA-His Sample	250	1014	$24.7x10^{-12}$

* Total NudF protein was determined by comparing protein bands of known BSA concentrations to the protein band of NudF. Total co-purified protein was determined by subtracting the amount of NudFHA-His protein from the total protein from Table 3.1.

The ability of rNudFHA-His to dephosphorylate IPP and DMAPP should not be discounted because rNudFHA-His may hydrolyze IPP and DMAPP and release pyrophosphate which cannot be detected in this enzyme assay. Gas chromatography was performed to analyze rNudFHA-His's and the co-purified proteins' ability to produce isoprenol and prenol in the presence of IPP and DMAPP (2.2.8). Results from the GC analysis did not demonstrate NudF activity since no alcohol could be detected in the samples analyzed suggesting NudF has no specificity towards IPP and DMAPP.

3.7 Quantitative Analysis of GST-NudF Activity

The HA and His tag that was incorporated at the carboxyl terminal end of the *nudF* gene may be interfering with NudF's activity since the tags follow the catalytic part of the sequence. Given that the carboxyl terminal epitope tag

might interfere with NudF activity a GST-NudF amino-terminal fusion protein was assembled (2.2.13). An IPTG inducible TAC promoter regulates fusion protein expression. The TAC promoter is repressed by the presence of LacI protein. IPTG induction alleviates LacI repression and thus stimulates transcription of the fusion protein. A glutathione sepharose column was used to purify the GST-NudF fusion protein (2.2.14). Glutathione is the specific substrate for GST and when immobilized it can capture GST and GST tagged proteins. Figure 3.11 shows Coomassie Blue stained mini gels of the flow through, wash and eluent samples collected from the GST and GST-NudF chromatography experiment. The molecular weight of GST is 26kD and NudF is approximately 26kD. Protein bands at the expected molecular weight can be visualized on the mini gels suggesting that GST protein and GST-NudF fusion protein are being produced in high quantities. Eluent 1 from each purification experiment was used for activity analysis.

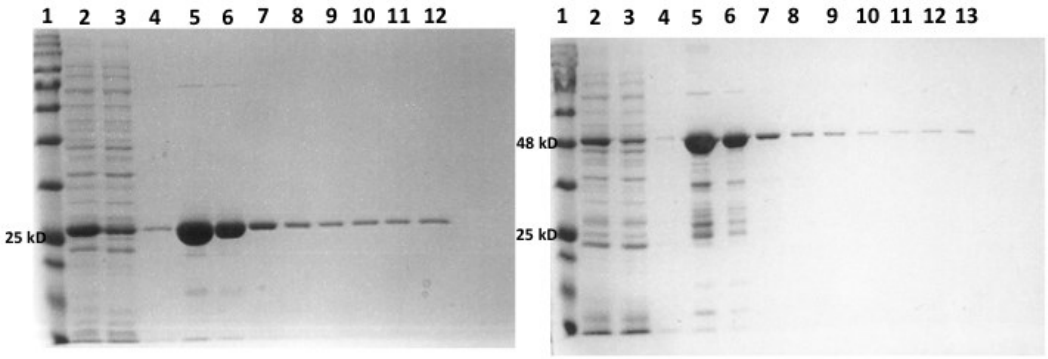


Figure 3.11 Recombinant GST and GST-NudF Purification SDS-PAGE was carried out on cell extract, flow through, wash, and eluent samples. The mini gels were stained with Coomassie Brilliant Blue. Left Gel: GST, Lane 1: MW ladder, Lane 2: Cell extract, Lane 3: Flow through, Lane 4: Wash and Lanes 5-12: Eluent fractions 1-8. Right: GST-NudF, Lane 1: MW ladder, Lane 2: Cell extract, Lane 3: Flow through, Lane 4: Wash, and Lanes 5-13: Eluent fractions 1-9.

A malachite green phosphatase assay (2.2.10) was carried out to determine activity of the purified fusion protein. Table 3.3 illustrates that the purified recombinant GST-NudF fusion sample has more activity towards IPP and DMAPP when compared to the purified GST sample. Specific activity for IPP is five times that of the control and specific activity for DMAPP is two times that of the control. These results suggest that NudF has specificity towards IPP and DMAPP where the turnover number (k_{cat}) of NudF for IPP is $5.5x10^{-3}$ s^{-1} and $3.4x10^{-3}$ s^{-1} for DMAPP.

Table 3.3 Specific activities of GST and GST-NudF

Sample	P_i (μM)	Enzyme Activity (moles/min)	Total Protein (μg)	Specific Activity
Purified GST	IPP: 3.1	$1.0x10^{-11}$	4	$2.6x10^{-12}$
	DMAPP: 4.5	$1.5x10^{-11}$		$3.7x10^{-12}$
Purified GST-NudF	IPP: 15.2	$5.1x10^{-11}$	4	$1.3x10^{-11}$
	DMAPP: 9.3	$3.1x10^{-11}$		$7.8x10^{-12}$

*10 μL of each purified sample was used. The amount of P_i is an average of triplicate samples. The enzyme activity was calculated by dividing moles of P_i by 30 mins. Total protein was determined by the Bradford method. Specific activity was calculated by dividing the enzyme activity by the total protein amount.

Given that NudF has activity against IPP and DMAPP it would be beneficial to determine whether NudF only has specificities towards these substrates or is it in fact, a general phosphatase. A pNPP assay was carried out to determine NudF's specificities (2.2.15). The compound-Nitrophenol Phosphate (pNPP) is a non-specific substrate used to assay phosphatase activity. Activity is

measured by the enzyme's ability to dephosphorylate pNPP to produce p-nitrophenol, a measureable colourmetric product that is proportional to the amount of free phosphate present. Figure 3.12 illustrates the activity of the general calf intestinal phosphatase, GST, and GST-NudF for pNPP. The data collected shows that GST and GST-NudF have no activity towards pNPP and thus suggests that GST-NudF is not a general phosphatase.

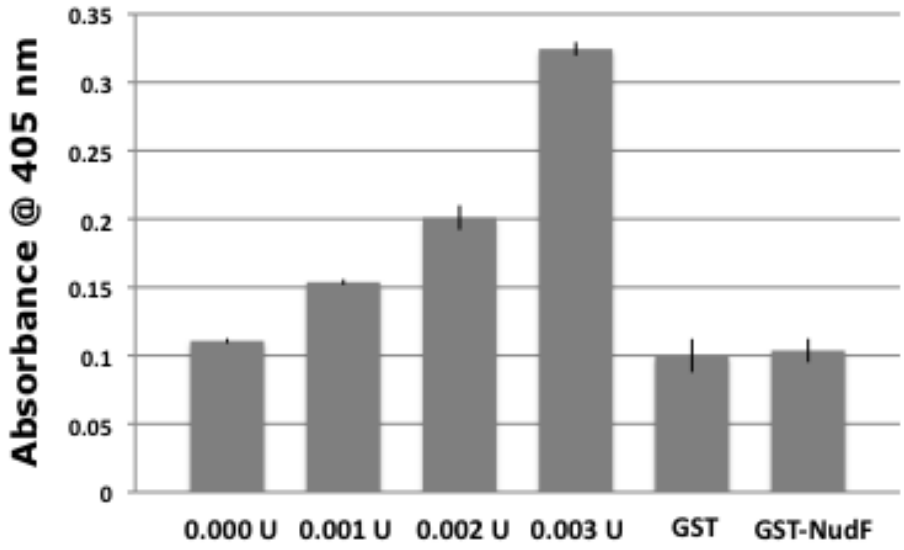


Figure 3.12 pNPP Phosphatase Assay A pNPP phosphatase assay was carried to determine GST-NudF specificities. Purified samples were incubated with 30 mM pNPP and the reaction was carried out for an hour. Varying units of calf intestinal phosphatase was used as a positive control. Each reaction was carried out in triplicates. Error bars represent standard deviation.

CHAPTER 4

Over Expression of *HMG1* in *S. cerevisiae*

4.1 *HMG1*

The *HMG1* gene encodes HMG-CoA reductase (HMGCR) in *S. cerevisiae*. It is the rate-limiting enzyme in the mevalonate pathway, a metabolic pathway responsible for the synthesis of isoprenoid intermediates (IPP and DMAPP), ergosterol, and other central biological components. More specifically HMGCR catalyzes the irreversible reduction of HMG-CoA into mevalonate, where NADPH is used as a reducing cofactor. To further optimize the synthesis of isoprenol the over expression of a hyperactive HMGCR should increase the synthesis of IPP and DMAPP and theoretically the production of isoprenol.

HMGCR is an integral membrane protein that is localized in the endoplasmic reticulum. HMGCR is comprised of 1054 a.a. and has two main domains, the amino-terminal membrane-binding domain and the carboxyl-terminal catalytic domain. Many reports have shown that when the catalytic domain of HMGCR is over expressed there is an enhancement in squalene synthesis, a downstream intermediate[13]. A 532 amino acid truncation at the amino terminus of HMGCR removes the membrane-binding domain. This deletion removes the regulatory sequences, preventing any negative feedback inhibition[32], and thus increasing flux through the mevalonate pathway.

4.2 Expression of t*hmg1MYC* in *S. cerevisiae*

Based on previous literature a truncated, *MYC* tagged, *HMG1* gene was expressed in *S. cerevisiae* (2.2.16). The truncated *HMG1* gene is under the control of a constitutive *PDC1* promoter allowing for overexpression of t*hmg1MYC*. Figure 4.1 illustrates that t*hmg1MYC* is expressed in *S. cerevisiae* since truncated HMGCRMyc protein production can be detected via western blot analysis of crude extracts; protein bands at the expected molecular weight can be observed (lane 4 and 8). A MYC antibody was used to detect the MYC epitope tag on t*hmg1MYC*. There are no bands present in the empty vector lane (lane 2). These results confirm that a truncated *HMG1* can be expressed in *S. cerevisiae* since protein production can be visualized.

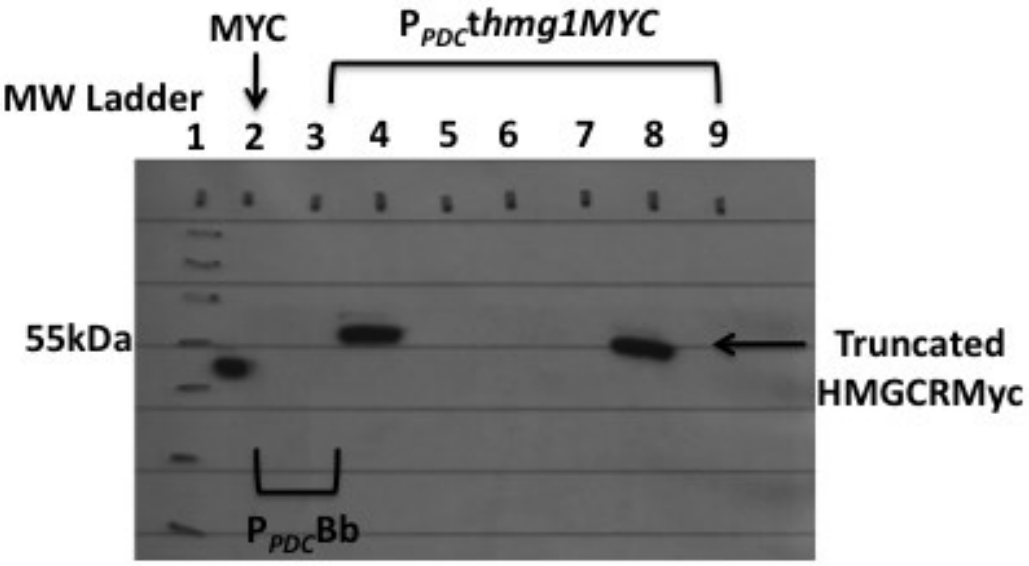


Figure 4.1 Expression of a truncated, Myc tagged, HMGCR Western blot was carried out on protein extractions from *S. cerevisiae* strains harbouring P$_{PDC}$*thmg1MYC and* P$_{PDC}$Bb. A MYC antibody was used for detection. Lane1: MW ladder, Lane 2: MYC positive control, Lane 3: P$_{PDC}$Bb, and Lanes 4-9: P$_{PDC}$*thmg1MYC* containing strain.

4.3 Quantitative Analysis of truncated HMGCRMyc Activity

An enzyme assay was carried out to investigate the activity of the truncated HMGCRMyc (2.2.17). Activity from crude extracts was determined by using HMG-CoA as a substrate and NADPH as a reductant. Changes in NADPH to $NADP^+$ can be measured via absorbance spectroscopy. NADPH absorbs light at 340 nm where as $NADP^+$ does not. Therefore the decrease in absorbance at 340 nm directly corresponds to the oxidation of NADPH and thus the change in absorbance at 340 nm will be proportional the amount of HMGCR present. Figure 4.2 illustrates the change in absorbance over time of the crude extracts harbouring the truncated HMGCRMyc and empty vector. There is no difference between the two and thus suggest that the HMGCR is not hyperactive.

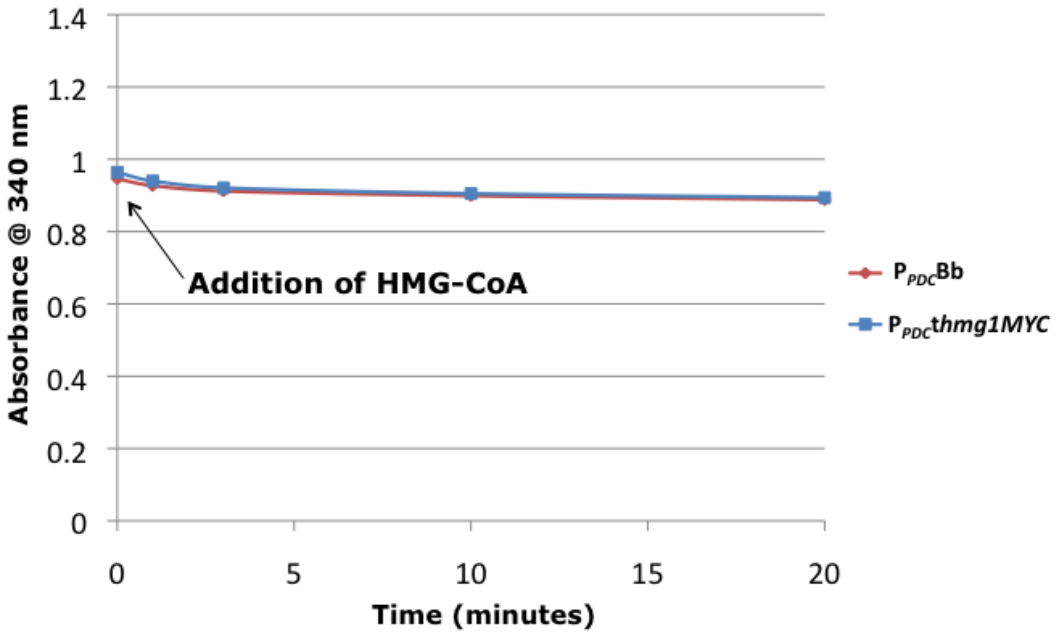


Figure 4.2 In vitro enzyme activity analysis of truncated HMGCRMyc Cell lysates of strains expressing a truncated HMG-CoA reductase and empty vector were prepared and incubated in 150 µM of NADPH and HMG-CoA. Absorbance at 340nm was measured at specific time intervals.

4.4 Expression of t*HMG1* and Quantitative Analysis of a Hyperactive HMGCR

The carboxyl-terminal Myc tag on HMGCRMyc is in close proximity to the catalytic domain and therefore might interfere with HMGCR activity. To overcome this potential limitation an untagged t*HMG1* gene was expressed in *S. cerevisiae* (2.2.18). Strains harbouring a single copy or multiple copies of t*HMG1* were generated. Confirmation of the t*HMG1* expression was first determined by growth on selectable plates. An enzyme assay was carried out on crude extract to measure t*HMG1* expression and selective activity (2.2.17). Figure 4.3 illustrates the enzyme activity curves of a strain containing a high

copy plasmid, P_{PDC}Bbt*HMG1* as well as a strain containing a single copy, YCplac22t*HMG1*. There is a significant difference between the change in absorbance of the high copy and single copy strain when compared to their controls. These observations confirm that a hyperactive HMGCR is present in the experimental strains and not present in the control strains. Figure 4.3 also shows difference between the high copy, P_{PDC}Bbt*HMG1* strain and the single copy, YCplac22t*HMG1* strain. These results show that the high copy strain contains more activity than the single copy strain because the P_{PDC}Bbt*HMG1* strain expresses more HMGCR. These observations can be confirmed by calculating specific activities of the strains. Table 4.1 verifies these observations, confirming that a hyperactive HMGCR is present in the experimental strains and absent in the control strains. Furthermore, Table 4.1 also illustrates that the high copy, P_{PDC}Bbt*HMG1* strain is more active against NADPH than the single copy, YCplac22t*HMG1* strain when specific activities are compared, $2.7X10^{-4}$ and $1.7X10^{-4}$, respectively. Again this is due to the presence of more HMGCR in the high copy strain.

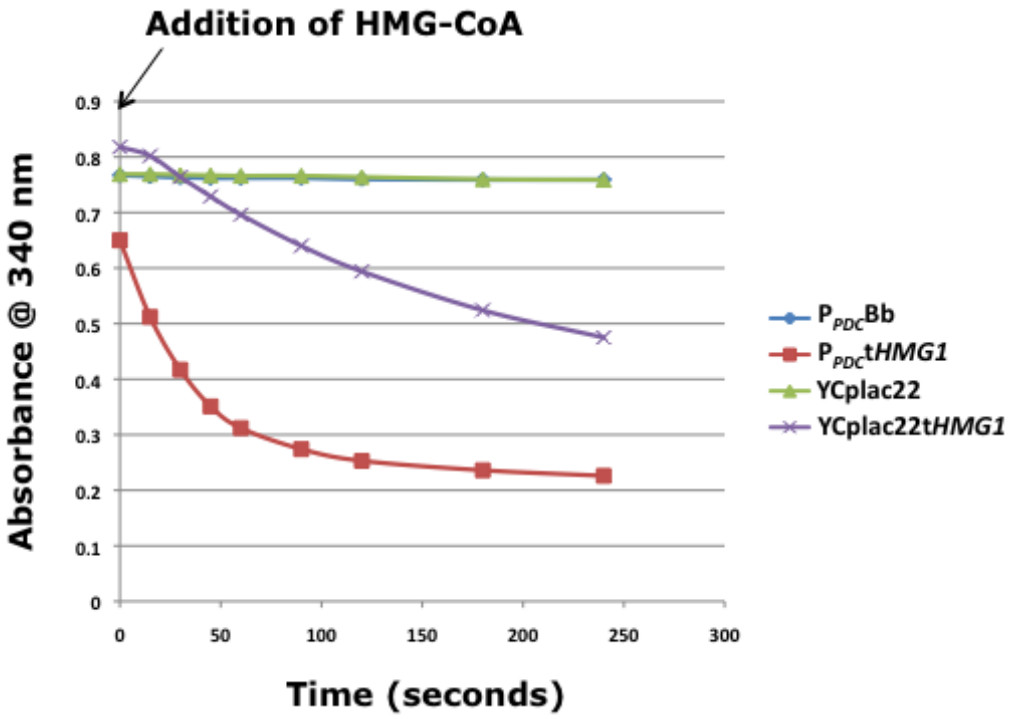


Figure 4.3: In vitro enzyme activity analysis of a hyperactive HMGCR
Cell lysates of strains expressing a hyperactive HMGCR (P_{PDC}t*HMG1* and YCplac22t*HMG1*) and empty vector (P_{PDC}Bb and YCplac22) were prepared and incubated in 150 µM of NADPH and HMG-CoA. Absorbance at 340nm was measured at specific time intervals.

Table 4.1 Specific activity of *in vitro* hyperactive HMG-CoA reductase

Strain	Initial Rate (0-60 secs)	Total Protein (µg)	Specific Activity
erg20::P_{MET3}*ERG20* P_{PDC}Bb	0.002	598	$2.8x10^{-6}$
erg20::P_{MET3}*ERG20* P_{PDC}-Bbt*HMG1*	0.330	1213	$2.7x10x^{-4}$
erg20::P_{MET3}*ERG20* YCplac22	<0.000	819	No measureable activity
erg20::P_{MET3}*ERG20* YCplac22*tHMG1*	0.132	772	$1.7x10^{-4}$

* The initial rate is the change in absorbance at 340nm between 0 and 60 secs. The initial rate in this case is also the enzyme activity and is an average of triplicates. The total protein was determined by the Bradford method. Specific activity was calculated by dividing the initial rate by the total protein amount.

CHAPTER 5

Conditional Regulation of *ERG20* in *S. cerevisiae*

5.1 *ERG20*

ERG20 is a *S. cerevisiae* gene that encodes for farnesyl pyrophosphate synthase. Farnesyl pyrophosphate synthase is an enzyme involved in the synthesis of farnesyl pyrophosphate (FPP), an intermediate involved in the biosynthesis of isoprenoids and other essential biological components. Farnesyl pyrophosphate synthase is responsible for the sequential catalytic condensation of two units of isopentenyl pyrophosphate (IPP) with one unit dimethylallyl pyrophosphate (DMAPP). One unit of IPP condenses with one unit of DMAPP to form geranyl pyrophosphate (GPP). GPP then condenses with the second unit of IPP to form FPP.

In order to engineer a *S. cerevisiae* strain that can produce a large amount of isoprenol the cytosolic concentration of IPP needs to be optimized. To do this the metabolic flux needs to be redirected in order to increase IPP concentrations. IPP is an important intermediate in the cell's development and as a consequence IPP is metabolized by farnesyl pyrophosphate synthase (FPP synthase) for the production of GPP and FPP and as a result the cytosolic concentration of IPP is limited. In order to accumulate IPP the expression of *ERG20* needs to be regulated. A point mutation in FPP synthase lowered its activity and consequently promoted excretion of prenyl alcohols into the culture medium, indicating an increased accumulation of IPP, DMAPP, and GPP[31]. Based on this study, the *ERG20* gene was conditionally regulated to promote accumulation of IPP and DMAPP.

5.2 Construction of a Methionine Regulated *ERG20 S. cerevisiae* Strain

Efficient homologous recombination in *S. cerevisiae* enabled the construction of a methionine-regulated *ERG20 S. cerevisiae* strain, *MET3-ERG20* (2.2.19). The methionine-regulated *ERG20 S. cerevisiae* strain was created so that a *MET3* promoter is regulating the expression of *ERG20*. The *MET3* promoter's activity is repressed by the presence of methionine[28]. Mao *et al.* reported that the *MET3* promoter was sensitive to methionine in the medium and despite the plasmid copy the activity of the enzyme they were studying was dependant on the concentration of methionine. This application offers a suitable technique in construction of a conditional-lethal strain. Since *ERG20* expression generates a vital enzyme it would be appropriate to create heterologous expression in *S. cerevisiae* strain where the expression of *ERG20* can be shut down on demand in the presence of methionine.

Strains in which the *ERG20* gene was correctly replaced with *MET3-ERG20* did not grow in the presence of methionine. Figure 5.1 illustrates that the experimental transformants grew well on -met -leu + dex plates and did not grow on – leu + dex plates. These results show that recombination occurred in the experimental transformants where the linear DNA fragment was successfully integrated into the chromosome. The experimental transformant's *ERG20* gene is under the transcriptional regulation of a *MET3* promoter since the transformants did not grow in the presence of methionine. This observation can also be concluded from the information on

Table 5.1 where the OD_{600} reading of the methionine regulated *ERG20* strain (*erg20::*P_{MET3}*ERG20*) is considerably less than the control after an overnight culturing in the presence of 2 mM methionine. These results also illustrate that the expression of *ERG20* is now under the regulation of a *MET3* promoter and thus in the presence methionine its expression can be repressed.

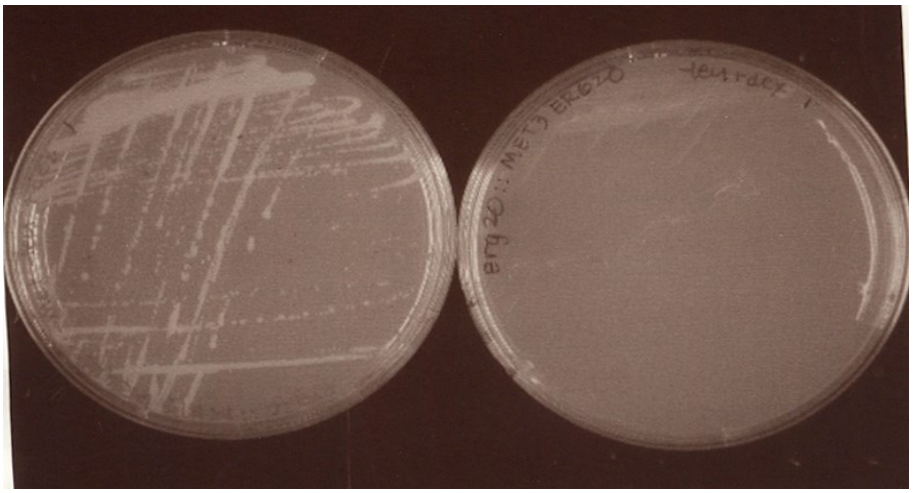


Figure 5.1 Heterologous expression of *ERG20* This strain was developed using homologous recombination. A *MET3* promoter controls the expression of *ERG20*. Left: Transformants plated on – met – leu + dex plates. Right: Transformants plated on – leu + dex plates.

Table 5.1 Cell density of *erg20*::P_{MET3}*ERG20* after methionine stimulation

Strain	OD_{600}
erg20::P_{MET3}*ERG20* + ddH_2O	8.725
erg20::P_{MET3}*ERG20* + 2mM met	6.375

* A 50 mL e*rg20*::*MET3ERG20* strain was grown in – met + dex media overnight. On the following day the culture diluted to an OD_{600} reading of 0.2. When the culture reached an OD_{600} reading of 0.5 it was split into two 20 mL cultures and stimulated overnight with 2 mM methionine and ddH_2O as a control.

5.3 IPP and DMAPP Detection

The e*rg20*::P*MET3ERG20 S. cerevisiae* strain was developed to accumulate IPP and DMAPP in the cytosol. To determine whether or not IPP is accumulating upon repression of P*MET3ERG20* several experiments were carried out. An e*rg20*::P*MET3ERG20 S. cerevisiae* strain harbouring a hyperactive HMGCR was used for analysis. Previous research has shown that isopentenyl pyrophosphate (IPP) could be separated and detected via HPLC-MS/MS[18] and DMAPP could be purified via reversed-phase HPLC[52]. Preliminary HPLC analysis (2.2.20) was carried out on DMAPP standard. Eluents corresponding to the peaks on the chromatogram were collected and analyzed with ESI-MS (2.2.22) to determine if this procedure could detect DMAPP. The mass corresponding to DMAPP was detected in the eluents of the solvent front peak and the following peak. These results suggest that the HPLC method was unable to effectively separate and detect DMAPP. From there it was deemed more effective to analyze IPP accumulation via ESI-MS. Preliminary ESI-MS analysis (2.2.22) of IPP/DMAPP extracted samples (2.2.21) did not result in detection of IPP or DMAPP in the methionine treated samples. Lastly, a CIP dephosphorylation assay (2.2.23) was carried out on prepared cell extracts. To ensure that revertants did not exist in the experimental strain, some of the cells were plated on selective media prior to methionine addition. The presence of CIP should convert the accumulated IPP into isoprenol. The presence of isoprenol was analyzed via GC (2.2.26). GC analysis did not detect any isoprenol in the treated and untreated samples

analyzed. These results suggest that CIP dephosphorylation assay is an ineffective way to analyze IPP accumulation.

5.4 IPP Accumulation Analysis

Since the previously described approaches were not successful in detecting IPP and DMAPP a C^{14}-Acetate metabolic labeling and thin layer chromatography (TLC) approach was employed (2.2.24). The experiment was adapted from Beyer *et al.*. Table 5.2 illustrates C^{14} counts that were measured from the IPP and FPP spots scraped from a cellulose TLC plate. The methionine treated strain has significantly less counts for spots corresponding to IPP and FPP when compared to the control strain. FPP counts decreased by over 39%, these results align with the expected decrease in *ERG20* transcription upon methionine treatment. However, IPP counts decreased by 100%. The data demonstrates that IPP accumulation is not occurring even though FPP counts had significantly decreased. The ratio of IPP to FPP decreased by a factor of 4 upon methionine treatment, where the untreated cultures have an approximate ratio of 1:4 and the treated cultures have an approximate ratio of 1:15. These results suggest that IPP is being metabolized instead.

Table 5.2 Liquid scintillation counting of scrapped TLC cellulose

Strain	Total Counts	IPP Counts	FPP Counts
erg20::P*MET3ERG20* YCplac22*tHMG1* + ddH_2O	5424400	25800 (0.5%)	96283 (1.8%)
erg20::P*MET3ERG20* YCplac22*tHMG1* + 2mM met	3424000	1760 (0.05%)	25570 (0.7%)

* 50 µL of the C^{14}-Acetate incubated culture was used to determine the total counts. IPP and FPP counts are the average of analytical triplicates.

CHAPTER 6

Isoprenol Production in *S. cerevisiae*

6.1 Construction of an Isoprenol Producing *S. cerevisiae* Strain

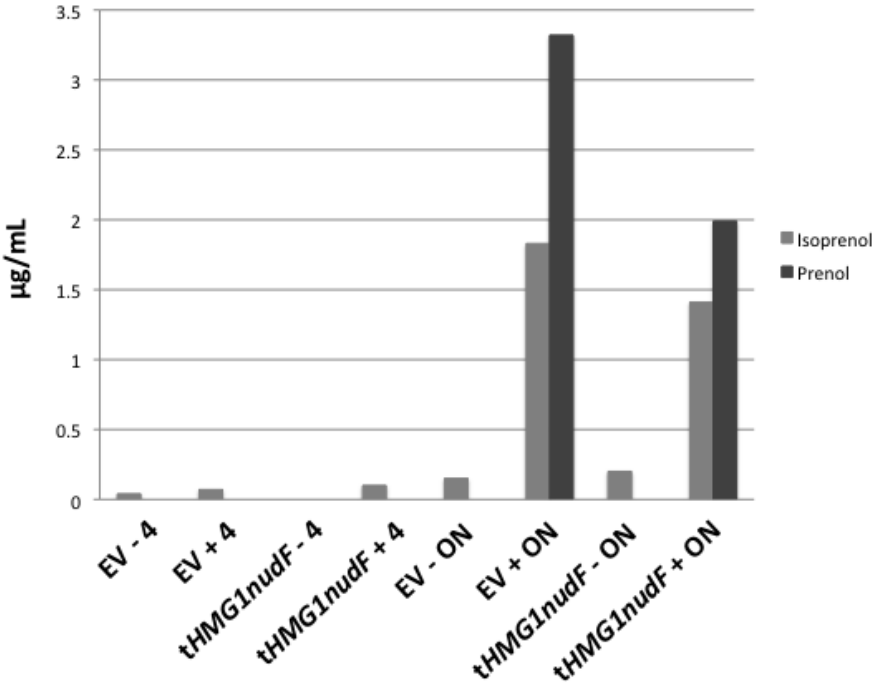
It has been shown that expression of *nudF* in *E. coli* resulted in the dephosphorylation of IPP to produce isoprenol[47]. It also has been demonstrated that the expression of a truncated *HMG1* (t*HMG1*) in *S. cerevisiae* encodes a hyperactive HMGCR, which produces more down stream products[13]. Over expression of *nudF* and t*HMG1* in *S. cerevisiae* should result in isoprenol production. However, IPP produced from the isoprenoid pathway is subsequently metabolized for cell growth. This inherent property of *S. cerevisiae* would prevent accumulation of IPP and therefore hinder optimal isoprenol production. Repression of *ERG20*, the gene that encodes the enzyme that metabolizes IPP and DMAPP, is expected to promote accumulation of IPP and DMAPP.

The *nudF* gene from *Bacillus subtilis* was over expressed in the W303 *S. cerevisiae* strain and an *in vitro* analysis showed that NudF protein is able to dephosphorylate IPP and DMAPP. A truncated *HMG1* gene was expressed in the W303 *S. cerevisiae* strain and an *in vivo* enzyme assay confirmed its hyperactivity and lastly a methionine regulated *ERG20 S. cerevisiae* strain was developed. Finally, the over expression of *nudF* and the expression of *tHMG1* was introduced to e*rg20*::P_{MET3}*ERG20* strain to generate a isoprenol producing strain. Methionine treatment of this strain is expected to promote isoprenol production.

6.2 Alcohol Analysis of the Engineered *S. cerevisiae* Strain

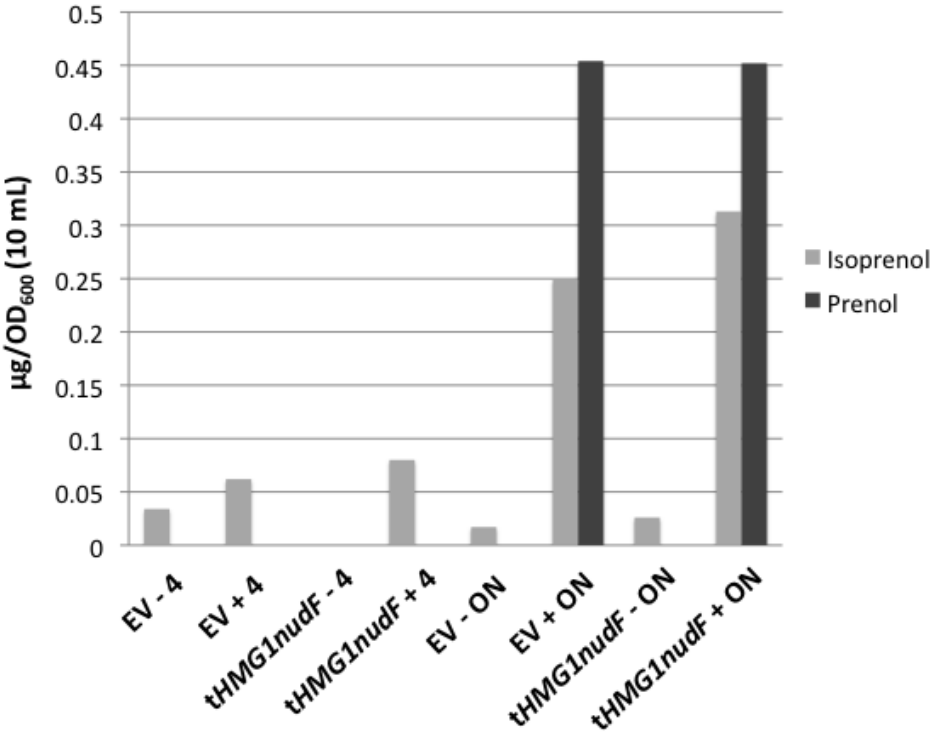
A methionine regulated *ERG20* strain (e*rg20*::P$_{MET3}$*ERG20)* expressing a low copy hyperactive HMGCR (YCplac22t*HMG1*) and an active *GAL1* regulated NudF (YCplac33*GAL1nudF*) was cloned (2.2.12 and 2.2.18) and used to assess isoprenol and prenol production as described previously (2.2.28 and 2.2.29). Figure 6.1 illustrates the concentration of isoprenol and prenol in the strains analyzed via GC. Very little isoprenol and prenol alcohol can be detected in the cultures analyzed. The data demonstrates that overnight methionine treatment, which represses expression of *ERG20,* appeared to generate the highest amount of both alcohols in the control (EV) and experimental (t*HMG1nudF*) strains. Small amounts of isoprenol can be detected in all of the strains analyzed except for the 4 hour untreated t*HMG1nudF* strain. More interestingly, prenol is only present in the strains treated with methionine overnight. However, when standard deviation is taken into account there appears to be no significant difference in alcohol production between the control and experimental strains analyzed. This data suggests the alcohols are only present when *ERG20* is inactivated but the presence of a hyperactive HMGCR and active NudF in *S. cerevisiae* does not lead to increase isoprenol or prenol production. Figure 6.2 also depicts isoprenol and prenol production in the same strains. The data in Figure 6.2 illustrates the amount of alcohol produced per OD_{600} of each strain analyzed. Untreated strains have higher OD_{600} readings than the treated strains since methionine represses cell

proliferation (Table 5.1). This data representation takes into account cell concentration and normalizes the data.



	EV - 4	**EV + 4**	**t*HMG1nudF* - 4**	**t*HMG1nudF* + 4**
Mean Isoprenol	0.04	0.07	0.00	0.10
Standard Deviation	0.07	0.12	0.00	0.03
Mean Prenol	0.00	0.00	0.00	0.00
Standard Deviation	0.00	0.00	0.00	0.00
	EV - ON	**EV + ON**	**t*HMG1nudF* - ON**	**t*HMG1nudF* + ON**
Mean Isoprenol	0.15	1.83	0.20	1.41
Standard Deviation	0.05	1.79	0.31	1.00
Mean Prenol	0.00	3.32	0.00	1.99
Standard Deviation	0.00	4.74	0.00	1.74

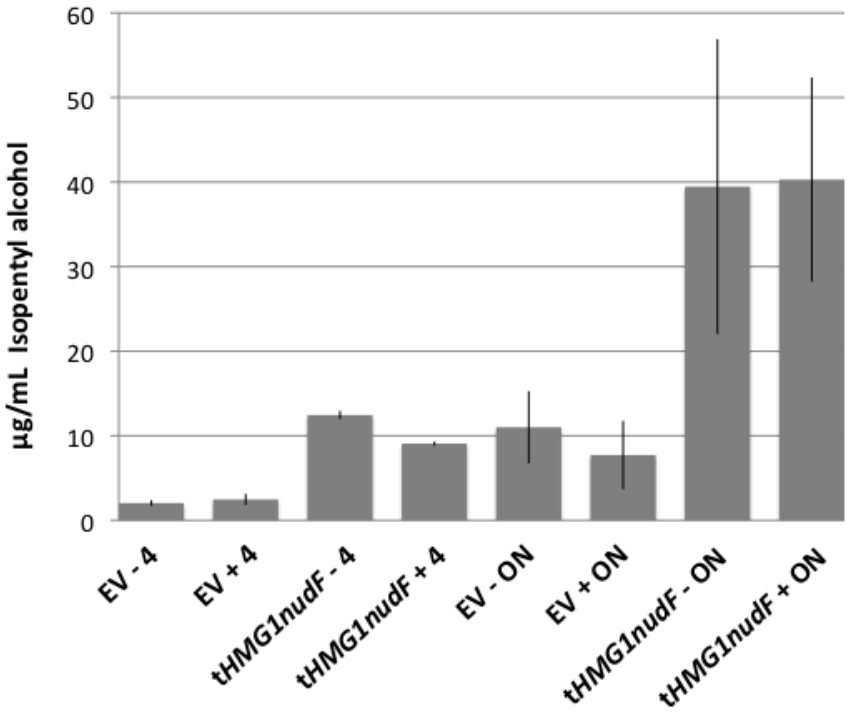
Figure 6.1 Isoprenol and prenol analysis (µg/mL) Empty vector strains (EV) and strains containing a hyperactive HMGCR and a active NudF (*thmg1nudF*) were treated with 2 % galactose and 2 mM methionine (+), or ddH_2O (-) for 4 hours (4) and overnight (ON). The data is an average of experimental triplicates analyzed.



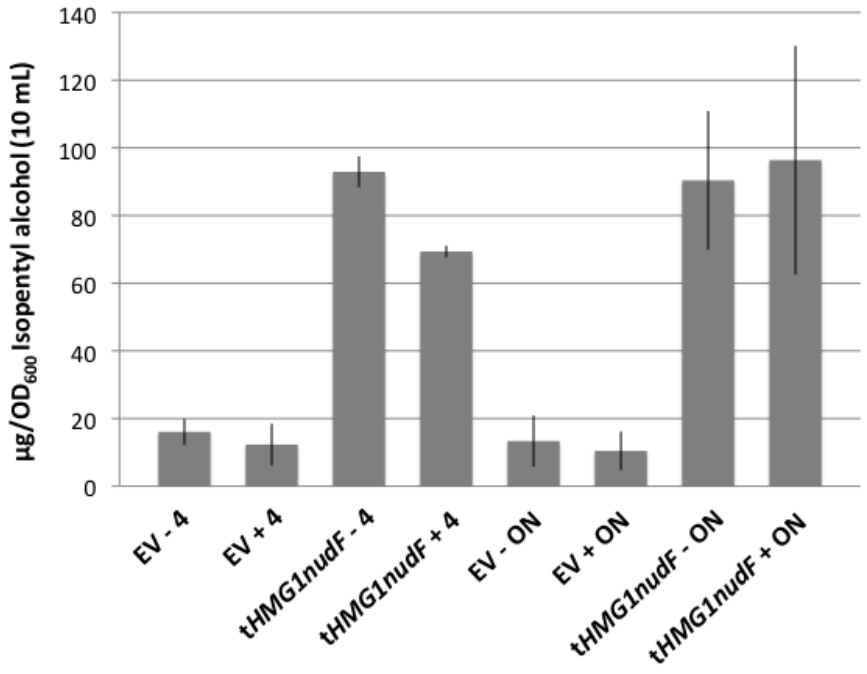
	EV - 4	**EV + 4**	**t*HMG1nudF* - 4**	**t*HMG1nudF* + 4**
Mean Isoprenol	0.034	0.062	0.00	0.08
Standard Deviation	0.06	0.11	0.00	0.02
Mean Prenol	0.00	0.00	0.00	0.00
Standard Deviation	0.00	0.00	0.00	0.00
	EV - ON	**EV + ON**	**t*HMG1nudF* - ON**	**t*HMG1nudF* + ON**
Mean Isoprenol	0.017	0.25	0.03	0.31
Standard Deviation	0.00	0.25	0.03	0.25
Mean Prenol	0.00	0.45	0.00	0.45
Standard Deviation	0.00	0.65	0.00	0.51

Figure 6.2 Isoprenol and prenol analysis (µg/OD_{600}) Empty vector strains (EV) and strains containing a hyperactive HMGCR and a active NudF (*thmg1nudF*) were treated with 2 % galactose and 2 mM methionine (+), or ddH_2O (-) for 4 hours (4) and overnight (ON). The data is an average of experimental triplicates analyzed.

During the process of developing an appropriate method for alcohol extraction and detection it was discovered that the engineered strains were producing isopentyl alcohol (3-methyl-1-butanol). Given this finding, the control and experimental strains were also analyzed for isopentyl alcohol production. Figure 6.3 illustrates the amount of isopentyl alcohol detected in the strains. All strains produced isopentyl alcohol. The engineered strains appeared to produce higher amounts of alcohol when compared to its empty vector control. Highest concentrations of isopentyl alcohol appeared in the overnight, untreated and treated engineered strains, 39.46 and 40.29 μg/mL, respectively. These results suggest that FPP synthase, encoded by *ERG20* has no role in the increased alcohol production, but the presence of a hyperactive HMGCR and an active NudF promoted isopentyl alcohol production. Figure 6.4 illustrates the amount of isopentyl alcohol produced per OD_{600}. The figure demonstrates that there is no significant difference in the amount of isopentyl alcohol production over time when cell concentration is taken into account.



6.3 Isopentyl alcohol analysis (µg/mL) Empty vector strains (EV) and strains containing a hyperactive HMGCR and a active NudF (*thmg1nudF*) were treated with 2 % galactose and 2 mM methionine (+), or ddH_2O (-) for 4 hours (4) and overnight (ON). The data is an average of experimental triplicates analyzed



6.4 Isopentyl alcohol analysis (µg/OD_{600}) Empty vector strains (EV) and strains containing a hyperactive HMGCR and a active NudF (*thmg1nudF*) were treated with 2 % galactose and 2 mM methionine (+), or ddH_2O (-) for 4 hours (4) and overnight (ON). The data is an average of experimental triplicates analyzed.

6.3 Toxicity of Isoprenol in *S. cerevisiae*

The presence of isoprenol could have a toxic effect on the growth of *S. cerevisiae*. Therefore we decided to investigate the potential for isoprenol to hinder the cells' normal metabolism and consequently decrease isoprenol production. Figure 6.5 represents the OD_{600} reading over time of W3O3 *S. cerevisiae* cultures containing varying concentrations of isoprenol. Figure 6.5 clearly shows that increasing amounts of isoprenol decreases cell growth; 0.75 % of isoprenol or higher completely halts *S. cerevisiae* growth.

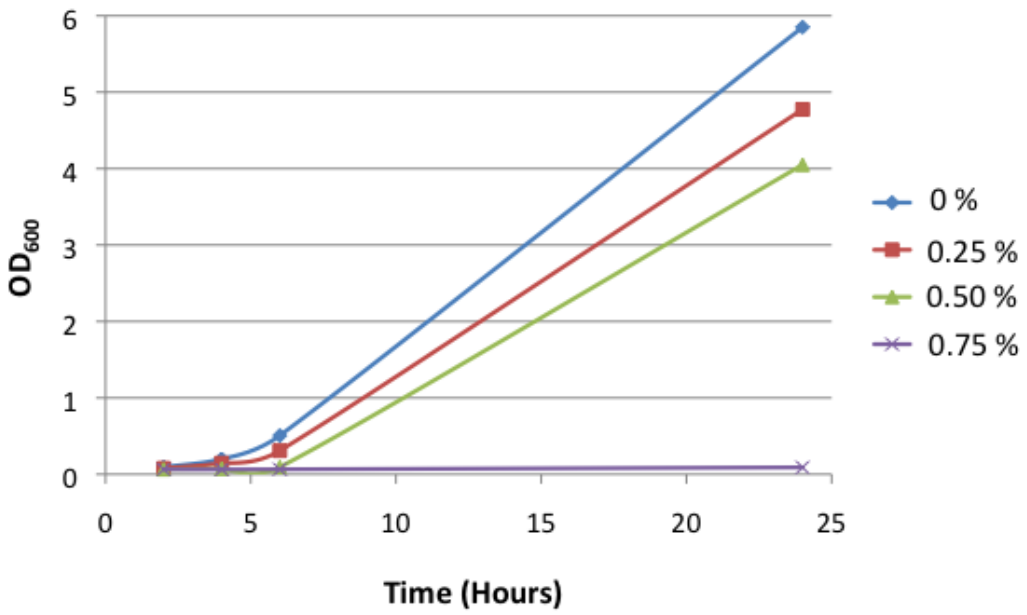


Figure 6.5 Isoprenol toxicity The W303 *S. cerevisiae* strain was incubated in varying concentrations isoprenol. The OD_{600} was measured at specific time intervals.

6.4 Potential for *S. cerevisiae* to Metabolize Isoprenol

The production of isoprenol was very limited in the engineered strain. Low production could be due to toxicity of alcohols as described previously (6.3), evaporation of alcohols, or *S. cerevisiae*'s ability to metabolize isoprenol. With this in mind, a W303 *S. cerevisiae* culture was grown in the presence of 0.5 % isoprenol and at each time interval a sample of the media was collected for GC-FID analysis (2.2.30 and 2.2.27) At 0.5 % isoprenol there was an increase in isoprenol concentration over time, 236 µg/mL at 2 hours and 270 µg/mL was detected after 24 hours. 10 µg/mL of prenol was also detected after a 24 hour period. These results suggest that isoprenol does not evaporate and

suggest that when in the presence of isoprenol *S. cerevisiae* has the ability to produce prenol.

Table 6.1 Changes in concentration of isoprenol in culture

Time	OD_{600}	Isoprenol (μg/mL)	Prenol (μg/mL)
2 hours	0.042	236	0
4 hours	0.055	266	0
6 hours	0.075	267	0
24 hours	0.733	270	10

* 25 mL culture W303 *S. cerevisiae* strain was grown in YEPD spiked with 0.5 % isoprenol

6.5 Expression of other Phosphatases

Isoprenol production in the engineered *S. cerevisiae* strain was lower than expected. Low levels of isoprenol production may be a result of multiple factors including poor NudF activity in *S. cerevisiae*. Therefore, other potential phosphatases were expressed in *S. cerevisiae* to see if increased production of isoprenol could be obtained. *S. cerevisiae* genes, *PHO8*, *LPP1*, and *DPP1* were independently expressed in the methionine regulated *ERG20* strain expressing a hyperactive HMGCR. The *PHO8* gene encodes for the Repressible Vacuolar Alkaline Phosphatase, *LPP1* encodes for the Lipid Phosphate Phosphatase and *DPP1* encodes for Diacylglycerol Pyrophosphate Phosphatase. LPP1 was cloned into both P_{PDC}Bb and pYES2 vectors and the other genes were cloned into pYES2. Transformation of *S. cerevisiae* with

these plasmids yielded no viable colonies suggesting that constitutive expression of these phosphatases was toxic.

CHAPTER 7

Discussion

7.1 General Summary

Successful metabolic engineering of *E. coli*, for production of isoprenol and prenol has been reported previously[47,51]. In brief, these studies assembled a synthetic pathway in *E. coli* to produce these alcohols. Currently, yield for isoprenol and prenol remain low, therefore effort has been directed towards optimizing their production. In 2007, Withers *et al.* first reported production of isopentenol. Withers *et al.* identified a *Bacillus subtilis* protein called NudF that had an affinity for IPP and DMAPP, converting them into their alcohol products. The discovery of NudF's enzymatic activity has been the basis for microbial production of isopentenol. As mentioned previously, isoprenol and prenol production has only been achieved in engineered *E. coli* strains. The mevalonate pathway synthesizes the precursors for these alcohols; this pathway is not naturally found in *E. coli* and consequently, imported. The mevalonate pathway exists in *S. cerevisiae;* therefore it was hypothesized that if metabolic engineering strategies were employed to increase IPP production and accumulation, and NudF was expressed, an isoprenol producing *S. cerevisiae* strain could be developed. However, the engineered strain did not produce isoprenol as expected, but surprisingly, produced isopentyl alcohol.

7.2 Expression of *nudF* in *S. cerevisiae* - Discussion

The specificity of NudF towards IPP and DMAPP has been previously reported[47]. Initially, an HA tagged NudF was expressed in *S. cerevisiae* and a HA-His tagged NudF was purified. NudFHA production was easily detected in *S. cerevisiae*, however, NudFHA-His appeared to have no activity towards IPP and DMAPP. NudFHA-His's lack of activity towards IPP and DMAPP suggests that the HA-His tag on NudF was interfering with its catalytic activity. Another drawback in this experiment was the poor purification of recombinant NudFHA-His. Very little protein was retained on the column during the chromatography experiments. The His tag may have interacted with the rest of the protein and inhibit its binding to the column, explaining why NudFHA-His was only detected by western blot analysis (Figure 3.3). The IMAC Tris buffer experiment (Figure 3.10) appeared to purify more NudFHA-His, this can be explained by the larger column and buffer system used in this experiment.

In order to re-examine NudF's activity towards IPP and DMAPP a GST-NudF fusion protein was assembled and purified. NudF showed some activity towards these substrates. These results confirm the negative affects of an HA-His tag on NudF activity. The low turnover number of GST-NudF for these substrates suggests that the NudF is not an efficient enzyme to use. This drawback could be due to the GST protein altering the native state of NudF. On the other hand, this result could explain why the yield of isopentenol still remains low in literature.

After numerous unsuccessful cloning experiments to constitutively over express *nudF* in *S. cerevisiae*, the gene was finally put under the control of a *GAL1* regulated promoter, where expression is induced by the presence of galactose. Constitutive expression of *nudF* in *S. cerevisiae* was deemed toxic; this may be due to the cell's inability to regulate growth with a foreign phosphatase present. There was an attempt to constitutively over express potential endogenous phosphatases in *S. cerevisiae*. *PHO8*, *DPP1*, and *LPP1* are genes from *S. cerevisiae* that encode phosphatases that have shown specificity towards isoprenoid intermediates[14,39,43]. It was hypothesized that expression of endogenous phosphatases would promote dephosphorylation of IPP and DMAPP into their alcohol derivatives. This attempt was also unsuccessful suggesting over expression of these phosphatases is also lethal to the cell.

7.3 Over Expression of *HMG1* in *S. cerevisiae* - Discussion

HMG1 encodes HMGCR, the rate-limiting enzyme in the mevalonate pathway. Expression of *HMG1* catalytic domain has been shown to increase the synthesis of downstream intermediates[29]. A truncated *HMG1* gene was expressed in *S. cerevisiae* to increase synthesis of IPP. In order to easily detect the production of the catalytic domain, a Myc tag was added to the carboxyl terminal end. Activity analysis of HMGCRMyc suggests that the tag interfered with HMGCR enzymatic activity. An untagged truncated *HMG1* gene was expressed and as expected, hyperactivity of the catalytic domain

was confirmed. HMGCR is a complex enzyme and therefore the presence of the Myc tag could have interfered with its activity in many ways. For example, the Myc tag could have distorted the native structure of the catalytic domain, making the substrate-binding site inaccessible.

7.4 Conditional Regulation of *ERG20* in *S. cerevisiae* – Discussion

To promote IPP and DMAPP accumulation, *ERG20* expression was put under the regulation of a methionine regulated *MET3* promoter. One of the major setbacks of this experiment was finding a method that would detect IPP and DMAPP. Preliminary HPLC analysis of the DMAPP standard was ineffective; this can be attributed to not having proper equipment to effectively separate DMAPP. ESI-MS analysis did not detect IPP or DMAPP. Non-detection of these intermediates could be a result of poor extraction methodology or growth of reverted cells. A CIP dephosphorylation assay was also carried out on cell extracts of the *ERG20* regulated strain expressing a hyperactive HMGCR; GC analysis did not detect any alcohol suggesting that this approach was ineffective. CIP should theoretically dephosphorylate IPP and DMAPP therefore the lack of alcohol detection is a result of poor extraction technique.

When the *ERG20* regulated strain expressing a hyperactive HMGCR was treated with methionine and analyzed for IPP using a metabolic labeling and TLC approach it was discovered that down regulation of *ERG20* expression unexpectedly decreased IPP counts by 100 % and also decreased the

proportion of IPP to FPP by a factor of 4. This discovery suggests that IPP is being metabolized elsewhere. In theory, the presence of a hyperactive HMGCR and the absence of FPP synthase should promote IPP accumulation. However, the data suggest that IPP is being metabolized by the remaining FPP synthase stores and potentially by an unknown catalyst. The significant decrease in IPP could be due to many factors. Given that the decrease in FPP was only 39%, it could be hypothesized that another enzyme in *S. cerevisiae* can act in the same manner as FPP synthase and metabolized the accumulated IPP into FPP. Another plausible explanation could be that the accumulated IPP is being used in another metabolic pathway. It can be safely assumed that DMAPP would accumulate along with IPP and that these intermediates could be dephosphorylated to produce trace amounts of their alcohol derivatives. It has been observed that the ratio of IPP to FPP affects downstream polyprenol synthesis, where the rise of FPP over IPP concentrations promoted an increase in polyprenol synthesis[31]. This could explain why there was a significant decrease in IPP counts in the cell.

7.5 Isoprenol Production in *S. cerevisiae* – Discussion

The isoprenol producing *S. cerevisiae* strain engineered in this study did not produce the desired product in an appreciable yield. There are a number of obvious explanations for this, however, the overall mechanism remains unclear and can be subject to interpretation.

First, low production of isoprenol could be due to toxicity. It has been demonstrated that low concentrations of isoprenol is toxic to *S. cerevisiae*, the W303 strain cannot tolerate 0.75 % isoprenol. This finding suggests that *S. cerevisiae* would be an unsuitable host organism to use for large-scale isoprenol production. 0.75 % is very low when compared to toxicity levels of other alcohols. For instance, ethanol can reach a concentration of 14 % before it becomes toxic to *S. cerevisiae*[42]. Isoprenol is very toxic to *S. cerevisiae* and can be explained by a couple of hypotheses. Isoprenol is not a known byproduct of *S. cerevisiae* and for that reason it is intolerant to isoprenol. Furthermore, isoprenol is a higher, unsaturated alcohol chain which may allow it to disrupt cell membranes more rapidly and stronger.

Low production of isoprenol could also be explained by *S. cerevisiae*'s ability to metabolize isoprenol. When the W303 strain was grown in 0.5 % isoprenol there was an increase in prenol over time, suggesting that *S. cerevisiae* has the ability to metabolize isoprenol. The production of prenol after 24 hours can be explained by the presence of an isomerase that is able to isomerize isoprenol into prenol. Isoprenol in the medium could also be competing with IPP and DMAPP for FPP synthase, allowing IPP and DMAPP to accumulate and be converted into their alcohol products. The amount of isoprenol detected (236 - 270 μg/mL) is significantly lower than what was expected (~427 mg/mL). This discrepancy can be due to several factors including, isoprenol evaporation, *S. cerevisiae*'s ability to metabolize isoprenol or localization of isoprenol in the cell.

S. cerevisiae can tolerate low concentrations of isoprenol and can potentially metabolize the alcohol; these findings do not fully explain why very little isoprenol was produced in the engineered strain. The pathway assembled in the engineered strain should have promoted alcohol production (Figure 7.1). Increasing the flux through the mevalonate pathway by expressing a hyperactive HMGCR and down regulating FPP synthase transcription should theoretically increase IPP and DMAPP synthesis and accumulation, respectively. The expression of NudF should selectively convert these intermediates into their alcohol derivatives. Limited production of isoprenol can be partially attributed to NudF's low activity towards IPP and DMAPP (7.2). Low activity can be due to the localization of NudF in the cells or *S. cerevisiae* ability to repress NudF activity.

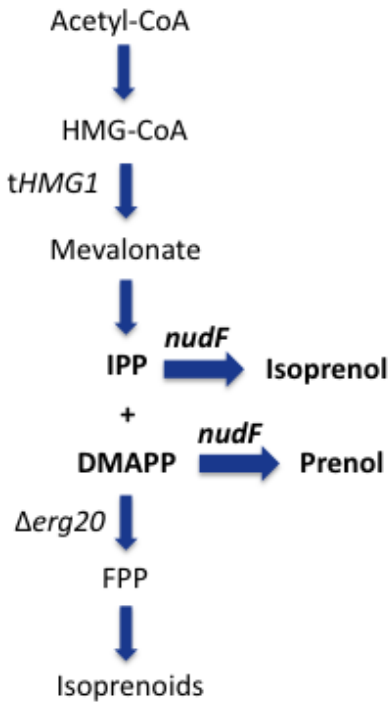


Figure 7.1 Simplified Schematic Illustration of the Engineered Metabolic Pathway Introduced into *S. cerevisiae* to Produce Isoprenol HMG-CoA = 3-hydroxy-3-methyl-glutaryl-coenzyme A; t*HMG1* = hyperactive HMG-CoA reductase; IPP = isopentenyl pyrophosphate; DMAPP = dimethylallyl pyrophosphate; *nudF* = NudF; Δ*erg20* = repressible expression of FPP synthase; FPP = farnesyl pyrophosphate.

The major limitation in this study is the unexpected decrease in cytosolic IPP when expression of *ERG20* was down regulated in the *ERG20* regulated *S. cerevisiae* strain expressing a hyperactive HMGCR (Detailed explanation in Section 7.4). Since IPP is not accumulating, there should be very little or no production of the desired alcohol product. IPP and DMAPP are transient intermediates in the mevalonate pathway therefore a subtle increase in cytosolic concentrations could signal the cell to halt isoprenoid production by redirecting glucose metabolism and subsequently increase metabolism of

these transient intermediates; negatively impacting NudF's ability to hydrolyze these intermediates into their alcohol products.

Despite this drawback, the engineered strain was able to produce a significant amount of isopentyl alcohol. Production of isopentyl alcohol as a potential biofuel has been reported previously[11]. The results suggest that the presence of a hyperactive HMGCR and NudF promoted the increased production of isopentyl alcohol (Figure 6.3). It is not known whether increase isopentyl alcohol production is due to both HMGCR and NudF or one of them individually. It was been proposed that prenol could be reduced to isopentyl alcohol in the presence of a NADPH dehydrogenase[8]. Prenol produced by NudF could be reduced to isopentyl alcohol by an endogenous dehydrogenase (Figure 7.2). However this theory does not explain why virtually no isoprenol or prenol was produced in the engineered strain, suggesting a different pathway is involved in isopentyl alcohol production. Amino acid metabolism can lead to the production of various branched chain alcohols including isopentyl alcohol[11]. The 2-ketoisocaproate intermediate in *E. coli* leucine synthesis was converted into isopentyl alcohol in the presence of an *S. cerevisiae* alcohol dehydrogenase and a *L. lactis* α-ketoisovalerate decarboxylase[11]. This work by Connor *et al.* suggest that this could be the pathway involved in isopentyl alcohol production in the engineered *S. cerevisiae*. It can be speculated that increased synthesis of isoprenoid intermediates negatively regulates the isoprenoid pathway and thus, glucose metabolism is redirected towards other pathways including amino acid

synthesis. Increase in amino acid synthesis, specifically leucine could promote the increase production of isopentyl alcohol due to the presence of endogenous alcohol dehydrogenases and carboxylases (Figure 7.3). This theory implies that NudF has no role in isopentyl alcohol production.

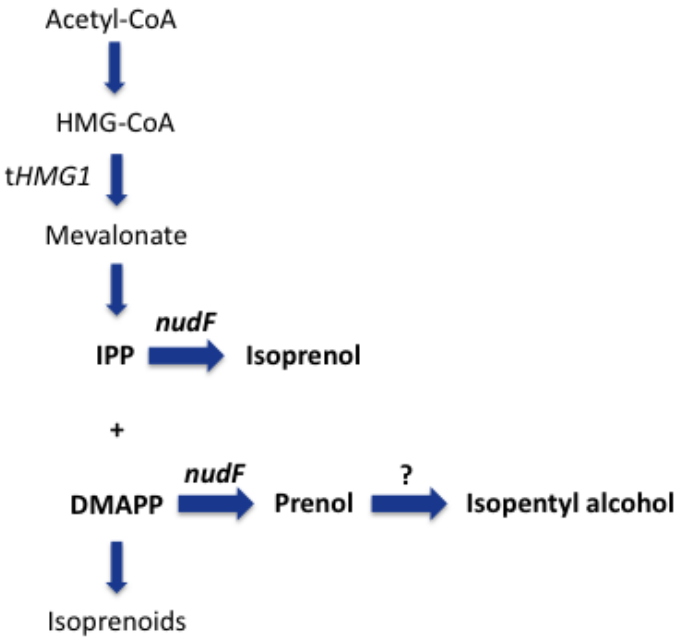


Figure 7.2 Simplified Schematic Illustration of Isopentyl Alcohol Production via Reductase Activity in *S. cerevisiae* HMG-CoA = 3-hydroxy-3-methyl-glutaryl-coenzyme A; t*HMG1* = hyperactive HMG-CoA reductase; IPP = isopentenyl pyrophosphate; DMAPP = dimethylallyl pyrophosphate; *nudF* = NudF.

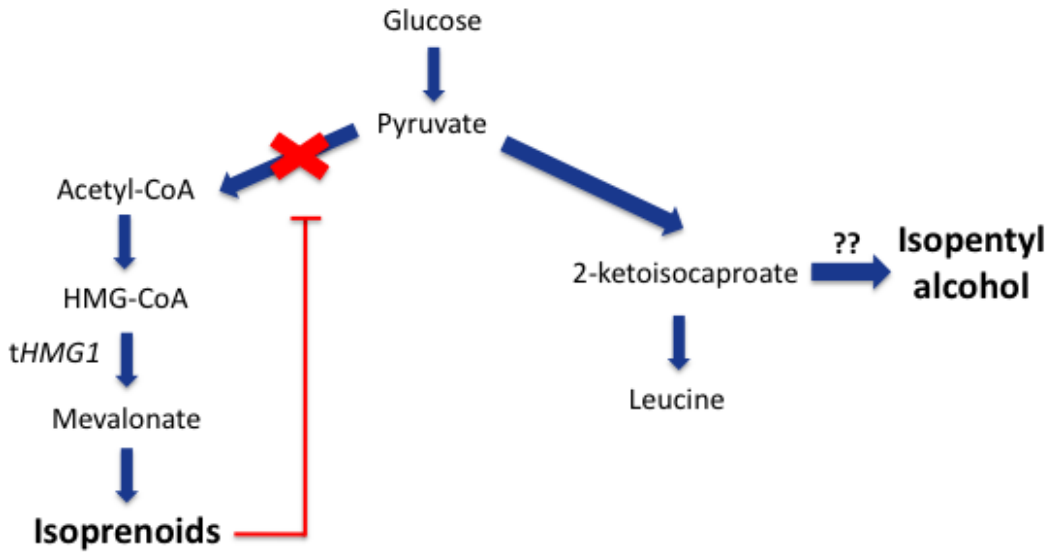


Figure 7.3 Simplified Schematic Illustration of Isopentyl Alcohol Production via Amino Acid Synthesis in *S. cerevisiae* HMG-CoA = 3-hydroxy-3-methyl-glutaryl-coenzyme A; t*HMG1* = hyperactive HMG-CoA reductase.

7.6 Future Directions

One of the major challenges of this research was constructing a *S. cerevisiae* strain that would increase and accumulate IPP. Plochocka *et al.* developed a *S. cerevisiae* strain that accumulated IPP. This strain had a point mutation at amino acid residue 197 in the *ERG20* gene; the lysine residue was substituted by glutamic acid. Since conditional regulation of *ERG20* was ineffective, it would be beneficial to use a *S. cerevisiae* strain bearing this point mutation in the *ERG20* gene. Expression of a hyperactive HMGCR and NudF in this strain should dramatically increase the production of isoprenol.

In vitro analysis of recombinant NudF activity illustrated low specificity towards IPP and DMAPP. It is unclear if NudF activity would be any different

in *S. cerevisiae*, but to further improve production of isoprenol a fusion protein between NudF and mevalonate-5-pyrophosphate decarboxylase could be created to increase NudF's proximity to IPP and thus isoprenol production. This decarboxylase is responsible for the last step in the conversion of mevalonate to IPP. A fusion protein between NudF and isopentenyl pyrophosphate isomerase could also be created to increase NudF's proximity to DMAPP, which could increase prenol production. Lastly, it would be beneficial to identify a better enzyme to replace NudF. Chou *et al.* screened thirteen Nudix hydrolases and found four other Nudix hydrolases that appeared to be more active towards IPP. The overexpression of NudB in Chou *et al.* engineered *E. coli* strain lead to the greatest conversion of IPP to isoprenol.

To further increase metabolic flux through the mevalonate pathway it would be useful to increase the acetyl-CoA pool in *S. cerevisiae*. In addition to the carbon precursors, ATP and NADPH cofactors are also required for isoprenoid synthesis. Previous reports improved NADPH availability in *S. cerevisiae* and saw an 85 % increase in isoprenoid synthesis through the mevalonate pathway, highlighting the importance of cofactor availability[36]. Increasing carbon precursors and cofactor availability would be an excellent addition to improving isoprenol production in *S. cerevisiae*.

During this research, it was unexpectedly discovered that the engineered strain produced a significant amount of isopentyl alcohol. The mechanism for

increased isopentyl alcohol production is unknown. It is not clear if NudF is required for this increased production therefore it would be useful to analyze alcohol production in a *S. cerevisiae* strain expressing only HMGCR.

It has been demonstrated that low concentrations of isoprenol is toxic to *S. cerevisiae*. Given that the original *S. cerevisiae* strain used was only able to tolerate 0.75 % of isoprenol it would be useful to determine a way to increase the strain's tolerance to isoprenol or identify a new strain that is able to tolerate the alcohol at higher concentrations.

7.7 Conclusion

The theory and strategy behind the development of an isoprenol producing *S. cerevisiae* strain was promising, however, introducing novel processes and redirecting existing pathways affects the entire cell. The result of this research emphasizes the existence of inherent limitations when manipulating complex microorganisms. While metabolic engineering has provided the opportunity for large-scale production of valuable chemicals, the establishment of appropriate pathways remains challenging due to confounding regulatory mechanisms in the native host.

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