Identifying genes to increase flux in the isoprenoid pathway in *S. cerevisiae*

A dissertation submitted for the partial fulfilment of
BS-MS dual degree in Science

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Certificate of Examination

This is to certify that the dissertation titled "**IDENTIFYING GENES TO INCREASE FLUX IN THE ISOPRENOID PATHWAY IN *S. CEREVISIAE***" submitted by Ms.ThoithoiLourembam (Reg. No. MS10094) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Shravan Kumar Mishra Dr. Rachna Chaba Prof. Anand Kumar Bachhawat
(Supervisor)

Dated: April 24th, 2015

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Prof. Anand K. Bachhawat at the Indian Institute of Science Education and Research, Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Thoithoi Lourembam

Dated: April 24th, 2015

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Prof. Anand K Bachhawat

(Supervisor)

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ABBREVIATIONS

Enzymes and Compounds

RtGGPPS	*R. toruloides* geranyl geranyl diphosphate synthase
RtPS	*R. toruloides* phytoene synthase
RtCRTI	*R. toruloides* phytoene dehydrogenase
AtPS	*A.thaliana* phytoene synthase
LC	Lycopene cyclase
PS	Phytoene synthase
GPP	Geranyl diphosphate
IDP/IPP	Isopentenyl diphosphate
DMAP/DMAPP	Dimethylallyl diphosphate
FDPS	Farnesyl diphosphate synthase
HMG-CoA reductase	3-hydroxy-3-methyl-glutaryl-CoA reductase
SNPs	Single nucleotide polymorphisms
tHMG1	Truncated HMG1
MVA pathway	Mevalonate pathway
PPPP	Prephytoene pyrophosphate
TERNase	Tris chloride EDTA-RNase

Chemicals

Amp	Ampicillin
ATP	Adenosine Triphosphate
dNTPs	2’ –deoxyadenosine 5’- Triphosphate
PEG	Poly Ethylene Glycol

Techniques

Agarose gel electrophoresis

HPLC (High-performance liquid chromatography)

Others

BLAST	Basic Local Alignment Search Tool
MSA	Multiple Sequence Alignment
ORF	Open Reading Frame
LB	Luria Bertani
SD	Synthetic Defined
YPD	Yeast peptone dextrose
TE	Tris chloride and EDTA
WT	Wild-type

Abstract

Isoprenoids (also called terpenoids) are a large and diverse class of naturally occurring organic compounds derived from five carbon isoprene units. Previous research in the lab has led to cloning and expression of the carotenoid producing enzymes of the red yeast in *S. cerevisiae* to yield a red pigmented strain. Current efforts are to identify genes and mutants which increase the flux in the isoprenoid pathway. In the present study, we have used two approaches towards this goal. The first approach is to find out the SNPs in natural isolates of *S. cerevisiae* that can lead to increase in flux by finding out SNPs that lead to increased pigmentation. The second approach is to develop a color based assay by exclusive biosynthesis of lycopene as a marker for identification of genes that increase flux in the isoprenoid pathway in *S. cerevisiae.* In the first approach, SNPs of different natural isolates of *S. cerevisiae* were examined for their impact on carotenoid pigmentation and a few candidate genes were examined. In the second approach, since mixtures of carotenoids interfere in a genetic screen (based on previous work in the lab), we developed a screen wherein only lycopene was biosynthesized by heterologous expression of *A. thaliana* phytoene synthase (AtPS) gene along with *R. toruloides* geranylgeranyl diphosphate synthase (RtGGPPS) and phytoene dehydrogenase (RtCRTI) gene. We show in this strain, a significant increase in the pigmentation level with over expression of tHMG1 (a known flux increasing gene) which validates genetic screen.

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Chapter 1

Introduction

1. Introduction

1.1 Terpenoids

Terpenoids are large and diverse class of naturally occurring organic compounds contributing more than 50,000 compounds to the natural products and its diversity [1]. These secondary metabolites are also called isoprenoids and derived from five-carbon isoprene units. Based on the number of carbon atoms present; they are classified as monoterpenes (C_{10} atoms), sesquiterpenes (C_{15} atoms), diterpenes (C_{20} atoms), triterpenes (C_{30} atoms) and tetraterpenes (C_{40} atoms). Isoprenoids are commercially valuable compounds. They are used in cosmetics (e.g. menthol), drugs (e.g artemisinin,taxol), biofuels (e.g. farnesene, bisabolene) and as coloring additives (e.g. astaxanthin and lycopene), and flavoring agents (e.g. Linalool, limonene)[2].

1.2 Biosynthesis of Terpenoids/Isoprenoids

Isoprenoids are synthesized by all forms of life- bacteria, plants and fungi. Two isoprenoid biosynthetic pathways exist that synthesize the precursors, isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP) [1]. These are -MEP pathway and the Mevalonate (MVA) pathway. MEP pathway is mainly employed in most prokaryotes and higher plants and occurs in chloroplast [3]. But MVA pathway occurs in cytosol and is employed by many prokaryotes and eukaryotes. The MVA pathway produces IPP from acetyl CoA, which is then isomerized to DMAPP. IPP then gives rise to higher order building blocks, geranyl pyrophosphate (GPP; C10), farnesyl pyrophosphate (FPP; C15) and geranyl geranyl pyrophosphate (GGPP; C20) [4] [1].

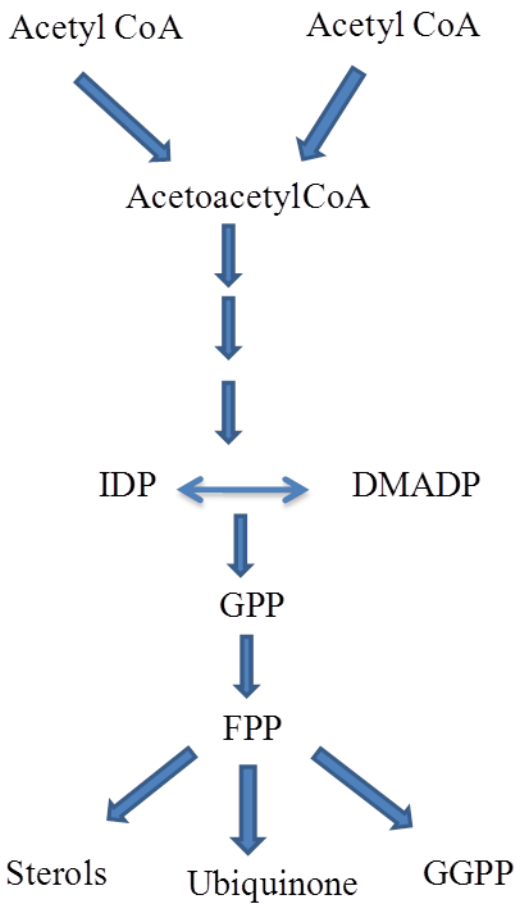


Fig. 1: Schematic overview of Mevalonate pathway.

1.3 Metabolic engineering of isoprenoids in yeast

There are two ways for the production of isoprenoids. The first approach is the extraction of isoprenoids from their natural sources and the second approach is the chemical synthesis of isoprenoids. The production of the isoprenoids from biological systems by metabolic engineering is emerging as an alternative to chemical synthesis whenever complex chemical structures are required or seasonal, regional and environmental variations affect extraction from their natural sources [2].

S.cerevisiae and *E. coli* have been engineered for the production of isoprenoids. The physiology, transcriptome, metabolome and genome of these organisms are well studied and they can be easily manipulated. *S. cerevisiae* is highly preferred host by the industry, as it can withstand high osmotic pressure and reduced pH compared to bacteria [1, 5].Furthermore, many higher eukaryotic genes express better in *S. cerevisiae*.

S. cerevisiae provides isoprenoid precursors such as GPP, FPP and GGPP for production of mono, sesequi, di and tetra terpenoids by metabolic engineering. Expression of plant derived terpene synthases in yeast cells showed that it was possible for enzymes to utilize these endogeneous precursors for production of terpenoids [6] (2).

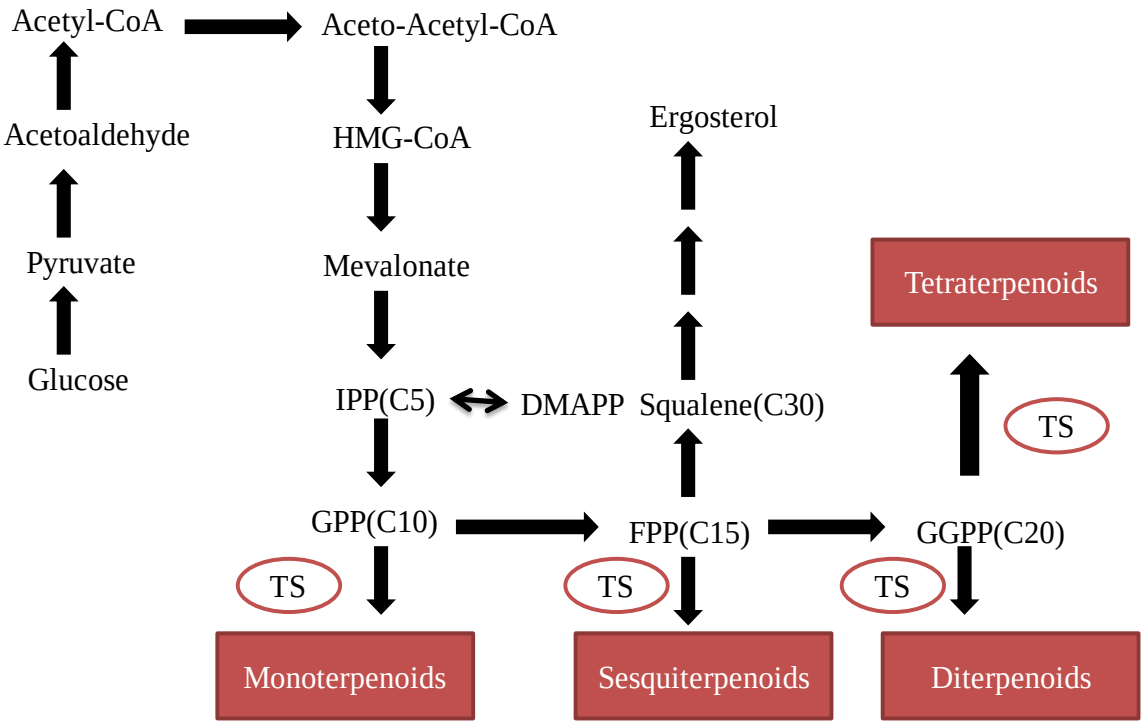


Fig. 2: Metabolic engineering of isoprenoid pathway of *S. cerevisiae* for terpenoids. Modified from [7]. TS- Terpene synthases.

Many terpenoids have been produced in *S. cerevisiae*by metabolic engineering.

Table 1: Commercially important terpenoids produced in *Saccharomyces cerevisiae* [7].

Monoterpenoids	**Diterpenoids**	**Sesquiterpenoids**	**Carotenoids**
a) Geraniol b) Linalool	a) Taxa-4(5),11(12)-diene, (Taxol precursor)	a) Amorpha-4,11-diene (Artemisinin precursor) b) Nootkatone	a) Lycopene b) β- carotene c) Astaxanthin

Strategies currently employed for increasing the yield of terpenoids in *S. cerevisiae* by metabolic engineering of isoprenoid pathway

Increasing the yield of terpenoids is critical for their commercial exploitation. Merely over expressing genes is inadequate, there is a need to remove the metabolic blocks and diversion of unwanted products. To increase the yield of terpenoids in *S. cerevisiae*, researchers have focused on the following regulatory enzymes of the isoprenoid pathway:

1. HMGR :

HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-CoA reductase; HMGR) is the key regulatory enzyme of the MVA pathway. Truncation of HMG1 (tHMG1) and over-expression of its catalytic domain has been found to increase the flux in the isoprenoid pathway [1].

2. ERG9:

ERG9 catalyzes first step of sterol biosynthesis. It catalyzes conversion of farnesyl diphosphate (FPP) to squalene and drains FPP pools away from the isoprenoid pathway. Hence, down-regulation of ERG9 is desirable. Down-regulation of ERG9 by its expression from weak promoter has been quite successful in increasing the flux towards the isoprenoids [1].

3. ERG20:

ERG20 catalyzes condensation of IPP and DMAPP to form GPP and subsequently FPP. Therefore up-regulation of ERG20 is desirable for the accumulation of FPP for increasing yield of terpenoids [1].

4. Upc2:

Upc2 is a transcription factor which regulates a number of ERG genes in the yeast ergosterol biosynthetic pathway. Over expression of constitutively active mutant of Upc2 (upc2-1) has been employed by researchers for increasing the flux in the pathway [1].

5. BTS1:

BTS1 encodes for geranyl geranyl diphosphate synthase which catalyses the conversion of FPP to GGPP. Therefore over expression of BTS1 is desirable and has been employed for increasing the yield of tetraterpenoids such as carotenoids [1].

Although these five genes have been identified as being important for flux, it is possible that other genes may also be participating but there are no ways to identify them. For this,

a visual genetic screen would be important to identify new genes that can increase the flux in the pathway.1

1.4 Metabolic engineering of *S. cerevisiae* for Carotenoid production

Cartenoids are the colored tetraterpenoids produced by some bacteria, plants and yeast. They are commercially valuable compounds. They are used as nutraceutical and have provitamin A activity [8, 9]. They are also used as food and cosmetic color additives. They also have anti-aging and antioxidant capacity. Being commercially important compounds, therefore their production by metabolic engineering route is desirable [2].

Carotenoids such as β-carotene, Lycopene and astaxanthin have been metabolically produced in *S. cerevisiae.*

In metabolically engineered *S. cervisiae* cells, for the production of carotenoids only three enzymes geranyl geranyl diphosphate synthase (GGPPS), phytoene synthase and phytoene dehydrogenase are expressed from carotenogenic organism. GGPPS catalyzes the conversion of farnesyl diphosphate (FPP) to geranyl geranyl diphosphate (GGPP) which is further converted to phytoene by bifunctional phytoene synthase. Phytoene is then converted to lycopene by phytoene dehydrogenase (CRTI) [1, 2,]. Bifunctional phytoene synthase further catalyzes the conversion of lycopene to γ-Carotene which is then converted to β-carotene [3].

Verwaal *et al*; 2006 first attempted to produce β-carotene in *S. cerevisiae* by expressing carotenoid genes of red yeast *Xanthophyllomyces dendrorhous*. They showed that overexpression of two enzymes of carotenoid pathway- phytoene synthase (CrtYB) and Phytoene dehydrogenase (CrtI) were sufficient for the carotenoid production in *S. cerevisiae.* They were able to achieve 5.9 mg/g concentration of β-carotene. Ukibe *et al*, 2009 successfully produced another carotenoid, Astaxanthin in *S.cerevisiae* by metabolic engineering. They co-expressed CrtS (cytochrome P50 oxidase) and CrtR (cytochrome P450 reductase) from *Xanthophyllomyces dendrorhous* along with the other carotenoid producing enzymes.

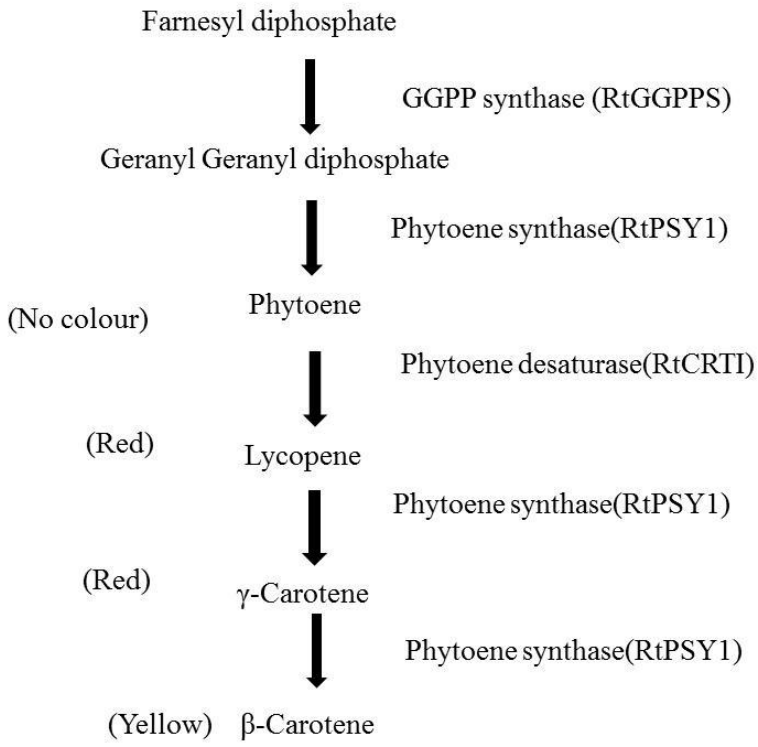


Fig. 3: Carotenoid pathway in *Rhodosporidium toruloides*

1.5 Carotenoid as a genetic screen

Carotenoids are coloured compounds and provide a visual phenotype to yeast colonies. This phenotype can be used in a genetic screen whose increased colour would reflect increased flux into the isoprenoid pathway. Attempts were made to use color of carotenoids as visual genetic screen by Ozyadin et al., 2013 and Yuan et al., 2014. However, they observed that although over expression of tHMG1 (regulatory enzyme of isoprenoid pathway) increases the flux in the isoprenoid pathway, an increase in pigmentation could not be observed.

The probable reason for this is the accumulation of phytoene. A second issue with the carotenogenic screen is that the enzymes are bifunctional leading to multiple carotenoids. This mixture of carotenoids could give a confusing picture about the flux.

1.6 Objective of the study

In this study, we aimed to identify genes/mutants that increase the flux in the pathway. For this, we have used two approaches.

(1) To find out the SNPs in natural isolates of *S. cerevisiae* to identify new genes involved in the isoprenoid flux.

(2) To develop a lycopene based visual genetic screen for identifying new genes involved in isoprenoid flux.

CHAPTER 2

MATERIALS AND METHODS

SECTION A: MATERIALS

2.1. CHEMICALS AND REAGENTS

All the chemicals used were obtained from commercial sources and were of analytical grade. Media components, fine chemicals and reagents were purchased from Sigma Aldrich, (St. Louis, USA), HiMedia, (Mumbai, India), Merck. India Ltd (Mumbai, India),USB Corporation (Ohio, USA) or Difco, USA. Oligonucleotides (primers) were designed using Gene Runner software and were purchased from Integrated DNA Technologies (IDT). Enzymes (Restriction enzymes, T4 DNA ligase, Calf Intestinal Phosphatase (CIP), Antarctic phosphatase, *Vent* DNA polymerase, *Taq* DNA polymerase and other modifying enzymes), their buffers, dNTPs, DNA and protein molecular weight markers were purchased from New England Biolabs Inc, (Beverly, MA, USA). Gel-extraction kits and plasmid miniprep columns were obtained from Fermantas.

2.2. STRAINS AND PLASMIDS

Escherichia coli DH5α was used as the cloning host. The genotype for the *E. coli* strain and the yeast strains used in the study are given in Table 2. The list of various plasmids used in this study is given in Table 3.

Table 2: List of bacterial and yeast strains used in the study

Sr. no.	Strain	Genotype	Source
1	DH5ά	*F' gyr A(Nal) recA1 relA endA1 thi-1 Lab strain Hsd 17(rk-mk+) gln V44 deoRΔ(lacZYA-argF) U169 [ϕ80dΔ(lacZ) M15]*	Lab strain
2	ABC 276	*S. cerevisiae MAT α ura 3-52 leu2Δ1 his3Δ200 trp1 lys2-801*	Lab strain

Table 3: List of Plasmids used in the study

Sr. no.	Clone No.	Plasmid name	Descriptions
1.	ABE 3569	pRS313TEF	The CEN-vector bearing HIS3 marker and TEF Promoter-MCS-CYC terminator for yeast expression and Amp^{r} marker for selection in *E. coli.*
2	ABE 443	p416TEF	The CEN-vector bearing URA3 marker and TEF Promoter-MCS-terminator for yeast expression and Amp^{r} marker for selection in *E. coli.* (Mumberg, et al., 1995)
3	ABC 4115	pRS416TEF-RtPSY1(t)	The CEN-vector bearing URA3 marker and TEF Promoter-MCS-terminator for yeast expression of RtPSY1 truncated gene from *R. toruloides* and Amp^{r} marker for selection in *E. coli.*
3	ABE 3663	pRS313TEF-tHMG1	The CEN-vector bearing HIS3 marker and TEF Promoter-MCS-terminator for yeast expression of tHMG1 gene from *S. cerevisiae* and Amp^{r} marker for selection in *E. coli.*
4	ABE 3518	pRS315TEF-RtGGPPS	The CEN-vector bearing LEU2 marker and TEF Promoter-MCS-terminator for yeast expression containing RtGGPPS gene from *R. toruloides* and Amp^{r} marker for selection in *E. coli.*
5	ABE 3500	pRS314TEF-RtCRTI	The CEN-vector bearing TRP1 marker and TEF Promoter-MCS-terminator for yeast expression containing RtCRTI gene from *R. toruloides* and Amp^{r} marker for selection in *E. coli.*

6	ABE 4159	p416TEF-AtPS	The CEN-vector bearing URA3 marker and TEF Promoter-MCS-terminator for yeast expression containing PS gene from *A. thaliana* and Amp^{r} marker for selection in *E. coli.*
7	ABE 3551	p416TEF(RtPSY 1+RtCRTI)	The CEN-vector bearing URA3 marker and TEF Promoter-MCS-terminator for yeast expression containing Rt(PSY1+CRTI) gene from *R. toruloides* and Amp^{r} marker for selection in *E. coli*.
8	ABE 3633	pRS315CYC-RtGGPPS	The CEN-vector bearing LEU2 marker and CYC Promoter-MCS-terminator for yeast expression containing RtGGPPS gene from *R. toruloides* and Amp^{r} marker for selection in *E. coli.*
9	ABE 3649	pRS314CYC-RtCRTI	The CEN-vector bearing TRP1 marker and CYC Promoter-MCS-terminator for yeast expression containing RtCRTI gene from *R. toruloides* and Amp^{r} marker for selection in *E. coli.*
10	ABE 4254	p416CYC-AtPS	The CEN-vector bearing URA3 marker and CYC Promoter-MCS-terminator for yeast expression containing Phytoene synthase gene from *A. thaliana* and Amp^{r} marker for selection in *E. coli*.
11	ABE 4211	p416TEF[RtPSY 1+RtCRTI+BTS1 (322134S)]	The CEN-vector bearing URA3 marker and TEF Promoter-MCS-terminator for yeast expression containing RtPSY1, RtCRTI and BTS1 gene from 322134S *S. cerevisiae* strain and Amp^{r} marker for selection in *E. coli.*
12	ABE 4225	p416TEF[RtPSY 1+RtCRTI+BTS1 (SK1)]	The CEN-vector bearing URA3 marker and TEF Promoter-MCS-terminator for yeast expression containing RtPSY1, RtCRTI and BTS1 gene from SK1 *S. cerevisiae* strain and

			Amp^{r} marker for selection in *E. coli.*
13	ABE 4242	pRS313TEF-Sut1	The CEN-vector bearing HIS3 marker and TEF Promoter-MCS-terminator for yeast expression containing Sut1 gene from *S. cerevisiae* and Amp^{r} marker for selection in *E. coli.*
14	ABE 4233	pRS313TEF-Ecm22	The CEN-vector bearing HIS3 marker and TEF Promoter-MCS-terminator for yeast expression containing Ecm22 gene from *S. cerevisiae* and Amp^{r} marker for selection in *E. coli.*
15	ABE 4243	pRS313TEF-Taf25	The CEN-vector bearing HIS3 marker and TEF Promoter-MCS-terminator for yeast expression containing Taf25 gene from *S. cerevisiae* and Amp^{r} marker for selection in *E. coli.*

2.3. OLIGONUCLEOTIDES

The list of various oligonucleotide primers used in this study is given in Table 4.

Table4: List of Oligonucleotides and their sequences in this study

Oligoname	**Sequence (5′to3′)**
PS (A. thaliana) FP	ACGCATGGATCCATGTCTTCAAGCTTAGTAGCAAG
PS (A. thaliana) RP	AAGCATCTCGAGTCATATCGATAGTCTTGAAC
BTS1+800bp promoter FP	AGCGCTCTGCAGCTGTTCAGATATAAGAAAAAG
BTS1 +800bp promoter RP	ATCGTTCTAGATCACAATTCGGATAAGTGGTC
PSY1 (*R. toruloides*) FP(truncated)	ATCGCGGATCCATGGGTGGTTTGGATTATTGG
PSY1 (*R. toruloides*) RP(truncated)	TCGACCTCGAGTTACAAAGCTTGCCAAGC
Sut1 FP BamH1	GACGTCGGATCCATGTCCACAAGCATTACAGTAAG
Sut1 RP Xho1	GATCGCCTCGAGCTAAAAATCAATGCTTTTATAG
Ecm22 FP BamH1	GTCAAGGGATCCATGACATCCGATGATGGGAAT

Ecm22 RP Xho1	GAGGTCCTCGAGTTACATAAAAGCTGAAAAGTTTG
Taf25 FP BamH1	GACGTCGGATCCATGGATTTTGAGGAAGATTACG
Taf25 RP Xho1	GATCGCCTCGAGCTAACGATAAAAGTCTGGGCG

2.4. MEDIA

All the media, buffers and stock solutions were prepared using Millipore elix5 deionized water unless otherwise mentioned. They were sterilized, as recommended, either by autoclaving at 15lb/inch^2 (psi) pressures at 121ºC for 15 minutes, or by using membrane filters (Advanced Microdevices Pvt. Ltd., India) of pore size 0.2-0.45µm (for heat labile compounds). Additional amino acid and nutrients were prepared as sterile stock and added as per requirements. Agar was added, if required, at a final concentration of 2.2%. Ampicillin was added at a final concentration of 100µg/ml.

Sr. no.	**Media**	**Composition (g/l)**	
1.	**LB (Luria–Bertani) Medium**	Yeast extract Tryptone NaCl pH of the above medium was adjusted to 7.0 with 1N NaOH	5 10 10
2.	YPD **(Yeast extract Peptone Dextrose) Medium**	Yeast extract Peptone Dextrose	10 20 20
3.	SD **(Synthetic Defined) Medium**	YNB (Yeast Nitrogen Base) (without amino acids and ammonium sulphate) $(NH_4)_2 SO_4$ Glucose	1.7 5 20

		Amino acids (LEU,HIS,LYS,TRP,URA) (as per requirement) Drop out mix (as per requirement) pH was adjusted to 6.0–6.5.	80 mg/l 2

2.5. BUFFERS AND STOCK SOLUTIONS

2.5.1. Ampicillin Stock Solution (50 mg/ml)

The required amount of ampicillin (sodium salt) was dissolved in the required volume of deionized water, and it was filter-sterilized using 0.2µm filter membrane. It was stored at -20°C in aliquots in micro centrifuge tubes.

2.5.2. 50% Glycerol (used for preparing –80°C stocks of *E. coli*)

Alkaline Lysis Buffers (Plasmid DNA isolation from *E. coli*)

Sr. no.	Chemical	Composition
1	**Solution-I** **(Resuspension Solution)**	50 mM Glucose 25 mMTris-HCl (pH 8.0) 10 mM EDTA (pH 8.0) Autoclaved and stored at 4ºC.
2	**Solution-II** **(Lysis Solution)** **(Freshly prepared)**	0.2 N NaOH (freshly diluted from a 10 N stock) 1% SDS (freshly diluted from a 10% stock) Stored at room temperature.
3	**Solution-III** **(Neutralization Solution)**	60ml 5 M Potassium acetate 11.5 ml Glacial acetic acid 28.5 ml Deionized water The resulting solution is 3 M w.r.t potassium and 5 M w.r.t acetate. Stored at 4ºC.

4	**TE Buffer** **(Tris-EDTA)** **(pH 8.0)**	10 mMTris-HCl (pH 8.0). 1 mM EDTA (pH 8.0).
5	**TERNAse** **(stock prepared at 10 mg/ml)**	Working stock 20 µg/ml in TE Buffer, pH 8.0.
6	**PCI** **(Phenol-chloroform isoamyl alcohol) Solution (100ml)**	50 ml Phenol [Equilibrated with Tris-HCl (pH 7.6)] 48 ml Chloroform 2 ml Isoamyl alcohol Stored at 4°C in dark brown bottle.

2.5.3. Agarose Gel Electrophoresis Reagents

Sr. no.	**Chemical**	**Composition**
1.	**1X TAE** **(Tris-acetate-EDTA)** **Buffer (per 1000 ml)** **(prepared from 50X TAE stock)**	40 mMTris-acetate. 1mM EDTA (pH 8.0). Autoclaved and stored at room temperature.
2.	**Orange-G dye** **(Gel loading dye, 6X)**	0.25% orange-G 30% glycerol
3.	**Ethidium Bromide** **(10 mg/ml stock)**	Final working concentration used at 0.5 µg/ml.
4.	**0.7-1% Agarose gel in 1× TAE**	

2.5.4. Solutions for preparation of chemical competent *E. coli* cells (Sambrook, 1989)

Sr. no.	Media	Composition
1.	**SOB**	20 g Bactotryptone 5 g Bacto yeast extract 0.5 g NaCl Above mentioned components were dissolved in 950 ml of water. 10 ml of 250 mM KCl was added and pH adjusted to 7 with 5N NaOH, volume was made up to 995 ml and autoclaved. Just before use, 5 ml of filter sterilized 2 M $MgCl_2$ was added.
2.	**SOC**	SOB + 20 mM Glucose
3.	**10% glycerol**	

2.5.5. Yeast Transformation Solutions (*S. cerevisiae*) (Ito, *et al.*, 1983)

a) 0.1 M Lithium acetate in TE (pH 7.5)

b) 50% PEG-3350 in 0.1 M Lithium acetate in TE (pH 7.5).

2.5.6. STES lysis mixture for plasmid / genomic DNA isolation from yeast

10 mMTris-HCl (pH 8.0)

1 mM EDTA (pH 8.0)

100 mMNaCl

1% SDS

2% Triton X-100

2.5.7. Solution for Hydroxlyamine mutagenesis [Forsburg Lab Protocol]

NaOH 90 mg

Hydroxylamine HCl 350 mg

Dissolved in 5 ml water and pH adjusted to 6.7. The solution was made fresh just before use.

SECTION B: METHODS

2.1 Growth and maintenance of bacteria and yeast strains

The *E. coli* strains DH5α was routinely grown at LB medium at 37°C. *E. coli* transformants were selected and maintained on LB medium supplemented with ampicillin.

The *S. cerevisiae* strains were regularly maintained on YPD medium and grown at 30°C. The yeast transformants were selected and maintained on SD medium with supplement as per requirements.

2.2 Recombinant DNA methodology (restriction digestion, ligation, transformationof *E. coli.* PCR amplification, etc)

All the molecular techniques used in the study for manipulation of DNA, protein, bacteria and yeast were according to standard protocols (Sambrook, 1989, Guthrie & Fink, 1991)[10,11] or as per manufacturers' protocol, unless specifically mentioned.

2.3 *Invitro* Hydroxylamine mutagenesis

Approximately, 10 µg plasmid DNA was dissolved in 0.5 ml of Hydroxylamine solution (90 mg NaOH, 350 mg hydroxylamine HCl in 5 ml water, pH around 6.7. freshly made up before use). This mixture was incubated at 37°C for 21 hrs. The hydroxylamine treated DNA was purified using Qiagen column. The pool of mutagenized plasmid was directly transformed into the appropriate yeast strain.

2.4 Transformation of yeast

The transformation of *S. cerevisiae* strains was carried out by lithium acetate method (Ito *et al*., 1983). *S. cerevisiae* cultures were grown in YPD at 30°C with shaking for 16-24 hrs and then reinoculated in fresh YPD to an initial OD600 of 0.1, cells were allowed to grow at 30°C for 5-6 hrs with shaking. Cells were harvested at 6000 rpm for 5 min, then were washed with sterile water followed by subsequent wash with 0.1 M lithium acetate solution (prepared in TE, pH 7.5) and were finally resuspended in the same solution. Cells were incubated at 30°C for 30 min with shaking. The cells were spun down, suspended in 0.1 M lithium acetate solution to a cell density of $1x10^9$cells/ml and divided

into 100µl aliquots. Approximately 50 µg (5 µl of 10 mg/ml stock solution) of heat denatured, salmon sperm carrier DNA, followed by 0.3 µg- 0.7µg of plasmid/DNA fragment were added to each aliquot and whole cell suspension was incubated at 30°C for 30 min. After the incubation, 0.3 ml of 50% PEG 3350 (prepared in 0.1 M lithium acetate, pH 7.5) was added to each tube, mixed well and again kept at 30°C for 45 min. The cell suspensions were subjected to heat shock at 42°C for 10 min. and the cells were allowed to cool to room temperature. The cells were pelleted down at 7000 rpm for 3 min. The cell pellet was resuspended in sterile water and appropriate volume of cell suspension was placed on selection plates.

2.5 Isolation of plasmid from yeast

Selected yeast transformants were inoculated in 3 ml of selection medium and the cultures were incubated at 30°C with shaking for 18-20 hrs. After the incubation, the cells were harvested at 8,500 rpm for 5 min at room temperature and the pellets were suspended in 200 µl of STES lysis solution. Equal amounts of sterile, acid-washed glass beads (425 to 600 µm, Cat# G8772, Sigma) were added and the cell suspensions were vortexed vigorously for 1 min at room temperature. The lysed suspensions were then treated with phenol-chloroform adding 200 µl of phenol-chloroform solution and then vortexing for 1-2 min at room temperature. The lysates were spun down at 12,000 rpm for 5 min at RT and the aqueous phase was collected in a fresh microfuge tube. 2 µl of this DNA was electro-transformed in *E. coli* and transformants were selected on LB plates (containing ampicillin). The *E. coli* transformants were then grown to isolate plasmids and verified by re-transformation into yeast.

2.6 Isolation of genomic DNA from yeast

Genomic DNA from *S. cerevisiae* strains was isolated by using the glass bead lysis method and the STES lysis buffer.

2.7 Growth assay by dilution spotting

For growth assay, the different *S. cerevisiae* strains carrying the plasmid were grown overnight in minimal medium and reinoculated in fresh medium to an OD_{600} of 0.1 and grown for 6 hours. The exponential phase cells were harvested washed with water and resuspended in water to an OD_{600} of 0.2. These were serially diluted to 2:10, 2:100,

2:1000, 2:10000. 10 µl of these cell resuspensions were spotted on minimal medium (containing leu/his/trp/lys as per required). The plates were incubated at 30ºC for 3 to 4 days and photographs were taken.

2.8 Bioinformatics analysis

The ORF sequences of HMG1, BTS1, ERG20, ERG9, UPC2 of the strains 322134S, SK1, Y55, UWOPS03-461.4, YJM981, DBVPG6044 were available at http://www.moseslab.csb.utoronto.ca/sgrp/

BLAST:

BLAST was used to find out similarity between phytoene synthase of *R. Toruloides* and phytoene synthases and lycopene cyclase from different organisms.

Multiple sequence alignment:

Multiple sequence alignment of monofunctional phytoene synthase from *A. thaliana*, bifunctional phytoene synthase from *R. toruloides*, phytoene synthase from *Pantoea ananatis* was performed using Clustal W2.

Snap gene viewer 2.7.1 was used for designing primer, finding restriction sites and gene mapping in cloning.

CHAPTER 3

RESULTS AND DISCUSSIONS

3. RESULTS AND DISCUSSIONS

3.1 Identification of SNPs in natural isolates of *S. cerevisiae* as a means of identifying new genes implicated in the isoprenoid flux in *S. cerevisiae*

26 wild type ura auxotrophic *S. cerevisiae* strains from different sources were provided by Dr. Himanshu, TIFR, Mumbai. Carotenogenic genes RtPSY1 and RtCRTI from *R. toruloides* both cloned under the same plasmid vector p416TEF were transformed in these strains. Selection of transformants was done on SD- URA plates. Approximately equal size colonies of 26 strains were selected and spotted at 0.02 O.D on SD- URA plates and color was coded [4].

Sl.no	Strain	Color	Sl.no.	Strain	Color	Sl.no.	Strain	Color
1	S288C	+++	11	Y12	++	21	YJM978	+++
2	Y11C17_ES	+++	12	UWOPS83-7873	++	22	YJM981	++
3	BC187	++	13	UWOPS87-2421	+++	23	YJM975	+++
4	YPS606	+++	14	UWOPS03-461.4	+++	24	DBVPG6044	+++
5	W303	Red	15	UWOPS05-217.3	++	25	DBVPG1373	+++
6	DBVPG1106	+++	16	UWOPS05-227.2	++	26	Y55	+++
7	SK1	+	17	322134S	++++	CONTROL	S288C	+
8	L-1374	+++	18	378604X	+++			
9	L-1528	+++	19	273614N	++			
10	NCYC110	+++	20	DBVPG6765	++			

Fig. 4: Comparison of colour in different *S. cerevisiae* strains. (Summary of earlier work)

+ sign indicates the pigmentation level

White pigment	+
Creamish pigment	++
Light yellow pigment	+++
Dark Yellow pigment	++++

The carotenoid formation in different 26 Ura auxotrophic *S. cerevisiae* strains after transformation of p416TEF (RtPSY1+RtCRTI) plasmid was compared. Selection of transformants was done on SD- URA plates. A wide variation in color was seen in these 26 *S. cerevisiae* strains. In order to identify the polymorphism in these natural isolates of

S. cerevisiae that may be responsible for differences in pigmentation, we decided to focus on the key regulatory genes of isoprenoid biosynthesis pathway in *S. cerevisiae* in a candidate gene approach. The candidate genes known to affect flux in the isoprenoid pathway are tHMG1, ERG20, ERG9, BTS1 and Upc2 transcription factor. It has been known that over-expression of tHMG1, BTS1 and Upc2-1 transcription factor; upregulation of ERG20 and down regulation of ERG9 increases the flux in the pathway. To identify whether differences in pigmentation in 26 *S. cerevisiae* strains is due to these 5 genes, we have chosen 5 highest carotenoid producing strains and 1 lowest carotenoid producing strain based upon the pigmentation level to carry out these comparisons [5].

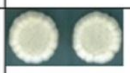
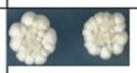
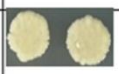
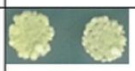
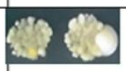
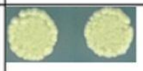
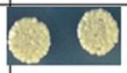
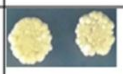
Sl no.	Strain	EXP. 1A	EXP. 1B	EXP.2	EXP.3	COLOR
1	SK1					+
2	UWOPS03-461.4					+++
3	322134S					++++
4	YJM981					++
5	DBVPG6044					+++
6	Y55					+++

Fig. 5: Comparison of pigment/color in 6 different *S. cerevisiae* strains (work done by Manisha Wadhwa). SK1- Lowest carotenoid producing strain, 322134S - Highest carotenoid producing strain

Sequence analysis of the key genes (HMG1, Upc2, ERG20, ERG9, BTS1) in the above 6 strains was performed using BLAST [Table 5]

Table 5. Identification of SNPs in different isolates of *S. cerevisiae* in five candidate genes.

Title: Sequence Analysis of key regulatory genes of the isoprenoid pathway in different *S. cerevisiae*strains										
Strains	**Genes**									
	HMG1(protein size=1054aa) , Seq. region=1-3165bp		**Upc2 (protein size=913aa), Seq.region=1036-2742bp**		**ERG9 (protein size=444aa) , Seq. region=1-1335bp**		**ERG20 (protein size=352aa) , Seq. region=1-1059bp**		**BTS1(protein size=335aa)Seq. region=1-1008bp**	
	Position of a.a	**Mutations**	**Position of a.a**	**Mutations**	**Position of a.a**	**Mutations**	**Position of a.a**	**Mutations**	**Position of a.a**	**Mutations**
3221345 (++++)	**3***	**Proline to Leucine**	**475***	**Lysine to Methionine**	**206***	**Glycine to Serine**			**51***	**Asparagine to Asparatic acid**
DBVPG 6044 (+++)	**3***	**Proline to Leucine**			**206***	**Glycine to Serine**				
	576*	**Serine to Alanine**								
	868*	**Threonine to Isoleucine**								
	1033*	**Proline to Leucine**								
SK1 (+)	**3***	**Proline to Leucine**	**457***	**Lysine to Arginine**	**206***	**Glycine to Serine**				
	16*	**Alanine to Valine**	**498***	**Alanine to Threonine**						
UWOPS03_461.4 (+++)	**3***	**Proline to Leucine**	**489***	**Lysine to Arginine**	**206***	**Glycine to Serine**	**151***	**Isoleucine to Leucine**		
Y55 (+++)	**3***	**Proline to Leucine**	**475***	**Lysine to Arginine**	**206***	**Glycine to Serine**			**Identical to BTS1 of S288c**	
	576*	**Serine to Alanine**								
	868*	**Threonine to Isoleucine**	**498***	**Alanine to Threonine**						
	1033*	**Proline to Leucine**								

Sequence analysis results showed a large number of SNPs in all the genes in the chosen strains. But in particular, there were two SNPs, one in BTS1 and another in Upc2 gene which were unique to only 322134S (high color producing) strain. To check whether these SNPs in BTS1 and Upc2 gene were really contributing to the high pigmentation level of 322134S strain, we adopted a strategy in which we cloned BTS1+800bp

promoter from both 322134S and SK1 strain in p416TEF (RtPSY1+RtCRTI) vector and transformed in least carotenoid producing strain SK1 as well as highest carotenoid producing strain 322134S strain. We also wanted to see the effect of an extra copy of BTS1 in both the strains and to check whether BTS1 from SK1 is diluting the actual pigmentation in 322134S or BTS1 from 322134S is increasing the color in SK1 keeping in mind that both 322134S and SK1 already has its own copy of BTS1.

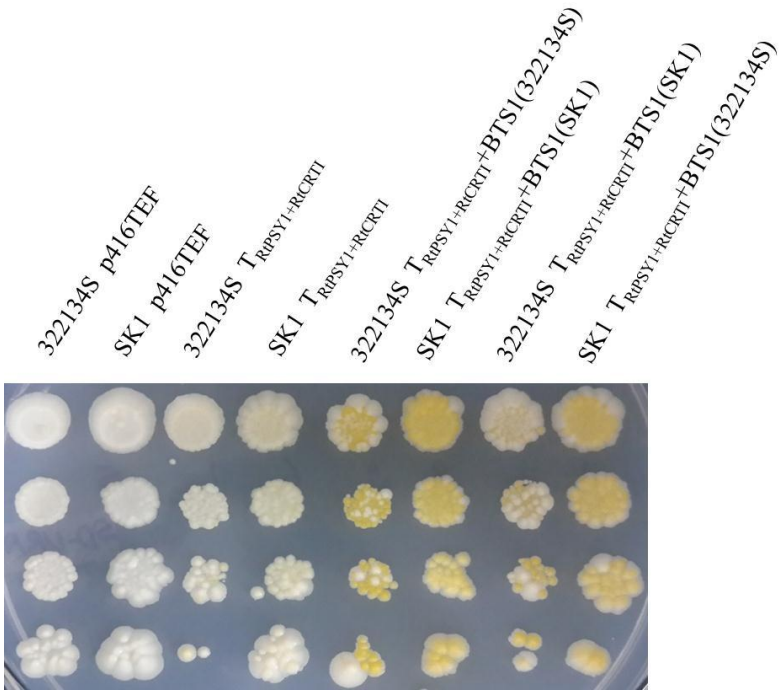


Fig. 6. Comparison of color of SK1 and 322134S strains with over-expression of BTS1 gene.

Upon expression of carotenogenic genes RtPSY1 + RtCRTI, 322134S and SK1 strain should give yellow and white color, respectively as observed in previous work (by Manisha Wadhwa). But now in this dilution spotting, both the strains are giving yellow color due to which this experiment could not be proceeded further [6].

3.2 Exclusive Biosynthesis of Lycopene:

To develop a color based visual assay for increasing flux in the isoprenoid pathway in *S. cerevisiae*, we followed up on previous work in the lab where carotenogenic genes from *R. toruloides* were expressed in *S. cerevisiae.* It was observed that expression of the carotenogenic enzymes-geranyl geranyl diphosphate synthase (RtGGPPS), phytoene synthase (RtPSY1) and phytoene dehydrogenase (RtCRTI) gave mixture of carotenoids

with different colors. As different carotenoids have different colors this creates hindrance for successful development of a genetic screen. Therefore, to develop a successful genetic screen, we needed a specific product which gives only one color. Lycopene being the first product in the isoprenoid pathway that gives color, it was chosen.

3.3 Pathway of Lycopene production in *S. cerevisiae*

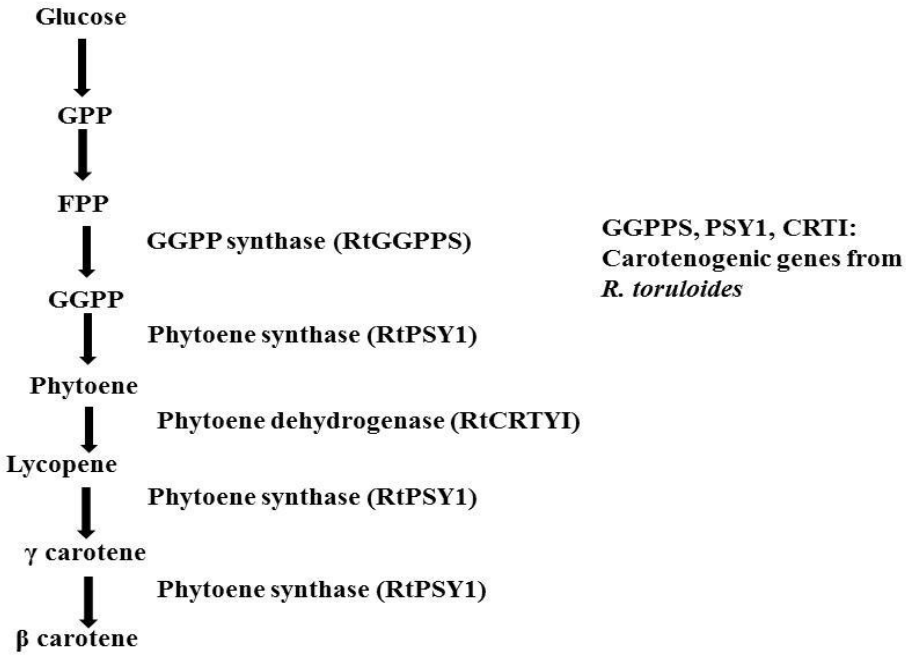


Fig. 7. Pathway showing heterologous Lycopene production in *S. cerevisiae*.

For the heterologous production of lycopene in *S. cerevisiae*, since precursor GGPP is produced in *S. cerevisiae* by the MVA pathway expression of only two carotenogenic genes RtPSY1 and RtCRTI from another carotenoid producing red yeast *R. toruloides* would yield lycopene. However, in addition to lycopene, other carotenoids are also formed owing to the bifunctional nature of the phytoene synthase enzyme which has both phytoene synthase and a lycopene cyclase activity.

3.4 There are two approaches for exclusive production of Lycopene in *S. cerevisiae*

A. Truncation of lycopene cyclase domain from bifunctional phytoene synthase of *R. toruloides.*

B. Expression of monofunctional phytoene synthase (AtPS) from *Arabidopsis thaliana* in *S. cerevisiae*

3.4(A) (i) Truncation of lycopene cyclase domain from bifunctional phytoene synthase of *R. toruloides*

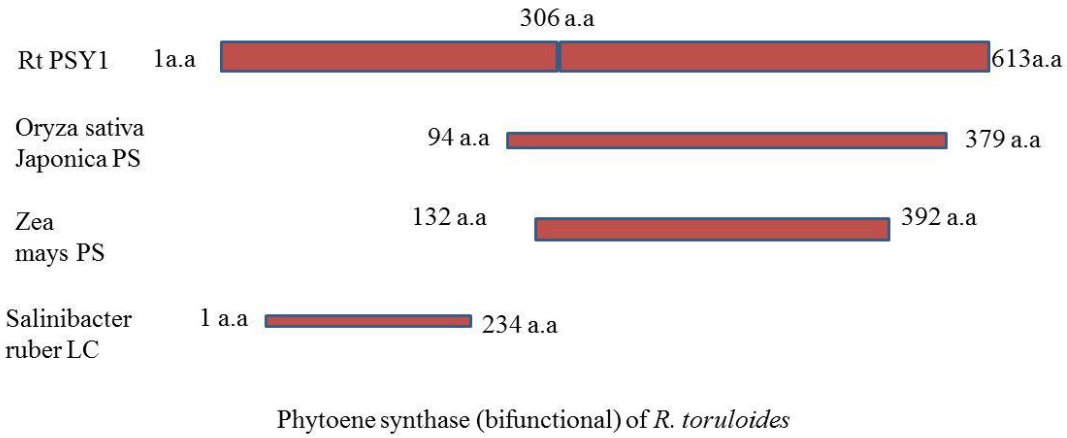


Figure 8: Identification of lycopene cyclase domain and phytoene synthase domain in bifunctional PS (*R. toruloides)*

R. toruloides phytoene synthase is bifunctional and 613 a.a long. It has two domains, lycopene cyclase (LC) domain and phytoene synthase (PS) domain.

From BLAST analysis, it is observed that lycopene cyclise of *Salini bacterruber* is aligned with the N-terminal of *R. toruloides* and phytoene synthase domains of *Oryza sativa Japonica* and *Zea mays* is aligned with C- terminal of *R. toruloides* [8] which suggests that lycopene cyclase and phytoene synthase of *R. toruloides* are present in N-terminal and C-terminal respectively.

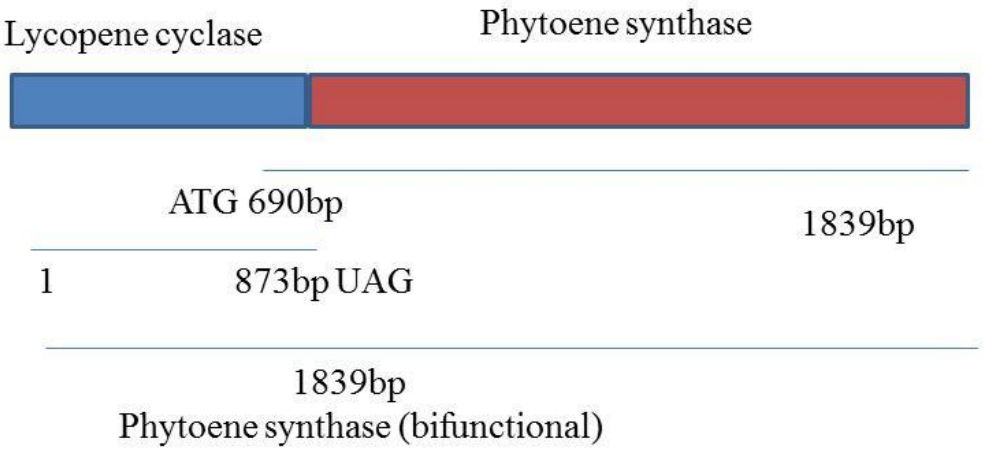


Fig. 9: Gene structure of bifunctional Phytoene synthase (Rt PSY1) of *R. toruloides*

The first domain i.e. lycopene cyclase catalyzes the conversion of lycopene to γ-Carotene and the second domain phytoene synthase catalyzes the conversion of GGPP to phytoene. Phytoene synthase domain of *R. toruloides* was PCR amplified using PS (*R. toruloides*) FP and PS (*R. toruloides*) RP and cloned under strong promoter p416TEF vector.

(ii) Expression of truncated phytoene synthase domain in *S. cerevisiae*

S. cerevisiae ABC276 strain is cotransformed with pRS315TEF-RtGGPPS, p416TEF-RtPS(t), pRS314TEF-RtCRTI and transformants were plated on SD-URA-TRP-LEU plates. But, their expression does not give any color as compared to control strain containing empty vectors p416TEF, pRS315TEF and pRS314TEF [10].

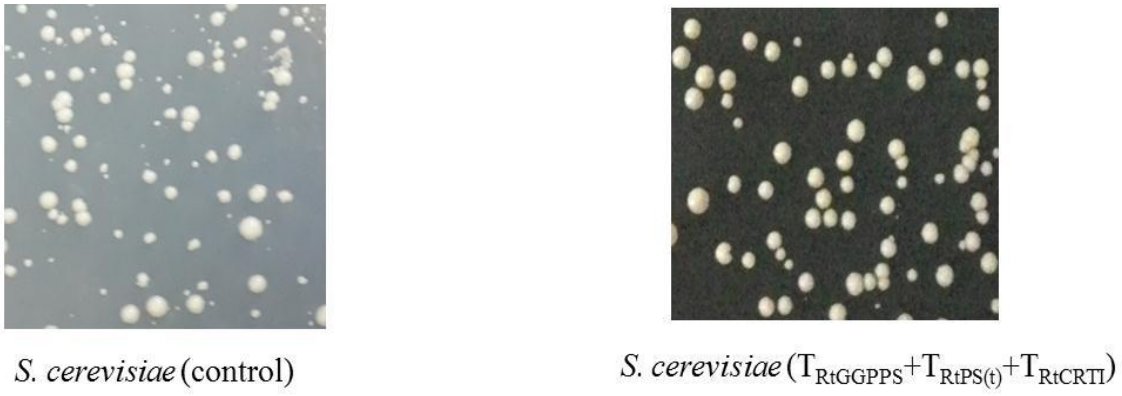


Fig. 10: Color of yeast transformants containing carotenogenic genes.

The possible reasons for absence of color in transformants are truncated phytoene synthase domain is not expressed in *S. cerevisiae* or truncated phytoene synthase is expressed but not functional. The other reason could be truncated phytoene synthase

domain cannot function without lycopene cyclase (LC) domain as it exists as bifunctional phytoene synthase. However, the exact reason is not known.

3.4(B) Expression of monofunctional Phytoene synthase (AtPS) from *Arabidopsis thaliana* in *S. cerevisiae*

Selection of monofunctional phytoene synthase from *A. thaliana* for lycopene biosynthesis:

Phytoene synthase is present in bacteria (*E. uredovora*, *E. herbicola*, etc), plant (*A. thaliana*, etc) and yeast (*Xanthophyllomyces dendrorhous*, *R. toruloides*, etc).For the biosynthesis of only lycopene, a monofunctional phytoene synthase could be an alternate enzyme. For the selection of monofunctional phytoene synthase, the organisms can be *E. herbicola* or *A. thaliana*. We have chosen *A. thaliana* because biosynthesis of lycopene by heterologous expression of *E. herbicola* monofunctional phytoene synthase in *R. toruloides* would need one more enzyme crtB [11,12]. In *A. thaliana*, only one gene Phytoene synthase is required for synthesis of lycopene [12].

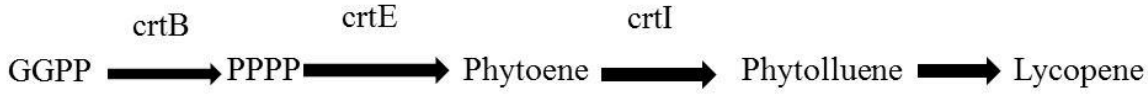


Fig. 11. Carotenoid pathway in *E. herbicola.*

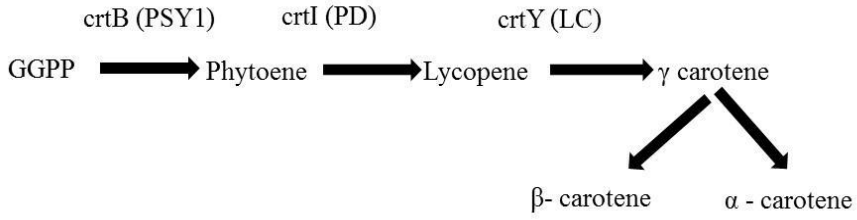


Fig. 12. Carotenoid pathway in *A. thaliana*

Multiple sequence alignment of *R. toruloides, A. thaliana* and *Pantoea* (*E. herbicola*) was done using ClustalW2 [13].

Monofunctional phytoene synthase of *A. thaliana* showed maximum similarity with the bifunctional phytoene synthase of *R. toruloides.* Hence, monofunctional phytoene synthase from *A. thaliana* has been chosen for lycopene biosynthesis in *R. toruloides.*

Using the ChloroP signal [13], we identified a chloroplast signal (120bp) in the N-terminal of the *A. thaliana* phytoene synthase.Then, monofunctional *A. thaliana* phytoene synthase (clone U16445) was PCR amplified using *A. thaliana* PS forward primer and PS reverse primer and cloned in vector p416TEF without chloroplast signal.

```
Arab_thal_PhySyn      ------------------------------------------------------------
Pant_anan_PhySyn      ------------------------------------------------------------
Rhod_toru_PhySyn      MGGLDYWLVHLRWTIPPALVLWSTFRKLRTRRDVYKTLFLVAIAVTATIPWDSYLIRHRI 60


Arab_thal_PhySyn      -------------------------PHYTOENESYNTHASEARABIDOPSISTHALIANA 35
Pant_anan_PhySyn      ------------------------------------------------------------
Rhod_toru_PhySyn      WSYPESSVVGPTLFAIPYEEIFFFFVQTYITATVYALFSRPVVHAVLLPRKPSDGRAARY 120


Arab_thal_PhySyn      MSSSVAVLWVATSSLNPDPMNNCGLVRVLESSRLFSPCQNQRLNKGKKKQIPTWSS---- 91
Pant_anan_PhySyn      ------------------------------------------------------------
Rhod_toru_PhySyn      IGTAAFLGIFALAWAKLEEGGEGTYLALIVGWVAPFLALLWFIASNHLLAMPRWAVGLPI 180


Arab_thal_PhySyn      ------------------SFVRNRSRRIGVVSSSLVASPSGEIALSSEEKVYNVVLKQAA 133
Pant_anan_PhySyn      ------------------------------------------------------MNNPS 5
Rhod_toru_PhySyn      LLPTLYLWECDARALQRGTWVIEKGTKLGLAFRGLEIEEAVFFLLTNVMIVFGLVACDYC 240
                                                                               : .

Arab_thal_PhySyn      LVNKQLRSSSY-------DLDVKKPQDVVLPGSLSLLVGEAYDRCGEVCAEYPKTFYLGT 186
Pant_anan_PhySyn      LLNHAVET-----------------------------------MAVGSKSFATAS 25
Rhod_toru_PhySyn      LAVHDLRSYDTRTSSVFPPLRDFLPILLNSPDAAQRQRIEDLQAAIDILSVHSKSFSTAS 300
                      *  : :.:                                          :  .*:*  .:

Arab_thal_PhySyn      LLMTPERRKAIWAIYVWCRRTDELVDGP--------NASHIT------------PMALDR 226
Pant_anan_PhySyn      KLFDAKTRRSVLMLYAWCRHCDDVIDDQTLGFQARQPALQTP------------EQRLMQ 73
Rhod_toru_PhySyn      QVFEGRLRLDLLSLYAWCRVCDDLIDNASTVAAAESNIDMISGCLDLLYPPSASTPTSLP 360
                       ::  . *  :  :*.***  *:::*.             .

Arab_thal_PhySyn      WEARLEDLFRG-RPFDMLDAALADTVARYPVDIQPFRDMIEGMRMDLKKSRYQNFDDLYL 285
Pant_anan_PhySyn      LEMKTRQAYAGSQMHEPAFAAFQEVAMAHDIAPAYAFDHLEGFAMDVREAQYSQLDDTLR 133
Rhod_toru_PhySyn      VRVSNKQIEAALPGLSEPERGAFHLLRLLPIARPPLDELLDGFRTDLSFLALSDSKGAKT 420
                       .   .:   .    .    .  .     :      : ::*:  *:     .: ..

Arab_thal_PhySyn      YCYYVAGTVG---LMSVPVMGIDPKSKATTESVYNAALALGIANQLTNILRDVGEDAR-- 340
Pant_anan_PhySyn      YCYHVAGVVG---LMMAQIMGVRDN------ATLDRACDLGLAFQLTNIARDIVDDAH-- 182
Rhod_toru_PhySyn      NGAANGNGNGKSSISAELPIKTDSDLLVYANNVASSVADLCVQLVWAHCTPYSRTPAQSV 480
                           ..  *   :     :    .       . . .  * :    ::        *:

Arab_thal_PhySyn      RGRVYLPQDELAQAGLSDEDIFAGKVTDKWRNFMKMQLKRARMFFDEAEKG--VTELSAA 398
Pant_anan_PhySyn      AGRCYLPASWLEHEGLNKENYAAPENRQALSRIARRLVQEAEPYYLSATAG--LAGLPLR 240
Rhod_toru_PhySyn      PRDPILSEAENAHVLAAAREMGQALQLVNIARDVPADLKIGRIYLPGRALDTPVPELTSD 540
                           *.     :     .:           .     :: .. :      .  :. *.

Arab_thal_PhySyn      SRWPVWASLLLYRRILDEIEANDYNNFT----KRAYVGKVKKIAALPLAYAKSVLKTSSS 454
Pant_anan_PhySyn      SAWAIATAKQVYRKIGVKVEQAGQQAWD----QRQSTTTPEKLTLLLAASGQALTSRMRA 296
Rhod_toru_PhySyn      RRALLARANEMAAHSKDAIEKLPQEARGGIRAACLVYLSIGDAVGKALDEGRVMERARVS 600
                          :  :  :  :    :*    :             .  . .      .: :     :

Arab_thal_PhySyn      RLSI--------- 458
Pant_anan_PhySyn      HPPRPAHLWQRPL 309
Rhod_toru_PhySyn      KGARARKAWQAL- 612
                      : .
```

Fig. 13: Multiple sequence alignment of phytoene synthase of *R. toruloides, A. thaliana and Pantoea ananatis.*

A) Control strain with empty vectors

B) $T_{RtGGPPS}+T_{PS(AT)}+T_{RtCRTI}$ PS (AT) from *A. thaliana*

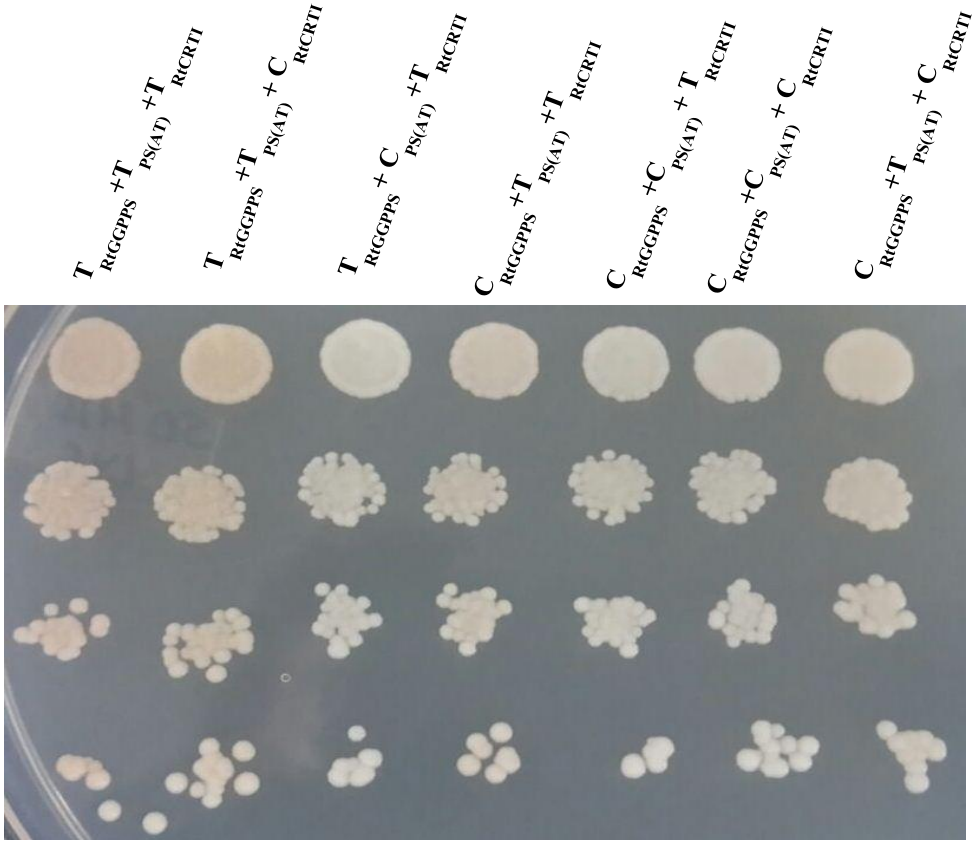
C) $T_{RtGGPPS}+T_{RtPSY1}+T_{RtCRTI}$ *(R. toruloides)*

Fig. 14. Color comparison of different *S. cerevisiae* strain combinations. A) Control strain *S. cerevisiae* with empty vector p416TEF B) Pink color colonies of *S. cerevisiae* containing RtGGPPS, RtCRTI and AtPS. C) Yellow color colonies of *S. cerevisiae* containing RtGGPPS, RtPSY1 and RtCRTI.

Expression of monofunctional phytoene synthase (At PS) from *A. thaliana* along with geranyl geranyl diphosphate synthase (RtGGPPS) and phytoene dehydrogenase (RtCrtI) in *S. cerevisiae* strain ABC276 gives light pinkish color suggesting the formation of lycopene [14]. But this has to be confirmed by HPLC.

3.5 Development of Lycopene based color/visual assay

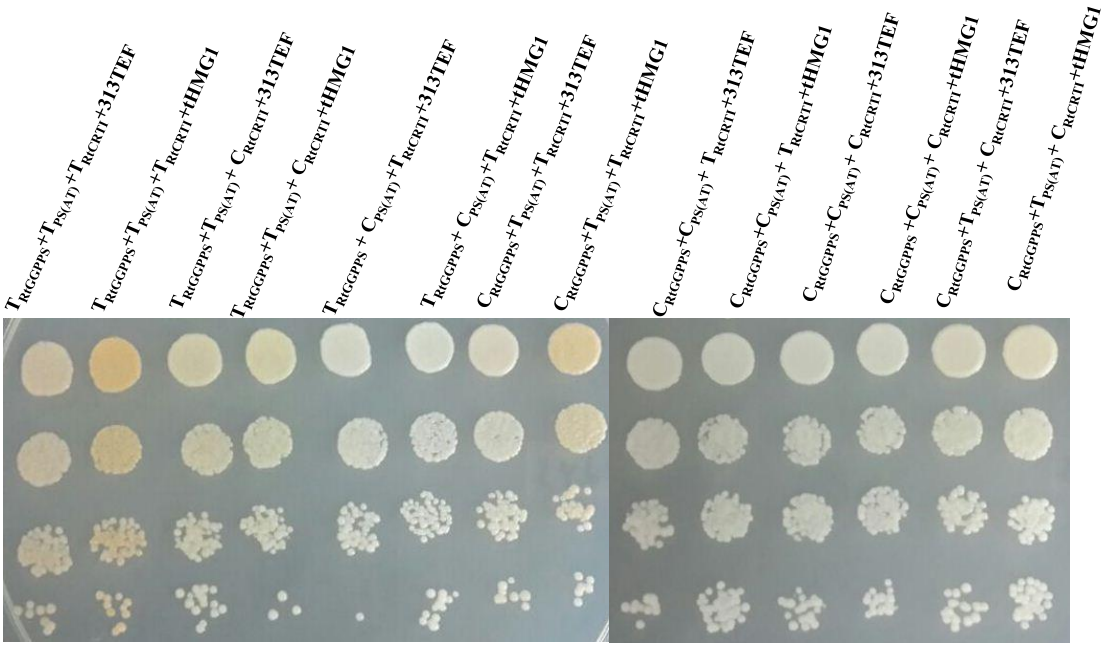
As pink color colonies suggested the formation of lycopene in yeast strain expressing carotenogenic genes, we aimed to develop the genetic screen using this pink color of the colonies. For this, carotenogenic genes RtGGPPS, AtPS, RtCRTI were cloned under strong (TEF) and weak (CYC) promoter and were transformed in *S. cerevisiae* ABC276 strain in different combinations and dilution spotted on SD-URA-TRP-LEU plates. Following strain combinations showed highest pigmentation: $T_{RtGGPPS}+T_{PS(AT)}+T_{RtCRTI}$, $T_{RtGGPPS}+T_{PS(AT)}+C_{RtCRTI}$, $C_{RtGGPPS}+T_{PS(AT)}+T_{RtCRTI}$ and $C_{RtGGPPS}+T_{PS(AT)}+C_{RtCRTI}$ [15]



SD-URA-TRP-LEU

Fig. 15. Dilution spotting to check phenotype (color) in *S. cerevisiae* strains expressing different combinations of carotenogenic genes under weak and strong promoters. T indicates the strong promoter TEF and C indicates the weak promoter CYC.

tHMG1, a regulatory enzyme whose over-expression is known to increase the flux in the isoprenoid pathway, is transformed in these different promoter combination strains and dilution spotted on SD-URA-HIS-TRP-LEU plates [16].



SD-URA-TRP-LEU-HIS

Fig. 16. Dilution spotting to check phenotype in above *S. cerevisiae* strains after over-expression of tHMG1.

Over-expression of tHMG1 showed increase in colour in two combination strains reflecting the increase in flux towards lycopene biosynthesis. The strains showing increased in colour were: T_{RtGGPS}+$T_{PS(AT)}$+$T_{RtCRTI}$and C_{RtGGPS}+$T_{PS(AT)}$+$T_{RtCRTI.}$ More intense color was observed in the strain T_{RtGGPS}+$T_{PS(AT)}$+T_{RtCRTI} .Hence, this strain T_{RtGGPS}+$T_{PS(AT)}$+T_{RtCRTI}has been chosen as the background strain for the color/visual based assay.

3.6 Validation of Lycopene based color screen with candidate genes

Mutagenesis of pathway specific candidate genes to increase flux in isoprenoid pathway:

We attempted to isolate mutants of transcription factors of isoprenoid pathway that increase flux in the pathway. Therefore, we have chosen three candidate genes- Ecm22, Sut1 and Taf25.Ecm22 is a sterol regulatory element binding protein which regulates

transcription of sterol biosynthetic genes. Sut1 is also a transcription factor which positively regulates genes involved in sterol uptake under anaerobic conditions [14, 15, 16]. Taf25 is a TATA global protein binding factor which affects a large number of genes in the cell. So here, the idea is to direct the flux from sterol biosynthesis towards isoprenoid biosynthesis.

Hydroxylamine mutagenesis of the candidate genes Sut1, Taf25, Ecm22 cloned under pRS313TEF (cloning done by Manisha) and tHMG1 was carried out. The mutated plasmids were transformed in *S. cerevisiae* background strain $T_{RtGGPPS}+T_{PS\ (AT)}+T_{RtCRTI}$ and mutants were selected on SD-URA-TRP-LEU-HIS plates [16].

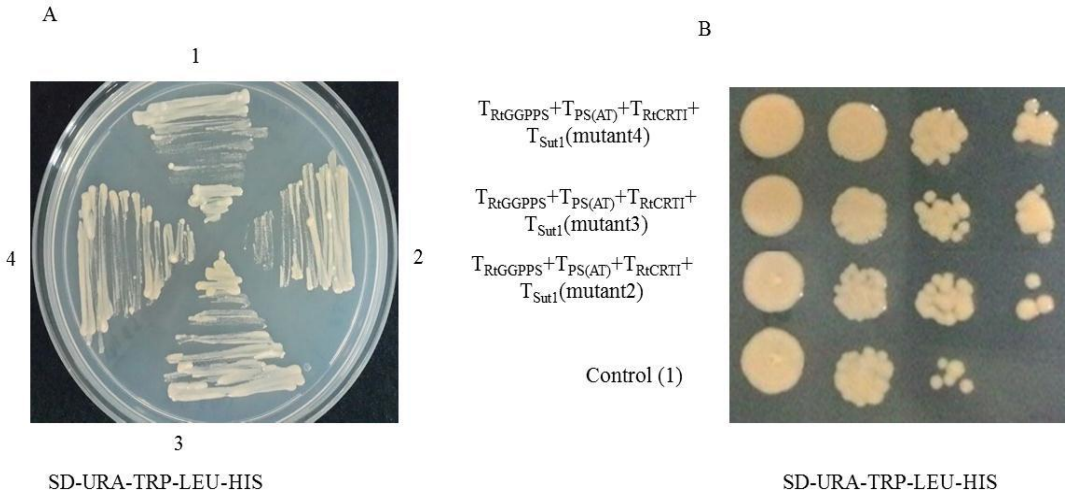


Fig. 17 A) Colony colours of the *S. cerevisiae* containing 1)$T_{RtGGPPS}+T_{PS(AT)}+T_{RtCRTI}+T_{Sut1}$(control)2,3,4) $T_{RtGGPPS}+T_{PS(AT)}+T_{RtCRTI}+T_{Sut1}$(mutated)

B) Dilution spotting to compare color of mutated Sut1 strains with the unmutated controlstrain.

Mutagenesis of Sut1: Transformants were screened for increase in color as compared to control strain [$T_{RtGGPPS}+T_{PS\ (AT)}+T_{RtCRTI}+T_{Sut1}$]. Three mutants colonies were selected and dilution spotted [17]. Mutant plasmids were isolated from these colonies. To check mutation, we have sent these plasmids for sequencing and results are awaited.

Mutagenesis of Taf25 and tHMG1: Transformants were screened for increase in color as compared to control strain [$T_{RtGGPPS}+T_{PS\ (AT)}+T_{RtCRTI}+T_{Sut1}$]. But no mutant colonies showed increase in color.

Mutagenesis of Ecm22: No growth on SD-URA-TRP-LEU-HIS plate. Mutagenesis is to be repeated.

CONCLUSION

In this study, we attempted to isolate mutants/genes which increase flux in the isoprenoid pathway by employing two approaches. In the first approach, we have identified SNPs in highest pigmented 322134S strain which are not found in any of the other *S. cerevisiae* strains and tried to find out their impact on the pigmentation levels. The pigmentation was due to expression of carotenogenic enzymes and difference in pigmentation is expected to be due to different fluxes. However, due to nonreproducable of earlier results (pigmentation levels failed to be similar in the strains) this work has not been proceeded further. Then in the second approach, we developed a lycopene based genetic screen in *S. cerevisiae* by heterologous expression of AtPS gene along with *R. toruloides* genes RtPSY1 and RtCRTI. We validated the lycopene based genetic screen using overexpression of tHMG1 (known flux increasing enzyme). Our genetic screen showed increased in pigmentation with over expression of tHMG1 suggesting an increase in colour which reflects increase in flux in the isoprenoid pathway. We also attempted to isolate mutants of transcription factors Sut1 and Ecm22of the pathway which could increase the flux using this screen. However, no mutants could be isolated. Thus, in conclusion, we have been able to develop a lycopene screen for isolating new genes/mutants affecting flux in the isoprenoid pathway and it is hoped that this would prove a useful tool in the field of research.

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