Evaluation of the promoters of the mevalonate biosynthetic pathway genes of the red yeast *Rhodosporidium toruloides*

A dissertation submitted for the partial fulfilment of

BS-MS dual degree in Science

By

Subhasis Behera
MS16141

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Certificate of Examination

This is to certify that dissertation titled **"Evaluation of the promoters of the mevalonate biosynthetic pathway genes of the red yeast *Rhodosporidium toruloides"*** submitted by Mr. Subhasis Behera (MS16141) 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: May 9, 2021

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Prof. Anand Kumar 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.

Subhasis Behera

Date: May 9, 2021

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 Kumar Bachhawat

(Supervisor)

Acknowledgement

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ABSTRACT

The mevalonate biosynthetic pathway (MVA) or the Isoprenoid pathway is essential in mammals, plants, and yeast to synthesise sterols and isoprenoids. There are also many valuable terpenoids that are produced from these pathways in different plant organisms. *Rhodosporidium toruloides* is emerging as a potential host organism for heterologously producing these terpenoids and isoprenoids. However, few tools are available to modify this yeast genetically. Also, there is limited knowledge regarding promoters and their regulation in *R. toruloides* in the MVA pathway. The goal was to evaluate *R. toruloides* promoters in *R. toruloides* and *S. cerevisiae* and to find any possible bifunctional promoter that may work in both yeasts. As part of this project, *in silico* work was followed by experiments towards this goal. *In silico* work was carried out to compare the promoters by phylogenetic footprinting to identify conserved motifs. Several promoters of the mevalonate pathway of *R. toruloides* were cloned, and evaluated in *S. cerevisiae,* followed by a directed evolution approach to make functional Rt HMG1 promoter in *S. cerevisiae.* We also evaluated selected promoters for their function in the native host, *R. toruloides*. Promoter deletions were also created of the RtHMG1 promoter and preliminary evaluation of a few of them was carried out. The GFP reporter was used to evaluate promoter strength of MVA pathway genes. The modified *R. toruloides* vector which was also developed in this project, would be helpful as a genetic engineering tool in *R. toruloides* based research.

CHAPTER 1

INTRODUCTION

1 Introduction

1.1 Mevalonate Biosynthetic Pathway of *Saccharomyces cerevisiae*

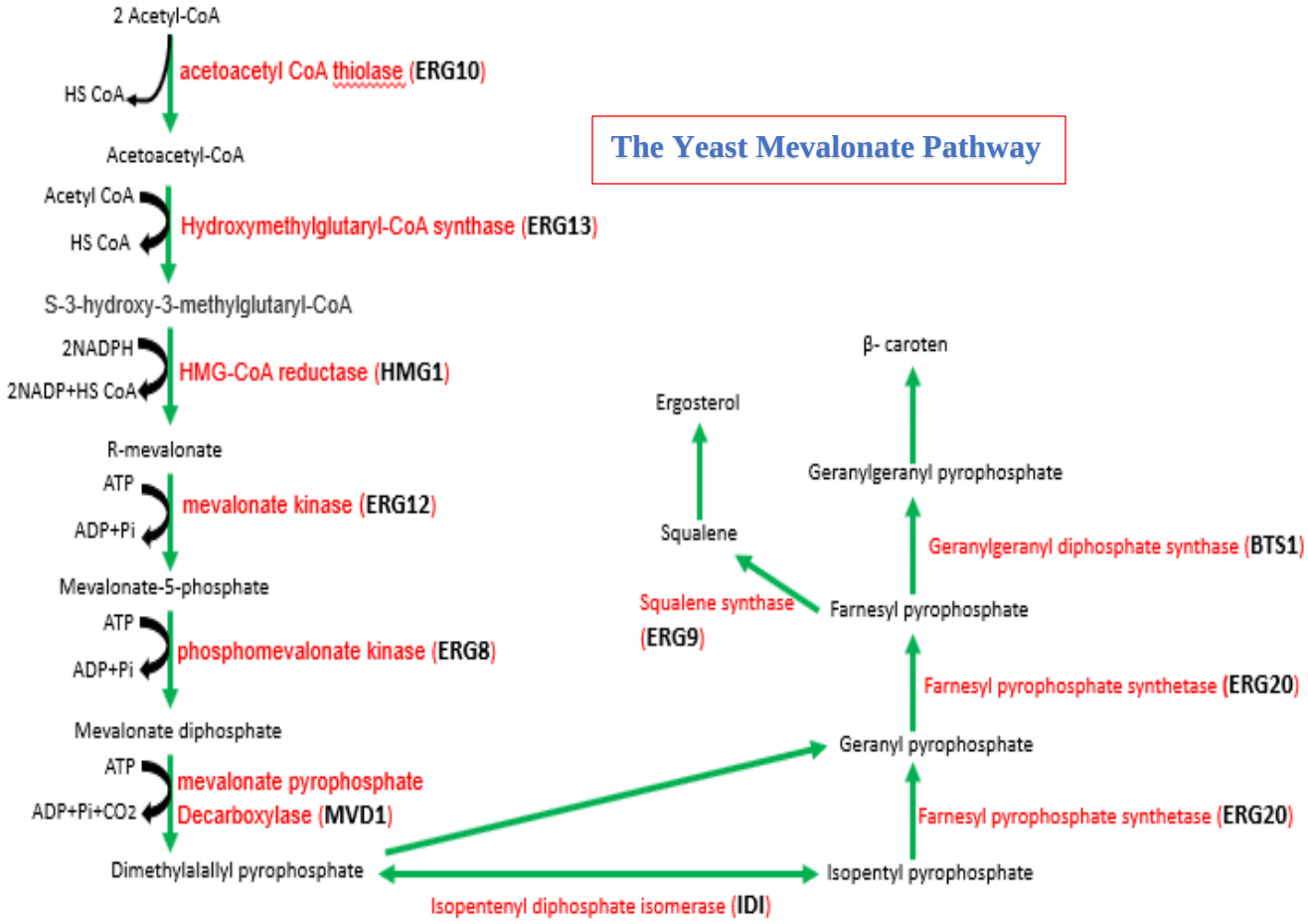


Figure 1: Schematic representation of yeast MVA pathway in *S. cerevisiae*

The upper mevalonate pathway (first three steps) produces R-mevalonate, whereas the lower mevalonate pathway produces five carbon Dimethylalallyl pyrophosphate (DMAPP) and Isopentenyl pyrophosphate (IPP) which are the precursors of isoprenoid. In the first step of the upper mevalonate pathway, two acetyl-CoA molecules condense to form acetoacetyl-CoA with the help of acetoacetyl CoA thiolase. In the second step, acetoacetyl-CoA condenses with one acetyl-CoA using hydroxymethylglutaryl-CoA synthase enzyme

to create S-3-hydroxy-3-methylglutaryl-CoA. HMG CoA is then converted into R-mevalonate in two NADPH reduction dependent step with the help of the enzyme HMG-CoA reductase. In the lower mevalonate pathway, R-mevalonate is phosphorylated into mevalonate-5-phosphate in ATP dependent manner by the mevalonate kinase enzyme. Phosphomevalonate kinase enzyme converts mevalonate-5-phosphate to mevalonate diphosphate in ATP driven reaction. Then mevalonate diphosphate is decarboxylated to dimethylalallyl pyrophosphate by mevalonate pyrophosphate decarboxylase enzyme that is also dependent on ATP. It is followed by an isomerization reaction, which leads to the formation of isopentyl pyrophosphate by isopentenyl diphosphate isomerase enzyme. Sequentially, isopentyl pyrophosphate is converted to geranyl pyrophosphate and farnesyl pyrophosphate by farnesyl pyrophosphate synthetase enzyme. Then geranylgeranyl diphosphate synthase enzyme converts farnesyl pyrophosphate to geranylgeranyl pyrophosphate. Farnesyl pyrophosphate can also branch out to be converted to squalene by squalene synthase eventually leading to ergosterol (Fig. 1).

1.2 Regulation of Mevalonate Pathway Genes of *S. cerevisiae*

Acetoacetyl CoA thiolase (encoded by ERG10) catalyzes 2 acetyl-CoA to acetoacetyl-CoA. When flux in mevalonate is disrupted, the sterol level decreases. In response to this, ERG10 mRNA is induced. ERG10 is regulated transcriptionally in response to sterol level (Dimster-Denk & Rine, 1996).

HMG CoA reductase catalyzes S-3-hydroxy-3-methylglutaryl-CoA to R-mevalonate. Yeast have two HMG CoA reductase enzyme encoded from HMG1 and HMG2 genes (Basson et al., 1986). HMG1 encoded enzyme is responsible for 83% activity in cell during aerobic condition, whereas HMG2 encoded enzyme is functional in anaerobic or stationary growth condition. In *Schizosaccharomyces pombe,* sterol regulatory element of genes respond to low oxygen (Hughes et al., 2005). Mainly, HMG1p is stable but HMG2p is rapidly degraded which is depend on signals from mevalonate. Mainly, HMG1p is responsible for regulation of HMG1 gene.

Squalene synthase catalyzes farnesyl pyrophosphate to squalene. Squalene synthase (ERG9) is activated by transcription factor Hap1/2/3/4p (heme activator protein), yAP-1

(yeast activator protein) and repressed by Ino2/4p (phospholipid transcription factor complex) (Kennedy et al., 1999).

1.3 *Rhodosporidium toruloides*: A Carotenoid Producing Yeast

R. toruloides is a red, basidiomycete yeast that is widely used as a host in synthetic biology (Tang et al., 2020). Researchers have done genome sequencing of various *Rhodosporidium* species. This organism has a high GC content of 60%, unlike the low GC content of *S. cerevisiae*. It has a high codon bias, so cloning is difficult. The transformation of this organism is possible by *Agrobacterium tumefaciens* mediated transformation method, but this is time-consuming as it takes two weeks. It intracellularly accumulates lipid up to 70% of its dry mass. Lipid can be used as a source of fuel in the place of petroleum. *R. toruloides* has been used as a host for lipids, isoprenoids and carotenoids production. Carotenoids are the raw material for the cosmetic, food, feed and health industries. Some of the notable features *of R. toruloides* are the ability to reach high cell densities and utilization of carbon and nitrogen from a variety of sources. It can make high-concentration of expensive products from low-value substrates. It has the ability to withstand a lot of stress and can grow at a variety of temperatures and pH levels (Park et al., 2018).

1.4 Need for Novel Promoters of *R. toruloides*

Promoters are the nucleotide sequence present upstream of the gene that helps in the regulation of gene expression. It can be constitutive or inducible. The promoters of GPD1, TPI1, PGK1, FBA1, PGI1 genes are constitutive; ANT is monodirectional, whereas H3-H4 is a bidirectional promoter. The VDAC2 promoter gives late-stage expression (Nora et al., 2019). There are few well-characterized inducible promoters of *Rhodosporidium* available in the database. For metabolic engineering purpose we needed independent, modular and tunable promoters. Some of the inducible promoters in *Rhodosporidium* are NAR1, CTR3, MET16 and ICL1 (Johns et al., 2016). Previously the strength of different promoters was evaluated in *R. toruloides*, and the strength was found to be PGI > PGK > FBA > TPI > GPD. The modified promoter of the D-amino acid oxidase gene (DAO1), $P_{DAO1\text{-}in1m1}$ was found to be induced by D-Alanine (Liu et al., 2015). The promoter of Lipid

droplet protein (LDP1) has high strength than RtGPD. Introns had an enhancing effect on ACC1, LDP1 and a repressing effect on ACL1, FAS1, DUR1 promoters (Liu et al., 2016).

1.5 Objective of the study

The project's overall goal is to evaluate mevalonate pathway promoters of *R. toruloides* and their expression levels and to understand their regulation. The project's second goal is to identify a bifunctional promoter that functions in *S. cerevisiae* and *R. toruloides*. Explicitly, the following are the objective of the study.

1. To carry out *in silico* analysis of promoters of the MVA pathway genes of *R. toruloides* and identification of possible conserved motifs.
2. To clone selected promoters of *R. toruloides* essential MVA pathway genes and evaluate the activity in *R. toruloides* and *S. cerevisiae*.
3. To create an *R. toruloides* promoter that functions in both *R. toruloides* and *S. cerevisiae*.

CHAPTER 2

MATERIALS AND METHODS

SECTION A: MATERIALS

2.1 CHEMICALS AND REAGENTS

All the chemicals and reagents used were obtained from commercial sources and were of analytical grade. Media components, fine chemicals, reagents were purchased from Sigma Aldrich (St. Louis, USA), HiMedia (Mumbai, India), Merck. India Ltd (Mumbai, India), USB Corporation (Ohio, USA) or Difco, USA. Enzymes (restriction enzymes, T4 DNA ligase, Vent DNA polymerase, Phusion DNA polymerase) and dNTPs were purchased from New England Biolabs Inc (Beverly, MA, USA). Primers were designed using SnapGene® software and were purchased from Sigma Aldrich (Bangalore, India). Gel-extraction kits and plasmid miniprep columns were obtained from Bioneer, Promega, Thermo Fisher Scientific or QIAGEN. Plastic and glasswares were purchased from Thermo Scientific®, Falcon®, Tarsons® and Thermo Fisher Scientific®.

2.2 STRAINS AND PLASMIDS

The *E. coli* strain DH5α was used as the cloning host in this study. The genotype for the E. coli strain and the yeast strains used in this study are given in Table 1. The list of various plasmids used in this study is given in Table 2.

Table 1: List of bacteria and yeast strains

Strain	Genotype	Source
ABE 460 (DH5α)	F' *gyr A96(Nal) recA1 relA1 endA1 thi-1 hsdR17 (rk-mk+) gln V44 deoR Δ(lacZYA-argF) U169 [ϕ80dΔ(lacZ) M15]*	Lab Strain
ABC 733 (BY4741)	*S.cerevisiae MATa, his3Δ1, leu2Δ0, met15Δ, ura3Δ0*	Lab Strain
AB 5345 (AGL1)	*A.tumefaciens EHA101 recA::bla pTiBo542ΔT Mop+ Cb^R*	Temasek Limited, Singapore

AB 5353 / AB5354	*R. toruloides ku70Δe (NCBI accession number – KF850470*	Temasek Limited, Singapore
AB 2867	*R. toruloides* Banno. MTCC No.-9565,ATCC-10657	Lab Strain

TABLE 2: List of Plasmids used

CLONE NO.	PLASMID NAME	DESCRIPTION
ABE 5684	p416TEF-ScGFP	*S. cerevisiae* vector cloned from 426.GFP in BamHI and EcoRI sites in 416 TEF(416 TEF Sc GFP)
ABE 5814	pRSII316	The CEN-vector bearing URA3 marker and Amp^r marker for selection in *E. coli*
ABE 5575	pRH2031	Rt vector bearing spectinomycin marker for selection in *E. Coli*

TABLE 3: List of Plasmids constructed

CLONE NO.	PLASMID NAME	DESCRIPTION
ABE 6083	pRSII316 ScGFP	Sc GFP cloned from p416 TEF-ScGFP between KpnI and BamHI sites in pRSII316 vector.
ABE 6205	pRH2031_mod	RtGFP cloned between BamHI and EcoRV sites in pRH2031 vector.
ABE 6206	pRSII316 HMG1 ScGFP	Rt HMG1 promoter cloned between SpeI and BamHI site upstream of Sc GFP in pRSII316 ScGFP vector.

ABE 6207	pRSII316 ERG13 ScGFP	Rt ERG13 promoter cloned between SpeI and BamHI site upstream of Sc GFP in pRSII316 ScGFP vector.
ABE 6208	pRSII316 ERG8 ScGFP	Rt ERG8 promoter cloned between SpeI and BamHI site upstream of Sc GFP in pRSII316 ScGFP vector
ABE 6209	pRSII316 MVD1 ScGFP	Rt MVD1 promoter cloned between SpeI and BamHI site upstream of Sc GFP in pRSII316 ScGFP vector.
ABE 6210	pRSII316 BTS1 ScGFP	Rt BTS1 promoter cloned between SpeI and BamHI site upstream of Sc GFP in pRSII316 ScGFP vector.
ABE 6211	pRH2031_mod HMG 1300	1300 bp length of Rt HMG1 promoter cloned in SpeI and BamHI sites upstream of RtGFP in pRH2031_mod vector.
ABE 6212	pRH2031_mod BTS1	1100 bp length of Rt BTS1 promoter cloned in SpeI and BamHI sites upstream of RtGFP in pRH2031_mod vector.
ABE 6213	pRH2031_mod ERG13	1500 bp length of Rt ERG13 promoter cloned in SpeI and BamHI sites upstream of RtGFP in pRH2031_mod vector.
ABE 6214	pRH2031_mod ERG8	1500 bp length of Rt ERG8 promoter cloned in SpeI and BamHI sites upstream of RtGFP in pRH2031_mod vector.
ABE 6215	pRH2031_mod ERG20	1500 bp length of Rt ERG20 promoter cloned in SpeI and BamHI sites upstream of RtGFP in pRH2031_mod vector.

ABE 6216	pRH2031_mod MVD1	1500 bp length of Rt MVD1 promoter cloned in SpeI and BamHI sites upstream of RtGFP in pRH2031_mod vector.
ABE 6217	pRH2031_mod HMG1 600	600 bp length of Rt HMG1 promoter cloned in SpeI and BamHI sites upstream of RtGFP in pRH2031_mod vector.
ABE 6218	pRH 2031_mod HMG1 400	400 bp length of Rt HMG1 promoter cloned in SpeI and BamHI sites upstream of RtGFP in pRH2031_mod vector.
AB 6219	AGL1 HMG1	pRH2031_mod HMG1 1300 was transformed to AGL1 by ATMT method.
AB 6220	AGL1 HMG1 600	pRH2031_mod HMG1 600 was transformed to AGL1 by ATMT method.
AB 6221	AGL1 HMG1 400	pRH2031_mod HMG1 400 was transformed to AGL1 by ATMT method.
AB 6222	AGL1 BTS1	pRH2031_mod BTS1 was transformed to AGL1 by ATMT method.
AB 6223	AGL1 pRH2031_mod	pRH2031_mod was transformed to AGL1 by ATMT method.

2.3 OLIGONUCLEOTIDES

The list of various oligonucleotide primers used in this study is given in Table 3.

TABLE 4: List of Oligonucleotides sequences used

N_HMG FP SpeI	ATAACTAGTGTTTGAGCCCGCTTGGTCG
N_HMG RP BamHI	GGATCCCGCTGCGCTTAGTGTGCG
N_BTS1 FP SpeI	ATAACTAGTCGTCTCTCGCCCACCAGATC

N_BTS1 RP BamHI	GGATCCCCTCAGACAAGCAGCAGCGAG
N_ERG13 FP SpeI	ATAACTAGTCGACGGAACGCTCATCTGCG
N_ERG13 RP BamHI	GGATCCTTCGTACGAGGTGAGGGAGG
ERG8 FP SpeI	ATAACTAGTGGACGTACCGGCGGAAAGAGC
ERG8 RP BamHI	GGATCCTGGCGGCGAAGCAGGTCGG
ERG20 FP SpeI	ATAACTAGTCATTCAGGCAGTCAGCAGGTTC
ERG20 RP BamHI	GGATCCGTCGAGGTGGAGCTGCTGG
MVD1 FP SpeI	ATAACTAGTGAGGGCGAGGCGGCGGAGGTAC
MVD1 RP BamHI	GGATCCCGTTCGAGGGGAAGGAGGGAGC
Rt FP pHMG -200 SpeI	ATAACTAGTGCGAAAGCAGGGCAGAGCACG
Rt FP pHMG -400 SpeI	ATAACTAGTCCGAGTCGTGGTGACGAGGAGG
Rt FP pHMG -600 SpeI	ATAACTAGTGTATCCGCCGCGCGGTTGAATTGACC
RTGFP RP EcoRV	GATATCTACTTGTAGAGCTCGTCCATGCCGAGG
RTGFP FP BamHI	ACTTCGGATCCATGAAGGGCGAGGAGCTCTTCACC

2.4 MEDIA

For the preparation of media, buffers and stock solutions used for this study, Millipore elix five deionized water was used, unless otherwise mentioned. They were sterilized, as recommended, either by autoclaving at 15 lb/inch2 (psi) pressures at 121ºC for 15 min or by using membrane filters (Advanced Microdevices Pvt. Ltd., India) of pore size 0.2-0.45 μm (for heat-labile compounds). Additional amino acids and nutrients were prepared as sterile stock and added as per requirements.

MEDIA	COMPONENTS	g/l
2.4.1 LB	Yeast extract	5 g/l
	Tryptone	10 g/l

(LURIA–BERTANI) MEDIUM	NaCl	10 g/l
	pH of the above medium was adjusted to 7.0 with 1N NaOH	
2.4.2 YPD (YEAST EXTRACT PEPTONE DEXTROSE) MEDIUM	Yeast extract	10 g/l
	Peptone	20 g/l
	Dextrose	20 g/l
2.4.3 SD (SYNTHETIC DEFINED) MEDIUM	YNB (Yeast Nitrogen Base)	1.7 g/l
	(without amino acids and ammonium sulphate)	
	$(NH4)_2SO_4$	5 g/l
	Dextrose	20 g/l
	Amino acids (as per requirement)	80 mg/l
	pH was adjusted to 6.0–6.5.	
2.4.4 2YT	Trypton	16 g/l
	Yeast Extract	10 g/l
	NaCl	5 g/l

2.5 BUFFER AND STOCK SOLUTION

2.5.1 Ampicillin Stock Solution (100 mg/ml)

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

2.5.2 Spectinomycin Stock Solution (50 mg/ml)

The required amount of spectinomycin was dissolved in the required volume of deionized water, and was filter-sterilized using a 0.2 μm filter membrane. It was stored at -20 °C.

2.5.3 Ceftoxime Stock Solution (100 mg/ml)

The required amount of ceftoxime was dissolved in the required volume of deionized water, and was filter-sterilized using a 0.2 µm filter membrane. It was stored at -20 °C.

2.5.4 Rifampicin Stock Solution (25 mg/ml)

The required amount of rifampicin was dissolved in the required volume of deionized water, and was filter-sterilized using a 0.2 µm filter membrane. It was stored at -20 °C.

2.5.5 Hygromycin Stock Solution (50 mg/ml)

The required amount of hygromycin was dissolved in the required volume of deionized water, and was filter-sterilized using a 0.2 µm filter membrane. It was stored at -20 °C.

2.5.6 50% Glycerol

The same volume of 100% glycerol and deionized water were mixed and was autoclaved. The solution was stored at room temperature.

2.5.7 Solution required for plasmid isolation

1) SOLUTION-I (RESUSPENSION BUFFER)	50 mM Glucose 25 mM Tris-HCl (pH 8.0) 10 mM EDTA (pH 8.0) Autoclaved and stored at 4ºC.
2) SOLUTION II (LYSIS BUFFER)	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 BUFFER)	5 M Potassium acetate 60 ml Glacial acetic acid 11.5 ml Deionized water 28.5 ml

	The resulting solution is 3M with respect to potassium and 5M with respect to acetate and was stored at 4ºC.
4) TRIS-EDTA (TE BUFFER)	10 mM Tris-HCl (pH 8.0) 1 mM EDTA (pH 8.0).
5) TE-RNASE (stock prepared at 10 mg/ml)	Working stock 20 µg/mL in TE Buffer (pH 8.0)

2.5.8 Agarose Gel Electrophoresis Reagents

1) 1X TAE (TRIS-ACETATEEDTA) BUFFER (PER 1000 ML) (PREPARED FROM 50X TAE STOCK	Stock: 40 mM Tris-acetate 1mM EDTA (pH 8.0) Autoclaved and stored at room temperature.
2) ORANGE-G DYE (GEL LOADING DYE, 6X)	0.25% orange-G and 30% glycerol
4) ETHIDIUM BROMIDE (10 mg/ml) STOCK	Final working concentration used at 0.5 µg/ml.
5) 0.7-1% AGAROSE GEL IN 1X TAE	

2.5.9 Solution for preparation of chemical competent *E. coli* cells

1) 0.1 M $CaCl_2$ PREPARED IN 15% GLYCEROL	$CaCl_2.2H_2O$ 2.94 g Glycerol 28 ml Deionized water 172 ml Autoclaved and stored at 4ºC

2) SOB	Bactotryptone 20 g Bacto yeast extract 5 g NaCl 0.5 g Above mentioned components were dissolved in 950 ml of water. 10 ml of 250 mM KCl was added and pH was 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.
3) SOC	SOB + 20 mM Glucose

2.5.10 Solution for preparation of competent *A. tumefaciens* cells

1) 20 mM $CaCl_2$ PREPARED IN 15% GLYCEROL	$CaCl_2.2H_2O$ 0.588 g Glycerol 30 ml Deionized water 170 ml Autoclaved and stored at 4ºC

2.5.11 Solution for genomic DNA isolation from yeast

10 mM Tris-HCl (pH 8.0)

1 mM EDTA (pH 8.0)

100 mM NaCl

1% SDS

2% Triton X-100

2.5.12 Solution for yeast transformation (*S. cerevisiae*)

1) 0.1 M Lithium acetate in TE (pH 7.5)

2) 50% PEG-3350 in 0.1 M Lithium acetate in TE (pH 7.5).

2.5.13 Solution for transformation of *R. toruloides*

- K-salts: 20.5% K_2HPO_4, 14.5% KH_2PO_4 (autoclaved)
- M-salts: 3% $MgSO_4*7H_2O$, 1.5% NaCl, 2.5% $(NH_4)_2SO_4$ (autoclaved)
- NH_4NO_3: 20% (autoclaved)
- $CaCl_2$: 1% (autoclaved)
- Z-Salts: 0.01% each of $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$, H_3BO_3, $MnSO_4.H_2O$ and $NaMbO_4.2H_2O$) (filtered through 0.2-micron membrane into an autoclaved bottle.)
- FeSO4: 0.01% (filtered through 0.2-micron membrane into an autoclaved bottle.)
- Glucose: 20% (1M) (autoclaved)
- Glycerol: 50% (autoclaved)

MinAB broth (for each litre)

- K-salts 10 ml
- M-salts 20 ml
- NH_4NO_3 (20%) 2.5 ml
- $CaCl_2$ (1%) 1 ml
- Z-Salts 5 ml
- $FeSO_4$ (0.01%) 10 ml

This buffer has pH 6.7. It should be filter-sterilized. Just before use, add 1 ml glucose (20%) to each 100 ml MinAB broth prepared above.

IM Broth (IMB)

- MinAB broth 500 ml
- 1 M MES (non-adjusted) (final 40 mM) 20 ml
- 50% glycerol (final 0.5% (W/V) glycerol) 5 ml Adjust pH to 5.5-5.7 with HCl
- Aliquot into 100 ml and autoclaved.

Just before use add 0.2 ml 0.1M AS (acetosyringon or 3'5'-dimethoxy-4'-hydroxyl-acetophenone) (final 200 μM) to 100 ml IMB.

IM Agar (IMA)

- IMB 200ml

- Bacto agar (2%) 4g

Autoclaved. Shake well when it remains hot after the autoclaving. Use directly or store at RT as stock. Add acetosyringone (AS) to 200μM and glucose to 5 mM making plates.

2.5.14 Solution for Hydroxylamine mutagenesis

- NaOH 90mg
- 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

2.6 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 28-30°C. The yeast transformants were selected and maintained on SD medium with supplement as per requirements.

2.7 Recombinant DNA methodology (Restriction digestion, ligation, transformation of *E. coli*, PCR amplification etc.)

All the molecular techniques used in the study for manipulation of DNA, bacteria, and yeast were according to standard protocols (Guthrie and Fink, 1991; Sambrook and Russell, 2001) or as per manufacturers' protocol unless specifically mentioned.

2.8 Genomic DNA isolation from yeast

Genomic DNA from *S. cerevisiae* strains was isolated as described previously (C, 1994) using the glass bead lysis method and the STES lysis buffer.

2.9 Transformation to *A. tumefaciens*

An aliquot of A. tumefaciens competent cell was taken. The transformation was carried out by adding 5 mg of plasmid DNA to competent cell and tap gently. Keep it back on ice. Almost

immediately, a heat shock was given at 37°C for 5 min. Then keep the eppendrof back in ice. Within 5 min, 1ml 2YT liquid medium and keep it at 28°C for 6 hours. After this centrifuge at 6000 rpm for 5 min, discard supernatant and spread 100 ml of resuspended above pellet with rifampicin selection plate. Incubate at 28°C to get colonies.

2.10 Transformation of yeast (*S. cerevisiae*)

The transformation of S. cerevisiae strains was carried out by the lithium acetate method. S. cerevisiae cultures were grown in YPD at 30°C with shaking for 16-24 hours and then reinoculated in fresh YPD to an initial OD_{600} of 0.2; cells were allowed to grow at 30°C for 4-5 hours 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. 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 1×10^9 cells/ml and divided into 100 µl aliquots. Approximately 50 µg (5µl of 10mg/ml stock solution) of heat-denatured, salmon sperm carrier DNA, followed by 0.3 µg- 0.7 µg of plasmid/DNA fragment were added to each aliquot, and the 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 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 the appropriate volume of cell suspension was plated on selection plates.

2.11 Transformation of yeast (*R. toruloides*)

The protocol was adopted from previous reports (Liu et al., 2013)

1. Inoculate 10 mL of YPD with *R. toruloides* (from the fresh plate), and 10 mL of MinAB supplemented with spectinomycin (50 mg/mL) with transformed *A. tumefaciens* AGL1 cells. (Note that both cells should be freshly prepared, not more than one week old)

2. Incubate both the culture at 28°C with shaking 280 rpm overnight (16-18 hours).

3. After overnight culture, inoculate the secondary culture of *R. toruloides* in 10 mL YPD and secondary culture of *A. tumefaciens* AGL1 in 10 mL IMB (pH- 5.5- 5.7) with 200 µM acetosyringone at 0.12 OD_{600}.

4. Incubate both the cultures at 28°C with shaking 280 rpm for 6 hours.

5. Take the IMA agar plate (with acetosyringone- 200µM), air dry for 30 min in a laminar flow and then cover the lids.

6. Place a sheet of Hybond membrane (catalogue no. RPN82B) onto the surface of IMA plates using a pair of forceps (sterilized by heating). Avoid trapping air bubbles by putting the membrane disk on one side of the plate and slowly lowering the membrane over the surface.

7. After 6 hours of growth, the yeast cell culture reaches an OD_{600} 0.6-0.7, and Agrobacterium reaches OD_{600} 0.3-0.4. Mix 100 µL yeast cells with 100 µL Agrobacterium cells in an Eppendorf tube.

8. Take 200µL cell mixtures prepared above and spot evenly into about ten spots over the surface of the membrane. Spread gently by rotating (while floating off the membrane) the L-spreader 1-2 circles. (Do not spread in more than two circles, which result in uneven spreading).

9. Air-dry the IMA plate for 30 min and then seal with parafilm.

10. Incubate at 24°C for 2-3 days (top-side-up). A good layer of cells is needed for efficient transformation. If not, lengthen the co-culture time.

11. Prepare the YPD plates with the appropriate antibiotic concentration (Hygromycin 150µg/ml and 300µg/ml Ceftoaxime). Dry for 30 min or more in a laminar flow.

12. Transfer the nylon membrane from the co-culture plates to YPD with antibiotics (Cef+, Hyg+). Air dry for 30 min-60 min.

13. Incubate 28-30°C for 4-5 days (upside-up). (colonies start appearing from the 3rd day).

2.12 Measurement of GFP

The yeast cells were harvested at 4°C, followed by washing with cold water and resuspended in water to an OD600 of 1.0. GFP fluorescence was measured in a spectrophotometer at Excitation of 488nm and Emission of 510 nm.

2.13 Hydroxylamine mutagenesis of plasmid

First of all, put on double gloves. Nearly 10 μg plasmid DNA was dissolved in 0.5 ml of Hydroxylamine solution (90 mg NaOH, 350 mg hydroxylamine HCl in 5 ml water (ice cold), pH around 6.5 freshly made up before use). It was incubated at 37°C for 20 hrs. The mutagenized plasmid DNA was purified using Qiagen column. Finally, the pool of mutagenized plasmid was directly transformed into the appropriate yeast strain.

2.14 Bioinformatics Analysis

1) Go to NCBI, search gene name and organism name. Click on gene name and blast mRNA sequence.

2) In blast tool choose “ref seq genome database (Ref Seq genome)”. Enter organism name *Rhodosporidium toruloides*. Run the blast.

3) Click on the first accession number under sequencing producing a significant alignment tab, where the E value is less. Search the gene name and find the location of the gene.

4) Subtract 1500 from where the gene starts, select region for the promoter and update the view. Get the promoter sequence. If the gene is located in the reverse strand, add 1500 where the gene starts. Get the promoter sequence and reverse complement it in the snap gene.

Similarly, the promoter sequence of different Rhodosporidium species was retrieved. Multiple sequence alignment was carried out by ClastalW.

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1 Phylogenetic footprinting of promoters of mevalonate pathway genes of Rt

Mevalonate pathway is an essential pathway involved in isoprenoid biosynthesis and is present in eukaryotes, archaea and some bacteria. *R. toruloides*, a red yeast shows increased metabolic flux in this pathway, hence its color. One of the major rate-controlling enzymes of this pathway is HMG CoA reductase that catalyzes the conversion of S-3-hydroxy-3-methylglutaryl-CoA to mevalonic acid. HMG CoA reductase is conserved across species; however, it shows higher efficiency in *R. toruloides*.

With the aim to develop molecular tools in *R. toruloides* and to identify *R. toruloides* promoters that can be used bifunctionally in *S. cerevisiae* and *R. toruloides*, phylogenetic footprinting was performed to identify conserved motifs in the promoter region of HMG1 and other vital genes of the pathway namely ERG13 and ERG8.

Multiple sequence alignment of 1200 nucleotide upstream of HMG1 gene (promoter region) for different *Rhodosporidium* species (*R. graminis, R. glutinis, R. toruloides*) was performed (Fig. 2). Phylogenetic footprinting was also performed for hydroxymethylglutaryl-CoA synthase and phosphomevolonate kinase.

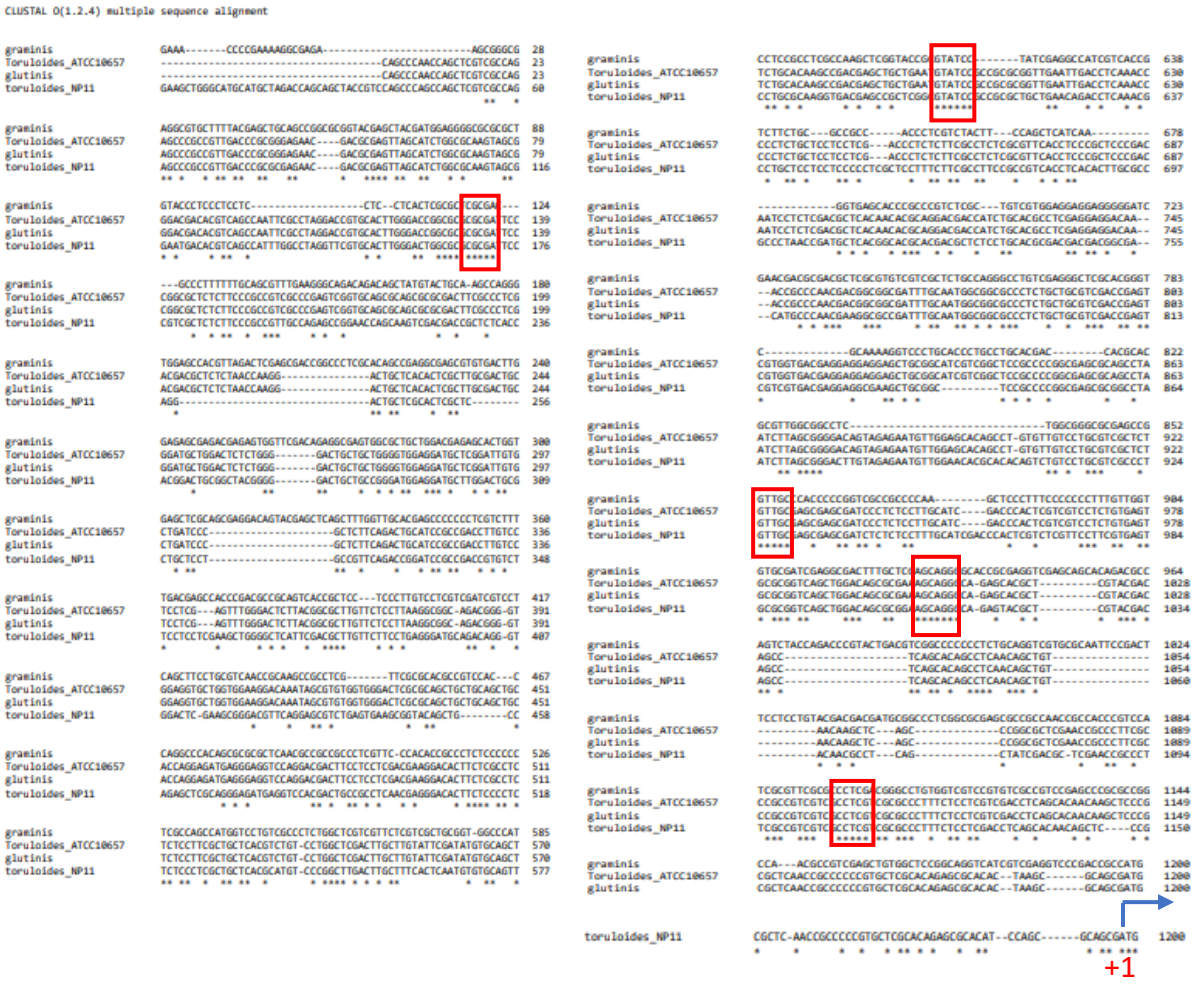


Figure 2: Phylogenetic footprinting of 1200bp region of HMG1 promoter of different *Rhodosporidium* species. The regions in the red boxes highlights the conserved motifs in the HMG1 Rt promoter.

Region -1067 to -1063 (CGCGA), -600 to -595 (GTATCC), -275 to -271 (GTTGC), -195 to -189 (AGCAGGG) and -96 to -92 (CCTCG) was found to be conserved upstream of HMG1 gene i.e., in its promoter region. This indicates regulation by a common factor which is conserved across *Rhodosporidium* species. Table 5 highlights the conserved motifs in the promoter region of HMG1, ERG13 and ERG8.

TABLE 5: Identification of motifs by phylogenetic footprinting of promoters

Species	promoter	Region (upstream of ATG)	Conserved motifs
Saccharomyces kluyveri *Saccharomyces mikatae* *Saccharomyces paradoxus* *Saccharomyces cerevisiae* *Saccharomyces kudriavzevii*	HMG1	-380 to -384 -193 to -189	CGTTT AGATC
Rhodosporidium mucilaginosa *Rhodosporidium graminis* *Rhodosporidium toruloides* *Rhodotorula sp. J31*	ERG13	-342 to -335 -368 to -363 -430 to -419 -442 to -435 -445 to -449 -465 to -456 -474 to -470 -482 to -476 -490 to -485 -508 to -504 -517 to -512 -544 to -539 -562 to -558 -574 to -570 -601 to -591 -661 to -651 -670 to -666 -691 to -686 -739 to -729 -747 to -741 -760 to -756 -909 to -902 -985 to -977 -1000 to -996 -1042 to -1026 -1087 to -1077 -1144 to -1137 -1168 to -1161 -1273 to -1266	GGTTCTGA GTTCCC CTCGGGTGGGAG GGCGGGTT ACGACG GGCGGTACGG TCGAC GTGGTAC GCGTAC CACGG ATGGAC CACCGG GGCGG GGCGA TGCTTCGACCT TTCTACATGCT CTCAA GTCACC GAGCACGAGCG TGCAGAC TGGGG ACTTTGCG CAGGTCATG CCGAC TGGATGCTCGAAGCCGA TTCCGCGCCGA CTCCACCT CGCTACCT GTCGTCGA

		-1288 to -1281	GGCGAGGT
		-1317 to,-1311	TCATCAC
		-1339 to -1335	TTCGT
Rhodosporidium toruloides *Rhodosporidium glutinis* *Rhodosporidium graminis*	ERG8	-19 to -15	CCGAC
		-75 to -68	CTGCTGGC
		-213 to -209	GCGTC
		-445 to -441	GTCGA
		-473 to -467	GTGCGTC
		-520 to -515	TGCTCG
		-579 to -573	GTCGA
		-616 to -612	AGCGT
		-646 to -642	GCTCC
		-698 to -694	CCTGT
		-797 to -791	CGATCCG
		-962 to -957	GGCAGG
		-1084 to -1079	CGAGCA
		-1139 to -1135	GCAAG
		-1193 to -1186	GTACCGCA
		-1300 to -1294	GCGAGGT
		-1363 to -1357	CGCTCGA

3.2 Creation of vector pRSII316 EGFP and Modified pRH2031

With the aim to develop a reporter to assess various *Rhodosporidium* promoters, two vectors were created one for each *S. cerevisiae* and *R. toruloides.* Various *R. toruloides* promoters were cloned upstream of the reporter gene EGFP to assess their efficiency.

For *S. cerevisiae*, pRSII316 EGFP vector was created by cloning EGFP CYC_{term} in pRSII316 vector. EGFP CYC_{term} was subcloned from pRS416 TEF EGFP using KpnI and BamHI sites (Fig. 3). In the upstream of EGFP different Rt promoters were cloned using SpeI and BamHI sites.

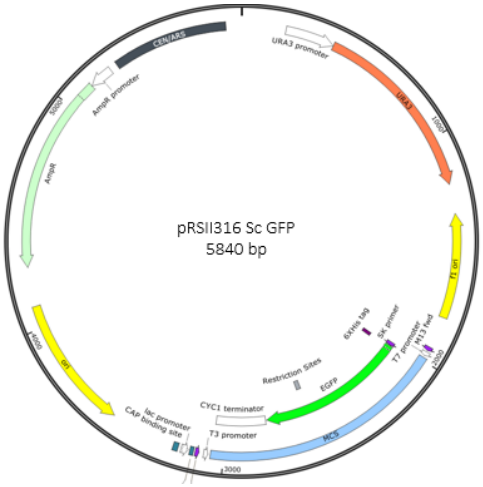


Fig 3: *S. cerevisiae* vector (pRSII316 Sc GFP)

In *Rhodosporidium* vector, pRH2031, there were no restriction enzyme sites available immediately upstream of Rt GFP ORF which hampered it being used for cloning purposes. The BamHI site was present after start codon ATG and two additional nucleotide base pairs. So, a forward primer was designed with BamHI site followed by start codon ATG and reverse primer EcoRV for the creation of modified pRH2031 vector (Fig. 4). The modified vector was then used to clone different *R. toruloides* promoters upstream of Rt GFP ORF using SpeI and BamHI enzymes (Fig. 5).

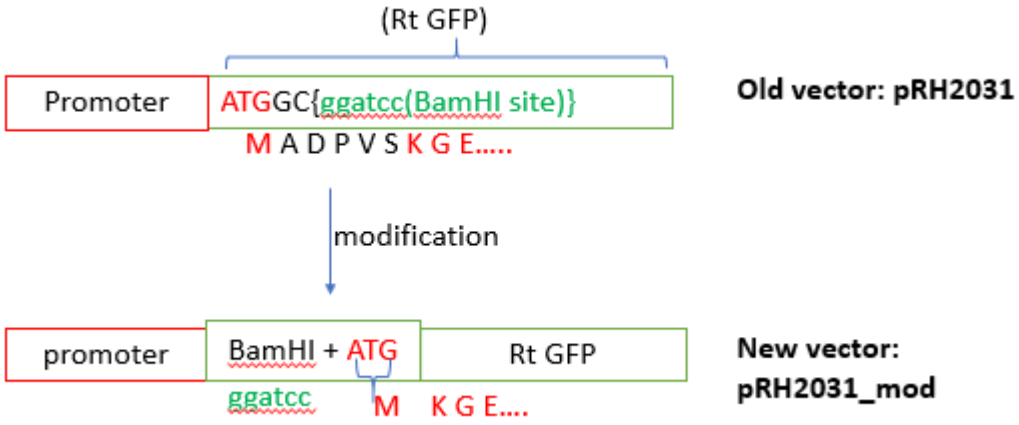


Fig 4: Modification of existing *R. toruloides* vector

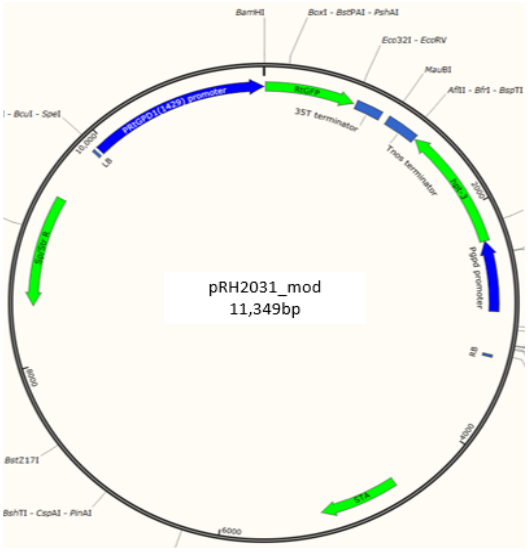


Fig 5: *R. toruloides* vector (pRH2031_mod)

3.3 Cloning and evaluation of promoters of MVA pathway genes of *R. toruloides*

To clone various *R. toruloides* promoters, 1500 bp upstream of gene (HMG1, ERG13, MVD1, ERG8, BTS1) were PCR amplified from *R. toruloides* genomic DNA and cloned in pRSII316 vector. The recombinant constructs containing the above different promoters were transformed to BY4741 *S. cerevisiae* strain. Final evaluation of promoter was done by measuring GFP expression using spectrophotometer (Fig. 6). Though the control shows high fluorescence, the expression of *R. toruloides* promoter was not observed in *S. cerevisiae* suggesting that *R. toruloides* promoters of mevalonate biosynthetic pathway gene (HMG1, BTS1, ERG13, ERG8, MVD1) are non-functional in *S. cerevisiae*.

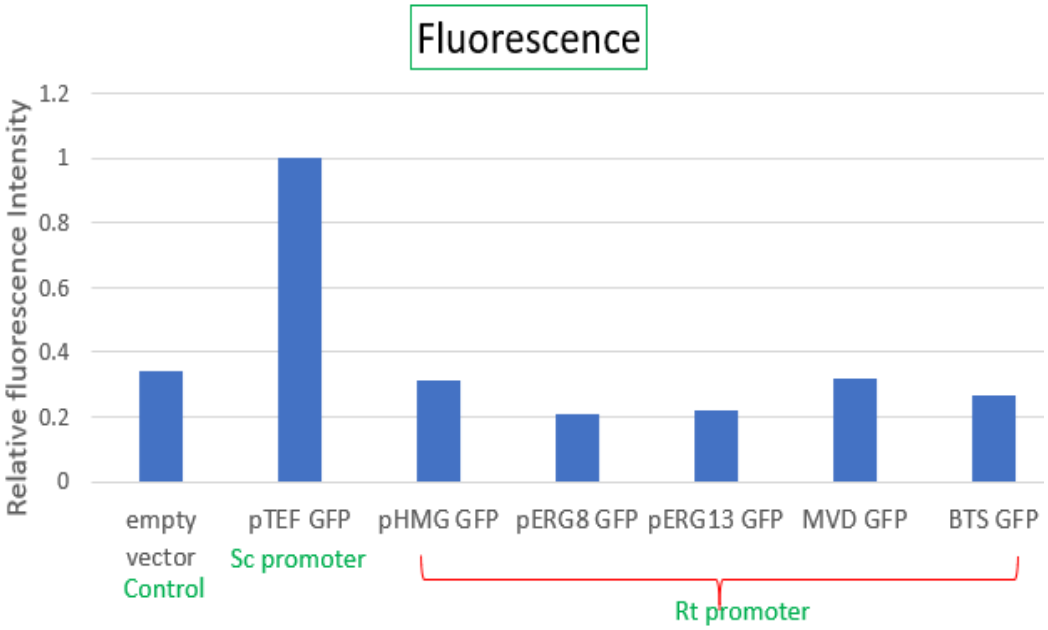
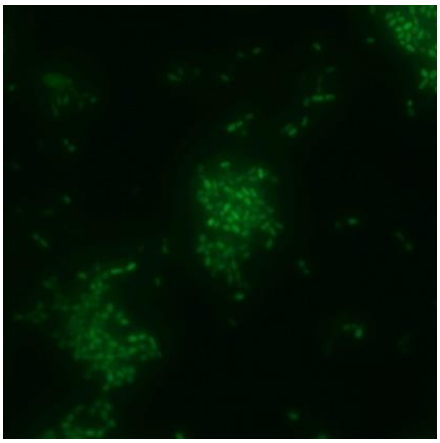
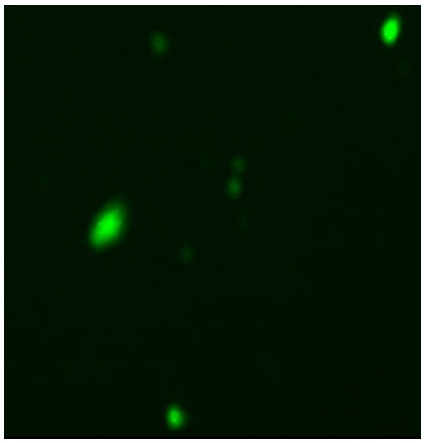


Fig 6: Evaluation of EGFP expression of *R. toruloides* promoter in *S. cerevisiae*. The data was from a single experiment and needs to be repeated.

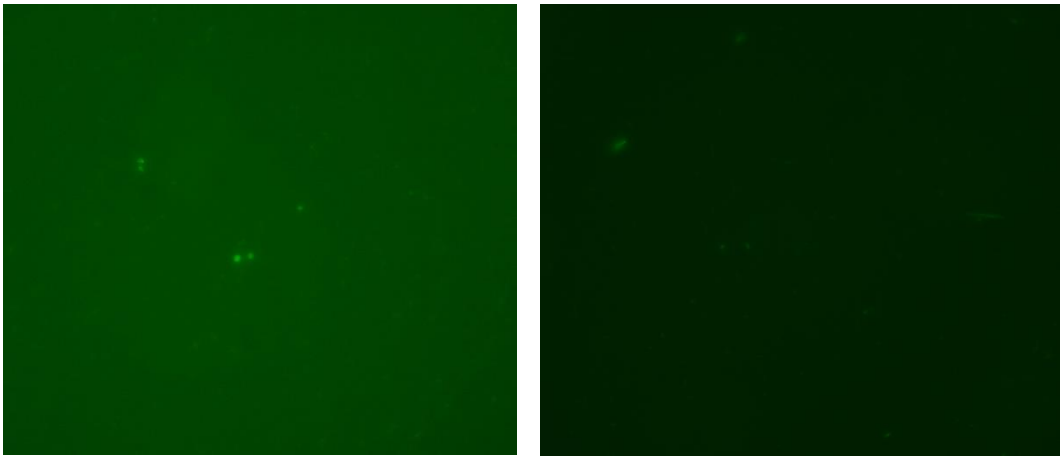
R. toruloides promoters were then cloned upstream of Rt GFP in pRH2031_mod (*R. toruloides* vector) using SpeI and BamHI and transformed in Ku70 *R. toruloides* using Agrobacterium-mediated transformation method. GFP expression was evaluated using fluorescence microscope. *R. toruloides* transformed with (HMG1, BTS1, GPD1) promoters showed GFP expression suggesting that promoters work in *R. toruloides* (Fig. 7).



(A)



(B)



(C) (D)

Fig 7: *R. toruloides* promoters (HMG1, BTS1, GPD1) in *R. toruloides* showing GFP expression; (A) pRH2031 vector (GPD1 promoter) GFP expression in 40x, (B), pRH2031_Mod vector (GPD1 promoter) GFP expression in 60x; (C) HMG promoter 1300 bp GFP expression in 20x; (D) BTS1 promoter GFP expression in 20x

3.4 Deletion Analysis of RtHMG1 promoter

Deletion analysis of HMG1 promoter was carried out to validate the conserved boxes identified in phylogenetic footprinting (Fig. 8). HMG1 promoter of length 1300, 600, 400 bp promoters each was cloned separately in pRH2031_mod vector showing GFP expression in *R. toruloides* and evaluated for GFP expression (Fig. 9). HMG1 promoter of length 1300, 600 and 400 bp showed GFP expression in *R. toruloides*. This suggests that 400 bp upstream of HMG1 works as functional promoter, length less than 400 bp was not evaluated in this study.

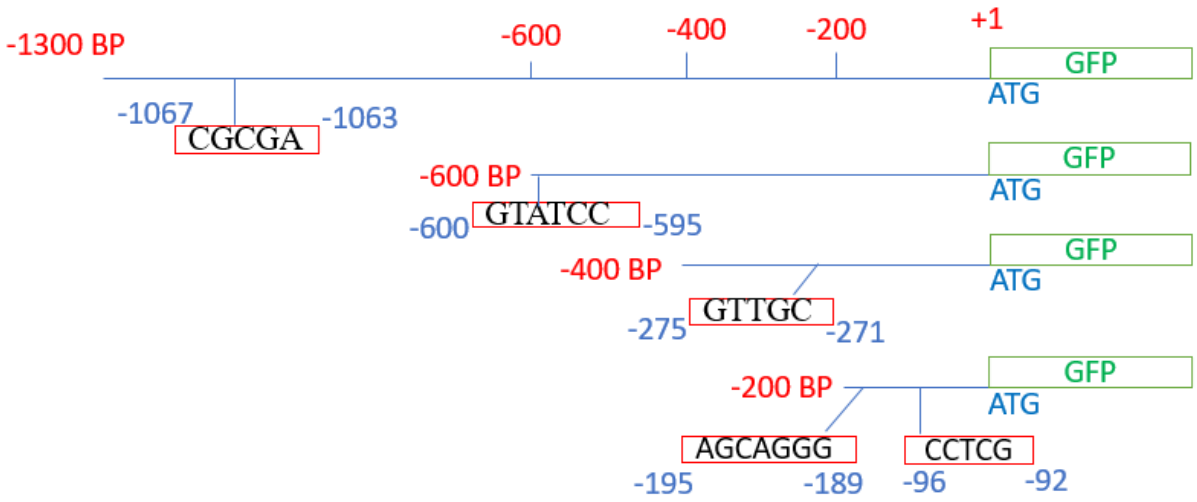
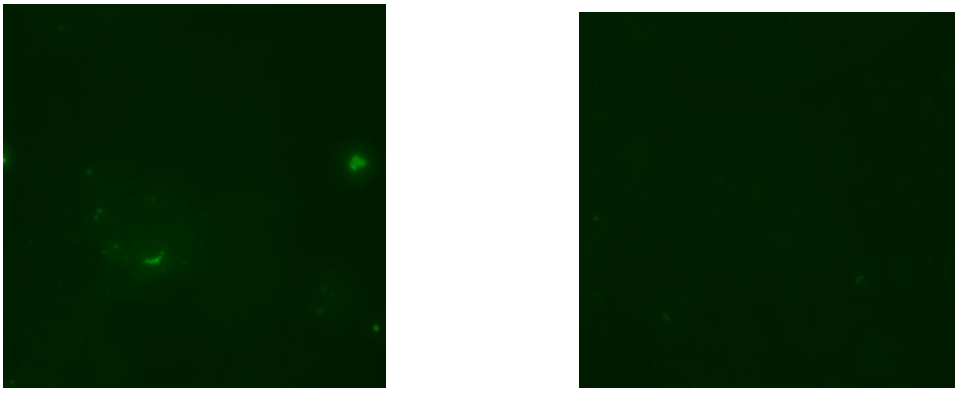
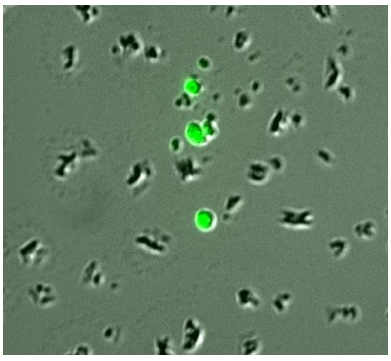


Fig 8: Deletion analysis of Rt HMG1 promoter of different length.



(A)

(B)



(C)

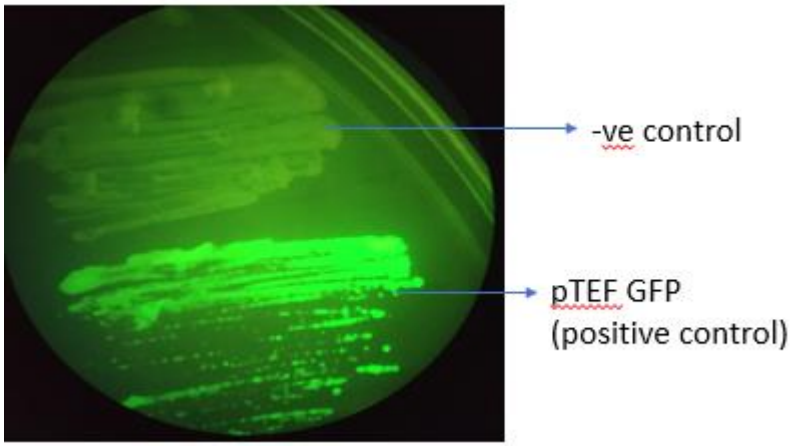
Fig 9: *R. toruloides* promoters (HMG1 -600 bp, -400 bp) in *R. toruloides* showing GFP expression; (A) HMG1 promoter 600 bp GFP expression in 20x; (B) HMG1 promoter 400

bp GFP expression in 20x; (C), HMG1 promoter 400 bp GFP expression in different field 60x

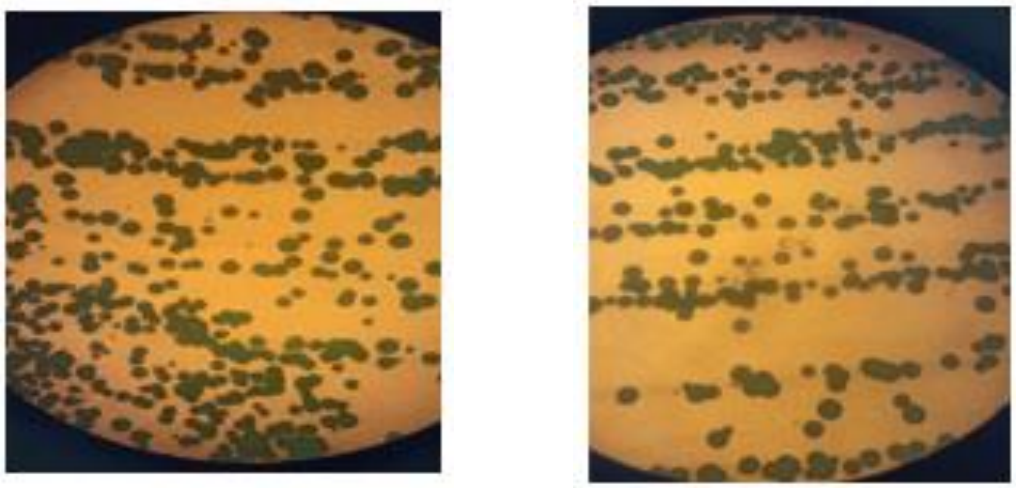
3.5 Directed Evolution of Rt HMG1 promoter in *S. cerevisiae*

Since the *R. toruloides* promoters we evaluated were non-functional in *S. cerevisiae*, directed evolution was used to try and isolate mutants showing higher promoter activity in *S. cerevisiae*. Random mutagenesis was carried out in the plasmid using hydroxylamine. *S. cerevisiae* transformants were screened for higher GFP expression. Ten plates were screened containing 1440, 1080, 1360, 1216, 1096, 1168, 824, 920, 808 and 1552 colonies using a fluorescence microscope (Fig. 10).

None of the mutants showed higher GFP expression compared to control.



(A)



(B) (C)

Fig 10: GFP screening of *S. cerevisiae* transformants; (A) Control (-/+) GFP expression, (B) Rt HMG1 promoter not showing GFP expression, (C) Mutated Rt HMG1 promoter not showing any signifcant GFP expression

3.6 Conclusion and Discussion

In this study, we cloned and evaluated *R. toruloides* promoters of the mevalonate biosynthetic pathway gene (HMG1, BTS1, ERG13, ERG8, MVD1) of red yeast in *S.* cerevisiae and also evaluated promoter of HMG1 and BTS1 in *R. toruloides*.

None of the *R. toruloides* promoters evaluated were functional in *S. cerevisiae*. This may be because of the very GC rich nature of these promoters. However, since these promoters were shown to be functional in Rhodosporidium, these promoters can be used as genetic tools for metabolic engineering heterologous pathways in Rhodosporidium. The use of low copy number plasmid vector used in *S. cerevisiae* for cloning and transformation of promoters might have effect on its fluorescence and the sensitivity of the measurements.

We attempted to make Rt HMG1 promoter functional in *S. cerevisiae* by directed evolution. The significant difference in nucleotide sequence and GC content of both organisms might have made *R. toruloides* promoter challenging to function in *S. cerevisiae*. Also, more number of colonies were needed to screen for desired mutant.

The ATMT methods randomly integrate the plasmid into the genome of *R. toruloides*, unlike homologous recombination. Also, the *R. toruloides* transformants are unstable in the hygromycin selection plate. We need to grow *R. toruloides* transformants in the selection plate for more generations to make it stable and then evaluate the promoters.

Phylogenetic footprinting of HMG1 promoter of different Rhodosporidium species showed presence of conserved motifs indicating a common mode of regulation conserved across the species. Deletion analysis of HMG1 promoter reveal that 400 bp HMG1 promoter would be sufficient to drive the gene expression through this promoter. Further analysis is required to determine the regulation and the role of the possible motifs in the promoter.

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