Yeast Metabolic Engineering for Production of Farnesol and Geranylgeraniol

Bio-Technical Resources (BTR)

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Isoprenoids Pathways

- **Isoprenoids:**
  - Most diverse group of naturally occurring compounds
  - Derived from 5-carbon molecule isopentenyl pyrophosphate (IPP)
- **Two distinct pathways for IPP synthesis:**
  - Acetyl-CoA to mevalonate, to IPP
  - Non-mevalonate pathway: Glyceraladehyde-3-P and pyruvate to deoxyxylolose-5-P, to IPP
  - *S. cerevisiae*, mevalonate-dependent pathway
- **IPP monomers are condensed to form isoprenoids of different lengths:** including valuable molecules such as carotenoids, ubiquinones, steroids, precursors for vitamin synthesis, and pharmaceuticals (CoQ10)
- **BTR's focus:** production farnesol and geranylgeraniol

Generation of Squalene Synthase Mutants Using Classical Mutagenesis and Screening

ATCC 28383, haploid wild type strain.
Mutagenize with nitrous acid

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Plate on YPD +cholesterol +nystatin. Incubate at 28° C

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Replica plate survivors to YPD. Incubate at 36°C

↓

Isolate mutants that do not grow in the absence of cholesterol. Screen for isoprenoid accumulation using tube cultures

↓

Identified three strains that exhibited significant farnesol accumulation under aerobic conditions

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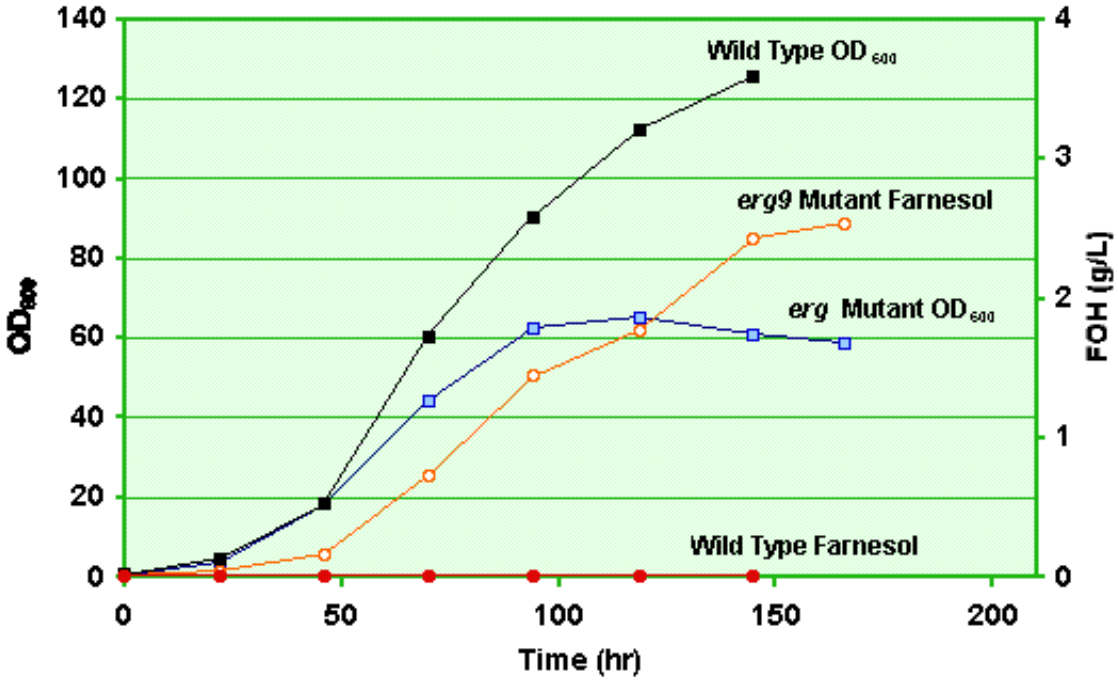
3 ERG9 Mutants: MBNA1-1, 1-9, 1-13

- Yeast normally cannot take up exogenous sterols unless grown under anaerobic conditions.

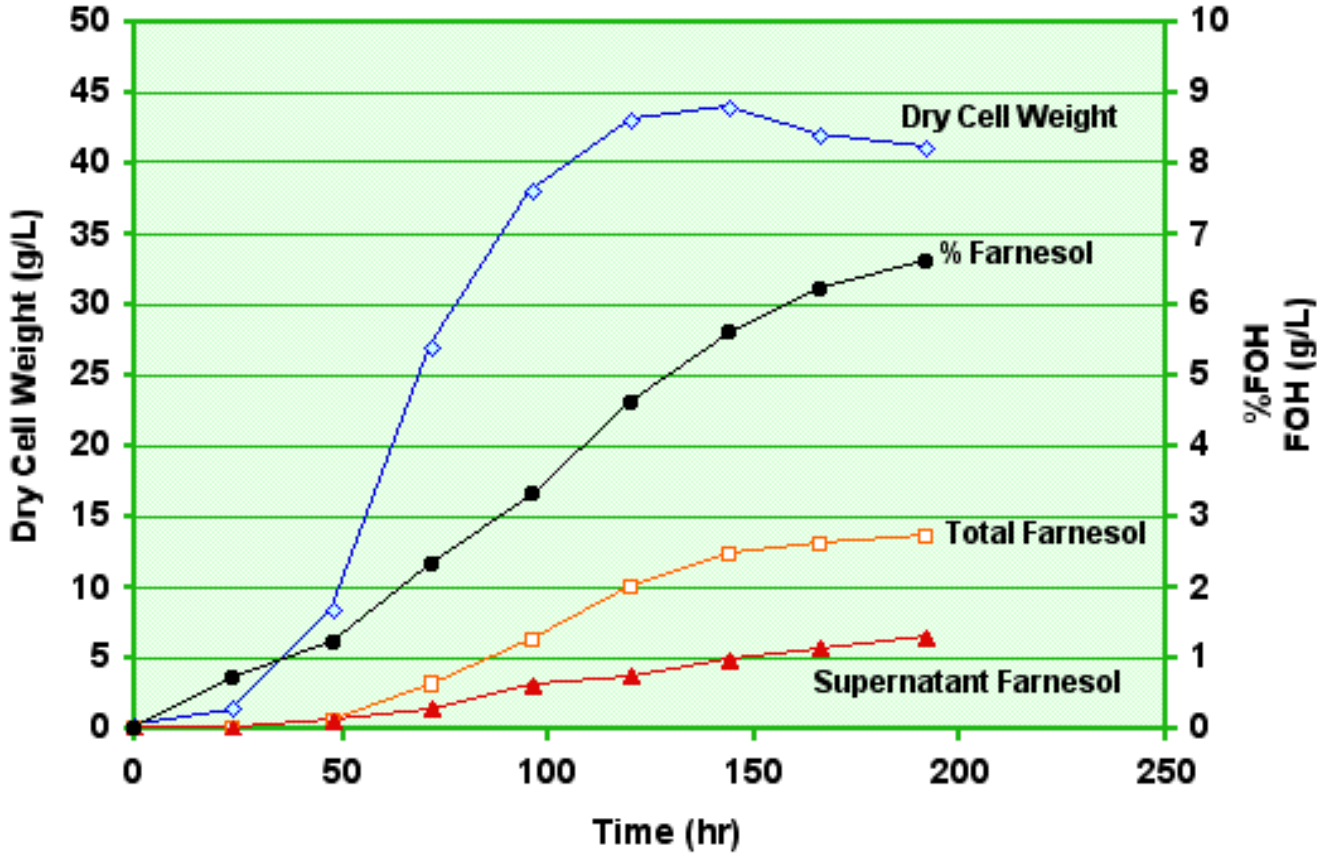
- Isolated ergosterol-dependent mutants
  - Mutation in *ERG9* (coding for squalene synthase)
  - Uncharacterized mutation(s) that confer the ability to take up sterols under aerobic conditions, referred as *sue* (Sterol Uptake Enhancement)

Wild type and *erg9* Mutant in Fed Batch Fermentations



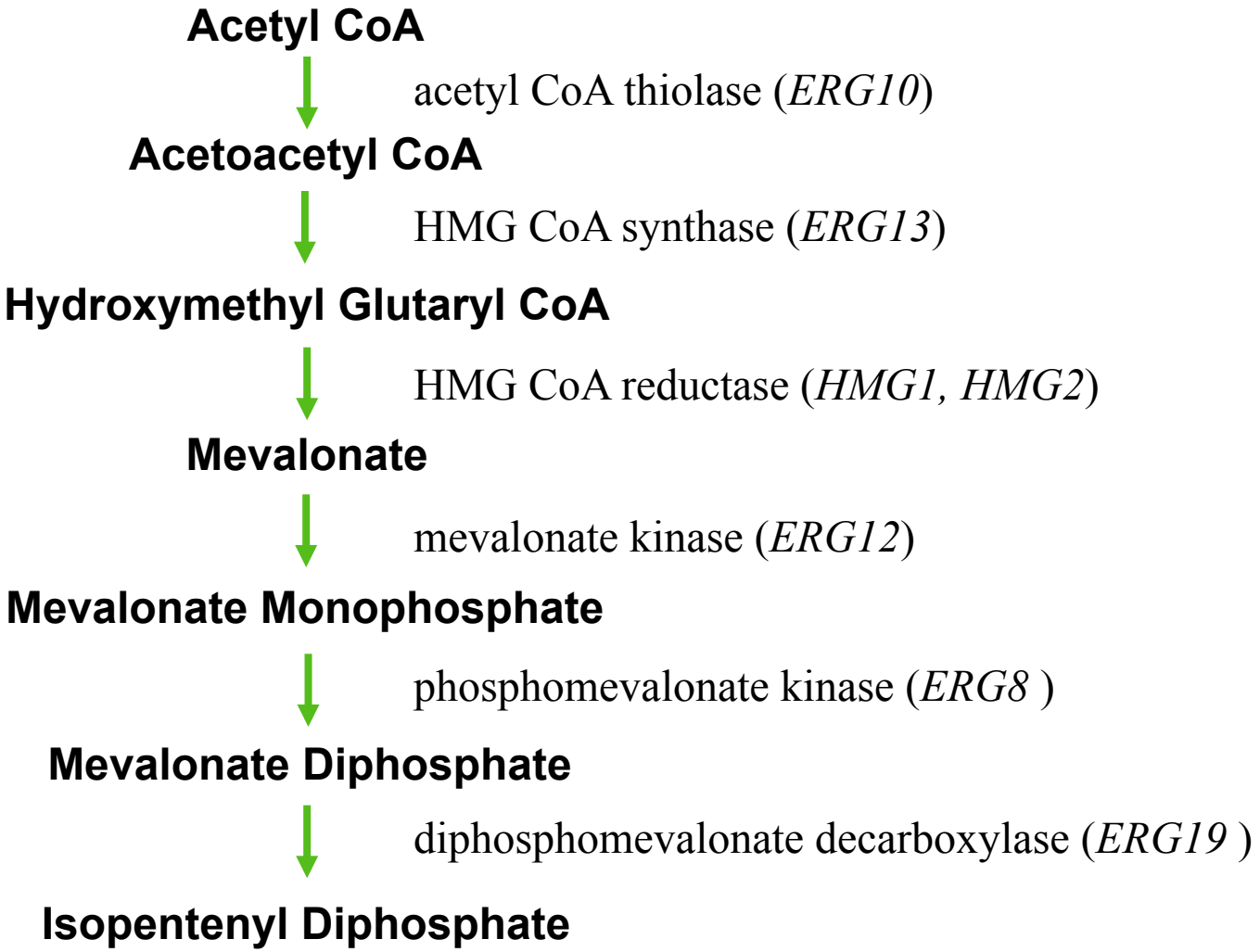
Total FOH	Dry Cell Weight	Total FOH % Dry Weight	FOH Supernatant	FOH Cell Pellet*	Cell FOH % Dry Weight
2.72 g/L	41 g/L	6.6%	1.3 g/L	1.42 g/L	3.5%

MBNA1-13 in Fed Batch Fermentation

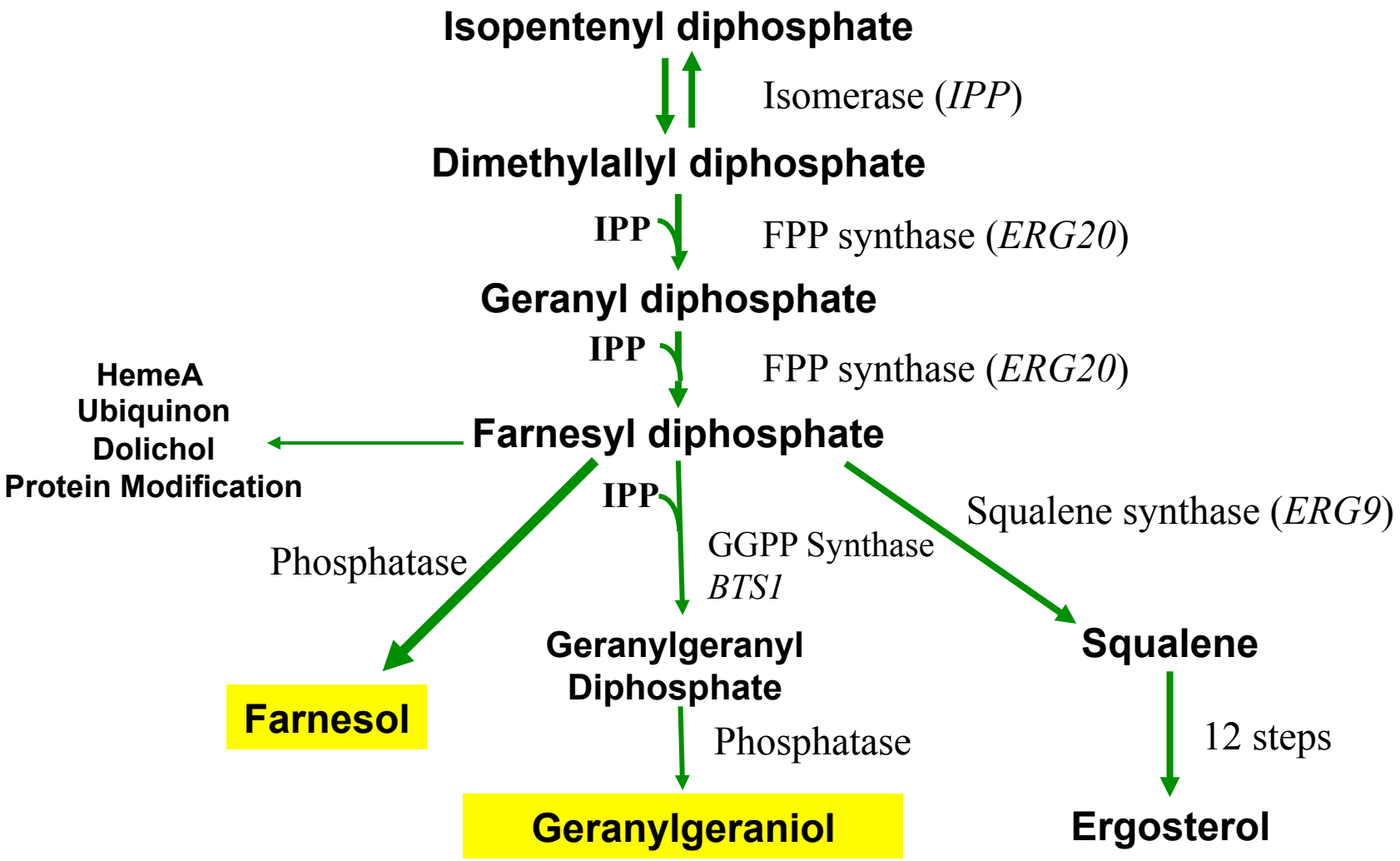


- Farnesol accumulated rapidly during the growth phases and accumulation continued during the non-growth phase.
- Farnesol was efficiently released from the cells as soon as it was produced.
- Dry cell weight reached approximately 44 g/L. Total farnesol level exceeded 6% of dry cell weight.

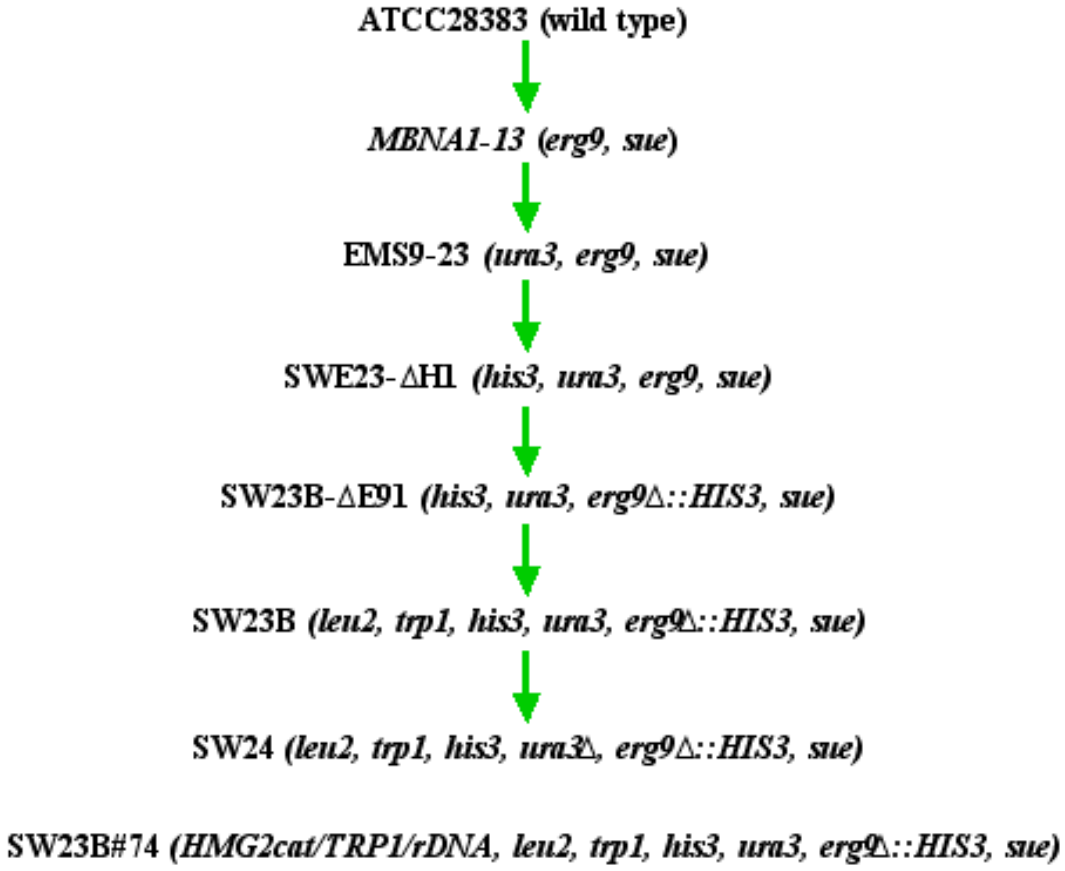
Isoprenoid Biosynthetic Pathway in *S. cerevisiae*



Isoprenoid Biosynthetic Pathway in *S. cerevisiae*



Strain Genotypes



- Strains derived from *erg9* mutant MBNA1-13.
- Auxotrophic mutations introduced for molecular manipulation of the isoprenoid pathway

Amplification of GGPP Synthase

Strain/plasmid	Amplified Gene	GGPP Synthase nmol/min.mg	Dry Cell Weight mg/ml	Farnesol µg/ml	GGOH µg/ml
EMS9-23/YEp352	Control	Not Detected	3.80	309	5.7
EMS9-23/pTWM110	*BTS1* *S. cerevisiae*	0.065	3.64	278	44.0
EMS9-23/pSW4A3	*crtE* *E. uredovora*	0.094	3.32	206	78.7
EMS9-23/pSW9-1A	*al-3* *N. crassa*	0.032	3.41	266	15.0
EMS9-23/pSW10-2B	*ggs* *G. fujikuroi*	0.030	3.58	283	28.6

- GGPP synthase genes: yeast *BTS1*, bacterial *crtE* from *Erwinia uredovora*, and two filamentous fungal homologs, *al-3* from *Neurospora crassa* and *ggs* from *Gibberella fujikuroi*.
- GGPP synthase genes were inserted into a high copy-number plasmid, and expressed using strong yeast promoters of *PGK* or *ADH1* genes
- Over-expression of GGPP synthases in *erg9* mutants led to higher accumulations of GGOH, the highest level achieved with *crtE* in shake flask cultures

Amplification of FPP Synthase

Strain/plasmid	Amplified Gene	FPP Synthase nmol/min.mg	Dry Cell Weight mg/ml	Farnesol µg/ml	GGOH µg/ml
SWE23-ΔE91/ YEp352	Control	2.1	2.9	228.0	4.0
SWE23-ΔE91/ pJMB19-31 #1	*ERG20 FPP Synthase*	44.0	3.0	171.0	37.0

- YEp352: empty vector, high copy number, *URA3* selection
- pJMB19-31: YEp352 containing *GPD* promoter/*ERG20* (FPP synthase)

- Overexpression of native FPP synthase gene (*ERG20*):
  - Strong yeast promoter, high-copy-number plasmid
  - Elevated FPP synthase activity
  - Greatly increased accumulation of GGOH, but no increase in farnesol accumulation.

Effect of Amplifying Deregulated HMG CoA Reductase (HMG2)

Shake Flask

Strain/plasmid	Amplified Gene	Reductase nmol/mg.min	Dry Wt. g/L	Farnesol g/L	Farnesol % dry wt.
EMS9-23/YEp352	control	1.1	3.3	0.21	6.4
EMS9-23/pRH124-31	*HMG2cat*	7.7	3.1	0.15	5.0

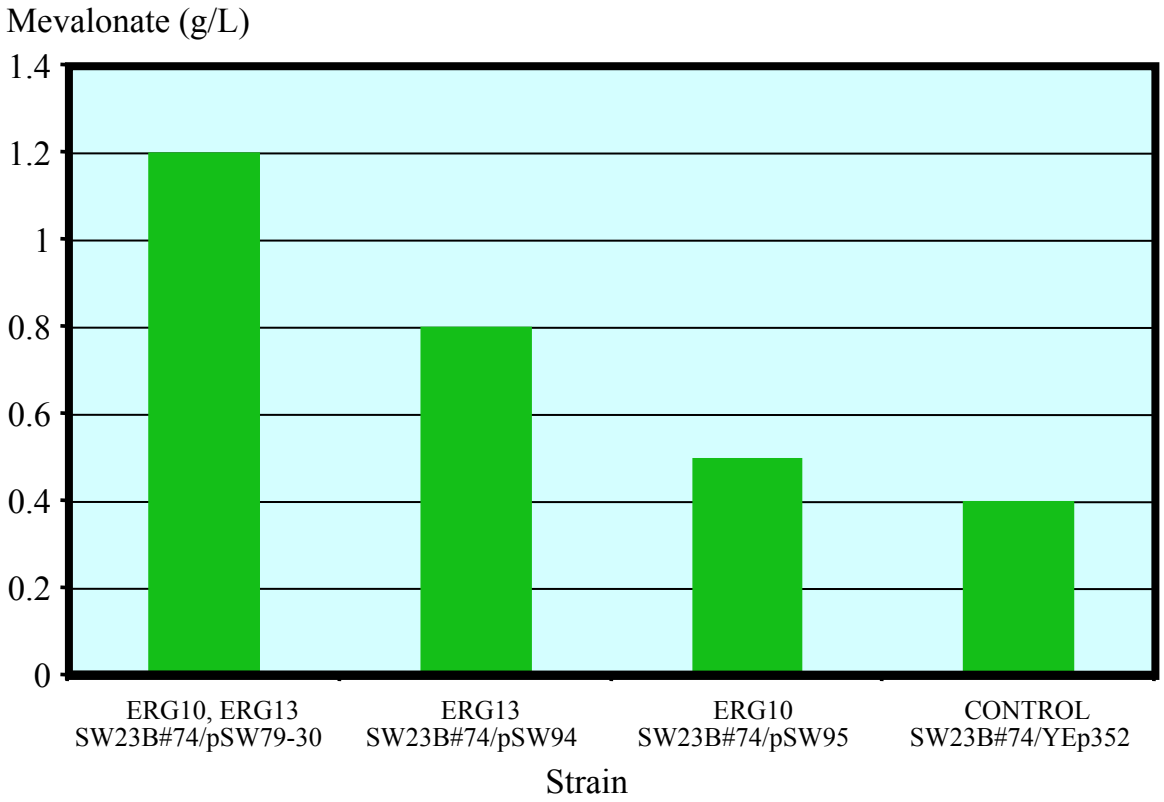
Fed-Batch Fermentation

Strain/plasmid	Amplified Gene	Reductase nmol/mg.min	Dry Wt. g/L	Farnesol g/L	Farnesol % dry wt.
SWE23-ΔE91/YEp352	control	4.0	37.2	2.2	6.0
SWE23-ΔE91/pRH124-31	*HMG2cat*	18.0	36.8	3.6	9.8

YEp352 = empty vector, high copy, *URA3* selection
pRH124-31 = YEp352, *GPD* promoter/*HMG2 cat* gene fusion (catalytic domain of the *HMG2* gene)

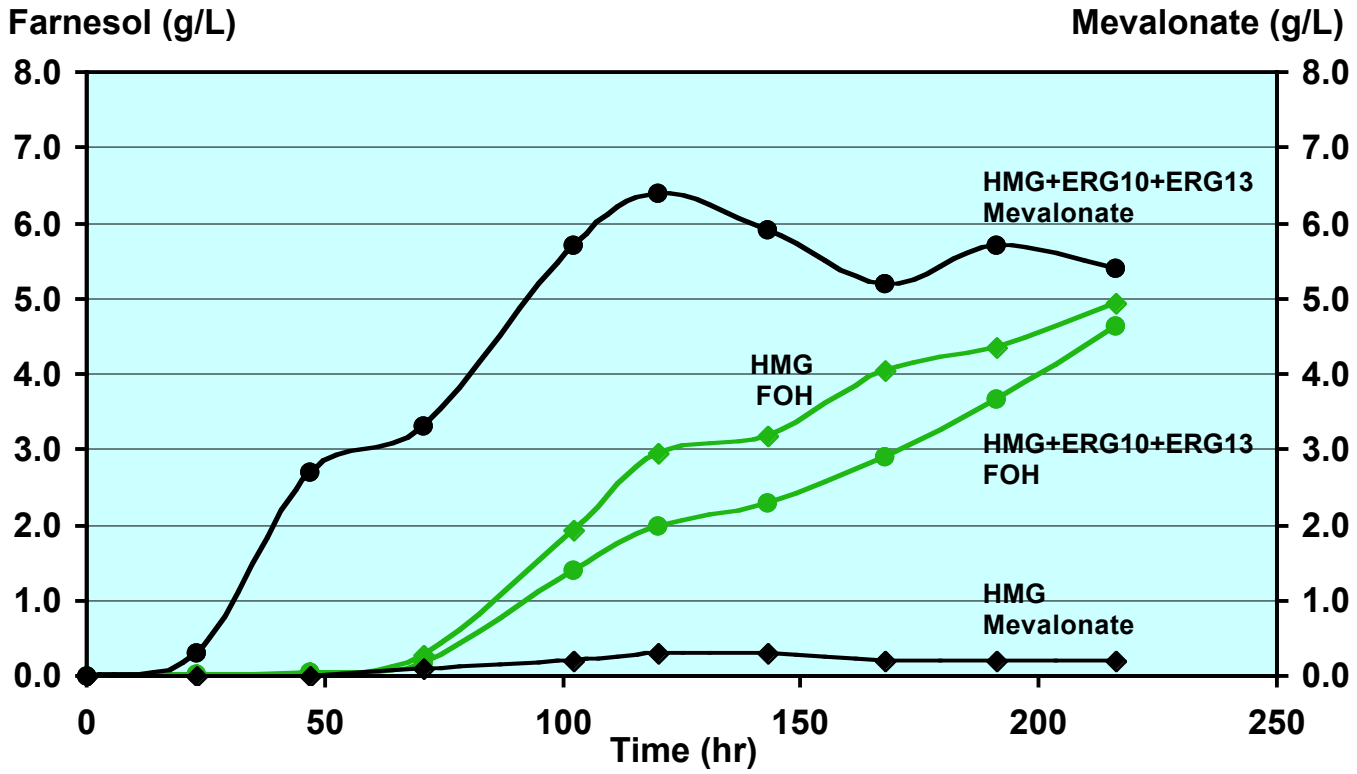
- Catalytic domain of HMG2 lacks the transcriptional and protein degradation signals that normally control the level of the enzyme.
- Strain with over-expressed catalytic domain exhibited lower farnesol accumulation than strains with normal HMG CoA reductase in shake flask cultures.
- However, in fermentors under fed-batch conditions, this strains accumulated significantly more farnesol than the control strains.

Effect of Amplifying Upper Isoprenoid Pathway Enzymes in an *erg9* Mutant



ERG10: Acetoacetyl CoA thiolase
ERG13: HMG CoA synthase
Control: SW23B#74 (8 copies of *HMG2cat*)

Farnesol and Mevalonate Accumulation in Strains with Amplified Upper Pathway Enzymes



- Over-expression of deregulated HMG CoA reductase led to accumulation of mevalonate in the medium.
- Amplifying genes of acetoacetyl CoA thiolase and HMG CoA synthase in strains with elevated HMG CoA reductase actitivity led to a dramatic increase in mevalonate accumulation, without significant change in farnesol accumulation.

Conclusions

- *erg9* inactivation plus mutation(s) conferring aerobic sterol uptake: accumulated >2.5 g/L farnesol in fermenters under fed-batch conditions
- Amplification of either GGPP synthase or FPP synthase in *erg9* mutants led to elevated accumulation of GGOH (geranylgeraniol), but the major isoprenoid produced was farnesol
- Amplification of de-regulated HMG CoA reductase activity in *erg9* mutants led to higher accumulation of farnesol, > 4 g/L in fermentors under fed-batch conditions
- Gene amplification for the first three steps of isoprenoid pathway, namely acetoacetyl CoA thiolase, HMG CoA synthase, and deregulated HMG CoA reductase, led to significant increases in mevalonate accumulation, but farnesol levels were not affected, suggesting metabolic restriction at the level of mevalonate kinase

Reference:
S. Takahashi, Y. Yeo, B.T. Greenhagen, T. McMullin, L. Song, J. Maurina-Brunker, R. Rosson, J.P. Noel, J. Chappell. Metabolic Engineering of Sesquiterpene Metabolism in Yeast. Biotechnology and Bioengineering. May 1; **97(1):**170-181 (2007)