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Monoterpene production by the carotenogenic yeast *Rhodosporidium toruloides*

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Abstract

Due to their high energy density and comparable physical properties, several monoterpenes have been investigated as potential renewable transportation fuels, either as drop-in replacements or petroleum blends, for heavy and light-weight vehicles as well as for aviation. Sustainable microbial production of these biofuels requires the ability to utilize cheap and readily available feedstocks such as lignocellulosic biomass, which can be depolymerized into fermentable carbon, such as glucose and xylose. However, common microbial production platforms such as the yeast *Saccharomyces cerevisiae* are not naturally capable of utilizing xylose, hence requiring extensive strain engineering and optimization to efficiently utilize lignocellulosic feedstocks. In contrast, the oleaginous red yeast *Rhodosporidium toruloides* is capable of efficiently metabolizing both xylose and glucose, which indicates that it is suitable to produce bio-based molecules from non-food plant lignocellulosic biomass. *R. toruloides* naturally produces C40 tetraterpenoid carotenoids as red pigment, indicating that the native *Rhodosporidium* mevalonate (MVA) pathway may provide a naturally high flux of carbon through the MVA pathway, enabling efficient production of heterologous terpenes. This work investigates *Rhodosporidium toruloides* as a platform for producing monoterpene biofuels from lignocellulosic biomass.

Sixteen monoterpene synthase originating form plants, bacteria and fungi making a total of nine different monoterpenes were expressed in *R. toruloides*, and evaluated monoterpene production by GC-MS. Eight of these constructs resulted in strains that produce different monoterpenes, either as individual compounds or as mixtures, with 1,8-cineole, ocimene, limonene, pinene, sabinene, and careen being produced at the highest level in that order. Highest titers of 1,8-cineole produced from the *HYP3* synthase from *Hypoxylon SP. E74060B* were observed in cultures containing a dodecane overlay. 1,8-cineole production reached 15.81 mg/L level in test tubes. This monoterpene has physical properties that indicate that it may be a good alternative gasoline, and additional testing of its fuel properties provide evidence that it may be a good biofuel for spark ignition engines. Our results demonstrated the first time use of *Rhodosporidium toruloides* as a microbial platform for the production of monoterpenes with potential biofuel applications from lignocellulosic biomass

Schematic diagram of *R. toruloides* native mevalonate MVA pathway

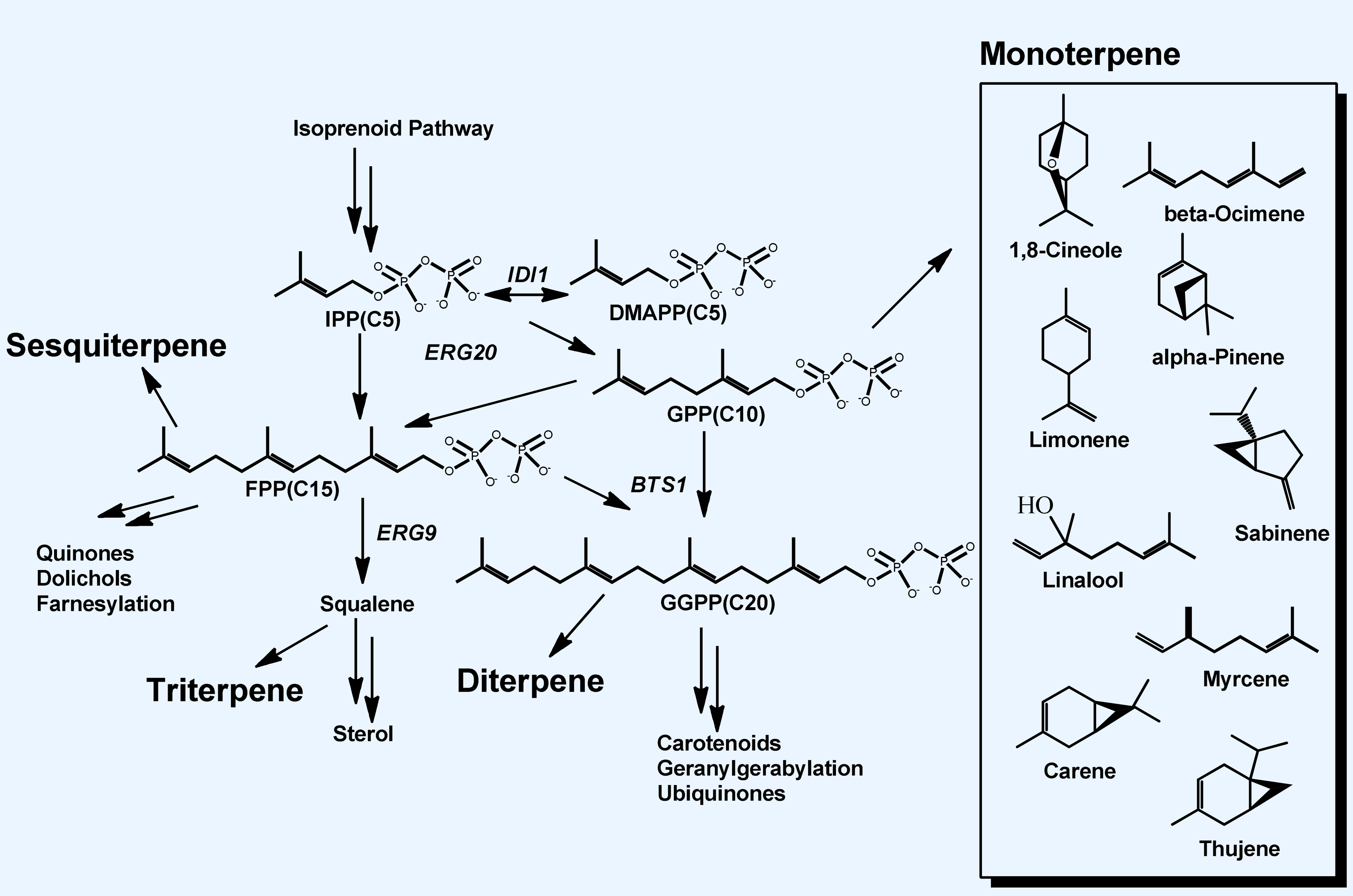

Figure 1. Schematic diagram of *R. toruloides* native mevalonate MVA pathway, which provides the opportunity for diverting intermediates GPP from MVA pathway for the biosynthesis of our desired monoterpene production.

Summary of Monoterpen synthases have been tested for monoterpene production in *R. toruloides*

Product	Gene Name	Organism	Gene bank Access Number	Enzyme Kinetic for GPP	Product in *R. toruloides*	
					Dodecane Overlay	SPME
1,8-Cineole	*Hyp3*	*Hypoxylon sp. E74060B*	KJ433271.1	K_m=2.5±0.6 μM K_{cat}=0.295 S^{-1}	++	++
	SSCG_00536 CnsA	*Streptomyces clavuligerus*	DS570626.1	K_m=0.17 μM K_{cat}=0.079 S^{-1}	+	+
Ocimene	*ama0e23*	*Antirrhinum majus*	AY195607.1	NA	ND	+
	LcTPS1	*Licea cubeba*	HQ651178.1	NA	ND	ND
limonene	*ag10*	*Abies grandis*	AF006193.1	NA	ND	Detectable
Pinene	*PT30*	*Pinus taeda*	AF543530.1	K_m=47±9μM	ND	+
	AG3.18	*Abies grandis*	U87909.1	K_m=6μM	ND	Detectable
Myrcene	*amaOc15*	*Antirrhinum majus*	AY195608.1	NA	ND	ND
	ama1e20	*Antirrhinum majus*	AY195609.1	NA	ND	ND
	AG2.2	*Abies grandis*	U87908.1	NA	ND	ND
Linalool	*PaTPS-Lin*	*Picea Abies*	AY473623.1	NA	ND	ND
Sabinene	*RlemTPS2*	*Citrus jambhiri*	AB266585.1	NA	ND	ND
	SabS1	*Salvia pomifera*	DQ785794.1	K_m=7.4 μM	+	+
Carene	*PaJF67*	*Picea abies*	AF461460	NA	ND	ND
	TpsB	*Salvia stenophylla*	AF527416.1	NA	ND	+
Thujene	*LcTPS2*	*Licea cubeba*	HQ651179.1	NA	ND	ND

ND: No detectable <1 mg/L; +: <5 mg/L; ++:5-20 mg/L

1,8-Cineole productions in *R. toruloides*

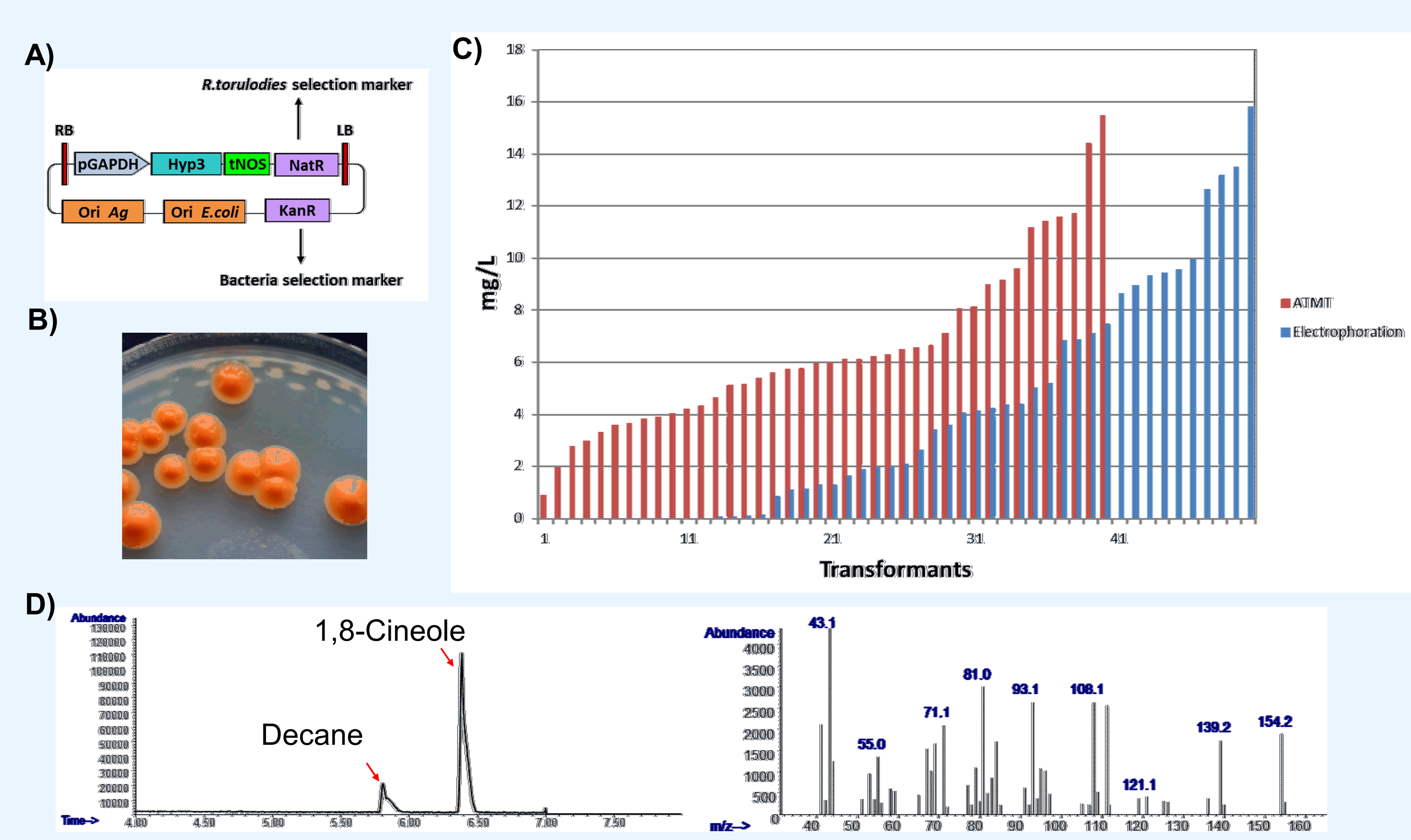

Figure 2.Cineole producation in *R.toruloides*. A) Binary vector containing cineole synthase *Hyp3* designed for integrating *R. toruloides* through ATMT transformation method, also could be served as template for PCR reaction amplification to transform *R. toruloides* through electroporation ransformation method. B) Picture of *R.torulodies* colonies C) The comparison of cineole production in *R. toruloides* by two transformation methods the ATMT (*Agrobacterium* mediated transformation) vs electroporation. D) GC chromatographs of *R. toruloides* engineered for expression of the cineole synthase *Hyp3*.

Comparison of the relevant fuel properties of the 1,8- cineole with and common biofuel ethanol

Fuel physical property	1,8-Cineole	Ethanol
Research Octane Number (RON)	99.2	109
Motor Octane Number (MON)	91.0	90
Sensitivity (RON-MON)	8.2	19
Energy Density [MJ/L]	38.0	20.2
Heat of Vaporization [kJ/kg]	255	919
Vapor Pressure [kPa @25 C]	0.25	7.833
Water Solubility [g/L @21 C]	3.5	Fully Miscible
Oxygen Content [% of total mass]	10.4	34.7

Octane Numbers for RON and MON determined by ASTM D2699 and D2700 respectively in collaboration with Intertek Group plc in Benicia CA. Physical properties gathered from the NREL Co-optima fuel properties database.

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