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Aromatic and Monoterpene Alcohol Accumulation by *Eremothecium ashbyi* Strains Differing in Riboflavinogenesis

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Abstract—We examined the accumulation of phenylethanol, geraniol, citronellol, and nerol by *Eremothecium ashbyi* Guillermond 1935 strains characterized by different levels of riboflavin synthesis. There was a significant positive correlation between riboflavin and monoterpene alcohol biosyntheses (Spearman's correlation coefficients = 0.81–1.00, $p \le 0.05$). Strain accumulation of the main secondary metabolites such as vitamin B_2 and aroma forming compounds was found to be accompanied with an increase in the lipid droplet quantities and the vacuole filling with lipophilic compounds. These phenomena may be used as an indirect measure of riboflavinogenesis intensity and essential oil synthesis.

Keywords: Eremothecium, essential oil, accumulation of phenethyl and monoterpene alcohols, riboflavinogenesis

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INTRODUCTION

Eremothecium ashbyi Guillermond 1935 is an industrially valuable microorganism, which synthesizes biologically active substances. It is a riboflavin overproducer because of its ability to suppress the action of iron ions, which inhibit all biosynthetic pathways for vitamin formation in other microorganisms [1–4]. The producer strain cultivation may lead to a riboflavin concentration of up to 3.5 g per liter of culture broth (CB) at day 7 [2, 4]. Earlier studies have also shown that this microorganism can excrete more than 350 mg of essential oil (similar to rose oil) per liter of CB. This oil is a mixture of volatile fragrant compounds (geraniol, citronellol, 2-phenyl ethanol, etc.), which are commonly used as bacteriostatic agents and flavorings in pharmaceutics [5, 6]. In human organism, these compounds participate in metabolic processes and possess almost no side effects. Moreover, many of them are precursors of physiologically active substances such as hormones, neurotransmitters, and other endogenous compounds. The global demand on perfumes, cosmetics, beverages, confectionery products, and household chemicals containing rose-smelling essential oils is constantly growing that leads to an increase in oil production.

Therefore, the development of the Eremothecium oil biotechnology as a novel technology for essential oil production requires fundamental and applied research at molecular, cellular, and organism levels, including the study of the producer genomics, proteomics, and metabolomics. The search for new sources of essential oil biotechnological raw materials also implies the improvement of methodological issues, in particular, the physiological and biochemical selection criteria. As a rule, the degree of mycromycete colony pigmentation is caused by the flavinogenesis level. The revelation of correlation between colony pigmentation and aromatic and monoterpene alcohol accumulation can be a simple visual criterion to determine highly active essential oil producers. This reduces selection process complexity.

This paper aims to investigate processes of fragrant compound accumulation by *Eremothecium ashyi* strains with different levels of riboflavin synthesis.

EXPERIMENTAL

In our study, we used *Eremothecium ashbyi* Guilliermond 1935 strains VKM F-124, VKM F-3009, and VKPM F-340 (Pinsk-86) from the All-Russian Collection of Microorganisms (Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Russia) and the Russian National Collection of Industrial Microorganisms (State Research Institute of Genetics and Selection of Industrial Microorganisms, Russia). Microorganisms were maintained on slant potato glucose, wort, and malt agars and Sabouraud and Czapek media.

The inoculum was grown at 28°C for 72–96 h on slant glucose peptone agar containing 2.0 g × L^{-1} of yeast extract, 2.0 g × L^{-1} of peptone, 10.0 g × L^{-1} of glucose and 20.0 g × L^{-1} of agar at pH from 6.5 to 6.7.

The submerged cultivation was then performed in flasks on a shaker at 200 rpm and 28°C for 24–72 h. We used liquid soybean sucrose medium containing 15.0 g × L^{-1} of sucrose, 20.0 g × L^{-1} of soybean meal, 10.0 g × L^{-1} of corn extract, and 1.0 g × L^{-1} of potassium hydrogenphosphate at pH levels from 6.0 to 7.0. The inoculum volume was 5% of the total medium volume.

The submerged fermentation was performed in glucose peptone medium (7.5 g × L^{-1} of glucose, 4.0 g × L^{-1} of peptone, 2.0 g × L^{-1} of sodium succinate, 0.5 g × L^{-1} of potassium hydrogen phosphate, and 0.14 g × L^{-1} of inositol at pH 6.5). The strains were cultured during continuous shaking at 220 rpm and a temperature of 28°C for 24–120 h. CB samples were collected in triplicate every 12 h to examine product accumulation dynamics.

We studied unstained and stained (methylene blue, iodine, Sudan III, and black ink) preparations using a BIOMED-6 microscope (Biomed, Russia) at magnifications of 4×, 10×, 40× and 100×.

In 12, 36, 48, 56, and 64h, we sampled CB for electron microscopy and fixed this material with 5% glutaraldehyde solution in phosphate buffer for 24 h. Buffer pH varied from 5.8 to 7.0 and depended on CB pH value. Samples were washed with phosphate buffer solution for 10 min and fixed with a 1% aqueous OsO_4 for 1.0–1.5 h. The standard electron microscopy preparation and embedding was performed, including an additional staining with 5% uranyl acetate solution in 70% ethanol for 12 h. Ultrathin sections were prepared using an ultramicrotome, stained with Reynolds' lead citrate for 5 min, and examined under a JEM-100 C microscope (JEOL, Japan) at magnifications from 15 to 41 thousand times.

The riboflavin content was determined on a SF-103 spectrophotometer (Akvilon, Russia) at a wavelength of 444 nm. Mycelium were lysed to transfer completely riboflavin to CB. One milliliter of 6 N hydrochloric acid was added to 59 mL of CB and kept at 100°C for 30 min; and then a mixture (lysate) was filtered. Riboflavin content in filtrates was determined using calibration curve. The curve was constructed using absorbance values of aqueous riboflavin solutions at concentrations of 0.05, 0.1, 0.3, 0.5, 0.7, 1.0, and 1.5 g × L^{-1} at 444 nm. The culture medium was used as a reference solution. No substances with absorption maxima at (444 ± 1) nm were detected in the culture media used for riboflavin synthesis by fungi.

Lipophilic substances, including aroma forming compounds (essential oil extract), were extracted from CB with hexane three times without cell separation [5, 6]. Hexane was then removed on a rotary evaporator under vacuum, and the residue was weighed on an analytical balance.

The component composition of fragrant compounds was determined via a Perkin Elmer Clarus 680 gas chromatograph (USA) equipped with a flame ionization detector and a polar column (INNOWAX, 60 m × 0.32 mm). The temperature was programmed to increase from 80 to 250°C at a rate of 2°C × min^{-1}. Samples (0.2 μL) were injected at a temperature of 250°C; the detection temperature was 250°C. Flow rate of carrier gas (helium) was 0.5 mL × min^{-1}.

The statistical data analysis was performed using Excel (Microsoft) and Statistica software. To estimate the variability of quantitative characteristics, we used standard deviation from arithmetic mean values. The correlations were determined via interval variables and Spearman's coefficients (when parameters had distributions different from the normal one). The analysis results were given as p-criteria, and difference was considered to be significant at $p \le 0.05$ (critical level of significance) [7, 8].

RESULTS AND DISCUSSION

In our study, we compared different *E. ashbyi* strains: industrial VKPM F-340, yellow VKM F-3009, and white VKM F-124 (a mutant obtained by E.F. Semenova [9]). The color intensity of strain colonies differed depending on the riboflavinogenesis level [10–12].

We examined the dynamics of biomass accumulation during strain submerged cultivation and revealed no significant differences from the known regularities. The log phase of growth was observed till 36 h and followed by a slowdown in growth rate (transition to stationary phase). Cell autolysis occurred at the end of fermentation (Fig. 1). Sixty-hour cultivation of strains VKPM F-340 and VKM F-3009 and a white mutant of strain VKM F-124 resulted in minimum (1.06 g × L^{-1}), medium (2.01 g × L^{-1}), and maximum (2.89 g × L^{-1}) values of biomass accumulation, respectively. The submerged microorganism cultivation led to medium acidification during log phase and alkalization during stationary and decline phases. After 60 h, strain VKM F-124 had higher level of biomass accumulation and medium alkalization than other strains. The degree of medium alkalization might be caused by the changes in acid-base balance of the accumulated biomass and the intensity of alkaline metabolite excretion.

The riboflavin synthesis and accumulation in culture medium started in log phase and increased during stationary and decline phases (Fig. 2). The riboflavin synthesis by strain VKPM F-340 was the most intense. Strain VKM F-3009 and the white mutant of strain VKM F-124 had less intense riboflavin synthesis (three and ten times, respectively) than strain VKM F-340.

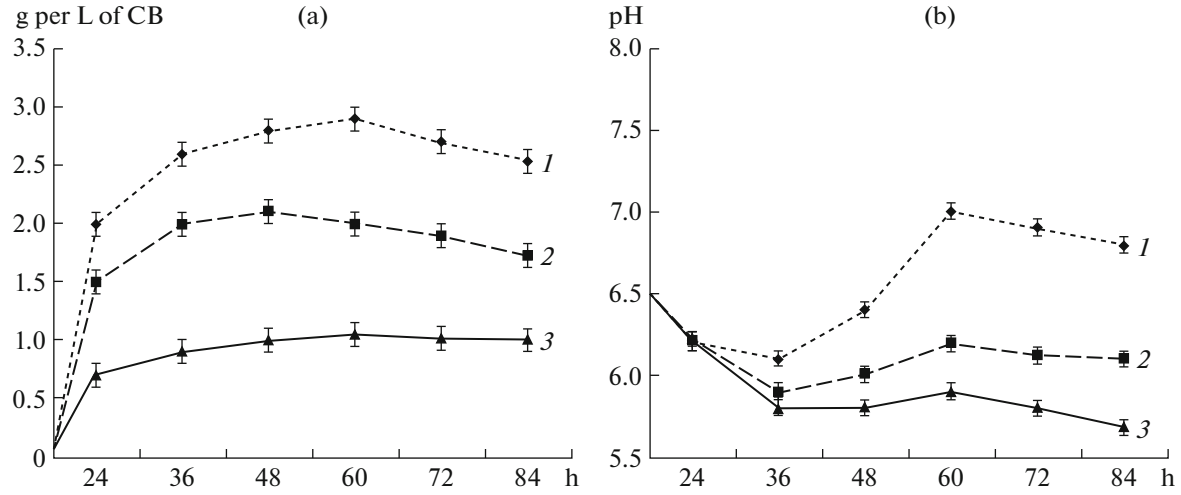

Fig. 1. (a) Dry biomass accumulation and (b) pH changes of CB during submerged cultivation of (*1*) VKM F-124, (*2*) VKM F-3009, and (*3*) VKPM F-340 strains of *E. ashbyi*.

The intensity of accumulation of the main aroma forming compounds (geraniol, citronellol, nerol, and 2-phenylethanol) was different among these strains. The most intense accumulation of these compounds was observed in stationary phase (Figs. 3 and 4). Significant differences in geraniol synthesis were noticed at certain stages of culture development. The highest increase in geraniol accumulation by strain VKM F-3009 was observed between 48 and 60 h while for strain VKPM F-340 and VKM F-124 it was revealed between 48 and 60 h. Nevertheless, the latter had the insignificant geraniol increase.

It was noted above that in 24 h essential oil and riboflavin started to accumulate within mycelia and in medium, and lipid bodies formed in micromycete vegetative hyphae (Fig. 5). Among all strains, the increase in the essential oil synthesis efficiency (Figs. 3 and 4) was accompanied with the growth in spherosome quantities and sizes.

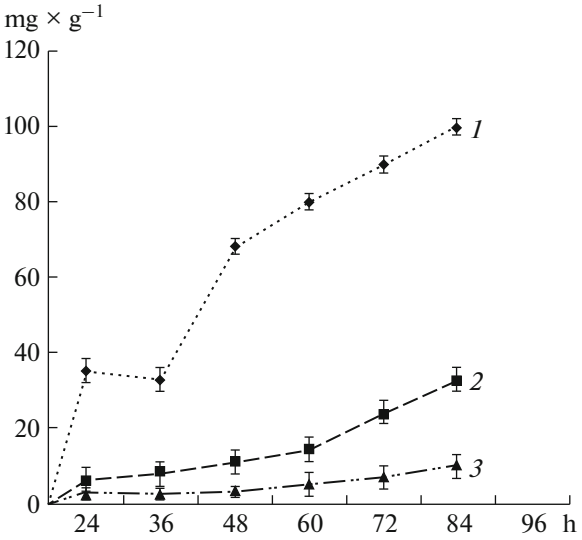

Fig. 2. Accumulation (mg × g^{-1}) of vitamin B_2 during cultivation of (*1*) VKPM F-340, (*2*) VKM F-3009, and (*3*) VKM F-124 strains of *E. ashbyi*.

The marked vacuolization (after 36–48 h) and the beginning of sporogenesis (after 48–60 h) were observed during the period of the highest accumulation of aroma forming compounds and vitamin B_2 (Fig. 6). Strain VKPM F-340, which possessed the highest intensity of essential oil synthesis, had more intense vacuolization (numerous small vacuoles) than other strains. The vacuolization of this strain started at earlier stages and finished at the later stages of growth than that of other strains. Strain VKPM F-340 possessed more intense protoplasm lipophilicity (osmophilicity in electron microscopy) than other strains. Protoplasm lipid bodies virtually disappeared after the release of lipophilic metabolites (phenylethyl and monoterpene alcohols) into the medium at the end of stationary phase. The excretion of aroma forming compounds might be a way to control their synthesis using the mechanisms of overflow or excess metabolite excretion [13, 14].

The content analysis of data on the metabolic pathways of riboflavin and aroma forming compound biosyntheses in eukaryotes allowed us to propose a hypothetical model, which describes the riboflavin and essential oil biogenesis in *E. ashbyi* and includes basic biochemical reactions for correlation determination among all reactions (Fig. 7) [8, 13–19]. According to this model, fatty acids, glucose, aspartate, asparagine, glycine, serine, and threonine are the main substrates for the riboflavin synthesis, whereas fatty acids, glucose, and leucine are substrates for the monoterpene alcohol synthesis (geraniol, nerol, and citronellol); and phenylalanine participates in the aromatic alcohol synthesis (β-phenylethanol). Acetyl-CoA, which is

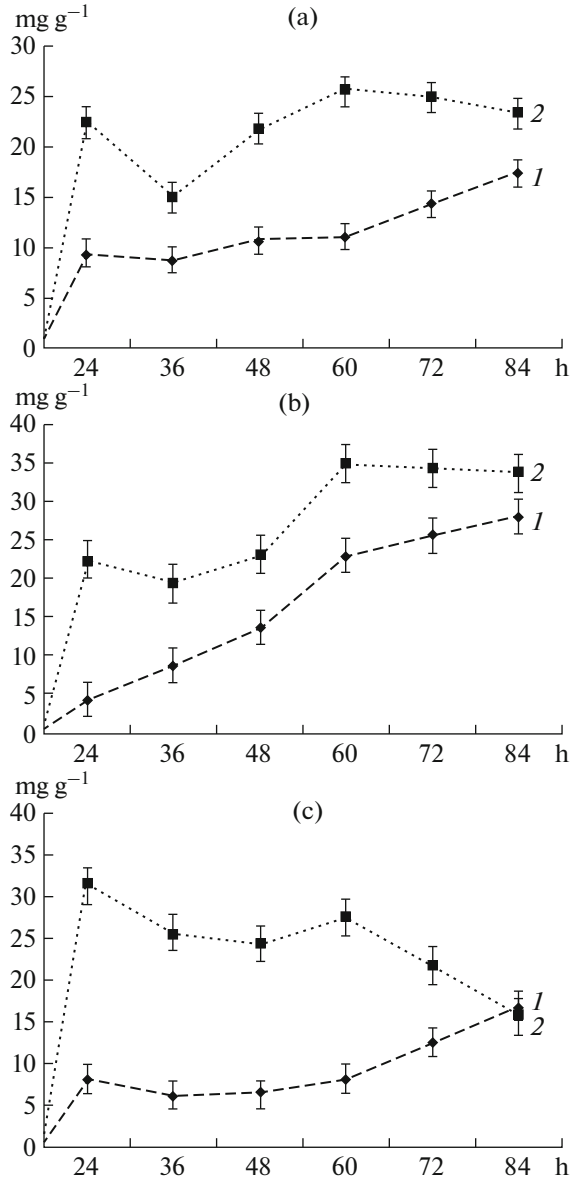

Fig. 3. Accumulation (mg × g^{-1}) of (*1*) geraniol and (*2*) β-phenylethanol during submerged cultivation of (a) VKM F-3009, (b) VKPM F-340, and (c) VKM F-124 strains of *E. ashbyi*.

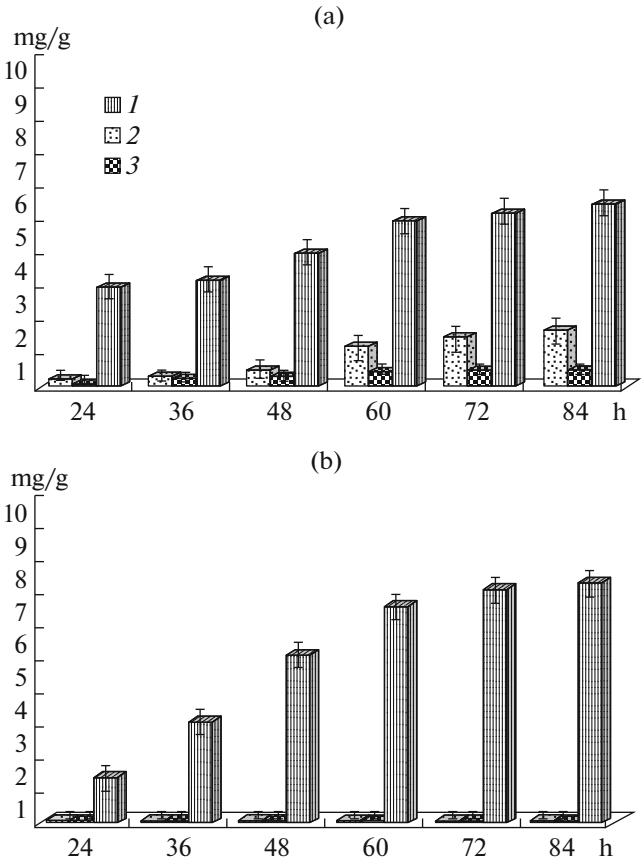

Fig. 4. Accumulation (mg × g^{-1}) of (a) citronellol and (b) nerol during submerged cultivation of (*1*) VKPM F-340, (*2*) VKM F-3009, and (*3*) VKM F-124 strains of *E. ashbyi*.

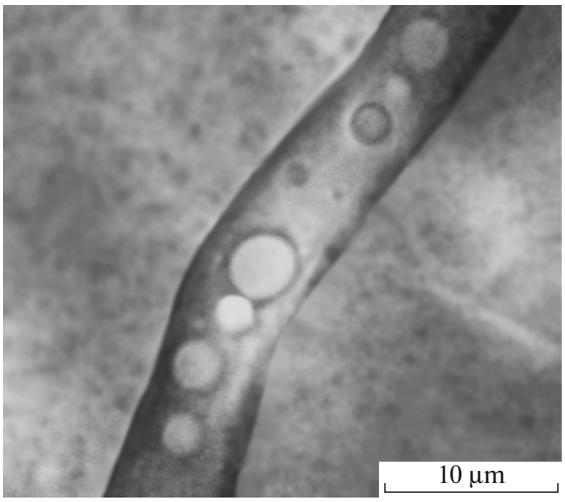

Fig. 5. Lipid droplets in a *E. ashbyi* hypha (Sudan III staining).

formed during glucose and fatty acid catabolism, is a key compound in vitamin and monoterpene syntheses. This metabolic model enables us to understand the relationships among the processes of the synthesis of flavins and terpene and aromatic alcohols, their direction, cellular localization, and the regulation mechanisms of *E. ashbyi* productivity.

Figs. 2–4 show that strain VKPM F-340 had the highest vitamin and aroma forming compound productivity. However, our study of the dynamics of formation of these compounds revealed that in 60 h the accumulation of monoterpenes had no changes and remained approximately the same, while that of riboflavin continued to increase. This might be caused by the decreased medium glucose concentration, which led to cessation or reduction of the rate of monoterpene alcohol synthesis (geraniol, nerol, and citronel-

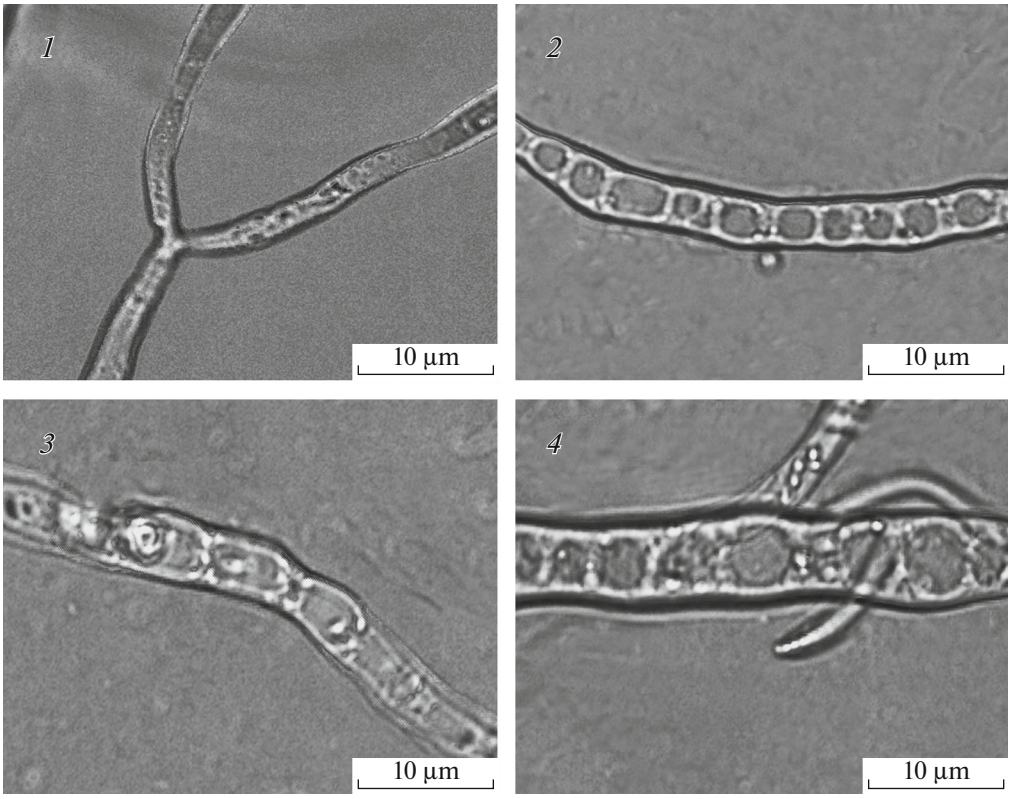

Fig. 6. Mycelium of *E. ashbyi* during (*1*) log phase, (*2*) a slowdown in the growth, (*3*) stationary phase, and (*4*) at the beginning of decline phase.

lol) and affected the synthesis of riboflavin and β-phenylethanol in a less degree. The latter can be synthesized from amino acids [14, 20–22]. β-Phenylethanol was detected in CB of all strains; this circumstance indirectly indicates the expression of active genes, for example, *ARO8a*, *ARO8b*, *ARO10*, or *ARO80*, which are responsible for aromatic amino acid catabolism [20].

Literature analysis [6, 12–14, 16, 20–22] and our data suggested that the riboflavin and monoterpene biosyntheses might be coupled in regard to their dynamics and cellular localization (compartmentation), and their gene regulation may also be interconnected. The correlation analysis of the content of riboflavin, geraniol, citronellol, and nerol and their total content in CB confirmed this assumption with a confidence level of 95.0–99.9% (table). The Spearman's coefficients showed strong (VKM F-124) and very strong (VKM F-3009 and VKPM F-340) direct (positive) simple (between riboflavin and monoterpene alco-

Spearman's correlation coefficients between riboflavin and monoterpene alcohol syntheses by *E. ashbyi* strains

Compound	Correlation coefficients			Significance level range
	Producer			
	VKM F-124	VKM F-3009	VKPM F-340	
Geraniol	0.81	0.94	0.94	0.005–0.052
Citronellol	0.97	1.00	0.94	0.001–0.050
Nerol	–	1.00	0.94	0.005–0.050
Geraniol + citronellol	0.90	0.94	0.94	0.005–0.015
Geraniol + nerol	0.81	1.00	0.94	0.005–0.052
Citronellol + nerol	0.97	1.00	0.94	0.005–0.050
MTAs[a]	0.90	1.00	0.94	0.005–0.050
PEA[b]/(citronellol + nerol)	–0.90	–0.94	–0.94	0.000–0.005

[a] MTAs are monoterpene alcohols, [b] PEA is phenylethyl alcohol, and "–" means that the compound was not detected.

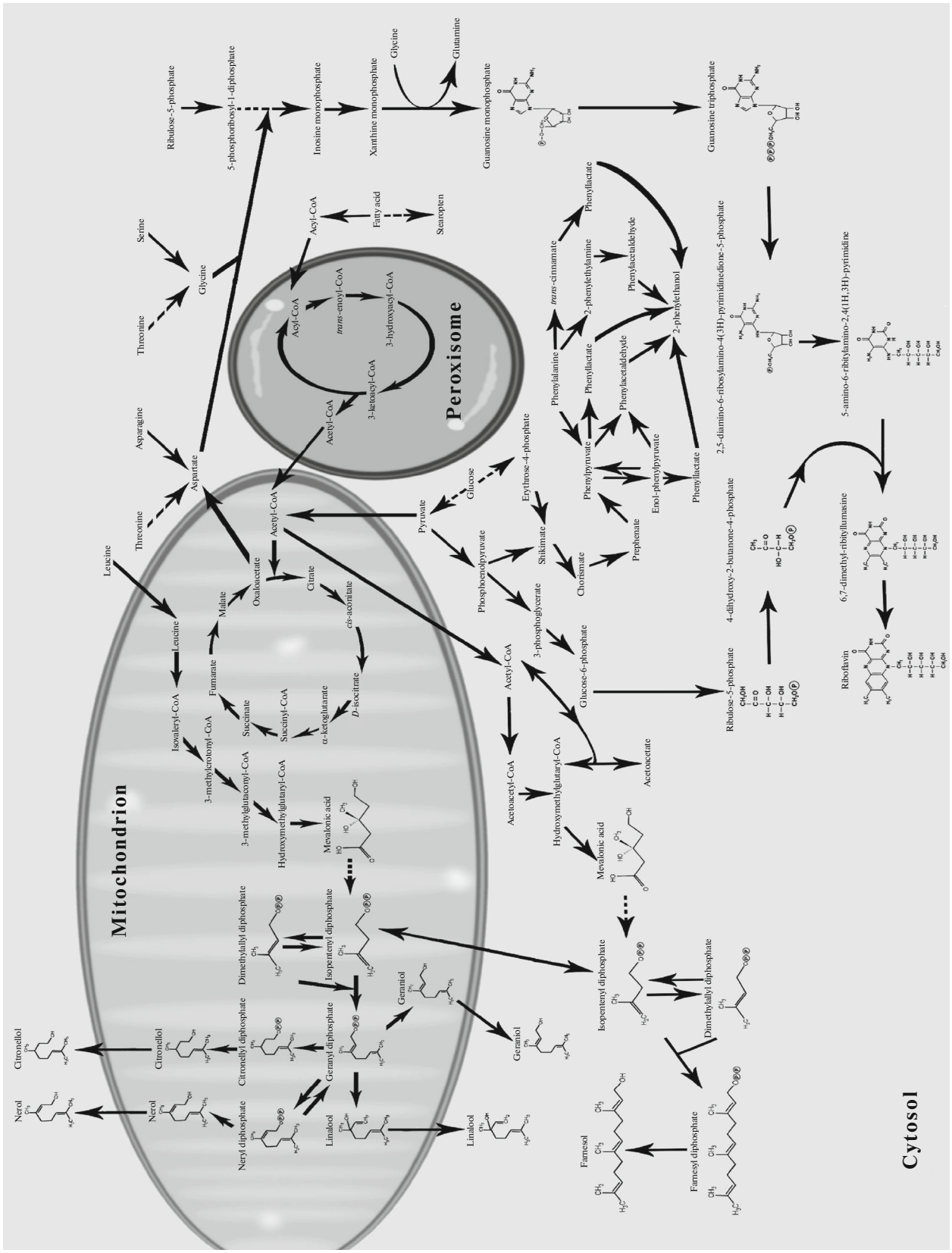

Fig. 7. Hypothetical metabolic model of biosynthesis of riboflavin, phenylethanol, and terpene alcohols by *E. ashbyi* cells.

hols), and multiple (between riboflavin and monoterpene alcohol combinations) correlations. Therefore, the high level of riboflavinogenesis can serve as a marker of high cell metabolic activity and an additional criterion for the selection of mutants with high level of aroma forming compound synthesis.

These results are of great importance for the development of methods for regulation of the biosynthesis of industrially important metabolites, such as riboflavin and phenyl ethyl and monoterpene alcohols. However, these data should be further evaluated using molecular biology techniques to determine possible methods of gene regulation of these processes.

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