Research Article

Study of the Effect of Squalene Epoxidase Activity on Squalene Biosynthesis by Yeast Saccharomyces Cerevisiae VGSh-2

Olga L. Meshcheryakova, Galina P. Shuvaeva, Tatyana V. Sviridova, Anna A. Tolkacheva, and Olga S. Korneeva

Voronezh State University of Engineering Technologies
ORCID
Anna A. Tolkacheva 0000-0003-0725-6482

Abstract. The researchers of this study investigated the biosynthesis of squalene by the yeast *S. cerevisiae* VGSH-2 through the activity of squalene epoxidase, which is a key enzyme in the conversion of squalene to ergosterol. It has been established that under aerobic conditions the antimycotic drug terbinafine promotes the switching of ergosterol formation to squalene synthesis. This switch occurs through specific inhibition of the squalene epoxidase of the yeast *S. cerevisiae* VGSH-2, thus increasing the biosynthetic ability of the yeast towards squalene. According to the results of this study, the optimal concentration of terbinofine in the nutrient medium was 0.3 μmol / cm^3. This concentration led to a 5-fold decrease in squalene epoxidase activity and a 7-8 times increase in squalene synthesis. The results obtained can be used to develop a competitive technology for the industrial production of squalene by microbial synthesis.

Keywords: squalene, yeast, biosynthesis, inhibition of activity, terbinafine, squalene epoxidase, *Saccharomices cerevisiae* VGSH-2

Corresponding Author: Olga S. Korneeva; email: korneeva-olgas@yandex.ru

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1. Introduction

The problem of obtaining biologically active substances, including functional lipids, is one of the up-to-date problems of modern biotechnology and pharmacology. The use of functional lipids, phytosterols, phytostanols and their esters in functional food technology has become a widely recognized, successful innovation, since each of these lipid classes has a specific targeted effect on human health. One of these functional lipids is the precursor of the biosynthesis of sterols and steroids - squalene, which has a unique pharmacological activity. It is a natural antioxidant [1], has antitumor [2, 3], radioprotective and immunomodulatory effects [4], and also helps to decrease blood cholesterol level [5].

Squalene is a hydrocarbon, a natural component of vegetable oils, such as olive oil, palm oil, wheat germ oil [6-10]. Squalene is commercially obtained from fish liver, especially shark liver [11], and purified for use in pharmaceuticals and vaccines. However,

the presence of cholesterol in the lipid composition makes it difficult to purify squalene and makes it one of the most scarce and expensive products. The microbiological synthesis of squalene can be one of the ways to obtain this hydrocarbon, and therefore the search for highly active producers of squalene biosynthesis, the development of modes of their cultivation and methods for the isolation of squalene is an urgent task of modern biotechnology.

It is known that yeast is able to synthesize squalene, which is an intermediate product in the biosynthesis of ergosterol, a precursor of lanosterol. The indicated transformation of squalene occurs under the effect of enzymes, the key of which is squalene epoxidase, which converts it into 2,3-oxido-squalene, which participates in the cyclization reaction and the formation of lanosterol, followed by its conversion into ergosterol [6]. One of the ways to increase the biosynthetic ability of yeast in relation to squalene is to inhibit the activity of squalene epoxidase. Considering that a promising producer of squalene is the yeast of the genus *Saccharomyces* due to its economy, safety, and initially high productive capacity for the synthesis of squalene in comparison with other yeasts [12, 13], the aim of this work was to study the effect of aeration conditions and terbinafine on the activity of squalene epoxidase of the yeast *S. cerevisiae* VGSH-2 to increase their own biosynthetic ability in relation to squalene.

2. Materials and research methods

The object of the study was the strain of the yeast *S. cerevisiae* VGSH-2, obtained by the method of induced mutagenesis with exposure to ultraviolet radiation (UVL) and UVL in combination with a chemical mutagen (ethylene imine) from the yeast *S. cerevisiae* p. XII. The strain was deposited in the placePlaceTypeMuseum of PlaceNamePure Cultures of the Department of Biochemistry and Biotechnology, Voronezh State University of Engineering Technologies, VSUIT [14].

As an inhibitor of squalene epoxidase, an alliamine-fungicidal preparation, terbinafine, was used, which has antifungal and general antimicrobial activity. This preparation has proven to be effective and safe because for quite a long time it has been used in medical practice as an antimycotic agent in the treatment of fungal infections [12, 15]. For the inhibition of the enzyme, a 1% solution of terbinafine in dimethyl sulfoxide was prepared.

The cultivation of the producer was carried out at the beginning in aerobic, then in anaerobic conditions in a nutrient medium of the following composition, g/dm^3: glucose

- 20, yeast extract - 5, peptone - 5, malt extract - 5. Determination of squalene content in each flask was carried out every 12 hours.

Cultivation of yeast under aerobic conditions. To obtain an inoculum, a daily culture of the yeast *S. cerevisiae* VGSH-2 was grown in a nutator with a rotation frequency of 250 rpm, at a temperature of 30 ± metricconverterProductID2°C2°C for 72 h, in shake flasks with a volume of 250 cm^3, containing 100 cm^3 of the nutrient medium pH 5.5.

Cultivation of yeast under anaerobic conditions. The inoculum of the yeast *S. cerevisiae* VGSH-2 was transferred into 250 cm^3 flasks containing 100 cm^3 of fresh nutrient medium. The flasks were sealed with a gas outlet and left under anaerobic conditions for 24-72 hours at a temperature of 30 ± metricconverterProductID2°C2°C and a pH of 5.5.

The yeast biomass was separated from the supernatant by centrifugation at 10,000 rpm for 10 min. The resulting precipitate was used to determine squalene and squalene epoxidase activity.

Determination of squalene epoxidase activity. The squalene epoxidase activity was determined by the TLC method [16]. For this, a standard analytical mixture of the following composition was used in a volume of 0.3 cm^3: yeast suspension with a mass concentration of 3 mg/cm^3); 100 μM Tris-HCl buffer, pH 7.5; 100 μM FAD; metricconverterProductID3 mM3 mM NADP; metricconverterProductID1 mM1 mM EDTA; l mM AMO-1618 and 32 μM ^{3}H-squalene. The resulting mixture was dispersed with 0.005% Tween 80. The reaction was carried out in a glass test tube with a screw cap at 30 °C in a water bath with constant shaking. After a pre-incubation for 10 minutes, the reaction was initiated by the addition of squalene, maintained for 30 minutes, and stopped by the addition of 0.6 cm^3 of 90% v/v ethanol, containing 15% KOH and 0.2% pyrogallol. The tubes were sealed and heated for 20 min at 75 °C for saponification. The unsaponified materials were extracted twice with 2.5 cm^3 of petroleum ether. The solvent was evaporated under a stream of nitrogen and the residue was dissolved in a small volume of diethyl ether and applied to precoated TLC plates with silica gel and ergosterol acetate as an ultraviolet visible marker for 2,3-oxido-squalene. The enzyme activity was expressed in pmol of 2,3-oxido-squalene formed per mg of cellular protein / min.

Isolation of squalene from yeast cells. The destruction of yeast cell walls was carried out by autolysis with the addition of isopropyl alcohol to the reaction mixture in a ratio of 1:3, respectively, at a temperature of 40° C for 60 h. Squalene was extracted with a chloroform/methanol mixture (2:1). The biomass was removed by filtration through cotton wool, and the filtrate was passed through activated molecular sieves to remove residual moisture. The biomass was washed 2-3 times with a fresh chloroform/methanol

mixture (20 cm^3 each time). The solvent was then removed from the extract using a rotary evaporator at metricconverterProductID500 mm500 mm Hg and a temperature of metricconverterProductID45 °C45 °C until a viscous oily substance was obtained. The crude extract was a two-layer product: the upper layer was a white fatty substance, the lower one was a pale-yellow oily substance.

Determination of squalene and ergosterol by HPLC. To construct the calibration dependence, squalene obtained from shark liver, additionally purified by the TLC method, was used. The analysis was performed on a liquid microcolumn chromatograph with a UV detector at wavelengths of 210 and 220 nm at metricconverterProductID40 °C40 °C for 12 minutes for each sample at a mobile phase flow rate of 200 μL/min. Acetonitrile with a mass fraction of 100% was used as an eluent. For chromatography, a reversed phase column (ProntoSil-120-5-C18 AQ. Size: 2.0x75 mm. Grain: 5.0 μm) was used.

The concentration of ergosterol was determined by the method of direct analysis of the extract using a normal-phase column with LichrospherSi silica gel metricconverter-ProductID150 mm150 mm in length, metricconverterProductID4.6 mm4.6 mm in inner diameter, and 5 μm in sorbent particles. The mobile phase was hexane-isopropanol (95:5), the spectrophotometric detection wavelength was 282 nm [17].

3. Research results and discussion

It is known that the content of ergosterol increases 2-3 times when the yeast is affected by various kinds of radiation. This effect leads to an increase in the metabolic activity of the producer, in particular, an increase in the synthesis of a number of hydrolytic enzymes, an increase in the content of ergosterol and, accordingly, of squalene, which may be explained by the inhibition of the amination process, accompanied by a change in cell metabolism and an increase in lipid synthesis [12]. On this basis, to study the inhibition of squalene epoxidase and squalene biosynthesis, there was used a mutant strain of the yeast *S. cerevisiae* VGSH-2.

The biogenetic sequence of the synthesis of steroid substances in yeast cells can be represented as the following scheme: acetyl-CoA - mevalonate - farnesyl pyrophosphate - squalene - lanosterol - ergosterol [18]. Since ergosterol performs several important functions in yeast cells, sterol synthesis must be under tight regulation. There are two possibilities for controlling yeast sterol levels. The first one is anaerobic, since yeast cannot synthesize the sterol ring in the absence of molecular oxygen, and it becomes permeable to exogenous sterols [16]. Under these conditions, depletion or enrichment

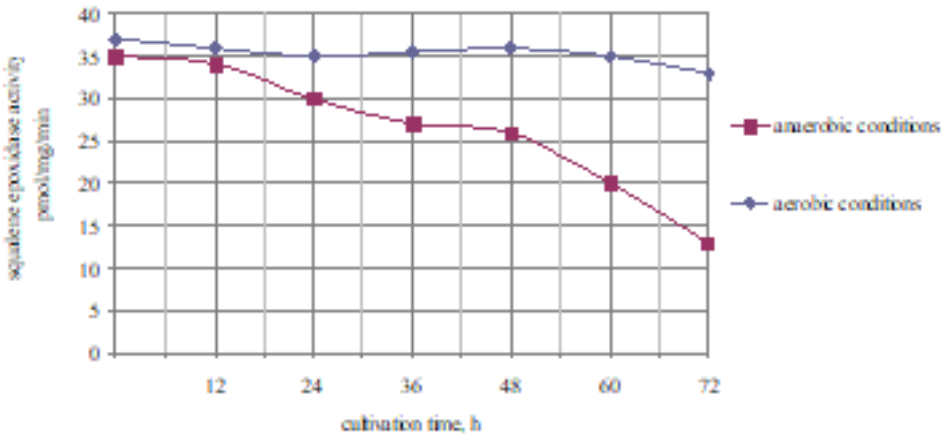

Figure 1: Effect of aeration conditions on the activity of squalene epoxidase *S. cerevisiae* VGSH-2.

of sterols and a decrease in the activity of key enzymes for converting squalene to lanosterol may occur.

The second possibility is associated with the use of inhibitors of key enzymes of ergosterol biosynthesis under aerobic conditions by blocking their active centers and, as a result, regulation of the formation of ergosterol precursor molecules. In an environment with molecular oxygen, with aeration of the environment, the squalene content in yeast cells is insignificant, since the process of accumulation of sterols prevails. Sterol synthesis under anaerobic conditions is not associated with yeast growth. The content of sterols increases with aging of the culture and sterol formation continues after the growth of cells stops, in the stationary phase.

A study of the effect of aeration on squalene epoxidase activity is shown in Fig. 1. The squalene epoxidase activity during yeast cultivation under anaerobic conditions was 26 pmol/mg/min, which is significantly less than the control activity under aerobic conditions (35 pmol/mg/min) (Fig. 1).

The squalene formation stage is the first reaction with molecular oxygen. In the presence of oxygen, squalene epoxidase is activated and promotes the rapid conversion of squalene to 2,3-oxido-squalene, and later to sterols. Under aerobic conditions, the activity of squalene epoxidase practically does not change, accumulation of cellular ergosterol is observed, and the content of squalene in yeast cells is insignificant, but sufficient for their vital activity, which is confirmed by studies of other scientists [14, 19]. This is due to the fact that oxygen is an activator of this enzyme and promotes the conversion of squalene to sterols. The results obtained confirm the activating effect of oxygen on the activity of squalene epoxidase. Thus, under anaerobic conditions,

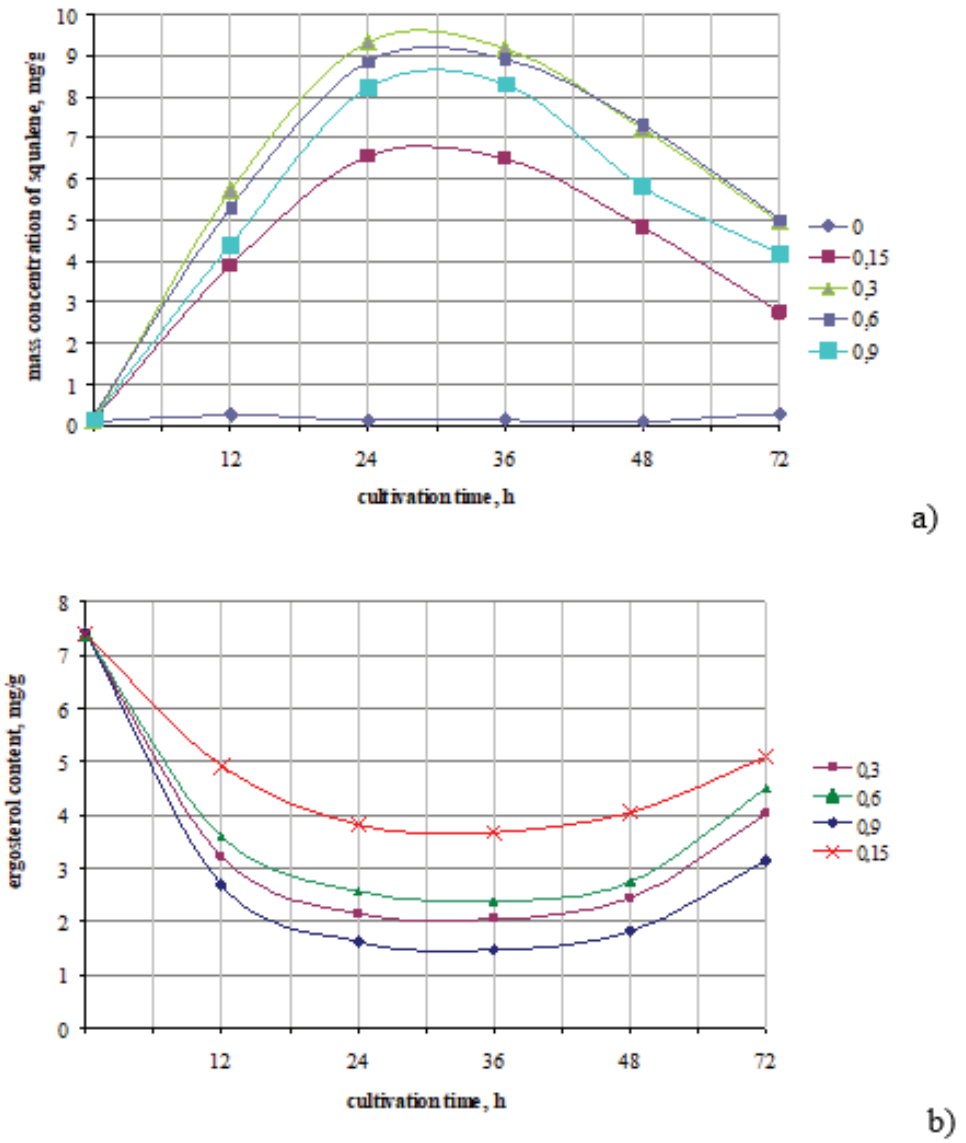

Figure 2: Synthesis of squalene (a) and ergosterol (b) in yeast cell *S. cerevisiae* VGSH-2 at various concentrations of terbinafine, μmol/cm^3.

after 42-48 hours of yeast cultivation, a decrease in the enzymatic activity of squalene epoxidase by 40-50% is observed.

To switch the process of ergosterol formation under aerobic conditions to the process of squalene synthesis, it is necessary to inactivate squalene epoxidase. Terbinafine selectively inhibits eukaryotic squalene epoxidase, not being an analogue of the substrate [20]. With the help of the naphthalene-containing center of its molecules, it suppresses the formation of this enzyme, which leads to the arrest of biosynthesis and depletion of the cellular reserves of ergosterol and the accumulation of squalene in the cell.

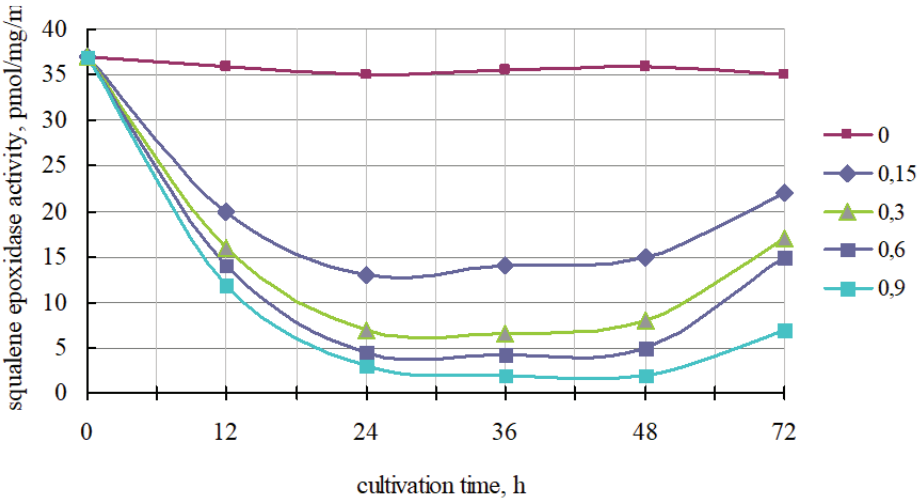

Figure 3: Effect of terbinafine concentration, μmol/cm^3, on squalene epoxidase activity

To reveal the effect of terbinafine on the activity of scalene-epoxidase and the accumulation of squalene in the producer's cells, it was sequentially introduced into nutrient media at concentrations from 0.15 to 0.9 μmol/cm^3. The culture was incubated at 30 ± metricconverterProductID2 °C2 °C for 72 h and pH 4.0-5.0. A control flask of *S. cerevisiae* VGSH-2 was grown under the same conditions in the absence of terbinafine.

Analysis of experimental data showed that when terbinafine was added to the nutrient medium in an amount of 0.3 μmol/cm^3 in yeast cells, the mass concentration of squalene increased to 9.15 mg/g, while the content of ergosterol decreased to 2.32 mg/g (Fig. 2, 3). Longer yeast cultivation led to a decrease in the amount of squalene and an increase in the synthesis of ergosterol, which may be associated with the use of squalene by yeast as a carbon source or its conversion into sterol.

In parallel, studies were carried out on the effect of the dosage of terbinafine on the activity of squalene epoxidase. It was found that when terbinafine was added to the medium in an amount of 0.9 μmol/cm^3, the greatest decrease in the squalene epoxidase activity was observed to 2.6 pmol/mg/min (Fig. 3). At the same time, a lower content of squalene and an almost complete absence of ergosterol were noted, which may occur as a result of a decrease in the viability of yeast cells due to the fungistatic effect of this preparation. With the content of terbinafine in the nutrient medium at a concentration of 0.3 μmol/cm^3, the squalene epoxidase activity was 7.0 pmol/mg/min. This process is dose-dependent; exceeding the required concentration of terbinafine in the nutrient medium has a fatal effect on yeast cells [21, 22].

Thus, the optimal concentration of terbinafine, which provides an increase in the synthesis of squalene by 7-8 times and a decrease in the activity of squalene epoxidase by 5 times, is 0.3 μmol/cm^3.

4. Conclusion

As a result of the research, the dependence of squalene biosynthesis by the yeast *S. cerevisiae* VGSH-2 on the activity of squalene epoxidase was established. It has been shown that under aerobic conditions, when the antimycotic preparation terbinafine is used in the nutrient medium, the activity of squalene epoxidase decreases by 5 times in the cells of the yeast *S. cerevisiae* VGSH-2 due to its inhibitory effect, which leads to an increase in the synthesis of squalene in 7-8 times.

The results obtained can be used to develop a competitive technology for the industrial production of squalene by microbial synthesis.

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