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OPTIMIZATION OF CONDITIONS FOR BIOCATALYTIC PRODUCTION OF STIGMAST-4-EN-3-ONE

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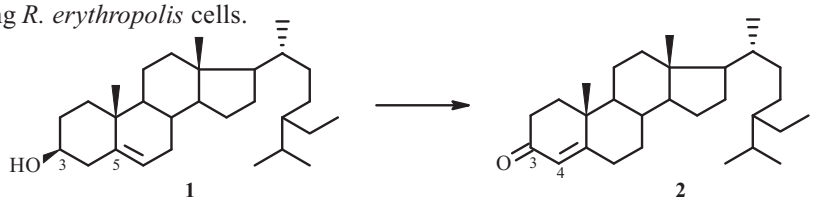
Pharmacologically active stigmast-4-en-3-one, the maximum formation of which (93.4%) was achieved in the presence of n-hexadecane and palmitic acid, was produced by biotransformation of β*-sitosterol (6 g/L) using rhodococcus cells.* β*-Sitosterol was added to the incubation medium as a mixture with* β*-cyclodextrin and Tween-80. The biotransformation processing conditions were optimized for* β*-sitosterol at high concentrations (10 g/L) using* R. erythropolis *IEGM 90 cells to catalyze formation of stigmast-4-en-3-one (75%).*

Keywords: phytosterols, biotransformation, *β*-sitosterol, stigmast-4-en-3-one, biological activity.

Modification of the polycyclic framework of available sterols, in particular *β*-sitosterol, which is obtained via chemical reprocessing of lumber mill wastes, is an effective method for preparing pharmacologically active compounds [1, 2]. *β*-Sitosterol and its derivatives exhibit angiogenic [3], antipyretic [4], antitumor [5], antimutagenic [6], and immunomodulating [7] activity and are used for prevention of cardiovascular diseases [8] and correction of lipid-fat exchange [9, 10]. Chemical methods for transforming *β*-sitosterol address mainly modification of the 3*β*-hydroxyl and the C-5 double bond. A relatively high (45–70%) level of *β*-sitosterol conversion is achieved through acylation of the sterol 3*β*-hydroxyl to form compounds with hypocholesterolemic activity [1, 11]. Multi-step methods for chemical synthesis of lactams, oximes, and aromatic derivatives of *β*-sitosterol in addition to compounds (in particular, ecdysteroids and brassinosteroids) with insecticidal and phytohormonal activity were developed [1]. However, the yields of desired products were < 25%. *β*-Sitosterol is transformed more effectively by using biocatalytic methods that enable *β*-sitosterol to be selectively modified with a high degree of regio- and stereoselectivity, including molecular centers that are difficultly accessible to chemical synthesis [12]. As a rule, the principal products are androst-4-en-3,17-dione and androsta-1,4-dien-3,17-dione (31.0–70.6%), key intermediates in the industrial synthesis of hormone preparations [13]. Furthermore, androstane hydroxyl derivatives (in particular 9*α*-hydroxyandrost-4-en-3,17-dione and testosterone) [14, 15] and products with a C-7 double bond, oxidized aliphatic chain, and opened ring D [16, 17] were prepared using biocatalytic methods and *β*-sitosterol.

The product of oxidative transformation of *β*-sitosterol (**1**), stigmast-4-en-3-one (**2**), the pharmacological effectiveness of which for treating hyperglycemia and androgen-dependent diseases was 8.4–39.2 times greater than that of the starting sterol, was reported [18–21]. Compound **2** was formed in the first step of bacterial transformation of *β*-sitosterol via oxidation of the 3*β*-hydroxyl and isomerization of the C-5 double bond to the C-4 position (Scheme 1). The maximum yield (40%) of the desired product was attained by using the actinobacterium species *Rhodococcus erythropolis* [22]. A chemical method for preparing **2** in 70% yield via Oppenauer oxidation of **1** using cyclohexanone as the oxidant was described [23]. We found earlier [24] that *R. erythropolis* converted **1** (0.5 g/L) in 80% yield into stigmast-4-en-3-one in the presence of *n*-hexadecane and palmitic acid. Herein we present optimized conditions for biotransformation of *β*-sitosterol at high concentrations (from 4 to 10 g/L) into **2** using *R. erythropolis* cells.



1 **2**

Scheme 1

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TABLE 1. Screening of Sterol-Transforming Activity of *R. erythropolis* Collection Strains with Added β-Sitosterol (10 g/L)*

Strain	Stigmast-4-en-3-one, %	Strain	Stigmast-4-en-3-one, %
Abiotic control	0	IEGM 270	46.9
IEGM 10	51.5	IEGM 487	45.5
IEGM 11	60.5	IEGM 490	75.4
IEGM 18	59.0	IEGM 503	54.2
IEGM 20	56.7	IEGM 507	59.7
IEGM 179	43.8	IEGM 609	55.4
IEGM 183	57.4	IEGM 682	48.7
IEGM 190	51.8	IEGM 683	51.4
IEGM 212	56.8	IEGM 698	49.2
IEGM 244	56.6	IEGM 745	52.4
IEGM 267	50.8	IEGM 766	59.6

*β-Sitosterol was added to the fermentation medium as a mixture with β-cyclodextrin (1:1) in 1.2% aqueous Tween-80.

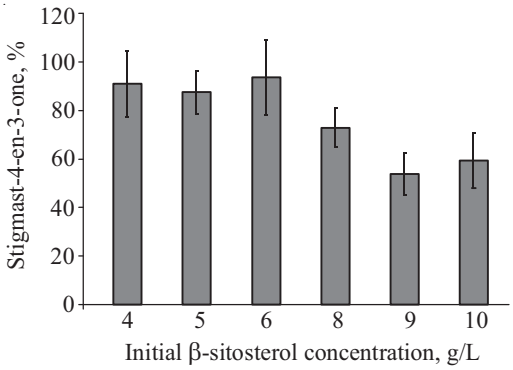

Fig. 1. Formation of stigmast-4-en-3-one by *R. erythropolis* IEGM 487 cells with added *n*-hexadecane (2.5 g/L), palmitic acid (0.15 g/L), and β-sitosterol (4–10 g/L) as a mixture with β-cyclodextrin (1:1) in 1.2% aqueous Tween-80 (80–200 mL).

The yield of the desired product was <50% if **1** was added as a solution in *i*-PrOH or synthetic surfactants (Tween-40, Tween-60, or Tween-80). The highest yield (47%) of **2** was obtained in experiments using Tween-80. Figure 1 shows that addition of **1** to the incubation medium as a mixture with Tween-80 and β-cyclodextrin increased significantly the effectiveness of producing **2** at high sterol concentrations.

The formation of **2** was maximum (93.4%) upon addition to the growth medium of β-sitosterol (6 g/L) in a mixture of β-cyclodextrin (1:1) in aqueous Tween-80 in the presence of *n*-hexadecane (2.5 g/L) and palmitic acid (0.15 g/L). Increasing the initial concentration of the substrate to 8–10 g/L reduced the sterol-transforming activity of *R. erythropolis* by 20–40%.

Table 1 presents results from screening of the biotransforming activity of various *R. erythropolis* strains upon adding β-sitosterol (10 g/L). The most active β-sitosterol biotransformer among the studied strains was *R. erythropolis* IEGM 490, which converted 75% of **1** into **2** (Table 1).

Stigmast-4-en-3-one was isolated effectively in 71.5% yield by column chromatography over silica gel with separation of 9.8 g/L of total reaction products that were acylated beforehand using Py and Ac_2O. The structure of **2** was confirmed using PMR spectroscopy.

EXPERIMENTAL

NMR spectra were recorded from $CDCl_3$ solutions with HMDS internal standard on a Varian Mercury Plus 300 (300 MHz) spectrometer. Melting points were measured on a PTP apparatus (Russia). Specific optical rotation was determined in EtOH solution on a Perkin–Elmer (USA) model 341 polarimeter at wavelength 589 nm.

The product mixture from biotransformation of β-sitosterol was analyzed by TLC using silica gel 60 F_{254} plates (Merck, Germany) with a fluorescent additive and a mixture of petroleum ether (60–80°C) and EtOAc (7:3). The formation of **2** was detected using an LG-58 UV-lamp (Russia). Residual **1** was determined after spraying H_2SO_4 (5%) and heating at 95–100°C for 2-3 min. The content of **2** in the total reaction products was estimated by UV spectroscopy on a Lambda EZ201 spectrometer (Perkin–Elmer, USA) [25] and GC/MS on an Agilent 6890N gas chromatograph with an HP-5MS SN US 15189741-1 quartz column and an MSD 5973N quadrupole mass spectrometer (Agilent Technology, USA). Column chromatography was performed using silica gel (60–200 μm, Merck, Germany) with a compound:sorbent ratio of ~1:20 with elution by hexane:EtOAc (98:2).

We used *R. erythropolis* strains (all IEGM) 10, 11, 18, 20, 179, 183, 190, 212, 244, 267, 270, 487, 490, 503, 507, 609, 682, 683, 698, 745 and 766 from the Regional Specialised Collection of Alkanotrophic Microorganisms (official acronym IEGM, number in the World Federation for Culture Collections, 768; http://www.iegm.ru/iegmcol/index.html) [26]. The biotransformation was carried out in mineral medium of composition (g/L) KNO_3 (1.0), KH_2PO_4 (1.0), $K_2HPO_4 \cdot 3H_2O$ (1.0), NaCl (1.0), $MgSO_4 \cdot 7H_2O$ (0.2), $CaCl_2 \cdot 2H_2O$ (0.02) with added yeast extract (1 g/L) (FGUP NPO Mikrogen, Russia) and Postgate microelement solution (0.1 vol%) [27]. Compound **1** (4–10 g/L) was added to the medium as a mixture with β-cyclodextrin (1:1) in aqueous Tween-80 (80–200 mL, 1.2%) [28] and *n*-hexadecane (2.5 g/L) and palmitic acid (0.15 g/L) in *i*-PrOH (1.0 mL). The inoculating material was a suspension (5 mL) of *R. erythropolis* cells in normal saline (OD_{600} = 2.0) that were grown beforehand on slanted meat-peptone agar and collected during the exponential growth phase. The biotransformation process was carried out over 5 d on a Sartorius orbital rocker (Germany) at 150 rpm and 28°C.

The biotransformation products of **1** were extracted by EtOAc (3 × 50 mL). The combined extracts were dried over Na_2SO_4. The solvent was removed in a rotary evaporator (Heidolph, Germany). The product mixture (9.8 g/L) that formed during biotransformation of **1** by *R. erythropolis* IEGM 490 cells was acylated using Py and Ac_2O [25]. Non-polar impurities (0.6 g) were separated by column chromatography using hexane as the eluent. β-Sitosterol acetate (2.90 g) and **2** (6.93 g) were isolated successively upon elution with EtOAc:hexane (1:50).

Stigmast-4-en-3-one. Yield 71.5%, R_f 0.60 (hexane:EtOAc, 7:3), mp 77–77.5°C, $[\alpha]_D^{22}$ +10.2° (*c* 0.5, $CHCl_3$). PMR spectrum (300 MHz, $CDCl_3$, δ, ppm, J/Hz): 0.71 (3H, s, CH_3-18), 0.83 (6H, d, J = 6.9, CH_3-26,27), 0.89 (3H, t, J = 7.1, CH_3-29), 0.95 (3H, d, J = 6.9, CH_3-21), 1.05 (3H, s, CH_3-19), 2.2-2.35 (2H, br.m, CH_2-2), 5.72 (1H, br.s, CH-4).

The physicochemical constants and PMR spectrum of the product agreed with the literature data [29, 30].

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