Research Article

Features of the Bioconversion of Pentacyclic Triterpenoid Oleanolic Acid Using Rhodococcus Actinobacteria

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Abstract. The ability of actinobacteria of the genus *Rhodococcus* to transform oleanolic acid (OA), a plant pentacyclic triterpenoid, was shown for the first time using bioresources of the Regional Specialized Collection of AlkanotrophicMicroorganisms (IEGM; WDCM #768;www.iegmcol.ru). The most promising strains (*R.opacus* IEGM 488 and *R.rhodochrous*IEGM 285) were selected, and these catalyzed80% bioconversion of OA (0.5 g/L) in the presence of *n*-hexadecane (0.1% v/v) for seven days. The process of OA bioconversion was accompanied by a gradual decrease in the culture medium pH. Adaptive responses of bacterial cells to the OA effects included the formation of compact cellular aggregates, a marked change in the surface-to-volume ratio of cells, and a significant increase in the Zeta potential values. The results demonstrated that the process of OA bioconversion was catalyzed by membrane-bound enzyme complexes. Participation of cytochrome P450-dependent monooxygenases in the oxidation of the OA moleculewas confirmedusing specific inhibitors. The obtained data expand our knowledge on the catalytic activity of actinobacteria of the genus *Rhodococcus* and their possible use as biocatalysts for the bioconversion of complex hydrophobic compounds. The results can also be used inthe searchfor promising OA derivatives to be used in the synthesis of biologically active agents.

Keywords: bioconversion, oleanolic acid, *Rhodococcus*, biologically active compounds

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1. Introduction

The drugs derived from secondary plant metabolites, including pentacyclic triterpenoids, make up about 25% of the total pharmaceutical market[1]. In nature, pentacyclic triterpenoids are represented by more than 20 structures formed by cyclization of their precursor squalene in the tissues of plants, fungi, marine invertebrates, and algae[2–5]. One of the most common plant pentacyclic triterpenoids is oleanolic acid (OA; Figure 1), which concentration in fruits and leaves of *Olea europaea*can reach

35 mg/g dry weight[6, 7]. OA is widely used to producederivatives with pronounced anti-inflammation, antitumor, antimicrobial, antiviral, and anti-diabetic activities[8–12]. Nowadays, valuable OA derivatives are often synthesizedby chemical methods requiring extreme temperatures and acidity, aggressive and expensive reagents, as well as protective groups of the reactive sites of the molecule[13,14]. Alternatively, biologically active derivatives ofOA can be produced via its biological conversion using the catalytic activity of microorganisms. Microbial conversion occursin environmentally friendly conditions with high stereo- and regioselectivity and in one technological stage. Moreover, bioconversion allows modification of molecules in positions that are not available for chemical reagents[15]. However, the processes of OA biological conversion described in the literature have significant disadvantages. Thus, the use of fungi is unsafe and impractical due to their ability to produce mycotoxins and the mycelial growth type [16–18]. The few known processes of bacterial transformation catalyzed by representatives of the genera *Bacillus*and*Nocardia*, with individual strains beingpathogenic, are characterized by relatively low (up to 63%) level of substrate conversion. In addition, the described bacterial biocatalysts show their activity at OA concentration not exceeding 0.3 g/L[19, 20]. In view of this, a search for non-pathogenic bacterial strains able to catalyze the process of highly effective OA conversion is needed.

One of the microbial groupsextensively explored in modern biotechnology is non-pathogenic actinobacteria of the genus *Rhodococcus* characterized by a great variety of transformable complex hydrophobic compounds. Due tohigh catalytic activity, metabolic lability and polytrophy, multi-purpose oxygenases, synthesis of biosurfactants, the non-mycelial type of growth and the absence of pronounced pathogenic properties make it promising to use unique metabolic systems of rhodococci for the directed conversion of OA[21–23]. It is worth noting that the ability of *Rhodococcus*actinobacteria to biologically convert pentacyclic triterpenoid betulinto betulone with pronounced biological activity was previously shown [24, 25].

2. Materials and Methods

2.1. Microorganisms

In this work, 70 strains of *Rhodococcus*actinobacteria from the Regional Specialized Collection of AlkanotrophicMicroorganisms (IEGM; WDCM #285; www.iegmcol.ru), belonging to species*R. aetherivorans*(1 strain), *R. cercidiphylli*(1 strain), *R. erythropolis*(14 strains),

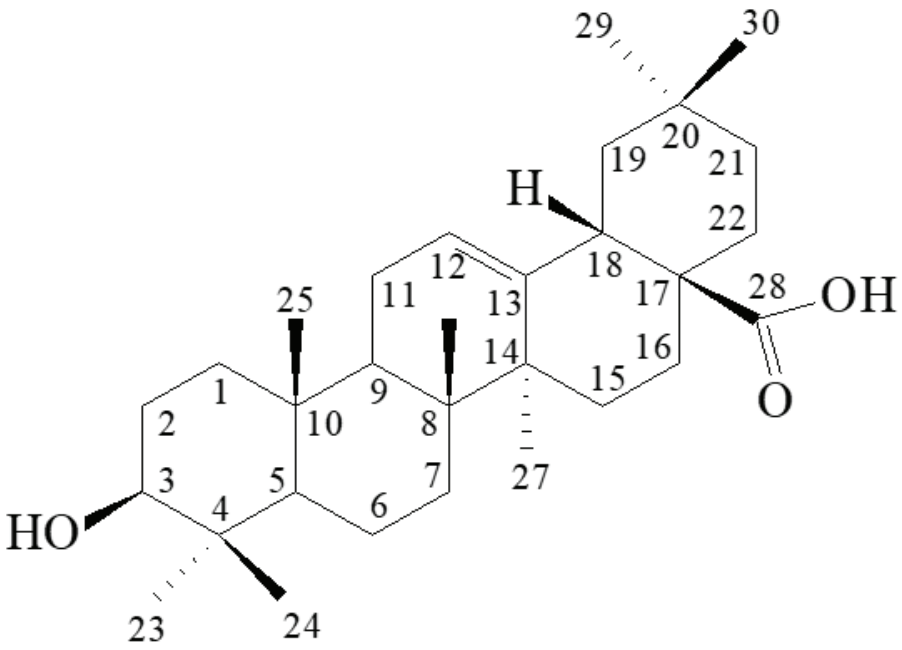

Figure 1: Structure of oleanolic acid.

R. fascians(2 strains), *R. jostii*(3 strains), *R. opacus*(15 strains), *R. qingshengii*(2 strains), *R. rhodochrous*(6 strains),and*R. ruber*(26 strains) were used (Table 1).

2.2. Chemicals

OA (≥98%, $C_{30}H_{48}O_3$, CAS 508-02-1, 3β-hydroxy-olean-12-ene-28-oic acid) purchased from Shanghai Yuanye Bio-Technology Co. (China) was used in the experiments. Ethyl acetate, hexane, and dimethyl sulfoxidewere of the highest commercially available grade (Cryochrom, Russia). *n*-Hexadecane was purchased from Reachim (Russia). (Trimethylsilyl)diazomethane solution in diethyl ester (2.0 M) was purchased from Sigma-Aldrich (USA).

2.3. Cultivation conditions

Batch cultivation of bacterial cultures was performed in 100 ml Erlenmeyer flasks containing 25 ml of the medium with shaking at 160 rpm on the Sertomat IS orbital shaker (Sartorius, Germany) at 28°C. In the experiments on bioconversion, the mineral medium of the following composition (g/L) was used: K_2HPO_4 – 1.0; KH_2PO_4 – 1.0; KNO_3 – 1.0; NaCl – 1.0; $MgSO_4$ – 0.2; $CaCl_2$ – 0.02. In addition, yeast extract (0.1 g/L), trace element solution (0.1% v/v), and *n*-hexadecane (0.1% v/v) as a co-substrate were used. OA dissolved in dimethyl sulfoxide (1:10 mg/μL) was added at a concentration of

Table 1: Collection strains used in the work.

Species	No. of strains	Strain number in the collection
R.aetherivorans	1	IEGM 911^T
R.cercidiphylli	1	IEGM 1184
R.erythropolis	14	IEGM 7^T, IEGM 21, IEGM 179, IEGM 191, IEGM 200, IEGM 202, IEGM 254, IEGM 266, IEGM 275, IEGM 490, IEGM 499, IEGM 507, IEGM 687, IEGM 766
R.fascians	2	IEGM 39, IEGM 930
R.jostii	3	IEGM 30, IEGM 60, IEGM 68
R. opacus	15	IEGM 56, IEGM 57, IEGM 58, IEGM 59, IEGM 246, IEGM 248, IEGM 249, IEGM 262, IEGM 264, IEGM 488, IEGM 489, IEGM 717, IEGM 765, IEGM 1157, IEGM 2226
R.qingshengii	2	IEGM 247, IEGM 267
R.rhodochrous	6	IEGM 66, IEGM 608, IEGM 647, IEGM 654, IEGM 655, IEGM 760
R.ruber	26	IEGM 72, IEGM 73, IEGM 74, IEGM 76, IEGM 79, IEGM 81, IEGM 84, IEGM 86, IEGM 87, IEGM 90, IEGM 91, IEGM 92, IEGM 93, IEGM 94, IEGM 107, IEGM 172, IEGM 219, IEGM 227, IEGM 229, IEGM 232, IEGM 233, IEGM 235, IEGM 237, IEGM 239, IEGM 240, IEGM 324

0.5 g/L. In some experiments, inhibitors of cytochrome P450-dependent oxygenases (ketokonazole, metyrapone) were used at a concentration of 1 mM[26]. An inoculated medium without OA was used as the biotic control, an uninoculated medium with *n*-hexadecane and OA was used as the abiotic control.

2.4. Cell viability test

Cell viability was determined by staining with iodonitrotetrazolium chloride (INT, Sigma-Aldrich, USA). 100 µL of culture and 50 µL of 0.2% aqueous solution of INT were added to 96-well polystyrene microplates. To completely staining, the samples were incubated at 28°C for 24 hours. In comparative experiments to determine the viability of the studied cultures, the optical density of samples was measured by a Multiskan Ascent spectrophotometer (Thermo Electron Corporation, USA).

2.5. Phase contrast microscopy

Cells were visualized using an Axiostar plus optical microscope (Carl Zeiss, Germany) in a phase contrast mode with x1000 magnification. Bacterial cell sizewas measured using the PixeraPRO 150ES camera (Pixera, USA) and the Video Test-Size 5.0 computer program (Akond, Russia). The volume (V) and area (S) of cells were calculated thing formulas [27]:

$$V = r^2\pi h\ (\mu m^3);\ (1)$$

$$S = 2r^2\pi + \pi rh\ (\mu м^2),\ (2)$$

where r is ½ of the cell width;

π is 3.14;

h is the cell length.

2.6. Zeta potential measurement

The bacterial culture pre-grown with or without OA was washed and resuspended in 10 mM KNO_3 to OD_{600}0.2 (Lambda EZ201 spectrophotometer, Perkin-Elmer, USA). The electrokinetic potential of the obtained samples was measured using the ZetaSizer Nano ZS (Malvern Instruments, UK).

2.7. Preparation of actinobacterial cell fractions

Actinobacterial cells pre-grown for 2 days in the meat-peptone broth were washed three times and resuspended in the phosphate-alkaline buffer (pH 7.0). The cell suspension was homogenized using a Soniprep 150 ultrasonic disintegrator (MSE, UK) at the amplitude of 10 μm for 45 min with mandatory water cooling. The cell homogenate was centrifuged at 6,000 rpm and4°C for 15 min to obtain cytoplasmic enzymes (supernatant) (I). Membrane-bound enzymes were solubilized by resuspending the precipitate in 100 mL of 1% Triton X-100 solution (Sigma-Aldrich, USA) in the phosphate-alkaline buffer (pH 7.0) and stirring on the orbital shaker for 30 min. The supernatant with extracted membrane-bound enzymes (II) was obtained by centrifugation. After sonication, the cell precipitate with enzymes strongly bound to the membrane and non-extractable with a detergent (III) was resuspended in 100 mL of the phosphate-alkaline buffer (pH 7.0). The prepared cell fractions included (I) a supernatant with cytoplasmic enzymes; (II)

a supernatant with extracted membrane-bound enzymes; and (III) a resuspended cell precipitate with non-extractable enzymes.

2.8. Qualitative and quantitative analysis of OA and its metabolites

To extract the residual OA and its possible derivatives, the fermentation medium (25 mL) was acidified with 10% HCl solution (achieving pH 4.0) and extracted three times with an equal volume of ethyl acetate. The quality control was carried out by thin layer chromatography (TLC) on ALUGRAM® XtraSILG/UV_{254} plates (MACHEREY-NAGEL GmbH&Co, Germany) in the hexane:ethyl acetate system (3:7, v/v) The plates were sprayed with 5% H_2SO_4 and heated for 2–3 min at 95–100°C. Detection of the studied compounds was performed at λ = 365 nm (UV irradiator LG/58, Russia). Quantitative analysis was performed by gas chromatography-mass spectrometry (GC-MS) using an Agilent 6890N gas chromatograph with a quartz column and an Agilent MSD 5973N quadrupole mass spectrometer (Agilent Technology, USA). Before GC-MS analysis, the samples were methylated using(trimethylsilyl)diazomethane (Sigma-Aldrich, USA).

3. Results and Discussion

3.1. Screening test

Rhodococcus actinobacteriastrains from IEGM collection were screened for their ability to catalyze the bioconversion of OA.It was found that most of the studied cultures were not able to use OA as the sole source of carbon and energy. Only individual strains (*R. jostii*IEGM 60, *R. opacus*IEGM 246, IEGM 262, IEGM 488, IEGM 1157, *R. rhodochrous*IEGM 66, and*R. ruber*IEGM 107, IEGM 324) showed catalyticactivities. Of these, strains*R.opacus*IEGM 488 and *R.rhodochrous* IEGM 66exhibitingthe greatest catalytic activity were selected for further experiments. *R. opacus* IEGM 488 was isolated from oil-contaminated water of the UnvaRiver, Perm Krai, Russia. This strain uses *n*-hexadecane and crude oil as sole sources of carbon [28]. Whereas*R. rhodochrous*IEGM 66 transforms thioanisol, forms aggregated with liqiud hydrocarbons (*n*-hexadecane), catalyzes the oxidation of prohiral sulfides into (S)-sulfoxides, and is resistant to *n*-hexane, ethanol, and heavy metals Cr^{6+} (10.0 мМ), Mo^{6+} (5.0 мМ). It should be noted that the abovestrain shows high transforming activity towards pentacyclic triterpenoid betulin andcatalyzesthe formation of biologically active betulone [29].

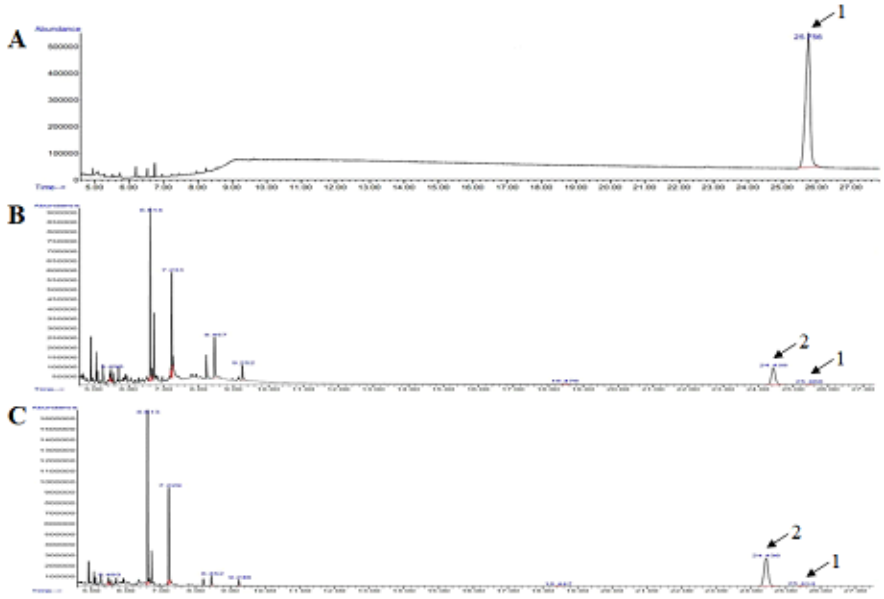

Figure 2: Chromatograms of OA (A) and its bioconversion products using*R.opacus* IEGM 488 (B) and *R.rhodochrous* IEGM 66 (C). 1 – OA, 2 – probable derivative of OA.

3.2. Bioconversion of OA using R. opacusIEGM 488 R. rhodochrousIEGM 66

It is known that the use of additional sources of carbon and energy, in particular *n*-hexadecane, can increase the bioconversion efficiency of complex hydrophobic compounds using actinobacteria[25, 30]. According to the dataobtained, *R.opacus*IEGM 488 and*R.rhodochrous* IEGM 66 catalyzed 80% conversion of 0.5 g/L OA in the presence of *n*-hexadecane for 7 days (Figure 2). It should be noted that actinobacteriareported in literature showed catalytic activity only at a concentration of OA not exceeding 0.3 g/L. For example, actinobacteria of the genus *Nocardia* catalyzed 70% conversion of 0.3 g/L OA for 13 days [19], and actinobacteria of the genus *Streptomyces* catalyzed 60% conversion of 0.04 g/L OA for 5 days[20].

TheOA biotransformation dynamics was studied.It was found that the maximum conversion level using *R.opacus* IEGM 488 and *R.rhodochrous* IEGM 66 occurred on 2 and 4 days of the experiments, respectively (Figure 3A,B). At the same time, the effect of OA on *R.opacus* IEGM 488 cells was manifested in a pronounced inhibition of their growth compared to biotic control throughout the experiment (Figure 3A). Whereas the addition of OA into the culture medium of *R.rhodochrous* IEGM 66 did not cause a decrease in the biomass growth (Figure 3B). It should be noted that OA concentration in the abiotic control remained almost unchanged, confirming the biocatalytic nature of the substrate transformation.

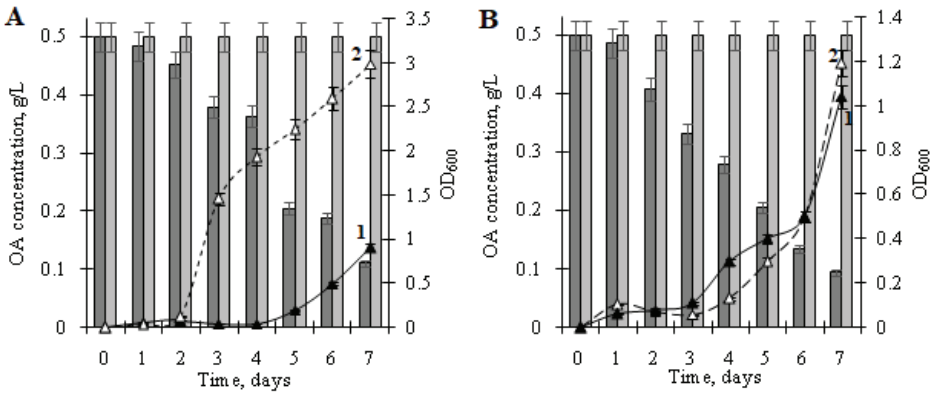

Figure 3: Dynamics of OA biotransformation using*R.opacus* IEGM 488 (A) and *R.rhodochrous* IEGM 66 (B). ■–OA concentration during biotransformation, ■– abiotic control, – biotic control.

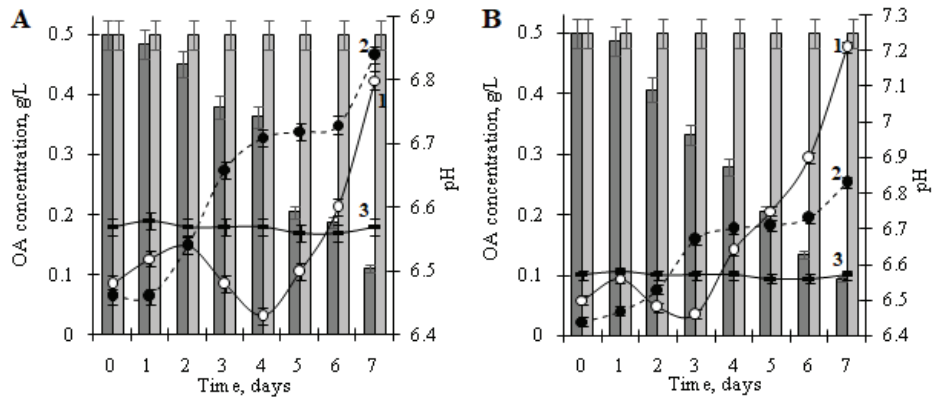

Figure 4: Dynamics of the mediumpH during OA bioconversion (– abiotic control, ■– OA concentration during bioconversion, ■– abiotic control.

Studying the dynamics of pH changes ofthe medium (Figure 4A,B) showed that when rhodococci were cultured in the presence of *n*-hexadecane without OA, the pH values gradually increased from 6.45 to 6.85.Itmightbe due to the gradual accumulation of bacterial cell products. Whereas in the presence of OA, the medium pH ranged from 6.4 to 6.6 in the first 3–4 days, and then dramatically increased to 6.8 in the case of *R.opacus* IEGM 488 (Figure 4A) and to 7.2 in the case of *R.rhodochrous* IEGM 66(Figure 4B). Such changes in the pH values to low-alkaline may be associated with a decrease in triterpenic acid concentration in the culture medium. The pH values in the abiotic control were almost unchanged,allowing to inferthat changes in the medium pHare associated with the catalytic activity of rhodococci.

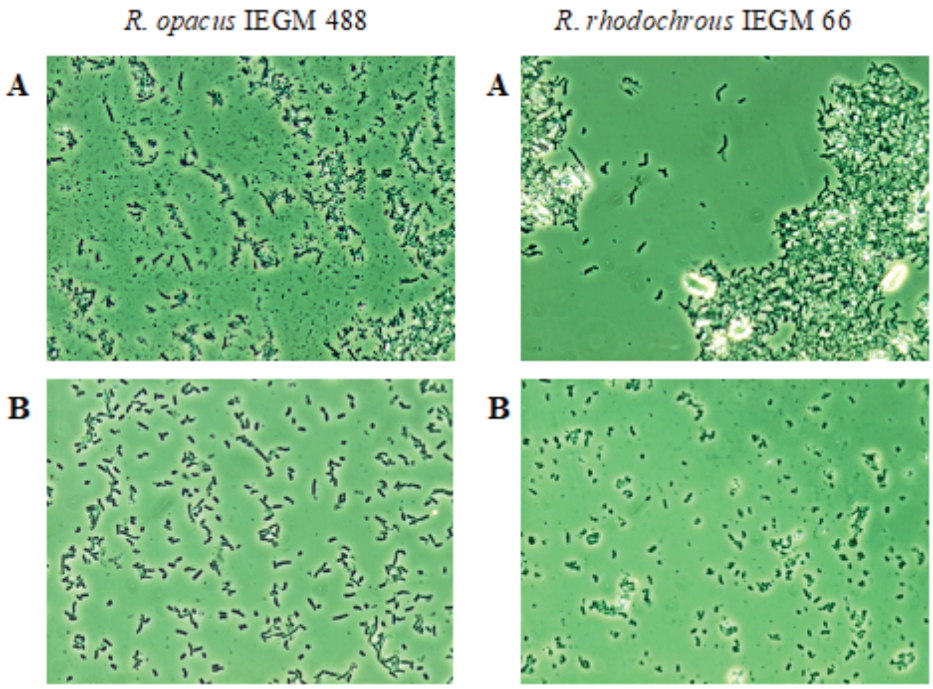

Figure 5: *opacus* IEGM 488 and *R. rhodochrous*IEGM 66 in phase contrast (x1000) with (A) or without (B) OA.

3.3. Effects of OA on Rhodococcus cells

According to phase-contrast microscopy data, rhodococci formed compact cellular aggregates in the presence of OA (Figure 5). The formation of aggregates seems to enablethe population to adapt and grow in conditions where single cells are not able to reproduce and transform OA.

Analysis ofmorphometric data of OA-exposed cells resulted in detection of changes in the cell length and width, and alsoin surface-to-volume ratio of cells(Table 2). In the case of *R. rhodochrous* IEGM 66, an increase in the ratio of cell surface area to cellvolume was detected. According to literature data [27], it promotes a more effective contact of cells with the substrate. Whereas in the case of *R. opacus* IEGM 488, the opposite protective reaction of rhodococciwas observed, tending toreducethe cell surface area availableto contact with OA.

Another reaction of rhodococci to the presence of OA was the changedsurface electrokinetic potential of cells (Table 2). It was found that the addition of OA led to a significant (68%) increase in the electrokinetic potential of bacterial cells. This Zeta potential shift may be due to possible destabilization of cell membranes influenced by toxicants [31].

Table 2: Effects of OA on morphological parameters of bacterial cells.

Cultivation conditions	Length, μm	Width, μm	Area (S), μm^2	Volume (V), μm^3	Relative area (S/V), μm^{-1}	Zeta potential, mV
*R. opacus*IEGM 488						
Medium with *n*-hexadecane	7.19±0.36	1.93±0.10	49.42±2.47	21.02±1.05	2.35±0.11	-45.0±0.05
Medium with *n*-hexadecane and OA	17.13±0.86	2.52±0.13	145.52±7.27	85.42±4.27	1.71±0.09	-14.35±0.07
*R. rhodochrous*IEGM 66						
Medium with *n*-hexadecane	9.61±0.48	2.84±0.14	98.35±4.91	60.83±3.04	1.62±0.08	-42.50±0.57
Medium with *n*-hexadecane and OA	12.28±0.61	2.59±0.13	110.40±5.52	64.66±3.23	1.71±0.09	-13.55±0.07

Table 3: Biotransformation of OA using cell fractions.

Cell fraction	*R.opacus*IEGM 488	*R.rhodochrous* IEGM 66
Whole cells	+*	+
Supernatant with cytoplasmic enzymes (I)	–	–
Supernatant with extracted membrane-bound enzymes (II)	+	+
Resuspended cell precipitate with non-extractable enzymes (III)	–	–
*Note. "+" – decrease in OA concentration, "-" – no decrease in OA concentration.		

3.4. Enzymes involved in the bioconversion of OA

According to literature data, the reactions of microbial oxidation of triterpenoids involve cholesterol oxidase[32], 3β-hydroxysteroid dehydrogenase[33], as well as enzymes of the CYP450 family[34, 35]. All these enzymes are localized mainly in the cytoplasm or are associated with the cell membrane. In this work, using cell fractions of rhodococci, it was shown that oxidation of OA was catalyzed by enzymes associated with the cell membrane and extracted with a detergent (Table 3).

Using the inhibitors of cytochrome P450-dependent oxygenase activity, we confirmed the involvement of these enzyme complexes in the process of actinobacterial transformation of OA. In particular, the use of metyrapone and ketokonazole resulted in the complete inhibition of the process of OA transformation, as evidenced by no metabolites in the post-culture liquid (Table 4).

Table 4: Effects of inhibitors of P450-dependent enzymes on the transforming activity of actinobacteria.

Inhibitor	*R.opacus*IEGM 488	*R.rhodochrous* IEGM 66
Metyrapone	Complete inhibition	Complete inhibition
Ketoconazole	Complete inhibition	Complete inhibition

4. Conclusion

The ability of actinobacteria of the genus *Rhodococcus* to bioconvertthe plant pentacyclic triterpenoid OA was shown. The comparative analysis of transformationactivities of 70 cultures of different rhodococcalspeciesresulted in selection of*R.opacus* IEGM 488 and *R.rhodochrous* IEGM 66. These strains catalyzed the effective (80%) conversion of a relatively high (0.5 g/L) concentration of OA in the presence of *n*-hexadecane (0.1% v/v)for 7 days. It is worth noting that bacterial biocatalysts reported in literature demonstrated their transformationactivities at a concentration of OA not exceeding 0.3 g/L [19, 20]. It was shown that the process of OA bioconversion using rhodococci was accompanied by a decrease in pHof the cultivation medium of biocatalysts. For the first time, the adaptive responses of rhodococci in the presence of OA were described. Along with the formation of compact cell aggregates, the most typical reactions of actinobacterial cells included a marked change in the surface-to-volume ratio of cells,and a significant increase in the Zeta potential values. Using separate cell fractions, it was found that the conversion of OA was catalyzed by membrane-bound enzyme complexes. Using inhibitors,the involvementof CYP450 family enzymes in the process of OA bioconversion was confirmed. The obtained data expand our knowledge on the catalytic activity of actinobacteria and their possible use as biocatalysts for biotransformation of complex hydrophobic compounds. Being currently searched for are OA derivatives with high prospects in synthesis of biologically active compounds.

5. Acknowledgments

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