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Bioconversion of ecotoxic dehydroabietic acid using *Rhodococcus* actinobacteria

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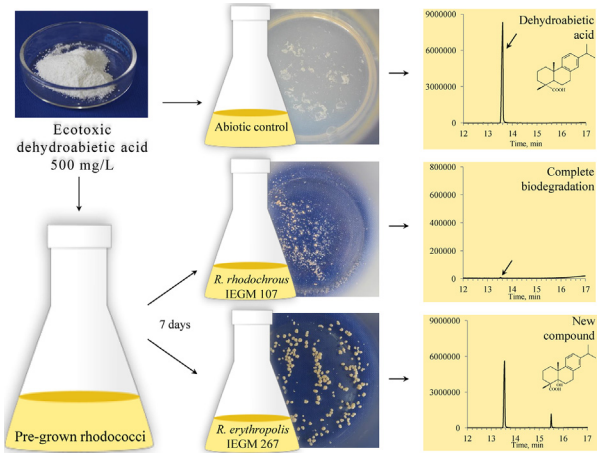
HIGHLIGHTS

- Actinobacteria are capable to bioconversion of 500 mg/L dehydroabietic acid (DHA).
- The addition of DHA leads to increased rhodococcal respiration.
- DHA induces morphological-physiological changes in *Rhodococcus* actinobacteria.
- A novel metabolite of DHA bioconversion by *R. erythropolis* IEGM 267 was identified.

GRAPHICAL ABSTRACT



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ABSTRACT

Actinobactrial strains *Rhodococcus erythropolis* IEGM 267 and *R. rhodochrous* IEGM 107 were used to study biodegradation of dehydroabietic acid (DHA), a toxic tricyclic diterpenoid. The experiments were carried out in batch cultures of pre-grown rhodococci in the presence of 0.1% (v/v) *n*-hexadecane under aerobic conditions for 7 days. It was shown that *R. erythropolis* IEGM 267 and *R. rhodochrous* IEGM 107 partially and completely degraded DHA (500 mg/L), respectively. Characteristic physicochemical (reduced zeta potential) and morphological-physiological (increased average size of single cells and cell aggregates, increased root-mean-square roughness) changes in DHA-exposed actinobacteria were revealed. Products of DHA bioconversion by *R. erythropolis* IEGM 267 were analyzed and exhibited a previously unidentified metabolite 5α-hydroxy-abieta-8,11,13-triene-18-oat. The obtained experimental data widen the knowledge on the catalytic activity of rhodococci and their possible contribution to decontamination of natural ecosystems from pollutants.

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1. Introduction

Resin acids are toxic tricyclic diterpenoids produced by coniferous trees of *Pinaceae* [1]. Those acids may account for up to 21.4% of the polar extractable fraction or 0.2–0.8% of the dry total weight of the wood [2]. A major component (up to 60%) of the resin fraction is dehydroabietic acid (DHA) **1** (Fig. 1) [3]. Mechanical and chemical pulping processes result in DHA release and in relatively high DHA concentrations (up to 500 mg/L) in the wastewater. Accumulated in natural ecosystems, DHA has a toxic effect on living organisms that can lead to ecological disbalances. It is established the acute toxicity towards various test organisms varies from 0.1 to 6.5 mg/L [1,4–6]. Consequently, to discover the effective methods to reduce the DHA concentration in the pulp and paper effluent is topical.

Due to natural bioaccumulation, conifer-produced DHA is found in marine and river water [7]; sediments and soil [8]; and living organisms [6]. In addition, the ability to produce DHA is customary for some representatives of streptomycetes and cyanobacteria [9,10]. The crucial role in natural detoxification of pollutants belongs to destructor microorganisms. Because DHA is widelspread in the environment, microorganisms of various phylogenetic groups (fungi, bacteria) with the ability to partially or completely degrade DHA [1,11].

Biological treatment of wastewater based on enzymatic activity of microorganisms and natural processes of pollutant degradation is currently considered to be an advanced method which does not require aggressive reagents, and biological reactions proceed in one technological stage [1]. In most environments, DHA are found in low concentrations [11]. Therefore, many reported microorganisms generally exhibit degradation activity at DHA concentration not exceeding 250 mg/L [12–17] in the culture medium. Of the bacterial isolates characterized so far, most are gram-negative *Proteobacteria* with representatives in α-, β- and γ-subclasses. Their biotechnological application is potentially dangerous because of possible pathogenicity. The gram-positive biodestrurtors are represented by few *Mycobacterium* and *Bacillus* strains [18–20]. According to the above, it is necessary to discover effective non-hazardous biocatalysts that retain effective degradation ability against higher DHA concentrations. One of the intensively studied groups of microorganisms in terms of biotechnological application are actinobacteria capable of transforming and degrading complex reculcitrant organic compounds at high concentrations [21,22]. Non-mycelial growth, lack of pathogenic properties, a flexible metabolic system and high oxygenase activity, all these determine the prospects for actinobacteria to be used as perspective biocatalysts [23–25]. At present, there are only a few studies describing actinobacteria in resin acid bioconversion [26,27]. This article reports on possible usage of actinobacteria from the Regional Specialised Collection of Microorganisms (IEGM; WDCM 768; http:/www.iegmcol.ru/) in effective DHA biodegradation.

2. Materials and methods

2.1. Microorganisms

In this work, 115 strains from the Regional Specialized Collection of Alkanotrophic Microorganisms representing the five species *Gordonia rubripertincta* (32 strains), *G. terrae* (21 strains), *R. erythropolis* (26 strains), *R. qingshengii* (3 strains), *R. rhodochrous* (3 strains) and *R. ruber* (30 strains) (Table 1) were used.

2.2. Chemicals

DHA (99.1%) was purchased from the Mosinter group limited (China). Ethyl acetate, hexane, and acetonitrile were of the highest commercially available grade (Cryochrom, Russia). Pure *n*-hexadecane was purchased from Reachim (Russia). (Trimethylsilyl)diazomethane solution 2.0 M in diethyl ether was purchased from Sigma Aldrich (USA).

Table 1
Collection strains used in the work.

Species	No. of strains	Strain number in the IEGM collection
G. rubripertincta	32	95, 96, 97, 98, 100, 101, 103, 104, 105, 106, 109, 110, 118, 119, 120, 121, 122, 124, 126, 127, 128, 132, 133, 134, 135, 138, 139, 140, 723, 724, 725, 730
G. terrae	21	130, 136, 143^T, 144, 145, 146, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164
R. erythropolis	26	7^T, 11, 12, 14, 18, 19, 20, 21, 22, 23, 24, 25, 26, 98, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 267
R. qingshengii	3	1016^T, 247, 550
R. rhodochrous	3	64, 66, 107
R. ruber	30	70^T, 74, 80, 81, 82, 83, 84, 85, 86, 87, 88, 90, 91, 92, 93, 94, 172, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231

2.3. Use of DHA as the sole carbon source

Batch cultivations of actinobacteria were performed in 250 mL Erlenmeyer flasks containing 100 mL of the medium with shaking (160 rpm) at 28 °C. The mineral medium contained (per L) KNO_3 1.0 g, K_2HPO_4 1.0 g, KH_2PO_4 1.0 g, NaCl 1.0 g, $MgSO_4 \times 7H_2O$ 0.2 g, $CaCl_2 \times 2H_2O$ 0.02 g, $FeCl_3$ 0.001 g, and 0.1% (v/v) trace element solution (1.5 g/L $FeCl_3 \times 7H_2O$, 0.1 g/L H_3BO_3, 0.01 g/L $ZnSO_4 \times 7H_2O$, 0.05 g/L $Co(NO_3)_2 \times 6H_2O$, 0.005 g/L $CuSO_4 \times 5H_2O$, and 0.005 g/L $MnCl_2 \times 4H_2O$). DHA concentrations of 20, 40, 60, 80, and 100 mg/L as a powder or a solution in ethanol (1:10 mg/μL) were added as a carbon source. The medium was sterilized at 121 °C and 1.06 kg/cm^2 for 30 min. Suspensions of actinobacterial cells (OD_{600} 1.0) pre-grown on agar (Oxoid, UK) for 2 days were used to inoculate media. The DHA biodegradation proceeded within 14 days. The sterile DHA-containing mineral medium was used as an abiotic control.

2.4. Screening test

In screening experiments on the ability of the collection strains to DHA biodegradation, an inoculum of a specific test strain and a mineral medium (according to paragraph 2.3) with 0.1% (v/v) yeast extract (10%) (Microgen, Russia) and 0.02–0.1% (v/v) *n*-hexadecane were used. DHA (500 mg/L) was dissolved in ethanol (1:10 mg/μL) and added after 48 h of cell cultivation. The DHA biodegradation proceeded for 7 days. The controls included (1) sterile DHA-containing mineral medium to evaluate the abiotic DHA stability; and (2) *n*-hexadecane- and bacterial cell-containing medium without DHA to differentiate possible bacterial metabolites.

2.5. Cell viability tests

To determine the number of living cells, the iodonitrotetrazolium violet (IV) staining method was used according to the following procedure: 100 μL of the test sample and 50 μL of 0.2% aqueous solution of IV were added in 96-well round-bottomed polystyrene microplates (Medpolymer, Russia). The IV was reduced to water insoluble formazan and this resulted in the development of red-violet staining, indicating actively respiring bacterial cells present in the sample. Samples were incubated at 28 °C for 24 h. After that period, the OD of the colored suspension was measured

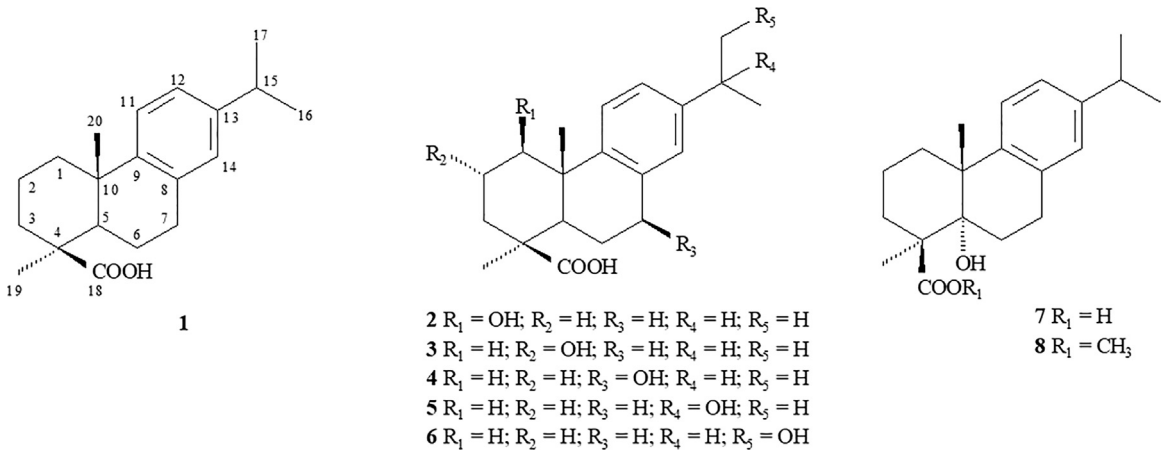

Fig. 1. Structures of DHA and DHA derivatives.

with a Multiscan Ascent microplate reader (Thermo Electron Corporation, Finland) at 630 nm wavelength [28].

2.6. Minimal inhibition concentration of DHA

Minimal inhibition concentration (MIC) of DHA against actinobacteria was determined by the micromethod of serial two-fold dilutions using 96-well round-bottomed polystyrene microplates. 100 μL of a sterile medium (LB) were inoculated into microplate wells. 100 μL of ethanol-dissolved DHA (working solution concentration, 10:100 mg/μL) were added to the first well of each row and mixed thoroughly. 100 μL of the solution from the resulting mixture was transferred to the next well. The procedure was repeated until a series of two-fold dilutions was formed. The DHA concentration in one row ranged from 0.7 mg/L to 50 g/L. 10 μL (5×10^5 cells/mL) of the test culture pre-grown in LB were added to the resulting mixture. The plates were incubated at 28 °C for 3 days.

2.7. Phase contrast microscopy

Phase contrast microscopy was performed using an Axiostar plus light microscope (Carl Zeiss, Germany). Microscopic images and measurements of cell aggregate sizes were obtained with a PRO-150ES digital camera (Pixera, USA) and the VideoTest, Size 5.0 software (St. Petersburg, Russia).

2.8. Combined confocal laser and atomic force scanning microscopy

The morphology of bacterial cells was analyzed using a combined microscopic system which consists of the Olympus FV1000 laser confocal scanning microscope (CLSM) (Olympus Corporation, Japan) and the Asylum MFP-3D-BIO atomic force microscope (AFM) (Asylum Research, USA). Prior to imaging, a drop (15–20 μL) of the cell suspension was placed on a glass cover slip, mixed with the same volume of LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen, USA) and allowed drying in darkness for 10–15 min. Then the cover slip was rinsed with deionized water and CLSM-scanned using an Olympus 100× oil immersion lens (numerical aperture, 1.4). SYTO 9 and propidium iodide included in LIVE/DEAD® were excited with the argon laser (λ = 488 nm) with a 505/525 nm barrier filter and the He–Ne laser (λ = 543 nm) with a 560/660 nm barrier filter, respectively. Image sizes of 0.12 × 0.12 mm (resolution, 1600 × 1600 pixels) were obtained at a speed of 40 nm/pixels. The images were analyzed by FV10-ASW 3.1 (Olympus Corporation, Japan). The cell volume and area were additionally calculated according to Neumann et al. [29]. The cell volume was calculated by the formula:

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

cell surface area:

$$a = 2r^2\pi + \pi rh (\mu m^2),$$

where r – ½ cell width, h – cell length, π – 3.14.

The CLSM image was imported into the Igor Pro 6.22A (WaveMetrics, USA) software used for AFM. AFM scanning of the preparations was carried out in a semi-contact mode in air. Silicon cantilevers AC240TS (Olympus, Japan) with resonance frequencies of 50–90 kHz and spring constants of 0.5–4.4 N/m were used.

2.9. Zeta potential measurement

Electrokinetic (zeta) potential of bacterial cells was measured by the dynamic light scattering using a ZetaSizer Nano ZS analyzer (Malvern Instruments, UK) with Malvern ZetaSizer, v. 2.2. The actinobacterial suspension grown with 0.1% *n*-hexadecane and DHA (500 mg/L) was washed twice and resuspended in 10 mM KNO_3 (pH 7.0) to achieve OD_{600} 0.2.

2.10. Respirometry analysis

Respiratory activities of bacterial cells were measured using a six-channel indirect cycle MicroOxymax® respirometer (Columbus Instruments, USA) connected to a PC. Experiments were performed in 300 mL Micro-Oxymax bottles. The bacterial cultures were constantly stirred (300 rpm, 28 ± 2 °C) on a multi-position magnetic stirrer RT 10 (Power IKAMAG, Germany). The amount and rate of O_2 consumed and CO_2 released (μL) were evaluated. The parameters of respiratory activity were recorded automatically every 42 min for 9 days.

2.11. DHA derivative isolation and identification

To isolate residual DHA and its metabolites, the culture media were acidified with 10% HCl solution and extracted three times with an equal volume of ethyl acetate. The combined organic layers were washed with an aqueous solution of 1% Na_2CO_3 and then with distilled water to a final pH of 7.0. The ethyl acetate extract was dried over anhydrous Na_2SO_4. The solvent was removed with a rotary evaporator (Heidolph, Germany). A mixture of DHA metabolites was methylated with (trimethylsilyl)diazomethane for 1 h. DHA biodegradation products were qualitatively analyzed by TLC on sil-

ica gel 60 plates (Merk, Germany). The sample spots were visualized by treating plates with ethyl acetate:*n*-hexane (3:7, v/v) and spraying with a solution of 5% H_2SO_4 followed by heating at 95–100 °C for 2–3 min. The GC–MS of the DHA biodegradation products were performed using an Agilent 6890N/5975B chromatograph (Agilent Technologies, USA) equipped with a HP-5MS capillary column (30 m × 0.25 mm, 0.25 μm) and operating in the electron impact ionization mode (70 eV). Helium was used as a carrier gas (1 mL/min). The column temperature was programmed from 120 to 320 °C. MS were recorded in the range of 40–460 *m/z* and compared with those from the NIST08 Library. The DHA biodegradation dynamic was quantitatively analyzed by HPLC using a LC Prominence 20 AD chromatograph (Shimadzu, Japan) fitted with a reversed-phase column Supelcosil™ LC-18 (150 × 4.5 mm, 5 μm) and a diode matrix detector (SPD-M20A). The mobile phase contained 70% acetonitrile solution; the flow rate was 1 mL/min; the detection wavelength at 190 nm; column temperature was 40 °C. The retention time for DHA and compound **8** was 14.99 ± 0.02 and 7.88 ± 0.02 min, respectively. The data were registered and processed using the LCSolution program (v. 1.25).

2.12. Preparative isolation of methyl 5α-hydroxy-abieta-8,11,13-triene-18-oate(**8**) from the mixture of DHA bioconversion products by R. erythropolis IEGM 267

To methylate the DHA biodegradation products, the concentrated extract (3 g) was dissolved in 70 mL acetone, than methyl iodide (4.26 g) and K_2CO_3 (4.14 g) were added to the solution. The reaction mixture was boiled with a reflux condenser under stirring for 1.5 h. After the reaction was completed, the mixture was filtered and the solvent was removed with a rotary evaporator. The residue was separated using a flash-chromatograph (BÜCHI, Switzerland) on a cartridge 40 mm in diameter and 150 mm long (BÜCHI, Switzerland) containing 90 g of silica gel 60. The ratio of the methylated extract and the sorbent was 1:30. The elution was carried out with the hexane:ethyl acetate mixture using the concentration gradient from 99:1 to 9:1. The isolated yield of compound **8** after colon chromatography was 17%.

The IR spectrum of the compound **8** dissolved in $CHCl_3$ was recorded on the Bruker 66/S IFS Fourier spectrometer (Bruker, Germany). The 1D and 2D NMR spectra of compound **8** dissolved in $CDCl_3$ were recorded on the Bruker AVANCE II (Bruker BioSpin GmbH, Germany, 400 and 100 MHz for ^{1}H- and ^{13}C-NMR spectra, respectively). Chemical shifts (δ) were stated in units of parts per million (ppm) relative to the TMS as an internal standard. The optical rotation was measured on the Perkin Elmer 341 polarimeter (Perkin Elmer, USA) using sodium D light for $CHCl_3$ solution. The melting point was measured using the OptiMelt MPA100 (Stanford Research Systems, USA) instrument at the heating rate of 1 °C/min.

3. Results and discussion

3.1. Screening test

Many aerobic bacterial isolates were isolated from pulp and paper industry effluent, biological wastewater treatment systems and from bottom and soil samples taken in the immediate area of pulp and paper mills. These isolates use resin acids as the sole carbon and energy source [14,30–32]. It has been shown that only *R. erythropolis* IEGM 267 from all strains studied is capable to use DHA at a concentration of 20 mg/L as the sole carbon source. The remaining cultures did not exhibit any growth in the presence of 20–100 mg/L DHA within 14 days of the experiment. The ability of actinobacteria to utilize DHA in the presence of 0.5% (v/v) *n*-hexadecane as the inducer of ecotoxicant decomposition has been previously described [26]. *n*-Hexadecane present in the culture medium promotes the induction of multi-purpose oxygenase systems in alkanotrophic actinobacteria [33]. Furthermore, due to the lack of the catabolic repression in actinobacteria, particularly in rhodococci, *n*-alkanes can be used as co-substrates in biotransformation processes of recalcitrant compounds [34]. In a number of studies on actinobacterial biotransformations of complex organic compounds, the *n*-hexadecane concentration of 0.1% (v/v) was proved to be optimal [35,36]. Screening of the actinobacterial catalytic activity against DHA was carried out under similar conditions, and DHA was added after 48 h of bacterial cell cultivation.

Ten strains capable of complete or partial degradation of DHA (Table 2) were selected. *R. rhodochrous* IEGM 107 possesses the greatest potential for natural system decontamination from resin acids. It catalyzed the high (>98%) DHA biodegradation. Five strains could degrade more than 50% of DHA. Their catalytic activities were distributed in the order of *G. rubripertincta* IEGM 104 > *G. terrae* IEGM 150 > *G. rubripertincta* IEGM 109 > *G. rubripertincta* IEGM 105 > *R. ruber* IEGM 80. TLC showed that *G. rubripertincta* IEGM 100, IEGM 120, IEGM 132 and *R. erythropolis* IEGM 267 catalyzed the DHA bioconversion to form a more polar product. According to GC–MS, the detected unknown metabolite gives a mass spectrum with a molecular-ion peak at *m/z* 330.3.

3.2. Study of the DHA biodegradation process using R. rhodochrous IEGM 107 and R. erythropolis IEGM 267

A detailed study of the DHA bioconversion process using the most active biocatalysts *R. rhodochrous* IEGM 107 (GenBank: MG585109) and *R. erythropolis* IEGM 267 (GenBank: MRBQ00000000.1) was carried out. It included sampling every 24 h for microscopic analyses, HPLC of residual DHA and its metabolites, viable cell determinations, measurements of respiratory activity and pH of the reaction medium.

As shown in Fig. 2A, a decrease in the viable cell number of *R. rhodochrous* IEGM 107 was observed during 48 h after DHA addition to the culture medium. Presumably, this experimental stage corresponded to the adaptation period of *R. rhodochrous* IEGM 107 to a DHA toxic effect, with its concentration decreased to 356 mg/L within 48 h. The next day marked a rapid increase in growth and catalytic activity of bacterial cells, with only 104 mg/L DHA observed in the culture medium. Then the DHA concentration decreased gradually, and the residual DHA was less than 5 mg/L on the day 9. Different dynamics of the viable cell number was observed during DHA biodegradation by *R. erythropolis* IEGM 267. As shown in Fig. 2B, the addition of DHA led to 1.5–2.8 times higher rhodococcus cell inhibition compared to the biotic control throughout the experiment. As revealed by HPLC, the gradual decrease in DHA concentration in the culture medium was accompanied by the metabolite *m/z* 330.3 formation, with its maximum (14%) concentration on day 8 of the experiment. The DHA effects on the viable cell number are consistent with MICs of DHA. For *R. rhodochrous* IEGM 107, the MIC value was 780 mg/L, indicating relatively high resistance to DHA toxic effects compared with many known biodestructors [30,31,37]. Conversily, *R. erythropolis* IEGM 267 were much less resistant to DHA toxic effect (MIC 24 mg/L).

According to phase-contrast microscopy analyses, rhodococci formed compact cellular aggregates of 10–50 μm in size with more than ten viable cells in the mineral medium in the presence of *n*-hexadecane. Subsequent addition of DHA resulted in the formation of cellular aggregates up to 0.5 mm in size for *R. rhodochrous* IEGM 107, while the size of *R. erythropolis* IEGM 267 aggregates achieved 5 mm.

The physiological state of actinobacteria in the presence of DHA was revealed by CLSM data. After 48 h of DHA addition, cells of *R. rhodochrous* IEGM 107 were still viable, but cells with red fluores-

Table 2
DHA biodegradation by actinobacteria in the presence of *n*-hexadecane.

Strain	Products of biodegradation*, %		Concentration residual DHA**, mg/L
	DHA	Metabolite *m/z* 330.3	
Abiotic control	100.0	–	490.0 ± 4.0
G. rubripertincta IEGM 100	90.5 ± 5.2	9.5 ± 0.9	443.0 ± 25.4
G. rubripertincta IEGM 104	9.0 ± 0.5	–	44.1 ± 2.4
G. rubripertincta IEGM 105	44.0 ± 5.2	–	215.0 ± 25.4
G. rubripertincta IEGM 109	41.0 ± 4.8	–	200.9 ± 23.5
G. rubripertincta IEGM 120	98.8 ± 0.9	1.2 ± 0.5	484.1 ± 4.4
G. rubripertincta IEGM 132	92.2 ± 3.5	8.2 ± 3.5	451.7 ± 17.5
G. terrae IEGM 150	35.0 ± 3.7	–	171.5 ± 18.1
R. erythropolis IEGM 267	86.0 ± 4.9	14.0 ± 1.2	421.4 ± 24.0
R. rhodochrous IEGM 107	≤ 2.0	–	4.0 ± 2.0
R. ruber IEGM 80	49.0 ± 5.7	–	240.0 ± 27.9

The GC–MS* and HPLC** data after 7-day cultivation of actinobacteria in the presence of DHA (500 mg/L) are shown.

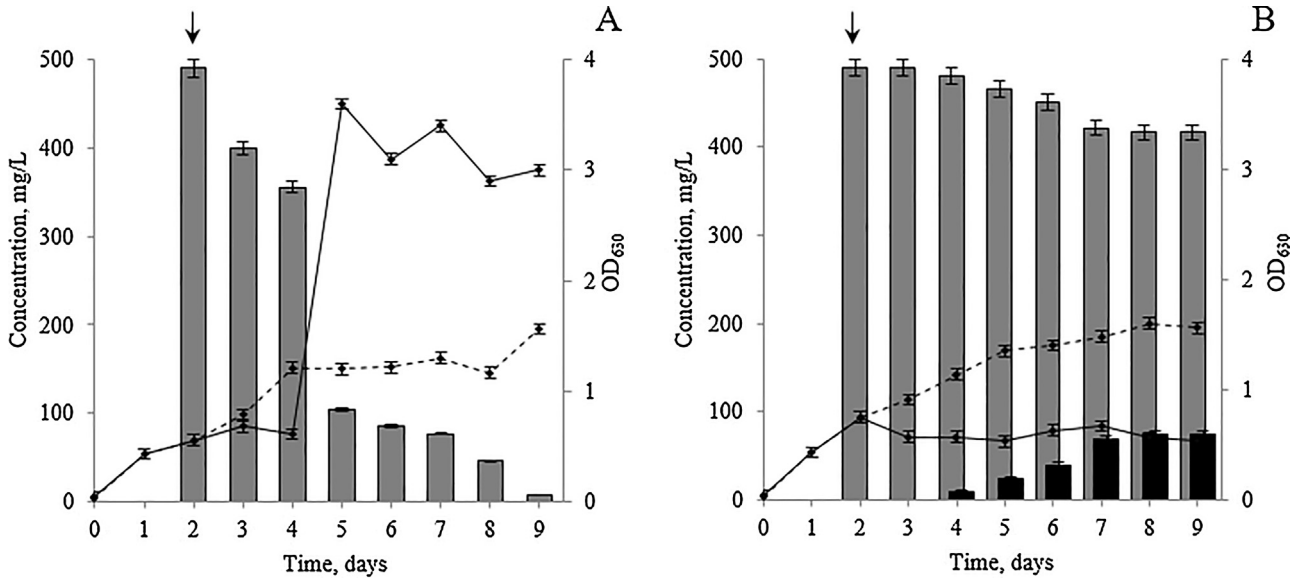

Fig. 2. Dynamics of DHA biodegradation by *R. rhodochrous* IEGM 107 (A) and *R. erythropolis* IEGM 267 (B).
Concentrations of ( ) DHA and ( ) metabolite *m/z* 330.3 (mg/L), ( ) bacterial growth (OD_{630}) during DHA biodegradation, ( ) bacterial growth in the presence of *n*-hexadecane. The arrow indicates the time points when DHA was added.

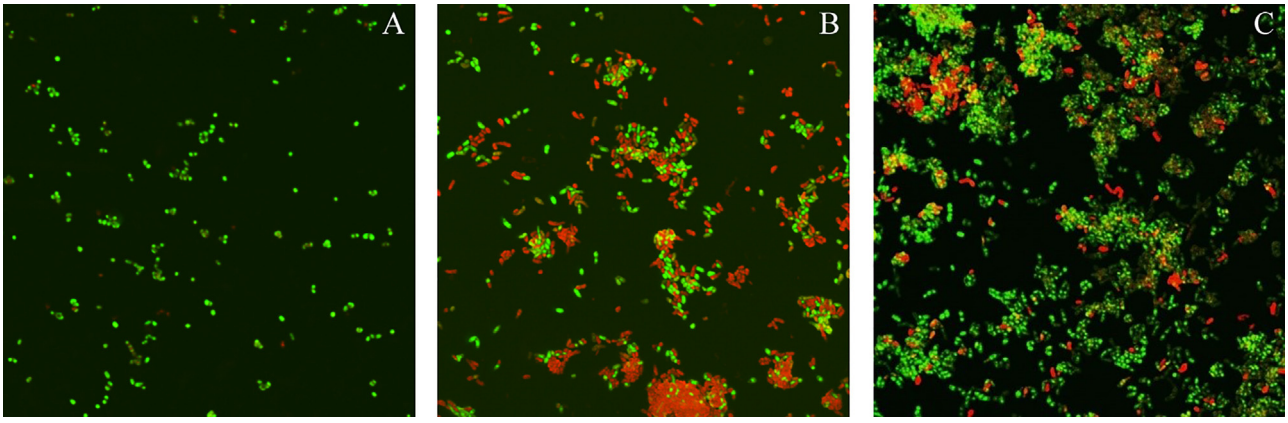

Fig. 3. CLSM images (100×) of *R. rhodochrous* IEGM 107 before DHA addition (A); after 48 h (B) and 72 h (C) of cultivation with DHA. Green cells are alive, red ones are dead. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cence were present in the CLSM images, indicating probably some toxic effect of the resin acid. After 72 h of cultivation in the presence of DHA, the number of viable cells increased (Fig. 3). Probably, this effect was due to cell adaptation to the ecotoxicant.

After 24 h cultivation of *R. erythropolis* IEGM 267 in the presence of 500 mg/L DHA, the damaged cells along with live and dead cells (Fig. 4) were found in the medium. A characteristic "double" (green-orange) luminescence of certain parts of a cell or of the whole cell may indicate the damaged membrane of viable cells. Similar effect

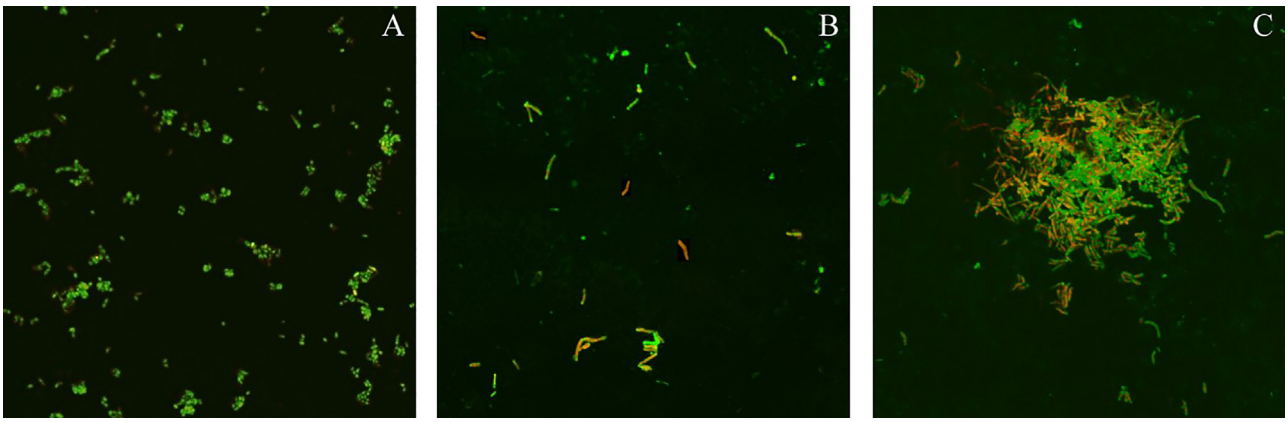

Fig. 4. CLSM images (100×) of *R. erythropolis* IEGM 267 before DHA addition (A); after 24 h (B) and 168 h (C) of cultivation with DHA. Green cells are alive, red ones are dead. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
DHA effects on morphometric parameters.

Conditions	Length, μm	Width, μm	Volume, μm^3	Area, μm^2
R. rhodochrous IEGM 107				
Mineral medium with *n*-hexadecane	1.38 ± 0.28	1.13 ± 0.19	1.38 ± 0.16	4.45 ± 0.28
Mineral medium with *n*-hexadecane plus DHA	1.82 ± 0.26	1.26 ± 0.24	2.26 ± 0.13	6.09 ± 0.23
R. erythropolis IEGM 267				
Mineral medium with *n*-hexadecane	3.10 ± 1.03	1.01 ± 0.09	2.65 ± 0.28	6.85 ± 0.18
Mineral medium with *n*-hexadecane plus DHA	6.83 ± 1.96	0.85 ± 0.11	3.87 ± 0.36	10.24 ± 0.24

Data are presented for living cells of actinobacteria after 7-day cultivation with 500 mg/L DHA and 0.1% (v/v) *n*-hexadecane.

(double luminescence) was observed when rhodococcal cells were stained with LIVE/DEAD dye after their exposure to high concentrations of organic solvents [38].

Morphometric studies using AFM analysis made it possible to record the average values of the cell size parameters. As shown in Table 3, the DHA additions induced changes in the cell size, in particular their increased length and width. The most pronounced size changes were observed when DHA was added to the culture medium of *R. erythropolis*IEGM 267. At the same time, an increase in the ratio of the cell surface area to its volume was noted, which, according to the literature data [29], promotes a more efficient cell-substrate contact.

AFM images of DHA-incubated *R. rhodochrous* IEGM 107 revealed extracellular fluid formation for many samples scanned (Fig. 5A). This phenomenon could not be a consequence of the lysis of bacterial cells because damaged cells were not found in the *Rhodococcus* population. When CLSM and AFM images were aligned, living cells were observed in the liquid. We assume that the extracellular fluid may be a biosurfactant or a mixture of a biosurfactant, *n*-hexadecane, and DHA. It is known, that biosurfactant synthesis contributes to the increased contact area of actinobacteria with hydrophobic compounds and their bioavailability [39]. Similar results were obtained by scanning actinobacterial cells during betulin biotransformation [40]. Atrat et al. suggested earlier that in β-sitosterol biotransformation by *Mycobacterium fortuitum* NRRL B-8119, a multicomponent mobile lipophilic mesophase was formed, which gradually dissolved and triggered the transport mechanism of the substrate into the cell [41]. Likewase, the AFM images of the obtained actinobacterial biosurfactant adsorbed on the glass exhibited micelles and vesicles formed around the bacterial cells [42]. Similar structures were seen in our images. AFM images of *R. erythropolis* IEGM 267 cells incubated with DHA showed no formation of extracellular fluid (Fig. 5B), while DHA granules were observed on the cell surface.

Table 4
Changes in the actinobacterial cell surface.

Culture conditions	Roughness, nm	Electrokinetic potential
R. rhodochrous IEGM 107		
Mineral medium with *n*-hexadecane	206.5 ± 10.7	−26.6 ± 0.9
Mineral medium with *n*-hexadecane plus DHA	365.9 ± 6.9	−27.3 ± 1.1
R. erythropolis IEGM 267		
Mineral medium with *n*-hexadecane	100.7 ± 9.8	−15.5 ± 1.4
Mineral medium with *n*-hexadecane plus DHA	289.3 ± 12.4	−19.8 ± 1.0

Data of AFM scanning and Zeta measurements of living actinobacterial cells after 7-day cultivation with 500 mg/L DHA plus 0.1% (v/v) *n*-hexadecane are shown.

DHA effects on actinobacteria were accompanied by changes in their cell surfaces. According to AFM, a characteristic feature of actinobacterial cell surface in the presence of DHA was increased root-mean-square roughness (Table 4). The electrokinetic (Zeta) potential data for bacterial cells suggest that addition of DHA has a more pronounced change in this parameter for *R. erythropolis* IEGM 267 (by 21.7%). A similar effect may be associated with a higher resistance of *R. rhodochrous* IEGM 107 to toxic effects of DHA compared to *R. erythropolis* IEGM 267 (MIC data).

Respiratory activity as one of the indicators of bacterial cell viability and intensity of metabolic processes was used to monitor DHA biodegradation in this study. As seen from Fig. 6A, DHA added to the culture medium on day 1 decreased the respiration rate. For *R. rhodochrous* IEGM 107, the decreased respiration rate correlated with the decreasednumber of viable cells. On day 5, the increased growth and also respiration rate was observed.

For *R. erythropolis* IEGM 267, the respiratory activity measurements during the DHA biodegradation showed that O_2 uptake and CO_2 release rates were independent of the number of viable cells over the experiment and correlated with the

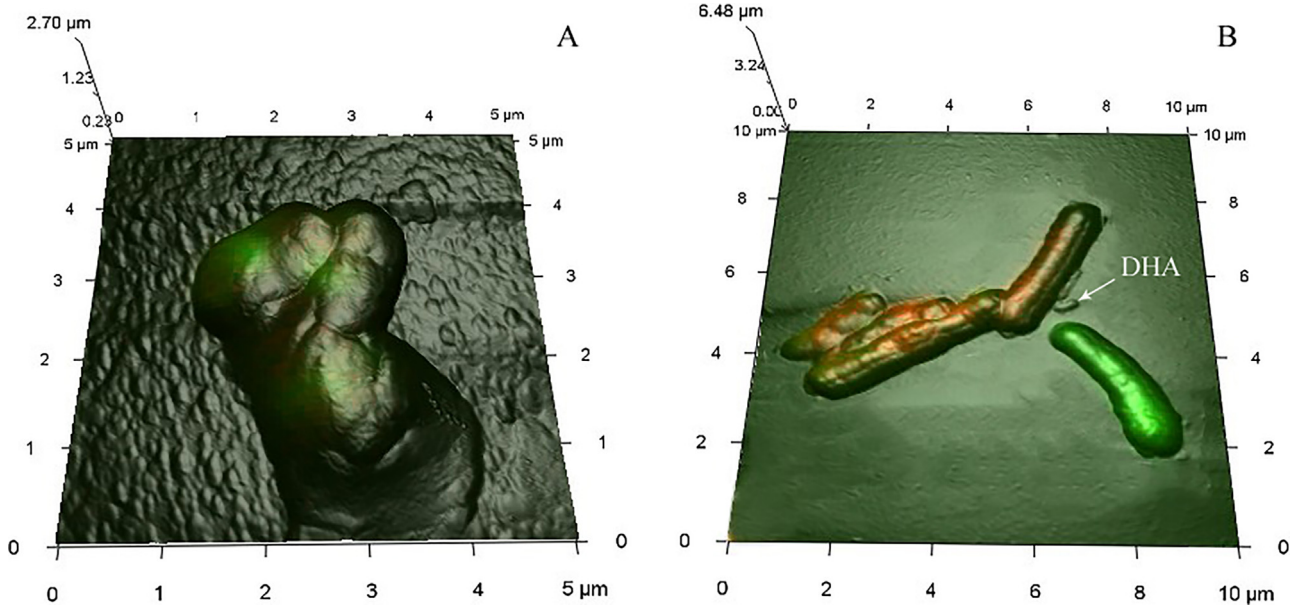

Fig. 5. AFM-CLSM images of *R. rhodochrous* IEGM 107 (A) and *R. erythropolis* IEGM 267 (B) after 168 h addition of DHA. The arrow indicates DHA granules.

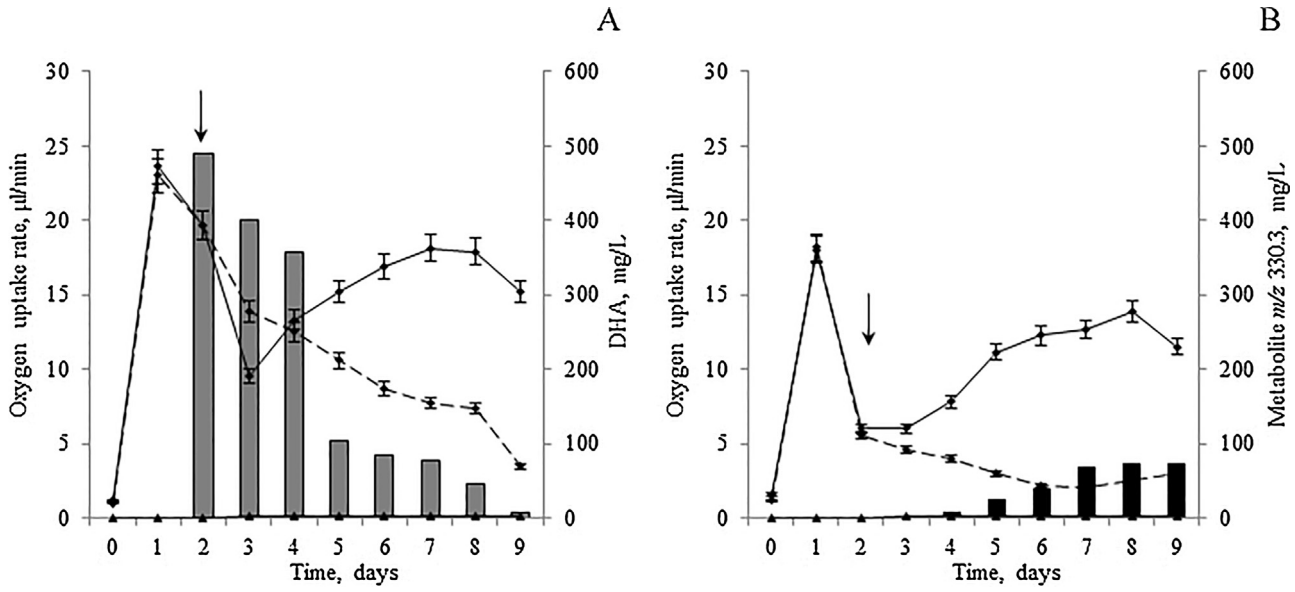

Fig. 6. Respiratory activities of *R. rhodochrous* IEGM 107 (A) and *R. erythropolis* IEGM 267 (B).
Oxygen uptake during DHA biodegradation (—●—) and growth in the presence of *n*-hexadecane (– ♦ –), abiotic control (—▲—), concentrations of (▭) DHA and (▬) metabolite *m/z* 330.3 (mg/L). The arrow indicates the time when DHA was added.

formation-accumulation of the DHA metabolite. Indeed, the highest respiration rates (13.41–14.28 μL/min) were measured after DHA addition to the nutrient medium on day 8 of the experiment (Fig. 6B) when the maximum level of metabolite formation was observed (see Fig. 1B). In general, the total amount of CO_2 released and O_2 consumed within 9 days of DHA biodegradation by *R. erythropolis* IEGM 267 (139088 μL of CO_2 and 149941 μL of O_2, respectively) was 1.5 times lower than those with *R. rhodochrous* IEGM 107 (223959 μL of CO_2 and 216549 μL of O_2, respectively). At the same time, the average respiration rate for *R. rhodochrous* IEGM 107 in the experiment (15 μL/min) was higher than that of *R. erythropolis* IEGM 267 (10 μL/min).

As previously shown, the level of actinobacterial catalytic activity is influenced by the pre-growth of bacterial cells in a medium with *n*-hexadecane [26,35,36]. In this respect, we studied the dependence of the metabolite *m/z* 330.3 formation on the initial concentration of *n*-alkane in the culture medium of *R. erythropolis* IEGM 267 (Fig. 7). According to our data, the addition of more than 0.1% (v/v) *n*-hexadecane led to a decreased degradation activity of rhodococci, with metabolite *m/z* 330.3 being not detected in the medium. At the same time, a noteworthy correlation of the growth and degradation activities with a decrease in the alkane concentration in the medium was observed. As shown in Fig. 7, rhodococci pre-grown with 0.06% (v/v) *n*-hexadecane demonstrated an increased metabolite *m/z* 330.3 formation up to 26.74% (133.7 mg/L). Whereas the OD_{630} value of the cell suspension stained with iodonitrotetrazolium did not exceed 0.57 before DHA addition, the monitored growth of rhodococci at the end of the experiment was on average by 40% higher compared with other experimental variants.

Table 5
^{1}H- and ^{13}C-NMR data of methyl 5α-hydroxy-abieta-8,11,13-triene-18-oate **8**.

Position	δ_C ^{13}C	DEPT	δ_H ^{1}H	HMBC
1	31.02	CH_2	2.03 m 2.11 m	H-1/C-2; H-1/C-3; H-1/C-5; H-1/C-9; H-1/C-20
2	17.49	CH_2	1.84 m	H-2/C-3; H-2/C-4; H-2/C-10
3	31.53	CH_2	2.41 m 1.58 m	H-3/C-1; H-3/C-2; H-3/C-4; H-3/C-5; H-3/C-18; H-3/C-19
4	50.62	C	–	
5	75.04	C	–	
6	25.31	CH_2	2.09 m 2.32 m	H-6/C-4; H-6/C-5; H-6/C-7; H-6/C-8; H-6/C-10
7	25.13	CH_2	2.92 m 3.06 m	H-7/C-5; H-7/C-8; H-7/C-9
8	133.72	C	–	
9	144.34	C	–	
10	42.22	C	–	
11	123.43	CH	7.14 d (8.2)	H-11/C-7; H-11/C-8; H-11/C-10; H-11/C-13
12	123.70	CH	7.01 brdd (8.2, 1.7)	H-12/C-9; H-12/C-14; H-12/C-15
13	145.01	C	–	
14	125.00	CH	6.94 brd (1.7)	H-14/C-9; H-14/C-12; H-14/C-15
15	32.95	CH	2.85 sept (6.9)	H-15/C-16,17; H-15/C-13; H-15/C-14; H-15/C-12
16 /17	23.42	$2CH_3$	1.25 d (6.9)	H-16,17/C-13; H-16,17/C-15; H-16/C-16,17; H-17/C-16,17
18	178.34	C	–	
19	19.22	CH_3	1.46	H-19/C-3; H-19/C-4; H-19/C-5; H-19/C-18
20	28.08	CH_3	1.33	H-20/C-3; H-20/C-5; H-20/C-9; H-20/C-10
21	51.45	CH_3OO	3.73	H-21/C-18
		OH	3.36	OH/C-4; OH/C-5; OH/C-10

Recorded at 400 (^{1}H-NMR) and 100 (^{13}C-NMR) MHz in $CDCl_3$; δ in ppm, s – singlet, d – doublet, dd – doublet of doublet, sept – septet, m – multiplet, *J* values (Hz) are indicated in brackets.

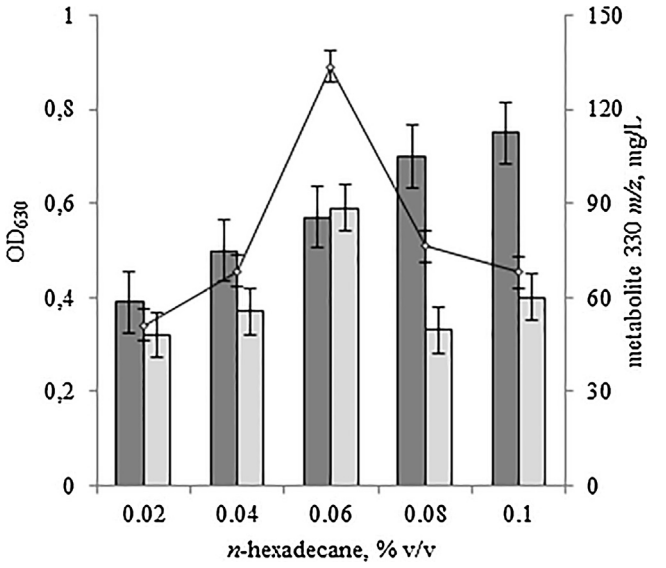

Fig. 7. Concentration (mg/L) of metabolite *m/z* 330.3 (—◇—) and growth of *R. erythropolis* IEGM 267 (OD_{630}) before DHA addition ( ) and after 168 h ( ) cultivation with DHA at different initial *n*-hexadecane concentrations (%, v/v) in the medium.

3.3. *Identification of metabolite* m/z *330.3*

Currently, it is important not only to find effective ways to neutralize toxic DHA, but also to obtain new compounds on its basis. The known DHA derivatives have a wide range of biological activities, i.e. antiulcer [43,44], antimicrobial [45], fungicidal [46,47], anxiolytic [48], antiviral [47,49], and antitumour [44,47,50], and also can be used as intermediates to synthesize bioactive compounds [51]. Numerous examples of biodegradation with the formation of stable metabolites (use of inhibitors) or targeted DHA bioconversions are described in the literature.

For example, 1β-hydroxy-DHA **2** isolated from the DHA culture medium by fungi *Aspergillus*, *Chephalosporum, Cunninghamella*, *Fusarium*, *Gibberella* and *Rhizopus* exhibits antimicrobial and inhibitory activities against α-glucosidase [15,16,52]. Selective antitumor and antimicrobial activities are exhibited by 2α-hydroxy-DHA **3**, the formation of which from DHA is catalyzed by *Mortierella isabellina* and *Mucor ramannianus* [53,54]. 7β-Hydroxy-DHA **4** formed during DHA biodegradation by *P. abietaniphila* has fungicidal and antitumour activities [13,45,55]. The formation of 15-hydroxy-DHA **5** is catalyzed by *C. elegans*, *G. fujkuroi*, *M. isabellina* and *R. erythropolis*. It has an anti-inflammatory effect and is used as an intermediate to synthesze antiviral and antitumor compounds [15,26,51,53]. 16-Hydroxy-DHA **6** produced in fungal DHA biodegradation inhibits α-glucosidase [15].

The metabolite **8** *m/z* 330.3 (M^+), is the methyl ether of a novel compound **7** obtained in this study. Methyl 5α-hydroxy-abieta-8,11,13-triene-18-oate **8** as colorless needle crystal (mp 59.6 °C (*n*-hexane), $[\alpha]_{589}^{22}$ + 24.0 (*c* 0.55, $CHCl_3$), R_f value 0.62 (hexane/ethyl acetate 7:3)) was isolated from the methylated extract by *silica gel flash chromatography*. This compound is a hydroxyl-containing DHA derivative, which was confirmed by the presence of the molecular-ion peak at *m/z* 330.3 in the GC—MS and NMR spectral data. So, IR absorption bands at 3480 cm^{-1} and 1721 cm^{-1} revealed hydroxyl and carbonyl groups in the structure of DHA derivative **8**. Its ^{13}C NMR spectrum (DEPT and APT) as well as ^{13}C NMR spectrum of DHA methyl ester [56] indicated the presence of 21 carbon signals, including two tertiary and two secondary methyl groups, a benzene ring, and low-field ester signals (Table 5). Among sp^3 carbon signals of compound **8,** DEPT measurements indicated only one tertiary (CH) carbon at δ_C 32.95 ppm assigned to C-15 and a new singlet of quaternary carbon at δ_C 75.04 ppm assigned to C-5 associated with a hydroxyl group. Alternatively ^{13}C NMR spectrum of the initial substrate included two tertiary sp^3 carbons C-5 and C-15. The ^{1}H NMR spectrum of compound **8** showed a new signal for C-5 hydroxyl group at δ_H 3.36 ppm and intact septet signal of H-15 at δ_H 2.85 ppm, along with the characteristic set of DHA methyl ester signals [57]. Based on 2D ^{1}H–^{1}H NMR and ^{13}C–^{1}H NMR spectra (COSY, NOESY, HMQC, and HMBC), compound **8** is suggested to be a methyl abieta-8,11,13-triene-18-oate derivative with a tertiary hydroxyl group at C-5. So, the most informative crosspeaks in

the HMBC spectra were H-1/C-2; H-1/C-3; H-1/C-20; H-3/C-5; H-3/C-18; H-3/C-19; H-6/C-5; H-6/C-7; H-7/C-8; H-7/C-9; H-19/C-4; H-19/C-5; H-19/C-18; H-20/C-10; OH/C-4; OH/C-5; OH/C-10. The NOESY spectrum of compound **8** confirmed the significant NOE interaction between the axial OHgroup and axial H-3, and weak spatial interactions of OH group with equatorial H-7 and both H-6 atoms, revealing the relative α-orientation of OH at C-5.

4. Conclusion

Based on the screening results of IEGM Collection bioresources, actinobacterial strains capable of complete (*R. rhodochrous* IEGM 107) or partial (*R. erythropolis* IEGM 267) biodegradation of ecotoxic DHA were selected. It was shown that the selected rhodococci were able to degrade DHA at a high initial concentration (500 mg/L) within relatively short period (7 days). Pre-growth of actinobacteria in the presence of *n*-hexadecane is essential for DHA degradation, with optimal *n*-hexadecane concentrations being 0.1 and 0.06% (v/v) for *R. rhodochrous* IEGM 107 and *R. erythropolis* IEGM 267, respectively. The DHA derivative was identified as the new natural product 5α-hydroxy-abieta-8,11,13-triene-18-oic acid. The data obtained expand our knowledge on catalytic activities of actinobacteria and can be used to develop advanced technologies for effective removal of resin acids from pulp mill effluents.

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