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BIOCATALYSIS

Bacterial Degradation of Ecotoxic Dehydroabietic Acid

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Abstract—The possibility of biodegrading dehydroabietic acid ($C_{20}H_{28}O_2$, CAS: 1740-19-8, abieta-8,11,13-trien-18-oic acid) (DAA), a toxic tricyclic diterpenoid, accumulated in the waste waters of the pulp and paper industry is studied using actinobacterial strain *Dietzia maris* IEGM 55^T. Cells of the strain *Dietzia maris* IEGM 55^T are shown to be resistant to DAA (MIC, 390 mg/L). The strain is not able to use DAA as its sole source of carbon and energy. DAA (500 mg/L) decays almost completely in 7 days when the bacteria are preliminarily grown with *n*-hexadecane. The DAA effect on the viability and respiratory activity of the bacteria is studied. Analysis of the antimicrobial activity shows that extracts of the obtained metabolites are not toxic, in contrast to the initial substrate. The resulting data expands our views on the catalytic activity of actinobacteria and their impact in decontaminating natural ecosystems that contain ecotoxicants.

Keywords: resin acid, dehydroabietic acid, biodegradation, *Dietzia maris*
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INTRODUCTION

Resin acids are widespread toxic tricyclic diterpenoids produced by coniferous plants of the family *Pinaciae*. Dehydroabietic acid ($C_{20}H_{28}O_2$, CAS: 1740-19-8, abieta-8,11,13-trien-18-oic acid;) (DAA) is the predominant component in the resin acids found in the galipot of coniferous plants (Fig. 1).

DAA has been detected in the waste waters of the pulp and paper industry in relatively high concentrations (up to 500 mg/L). DAA released into the environment has a chronic toxic effect on humans and upsets the ecological balance. The main indicators of DAA acute toxicity (LD_{50}) for different test organisms range from 0.1 to 6.5 mg/L [1–4].

Chemical means for treating industrial wastewaters do not allow the complete destruction of DAA, are ineffective, and require the use of aggressive reagents. Priority is therefore given to biotechnological means based on microbial enzymatic activity and natural processes of ecopollutant destruction. Biocatalysis enables us to effectively neutralize resin acids under "soft" conditions (physiological temperatures, normal pressure, and neutral pH values) [1]. Microorganisms belonging to different phylogenetic groups (fungi, bacteria) and capable of partial or complete DAA degradation are well known. Familiar DAA destructors include representatives of bacterial genera (*Pseudomonas*, *Sphingomonas, Zoogloea, Flavobacterium*) [5–8] and mycelial fungi (*Mortierella*, *Mucor*, *Fusarium*) [9]. Despite the great diversity of biodestructors, the most effective bacterial and fungal cultures are active in DAA concentrations not exceeding 180 mg/L [10–12] and 250 mg/L [13–15], respectively. The search for new biodestructors that are active in high DAA concentrations is thus of great relevance.

Alkanotrophic actinobacteria capable of using aliphatic, monoaromatic, and polyaromatic hydrocarbons as sources of carbon and energy are one of the main groups of biodestructors of ecotoxicants [16–19]. It is known that their system for the primary oxidation of hydrocarbons contains P450-dependent enzymes that probably participate in DAA oxidation [20–22]. Similar systems (P450-dependent monooxygenases) have recently been found in DAA-degrading

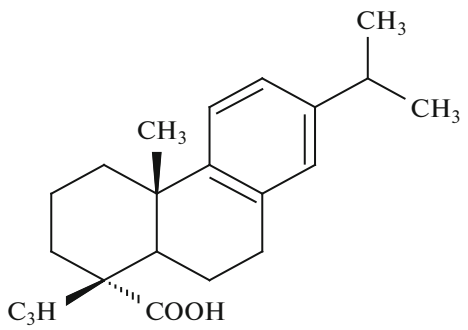

Fig. 1. Structural formula of DAA.

Table 1. Investigated strains

Species	Collection number
Dietzia maris	IEGM 44, IEGM 46, IEGM 52, IEGM 53, IEGM 54, IEGM 55^T, IEGM 166, IEGM 167, IEGM 168, IEGM 169, IEGM 290, IEGM 291, IEGM 293, IEGM 294, IEGM 295, IEGM 297, IEGM 298

Pseudomonas abietaniphila BKME-9 and *Streptomyces griseolus* 2ZBX [11, 23]. Nonmycelial growth, a lack of pathogenic properties, the lability of the metabolic system, and the specificity of multipurpose oxygenase enzymatic systems make actinobacteria promising catalyzers for DAA biodegradation.

The aim of this work was to assess the possibility of using actinobacterial strains of *Dietzia maris* for DAA biodegradation.

EXPERIMENTAL

This work was performed with 17 strains of *Dietzia maris* (Table 1) isolated from oil-contaminated soil and water and cataloged in the Regional Specialized Collection of Alkanotrophic Microorganisms (IEGM no. 768 in the World Federation of Culture Collections, www.iegm.ru; USU no. 73559, www.ckp-rf.ru/usu/73559).

The minimum inhibitory concentrations (MICs) of DAA for the strains were determined by means of double serial dilution with a 96-well polystyrene plate [24]. The wells were filled with 100 μL of meat-peptone broth (MPB). The DAA (10 mg) was dissolved in 100 μL of ethanol, and the solution was added to the first well in the range and thoroughly mixed. Then 100 μL of the mixture were collected and transferred to the next well. This manipulation was repeated to obtain double dilution series. DAA concentration range in the series was of 0.0244 to 50 g/L. Double dilutions of the DAA solvent (ethanol) of the same concentrations were prepared to assess the effect of ethanol on the level of MICs. Suspensions of the cultures preliminary grown in MPB (10 μL) were added to the mixtures to reach concentrations of 5×10^5 cells/mL. The plates were incubated for 3 days at 28°C.

In biodegradation experiments, the strains were batch cultivated in 250-mL Erlenmeyer flasks with 100 mL of the medium under constant stirring (160 rpm) at 28°C. The experiments were performed using a liquid mineral medium containing (g/L) KNO_3, 1.0; K_2HPO_4, 1.0; KH_2PO_4, 1,0; NaCl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.02; $FeCl_3$, 0.001. The medium was supplemented with 0.1% vol of trace element solutions [25]. The bacteria grown on meat-peptone agar (Oxoid, UK) for 48 h and suspended in a physiological solution were added to the nutrient medium to reach a concentration of 0.9×10^7 cells/mL. The medium was supplemented with 1 : 10 DAA (99.1%, Mosinter Group Limited, China) ethanol solutions (20 to 500 mg/L). Biodegradation was studied in experiments with DAA as the sole source of carbon and with DAA added after preliminary cultivation of the strains in 0.1% vol of *n*-hexadecane and 0.1 g/L of yeast extract (YE) for 2 days. Sterile DAA solution in the medium (to assess the abiotic degradation of DAA) and a nutrient medium without DAA but containing *n*-hexadecane, yeast extract, and bacterial cells (to control the metabolites formed during DAA degradation) were used as controls. The duration of DAA degradation was 7 days. The optical density (OD) of the cell suspension was measured using a Lambda EZ201 spectrophotometer (PerkinElmer, USA) at 660 nm. The pH values of the nutrient medium were measured with a pH-150m pH meter (GZIP, Belarus) throughout the experiment.

Staining with iodonitrotetrazolium violet (INT) was used to assess cell viability. To accomplish this, 100 μL of culture fluid were stained directly in a 96-well polystyrene plate by adding 50 μL of 0.2% INT aqueous solution to the wells. The INT was reduced, resulting in the formation of water-insoluble formazan and red-violet coloration confirming presence of active bacterial cells actively respirating cells. To achieve complete reduction of the dye, the samples were incubated at 28°C. After 24 hours of incubation, the OD of the stained solution was measured using a Multiscan Ascent microplate photometer (Thermo Electron Corporation, Finland) at 630 nm [26].

Microscopy was performed using an Axiostar plus phase contrast microscope (Carl Zeiss, Germany). The specimens were preliminarily fixed and stained with 10% Ziehl carbol fuchsin. Photodocumentation and measuring the size of cell aggregates were done using a Pixera camera (United States) and the Video-Test-Razmer 5.0 software (Russia).

The morphology of the bacterial cells was studied using the Olympus FV 1000 confocal laser scanning microscope (CLSM) (Olympus Corporation, Japan) at Perm State University. Drops (15–20 μL) of the cell suspension were mixed with equal volumes of LIVE/DEAD® BacLight™ Bacterial Viability Kit fluorescent dye (Invitrogen, United States), placed on the cover glass, and dried in air in the dark for 10–15 min. Each specimen was washed with deionized water and scanned using the CLSM with an immersion objective (magnification, 100×; numerical aperture, 1.4). An argon laser (λ = 488 nm) with a 505/525-nm barrier filter and a helium–neon laser (λ = 543 nm) with a 560/660-nm barrier filter were used to excite the fluorescence of the SYTO9 and propidium iodide, respectively, contained in the LIVE/DEAD® dye. The image

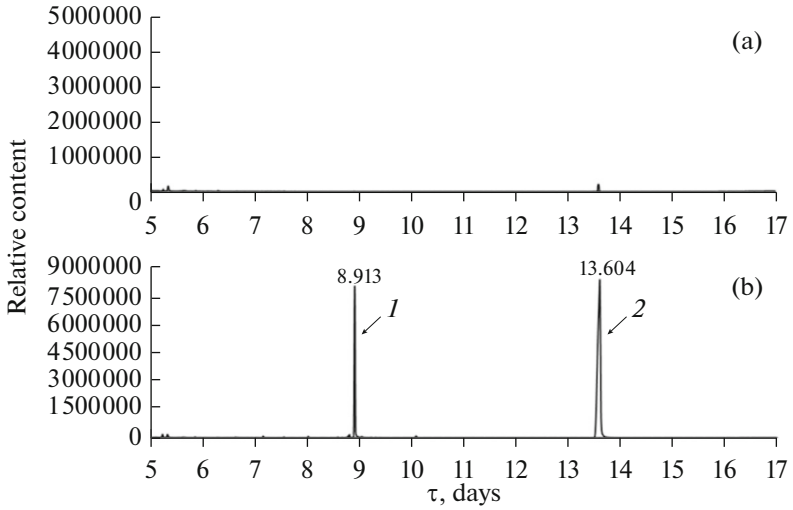

Fig. 2. Results of (a) GC–MS analysis performed during DAA biodegradation (500 mg/L) by *D. maris* IEGM 55^T and (b) abiotic control. Peaks correspond to (*1*) *n*-hexadecane and (*2*) DAA.

size was 0.12 × 0.12 mm (resolution, 1600 × 1600 pixels). The rate at which images were obtained was 40 nm/pixel. Qualitative analysis was carried out by the presence of green (live) or red (dead) cell fluorescence. Quantitative morphometric analysis of the images was performed using the FV10-ASW 3.1 software (Olympus Corporation, Japan). The volumes and areas of the cells were calculated according to [27] using the formulas

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

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

where r is half a cell's width, h is cell length, and $\pi = 3.14$.

The respiration of the strains was determined using a Micro-Oxymax^R 6-chamber closed circuit respirometer connected a computer (Columbus Instruments, United States). The experiments were performed in 300 mL-glass flasks (Micro-Oxymax, United States). Quantities (μL) of the utilized oxygen and released CO_2 were assayed with constant stirring (300 rpm, 28 ± 2°C) using an RT 10 magnetic stirrer (Power IKAMAG, Germany). The parameters of respiration were registered every 42 min for 9 days.

To extract DAA metabolites, the medium was acidified with 10% HCl and thrice extracted with equal volumes of ethyl acetate. The combined extracts were washed with 1% $NaHCO_3$ aqueous solution and distilled water (pH 7.0). The resulting ethyl acetate extract was dehydrated with Na_2SO_4. The solvent was removed using a Laborota 4000 rotary evaporator (Heidolph, Germany). The formation of metabolites was controlled by thin layer chromatography on silica gel plates (Merck, Germany). Samples of the extracts were preliminarily treated with trimethylsilyldiazomethane to prepare them for gas chromatography–mass spectrometry (GC–MS). Qualitative analysis was performed via GC–MS using an Agilent 6890N/5975B gas chromatograph (Agilent Technologies, United States) equipped with HP-5ms capillary column (30 m × 0.25 mm × 0.25 μm) in the electron impact ionization mode (70 eV). Helium was used as the carrier gas (1 ml/min). Quantitative DAA analysis was performed via reversed-phase high-performance liquid chromatography (RP-HPLC) using a Prominence 20AD chromatograph (Shimadzu, Japan) equipped with a Supelcosil^{TM} LC-18 chromatographic column (150 × 4 mm × 5 μm) and an SPD-M20A diode array detector. Acetonitrile (70%) was used as the eluent.

The antimicrobial activities of DAA and the obtained extracts of metabolites were assessed via double serial dilution as described above using test cultures *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Micrococcus luteus* NCIMB 196, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853. With the exception of *M. luteus* NCIMB 196, which was incubated at 28°C, the cultures were incubated at 37°C. Antibiotics (ampicillin, erythromycin) were used for comparison.

RESULTS AND DISCUSSION

Screening of the strains deposited in the collection allowed us to select strain *D. maris* IEGM 55^T capable of degrading DAA. According to our data, the DAA MIC for this strain was 390 mg/L, exceeding the known MIC values for DAA destructors described earlier [7, 15]. Despite the relatively high resistance of *D. maris* IEGM 55^T to DAA, this strain did not use resin acid as its sole source of carbon.

According to the literature data, some biodestructor microorganisms display catalytic activity in relation to resin acids only when there are additional growth substrates. For example, some members of the

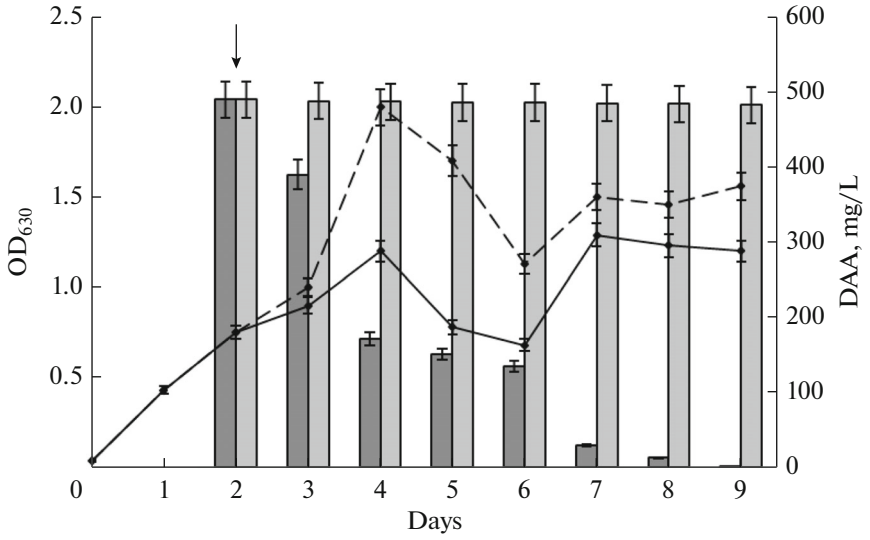

Fig. 3. DAA concentration ( ) and growth activity of *D. maris* IEGM 55^T with *n*-hexadecane and 500 mg/L of DAA (—●—), with *n*-hexadecane without DAA addition (–◆–); abiotic control ( ); ↓ corresponds to DAA addition.

genera *Pseudomonas* and *Bacillus* are capable of DAA utilization only in nutrient-rich media [22]. Cells were therefore preliminarily grown with *n*-hexadecane and yeast extract for 48 h to increase the destructive activity of the bacteria in relation to DAA (500 mg/L). According to our GC–MS results, *n*-hexadecane was not registered in the culture fluid after 7 days of degradation, while the residual content of DAA was less than 2% (Fig. 2). It should be noted that most known bacterial and fungal biodestructors utilize DAA (concentrations exceeding 250 mg/L) for 10–14 days, while only some representatives of *Proteobacteria* biodestruct 100 mg/L of DAA in 3 days [8, 11, 14, 28, 29].

Fig. 4. Aggregation of cells of *D. maris* IEGM 55^T: (a) with *n*-hexadecane; (b) after adding DAA. The data correspond to the ninth day of the experiment.

The growth and destruction of the strain *D. maris* IEGM 55^T were studied. The highest level of DAA destruction was observed simultaneously with the highest growth activity (at 4 and 7 days of the experiment) (Fig. 3). It was shown that the OD_{630} values with DAA were less than those in the control experiment, testifying to the activity of 500 mg/L of DAA in inhibiting growth.

It should be noted that with *n*-hexadecane as the sole source of carbon, the bacteria formed compact cell aggregates (of 15 to 30 μm) (Figs. 4a and 5a), while adding DAA resulted in the formation of larger aggregates 460 to 1300 μm in size (Figs. 4b and 5b). Aggregation probably allows the population of bacteria to adapt to conditions under which separate cells are not able to utilize toxic hydrophobic substrates.

The physiological state of bacterial cells in the presence of DAA was assessed based on the results obtained via confocal microscopy. Most of the cells remained viable (green fluorescence) after adding DAA (500 mg/L) (Fig. 6). The presence of cells with red fluorescence testified to the damage of cell membranes, indirectly confirming the toxic effect of the DAA concentration that was used.

Morphometric analysis revealed the average values of the main parameters of viable cells in the presence of DAA, which induced changes in cell sizes (particularly shorter lengths and narrower widths) (Table 2). The ratio of cell surface area to volume increased; according to the literature, this allows for a better contact between the cells and substrates [19]. Changes in

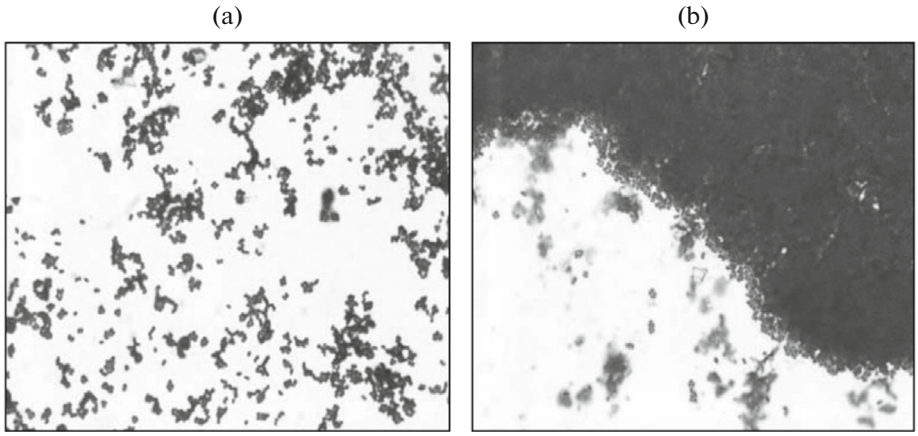

Fig. 5. Cell aggregates of *D. maris* IEGM 55^T in phase contrast mode: (a) aggregates formed with *n*-hexadecane; (b) fragment of an aggregate formed after adding 500 mg/L of DAA.

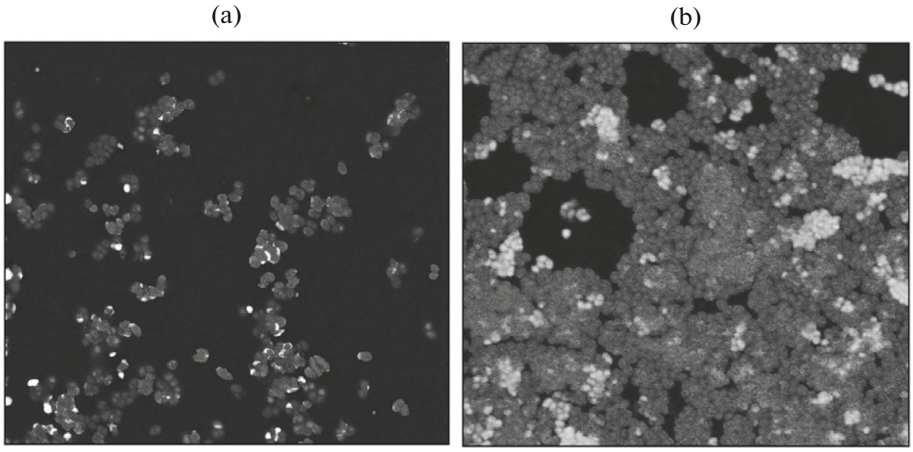

Fig. 6. Cell aggregates of *D. maris* IEGM 55^T under confocal laser scanning microscopy: (a) aggregates formed with *n*-hexadecane; (b) fragment of the aggregate formed after adding DAA. In our black-and-white images, viable cells with green fluorescence are gray; dead cells with red fluorescence are light grey.

morphophysiological properties could probably be considered as a mechanism of the actinobacterial cell adaptation to the toxic effect of the studied acid.

The dynamics of the bacteria's respiratory activity was characterized by stable O_2 consumption and CO_2 release during DAA biogradation. The rates curves of O_2 consumption and CO_2 release virtually mirrored one another. Adding of DAA increased the respiratory activity of the bacteria (Fig. 7). The highest respiratory activity (34.0–35.5 μL/min of O_2 and 35.24–36.79 μL/min of CO_2) was registered on the fourth day of the experiment. As noted above (Fig. 2), DAA biodegradation and growth activity during this period were also high. The subsequent reduction in respiratory activity correlated with a drop in DAA concentration probably due to depletion of the source of carbon (both *n*-hexadecane and DAA). Neither respiration nor DAA degradation were detected in the abiotic control. The degradation of the resin acid was thus caused by the catalytic activity of the bacteria.

The pH of the fermentation medium on day one after adding DAA predictably shifted to a weakly acid (pH 6.26) region of the spectrum. As the concentration of DAA in the medium fell, the pH values rose to

Table 2. Morphometric parameters of *D. maris* IEGM 55^T cells during DAA biodestruction

Carbon source	Length, μm	Width, μm	Volume, μm^3	Area, μm^2
n-Hexadecane	1.74 ± 0.15	1.38 ± 0.13	2.6 ± 0.023	6.75 ± 0.38
n-Hexadecane + DAA	1.24 ± 0.14	1.09 ± 0.11	1.15 ± 0.01	3.98 ± 0.23

Presented data correspond to the ninth day of the experiment.

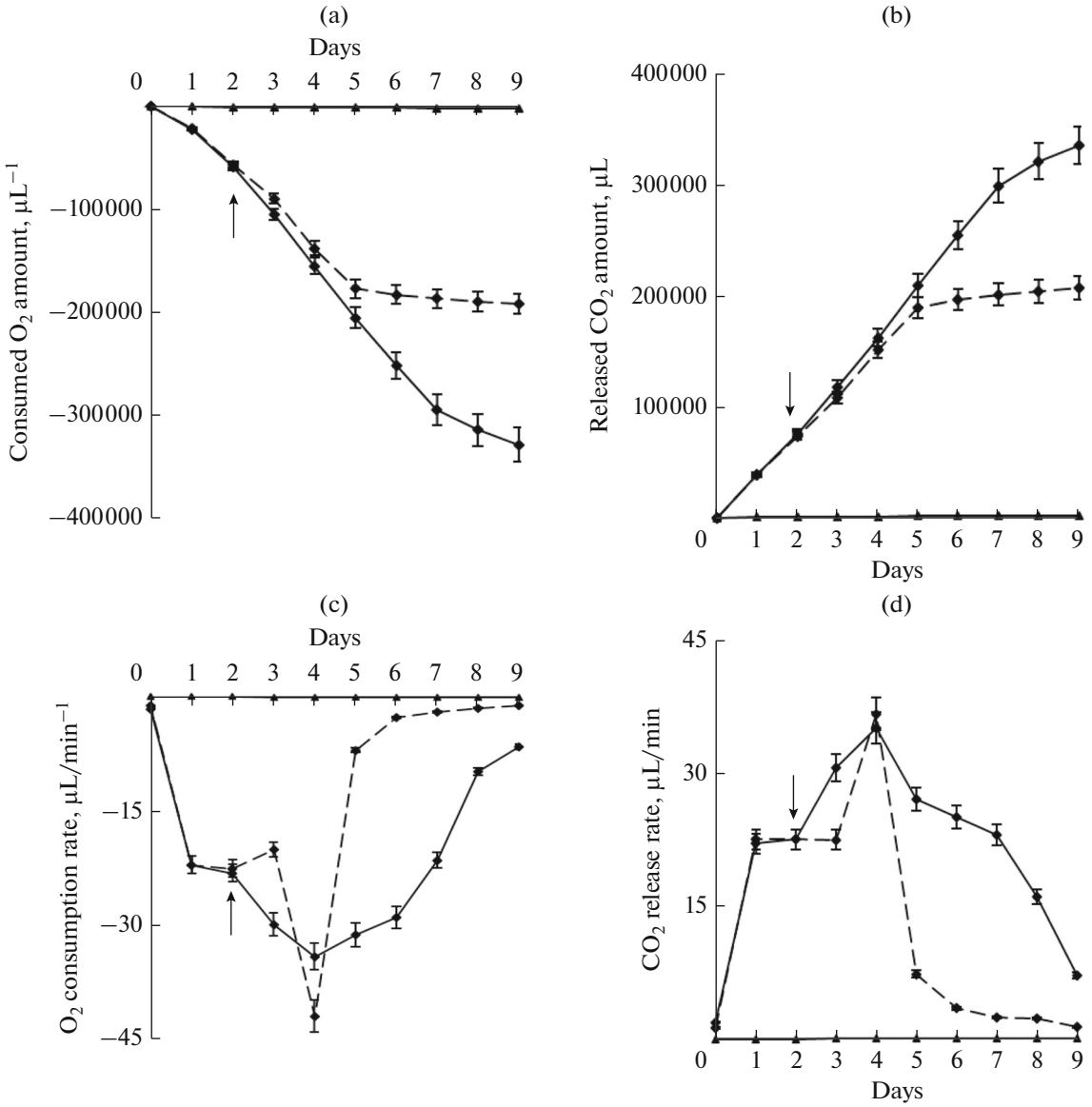

Fig. 7. (a) Dynamics of O_2 consumption, (b) CO_2 release and (c, d) respiratory activity of *D. maris* IEGM 55^T cells with *n*-hexadecane (–◆–), after adding DAA (–●–), and abiotic control (–▲–); ↓ marks the addition of DAA.

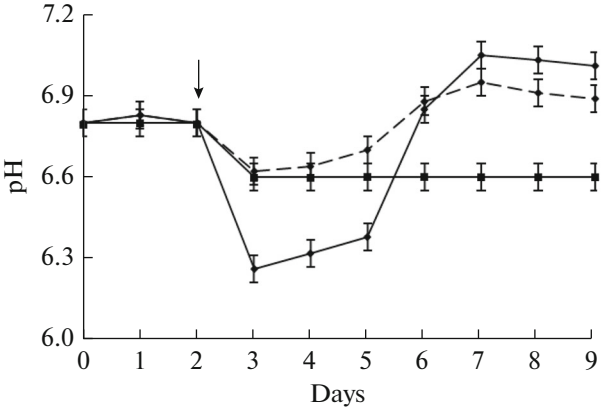

Fig. 8. Changes in pH of the culture medium *D. maris* IEGM 55^T with *n*-hexadecane (–◆–), after adding DAA (–●–), and abiotic control (–▲–); ↓ marks the addition of DAA.

neutral (pH 7.01–7.03). The observed changes in pH of the reaction medium were associated with the bacterial catalytic activity, since the pH of the abiotic control changed slightly after adding DAA (Fig. 8).

Analysis of the antimicrobial activity of DAA and ethyl acetate extracts of the metabolites obtained after the experiments revealed that gram-positive bacteria were most sensitive to DAA (Table 3). The MICs for *S. aureus* ATCC 25923 and *M. luteus* NCIMB 196 were 0.097 and 0.024 mg/mL, respectively. The low concentration of DAA (0.012 mg/mL) completely inhibited the growth of *B. subtilis* ATCC 6633. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were more resistant to DAA, with MIC values of 1.56 and 12.5 mg/mL, respectively.

The antimicrobial activity of ethyl acetate extracts of the obtained metabolites was lower than that of

Table 3. Antimicrobial activity (MIC) of DAA and extracts of its metabolites (mg/mL)

Test culture	DAA	Extract	Ethanol	Ampicillin	Erythromycin
S. aureus ATCC 25923	0.097	12.5	12.5	0.000047	0.0007
M. luteus NCIMB 196	0.024	0.78	12.5	0.012	0.0015
B. subtilis ATCC 6633	0.012	1.56	25	0.024	0.000047
E. coli ATCC 25922	1.56	12.5	12.5	0.024	0.19
P. aeruginosa ATCC 27853	12.5	–*	12.5	–*	0.19

*No antimicrobial activity was detected.

DAA. The MIC values for *E. coli* ATCC 25922, *M. luteus* NCIMB 196, and *S. aureus* ATCC 25923 were 8, 32, and 128 times higher. The MIC value for *B. subtilis* ATCC 6633 changed from 0.012 to 1.56 mg/mL. The inhibitory activity in relation to *P. aeruginosa* ATCC 27853 was detected at more than 50.0 mg/mL of the extract. Our results thus testify indirectly to the possibility of DAA detoxification using *D. maris* IEGM 55^T cells.

CONCLUSIONS

Our work using the bioresources of the Regional Specialized Collection of Alkanotrophic Microorganisms allowed us to select strain *D. maris* IEGM 55^T, which displayed high activity (up to 98%) in DAA biodegradation with 500 mg/L of the acid. It was shown that in contrast to DAA, extracts of the resulting metabolites were nontoxic. Our results show that *D. maris* IEGM 55^T could be a promising effective biocatalyzer for the degradation of toxic DAA.

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