Diclofenac as a factor in the change of *Rhodococcus* metabolism

E A Tyumina¹, G A Bazhutin², E V Vikhareva³, A A Selyaninov⁴ and I B Ivshina¹,²,³

¹Perm State National Research University, Perm, Russia
²Perm State Humanitarian Pedagogical University, Perm, Russia
³Perm State Pharmaceutical Academy, Perm, Russia
⁴Perm State National Research Polytechnic University, Perm, Russia
⁵Institute of Ecology and Genetics of Microorganisms, Ural Branch Russian Academy of Sciences, Perm, Russia

E-mail: elenatyumina@mail.ru

Abstract. The non-steroidal anti-inflammatory drug diclofenac (2-[2-(2,6-dichloroanilino)phenyl] acetic acid) is one of the most frequently detected pharmaceuticals in the environment. The ability of actinobacteria of the genus *Rhodococcus* to biodegrade diclofenac (170 μM) as the sodium salt has been revealed. Cells of *R. ruber* IEGM 231 pre-grown with diclofenac (1.7 μM) removed diclofenac (about 50% degradation) within 56 days in the presence of glucose. Diclofenac induced changes in the morphometric characteristics of rhodococci. The products of diclofenac biodegradation did not have pronounced phytotoxicity.

1. Introduction

Diclofenac (DCF; C14H11Cl2NO2; CAS No. 15307-86-5; 2-[2-(2,6-dichloroanilino)phenyl] acetic acid) is one of the most popular non-steroidal anti-inflammatory drugs (NSAIDs) with pronounced analgesic, anti-inflammatory and antipyretic activities. The annual consumption of DCF is 940 tons per year [1]. It is known that in the human body DCF is not completely metabolized, and part of the administered dose is released unchanged [2]. In sewage treatment plants, the efficiency of removal of DCF does not exceed 60% [3]. In this regard, DCF migrates to natural ecosystems and it is the most frequently detected pharmaceutical in the environment [4]. In water and terrestrial ecosystems, DCF occurs in concentrations ranging from 0.02 ng/L to 20.00 μg/L [4]. Data on the detection of DCF in water of Russia are few. In rivers and reservoirs of Moscow DCF was detected at concentrations of 0.025–0.35 ng/L, and in St. Petersburg sewage DCF concentrations were up to 550 ng/L [5, 6].

DCF is an aromatic chlorinated nitrogen-containing compound, a derivative of aromatic carboxylic acid. The molecular structure of DCF causes its toxicity, persistence and high resistance to degradation.

The toxic effects of this NSAID on birds [7], hydrocoles [8], plants [9] have already been observed. In humans, DCF can cause disruptions in liver and gastrointestinal tract, as well as adverse cerebral and cerebellar effects [10, 11].

In connection with the inefficiency of traditional methods of pharmaceutical waste disposal, the search for alternative technologies is relevant. The use of enzymatic activity of microorganisms is
promising. However, works devoted to microbial biodegradation of DCF are few. There are a few works on the biodegradation of DCF by Actinoplanes [12], Brevibacterium [13], Labrys [14], and Raoultella [3].

Previously, we selected strains of actinobacteria which are active biodestructors of DCF [15]. To understand the mechanisms of DCF biodegradation, the complete genome of DCF degrading bacterium R. ruber IEGM 231 which is also active biodegrader of a wide range of complex organic compounds, has been deciphered [16]. Among the revealed abundance (more than 6000) of catabolic genes, genes encoding oxygenases/hydroxylases (75), dioxygenases (45), cytochromes P450 (21), dehydrogenases (285), laccases and other enzymes were found. Using the obtained data, we proceeded from the assumption that the multipurpose oxygenase systems of actinobacteria will catalyze the processes of direct oxidation of DCF, including the introduction of hydroxyl groups in the aromatic ring until the chemical structure of this ecotoxicant is completely destroyed.

In this paper, the results of the study of DCF biodegradation by R. ruber IEGM 231 are presented.

2. Materials and methods
The strain R. ruber IEGM 231 (DDBJ/EMBL/GenBank under accession numbers CCSD0100001 to CCSD01000115) isolated from spring water (Perm, Russia) is an active biodegrader of complex hydrophobic organic compounds, including the analgesic n-acetaminophen [15]. Strain is deposited in the Regional Specialized Collection of Alkanotrophic Microorganisms (IEGM, WDCM 768, http://www.iegmcol.ru/strains/rhodoc/ruber/r_ruber231.html).

Experiments on DCF biodegradation were carried out using the mineral medium RS [17]. DCF was added as the sole carbon and energy source at a concentration of 170 μM. As a co-substrate, glucose (28 mM) was added. Rhodococci were grown in LB broth supplemented with 1.7 μM DCF. Broth cultures were centrifuged at 3,000 rpm for 10 min, washed twice with 10 mM potassium phosphate buffer (pH 7.0) and re-suspended in the same buffer to a concentration of 7.5×10⁸ cells/ml.

A mathematical prediction of the duration of DCF biodegradation process was carried out based on data on removal of DCF. As a mathematical model of the change in the DN concentration, a first-order kinetic equation was used (1).

\[ \frac{dx}{dt} = -kx \]  

The controls included (a) sterile DCF-containing medium to evaluate abiotic DCF degradation; (b) DCF-containing medium with Rhodococcus cells killed by threefold autoclaving at 120 °C for 20 min to evaluate DCF sorption by cell biomass; (c) Rhodococcus-inoculated glucose-containing medium without DCF to differentiate bacterial metabolites resulting from DCF and glucose.

The combined atomic-force and confocal laser scanning system including the MFP-3D-BIOTM atomic force microscope (AFM; Asylum Research Inc., USA) and the Olympus Fluo View 1000 confocal laser microscope (Olympus, Japan) was applied.

The DCF concentrations in the cultivation media during the biodegradation process were measured by high-performance liquid chromatography using a LC Prominence chromatograph (Shimadzu, Japan) equipped with a Phenomenex Jupiter 5u reversed-phase C18 300 A column 250 × 4.6 mm 5 micron (Phenomenex, USA). Mobile phase was phosphate buffer (pH 3.5) – acetonitrile (60:40). Mobile phase flow rate was 1.0 mL/min. DCF was detected at 276 nm.

Products of DCF biodegradation were analyzed by gas chromatography-mass spectrometry (GC-MS) using an Agilent 6890-5973N chromatograph (Agilent Technologies, USA) equipped with a HP-5MS capillary column (30 m, inner diameter, 0.25 mm) operating in the ionization mode with an electronic impact at 70 eV. The extraction of DCF and its metabolites was carried out by chloroform.

The toxicity of DCF and its biodegradation products was assessed by phytotoxicity test [18] using Avena sativa L. Biological activity of individual products was predicted by PASS Online.

All experiments were performed in three replicates.
3. Results and discussion

3.1. Dynamics and kinetics of DCF biodegradation

*R. ruber* IEGM 231 was able to use DCF as the sole source of carbon and energy. In order to enhance the DCF bioconversion process, approaches of pre-growing bacteria in the presence of a low concentration of DCF (1.7 µM), as well as fractional supplementation of glucose as an additional source of carbon, were used. It was found that the residual content of DCF in the post-fermentation culture medium of *R. ruber* IEGM 231 on 56 day of the experiment was about 50% (figure 1).

The kinetic curves for the residual concentration of DCF, obtained by least-squares procedure, corresponded to the experimental data (figure 2). The values of the DCF biodegradation rate constant $k$ and the half-life $t_{1/2}$ were $k = 0.012\pm0.004$ days$^{-1}$ and $t_{1/2} = 56.82\pm1.71$ days. The prediction of the time to decrease DCF concentration to 1% from the initial DCF concentration with a probability of 95% was 526 days, using the hypothesis of the lognormal distribution law for the constant $k$.

![Figure 1. Biodegradation of DCF by *R. ruber* IEGM 231 (1). 2 – cellular dry weight, 3 – biosorption control, 4 – abiotic control.](image1)

![Figure 2. Prediction of changes in DCF concentration with a probability of 95% and lognormal distribution law. 1 – upper limit; 2 – lower limit; 3 – selective analog of mathematical expectation.](image2)
3.2. Change in morphometric characteristics of rhodococci exposed to DCF
The most typical reactions of R. ruber IEGM 231 cells exposed to DCF were an increase in cell length (figure 3B1), a change in the surface profile (figure 3B2), and an increase in the mean square roughness of cell surface (figure 3B3).

![Figure 3](image1.png)

**Figure 3.** AFM-images (1), surface profile (2) and frequency of distribution of surface roughness (3) of R. ruber IEGM 231. A – control cells, B – cells exposed to diclofenac.

3.3. DCF biodegradation products, their phytotoxicity and biological activity
The results of GC-MS revealed that among the primary metabolites of DCF biodegradation, 4'-hydroxydiclofenac, 5-hydroxydiclofenac and p-benzoquinone imine of 5-hydroxydiclofenac, were found. According to figure 4, they did not show significant phytotoxicity. It should be noted that these compounds undergo further decomposition to simple aliphatic compounds.

![Figure 4](image2.png)

**Figure 4.** Morphometric changes of Avena sativa L. exposed to DCF and the products of its biodegradation.
We predicted the biological activity of 5-hydroxydiclofenac of p-benzoquinone by PASS Online. Biological activity is described in PASS in a qualitative way ("yes"/"no"). The output of the prediction, in addition to the activity names, includes estimates of the probability of occurrence (Pa) and the absence of each activity (Pi) having values from 0 to 1. Calculations of the prediction of biological activity of p-benzoquinone carried out in PASS are summarized in table 1.

It is assumed that p-benzoquinone is CYP2J, GST A substrate, has broad inhibitory properties, can be used in the treatment of phobic disorders, rhinitis and can also have antiseboric activity.

**Table 1.** Prediction of biological activity of 5-hydroxyDCF p-benzoquinone imine by PASS Online.

| Pa   | Pi   | Activity                                      |
|------|------|-----------------------------------------------|
| 0.929| 0.003| CYP2J2 substrate                              |
| 0.925| 0.003| CYP2J substrate                               |
| 0.887| 0.006| Chlordecone reductase inhibitor               |
| 0.837| 0.009| Gluconate 2-dehydrogenase (acceptor) inhibitor|
| 0.807| 0.011| HIF1A expression inhibitor                    |
| 0.797| 0.012| Anaphylatoxin receptor antagonist             |
| 0.758| 0.021| Glycosylphosphatidylinositol phospholipase D inhibitor |
| 0.757| 0.008| Linoleate diol synthase inhibitor             |
| 0.744| 0.017| GST A substrate                               |
| 0.744| 0.053| Ubiquinol-cytochrome-c reductase inhibitor    |
| 0.728| 0.023| NADPH peroxidase inhibitor                    |
| 0.728| 0.064| Phobic disorders treatment                    |
| 0.719| 0.003| Rhinitis treatment                            |
| 0.707| 0.036| Antiseborrheic                                |
| 0.703| 0.054| Membrane integrity agonist                   |

**4. Conclusions**

We have found that *R. ruber* IEGM 231 exhibited resistance to a high concentration of DCF (170 μM). Discovered cellular characteristics could be considered as mechanisms of cell adaptation and, as a result, increase their resistance to the negative effects of ecotoxic DCF. During the biodegradation of DCF, products that did not have phytotoxicity were formed.

*R. ruber* IEGM 231 could be recommended for the production of a stable biocatalyst for use in wastewater treatment plants and for bioremediation of contaminated sites from pharmaceutical toxicants.

**Acknowledgments**

The study was supported by the Program of Fundamental Research of the Ural Branch of the Russian Academy of Sciences (18-4-8-21) and the Russian Foundation for Basic Research and the Ministry of Education and Science of the Perm Territory (grant number 17-44-590567).

**References**

[1] Zhang Y, Geissen S-U and Gal C 2008 Chemosphere **73** 1151–61
[2] Vieno N and Sillanpää M 2014 Environ. Int. **69** 28–39
[3] Palyzová A, Zahradník J, Marešová H, Sokolová L, Kyslíková E, Grulich M, Štěpánek V, Rezanka T and Kyslík P 2018 Folia Microbiol. (Praha) **63** 273–82
[4] aus der Beek T, Weber F A, Bergmann A, Hickmann S, Ebert I, Hein A and Küster A 2016 Environ. Toxicol. Chem. **35** 823–35
[5] Barenboim G M, Chiganova M A and Berezovskaya I V 2014 Water Sector Russia: Probl.
[6] Russkikh Ya V, Chernova E N, Nikiforov V A and Zhakovskaya Z A 2014 *Ecolog.* **1-2** 77–83
[7] Oaks J L et al 2004 *Nature* **427** 630–33
[8] Guiloski I C, Ribas J L C, da Silva Pereira L, Neves A P P and de Assis H C S 2015 *Ecotoxicol. Environ. Saf.* **114** 204–11
[9] Kummerová M, Zezulka Š, Babula P and Tríska J 2015 *J. Hazard. Mater.* **302** 351–61
[10] Bjorkman D 1998 *Am. J. Med.* **105** 17S–21S
[11] Aygün D, Kaplan S, Odaci E, Onger M E and Altunkaynak M E 2012 *Histol. Histopathol.* **27** 417–36
[12] Osorio-Lozada A, Surapaneni S, Skiles G L and Subramanian R 2008 *Drug Metab. Dispos.* **36** 234–40
[13] Bessa V S, Moreira I S, Tiritan M E and Castro P M L 2017 *Int. Biodeter. Biodegr.* **120** 135–42
[14] Moreira S, Bessa V S, Murgolo S, Piccirillo C, Mascolo G and Castro P M L 2018 *Ecotoxicol. Environ. Saf.* **152** 104–13
[15] Ivshina I B, Tyumina E A and Vikhareva E V 2017 *Microbial biotechnologies: fundamental and applied aspects* vol 9 (Minsk: Belaruskaya Navuka) pp 63–78
[16] Ivshina I B, Kuyukina M S, Krivoruchko A V, Barbe V and Fischer C 2014 *Genome Announc.* **2**(6) e01297–14
[17] Catalogue of Strains of Regional Specialized Collection of Alkanotrophic Microorganisms (accessed May 15rd, 2018. Available from: http://www.iegmcoll/strains/index.html)
[18] Liwarska-Bizukojc E and Urbaniak M 2007 *Biotechnologia* **76** 203–14