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ORIGINAL ARTICLE

Potential of the strain *Raoultella* sp. KDF8 for removal of analgesics

Andrea Palyzová[1] · Jiří Zahradník[2,3] · Helena Marešová[1] · Lucie Sokolová[1] · Eva Kyslíková[1] · Michal Grulich[1] · Václav Štěpánek[1] · Tomáš Řezanka[1] · Pavel Kyslík[1]

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Abstract The bacterial strain KDF8 capable of growth in the presence of diclofenac and codeine analgesics was obtained after chemical mutagenesis of nature isolates from polluted soils. The strain KDF8 was identified as *Raoultella* sp. based on its morphology, biochemical properties, and 16S rRNA gene sequence. It was deposited in the Czech Collection of Microorganisms under the number CCM 8678. A growing culture efficiently removed diclofenac (92% removal) and partially also codeine (about 30% degradation) from culture supernatants within 72 h at 28 °C. The degradation of six analgesics by the whole cell catalyst was investigated in detail. The maximum degradation of diclofenac (91%) by the catalyst was achieved at pH^{INI} of 7 (1 g/L diclofenac). The specific removal rate at high concentrations of diclofenac and codeine increased up to 16.5 mg/g_{CDW} per h and 5.1 mg/g_{CDW} per h, respectively. HPLC analysis identified 4′-hydroxydiclofenac as a major metabolite of diclofenac transformation and 14-hydroxycodeinone as codeine transformation product. The analgesics ibuprofen and ketoprofen were also removed, albeit to a lower extent of 3.2 and 2.0 mg/g_{CDW} per h, respectively. Naproxen and mefenamic acid were not degraded.

✉ Helena Marešová
maresova@biomed.cas.cz

[1] Institute of Microbiology of the Czech Academy of Sciences, Vídeňská 1083, 14220 Prague 4, Czech Republic

[2] Department of Genetics and Microbiology, Faculty of Science, Charles University in Prague, Viničná 5, 12840 Prague 2, Czech Republic

[3] Laboratory of Biomolecular Recognition, Institute of Biotechnology, v.v.i., BIOCEV, Průmyslová 595, 25250, Vestec, Czech Republic

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Introduction

The accumulation of newly emerging pollutants such as active pharmaceutical ingredients (APIs) and their metabolites in the aquatic environment has recently become a serious problem. Since APIs are only partially removed during wastewater treatment (Zhang et al. 2008), they are detected in groundwater, surface water, and wastewater effluents, as well as in drinking water at concentrations ranging from a few nanograms per liter (Jones et al. 2005) to 15 μg/L (Jux et al. 2002). The elimination of these pollutants is required because of their toxicity to organisms (Gavrilescu et al. 2015). Chronic toxicity in invertebrates (Ferrari et al. 2003) and hatching delay of zebra fish embryos (Hallare et al. 2004) were reported for concentrations in the milligram per liter range. Physicochemical techniques for the removal of APIs, based upon ozonation and oxidation processes, have specific disadvantages such as formation of toxic products (Guzzella et al. 2002; Ikehata et al. 2006; Richardson et al. 2007). Similarly, conventional techniques for wastewater treatment such as filtration or application of activated sludge do not efficiently remove APIs, which results in finding APIs in environmental samples including surface water, groundwater, and drinking water (Gomez et al. 2007; Benotti et al. 2009; Behera et al. 2011; Vieno and Sillanpää 2014). Based on a monitoring program carried out in five municipal sewage treatment plants, pharmaceutical pollutants were divided into several groups according to their removal efficiency (Bueno et al. 2012). Studies show that widely used analgesics are present in relevant concentrations in all the groups. Naproxen (NAP) and ibuprofen (IPF) are ranked among the nonsteroidal anti-

inflammatory drugs (NSAIDs) with high removal efficiency of 80%. In addition, most opioid and non-opioid analgesics show acceptable removal efficiency, e.g., diclofenac (DCF) and codeine (CD) around 60%. Low removal efficiency was reported for ketoprofen (KTP, 40%) and mefenamic acid (MA, 37%).

Despite the vast knowledge on the distribution of analgesics in the environment, very little is known about their fate especially in terms of microbial co-metabolism or biotransformation (Liu et al. 2001). The bacterial removal of non-opioid analgesics (e.g., diclofenac) was described during ongoing manganese oxidation by *Pseudomonas putida* MnB6: the degradation was markedly accelerated by the presence of manganese-oxidizing bacterial biomass (Meerburg et al. 2012). Data on the diclofenac toxicity to bacterial strain *Raoultella* sp. DD4 were described by Domaradzka et al. (2016). This strain was reported to be resistant to high concentrations of diclofenac (up to 3.0 g/L) but this compound was not a sufficient carbon and energy source for the strain. In addition, Murdoch and Hay (2005, 2013, 2015) described the metabolic pathways of ibuprofen utilization by *Sphingomonas* Ibu-2 and *Variovorax* Ibu-1 isolated from a sewage treatment plant. In general, ibuprofen is known for its easy biodegradability (removal efficiency of over 95%) in the lab-scale and in wastewater treatment plants when compared to diclofenac with removal efficiency of 28% in a pilot-scale membrane bioreactor (Radjenovic et al. 2009).

So far, little is known about biodegradation or utilization pathways of these pharmaceuticals in bacteria. Langenhoff et al. (2013) described ibuprofen degradation below the detection limit (0.01 μg/L) in a pilot membrane bioreactor whereas diclofenac was not removed. In shake flask cultures, the pharmaceuticals were also aerobically degraded by the microbial consortium of an activated sludge**:** ibuprofen was degraded faster than diclofenac and the process was not enhanced by the addition of primary substrates. Ibuprofen at a concentration of 250 mg/L was degraded below the detection limit within 4 days and diclofenac concentration (300 mg/L) was lowered by 75% within 3 weeks.

As regards opioid analgesics (e.g., codeine), bacterial utilization was described with the strain *P. putida* M10 which used morphine and codeine as a sole carbon and energy source (Bruce et al. 1990). Usually, most of the research has focused on codeine biotransformation by bacterial strains *Arthrobacter* sp. (Liras and Umbreit 1975a), *Pseudomonas testosteroni* (Liras et al. 1975b), *Bacillus* sp. (Madyastha and Reddy 1994), *Mycobacterium neoaurum* MTP650 (Zhang et al. 2005), *Streptomyces griseus* NRRL B8090 (Harder and Kunz 1989) and *Rhizobium radiobacter* R89-1 (Kyslíková et al. 2013).

In principle, biological techniques based on microorganisms could be more robust and effective for removal of pharmaceutical pollutants than currently used physico-chemical techniques. A whole cell catalyst is usually an option when an enzyme or several enzymes are involved in a biocatalysis. So far, only a limited number of whole cell catalysts have been used in industry because of features such as slow growth of microorganisms, strain sensitivity to the concentrations of reactants, nutrient requirements, and enzyme robustness and performance (Carballeira et al. 2009).

Our study deals with the isolation of bacterial strains capable of degrading various analgesics. A selection pressure of two selected analgesics (DCF, CD) was applied to microbiomes to obtain a bacterial strain having high analgesic degradation ability. The strain was identified, modified, and biochemically characterized. Effects of initial concentrations of DCF and CD, pH, and temperature were studied to obtain the optimal conditions for bacterial growth and analgesic degradation by whole cell catalyst. This is the first report on a single bacterial degrader capable of laboratory degradation of several different xenobiotics, active pharmaceutical compounds, at high concentrations.

Materials and methods

Chemicals and microorganisms

Analgesics (DCF sodium salt, IPF, KTP, NAP sodium salt, MA) and metabolite 4′-hydroxydiclofenac (4′-OH DCF) were purchased from Sigma Chemicals (USA). Codeine (CD) and 14-hydroxycodeinone (14-OH CD) was provided by Zentiva k.s., Czech Republic. Organic solvents were obtained from Merck (Germany) and Millinckrodt Baker (USA). The media components were purchased from Hi Media Laboratory (India), Oxoid (UK), and Difco (USA). The microorganisms under study were collected from soils polluted with chemical waste (microbiome A, sewage disposal plant, central Bohemia) and composted waste from a factory producing poppy seeds (microbiome B, Agrobyškovice s.r.o., Neratovice, Czech Republic; 50° 25′ 85.65″ N, 14° 48′ 53.81″ E).

Media and culture conditions

The cultures of bacteria were grown in a Luria-Bertani broth (LB; 1% tryptone, 0.5% yeast extract, 1% NaCl, pH of 7.0). Basal salts broth medium used in the study (BSB; in g/L): 0.1 NaCl, 1.03 K_2HPO_4, 0.75 KH_2PO_4 and 1.0 NH_4Cl, pH 6.7) was supplemented with trace elements (BSBTE; in mg/L): $MgSO_4.7H_2O$ 200, $CaCl_2.2H_2O$ 10, $ZnSO_4.7H_2O$ 6.6, $MnSO_4.4H_2O$ 0.17, $FeSO_4.7H_2O$ 1.5, $CoCl_2.6H_2O$ 0.48, $CuSO_4.5H_2O$ 0.47, and $Na_2MoO_4.2H_2O$ 0.45. To prepare solid media for screening or short-term strain maintenance, 22 g/L agar was added to the LB or BSB media. Stock ethanol solutions (1 g of analgesic dissolved in 10 mL of pure ethanol) of

DCF, IPF, KTP, MA, and NAP and water solution of CD were used to supplement a medium.

Standard cultures were grown in 20 or 100 mL of a given medium (100- or 500-mL culture flask) on an orbital shaker (200 rpm) at 28 °C. One percent inoculum was used to start cultures.

Tolerance of microbiomes to analgesics DCF, IPF, KTP, MA, NAP, and CD

The microbiome A or B was cultured in 100 mL of BSBTE medium supplemented with an analgesic (1 g/L) and the cultures grew at 28 °C up to 72 h. At the time of inoculation and after cultivation, the cultures were adequately diluted and spread onto LB plates for determination of the number of colony-forming units (CFU).

Isolation of improved DCF- or CD-degrading mutants

Soil microbiomes A and B were cultured in LB medium (culture conditions: 28 °C, 24 h, 200 rpm), the final culture was adequately diluted and spread onto solid medium BSB supplemented with DCF or CD (1 g/L). Single colonies were isolated and subjected to growth in 20 mL of BSBTE medium supplemented with DCF or CD (1 g/L) at 28 °C for 72 h. The cultures of isolates growing in the presence of analgesics were analyzed by HPLC to determine the residual concentrations of both compounds in culture supernatants. A mixed population of four selected isolates growing in the liquid BSBTE medium in the presence of analgesics and capable of degrading the drugs was treated with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) as a mutagenic agent according to Foster (1991). The mutants were analyzed for enhanced DCF or CD removal efficiency by growth in the liquid BSBTE medium (20 mL) supplemented with the analgesic (1 g/L) for 72 h at 28 °C.

Taxonomy of isolated microorganisms

Molecular identification was done by sequencing 16S rRNA gene amplified by polymerase chain reaction (PCR) using 16S rRNA gene primers Fwd27 and Rev1492 (Lane 1991). PCR amplicons were purified using a High Pure PCR Product Purification Kit (Roche, Switzerland) following the manufacturer's protocol. The PCR amplicons were sequenced on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, USA). The sequences so obtained were edited by Chromas Lite software (Technelysium Pty Ltd., Australia) and assembled using SeqMan (DNASTAR, Inc., USA). Searching for 16S rRNA gene sequence similarity was performed at the GenBank data library using the BLASTN program (NCBI, USA). The evolutionary history of aligned sequences with the Clustal W program was inferred using the Neighbor-Joining method and the Maximum Composite Likelihood model integrated in the MEGA7 program (Kumar et al. 2016).

Effect of incubation temperature on analgesic removal

Flasks containing 100 mL of BSBTE medium supplemented with 1.0 g/L of the analgesic (DCF or CD) were inoculated with pelleted and washed (BSB medium) biomass from precultures grown in LB medium (24 h at 28 °C). The cultures were grown for 72 h at 20, 28 or 37 °C. At regular intervals, culture aliquots were taken to determine a biomass cell dry mass (CDW), culture density at 600 nm (A_{600}), the number of CFU, pH, and residual concentration of an analgesic that was determined in a culture supernatant by HPLC technique. All experiments were conducted in triplicate.

Biotransformation of analgesics by whole cell catalyst

A biomass harvested from the exponential phase of growth of the culture in LB medium was washed with BSB medium and resuspended in BSBTE medium to obtain suspensions with a final concentration of 10% wet mass (WW). Small-scale biotransformations were performed as follows: 3 mL aliquots of the biomass suspension were transferred into the 6-well cell culture plates. DCF and CD were supplemented at a final concentration ranging from 1.0 to 5 g/L and 0.2 to 1.0 g/L, respectively. For the IPF, KTP, NAP, or MA analgesics, the reaction mixtures were supplemented with 1 g/L of the compound. Reaction mixtures were incubated for 96 h on a shaker (200 rpm) at 28 °C.

The effect of initial pH on removal of DCF by whole cell catalyst

The initial pH (pH^{INI}) of the reaction mixture (BSBTE medium supplemented with 1 g/L of DCF, 10% WW of biomass) ranged from 3 to 10. The mixtures (3 mL) were incubated for 96 h on a shaker (200 rpm) at 28 °C without pH maintenance. At 48-h intervals, aliquots were taken from the reaction mixtures, CFU of the catalyst was assayed, and the residual concentration of the analgesic was determined by HPLC in the supernatant of the reaction mixture and in pelleted and washed catalyst. The experiments were performed in triplicate.

HPLC analyses of analgesics

Preparation of samples for HPLC analyses was performed as follows: 1 mL samples (supernatant of a culture or reaction mixture supernatant and the pelleted and washed biomass) were extracted with 1 mL of *tert*-butyl methyl ether (TBME) under vigorous shaking (10 mL vials, vortex mixer, 10 min). Organic and water phases were separated by centrifugation

(12,000*g*, 10 min) and concentrations of substrates/products of biotransformation were determined in the organic phase by HPLC using a Dionex apparatus equipped with a diode array detector and a Lichroprep RPC18 column (250 mm × 4.6 mm) working at room temperature under isocratic elution. The mobile phase consisted of water and methanol (3:7 *v*/*v*, 0.2% phosphoric acid) for DCF, 4′-OH DCF, KTP, IPF analyses (flow rate of 0.8 mL/min, 28 °C), and water and methanol (4:1 *v*/*v*, 0.1% trifluoroacetic acid) for CD and 14-OH CD (flow rate of 1.0 mL/min, 40 °C). Detection of all compounds was done at 220 and 275 nm. Elution times for reactants were as follows: DCF 12.7 min, 4′-OH DCF 9.4 min, KTP 6.0 min, IPF 9.8, CD 5.1 min, and 14-OH CD 6.3 min.

Results

Screening, isolation, and characterization of bacterial degraders of analgesics

Tolerance studies of microbiomes A and B were performed in BSBTE medium supplemented with single DCF, IPF, KTP, MA, NAP, or CD analgesics in a concentration of 1 g/L. Glycerol was used as a source of carbon and energy in a control culture. In cultures with DCF and CD, the number of CFU decreased markedly and reached the value ranging from 10^5 to 10^6 CFU/mL. Comparable numbers of CFU in analgesic-free culture with glycerol (10^9 CFU/mL) and the cultures supplemented with four other analgesics (10^8 CFU/mL) were determined (Fig. 1). DCF and CD analgesics exhibiting the strongest negative effects on viability of the microbiomes were selected for further experiments.

350 and 50 isolates obtained in the screening study grew on a solid medium supplemented with DCF and CD, respectively. Among these 400 soil isolates, only four strains were capable of growth in liquid BSBTE medium supplemented with the analgesics. Three isolates from microbiome A exhibiting 59% removal of DCF were identified as *P. putida*, *Pseudomonas jesenii*, and *Klebsiella* sp. One isolate from microbiome B (18% removal of CD) was identified as *Raoultella* sp. The mixture of these four strains was subjected to chemical mutagenesis by MNNG to improve at random their removal abilities. The mutants were screened in BSBTE medium supplemented with DCF or CD. Biomass was separated, and extracted culture supernatants were analyzed by HPLC. Among 100 tested mutants, a single strain showed greatly improved removal of DCF (92%) and exhibited also 30% removal of CD. The isolate was designated as KDF8 and used for further research. The phenotype of the strain KDF8 was characterized and its biochemical and morphological traits are shown in Table 1.

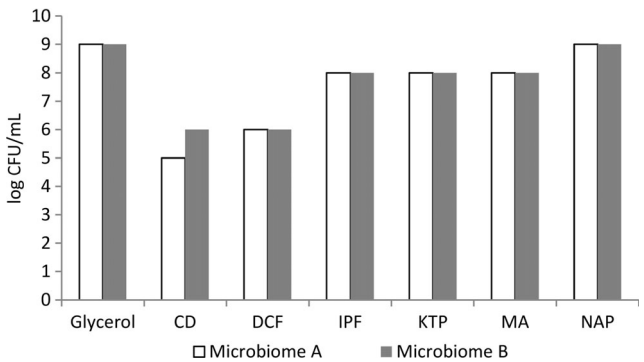

Fig. 1 The tolerance of microbiome A (□) or B (■) grown in liquid BSBTE medium (28 °C) supplemented with 1 g/L of the analgesics DCF, CD, IPF, KTP, MA and NAP and glycerol. The number of colony forming units (CFU) was determined at 0 h and after 72 h. Values represent the average of three independent experiments ± SD

Molecular classification of the strain KDF8 and the BLAST search showed that the strain belongs to the family Enterobacteriaceae, namely to the genus *Raoultella*. The partial nucleotide sequence of 16S rRNA gene region 1.35 kb in length showed the maximum nucleotide identity (over 99%) with the strains *R. planticola*, *R. ornithinolytica* and *Klebsiella* sp. A phylogenetic tree (Fig. 2) shows the phylogenetic position of strain KDF8. The strain *Raoultella* sp. KDF8 was deposited in the Culture Collection of Microorganisms under

Table 1 Morphological and biochemical characteristics of the strain KDF8

Test	Results
Colony color/shape	White, circular
Surface	Smooth, glossy
Growth at 37 °C	+
Motility	−
Indole	+
Ornithine decarboxylase	−
Voges-Proskauer reaction	+
Catalase	+
Oxidase	−
Urease	+
Gelatinase	−
Sugar utilization	
Glucose	+
Sucrose	+
Mannitol	+
Cellobiose	+
Rhamnose	+
Sorbitol	+
Trehalose	+
Raffinose	+
Dulcitol	−

+ positive characteristic; − negative characteristic

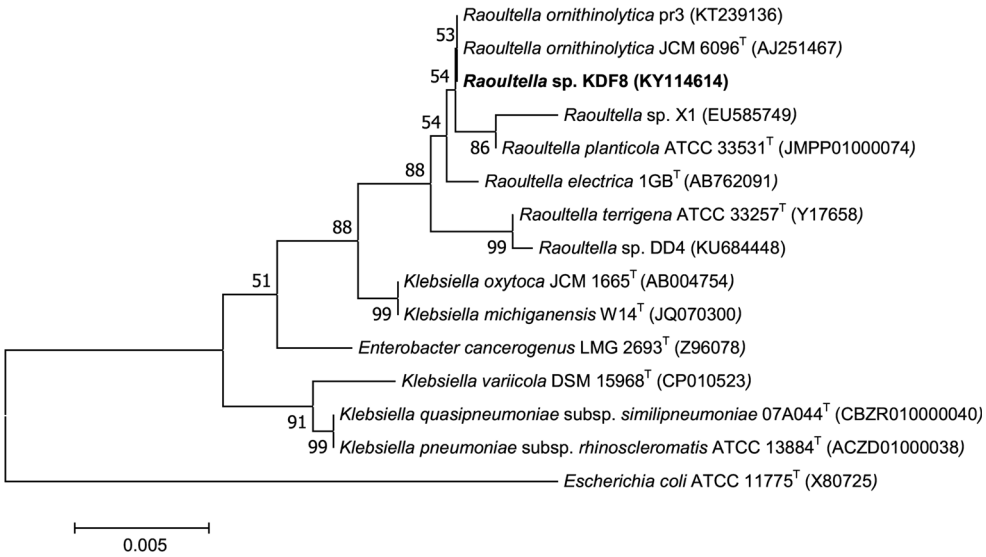

Fig. 2 Phylogenetic tree of *Raoultella* sp. KDF8 (highlighted in bold) and related strains based on 16S rRNA gene sequences. Numbers at the nodes are percentages of 1000 bootstrap replicates. The scale bar represents 0.5% nucleotide sequence divergence. GenBank accession numbers are indicated in parentheses; type strains are tagged with a superscripted T. *Escherichia coli* ATCC 11775^T is used as an outgroup

the number CCM 8678 and the sequence of the 16S rRNA gene was filed in the GenBank database under the accession number KY114614.

Growth of *Raoultella* sp. KDF8 in analgesic-supplemented mineral medium

The effect of DCF or CD at a concentration of 1.0 g/L on bacterial growth was tested in BSBTE medium. As a control for DCF-containing cultures, the strain was grown in a medium supplemented only with the solvent (ethanol, 1% *v*/*v*). The effect of analgesics on growth in liquid media was determined after 72 h. As shown in Table 2, the cultures reached biomass concentrations of 1.0 g_{CDW}/L for DCF or 0.15 g_{CDW}/L for CD. In the control culture, the maximum biomass concentration reached 0.5 g_{CDW}/L.

The growth curves and the time courses of the analgesic removals are shown in Fig. 3. The culture in the medium supplemented with DCF reached the stationary phase after approximately 48 h (Fig. 3a). DCF was being removed by the exponentially growing culture (μ of 0.09 1/h) as well as by the culture in the stationary phase of growth (culturing time from 50th to 72nd h). The complete removal of DCF (92%) within 72 h indicated that the culture was efficient in DCF removal from the supernatant and the strain can grow at 1 g/L of DCF.

The maximum biomass concentration of 0.15 g_{CDW}/L was reached in the culture in CD-supplemented medium BSBTE after 72 h of growth. The very slow growth was accompanied with only 30% removal of the CD (Fig. 3b).

The effect of a temperature (20, 28 and 37 °C) on culture growth was studied in a medium supplemented with DCF at a concentration of 1 g/L (Table 3). The temperature of 28 °C was optimal for DCF removal. While the lower temperature slowed down the growth rate as well as the DCF removal (55%), the temperature of 37 °C reduced significantly the biomass concentration (CDW of 0.22 g/L).

Table 2 Effect of DCF and CD on the growth of the strain KDF8 in BSBTE medium

	Diclofenac		Codeine
Concentration (g/L)	0.0	1.0	1.0
A_{600}	1.4 ± 0.20	2.4 ± 0.20	0.4 ± 0.20
CDW (g/L)	0.5 ± 0.07	1.0 ± 0.10	0.15 ± 0.02
pH^F	5.8	4.8	6.6

Cell dry mass (CDW) and culture density (A_{600}) of the culture after 72 h of growth. pH^F stands for final pH value of a culture. Values represent the average of three independent experiments ± SD

Biotransformation of analgesics by whole cell catalyst *Raoultella* sp. KDF8

The effect of pH^{INI} on the efficiency of DCF removal was studied for 96 h over a wide range of values from 3 to 10 in BSBTE medium at 28 °C. To determine a total degradation of DCF, the effect of pH^{INI} on DCF sorption to cells was also studied (Table 4). The optimal pH^{INI} for DCF removal ranges from 7 to 9, with biodegradation of DCF ranging from 91.5 to 60%. The data show that the final pH of the mixtures (pH^F) after 96 h reached a value ranging from 3.4 to 4.6.

The potential of the strain to biotransform the analgesic (concentration range of DCF 1.0–5.0 g/L; CD 0.2 and 1.0 g/L) was studied for up to 96 h in a reaction mixture consisting of 10% (WW) biomass suspension (7.6 g_{CDW}/L) in BSBTE medium

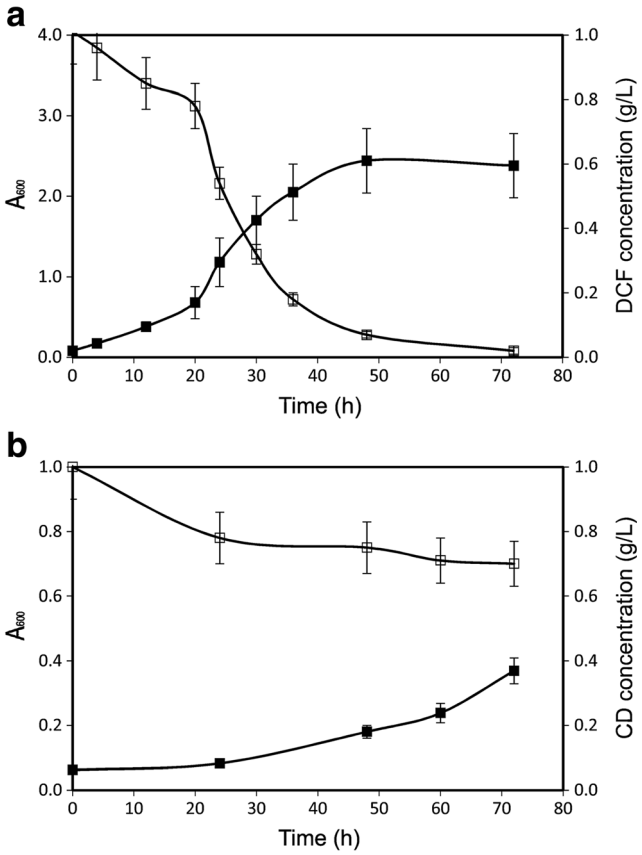

Fig. 3 Growth of a *Raoultella* sp. KDF8 strain culture in shake flasks (28 °C) with 100 mL of BSBTE medium supplemented with 1 g/L DCF (**a**) or CD (**b**): culture density (A_{600}, ■) and residual concentration of DCF or CD in culture supernatant (□)

(pH^{INI} 7) at 28 °C (Table 5). At the beginning and after 96 h, the CDW of a reaction mixture with 1 g/L of DCF was determined: the final value increased by 23%. Significant differences were observed between the removal of these two analgesics from mixture supernatant: 96% removal of DCF and 45% removal of CD were assayed at a concentration of 1 and 0.2 g/L, respectively. The percent removal decreased with increasing concentrations of both drugs. However, at the highest concentrations, the specific removal rates of DCF and CD were maximal and reached 16.5 and 5.1 mg/g_{CDW} per h, respectively. The comparison of the number of CFU assayed at the beginning of the reaction with DCF (10^{11} CFU/mL) and after 96 h (10^{10} CFU/mL) confirmed the viability of cells.

Analogous experiments were performed with other analgesics, i.e., IPF, KTP, MA, and NAP, at an optimal concentration of 1 g/L. The degradation ability of the catalyst was only observed in reactions supplemented with IPF and KTP (Table 6): 78% of the IPF and 60% of KTP were removed with the specific removal rate of 3.2 and 2.0 mg/g_{CDW} per h, respectively.

Table 3 Effect of incubation temperature on culture growth and DCF removal

Temperature (°C)	μ (1/h)	CDW (g/L)	DCF removal (%)
20	0.05 ± 0.01	0.72 ± 0.05	55 ± 2.05
28	0.09 ± 0.01	1.00 ± 0.12	92 ± 2.05
37	0.03 ± 0.005	0.22 ± 0.03	82 ± 1.63

Cultures of the strain KDF8 grew in 100 mL of BSBTE medium supplemented with 1 g/L DCF for 72 h. CDW and μ stand for cell dry mass and specific growth rate, respectively.Values represent the average of three independent experiments ± SD

Products of diclofenac or codeine biotransformation by whole cell catalyst

At the concentration of 1 g/L DCF or 0.2 g/L CD in the BSBTE medium, metabolites were detected by HPLC in supernatants of reaction mixtures at 24-h intervals (Fig. 4). During the first 24 h of biotransformation the concentration of DCF was decreasing and the product of transformation appeared: 4′-OH DCF reached the maximal concentration at 48 h of incubation, while DCF concentration dropped below the level of quantification (Fig. 4a). After 72 h of incubation, the concentration of 4′-OH DCF dropped also below the level of quantification. Figure 4b shows the time course of the formation of the compound 14-OH CD from CD, the concentration of which correspondingly decreased. After 72 h, the concentration of 14-OH CD was still detected in the reaction mixture.

Discussion

Because of their high overall consumption, analgesics are emerging as a new group of environmental contaminants. Most popular non-opioid analgesics (e.g., DCF, IPF, KTP, and others) are often detected in wastewaters, effluents, and surface waters. Recently, DCF has been included on the European Commission watch list of substances (Commission implementing decision (EU) 2015/495) that require environmental monitoring in the member states. In many cases, the effectiveness of sewage treatment plants for removing pharmaceuticals is limited (Gomez et al. 2007). To date, only a few bacterial isolates capable of degradation of analgesics at low concentrations have been described and isolated from the sludge of a sewage treatment plant: there is a report on ibuprofen metabolism by the bacterial strains *Variovorax* and *Sphingomonas* (Murdoch and Hay 2005, 2013, 2015). Regarding morphine alkaloids (e.g., the CD), these compounds were utilized as sole carbon and energy sources by the strain of *P. putida* isolated from industrial waste liquors (Bruce et al. 1990).

In this study, we described a new strain, *Raoultella* sp. KDF8, obtained by chemical mutagenesis of the nature

Table 4 Effect of pH^{INI} on DCF sorption to whole cell catalyst

pH^{INI}/pH^{F}	3/3.4	4/3.8	5/3.9	6/3.8	7/4.1	8/4.4	9/4.3	10/4.6
DCF in pellet (%)	56.8 ± 6.6	54 ± 5.5	69 ± 6.0	66.6 ± 5.5	8.5 ± 0.6	21.6 ± 2.5	40 ± 3.0	96 ± 2.5
Total degradation (%)	43.2	46	31	33.4	91.5	78.4	60	4

pH^{INI} and pH^{F} stand for the initial and final pH of the reaction mixture, respectively. Degradation was performed in BSBTE medium supplemented with 1 g/L DCF. Reaction time was 96 h. Total degradation represents the difference between initial and residual concentration (in pellet) of DCF. Values represent the average of three independent experiments ± SD

bacterium isolated from contaminated soil. As a whole cell catalyst, the strain efficiently removes analgesics.

Studies of DCF toxicity (EC50 is 1.95 g/L) on the growth of the strain *Raoultella* were performed by Domaradzka et al. (2016). The strain DD4 grew in a medium supplemented with high concentration of DCF (up to 3 g/L) but the strain was capable of degrading only 10% of 6 mg/L of DCF over 28 days. Our work deals with the development of a new, whole cell catalyst, a bacterial strain KDF8, capable of removal of different analgesics at much higher concentrations and with higher efficiency.

Sequencing and BLAST analysis of a 16S rRNA gene fragment revealed that KDF8 was 99% identical to *Raoultella* species and physiological and biochemical characterization confirmed this classification. As a consequence of the proposal of Drancourt et al. (2001) that, e.g., *Klebsiella planticola* and *K. ornithinolytica* should be reclassified into the genus *Raoultella*, we consider the strain designation as *Raoultella* sp. to be valid.

Species of the genus *Raoultella* have recently been identified as biodegraders of diverse aromatic compounds such as 2,4,6-trinitrotoluene (Claus et al. 2007), dimethoate (Liang et al. 2009), 2,4,5-trichlorophenoxyacetic acid (Zharikova et al. 2006), atrazine (Swissa et al. 2014) and sex steroid hormones (Ji et al. 2007). The strain *Raoultella* sp. KDF8 utilizes codeine as a carbon and energy source for growth and efficiently co-metabolizes DCF. We expect that IPF and KTP may be analogously co-metabolized. These novel traits expand the potential of the genus *Raoultella* as a whole cell catalyst for removal of xenobiotics from the environment. Co-metabolism of xenobiotics, especially by bacteria isolated from sites contaminated with one or multiple pollutants, is a common feature (Liu et al. 2001). However, we do not have at present any experimental data confirming any metabolic reactions of the analgesics in cultures of the strain KDF8 although certain intermediates were identified in the course of removal of DCF and CD.

As regards the effect of pH, our results do not correspond with literature data (Vieno and Sillanpää 2014) that the lower pH enhances the DCF removal: when the pH^{INI} is below the value of 7, DCF is not removed and more than a half of its concentration stays in the pellets. We hypothesize that in the pH range from 7 to 9, the DCF is efficiently metabolized, which correlates with its removal from the mixture supernatant and its low content in pellet.

Strain KDF8 removes also CD (about 30% under optimum conditions at a concentration of 1 g/L). CD belongs to compounds highly toxic for organisms and the toxicity of its metabolites is also known (Jairaj et al. 2003). Simultaneously, the strain KDF8 shows the ability to grow very slowly in the presence of CD that is used as a source of carbon and energy. Our finding may correlate with the results of morphine and codeine utilization by *P. putida* M10 (Bruce et al. 1990).

As a whole cell catalyst, the biomass of the strain *Raoultella* KDF8 efficiently removes four analgesics (DCF, IPF, KTP and CD) at pH^{INI} 7 after 96 h. The specific removal rate of DCF or CD was faster at higher concentrations whereas lower concentrations significantly increased the percentage of the degraded analgesic. 4′-OH DCF and 14-OH CD were the main products identified in the transformation experiment of DCF and CD by the whole cell catalyst. This is consistent with studies of transformation of DCF, where 4′-OH DCF and other oxidative

Table 5 Removal of DCF and CD from reaction mixture supernatant by whole cell catalyst: concentration effect of analgesics on specific removal rates

Substrate	Diclofenac					Codeine	
Concentration (g/L)	1.0	2.0	3.0	4.0	5.0	0.2	1.0
Degradation (%)	96 ± 1.95	88 ± 1.27	71 ± 0.84	64 ± 1.10	63 ± 0.75	45 ± 2.8	27 ± 2.0
SRR (mg/g_{CDW} per h)	5.2 ± 0.3	5.0 ± 0.3	10.0 ± 0.4	13.5 ± 0.6	16.5 ± 0.4	0.6 ± 0.03	5.1 ± 0.1

Analgesic removal by 10% (WW) cell suspension of *Raoultella* sp. KDF8 was studied in BSBTE medium (volumes of 3 mL, pH^{INI} 7) after 96 h at 28 °C. SRR stands for specific removal rate. Values represent the average of three independent experiments ± SD

Table 6 Removal and specific removal rates of IPF, KTP, NAP and MA from reaction mixture supernatants by whole cell catalyst

Substrates	CDW (g/L)	Removal (%)	SRR (mg/g_{CDW} per h)
IPF	5.0 ± 0.16	78 ± 2.43	3.2 ± 0.20
KTP	5.5 ± 0.17	60 ± 1.76	2.0 ± 0.10
NAP	5.2 ± 0.15	n.d.	n.d.
MA	5.0 ± 0.16	n.d.	n.d.

Analgesic (1 g/L) removal by a cell suspension of *Raoultella* sp. KDF8 was studied in BSBTE medium (3 mL volumes) after 96 h at 28 °C. CDW and SRR stand for biomass concentration of a suspension and specific removal rate for an analgesic, respectively. Values represent the average of three independent experiments ± SD. *n.d.*, not detected

metabolites, such as 5-hydroxydiclofenac (Gröning et al. 2007), 4′,5-dihydroxy and 3-hydroxy diclofenac were also identified in fractions of microsomes (Kumar et al. 2002), human hepatocytes (Bort et al. 1999) and in fish bile (Kallio et al. 2010). While the concentration of the main product 14-OH CD remains constant, 4′-OH DCF reaches the maximum in the 48th of the reaction, and then its concentration decreases significantly. Bouju et al. (2016) show that the degradation pathway during biological treatment of wastewater of diclofenac is much slower and more limited (up to 40% after 18 days) than the DCF removal via 4′-OH DCF described in this work. However, we did not study DCF removal at low concentrations and we cannot exclude the absence of induction of specific metabolic pathways under these conditions. The use of a whole cell catalyst for CD conversion by the strain *Rhizobium radiobacter* R89-1 has been described by Kyslíková et al. (2013). Our results are in agreement with their data that report increased conversion of CD at higher concentrations of the drug. Moreover, the biomass of the strain KDF8 was capable of removing other analgesics such as IPF or KTP, although with lower efficiency when compared with DCF.

In conclusion, the catalyst *Raoultella* sp. KDF8 has a potential for the degradation of analgesics with very different structures and may therefore have direct application in sewage treatment plants. This unique ability of the strain *Raoultella* sp. KDF8 suggests a high versatility of its metabolic pathways. Further investigation will be focused on deciphering degradation pathways by proteomics and metabolomics.

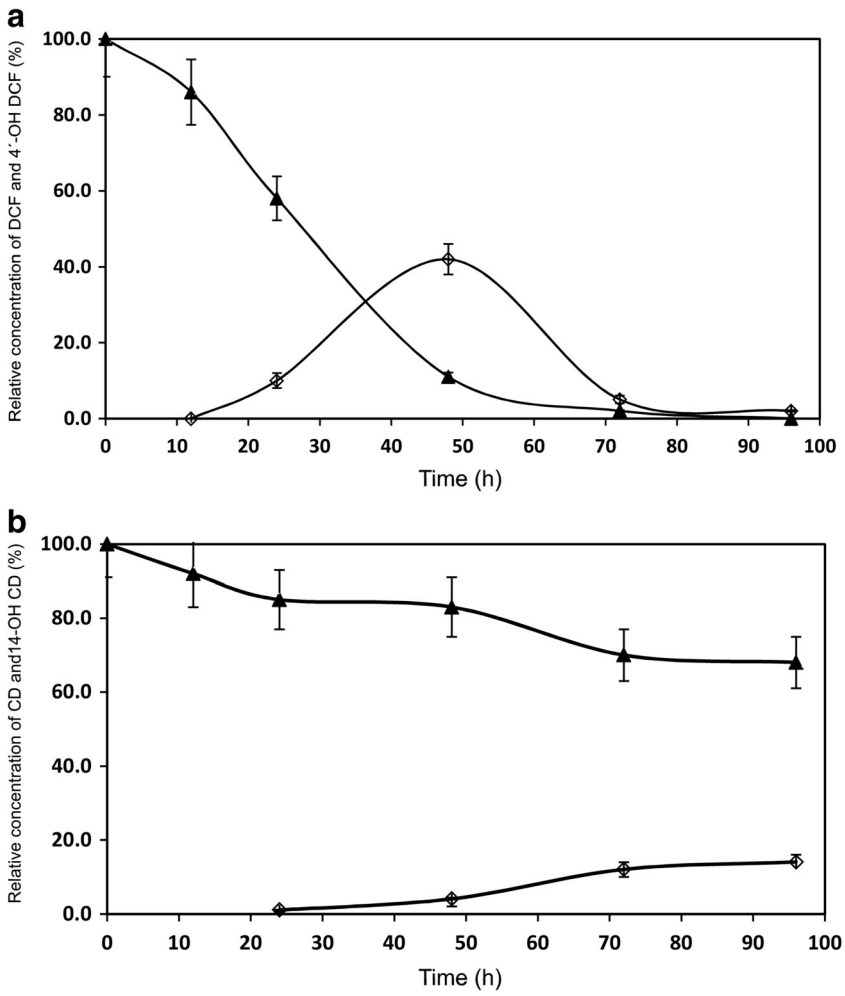

Fig. 4 Time-dependent profiles of biotransformation products of analgesics. Reaction mixture consisted of 1 g/L DCF or 0.2 g/L CD and 10% (WW) biomass suspension in BSBTE medium. **a** Relative concentration of DCF (▲) and 4′-OH DCF (◊). **b** Relative concentration of CD (▲) and 14-OH CD (◊)

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References

Behera SK, Kim HW, Oh JE, Park HS (2011) Occurrence and removal of antibiotics, hormones and several other pharmaceuticals in wastewater treatment plants of the largest industrial city of Korea. Sci Total Environ 409:4351–4360. https://doi.org/10.1016/j.scitotenv.2011.07.015

Benotti MJ, Trendholm RA, Vanderford BJ, Holady JC, Standford BD, Snyder SA (2009) Pharmaceuticals and endocrine disrupting compounds in U.S. drinking water. Environ Sci Technol 43:597–603

Bort R, Mace K, Boobis A, Gomez-Lechon MJ, Pfeifer A, Castell J (1999) Hepatic metabolism of diclofenac: role of human CYP in the minor oxidative pathways. Biochem Pharmacol 58(5):787–796. https://doi.org/10.1016/s0006-2952(99)00167-7

Bouju H, Nastold P, Beck B, Hollender J, Corvini PFX, Wintgens T (2016) Elucidation of biotransformation of diclofenac and 4′ hydroxydiclofenac during biological wastewater treatment. J Hazard Mater 301:443–452. https://doi.org/10.1016/j.jhazmat.2015.08.054

Bruce NC, Wilmot CJ, Jordan KN, Trebilcock AE, Gray Stevens LD, Lowe CR (1990) Microbial degradation of the morphine alkaloids: identification of morphinone as an intermediate in the metabolism of morphine by *Pseudomonas putida* M10. Arch Microbiol 154:465–470

Bueno MJM, Gomez MJ, Herrera S, Hernando MD, Agüera A, Fernández-Alba AR (2012) Occurrence and persistence of organic emerging contaminants and priority pollutants in five sewage treatment plants of Spain: two years pilot survey monitoring. Environ Pollut 164:267–273. https://doi.org/10.1016/j.envpol.2012.01.038

Carballeira JD, Quezada MA, Hoyos P, Simeó Y, Hernaiz MJ, Alcantara AR, Sinisterra JV (2009) Microbial cells as catalyst for stereoselective re-ox reaction. Biotechnol Adv 27:686–714. https://doi.org/10.1016/j.biotechadv.2009.05.001

Claus H, Bausinger T, Lehmler I, Perre N, Fels G, Dehner U, Preuß J, König H (2007) Transformation of 2,4,6-trinitrotoluene (TNT) by *Raoultella terrigena*. Biodegradation 18:27–35. https://doi.org/10.1007/s10532-005-9033-7

Commission implementing decision (EU) 2015/495 of 20 March 2015 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council. Official Journal C311/8, 25/10/2013

Domaradzka D, Guzik U, Hupert-Kocurek K, Wojcieszynska D (2016) Toxicity of diclofenac and its biotransformation by *Raoultella* sp DD4. Pol J Environ Stud 25(5):2211–2216. 10.15244/pjoes/62681

Drancourt M, Bollet C, Carta A, Rousselier P (2001) Phylogenetic analyses of *Klebsiella* species delineate *Klebsiella* and *Raoultella* gen. nov., with description of *Raoultella ornithinolytica* comb. nov., *Raoultella terrigena* comb. nov and *Raoultella planticola* comb. nov. Int J Syst Bacteriol 51:925–932. https://doi.org/10.1099/00207713-51-3-925

Ferrari B, Paxeus N, Lo Giudice R, Pollio A, Garric J (2003) Ecotoxicological impact of pharmaceuticals found in treated wastewaters: study of carbamazepine, clofibric acid, and diclofenac. Ecotoxicol Environ Saf 56(3):450–450. https://doi.org/10.1016/s0147-6513(03)00111-8

Foster PL (1991) *In vivo* mutagenesis. Methods Enzymol 204:114–125

Gavrilescu M, Demnerová K, Aamand J, Agathos S, Fava F (2015) Emerging pollutants in the environment: present and future challenges in biomonitoring, ecological risks and bioremediation. New Biotechnol 32:147–156. https://doi.org/10.1016/j.nbt.2014.01.001

Gomez MJ, Bueno MJM, Lacorte S, Fernandez-Alba AR, Agüera A (2007) Pilot survey monitoring pharmaceuticals and related compounds in a sewage treatment plant located on the Mediterranean coast. Chemosphere 66:993–1002. https://doi.org/10.1016/j.chemosphere.2006.07.051

Gröning J, Held C, Garten C, Claussnitzer U, Kaschabek SR, Schlomann M (2007) Transformation of diclofenac by the indigenous microflora of river sediments and identification of a major intermediate. Chemosphere 69(4):509–516. https://doi.org/10.1016/j.chemosphere.2007.03.037

Guzzella L, Feretti D, Monarca S (2002) Advanced oxidation and adsorption technologies for organic micropollutant removal from lake water used as drinking-water supply. Water Res 36:4307–4318

Hallare AV, Köhler HR, Triebskorn R (2004) Developmental toxicity and stress protein responses in zebrafish embryos after exposure to diclofenac and its solvent, DMSO. Chemosphere 56:659–666. https://doi.org/10.1016/j.chemosphere.2004.04.007

Harder PA, Kunz DA (1989) Bacterial hydroxylation of codeine. United States Patent No 4,798,792

Ikehata K, Naghashkar N, Ei-Din M (2006) Degradation of aqueous pharmaceuticals by ozonation and advanced oxidation processes: a review. Ozone Sci Eng 28:353–414

Jairaj M, Watson DG, Grant MH, Skellern GG (2003) The toxicity of opiates and their metabolites in HepG2 cells. Chem Biol Interact 146:121–129. https://doi.org/10.1016/S0009-2797(03)00091-7

Ji S, Liu Z, Liu Z, Ren H (2007) Isolation and characterization of a bacterial strain that efficiently degrades sex steroid hormones. Front Environ Sci Engin China 1:325–328

Jones OA, Lester JN, Voulvoulis N (2005) Pharmaceuticals: a threat to drinking water. Trends Biotechnol 23(4):163–167. https://doi.org/10.1016/j.tibtech.2005.02.001

Jux U, Baginski RM, Arnold HG, Kronke M, Seng PN (2002) Detection of pharmaceutical contaminations of river, pond, and tap water from Cologne (Germany) and surroundings. Int J Hyg Environ Health 205(5):393–398. https://doi.org/10.1078/1438-4639-00166

Kallio JM, Lahti M, Oikari A, Kronberg L (2010) Metabolites of the aquatic pollutant diclofenac in fish bile. Environ Sci Technol 44(19):7213–7219. https://doi.org/10.1021/es903402c

Kumar S, Samuel K, Subramanian R, Braun MP, Stearns RA, Chiu SL, Evans DC, Baillie TA (2002) Extrapolation of diclofenac clearance from in vitro microsomal metabolism data: role of acyl glucuronidation and sequential oxidative metabolism of the acyl glucuronide. J Pharmacol Exp Ther 303(3):969–978. https://doi.org/10.1124/jpet.102.038992

Kumar S, Stecher G, Tamura K (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33(7):1870–18744. https://doi.org/10.1093/molbev/msw054

Kyslíková E, Babiak P, Štepánek V, Zahradník J, Palyzová A, Marešová H, Valešová R, Hájíček J, Kyslík P (2013) Biotransformation of codeine to 14-OH-codeine derivatives by *Rhizobium radiobacter* R89-1. J Mol Catal B Enzym 87:1–5. https://doi.org/10.1016/j.molcatb.2012.10.004

Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, New York, pp 115–175

Langenhoff A, Inderfurth N, Veuskens T, Schraa G, Blokland M, Kujawa-Roeleveld K, Rijnaarts H (2013) Microbial removal of the pharmaceutical compounds ibuprofen and diclofenac from wastewater. Bio Med Res Int. https://doi.org/10.1155/2013/325806

Liang Y, Zeng F, Qiu G, Lu X, Liu X, Gao H (2009) Co-metabolic degradation of dimethoate by *Raoultella* sp. X1. Biodegradation 20:363–373. https://doi.org/10.1007/s10532-008-9227-x

Liras P, Umbreit WW (1975) Transformation of morphine by resting cells and cell-free systems of *Arthrobacter* sp. Appl Microbiol 30:262–266

Liras P, Kasparian SS, Umbreit WW (1975) Enzymatic transformation of morhine by hydroxysteroid dehydrogenase from *Pseudomonas testosterone*. Appl Microbiol 30:650–656

Liu CY, Speitel GE, Georgiou G (2001) Kinetics of methyl *t*-butyl ether co-metabolism at low concentration by pure cultures of butane-degrading bacteria. Appl Environ Microbiol 67:2197–2201. https://doi.org/10.1128/AEM.67.5.2197-2201.2001

Madyastha KM, Reddy GVB (1994) *Mucor piriformis*, an efficient *N*-dealkylating reagent for thebaine and its *N*-variants. J Chem Soc Perkin Trans 1:911–912. doi: https://doi.org/10.1039/p19940000911

Meerburg F, Hennebel T, Vanhaecke L, Verstraete W, Boon N (2012) Diclofenac and 2-anilinophenylacetate degradation by combined activity of biogenic manganese oxides and silver. Microb Biotechnol 5:388–395. https://doi.org/10.1111/j.1751-7915.2011.00323.x

Murdoch RW, Hay AG (2005) Formation of catechols via removal of acid side chains from ibuprofen and related aromatic acids. Appl Environ Microbiol 71:6121–6125. https://doi.org/10.1128/AEM.71.10.6121-6125.2005

Murdoch RW, Hay AG (2013) Genetics and chemical characterization of ibuprofen degradation by *Sphingomonas* Ibu-2. Microbiology 159:621–632. https://doi.org/10.1099/mic.0.062273-0

Murdoch RW, Hay AG (2015) The biotransformation of ibuprofen to trihydroxyibuprofen in activated sludge and by *Variovorax* Ibu-1. Biodegradation 26:105–113. https://doi.org/10.1007/s10532-015-9719-4

Radjenovic J, Petrovic M, Barcelo D (2009) Fate and distribution of pharmaceuticals in wastewater and sewage sludge of the conventional activated sludge (CAS) and advanced membrane bioreactor (MBR) treatment. Water Res 43(3):831–841. https://doi.org/10.1016/j.watres.2008.11.043

Richardson SD, Plewa MJ, Wagner ED, Schoeny R, deMarini DM (2007) Occurrence, genotoxicity, and carcinogenicity of regulated and emerging disinfection by-products in drinking water: a review and roadmap for research. Mutat Res-Rev Mutat 636:178–242. https://doi.org/10.1016/j.mrrev.2007.09.001

Swissa N, Nitzan Y, Langzam Y, Cahan R (2014) Atrazine biodegradation by a monoculture of *Raoultella planticola* isolated from a herbicides wastewater treatment facility. Int Biodeter Biodegr 92:6–11. doi: https://doi.org/10.1016/j.ibiod.2014.04.003

Vieno N, Sillanpää M (2014) Fate of diclofenac in municipal wastewater treatment plant—a review. Environ Int 69:28–39. https://doi.org/10.1016/j.envint.2014.03.021

Zhang Q, Rich JO, Cotterill IC, Pantaleone DP, Michels PC (2005) 14-Hydroxylation of opiates: catalytic direct autoxidation of codeinone to 14-hydroxycodeinone. J Am Chem Soc 127:7286–7287. https://doi.org/10.1021/ja051682z

Zhang YJ, Geissen SU, Gal C (2008) Carbamazepine and diclofenac: removal in wastewater treatment plants and occurrence in water bodies. Chemosphere 73(8):1151–1161. https://doi.org/10.1016/j.chemosphere.2008.07.086

Zharikova NV, Markusheva TV, Galkin EG, Korobov VV, Zhurenko EY, Sitdikova LR, Kolganova TV, Kuznetsov BB, Turova TP (2006) *Raoultella planticola*, a new strain degrading 2,4,5-trichlorophenoxyacetic acid. Appl Biochem Micro 42:258–262