

Enzymes Involved in Naproxen Degradation by *Planococcus* sp. S5

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Abstract

Naproxen is a one of the most popular non-steroidal anti-inflammatory drugs (NSAIDs) entering the environment as a result of high consumption. For this reason, there is an emerging need to recognize mechanisms of its degradation and enzymes engaged in this process. *Planococcus* sp. S5 is a gram positive strain able to degrade naproxen in monosubstrate culture (27%). However, naproxen is not a sufficient growth substrate for this strain. In the presence of benzoate, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid or vanillic acid as growth substrates, the degradation of 21.5%, 71.71%, 14.75% and 8.16% of naproxen was observed respectively. It was shown that the activity of monooxygenase, hydroxyquinol 1,2-dioxygenase, protocatechuate 3,4-dioxygenase and protocatechuate 4,5-dioxygenase in strain S5 was induced after growth of the strain with naproxen and 4-hydroxybenzoate. Moreover, in the presence of naproxen activity of gentisate 1,2-dioxygenase, enzyme engaged in 4-hydroxybenzoate metabolism, was completely inhibited. The obtained results suggest that monooxygenase and hydroxyquinol 1,2-dioxygenase are the main enzymes in naproxen degradation by *Planococcus* sp. S5.

Key words: *Planococcus*, biodegradation, naproxen, enzymes induction, aromatic plant compounds

Introduction

In recent years, non-steroidal anti-inflammatory drugs (NSAIDs) have been widely detected in the environment. These biologically active compounds and their continuous inflow into the environment may lead to their accumulation in the environment and chronic exposure of organisms. As a result, this may cause potential negative effects on living organisms. While the transformation mechanisms of non-steroidal anti-inflammatory drugs in the human body and in other animals have been extensively studied, the degradation of these drugs by bacteria has been seldom investigated and remains largely unknown (Quintana *et al.*, 2005; Marco-Urrea *et al.*, 2010; Wojcieszynska *et al.*, 2014). It is suggested that the transformation of naproxen by *Trametes versicolor* or activated sludge may occur by 2-(6-hydroxynaphthalen-2-yl)propanoic acid and 1-(6-methoxynaphthalen-2-yl)ethanone formation (Quintana *et al.*, 2005; Marco-Urrea *et al.*, 2010; Lahti and Oikari, 2011). Marco-Urrea *et al.* (2010) suggested participation of laccase and cytochrome P-450 system in biotransformation of this drug in fungi. Among bacteria, only *Stenotrophomonas maltophilia* KB2 is

known to be able to cometabolically degrade naproxen (Wojcieszynska *et al.*, 2014). After growth of strain KB2 on naproxen activity of phenol monooxygenase, naphthalene dioxygenase, hydroxyquinol 1,2-dioxygenase and gentisate 1,2-dioxygenase was observed. It suggests that degradation of this compound consists in its hydroxylation and aromatic ring cleavage (Wojcieszynska *et al.*, 2014). In monosubstrate culture naproxen is degraded by strain KB2 with low efficiency. The addition of a carbon source, such as glucose, acetate or powdered milk, increases the level of naproxen degradation (Quintana *et al.*, 2005; Lahti and Oikari, 2011; Wojcieszynska *et al.*, 2014). However, the non-aromatic substrates do not induce enzymes of aromatic ring degradation/transformation pathways. It is observed that the introduction of aromatic compounds (*e.g.* phenol) as an additional carbon source increase the degradation of toxic, persistent aromatic compounds (Kulkarni and Chaudhari, 2006; Greń *et al.*, 2010; Wojcieszynska *et al.*, 2014). However, the introduction of phenol or its derivatives into the environment may constitute an additional ballast and disturb ecological balance. Therefore, a good alternative seems to be the use of naturally occurring aromatic compounds, such as protocatechuic

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acid produced by *Prunus domestica*, *Melissa officinalis* or *Vitis vinifera* or vanillic acid identified in *Juglans regia* or *Chenopodium murales* (Ghareib *et al.*, 2010; Kakkar and Bais, 2014). The objective of our study is to examine the ability of *Planococcus* sp. S5 to degrade naproxen in either mono- or disubstrate cultures with plant aromatic compounds as growth substrates. *Planococcus* sp. S5 was chosen for this study since it degrades salicylate (Łabużek *et al.*, 2003), which is not only the intermediate of naphthalene degradation and mimetic of naproxen, but also belongs to non-steroidal anti-inflammatory drugs. Additionally, the aim of this study is to identify enzymes engaged in naproxen degradation by *Planococcus* sp. S5 in cometabolic conditions.

Experimental

Materials and Methods

Media and culture conditions. *Planococcus* sp. S5 was routinely cultivated in BBL nutrient broth at 30°C and 130 rpm for 24 hours. Then 6 mg/l naproxen was added to the culture. After 48 hours, cells were harvested by centrifugation (5.000 × g at 4°C for 15 min), washed with fresh sterile medium and used as inoculum. Degradation of naproxen in a monosubstrate, as well as cometabolic systems, was performed in 500 ml Erlenmeyer flasks containing 250 ml of the mineral salts medium (Greń *et al.*, 2010) inoculated with cells to a final optical density of about 1.5 and 0.5 at $\lambda = 600$ nm (OD600) for the monosubstrate and cometabolic systems, respectively. For degradation experiments two control cultures were prepared. The uninoculated control (I) consisted of 250 ml of sterile mineral salts medium, while the heat-killed control (II) consisted of 250 ml of autoclaved culture prepared under conditions identical to those of the experimental cultures. Naproxen was added to each flask to obtain a final concentration of 6 mg/l, and all cultures were incubated with shaking at 30°C for 28 days.

For studies on the cometabolic transformation of naproxen, as well as the induction of enzymes, 3 mM aromatic compound of plant origin: benzoate (BA), 4-hydroxybenzoic acid (4-HB), 3,4-dihydroxybenzoic acid (3,4-DHB) or vanillic acid (VA) was added. Cultures in 250 ml of sterile mineral salt medium supplemented with appropriate growth substrate and 6 mg/l naproxen were inoculated with cells to a final optical density of about 0.5 at $\lambda = 600$ nm (OD600), and incubated at 30°C with shaking at 130 rpm. If the complete degradation of the suitable growth substrate was observed, a successive dose of plant aromatic compound was introduced and the culture was left for incubation until it reached OD600 = 1.0. All cultures were grown in triplicate.

Analytical methods. To study the degradation of naproxen, 1 ml samples were taken periodically (every 7 days) from the culture medium and centrifuged (6.000 × g, 15 min). The concentration of aromatic substrates in the culture supernatant was determined by HPLC (Merck HITACHI) equipped with a LiChrospher® RP-18 column (4 × 250 mm), liChroCART® 250-4 Nucleosil 5 C18 and a DAD detector (Merck HITACHI). The mobile phase was acetonitrile and 1% acetic acid (50:50 v/v) at a flow rate of 1 ml/min. The detection wavelength was set at 260 nm. Naproxen and plant aromatic compounds in the supernatant were identified and quantified by comparing the HPLC retention times and UV-visible spectra with those of the external standards.

Preparation of cell extracts. After 28 days in culture, cells of *Planococcus* sp. S5 were harvested by centrifugation (4.500 × g for 15 min at 4°C) and the pellet was washed with 50 mM phosphate buffer, pH 7.0, and resuspended in the same buffer. Cell-free extracts were prepared by sonication of the whole cell suspension (6 times for 15 s) and centrifugation at 9.000 × g for 30 min at 4°C. Clear supernatant was used as a crude cell extract for enzyme assays.

Enzyme assays. Monooxygenase activity was determined spectrophotometrically by measuring NADH oxidation ($\epsilon^{340} = 6.220/\text{M cm}$) (Divari *et al.*, 2003). In order to determine the activity of dioxygenase-catalysed dihydroxylation, the formation of *cis,cis*-dihydrodiol was measured at 262 nm ($\epsilon^{262} = 8.230/\text{M cm}$) (Cidaria *et al.*, 1994). The activity of catechol 1,2-dioxygenase was measured spectrophotometrically by the formation of *cis,cis*-muconic acid at 260 nm ($\epsilon^{260} = 16.800/\text{M cm}$). In order to determine catechol 2,3-dioxygenase activity, the formation of 2-hydroxymuconic semialdehyde was measured at 375 nm ($\epsilon^{375} = 36.000/\text{M cm}$) (Wojcieszńska *et al.*, 2011). The activity of protocatechuate 3,4-dioxygenase was assayed by measuring oxygen consumption (Hou *et al.*, 1976). The activity of protocatechuate 4,5-dioxygenase was measured spectrophotometrically by the formation of 2-hydroxy-4-carboxymuconic semialdehyde at 410 nm ($\epsilon^{410} = 9.700/\text{M cm}$) (Wojcieszńska *et al.*, 2011). In order to determine gentisate 1,2-dioxygenase activity, the formation of maleylpyruvate was measured at 330 nm ($\epsilon^{330} = 10.800/\text{M cm}$) (Feng *et al.*, 1999). The activity of hydroxyquinol 1,2-dioxygenase was measured spectrophotometrically by the formation of maleylacetate at 243 nm ($\epsilon^{243} = 44.520/\text{M cm}$) (Wei *et al.*, 2010).

One unit of enzyme activity was defined as the amount of enzyme required to generate 1 μmol of product per minute. Protein concentration in the crude extract was determined by the Bradford method using bovine serum albumin as a standard (Wojcieszńska *et al.*, 2011). All experiments were performed in three

replicates. The values of enzyme activities were analyzed by one-way ANOVA ($p < 0.05$) using STATISTICA 10.0 PL software package.

Results and Discussion

Naproxen degradation in mono- and disubstrate cultures. Naproxen belongs to the polar acidic drugs that are often used by human population as a non-steroidal anti-inflammatory drug without prescription (Grenni *et al.*, 2014). For this reason, a great amount of naproxen or its metabolites is excreted and enters sewage treatment plants, where they are barely reduced and, consequently, released into the environment. Although these compounds are microcontaminants, detected in the range 0.01–2.6 $\mu\text{g/l}$, they can exert toxic effects on non-target organisms (Rodriguez-Rodriguez *et al.*, 2010; Grenni *et al.*, 2013; Curie *et al.*, 2014). That is why preliminary studies on microorganisms able to degrade naproxen, as well as enzymes involved in its degradation, are so important.

The presented paper is the first report on the degradation of naproxen by gram-positive bacterium – *Planococcus* sp. S5. As we demonstrated previously, strain S5 is able to grow on salicylate, benzoate or phenol and express either catechol 1,2-dioxygenase or catechol 2,3-dioxygenase depending on the inductor (Łabużek *et al.*, 2003; Hupert-Kocurek *et al.*, 2012). Since naproxen, the derivative of naphthalene, may be metabolized by acetylic acid or benzoic acid as intermediates (Annweiler *et al.*, 2000), the degradation potential of strain S5 suggests that this strain is a good candidate for naproxen biotransformation.

The chemical oxidation of naproxen in abiotic control, as well as adsorption of this drug on bacterial cells was not observed (data not shown). In monosubstrate culture, approximately 27.5% of naproxen was removed after 28 days. However, this compound was an insufficient carbon source for strain S5 and decrease in bacterial growth was observed (Fig. 1). This gave rise to the need for introducing an additional source of carbon into the culture. Due to the fact that aromatic plant compounds show a similar structure to naproxen, the use of such compounds as growth substrates may cause the induction of enzymes engaged in the metabolism of aromatic ring. Moreover, aromatic plant compounds are substrates naturally present in the environment. The results of studies on the transformation of naproxen in the presence of benzoate, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid or vanillic acid have shown that 4-hydroxybenzoic acid is a good growth substrate for cometabolic degradation of this drug. The increased efficiency of naproxen degradation was observed only in the presence of 4-hydroxybenzoic acid as an additional

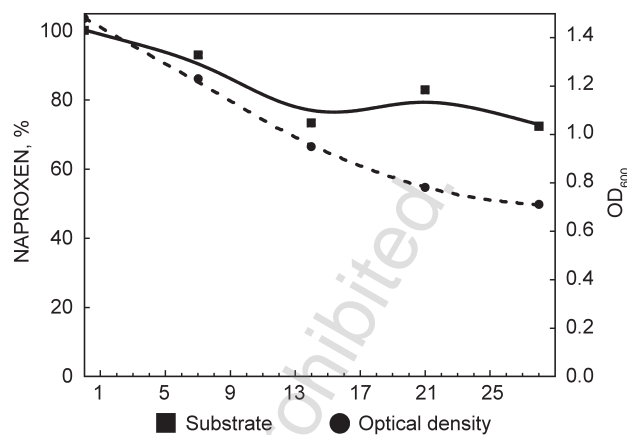


Fig. 1. Degradation of 6 mg/l naproxen by *Planococcus* sp. S5 and changes of microbial biomass monitored as optical density at 600 nm

carbon source (Fig. 2). This confirms the results of our studies on nitrophenol degradation by *Stenotrophomonas maltophilia* KB2. Addition of 4-hydroxybenzoic acid into the culture allowed the transformation of about 30% of mononitrophenols (Greń *et al.*, 2010).

Induction of degradation enzyme. Knowledge on enzymes engaged in naproxen degradation by microorganisms is very limited. Therefore, the aim of our study was to determine if/how the presence of naproxen affects the induction of key enzymes in aromatic degradation pathways. *Planococcus* sp. S5 is known to synthesizes two types of catechol dioxygenases: catechol 1,2-dioxygenase (in the presence of salicylate, benzoate or low concentration of phenol) and catechol 2,3-dioxygenase which synthesis is induced by salicylate or phenol (Łabużek *et al.*, 2003; Hupert-Kocurek *et al.*, 2012). Due to the low biomass obtained in the culture of strain S5 with naproxen as the only carbon source, as well as very low rate of naproxen degradation in the cultures with benzoate, 3,4-dihydroxybenzoic acid or vanillic acid as a growth substrate, degradation enzymes were isolated from cells grown in the presence of 4-hydroxybenzoate or 4-hydroxybenzoate and naproxen. After growth of the strain with 4-hydroxybenzoate activity of monooxygenase, hydroxyquinol 1,2-dioxygenase, protocatechuate 3,4-dioxygenase and gentisate 1,2-dioxygenase was observed (Table I, Fig. 3A). Monooxygenase is known to be engaged in hydroxylation of the aromatic ring of 4-hydroxybenzoate to 3,4-dihydroxybenzoic acid or 1,2,4-benzenetriol (Sze and Dagley, 1984; Eppink *et al.*, 1997; Wang *et al.*, 2002). The aromatic ring of 3,4-dihydroxybenzoate formed as a result of monooxygenase activity may be then cleaved by protocatechuate 3,4-dioxygenase, whereas degradation of 1,2,4-benzenetriol is catalyzed by hydroxyquinol 1,2-dioxygenase (Sze and Dagley, 1984; Park *et al.*, 2006). Additionally, hydroxylation of 4-hydroxybenzoate may lead to gentisic acid formation

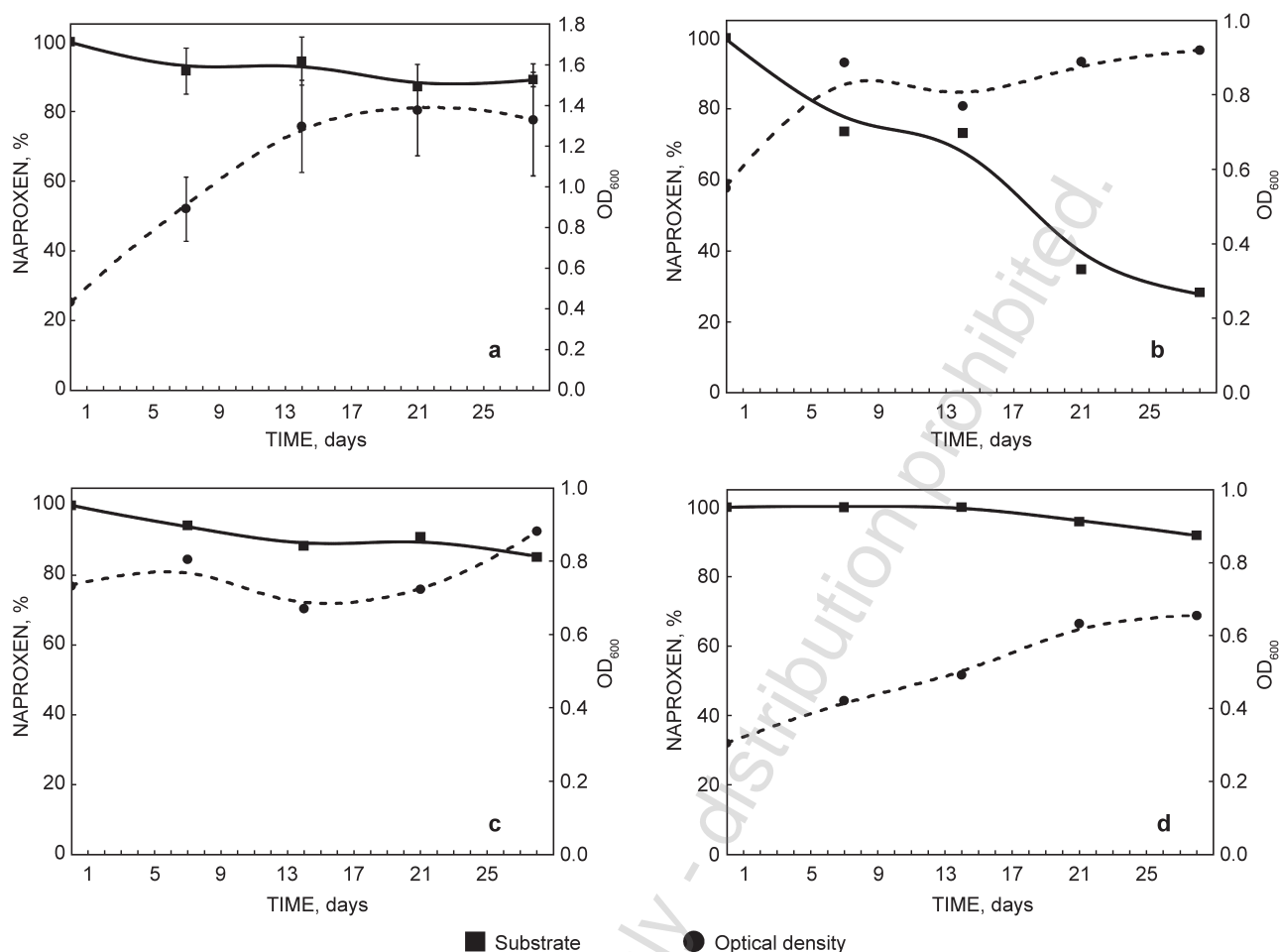


Fig. 2. Degradation of 6 mg/l naproxen by *Planococcus* sp. S5 in cometabolic systems and changes of microbial biomass monitored as optical density at 600 nm (a – with 3 mM benzoate; b – with 3 mM 4-hydroxybenzoate; c – with 3 mM 3,4-dihydroxybenzoate; d – with 3 mM vanillic acid)

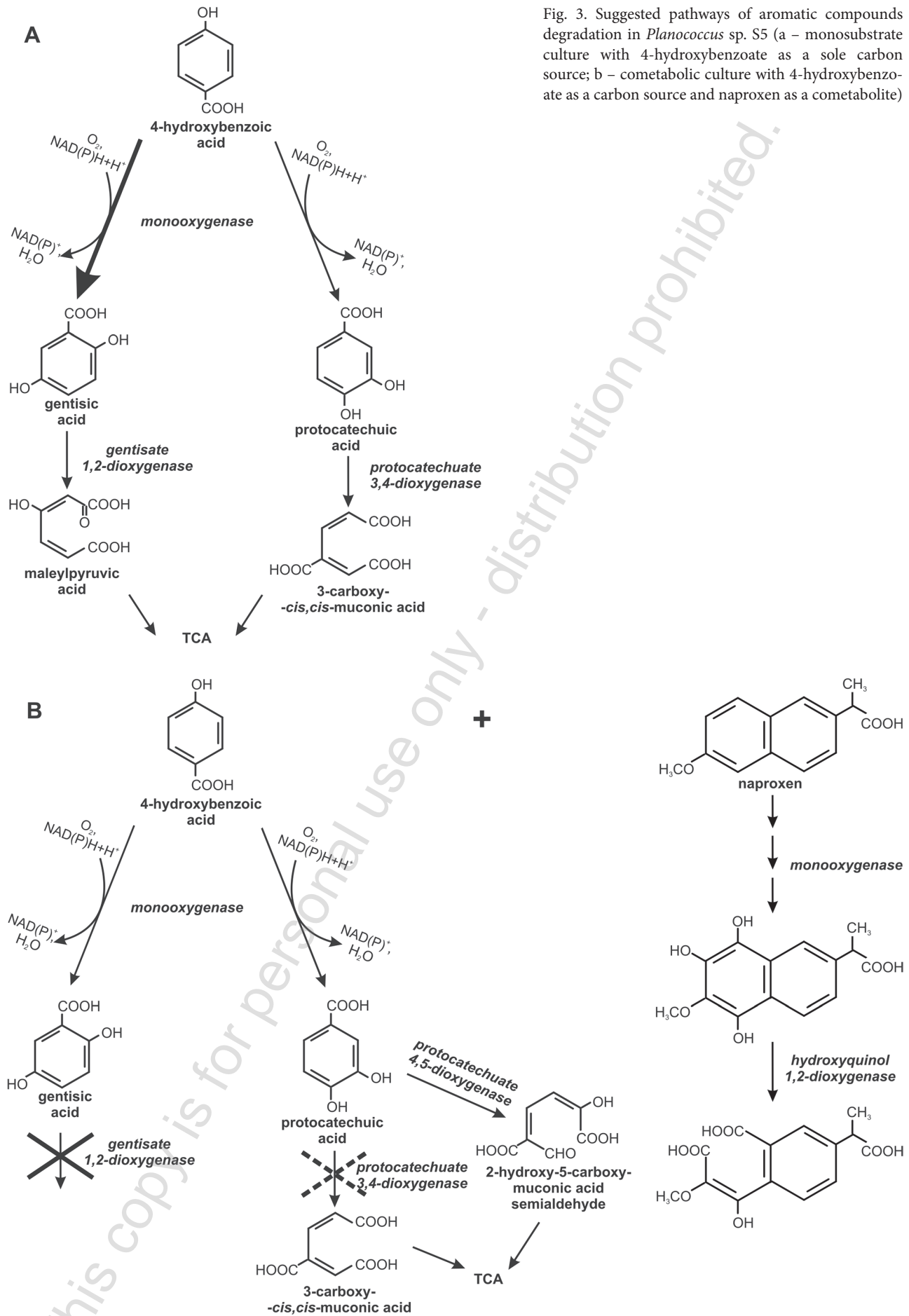
that is connected with the intramolecular migration (NIH Shift) of carboxylic group (Fairley *et al.*, 2002; Deveryshetty *et al.*, 2007). After growth of *Planococcus*

Table I
Specific activity of enzymes in the presence of 4-hydroxybenzoate or naproxen and 4-hydroxybenzoate

Enzyme	Specific enzyme activity (U/mg protein)	
	4-HB	naproxen + 4-HB
monooxygenase	25.74 ± 1.90	25.66 ± 7.40
naphthalene dioxygenase	0.0 ± 0.0	0.0 ± 0.0
hydroxyquinol 1,2-dioxygenase	46.56 ± 0.0	215.25 ± 90.74
catechol 1,2-dioxygenase	0.0 ± 0.0	0.0 ± 0.0
catechol 2,3-dioxygenase	0.0 ± 0.0	0.0 ± 0.0
protocatechuate 3,4-dioxygenase	58.55 ± 0.0*	30.47 ± 2.12*
protocatechuate 4,5-dioxygenase	0.0 ± 0.0*	3.68 ± 1.74*
gentisate 1,2-dioxygenase	104.16 ± 29.46*	0.0 ± 0.0*

* The asterisk indicates the values of enzymes activities which differ significantly ($p < 0.05$) in dependence on the growth substrate

sp. S5 with 4-hydroxybenzoate and naproxen changes in the activity of enzymes was observed (Table I, Fig. 3B). Activity of hydroxyquinol 1,2-dioxygenase increased approximately fourfold, while protocatechuate 3,4-dioxygenase and gentisate 1,2-dioxygenase, enzymes engaged in 4-hydroxybenzoate cleavage, were inhibited (Table I). As it is shown in Table I, in the presence of naproxen, protocatechuate 3,4-dioxygenase showed about 52 % of its initial activity while gentisate 1,2-dioxygenase was completely inhibited. The increase in hydroxyquinol 1,2-dioxygenase activity could be connected with the engagement of this enzyme in naproxen degradation (Wojcieszynska *et al.*, 2014). Decrease of protocatechuate 3,4-dioxygenase activity was observed by Luo *et al.* (2008) in the presence of naphthalene, which is the structural mimetic of naproxen. However, in *Planococcus* sp. S5 culture with naproxen and 4-hydroxybenzoic acid activity of protocatechuate 4,5-dioxygenase was observed (Table I). Activity of this enzyme was also observed by Yun *et al.* (2004) during their studies on 4-hydroxybenzoic acid degradation. We assume that induction of protocatechuate 4,5-dioxygenase



in the presence of naproxen and 4-hydroxybenzoate could be the response of bacterial strain to stress connected with inhibition of the main enzyme engaged in 4-hydroxybenzoate degradation (Fig. 3).

In conclusion, *Planococcus* sp. S5 has the ability of efficient degradation of naproxen in the presence of 4-hydroxybenzoate as a carbon source. In this condition, activity of monooxygenase, hydroxyquinol 1,2-dioxygenase, and two different protocatechuate dioxygenases is observed. The presence of various metabolic pathways and induction of different oxygenases involved in the degradation of aromatic compounds enable the use of *Planococcus* sp. S5 in the degradation of various aromatic pollutants including non-steroidal anti-inflammatory drugs.

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