re-BUS

You have downloaded a document from
RE-BUŚ
repository of the University of Silesia in Katowice

Title: Modulation of FAD-dependent monooxygenase activity from aromatic compounds-degrading Stenotrophomonas maltophilia strain KB2

Author: Danuta Wojcieszyńska, Izabela Greń, Katarzyna Hupert-Kocurek, Urszula Guzik.

Citation style: Wojcieszyńska Danuta, Greń Izabela, Hupert-Kocurek Katarzyna, Guzik Urszula. (2011). Modulation of FAD-dependent monooxygenase activity from aromatic compounds-degrading Stenotrophomonas maltophilia strain KB2. "Acta Biochimica Polonica" (2011, no. 3, s. 421-426).

Uznanie autorstwa - Na tych samych warunkach - Licencja ta pozwala na kopiowanie, zmienianie, rozprowadzanie, przedstawianie i wykonywanie utworu tak długo, jak tylko na utwory zależne będzie udzielana taka sama licencja.

UNIWERSYTET ŚLĄSKI W KATOWICACH
Biblioteka Uniwersytetu Śląskiego
Ministerstwo Nauki i Szkolnictwa Wyższego

ACTA ABP BIOCHIMICA POLONICA

Vol. 58, No 3/2011
421–426
on-line at: www.actabp.pl

Regular paper

Modulation of FAD-dependent monooxygenase activity from aromatic compounds-degrading *Stenotrophomonas maltophilia* strain KB2

Danuta Wojcieszyńska, Izabela Greń, Katarzyna Hupert-Kocurek and Urszula Guzik✉

Department of Biochemistry, University of Silesia in Katowice, Katowice, Poland

The purpose of this study was purification and characterization of phenol monooxygenase from *Stenotrophomonas maltophilia* strain KB2, enzyme that catabolises phenol and its derivatives through the initial hydroxylation to catechols. The enzyme requires NADH and FAD as a cofactors for activity, catalyses hydroxylation of a wide range of monocyclic phenols, aromatic acids and dihydroxylated derivatives of benzene except for catechol. High activity of this monooxygenase was observed in cell extract of strain KB2 grown on phenol, 2-methylphenol, 3-metylphenol or 4-methylphenol. Ionic surfactants as well as cytochrome P450 inhibitors or 1,4-dioxane, acetone and n-butyl acetate inhibited the enzyme activity, while non-ionic surfactants, chloroethane, ethylbenzene, ethyl acetate, cyclohexane, and benzene enhanced it. These results indicate that the phenol monooxygenase from *Stenotrophomonas maltophilia* strain KB2 holds great potential for bioremediation.

Keywords: monooxygenase, *Stenotrophomonas*, phenols, inhibitors

Received: 16 May, 2011; revised: 11 July, 2011; accepted: 13 September, 2011; available on-line: 16 September, 2011

INTRODUCTION

Phenols are widely distributed environmental pollutants generating strong interest in their bioremediation. Numerous bacterial strains are capable of mineralizing aromatic compounds. The wide range of substrates that can be transformed by microorganisms makes them a powerful tool for the bioremediation of environmentally harmful substances. Degradation of phenolic derivatives by aerobic bacteria generally proceeds by series of oxygenations. The initial step in the bacterial degradation of these compounds is the conversion of aromatic substrates into dihydroxylated intermediates by the action of monooxygenases. The next step is catalysed by dioxygenases that cleave the carbon–carbon bond and open the ring. The product of the ring cleavage is finally transformed into intermediates that can enter the citrate cycle (Cafaro *et al.,* 2004; Murray *et al.,* 2007; Arora *et al.,* 2010; Dresen *et al.,* 2010; Fujieda *et al.,* 2010; Plazmiňo *et al.,* 2010).

Aromatic monooxygenases catalysing hydroxylation of aromatic compounds are divided into two groups: activated-ring and nonactivated-ring ones. The active sites of these enzymes usually contain FAD as a prosthetic group and a dinuclear iron centre in which two iron atoms are bridged by an oxygen atom (Fe-O-Fe) (Divari *et al.,* 2003; Murray *et al.,* 2007). They activate molecular oxygen though the formation of a reactive flavin (hydro) peroxide which attacks the substrate by an electrophilic mechanism (Moonen *et al.,* 2002; Arora *et al.,* 2010; Dresen *et al.,* 2010; Plazmiňo *et al.,* 2010).

In this paper, we describe the purification and characterization of a novel NADH-dependent, FAD-containing monooxygenase from *Stenotrophomonas maltophilia* strain KB2, an interesting model for studying catabolism of phenols because of its unique ability to transform a wide range of these compounds compared with other similar microorganisms (Guzik *et al.,* 2009; Greń *et al.,* 2010). This study includes determination of the substrate specificity and catalytic properties of the enzyme. As in the environment compounds that inhibit monooxygenase activity are frequently present together with the substrates, effect of various surfactants, organic solvents, and cytochrome P450 inhibitors on the enzyme activity is also presented.

EXPERIMENTAL PROCEDURES

Media and culture conditions. *Stenotrophomonas maltophilia* KB2 (NCBI accession number DQ230920) is a Gram-negative, aromatic compound-degrading bacterium isolated from activated sludge of a sewage treatment plant in Bytom–Miechowice in Poland as described previously (Guzik *et al.,* 2009). Strain KB2 was induced in mineral salts medium (MSM) as described previously (Wojcieszyńska *et al.,* 2011) in the presence of 10 mM phenol. Cultures were incubated at 30°C and agitated at 130 rpm.

Preparation of enzyme. Cells were harvested in the late exponential growth phase and centrifuged at 5000 × *g* for 15 min at 4°C. The cells were washed with 50 mM phosphate buffer, pH 7.0, and resuspended in the same buffer. Cell suspension was sonicated 6 times for 15 s and centrifuged at 9500 × *g* for 20 min at 4°C. The supernatant was ultracentrifuged at 150 000 × *g* for 1.5 h at 4°C. The pellet, containing membrane-associated proteins, was resuspended in 40 mM phosphate buffer and used for enzyme assays or for further purification. Nu-

✉e-mail: urszula.guzik@us.edu.pl

Abbreviations: 2-AP, 2-aminophenol; 3-AP, 3-aminophenol; 4-AP, 4-aminophenol; 2-CP, 2-chlorophenol; 3-CP, 3-chlorophenol; 4-CP, 4-chlorophenol; 2-NP, 2-nitrophenol; 3-NP, 3-nitrophenol; 4-NP, 4-nitrophenol; 2-MP, 2-methylphenol; 3-MP, 3-methylphenol; 4-MP, 4-methylphenol

cleic acids were removed by centrifugation at 9500×*g* for 10 min after addition of 0.05 g/100 ml protamine sulphate. Obtained supernatant was fractionated with ammonium sulphate. Solid $(NH_4)_2SO_4$ was added to the extract to 20% saturation with constant stirring. After 30 min the precipitate was removed by centrifugation at 9500×*g* for 10 min. The supernatant was loaded onto Desalt Spin Columns equilibrated previously with 40 mM phosphate buffer. The elution was obtained with the same buffer. The eluate was loaded onto a column Sephadex G-100 (1.6 cm×82 cm) equilibrated with 40 mM phosphate buffer, pH 7.2. Elution was carried out with the same buffer at a flow rate of 1 ml/min and 2.5-ml fractions were collected.

Molecular mass determination. The value of the monooxygenase was determined by size exclusion chromatography on Sephadex G-100 in 50 mM phosphate buffer, pH 7.0, at 4°C. Elution volumes were determined by OD_{280} measurements and, in the case of the monooxygenase, by enzyme activity. The molecular mass markers used as standards were as follows: ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), bovine serum albumin (67 kDa), and trypsin (24 kDa).

Enzyme assays. The monooxygenase activity was determined spectrophotometrically by measuring NADH oxidation ($\varepsilon_{340} = 6220 \cdot M^{-1}\ cm^{-1}$) in 40 mM phosphate buffer (pH 7.0) containing 44 μM FAD, 4 mM NADH and 10 mM phenol derivative. One unit of enzyme activity was defined as the amount of NADH (μmol) the enzyme oxidized in the presence of substrate per minute at 30°C (Divari *et al.,* 2003). Protein concentration of all examined fractions was determined by the Bradford method (Bradford, 1976).

The reductase activity of monooxygenase was assayed spectrophotometrically at 30°C by monitoring the reduction of cytochrome *c* at 550 nm or dichlorophenolindophenol and nitroblue tetrazolium at 600 nm with a CECIL UV-Vis spectrophotometer (Cafaro *et al.,* 2004). FAD reduction was measured in 40 mM phosphate buffer (pH 7.2) containing 40 μM FAD, 1 mM NADH, and various amounts of protein at 30°C under anaerobic conditions. Anaerobic conditions were achieved by introducing the reaction mixture into a gastight cuvette inside an anaerobic glove box filled with nitrogen (Xun, 1996).

pH and temperature optima of monooxygenase. The effect of pH on the enzyme activity was determined by measuring the activity at 30°C over the pH range of 4.0 to 14.0 using the following buffers: 0.05 M phosphate/citrate (pH 4.0 to 4.5), 0.05 M Sörensen (pH 5.0), 0.05 M phosphate (pH 5.7 to 8.0), 0.05 glycine (pH 10.0), 0.05 Britton-Robinson (pH 11.00 to 12.00), and 0.05 ammonia/sodium hydroxide (pH 13.00–14.00).

The optimum temperature was determined by assaying the enzyme activity at various temperatures (5 to 40°C) in 50 mM phosphate buffer (pH 7.0). The enzyme and substrate solutions were pre-incubated, mixed, and the enzymatic reaction was followed at the same temperature.

Substrate specificity and kinetic studies. The catalytic parameters (Michaelis–Menten constant, K_m, and maximum velocity, V_{max},) were calculated by measuring initial linear rates of the enzymatic reaction after the

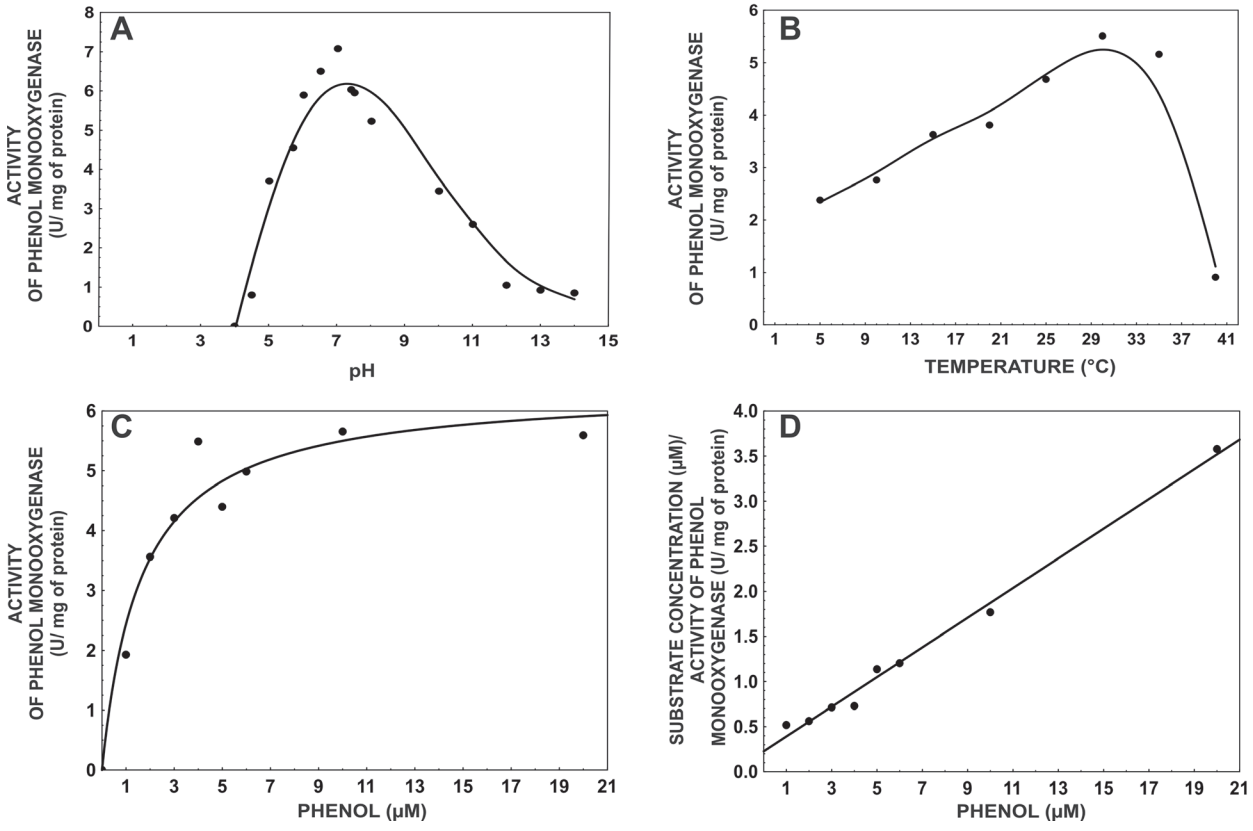

Figure 1. Effect of various factors on phenol monooxygenase activity in *Stenotrophomonas maltophilia* KB2 cell extract
(A) pH; **(B)** temperature; and **(C, D)** substrate concentration. Data points represent average of three independent experiments.

Table 1. Purification of phenol monooxygenase

Fraction	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Purification (fold)	Yield (%)
F1- cell extract	19.05	4.35	4.38 ± 1.09	1.00	100
F2- centrifuge (1718 × *g*) fraction	34.49	0.94	36.69 ± 3.56	8.4	181
F3- ultracentrifuge (150 000 × *g*) fraction	16.85	0.36	46.82 ± 5.26	11.0	88
F4- protamine sulphate treatment	25.30	0.45	56.23 ± 0.00	13.0	133
F5- ammonium sulphate fraction	29.09	0.19	153.13 ± 25.41	35.00	153
F6- desalt spin column fraction	7.47	0.05	149.50 ± 34.53	34.13	39
F7′-peak 159 (Sephadex G-100 column)	2.29	0.02	114.43 ± 0.00	26.13	12

addition of different concentrations of phenol ranging from 0 to 20 μM at 30 °C. Three independent measurements were carried out for each substrate concentration. K_m and V_{max}, were calculated from Hanes-Wolf equation.

The enzyme activity towards various substituted aromatic compounds was evaluated by incubating the enzyme with the respective aromatic substrate for 3 min and assaying the enzyme activity. The following substrates were assayed: aromatic acids: benzoic, protocatechuic, salicylic, 4-hydroxybenzoic, phthalic, cinnamic, 2-chlorophenylacetic, gallic, vanillic, and syringic acid; dihydroxy-substituted derivatives: hyroquinone, resorcinol and catechol; and a methyl derivative of catechol, guaiacol; derivatives of phenol: 2-chlorophenol (2-CP), 3-chlorophenol (3-CP), 4-chlorophenol (4-CP), 2-aminophenol (2-AP), 3-aminophenol (3-AP), 4-aminophenol (4-AP), 2-nitrophenol (2-NP), 3-nitrophenol (3-NP), 4-nitrophenol (4-NP), 2-methylphenol (2-MP), 3-methylphenol (3-MP), and 4-methylphenol (4-MP). All those substrates were used at 10 mM.

Effect of surfactants, organic solvents, and cytochrome P450 inhibitors on enzyme activity. The effect of various surfactants, organic solvents as well as cytochrome P450 inhibitors on the enzyme activity was evaluated by incubating the enzyme with the respective agent for 3 min and then assaying the residual enzyme activity with phenol as a substrate. The nonionic and ionic surfactants studied were: Triton X-100, Tween 20, oleic acid, stearic acid and sulphanilic acid. From among organic solvents, chloroethane, xylene, ethylbenzene, 1,4-dioxane, acetone, n-butyl acetate, ethyl acetate, cyclohexane and benzene were examined. As inhibitors of cytochrome P450, menadione, β-naphthoflavone as well as sodium azide were tested.

RESULTS AND DISCUSSION

Bacterial-derived enzymatic systems are of growing importance because of their potential use in bioremediation. Understanding the biochemical and structural properties of enzymes of degradation pathways is essential for their future potential applications. In this paper, we describe the purification and biochemical characterization of a novel NADH-dependent, FAD-containing phenol monooxygenase isolated from cultures of *S. maltophilia* KB2 induced by phenol.

In order to localise the monooxygenase activity in *S. maltophilia* KB2, the hydroxylation activity of the en-

Table 2. Substrate specificity of phenol monooxygenase

Substrate	Activity, %
Phenol	100.00
Benzoate	74.36 ± 2.10
Salicylate	136.72 ± 5.60
4-Hydroxybenzoate	128.38 ± 7.52
Phthalic acid	87.02 ± 6.10
Cinnamic acid	131.54 ± 9.29
Chlorophenoxyacetic acid	137.95 ± 5.88
Gallate	100.47 ± 14.10
Vanilic acid	5.81 ± 0.76
Syryngic acid	4.12 ± 0.84
Protocatechuate	74.36 ± 2.10
Hydroquinone	98.75 ± 15.16
Guaiacol	62.59 ± 14.64
Resorcinol	74.63 ± 4.25
Catechol	0.00 ± 0.00
2-CP	34.00
3-CP	22.03
4-CP	41.24
2-NP	33.33
3-NP	18.79
4-NP	43.36
2-MP	39.83
3-MP	28.95
4-MP	71.61
2-AP	35.45
3-AP	37.57
4-AP	47.74

Table 3. Effect of various surfactants on phenol monooxygenase activity

Compound	Concentration (mM)	Relative activity (%)
None		100
Triton X-100	1	26.88 ± 4.90
	2	37.29 ± 0.00
	5	38.48 ± 1.23
	10	406.64 ± 0.00
Tween 20	1	104.69 ± 16.81
	2	230.99 ± 20.50
	5	163.28 ± 18.76
	10	158.81 ± 1.50
Oleic acid	1	0.00 ± 4.61
	2	9.59 ± 2.90
	5	0.00 ± 0.00
	10	0.00 ± 0.00
Stearic acid	1	71.45 ± 19.54
	2	0.00 ± 0.00
	5	0.00 ± 0.00
	10	0.00 ± 0.00
Sulphanilic acid	1	74.25 ± 0.65
	2	66.19 ± 1.99
	5	57.77 ± 8.49
	10	7.88 ± 3.59

zyme towards phenols was tested in two fractions of crude extracts of bacterial cells obtained after ultracentrifugation at 150 000 × *g* the supernatant containing soluble proteins and the pellet of membrane-associated proteins. Monooxygenase activity was detected only in the pellet fraction. In contrast to phenol hydroxylase from *Acinetobacter radioresistens* (Divari *et al.,* 2003) and 2,4-dichlorophenol hydroxylase from *Acinetobacter* sp. (Beadle & Smith, 1982), the monooxygenase from *S. maltophilia* KB2 seems to be an insoluble membrane protein.

The purification procedure described in the Experimental Procedures section enriched the monooxygenase approximately 34-fold (Table 1). The active fraction (114 U/mg protein) obtained from Sephadex G-100 had a retention time of 152 min. The molecular mass of the purified monooxygenase was estimated to be 34 kDa by gel filtration. The low molecular mass of the enzyme suggested its monomeric structure, as reported previously for other hydroxylases (Batie *et al*., 1987; Xun 1996), but in contrast with many monooxygenases that form dimers and tetramers of high mass (up to 240 kDa) (Beadle & Smith, 1982). The effect of temperature and pH on the phenol monooxygenase activity from KB2 strain was determined in cell extracts. The highest activity of the enzyme was observed at pH 7.2 (Fig. 1A). Moonen *et al.* (2002) described a hydroxylase from a *Flavobacterium* strain which was optimally active around pH 8.3, and the most stable around pH 7.0, as at pH above 7.5 the enzyme easily lost the FAD molecule.

The monooxygenase from KB2 strain was rather sensitive to thermal inactivation and can be categorised as a mesophilic protein. The optimal temperature for phenol oxidation was 30 °C. Above 35 °C thermoinactivation of the enzyme was observed (Fig. 1B). Similar results were found for 4-monooxygenase from *Burkholderia cepacia* AC 1100 strain and hydroquinone hydroxylase from *Candida parapsilosis* CBS604. The optimal temperature for those enzymes was 35 °C and 37 °C, respectively (Xun, 1996; Eppink *et al.,* 2000).

The phenol oxidation by the monooxygenase from KB2 displays typical Michaelis–Menten kinetics. The K_m and V_{max} values were 1.62 μM and 6.39 μM/min, respectively (Fig. 1C, D). FAD was found to accelerate the reaction. It was also found that the presence of NADPH instead of NADH reduced significantly the monooxygenase activity, in contrast to the effect observed for chlorophenol 4-monooxygenase from *Burkholderia cepacia* AC1100 (Xun, 1996) and 2,4-dichlorophenol hydroxylase from *Acinetobacter* sp. (Beadle & Smith 1982). A similar effect of NADH on xylene monooxygenase from *Pseudomonas putida* mt-2 was observed by Shaw and Harayama (1992). Studies on 4-hydroxybenzoate-3-hydroxylase have provided some insight into the mode of the coenzyme recognition (Eswaramoorthy *et al.,* 2006). They postulate significant role of Arg-44, His-162 and Arg-269 in the interaction of the enzyme with NADPH. Arg-44 was involved in binding the adenosyl moiety and His-162 and Arg-269 in binding the pyrophosphate moiety of NADPH while the acidic group of (Asp/Glu) was required for the recognition of the 2'-OH group of NADH (Moonen *et al.,* 2002; Eswaramoorthy *et al.,* 2006).

Flavoprotein monooxygenases that are active with phenolic compounds usually contain FAD as a prosthetic group. They activate molecular oxygen through the formation of a reactive flavin (hydro)peroxide which attacks the substrate by an electrophilic mechanism (Moonen *et al.,* 2002; Kirchner *et al.,* 2003; Eswaramoorthy *et al.,* 2006; Dresen *et al.,* 2010). Phenol monooxygenases generate reduced flavine at the expense of NAD(P)H and next transfer electrons to the iron ions (Shaw & Harayama, 1992; Moonen *et al.,* 2002). The FAD reduction by the studied enzyme was estimated to be 10 U/mg of purified enzyme and indicated that FAD was the prosthetic group of this monooxygenase.

The monooxygenase from KB2 strain was also tested for its ability to reduce several artificial electron acceptor such as cytochrome *c,* nitrotetrazolium blue and 2,6-dichlorophenolindophenol, and was found to reduce only cytochrome *c*. The specific activity of the enzyme toward cytochrome *c* was 7.55 U/mg of purified enzyme. The reaction catalysed by the phenol monooxygenase of KB2 strain is typical for a monooxygenase with an external electron donor such as 2,4-dichlorophenol monooxygenase or xylene monooxygenase (Beadle & Smith, 1982; Shaw & Harayama, 1992; Kirchner *et al.,* 2003; Eswaramoorthy *et al.,* 2006; Arora *et al.,* 2010; Plazmiño *et al.,* 2010).

Many monooxygenases have narrow substrate specificities and regioselectivities, such as 3-hydroxyphenylacetate 6-hydroxylase from *Flavobacterium*. Besides 3-hydroxyphenylacetate, which was converted to 2,5-dihydroxyphenylacetate, the only alternative substrate was 3,4-dihydroxyphenylacetate (Moonen *et al.,* 2002). Because monooxygenases are not only used in the biocata-

Table 4. Effect of various organic solvents on phenol monooxygenase activity

Compound	Concentration (mM)	Relative activity (%)
None		100
Chloroethane	1	140.69 ± 23.11
	2	218.79 ± 0.70
	3	574.93 ± 49.41
Xylene	1	131.98 ± 7.29
	2	109.22 ± 7.69
	3	106.55 ± 5.70
Ethylbenzene	1	133.17 ± 1.17
	2	138.01 ± 1.04
	3	138.19 ± 7.09
1,4-Dioxane	1	138.11 ± 9.27
	2	33.21 ± 1.56
	3	5.15 ± 0.99
Acetone	1	162.22 ± 8.28
	2	81.56 ± 1.60
	3	11.45 ± 0.22
n-Butyl acetate	1	273.98 ± 43.94
	2	283.15 ± 6.30
	3	57.98 ± 1.21
Ethyl acetate	1	422.63 ± 31.23
	2	438.55 ± 73.40
Cyclohexane	0.1	46.82 ± 4.67
	0.2	132.51 ± 15.38
	0.3	283.79 ± 27.87
	0.4	331.29 ± 1.25
Benzene	1	122.23 ± 6.77
	2	141.77 ± 18.16
	3	119.37 ± 5.16
	4	154.61 ± 27.61

lytic production of fine chemicals, pharmaceuticals and food ingredients but also are of interest for bioremediation, we sought for an enzyme which would metabolize a wide range of benzene derivatives, causing sometimes serious local contamination of the environment.

By measuring NADH oxidation in the presence of different aromatic compounds, the substrate specificity of the monooxygenase was tested. As shown in Table 2, the monooxygenase from *S. maltophilia* KB2 acted on a broad range of substrates, including methylated phenols, aromatic acids and their dihydroxylated derivatives. The enzyme showed preferences for hydroxylation of phenols with a substituent in the *para*-position such as 4-CP, 4-AP, 4-NP and 4-MP, which suggests that the *para*-position enabled *orto*-hydroxylation without a simultaneous removal of the substituent group. During the enzymatic conversion of dihydroxybenzenes we observed that catechol was a poor substrate of the enzyme (Table 2). As catechol is usually formed during phenol hydroxylation, the enzyme catalysing this reaction probably has a regulatory site responsible for inhibition of the enzyme in the presence of excess product. Results in Table 2 obtained for *meta*-substituted phenols indicate low affinity of enzyme active site for these substrates. We assumed that substituents in the *meta*-position might comprise a steric hindrance for the monooxygenase activity. The monooxygenase from KB2 strain metabolised all aromatic acids used in this study (Table 2), albeit vanilic and syryngic acids only poor. That was probably caused by the presence of the methoxy group in their structure (Ueng *et al.,* 2000).

Most of the surfactants studied here affected the enzyme activity at all concentrations tested (Table 3). At lower concentrations of Triton X-100 there was a decrease in enzyme activity, whereas high concentration (10 mM) of this surfactant enhanced the activity of the enzyme. Tween 20 did not affect the enzyme activity at its lowest concentration, whereas enhanced activity of the monooxygenase was observed at higher concentrations of this surfactant. Different results were obtained by Lattard *et al.* (2002) and Myasoedova *et al.* (2007), who did not observe an effect of nonionic detergents on monooxygenase activity. The increase in activity in the presence of surfactants may be attributed to increased substrate accessibility of the enzyme (Chellappan *et al.,* 2010). In the presence of oleic, stearic and sulphanilic acids the monooxygenase activity decreased significantly. The inhibition of the enzyme at higher concentrations of ionic surfactants may be due to a combined effect of factors such as reduction in the hydrophobic interactions that play a crucial role in holding together the protein structure and direct interactions with the cell membrane.

Organic solvents affect enzyme activity *via* interactions with water, enzyme, substrate and product. It is well documented that hydrophobic solvents are usually better than hydrophilic ones, as the latter have a greater tendency to strip tightly bound water from the enzyme molecule (Shuster & Fishman, 2009). In our studies pronounced stimulatory effects of chloroethane, ethylbenzene, ethyl acetate, cyclohexane and benzene on monooxygenase activity were observed (Table 4). The enzyme activity was slightly stimulated in the presence of ethylbenzene. Xylene had almost no effect, while 1,4-dioxane, acetone, and n-butyl acetate inhibited the enzyme (Table 4).

Multicomponent monooxygenases usually consist of three protein components: a hydroxylase, a cofactorless regulatory protein, and a FAD-containing reductase. However, there is also a large group of P450-containing monooxygenases where P450 acts as the terminal oxidase (Degtyarenko, 1995; Sazinsky & Lippard, 2006). In our studies menadione, β-naphthoflavone and sodium azide were used as specific cytochrome P450 inhibitors. The concentrations of the inhibitors and percentage inhibition of hydroxylation are shown in Table 5. The partial or complete inhibition of the enzyme activity observed with menadione and β-naphthoflavone clearly showed that the activity of monooxygenase of KB2 strain depends on cytochrome P450. We observed increased activity at the lowest concentration of menadion, but this effect is difficult to explain. Sodium azide slightly in-

Table 5. Effect of cytochrome P450 inhibitors on phenol monooxygenase activity

Compound	Concentration (mM)	Relative activity (%)
None		100
Menadione	1	260.69 ± 10.23
	2	19.35 ± 4.21
	3	0.00 ± 0.00
	4	0.00 ± 0.00
	5	6.33 ± 0.42
β-Naphthoflavone	0.5	0.00 ± 0.00
	1	0.00 ± 0.00
	2	0.00 ± 0.00
Sodium azide	1	105.82 ± 3.24
	2	113.1 ± 11.79
	5	118.1 ± 8.28
	10	88.31 ± 43.84

hibited the enzyme activity (Table 5) even though this heme ligand is generally regarded as a P450 inhibitor (Matsunaga *et al.,* 1998). The mechanism of inhibitor action may involve their conversion into reactive intermediates which covalently bind to the active site of the enzyme blocking its interaction with the substrate (Beliaev *et al*., 2009). In our work we have shown that phenol monooxygenase from *S. maltophilia* KB2 is a typical flavoprotein monooxygenase that catalyses hydroxylation of a wide range of monocyclic phenols. Because of the high efficiency of substrate hydroxylation, the enzyme is an attractive candidate for bioremediation and biotechnological application. The results presented in this paper are a good starting point for further studies on the physicochemical properties of this enzyme and the structural features which determine its substrate specificity.

Acknowledgement

Katarzyna Lepsza is acknowledged for excellent technical assistance.

This work was supported by the Ministry of Science and Higher Education (grant N N305 212337).

REFERENCES

Arora PK, Srivastava A, Singh VP (2010) Application of monooxygenase in dehalogenation, desulphurization, denitrification and hydroxylation of aromatic compounds. *J Bioremed Biodegrad* 1-112 DOI: 10.4172/2155-6199.1000112.

Batie CJ, Haie E, Ballou DP (1987) Purification and characterisation of phtalate oxygenase and phtalate oxygenase reductase from *Pseudomonas cepacia. J Biol Chem* **4:** 1510–1518.

Beadle CA, Smith AR (1982) The purification and properties of 2,4-dichlorofenol hydroxylase from strain *Acinetobacter* species. *Eur J Biochem* **123:** 323–332.

Beliaev A, Ferreira H, Learmonth DA, Soares-da-Silva P (2009) Dopamine β-monooxygenase: mechanism, substrates and inhibitors. *Curr Enzym Inhib* **5:** 27–43.

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **7:** 248–258.

Cafaro V, Izzo V, Scognamiglio R, Notomista E, Capasso P, Casbarra A, Pucci P, Di Donato A (2004) Phenol hydroxylase and toluene/*o*-xylene monooxygenase from *Pseudomonas stutzeri* OX1: interplay between two enzymes. *Appl Environ Microbiol* **70:** 2211–2219.

Chellappan S, Jasmin C, Basheer SM, Kishore A, Elyas KK, Bhat SG, Chandrasekaran M (2010) Characterization of an extracellular alkaline serine protease from marine *Engyodontium album* BTMFS10. *J Ind Microbiol Biotechnol* DOI: 10.1007/s10295-010-0914-3.

Degtyarenko KN (1995) Structural domains of P450-containing monooxygenase systems. *Protein Eng* **8:** 737–747.

Divari S, Valetti F. Caposito P, Pessione E, Cavaletto M, Griva E, Gribaudo G, Gilardi G, Giunta C (2003) The oxygenase component of phenol hydroxylase from *Acinetobacter radioresistens* S13. *J Biochem* **270:** 2244–2253.

Dresen C, Lin LY-C, D'Angelo I, Tocheva EI, Strynadka N, Eltis LD (2010) A flavin-dependent monooxygenase from *Mycobacterium tuberculosis* involved in cholesterol catabolism. *J Biol Chem* **284:** 2264–2275.

Eppink MHM, Cammaart E, van Wassenaar D, Middelhoven WJ, van Berkel WJH (2000) Purification and properties of hydroquinone hydroxylase, FAD-dependent monooxygenase involved in the catabolism of 4-hydroxybenzoate in *Candida parapsilosis* CBS604. *Eur J Biochem* **267:** 6832–6840.

Eswaramoorthy S, Bonanno JB, Burley SK, Swaminathan S (2006) Mechanism of action of a flavin-containing monooxygenase. *Proc Natl Acad Sci* **106:** 9832–9837.

Fujieda N, Yakiyama A, Itoh S (2010) Five monomeric hemocyanin subunits from *Portunus trituberculatus*: purification, spectroscopic characterization, and quantitative evaluation of phenol monooxygenase activity. *Biochim Biophys Act* **1804:** 2128–2135.

Greń I, Wojcieszyńska D, Guzik U, Perkosz M, Hupert-Kocurek K (2010) Enhanced biotransformation of mononitrophenols by *Stenotrophomonas maltophilia* KB2 in the presence of aromatic compounds of plant origin. *World J Microbiol Biotechnol* **26:** 289–295.

Guzik U, Greń I. Wojcieszyńska D, Łabużek S (2009) Isolation and characterization of a novel strain of *Stenotrophomonas maltophilia* possessing various dioxygenases for monocyclic hydrocarbon degradation. *Braz J Microbiol* **40:** 285–291.

Kirchner U, Westphal AH, Müller R, van Berkel WJH (2003) Phenol hydroxylase from *Bacillus thermoglucosidasidasius* A7 a two-protein component monooxygenase with dual role for FAD. *J Biol Chem* **278:** 47543–47553.

Lattard V, Longin-Sauvageon Ch, Lachuer J, Delatour P, Benoit E (2002) Cloning, sequencing, and tissue-dependent expression of flavin-containing monooxygenase (FMO) 1 and FMO3 in the dog. *Drug Metab Dispos* **30:** 119–128.

Matsunaga I, Yamada M, Kusunose E, Miki T, Ichihara K (1998) Further characterization of hydrogen peroxide-dependent fatty acid α-hydroxylase from *Sphingomonas paucimobilis. J Biochem* **124:** 105-110.

Moonen MJ, Fraaije MW, Rietjens IM, Laane C, Berkel WJ (2002) Flavoenzyme-catalyzed oxygenation and oxidations of phenolic compounds. *Adv Synth Catal* **344:** 1023–1035.

Murray LJ, Garcia-Serres R, McCormick MS, Davydov R, Naik SG, Kim S-H, Hoffman BM, Huynh BH, Lippard SJ (2007) Dioxygen activation at non-heme diiron centers: oxidation of proximal residue in the I100W variant of toluene/*o*-xylene monooxygenase hydroxylase. *Biochem* **46:** 14795–14809.

Myasoedova KN, Arutyunyan AM, Magretova NN (2007) Reconstitution of mooxygenase activity of membrane cytochromes P450 *in vitro* and detergents. *Biochem Biophys Mol Bol* **415:** 262–267.

Plazmiño DE, Winkler M, Glieder A, Fraaije MW (2010) Monooxygenase as biocatalysts: classification, mechanistic aspects and biotechnological applications. *J Biotechnol* **146:** 9–24.

Sazinsky M, Lippard SJ (2006) Correlating structure with function in bacterial multicomponent monooxygenases and related diiron proteins. *Acc Chem Res* **39:** 558-566.

Shaw J, Harayama S (1992) Purification and characterisation of the NADH: acceptor reductase component of xylene monooxygenase encoded by the TOL plasmid pWW0 of *Pseudomonas putida* mt-2. *Eur J Biochem* **209:** 51–61.

Shuster V, Fishman A (2009) Isolation, cloning and characterization of a tyrosinase with improved activity in organic solvents from *Bacillus megaterium. J Mol Microbiol Biotechnol* **17:** 188-200.

Ueng YF, Chen ChCH, Chen ChF (2000) Inhibition of benzo(a)pyrene hydroxylation by lignans isolated from *Justicia procumbens. J Food Drug Anal* **8:** 309–314.

Wojcieszyńska D, Guzik U, Greń I, Perkosz M, Hupert-Kocurek K (2011) Induction of aromatic ring — cleavage dioxygenases in *Stenotrophomonas maltophilia* strain KB2 in cometabolic systems. *World J Microbiol Biotechnol* **27:** 805–811.

Xun L (1996) Purification and characterization of chlorophenol 4-monooxygenase from *Burkholderia cepacia* AC 1100. *J Bacteriol* **178:** 2645–2649.