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Screening of Bacterial Isolates from Polluted Soils Exhibiting Catalase and Peroxidase Activity and Diversity of their Responses to Oxidative Stress

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Abstract For the survival of individual isolates of gram-negative bacteria *Pseudomonas putida*, *Achromobacter xylosoxidans*, and the gram-positive bacterium *Bacillus megaterium*, in an environment polluted with crude oil products, the production of catalases exhibiting both catalase and dianisidine-peroxidase activity is important. Electrophoretic resolution of cell-free extracts of aerobically grown strains in Luria–Bertani medium during exponential phase revealed distinctive expression of catalatic and peroxidatic activities detected with 3,3′-diaminobenzidine tetrahydrochloride. A considerable diversity in microbial catalase and peroxidase responses to 20 or 40 mM H_2O_2 stress, resulted from hydroperoxidase's variant of original isolates, indicating an environmental selective pressure. However, catalase was important for the adaptation of cultures to high concentration of 60 mM H_2O_2. Appreciable differences in the sensitivity to toxic effect of H_2O_2 (20 or 40 mM) treatment between individual isolates and their adapted variants during growth were observed until the middle of exponential phase, but they were insignificant at the entry to stationary phase. Isolates also exhibited a considerable diversity in catalases responses to phenolic contaminants 1 and 2 mM *o*- or *p*-phenylenediamine. Catalase activity of bacterium *P. putida* was visibly stimulated only by *p*-phenylenediamine and not by its positional isomer *o*-PDA. This study contributes to a better understanding of the role catalases play in bacterial responses to a polluted environment.

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Introduction

Catalases are involved as one of the mechanisms used, to protect cells against the damage caused by reactive oxygen species (ROS) to cellular components, including nucleic acids, lipids, and proteins [13]. They decompose very efficiently the substances containing the peroxidic bond, preferentially, hydrogen peroxide, to oxygen and water, thus forming an essential part of the defense mechanism against oxidative stress [9]. Typical catalases reveal only negligible peroxidatic activity [18]. Bifunctional enzymes combining both catalatic and peroxidatic activities known as catalase-peroxidase are found in prokaryotes and marginally in some fungi and some protists [24]. Lower catalase and higher peroxidase activity with large aromatic l-electron substrates is characteristic for this enzyme.

Reactive oxygen species occur as a consequence of normal metabolism of microorganisms but they can also emerge from external sources. Many pollutants in the soils are subjected to oxidation processes, resulting in the formation of reactive intermediates that react with oxygen producing the various reactive oxygen species. Survival of microorganisms as a response to environmental oxidative stress is accompanied with the accurate regulation of catalase and peroxidase activities, including a possible alteration in the expression of isozymes [12]. Soil bacterium *Comamonas terrigena* N3H of the family *Comamonadaceae* is known for its broad catabolic diversity in the degradation of various xenobiotics. Isolated from soil contaminated with crude oil products, *Comamonas terrigena* N3H, exhibited much higher total catalase activity

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than the same species from unpolluted soil. Electrophoretic resolution of isolate cell-free extract revealed bands of catalase-1 and catalase-2, whereas in the control, only catalase-1 was detected [11]. Isolates of *Comamonas* spp. selected from soil polluted with crude oil products and similarly, isolates of *Aspergillus niger* isolated from mine surroundings contaminated with heavy metals exhibited higher catalases activities and were more tolerant to the exogenic toxic stress than the same species from control area [5, 11].

Obviously, catalases are not only essential for the protection against oxidative stress, but with peroxidation they can also participate in the metabolic changes for example of some phenolic compounds, ubiquitous contaminants in soil and water [3]. *C. terrigena* N3H lacks the key enzyme for degradation of phenols, phenolhydroxylase. Phenol peroxidation, the formation of the secondary product 2,2′-biphenyl, followed fluorimetrically was attributed to the peroxidatic action of a catalases [23]. *Mycobacterium* sp. strain PYR-1 degrades polycyclic aromatic hydrocarbons, environmental pollutants. It was shown, that inducible catalase-peroxidase of katG gene, of this culture is involved in molecular mechanisms of degradation of these pollutants [20].

In this report, bacterial isolates, from soils with long-term pollution by crude oil products and which exhibited distinct catalase and *o*-dianisidine-peroxidase activities were screened and identified. The diversity of catalase responses to exogenic oxidative stress under laboratory conditions, in the exponential phase of growth when cells are able to break down, the pollutants was followed [10]. Enzymes that participate in the protection of cells against oxidative stress damage are also involved in the metabolic oxidation of large aromatic substrates; therefore, our results can contribute to a better understanding of the physiological role of catalases in bacterial responses to polluted environments.

Materials and Methods

Chemicals and Bacterial Cultures

Chemicals, including tryptone, hydrogen peroxide, potassium ferricyanide, ferric chloride and 3, 3′-diaminobenzidine tetra hydrochloride (DAB) were purchased from Sigma Chemical Co. (USA), *o*-dianisidine dihydrochloride from Biomedicals, LLC (Germany) and meat extract-peptone agar from Imuna (Slovakia). Chemicals for polyacrylamide gel electrophoresis were obtained from BioRad (USA). The isolates were selected from soils long-term contaminated with petroleum refinery products. Serial dilutions of the suspension (10% soil in sterile 0.9% NaCl), were spread on nutrient agar plates containing 20-mM H_2O_2 at 28°C. The isolates of *Pseudomonas putida* SL1, *Achromobacter xylosoxidans* SL1, and *Bacillus megaterium* SL1 with distinct production of catalases chosen for the next experiments were selected in three steps. First, 62 colonies exhibiting sufficient catalase and dianisidine-peroxidase activity on nutrient agar plates were obtained. The enzymatic activities of cultivated isolates were determined by spectrophotometry methods. Twelve isolates were chosen following gel electrophoresis and staining of microbial catalases. The final three strains were chosen based their mainly on their distinctive expression of peroxidatic activities. The isolates were identified by means of standard morphologic and biochemical procedures at the laboratories of Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic. The identity of isolates was also confirmed by PCR amplification of the V3 region of the bacterial 16S rRNA gene.

Growth Conditions and Stress Treatments

Bacterial cultures were grown in Luria–Bertani medium (10 g tryptone, 5 g yeast extract and 5 g NaCl) at 37°C. Overnight culture (0.5 ml) in Luria–Bertani medium was transferred to a flask containing 50 ml of the growth medium and shaken at 200 rpm, at 37°C. The growth of all strains was monitored by the absorbance at 610 nm. When growth reached the one-fourth in the beginning (BE), or the middle of exponential phase (ME), or the entry to stationary phase (ES), the cells were exposed to added stress agents for 1 h. Then they were harvested by centrifugation at 5,000*g* for 20 min at 4°C, and washed.

The stress conditions were achieved by exposing cells to one of the two oxidative agents: at concentration of 20 and 40 mM H_2O_2, 1 and 2 mM *o*- or *p*- phenylenediamine (*o*-PDA and *p*-PDA). Viable cell counts were determined by serial dilution of the samples and dropping 100 µl of dilution in quadruplicates on nutrient agar plates.

Stepwise H_2O_2-Adapted Cultures

The nutrient medium was inoculated with bacterial isolate and cultivated at 37°C. When growth was visible, samples were subcultivated (2%, v/v) into identical fresh media containing H_2O_2. After performing a number of subcultivations with gradually increased concentrations of the oxidative stressor, samples (0.1 ml) were plated on nutrient agar and incubated at 37°C for 48 h. Individual colonies were selected for further growth in liquid or solid medium. The final concentration of H_2O_2 in the cultivation medium was 60 mM.

Enzymes Activity Determination

Mechanical disruption of frozen cells (−80°C) was performed in 50 mM sodium phosphate, 2 mM EDTA (pH 8.2) at 4°C with glass beads (diameter 0.4 µm). The homogenates were centrifuged at 15,000*g* for 10 min. Supernatants were used for the determination of enzymatic activities. ~~Catalase was determined at pH 7.0 by monitoring the decomposition of H_2O_2 at 240 nm with an extinction coefficient of 43.6 $M^{-1}cm^{-1}$ [16]. Peroxidase was assayed at pH 5.5 by monitoring the oxidation of *o*-dianisidine at 460 nm with extinction coefficient of 11.3 × 10^3 $M^{-1}cm^{-1}$ [7].~~ Protein concentration was determined by the method of Bradford with bovine serum albumin as a standard [4].

Gel Electrophoresis and Staining

Native polyacrylamide gels with the linear gradient (4–18%) were stained for either catalase or peroxidase activity. Catalase activity was visualized after a brief incubation in 0.003% hydrogen peroxide and followed by a freshly prepared a mixture of 2% potassium ferricyanide and 2% ferric chloride [22]. Peroxidase activity was visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB) using method described by Wayne and Diaz [21].

Results and Discussion

The growth of *P. putida*, *A. xylosoxidans*, and *B. megaterium* isolates under standard cultivation conditions in LB medium reached the stationary phase in 24 h. Aerobically grown strains during the exponential phase exhibited a gradual increase of both catalase and dianisidine-peroxidase activity (Fig. 1). The increase in total catalase activity in aging microbial isolates is not surprising. The growth of cells entering the stationary phase is subjected to the effect of two kinds of stresses, oxidative, and starvation. They may cause an accumulation of endogenous oxidants, resulting in an increase in respiratory activity and the induction of a new catalase [14, 15]. The levels of peroxidase activity in extracts of all isolates were similar, but only *A. xylosoxidans* cells exhibited a relatively high level of catalase activity.

Electrophoretic resolution of cell-free extracts of individual strains grown in the exponential phase revealed differences in expression of catalatic and peroxidatic activities. *P. putida* showed two bands of catalase: CAT1 and CAT2. The CAT1 isozyme with a lower molecular weight also exhibited peroxidatic activity by staining with DAB (Fig. 2a, b, lanes 2–4). *A. xylosoxidans* showed catalase bands corresponding to CAT1, which also showed peroxidatic activity. Induction of a second enzyme with a higher molecular weight, stained only for peroxidatic activity and was observed in the entrance to stationary phase (Fig. 2a, b, lanes 5–7). *B. megaterium* extracts also expressed two bands of catalase activity CAT1 and CAT3 and again, the enzyme with the lower molecular weight exhibited peroxidase activity. During growth, bands corresponding to CAT2, which showed only peroxidatic activity also appeared (Fig. 2a, b, lanes 8–10).

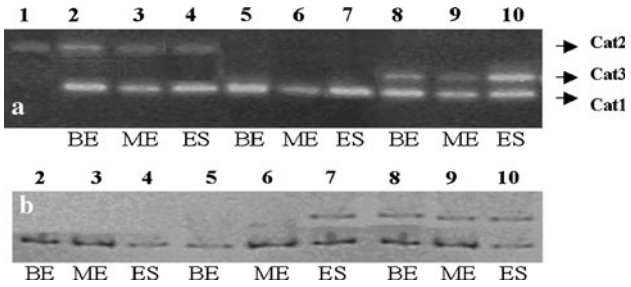

Fig. 2 Catalases **a** and peroxidases **b** in crude extracts of grown isolates indicated by native polyacrylamide gel electrophoresis (4–18%). *Lane 1* a typical catalase from bovine liver (232 kDA); *lanes 2–4 P. putida*; *lanes 5–7 A. xylosoxidans*; *lanes* 8–10 *B. megaterium*. Extracts were prepared from beginning (*BE*) and middle (*ME*) of the exponential phase and entering of the stationary phase (*ES*)

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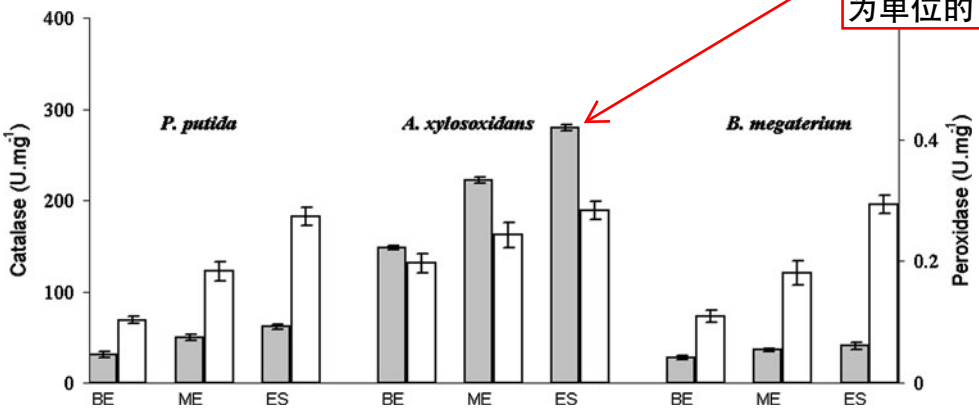

Fig. 1 Catalase (*grey columns*) and peroxidase (*empty columns*) activities in crude extracts of isolates growing in Luria–Bertani medium. Activities in beginning (*BE*) and middle (*ME*) of the exponential phase and entering of the stationary phase (*ES*) of growth. Data are the means ± SD of three replicates

It seems reasonable to suppose that the CAT1 bands (*P. putida, A. xylosoxidans and B. megaterium*) which exhibit both catalase and peroxidase activity represent a catalase-peroxidase. A new band in position CAT2 from *A. xylosoxidans* and similar bands from *B. megaterium* (CAT2) exhibited only peroxidatic activity and probably represent a different type of peroxidase. However, final conclusions on the chemical nature of the bacterial hydroperoxidases must await the isolation, purification and molecular characterization of the respective enzymes [17].

We compared the sensitivity of individual isolates to exogenous hydrogen peroxide stress during growth. During different growth phases, the isolates were stressed by high doses of H_2O_2 for 1 h and their survival was then recorded by counts of viable cells (Table 1). The resistance of cultures to exogenic treatment of H_2O_2 (20 or 40 mM) during growth, including entry to stationary phase, gradually increased. These results are in line with those of Dowds et al. [8]. They reported that cells of *Bacillus subtilis* in stationary phase became totally resistant to 10-mM H_2O_2, in contrast to the early log phase. Two of the eight proteins induced by 50-μM H_2O_2 were also induced in the absence of oxidative stress on entry into the late log phase of growth.

To obtain variants of *P. putida*, *A. xylosoxidans* and *B. megaterium* more resistant to H_2O_2 stress, individual isolates were subcultivated six times with gradually increasing concentrations of agent up to 60 mM. We compared the sensitivity of individual isolates and their H_2O_2-adapted variants to exogenous hydrogen peroxide stress during growth. Appreciable differences in sensitivity to the toxic effect of H_2O_2 (20, 40 mM) treatment were observed until the middle of exponential phase. The survival of adapted cultures of *P. putida* and *A. xylosoxidans* in comparison to original isolates after 40 mM H_2O_2 treatment was higher, approximately 1.7 and 1.5 times, respectively. At the entry to the stationary growth phase the differences in survival of adapted cultures in comparison to original isolate were negligible (Table 1).

The three isolates show a diversity in microbial catalases responses involved to oxidative stress treatments. Analysis of *P. putida* and *B. megaterium* enzymes in the middle growth phase revealed high levels of peroxidatic activity, while the catalase activity was inhibited in *A. xylosoxidans*, on the other hand catalatic activity was stimulated and peroxidatic activity was decreased (Fig. 3). The early stimulation of peroxidatic activity in *P. putida* and *B. megaterium* cells supports the metabolic changes leading to pollutant decay, whereas the increase of catalase activity in the *A. xylosoxidans* isolate is more characteristic of defensive mechanisms against oxidative stress. Pollution also led to a diversity of catalase responses to oxidative stress in isolates of *Comamonas* [2, 11]. The level of catalase activity in crude extracts of *C. terrigena* N3H and

Table 1 Sensitivity of the cultures to exogenous H_2O_2 stress

Culture	Phase of growth	Survival after H_2O_2 (20 mM)	Treatment[a] (40mM)
P. putida isolate	BE	51.2 ± 4.2	40.9 ± 5.2
	ME	61.3 ± 3.1	5.24 ± 3.4
	ES	81.3 ± 2.1	74.3 ± 2.3
P.putida H_2O_2 adapted	BE	62.3 ± 4.0	51.0 ± 4.7
	ME	90.4 ± 2.4	86.3 ± 2.2
	ES	91.4 ± 2.4	87.3 ± 1.7
A. xylosoxidans isolate	BE	61 2 ± 47	48 2 ± 5.1
	ME	81.4 ± 2.0	56.6 ± 2.1
	ES	91.0 ± 2.2	78.0 ± 2.6
A. xylosoxidans H_2O_2 adapted	BE	68.1 ± 5.7	54.3 ± 5.7
	ME	94.3 ± 2.6	90.0 ± 2 7
	ES	924 ± 23	89 4 ± 2 8
B. megaterium isolate	BE	70.4 ± 4.6	52.4 ± 4.7
	ME	81.0 ± 2.6	66.0 ± 2.9
	ES	89.2 ± 2.1	79.2 ± 2.3
B. megaterium H_2O_2 adapted	BE	78.3 ± 3.9	60.4 ± 4.1
	ME	88.3 ± 2.2	74.0 ± 2.1
	ES	89.1 ± 2.1	81.2 ± 2.1

Given are mean values ± SD for three independent measurements

BE beginning of the exponential phase, *ME* middle of the exponential phase, *ES* entering of the stationary phase

[a] Percentage of survival compared to control cultures that were grown without oxidative stress

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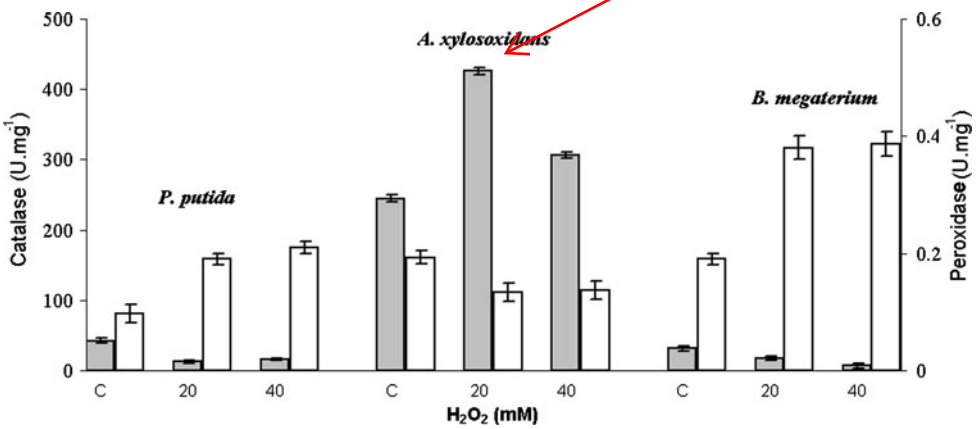

Fig. 3 The effect of H_2O_2 stress on catalase (*grey columns*) and peroxidase (*empty columns*) activities of cultures grown at the middle of exponential phase. Control (*C*)—activities in cultures without the stress effect. Data are the means ± SD of three replicates

Table 2 Relative changes of catalase and peroxidase activity in H_2O_2-adapted cultures in comparison to original isolates

Culture	Phase of growth	Catalase activity (%)[a]	Peroxidase activity (%)[a]
P. pulida H_2O_2 adapted	BE	822 ± 36	9 ± 3
	ME	532 ± 27	25 ± 4
	ES	695 ± 27	51 ± 2
A. xylosoxidans H_2O_2 adapted	BE	220 ± 17	80 ± 7
	ME	225 ± 12	70 ± 6
	ES	180 ± 10	50 ± 6
B. megaterium H_2O_2 adapted	BE	110 ± 11	140 ± 10
	ME	115 ± 7	120 ± 9
	ES	130 ± 5	130 ± 6

Given are mean values ± SD for three independent measurements

BE beginning of the exponential phase, *ME* middle of the exponential phase, *ES* entering of the stationary phase

[a] Percentage compared to values of original isolate

C. terrigena N1C isolated from crude oil-contaminated soil greatly exceeded the corresponding activity from the control in all growth phases. The expression of isozymes of catalase-1 and catalase-2 in these cultures was distinctive as well.

The potential diversity of catalase responses to oxidative stress can be ascertained by sequence analysis of highly conserved catalase sequence motifs. Constitutively expressed catalase-1 represents the major source for defence of *C. terrigena* N3H cells against toxic peroxides. Boháčová et al. found that the specific PCR product for catalase-1 in *C. terrigena* N3H isolate was identical with the corresponding sequence of the *cat-1* gene of *C. terrigena* N1C, which was isolated from polluted soil as well [2]. However, in other strains not exposed to pollution or to ROS, this sequence motif could not be found. In spite of this, the different catalases activity in *C. terrigena* N3H and N1C cells can possibly be attributed to the regulation of isozyme expression.

The adapted variants of *P. putida* and *A. xylosoxidans* cultures exhibited a much higher level of total catalase activity than the original isolates The data suggest that the adapted strains reached higher levels of activity at an earlier point in the growth cycle. (Table 2). This suggests that catalase is important for adaptation of cultures to high concentrations on oxidative stressor. The stimulation of catalase activity is accompanied by a decrease of

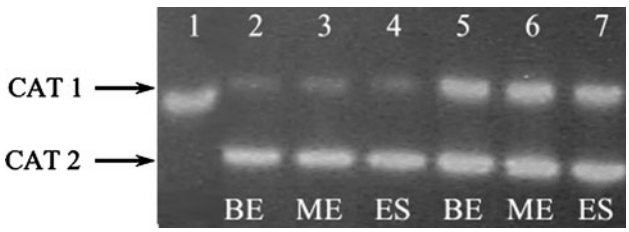

Fig. 4 Catalase in cell-free extracts of *Pseudomonas putida* isolate and its H_2O_2 adapted subculture during the growth phase indicated by PAGE (4–18%). *Lane 1* a typical catalase from bovine liver (232 kDA). Homogenates were prepared from beginning (*BE*), middle (*ME*) of the exponential phase and entering of the stationary phase (*ES*) of isolate (*lanes 2*, *3*, *4*) and its adapted subculture (*lanes 5*, *6*, *7*). The amount of loaded protein on the gel lines was the same

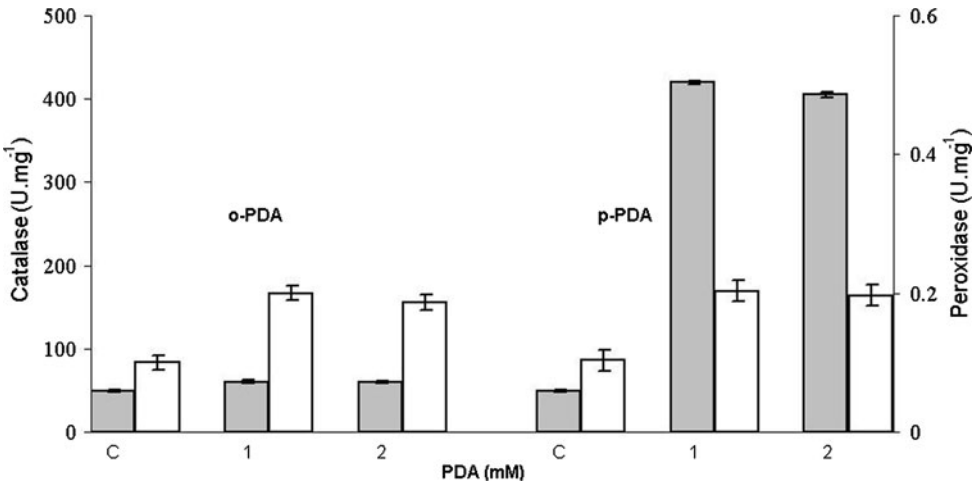

Fig. 5 The effect of *o*- or p-phenylenediamine on catalase (*grey columns*) and peroxidase (*empty columns*) activity of *Pseudomonas putida* isolate grown at the middle of exponential phase. Control (*C*)—activities in cultures without the stress effect. Data are the means ± SD of three replicates

peroxidase activity, most significantly in *P. putida* cells. Electrophoresis showed that on the protective catalase response is represented mainly by the inducible catalase CAT1 (Fig. 4). H_2O_2-adaptation of the *B. megaterium* isolate, which produces two isozymes with peroxidatic activity, led only to a relatively slight increase in catalase and peroxidase activities (Table 2). The data show, that the diversity of the catalases responses to oxidative stress also depends on the concentration of the oxidative stressor. It seems reasonable to suppose, that low concentrations of pollutants will induce the metabolic oxidation, respectively degradation changes of pollutants. Triphathi et al. showed that catalase and ascorbate peroxidase activities increased only at 2.5 μM Cu^{+2} and 25 μM Zn^{+2} when oxidative stress was mild and were inhibited at 10 μM Cu^{2+} under intensive oxidative stress [19].

Phenol compounds are present in waste arising from combustion and from the processing of fossil fuels [19]. The response of microbial catalases exhibited specificity to positional isomers *o*- and *p*-phenylenediamine (1 and 2 mM). While pollutants markedly increased peroxidase activity in all isolates, catalase activity was significantly stimulated only by treatment of positional isomer *p*-PDA in *P. putida* cells but not by *o*-PDA (Fig. 5). This diversity of catalases responses to specific pollutants can affect for example growth of isolate, or the degradability or toxicity of the pollutant [1].

The response of microbial isolates to environmental factors involves complex relations between biotic and abiotic components of the environment [5, 6]. These relations can lead to different responses to different conditions. We have shown that the expression of microbial catalases resulting from a polluted environment ca be involved not only in resistance to reactive oxygen species, but also in the degradation of specific pollutants. Future investigations will focus on determining the role of catalases in the mechanisms by which specific pollutants can be degraded.

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