Scientific Research and Essay Vol. 2 (7), pp. 244-250, July 2007
Available online at http://www.academicjournals.org/SRE
ISSN 1992-2248 © 2007 Academic Journals

Full Length Research Paper

Toxicity of phenolic compounds to oxidoreductases of *Acinetobacter* species isolated from a tropical soil

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Accepted 15 June, 2007

The effects of 2-nitrophenol, 4-nitrophenol, 2,4-dinitrophenol and 4-bromophenol on the activities of periplasmic nitrate reductase and dehydrogenase enzymes of *Acinetobacter* sp. isolated from a tropical soil were assessed via reduction of nitrate and 2,3,5-triphenyltetrazoluim chloride, respectively. There were significant differences in the response of dehydrogenase enzyme but not periplasmic nitrate reductase to the various compounds. However, at different concentrations, the phenolic compounds had significant effects on periplasmic nitrate reductase but not dehydrogenase activity. Estimations of the degree of inhibition/stimulation of both enzyme activities showed a significant dose-dependent response. 2,4-Dinitrophenol showed decreasing progressive stimulation of dehydrogenase enzyme with increasing concentration (40 – 200 mg/l) while 2-nitrophenol, 4-nitrophenol and 4-bromophenol showed increasing progressive inhibition of both enzyme activities. The IC_{50} of the phenolic compounds to the periplasmic nitrate reductase activity were comparable (33.46 – 44.75 mg/l) but lower than those for the dehydrogenase activity (>200 mg/l). This suggested greater sensitivity of periplasmic nitrate reductase than dehydrogenase enzyme to the phenolic compounds. This study suggested that the toxic effects of chemicals on specific microbiological activities are better studied using the appropriate enzymes involved rather than the dehydrogenase activity.

Key words: *Acinetobacter* sp., dehydrogenase, periplasmic nitrate reductase, phenolic compounds.

INTRODUCTION

Oxidoreductases comprise a large class of enzymes that catalyse biological oxidation-reduction reactions. These reactions mediated by microbes, control organic oxidations and element cycling in nature. They are a major method of solubilisation of metals in the environment and are important concepts in pollution, pollution prevention, prediction of fate and transport of pollutants (Manahan, 1994). Nitrate reductases and dehydrogenases are examples of such enzymes (Toseland et al., 2005).

Nitrate reduction is a bacterial physiological function that has important agricultural, environmental and public health implications. Three types of bacterial nitrate reductases which differ at the level of cellular location, structure, biochemical properties, regulation and gene organization have been described (Moreno-Vivian et al., 1999; Potter et al., 2001). These are membrane bound respiratory nitrate reductase (NAR), cytoplasmic assimilatory nitrate reductase (NAS) and periplasmic dissimilatory nitrate reductase (NAP). The NAP, first reported for phototrophic and denitrifying bacteria is widespread among many Gram negative bacteria including *Alcaligenes eutrophus* (Siddiqui et al., 1993), *Paracoccus pantotrophus* (Ellington et al., 2002) and *Wolinella succinogenes* (Simon et al., 2003). NAP systems are normally unaffected by ammonium or oxygen and are expressed during growth on highly reduced substrates (Moreno-Vivian et al., 1999). Different physiological functions have been proposed for the enzyme; however, there are clear evidences that the enzyme is a dissimilatory enzyme used for redox balancing or the dissipation of excess redox energy during oxidative metabolism of reduced carbon substrates (Ellington et al., 2002). Denitrification originally described as an anaerobic respiratory process has been found to occur in aerobic conditions and a NAP seems to be the major enzyme (Bedzyk et al., 1999). This is evidenced by the fact that reduction of nitrate in the periplasm is not sensitive to the oxygen inhibition of nitrate transport across the cytoplasmic membrane that prevents reduction

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by the membrane-bound enzyme (Denis et al., 1990). Consequently, the possession of a periplasmic nitrate reductase has been linked to the ability to respire nitrate in the presence of oxygen in laboratory strains (Carter et al., 1995). Since the loss of nitrate from agricultural soil reduces crop yield as well as affects the fauna and flora on and in the soil, denitrification is not a desirable process from the agricultural point of view (Aber, 1992).

Soil quality changes resulting from environmental perturbations or management practices have been assessed through the use of soil enzymes. One of such enzymes is the dehydrogenase enzyme whose activity is considered an indicator of oxidative metabolism in soils and thus of the microbiological activity (Quilchano and Maranon, 2002). It represents the intracellular flux of electrons to oxygen due to the activity of several intracellular enzymes catalysing the transfer of hydrogen and electrons from one compound to another (Nannipieri et al., 1990).

Phenolic compounds are common constituents of aqueous effluents from the industrial processes such as resin production, oil refining and coking plants (Kibret et al., 2000). They are components of pesticides and herbicides such as Atrazine, Nitrofen, Parathion, Carbofuran and Bifenox. They can thus result from the degradation of herbicides and pesticides by plants and micro-organisms as well as by physical and chemical factors (Bukowska and Kowalska, 2003). Their toxicity to microbes has been reported (Mort and Dean-Ross, 1994; Bukowska and Kowalska, 2003). They are membrane damaging microbiocides and their overall toxic effects is caused by distinct and complex mechanisms such as narcosis, the inhibition of growth and the uncoupling of adenosine triphosphate synthesis (Choi and Gu, 2001). At sufficiently high concentrations, phenols have been shown to be toxic even for species capable of using it as a growth substrate (Santos et al., 2001). The activities of soil micro-organisms have been observed to be inhibited by nitrophenols and some other phenolic compounds (Megharaj et al., 1991, 1992).

This study aim at assessing the impacts of some phenolic compounds on periplasmic nitrate reductase and dehydrogenase enzymes of an aerobic denitrifier isolated from an agricultural soil.

MATERIALS AND METHODS

Isolation of organism

Soil samples were collected randomly from an agricultural farm using a sterile metal cylindrical tool at a depth of 5 cm below the soil surface. Samples were put in a sterile glass container and delivered to the laboratory within 20 min of collection. The samples were homogenised and sieved through a sterile 2 mm mesh. The sieved sample (2.0 g) was dissolved in 18 ml of sterile physiological saline and serially diluted up to 10^{-5} dilutions. One tenth of a millilitre (0.1 ml) of the 10^{-2} to 10^{-5} dilutions was inoculated separately onto nutrient agar and defined agar plates. The defined agar medium was modified from Celen and Kilic (2004), and has the following composition (g/l): succinic acid, 3.54; NaOH, 1.2; NH_4Cl, 0.535; Na_2HPO_4, 18; KH_2PO_4, 1.0; NaCl, 2.5; $MgSO_4$, 0.1; $FeSO_4$, 1.11; fungicide (Ketoconazole), 0.05; agar 17; pH 7.2. The nutrient agar plates were incubated for 24 h while the modified defined medium plates were incubated for 96 h on the laboratory bench at room temperature (28 ± 2°C). Bacterial populations in the soil sample (colony forming units per gram of soil) were determined by enumerating the colonies that formed on both the nutrient agar and defined medium agar plates and the percentage of aerobic denitrifiers calculated there from.

Purification and screening for nitrate reduction

Colonies on the modified defined medium were purified by subculturing onto fresh plates until pure cultures were obtained. Purified cultures were stored on slants of the same medium and kept in the refrigerator (4°C). Purified cultures were screened for the ability to reduce nitrate using the nitrate reduction assay modified from Celen and Kilic (2004). The isolates were grown on defined medium plates for two days at room temperature. Smears of the selected organism were made on a white tile at different points and 0.1 ml of potassium nitrate was dropped on the smears. This was left for 10 min at room temperature. Thereafter, 0.1 ml of 1% sulphanilamide in 5% phosphoric acid was added and left for another 10 min. Finally, 0.1 ml of 0.1% naphthylene ethylene diamine dihydrochloride (NEDD) was added. The occurrence of a reddish pink colour indicated the existence of nitrite, and therefore the aerobic reducetion of nitrate to nitrite by the bacteria. One of the isolates that carried out aerobic nitrate reduction was selected for further study. The morphological and biochemical characterisation of the isolate up to the generic level was performed following the schemes of Holt et al. (1994).

Preparation of inoculum for toxicity assays

The organism was plated on the modified defined medium (Celen and Kilic, 2004) containing 10 mM KNO_3 instead of the 10 mM NH_4Cl and incubated at room temperature for two days. The culture was washed off the surface of the agar with sterile phosphate buffered saline (PBS), pH 7.2, and the cell suspension transferred into a 20 ml screw-capped test tube. The cells were washed twice with the PBS and harvested by centrifugation at 3500 rpm for 10 min. Harvested cells were standardized by resuspending in PBS and adjusting the turbidity to give an optical density of 0.4 at 600 nm. The cell suspension served as the standardized inoculum for the studies. The dry weight of the cell suspension was estimated by drying a 2 ml aliquot of the standardized cell suspension to constant weight in a pre-weighed crucible in an oven at 105°C.

Periplasmic nitrate reductase activity assay

Portions (0.2 ml) of the standardized cell suspensions were inoculated into sterile triplicate 20 ml screw-capped glass tubes containing 4.8 ml of the modified defined medium supplemented with a particular concentration (40 - 200 mg/l) of 2-nitrophenol, 2,4-dinitrophenol, 4-nitrophenol or 4-bromophenol. The control consisted of the inoculated medium without phenolic compounds. The cultures were incubated at room temperature for 4 h on a shaker at 150 rpm. Thereafter the tubes were centrifuged at 3500 rpm for 10 min. Nitrite concentrations in the supernatants were determined according to Promega (1995). One millilitre of the supernatant was mixed

with 1 ml of 1% sulphanilamide in 5% phosphoric acid in a 10 ml screw-capped test tube. This was allowed to stand for 10 min at room temperature. Thereafter, 1 ml of 0.1% naphthylene ethylene diamine dihydrochloride (NEDD) in distilled water was added and the set up allowed standing for another ten minutes at room temperature before the absorbance was read in a spectrophotometer at 540 nm. Nitrite concentrations in the samples were estimated by reference to a standard dose-response curve. Periplasmic nitrate reductase (NAP) activity was expressed relative to the amount of nitrite formed.

Dehydrogenase activity assay

Portions (0.2 ml) of the standardized cell suspensions were inoculated into sterile triplicate 20 ml screw-capped glass tubes containing 4.7 ml of the modified defined medium supplemented with the phenolic compounds at different concentrations (40 – 200 mg/l). One tenth of a millilitre (0.1 ml) of 1.5 % 2,3,5-triphenyltetrazolium chloride (TTC) in the modified defined medium was added and then incubated statically at room temperature for 4 h. The control consisted of the organism, the medium and TTC without phenolic compounds. The triphenyl formazan (TPF) produced was extracted in 3 ml amyl alcohol and the absorbances measured spectrophotometrically at 445 nm. The amount of formazan produced was determined by reference to a standard dose-response curve. Dehydrogenase activity (DHA) was expressed as milligrams of TPF formed per mg dry weight of cell biomass per hour.

Calculation of inhibition or stimulation of enzyme activity

Inhibitions or stimulations of the oxidoreductase activities were calculated relative to the controls. Where applicable, the IC_{50} of the phenolic compounds were determined by fitting the percentage inhibition values to simple equations using Table 2D Curve (Systat Incorporation USA) and calculating the concentrations of phenolic compounds at 50 % inhibition of enzyme activity.

Statistical analysis

Data generated were subjected to multiple factor analyses of variance (ANOVA). Relationships between the effects of phenolic compounds on enzyme activities were analysed using regression analyses and the Pearson's product-moment correlation coefficient.

RESULTS AND DISCUSSION

The microbial population of the soil sample on nutrient agar and the modified defined medium agar plates were 4.4×10^6 cfu/g soil and 2.8×10^6 cfu/g soil, respectively. The percentage of aerobic denitrifiers in the sample was found to be 63.64 %. This indicates that more than half of the bacterial population in the soil expressed periplasmic nitrate reductase (NAP) activity, in line with the observation that this enzyme activity is widespread among microbial populations from different environments (Carter et al., 1995). The organism used for the study was identified as *Acinetobacter* sp. *Acinetobacter* species are Gram negative bacteria that are nutritionally versatile. They have been attracting growing interest in both environmental and biotechnological applications because they are known to be involved in biodegradation of a number of different pollutants and in the production of a number of economically valuable products (Abd-El-Haleem, 2003).

The reduction of nitrate observed in the test system by the isolated organism is attributed to the activity of periplasmic nitrate reductase (NAP) enzyme because of the experimental conditions under which the study was carried out. The activity of cytoplasmic nitrate reductase (NAS) is repressed by ammonium that was present in the isolation medium and membrane-bound nitrate reductase (NAR) cannot reduce nitrate to nitrite under aerobic conditions (Celen and Kilic, 2004; Simon et al., 2003; Potter et al., 2001). The fact that the isolate grew on a medium containing NH_4Cl and could reduce nitrate aerobically indicates that denitrification by the organism was due to its possession of a periplasmic nitrate reductase.

The effects of different concentrations of phenolic compounds on the periplasmic nitrate reductase and dehydrogenase activities of *Acinetobacter* species are shown in Figure 1. The relationships between concentrations of the phenolic compounds and activities of NAP and dehydrogenase enzymes are described by simple equations derived from Figure 1 as shown in Table 1. Apart from 2,4-dinitrophenol which had no good fit for the dehydrogenase activity, high R^2 values ($0.881 \le R^2 \le 0.999$) were obtained in the regression analysis (Table 1). This implies a dose-dependent response of both enzymes to the concentrations of the phenolic compounds and indicated that the concentrations of the respective phenolic compounds strongly determine the activities of both oxidoreductases. The result of the analysis of variance (ANOVA) showed that despite the significant differences ($P < 0.05$) on the DHA of *Acinetobacter* species due to the presence of the phenolic compounds, there were no significant differences ($P > 0.05$) in nitrite production by the various compounds. However, the different concentrations of the phenolic compounds had significant effects on the periplasmic nitrate reductase but not on dehydrogenase activity. The association between the activities of NAP and dehydrogenase enzymes of *Acinetobacter* species using the Pearson's product moment correlation coefficient indicated that both microbial processes showed a positive correlation. This correlation was strong ($0.63 \le r \le 0.90$) for all the other phenolic compounds apart from 2,4-dinitrophenol ($r = 0.04$). This is not surprising since both aerobic denitrification and dehydrogenase activities are membrane associated microbial processes which have been reported to be subject to phenol toxicity (Irya et al., 2003; Choi and Gu, 2001).

Estimations of the degree of inhibition/stimulation of periplasmic nitrate reductase and dehydrogenase activities by the different concentrations of phenols are shown in Figure 2. The dose-dependent responses of both enzymes to the concentrations of the phenolic compounds were significant ($P < 0.05$). Apart from 2,4- dinitrophenol,

Table 1. Relationships between concentrations of the phenolic compounds and activities of NAP and dehydrogenase enzymes.

Phenolic compound	Model	a	b	R^2
Dehydrogenase				
2-Nitrophenol	$y = \frac{1}{a + bx^{0.5}}$	10.9494	0.3458	0.996
4-Nitrophenol	$y = \frac{1}{a + bx^{1.5}}$	10.6979	0.0017	0.881
4-Bromophenol	$y = \frac{1}{a + bx^{3}}$	10.8017	0.0046	0.998
Periplasmic nitrate reductase				
2-Nitrophenol	$y = \frac{1}{a + bx^{2}}$	1.0916	0.0003	0.979
2,4-Dinitrophenol	$y = \frac{1}{a + bx^{2}}$	1.0579	0.0002	0.988
4-Nitrophenol	$y = \frac{1}{a + bx^{2}}$	1.0708	0.0010	0.999
4-Bromophenol	$y = \frac{1}{a + bx^{3}}$	1.0634	0.0073	0.999

y = respective enzyme activity, x = concentration of respective phenolic compound a and b are model parameters.

which showed decreasing progressive stimulation of dehydrogenase activity (28.33 – 6.67%) with increasing concentrations of phenolic compound from 40 to 200 mg/l, all the other phenols progressively inhibited dehydrogenase activity as the concentrations were increased.

Furthermore, all the phenolic compounds progressively inhibited periplasmic nitrate reductase activity as their concentrations were increased from 40 - 200 mg/l. The inhibition of periplasmic nitrate reductase activity means inhibition of denitrification while its stimulation means otherwise. Since denitrification which removes bioavailable nitrogen from soil is not a desirable process in agriculture (Aber, 1992), the presence of these phenolic compounds in agrochemicals applied to soil at concentrations inhibitory to denitrification will help to conserve soil nitrate and enhance agricultural productivity. However if nitrates are in excess of plant requirements, due to their aqueous mobility, they can find their way into rivers and streams where they cause eutrophication. Excess nitrates in the soil can also be converted to its acid form, thus reducing soil quality and lead to stunted growth of plants.

Stimulation of dehydrogenase activity by 2,4-dinitrophenol could indicate the use of the phenol as a growth substrate by the *Acinetobacter* strain. Several *Acinetobacter* isolates have been reported to degrade phenol and other substituted aromatics (Abd El-Haleem, 2003; Abd El-Haleem et al., 2003). This progressively decreasing stimulation of dehydrogenase activity with increasing 2, 4-dinitrophenol concentration is in line with the well documented inhibitory nature of phenols at high concentrations for organisms which can use phenols as growth substrates (Acuña-Argüelles et al., 2003; Ruiz-Ordaz et al., 1998). Variations in response of microbial processes to exposure to phenolic compounds have been reported. Stotzky and Norman (1961) observed that nitrophenols repressed soil respiration and microbial activity, while Steward et al. (1996) reported that there was no significant impact of bromophenols on the microbial biomass and their activities.

The inhibition of periplasmic nitrate reductase and dehydrogenase activities is attributable to the toxicity of the phenolic compounds to both enzymes. Dehydrogenase enzymes are membrane associated and phenols have been reported to affect membranes (Irya et al., 2003). This inhibition of denitrification is in line with the observations of Aber (1992), who reported that the intro-

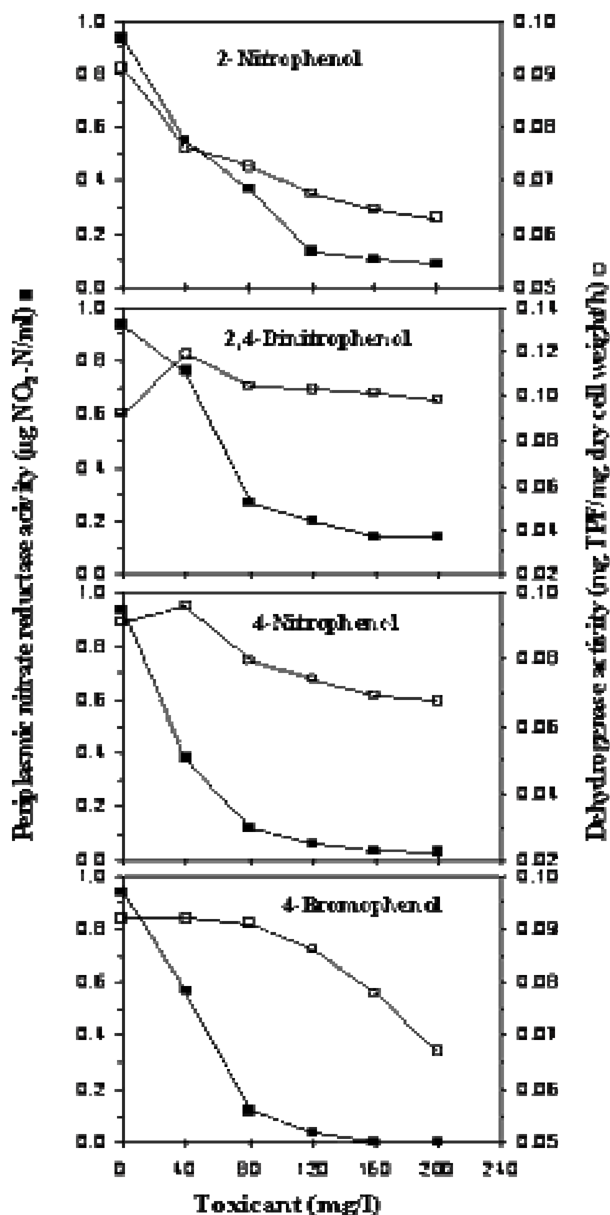

Figure 1. Effects of phenolic compounds on periplasmic nitrate reductase and dehydrogenase activities of *Acinetobacter* species.

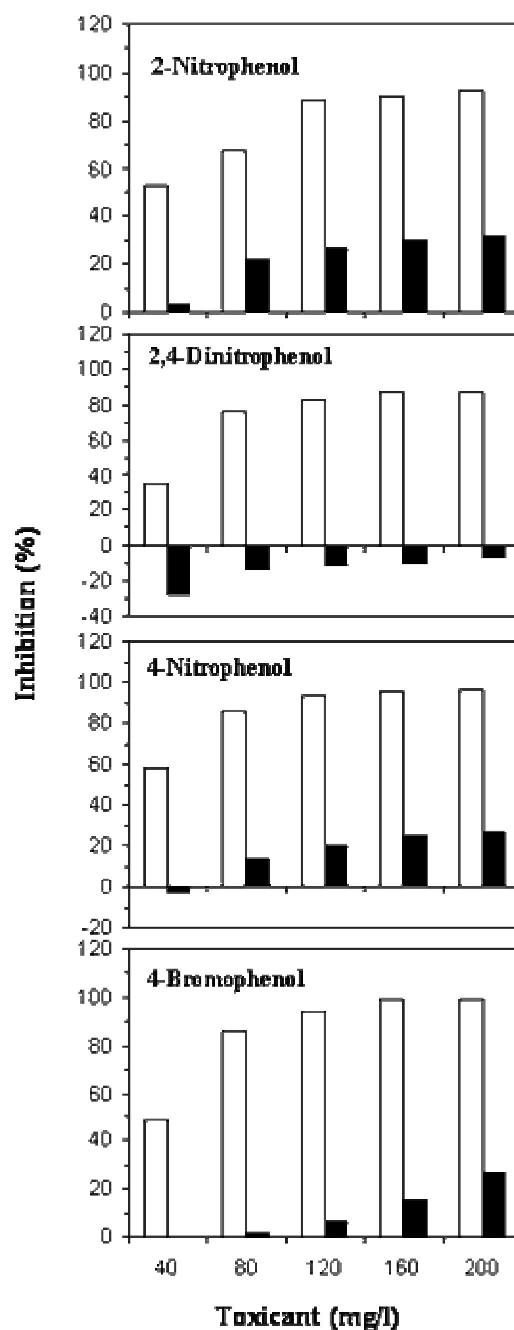

Figure 2. Relative effects of phenols on periplasmic nitrate reductase (□) and dehydrogenase (■) activities of *Acinetobacter* species (> 0% = Inhibition; < 0% = Stimulation).

duction of organopesticides containing phenolic compounds into agricultural soil, depressed the process of denitrification.

The median inhibitory concentrations (IC_{50}) values of the phenolic compounds for the periplasmic nitrate reductase (Table 2) were calculated from the percentage inhibition values versus toxicant concentration plots (not shown) by fitting data into simple equations (equations 1 - 3), where *y* is the % inhibition, *x* is the concentration of the phenolic compound, *a* and *b* are model parameters. Due to the high correlation coefficients obtained from the plots (Table 2), the toxicity of the various phenols to periplasmic nitrate reductase could be predicted from the simple equations outlined below. Similar simple equations have been used to predict the toxicity of amphiprotic compou-

Table 2. Median inhibitory concentrations (IC_{50}) of phenolic compounds

Phenoli compounds	IC_{50} (mg/l)[a]	
	Nitrate reductase activity	Dehydrogenase activity)
2-Nitrophenol	37.60 (0.959)	> 200
2,4-Dinitrophenol	45.62 (0.999)	>200[b]
4-Nitrophenol	35.49 (0.999)	>200
4-Bromophenol	40.35 (0.999)	>200

[a]Figures in parenthesis are correlation coefficients (R^2);
[b]2,4-Dinitrophenol was stimulatory to dehydrogenase activity all concentrations studied

nds to the inhibition of the dehydrogenase activity of *Pseudomonas putida* (Gul and Ozturk, 1998). Such predictions are useful in controlling the use of chemicals to prevent the release of harmful levels of potentially toxic ones into the environment. The median inhibitory concentrations of the various compounds for the periplasmic nitrate reductase enzyme were comparable (35.49 – 45.62 mg/l), while the values were all greater than 200 mg/l for dehydrogenase enzyme activity. The order of toxicity for periplasmic nitrate reductase activity is 2-nitrophenol>4-nitrophenol>2,4-dinitrophenol>4-bromophenol. The lower IC_{50} values obtained for periplasmic nitrate reductase compared with dehydrogenase activity indicates that periplasmic nitrate reductase is more sensitive to the toxicity of the various phenolic compounds. This higher sensitivity of periplasmic nitrate reductase may not be unrelated to the fact that the enzyme activity takes place in the periplasmic membrane while the dehydrogenase enzyme, though membrane-associated, is active intracellularly. Phenols and phenolic compounds are membrane damaging microbiocides (Choi and Gu, 2001). Determination of periplasmic nitrate reductase activity was estimated by the measurement of the activity of a specific enzyme acting on a particular substrate (nitrate) while the determination of dehydrogenase activity was a measure of the activities of a battery of enzymes that transfer hydrogen and electron from substrate to appropriate acceptor (Rossel et al., 1997).

$$2-\text{nitrophenol};\ y^{-1} = a + bx \qquad (1)$$

$$2,4-\text{dinitrophenol};\ y^{2} = a + \frac{b}{x^{2}} \qquad (2)$$

$$4-\text{nitrophenol};\ 4\text{-bromophenol};\ \ln y = a + \frac{b}{x^{2}} \qquad (3)$$

Since dehydrogenase measures total microbial activity, a comparison of the IC_{50} values for cell inhibition studies showed that *Acinetobacter* sp. with a 4 h IC_{50} of >200 mg/l was more tolerant to 2-nitrophenol and 4-nitrophenol than *P. putida* reported to have a 16 h IC_{50} of 0.9 and 4.0 mg/l (Bringmann and Kuehn, 1977) and the algae *Chlorella vulgaris* with a 16 h IC_{50} of 1.53 and 6.97 mg/l (Kramner et al., 1986) for the respective phenolic compounds.

Findings from this study shows that much as the dependence on the dehydrogenase activity assay is a measure of the microbiological activity, toxicity effects of chemicals on specific oxidative microbial metabolism such as aerobic denitrification are better studied using the specific enzyme involved.

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