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Influence of exposure time on phenol toxicity to refinery wastewater bacteria

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Influence of exposure time on the response of pure cultures of bacteria and microbial community of petroleum refinery wastewater to toxicity of phenol was assessed through TTC-dehydrogenase activity (DHA) inhibition test. At sufficient concentrations, exposure of these bacterial cells to phenol resulted in inhibition of dehydrogenase activity. In *Pseudomonas* sp. RWW2 and *Escherichia* sp. DISK2, phenol progressively inhibited dehydrogenase activity at 200 - 1400 mg/l at all the exposure time. However, in *Bacillus* sp. DISK1, *Pseudomonas* sp. DAF1 and microbial community, increase in exposure time resulted in stimulation of dehydrogenase activity at lower concentrations of phenol. The toxicity threshold concentrations of phenol vary among the bacterial strains and the exposure time and indicate that bacteria could acclimate to phenol with increase in exposure time. At concentrations higher than 800 mg/l, phenol toxicity was not overcome in *Pseudomonas* and *Escherichia* species as well as the microbial community. It is suggested that for acute TTC-dehydrogenase assay involving bacteria, reliable and reproducible result would be best achieved within 48 h.

Key words: Dehydrogenase activity, petroleum refinery wastewater, dose-response models.

INTRODUCTION

Phenol is a common constituent of wastewater such as those produced from petroleum refineries, coal gasification plants, phenolic resin industries, plastics manufacture, pharmaceutical industries, etc. Wide ranges of microorganisms have been reported to degrade phenol mainly at low concentration. However, biodegradation of phenol is often inhibited by toxicity exerted at high concentration (Oboirien et al., 2005; Okpokwasili and Nweke, 2006; Khleifat, 2006; Agarry and Solomon, 2008; Agarry et al., 2008). Phenol is a membrane-damaging microbiocide causing changes in the lipid-to-lipid and lipid-to-protein ratios in the membrane, membrane permeability and activity of membrane-associated proteins (Keweloh et al., 1990; Heipieper et al., 1991, 1992). The loss of cytoplasmic membrane integrity results in disruption of energy transduction, disturbance of membrane barrier function, inhibition of membrane protein function and subsequent cell death.

Due to its toxicity to microorganisms, phenol may often cause the breakdown of wastewater treatment systems by inhibition of microbial growth (Ren and Frymier, 2003). Thus, sensitive bacterial assays for toxicity assessments are important. Parameters used to evaluate toxic effects of chemicals on bacterial populations include inhibitions of growth, respiratory activities, bioluminescence, activities and biosynthesis of specific and non-specific enzymes. The estimation of respiratory activities has been used primarily to assess toxicity of chemicals to bacteria (Dutka et al., 1983; King, 1984; King and Painter, 1986; King and Dutka, 1986; Elnabarawy et al., 1988; Strotmann et al., 1993; Okolo et al., 2007). The oxygen uptake rates and reduction of redox indicators are followed polarographically and spectrophotometrically respectively.

Redox indicators are used as artificial electron acceptor in dehydrogenase assay to determine intracellular flux of electrons from electron donors to acceptors in the presence of toxicants. The most widely used indicator is 2, 3, 5-triphenyltetrazolium chloride (TTC) which is reduced to red coloured 2, 3, 5-triphenyl formazan (TPF) by microbial dehydrogenases, a battery of enzymes that catalyze the movement of electron from substrate to

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electron acceptors. The general reaction is:

$$TTC \xrightarrow{+2e^- +2H^+} TPF$$

Dehydrogenase activity measurement is based on quantification of TPF produced from the reduction of TTC. Reduction of TTC has been used as a measure of microbial growth or viability in the presence or absence of toxicant (Tengerdy et al., 1967; Botsford et al., 1997; Eloff, 1998; Gabrielson et al., 2002). Dehydrogenase measurement is usually a rapid assay in which results can be obtained within few hours. It could also be used for acute toxicity assays that last up to 96 h. However, time is an important factor, as the production of TPF is affected by longer incubation time. Therefore, for a better result, it is important to determine the optimum incubation time during assessment of acute toxicity of chemicals to microorganisms. This would have bearing on the evaluation of responses of bacteria to toxic chemicals. In this study, we investigated the influence of exposure time on the reduction of TTC, to assess phenol toxicity and acclimation potential of pure cultures of bacteria and microbial community derived from a petroleum refinery wastewater.

MATERIALS AND METHODS

Wastewater samples, microbial community and bacterial strains

Microbial community and pure cultures of bacteria from Port Harcourt crude oil refinery wastewater were used in this study. The untreated wastewater samples include the process wastewater derived from the refining process (PWW) and the raw wastewater (RWW) which is a combination of PWW and sewage that is channeled to the dissolved air floatation unit (DAF) for physical removal of oil droplet and then to the rotary biodisk (DISK) for biological treatment. The treated wastewater samples include treated wastewater, which is refinery effluent that has undergone both chemical and biological treatment to eliminate or reduce contents, and the observation pond wastewater (OPWW). The phenolic contents of the raw wastewater, DAF unit, rotary biodisk and treated wastewater were 71.2, 71.2, 13.6 and 9.43 mg/l respectively. Water samples were collected in sterile bottles, stored in a cooler and taken to the laboratory for microbiological analyses. The samples were analyzed within 6 h of collection to avoid deterioration of sample. The phenol-degrading bacteria were isolated on mineral salts agar supplemented with phenol as the only source of carbon and energy. The mineral salt medium contains (mg per litre of deionized water): Phenol, 235; KH_2PO_4, 420; K_2HPO_4, 375; $(NH_4)_2SO_4$, 244; NaCl, 30; $CaCl_2$, 30; $MgSO_4$, 30; $FeCl_2$, 3 (Hill and Robinson, 1975). The phenol-degrading bacteria growing on the mineral salts-phenol agar were purified on nutrient agar (Lab M) and stored in nutrient agar slants at 4°C. The isolates were characterized biochemically using standard microbiological methods. Identification to generic level followed the scheme of Holt et al. (1994). The phenol-degrading bacterial strains, *Pseudomonas* sp. DAF1 and *Pseudomonas* sp. RWW2 were isolated from the dissolved air floatation unit and the raw wastewater respectively. *Bacillus* sp. DISK1 and *Escherichia* sp. DISK2 were isolated from the rotary biodisk wastewater. The bacterial strains represent the preponderant morphotypes in their respective sources. The microbial community was that of the rotary biodisk wastewater that was stored at room temperature until the phenol content was completely degraded.

Preparation of inocula for toxicity assay

The bacterial strains were grown to mid exponential phase in nutrient broth (HIMEDIA) on a rotary shaker incubator (150 rpm) at room temperature (28 ± 2°C). The cells were harvested by centrifugation at 4000 rpm for 10 minutes. Harvested cells were washed twice in sterile deionized distilled water and resuspended in the same water. The cell suspensions were standardized in a spectrophotometer to an optical density of 0.6 at 420 nm. The dry weights of the standardized cells were determined by drying 10 ml portion of cell suspension to constant weight in an oven at 110°C. The standardized cell suspensions were used as inocula for dehydrogenase assay.

Dehydrogenase activity assay

Dehydrogenase activity assay was done using TTC as the artificial electron acceptor, which is reduced to red coloured triphenyl formazan (TPF). The assays were done in 3 ml volumes of nutrient broth-glucose-TTC medium supplemented with varying concentrations of phenol in 20 ml screw-capped test tubes. Portions (0.3 ml) of the bacterial cell suspension or wastewater (for microbial community) were inoculated into triplicate test tubes containing 2.5 ml of z-buffered nutrient broth-glucose medium amended with graded concentrations (100 – 1400 mg/l) of phenol. Thereafter, 0.2 ml of 0.4 % (w/v) TTC in deionized distilled water was added to each tube. The final concentrations of nutrient broth, glucose in the medium were 2 mg/ml each. The controls consisted of the isolates and media without phenol. Four sets of triplicate test tubes were prepared for each bacterium and the microbial community. The reaction mixtures were incubated statically at room temperature for 24, 48, 72 and 96 h to determine the effect of exposure time on dehydrogenase activity. The TPF produced after each exposure period were extracted in 4 ml of amyl alcohol and determined spectrophotometrically at 500 nm. The amount of formazan produced was determined from a standard dose-response curve (R^2 = 0.9999).

Data analysis

Data were expressed as the mean and standard deviations. Dehydrogenase activity was expressed as micrograms of TPF formed per mg dry cell weight of cell biomass per hour. Dehy-drogenase activity and phenol inhibition of dehydrogenase activity were calculated relative to the control as shown in equation 1. To estimate the toxicity thresholds, IC_{20}, IC_{50} and IC_{80} which are the concentrations of phenol that inhibited dehydrogenase activity by 20, 50 and 80% respectively, the data generated from equation 1 were fitted into logistic (equation 2) and sigmoid (equation 3) dose-response models. For the responses with stimulation of dehydrogenase activity, data were fitted into asymmetric logistic dose-response model (equation 4). The kinetic parameters were estimated by iterative minimization of least squares using Levenberg-Marquardt algorithm of Table Curve 2D. All regressions were done using the mean data and standard deviations. The toxicity thresholds for each bacterium and microbial community at the different exposure time were compared pairwise using student's t-test with the levels of significance set at $P < 0.05$.

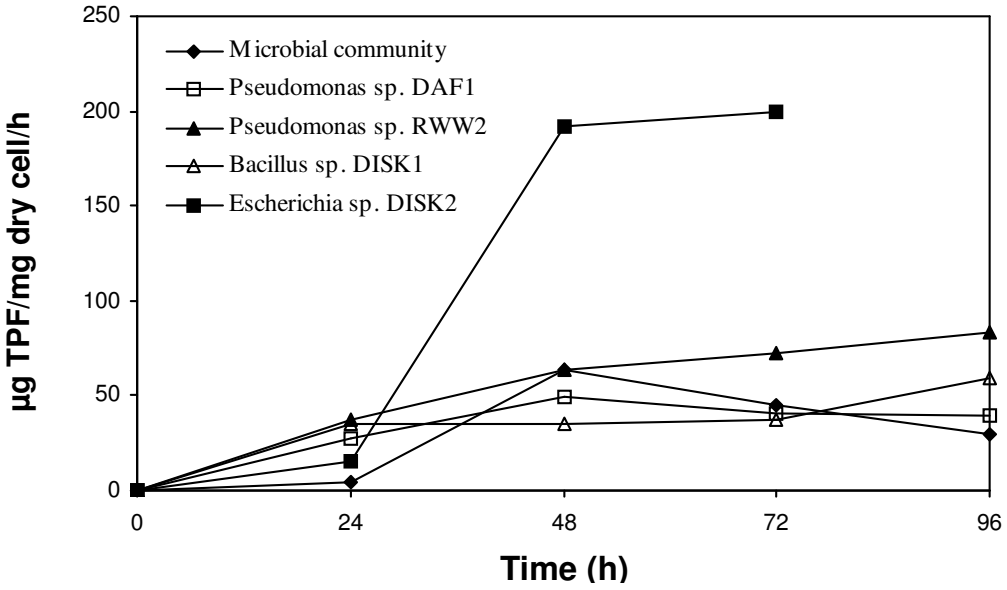

Figure 1. Effect of exposure time on formazan production in pure cultures of bacteria and microbial community of petroleum refinery wastewater in the absence of phenol.

$$\text{DHA (\% of control)} = \frac{T_A}{C_A} \times 100 \quad (1)$$

$$\text{DHA (\% of control)} = \frac{a}{1+\left(\frac{x}{b}\right)^c} \quad (2)$$

Where C_A is the absorbance of TPF produced in uninhibited control (without phenol), T_A is the absorbance of TPF produced in inhibited test (with different concentrations of phenol), x is the concentration of phenol, a is the uninhibited value of enzyme activity (100 %), b is IC_{50} and c is dimensionless toxicity parameter

$$\text{DHA (\% of control)} = \frac{a}{1+\exp(b(x-c))} \quad (3)$$

Where b is slope parameter indicating the inhibition rate and c is IC_{50}

$$\text{DHA (\% of control)} = an(1+n)^{-d-1} d^{-d} (d+1)^{d+1}$$
$$n = \exp(-(x + c * \ln(d) - b)/c) \quad (4)$$

Where a, b, c and d are model parameters

RESULTS AND DISCUSSION

The effects of exposure time on the production of triphenyl formazan in pure cultures of phenol-degrading bacteria and microbial community in the absence of phenol is shown in Figure 1. In *Pseudomonas* sp. RWW2, production of triphenyl formazan increased steadily up to 96 h of incubation. In *Pseudomonas* sp. DAF1 and microbial community, formazan production increased up to 48 h and thereafter decreased with further incubation up to 96 h. Triphenyl formazan production in *Escherichia* sp. DISK2 increased sharply at 48 h of incubation and increased less sharply at 72 h. In *Bacillus* sp. DISK1, the production of formazan seem not to be affected as incubation time increased from 24 to 72 h. However, there was sharp increase in formazan production at 0 – 600 mg/l phenol by 96th h of incubation. Interestingly, there was stimulation of dehydrogenase activity in *Bacillus* sp. DISK1 at 24, 48 and 72 h of incubation by phenol concentrations up to 900 mg/l.

The effects of phenol on the production of triphenyl formazan with time and in relation to the controls are shown in Figures 2 - 4. Generally, phenol inhibited the production of triphenyl formazan and thus dehydrogenase activity. The dehydrogenase activity in the microbial community was completely inhibited by 1000 mg/l phenol at 24 h of exposure. However, as the exposure time increased, the microbial community exhibited little tolerance as indicated by relative increase in dehydrogenase activity at 48 h when 1400 mg/l of phenol almost completely inhibited dehydrogenase activity. At 1400 mg/l phenol, the dehydrogenase activity of the microbial community were 4.52 ± 0.14 and 3.73 ± 0.41 µg TPF/ mg cell dry wt./h at 72 and 96 h of incubation respectively. Similar trend of response was observed in pure cultures of bacteria. High concentrations of phenol had serious effect on the dehydrogenase activity of the bacteria. At 1400, 1200 and 2000 mg/l, inhibition of dehydrogenase activity in *Pseudomonas* sp. RWW2, *Escherichia* sp. DISK2 and *Bacillus* sp. DISK1 respectively was not overcome by

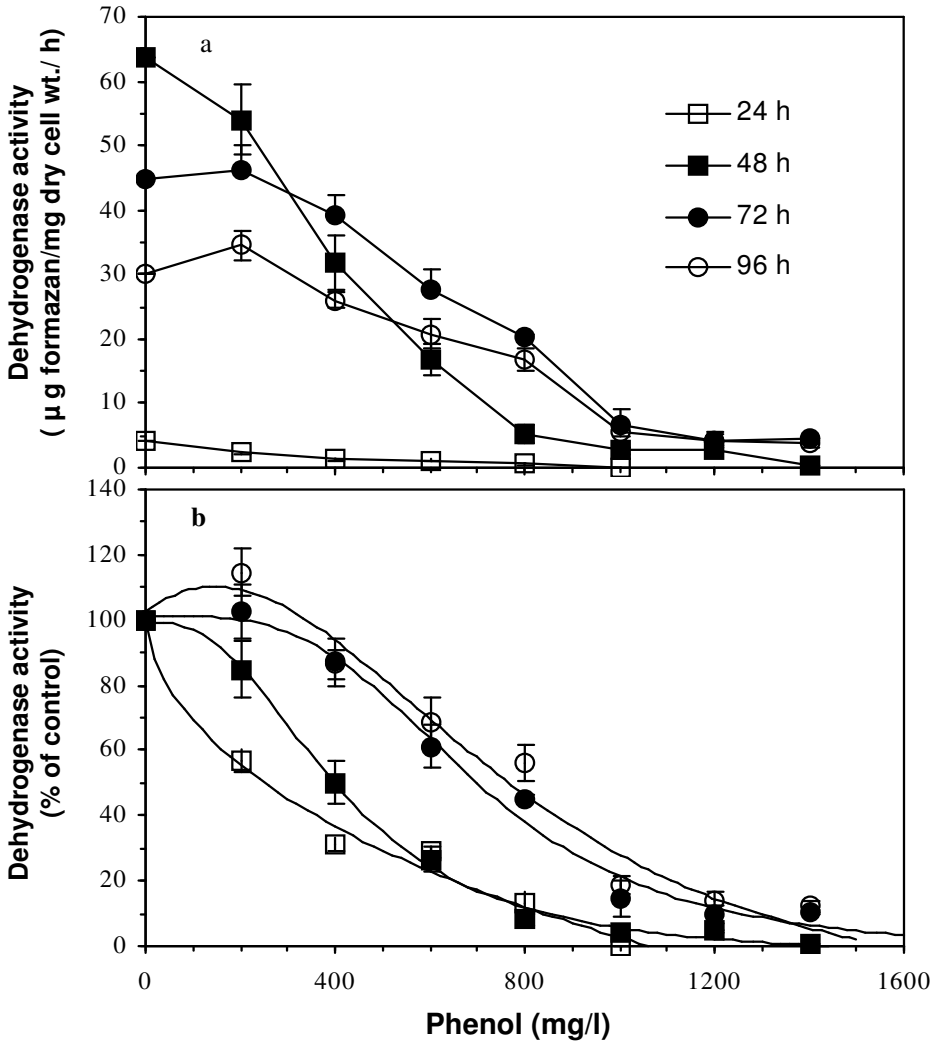

Figure 2. Effects of phenol on the dehydrogenase activity in microbial community of petroleum refinery wastewater at different exposure time. Data represents absolute values (a) and relative values (b).

increase in incubation time.

The production of triphenyl formazan in the bacteria is a measure of respiration and growth. The bacteria grew up to 96 h of incubation. However, the growth of the organisms was vigorous within 24 and 48 h of incubation. Generally, phenol inhibited growth of the organisms as was indicated by reduced formazan production. However, some bacteria have adaptive capability to the toxicity of phenol. *Pseudomonas* sp. DAF1 tolerated phenol at concentrations ranging from 200 - 800 mg/l with increase in the incubation time. The increase in formazan production at these concentrations at 72 and 96 h of incubation indicated that the bacteria overcame the toxicity of phenol. This is attributed to acclimation of the organisms to phenol. Acclimation of *Pseudomonas* species to phenol has been reported and acclimated cells used in biodegradation of phenol at high concentrations (Mamma et al., 2004; Kumar et al., 2005). Similar acclimation response was observed in the microbial community at 200 mg/l phenol. Although there was increase in triphenyl formazan (TPF) production in *Pseudomonas* sp. RWW2 and *Escherichia* sp. DISK2 as incubation period increases, the relative dehydrogenase activity decreased. Also, dehydrogenase activity in *Bacillus* sp. DISK1 decreased relative to control at 96 h of incubation. This observation is as a result of increase in formazan production in the absence of phenol, without proportionate increase in formazan production in the presence of phenol.

The dose - dependent responses of dehydrogenase activity to concentrations of phenol were significant ($P < 0.05$). The progressive decrease in dehydrogenase activity with increasing phenol concentrations is in line with the well - documented inhibitory nature of phenol at high concentration even for organisms that can use phenols as growth substrates (Santos et al., 2001; Goudar et

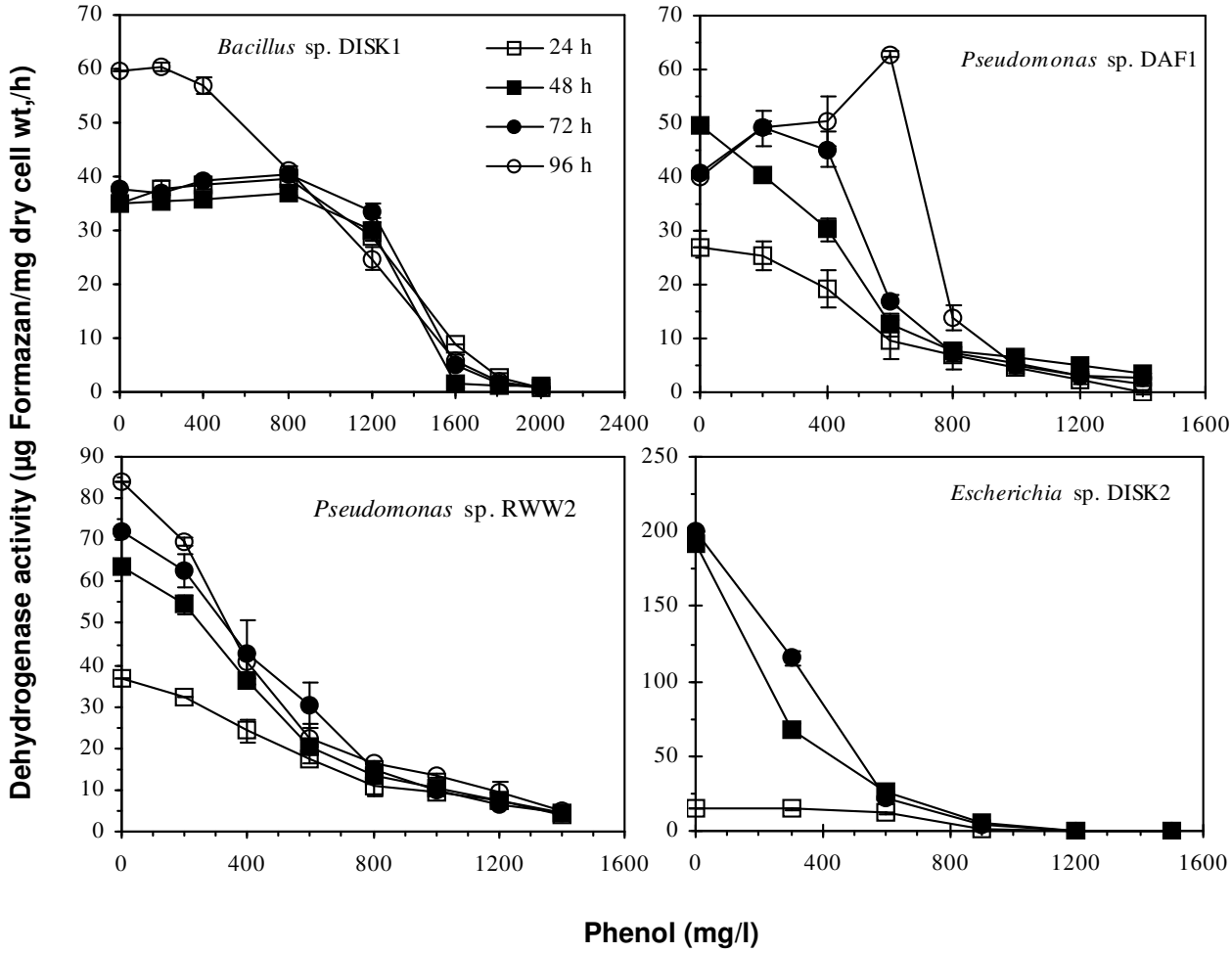

Figure 3. Effects of phenol on formazan production in pure cultures of bacteria at different exposure time.

al., 2000; Okpokwasili and Nweke, 2006; Saravanan et al., 2008; Agarry et al., 2008; Agarry and Solomon, 2008). Dehydrogenase enzymes are membrane-associated and phenols have been reported to affect membrane (Heipieper et al., 1991, 1992; Keweloh et al., 1990). Dehydrogenase activity is mediated by dehydrogenases, a wide group of enzymes, which are present in all living cells and are essential in catalyzing the biological oxidation of organic compounds. They catalyze the transfer of hydrogen and electron through a chain of electron acceptors to oxygen as terminal electron acceptor to form water (Rogers and Li, 1985; Chander and Brookes, 1991). The artificial electron acceptor, 2, 3, 5-triphenyltetrazolium chloride (TTC) has been widely used as direct measurement of microbial growth (Tengerdy et al., 1967; Ghaly and Ben-Hassan, 1993). Abbondanzi et al. (2003) has also suggested good correlation of TTC – dehydrogenase activity with microbial growth. Thus, dehydrogenase activity can be a measure of microbial growth and respiration. Dehydrogenase activity in the presence of phenol is a measure of effect of phenol on the growth and respiration of pure cultures of bacteria and microbial community.

The toxicity threshold concentrations of phenol against the bacterial strains with respect to incubation time are shown in Table 1. The organisms show variable response pattern to elongation of incubation time. In *Pseudomonas* sp. DAF1, the toxicity thresholds decreased at 48th h of incubation and increased thereafter at 72 and 96th h of incubation. In *Pseudomonas* sp. RWW2, toxicity thresholds decreased as incubation time is extended to 48 h, increased at 72 h and came down with further incubation at 96th h of incubation. In *Bacillus* sp. DISK1, the toxicity thresholds increased steadily up to 72 h of incubation and decreased thereafter below 24 h values at 96 h of incubation. For *Escherichia* sp. DISK2, the toxicity threshold concentrations of phenol decreased as incubation time is increased from 24 to 48 h. It increased with further increase in incubation time at 72 h albeit below the 24 h value. The microbial community had interestingly different response pattern to toxicity of phenol. In the microbial community, toxicity thresholds increased steadily from 24 - 96 h of incubation. The significant difference (at $P < 0.05$) between the threshold concentrations of phenol

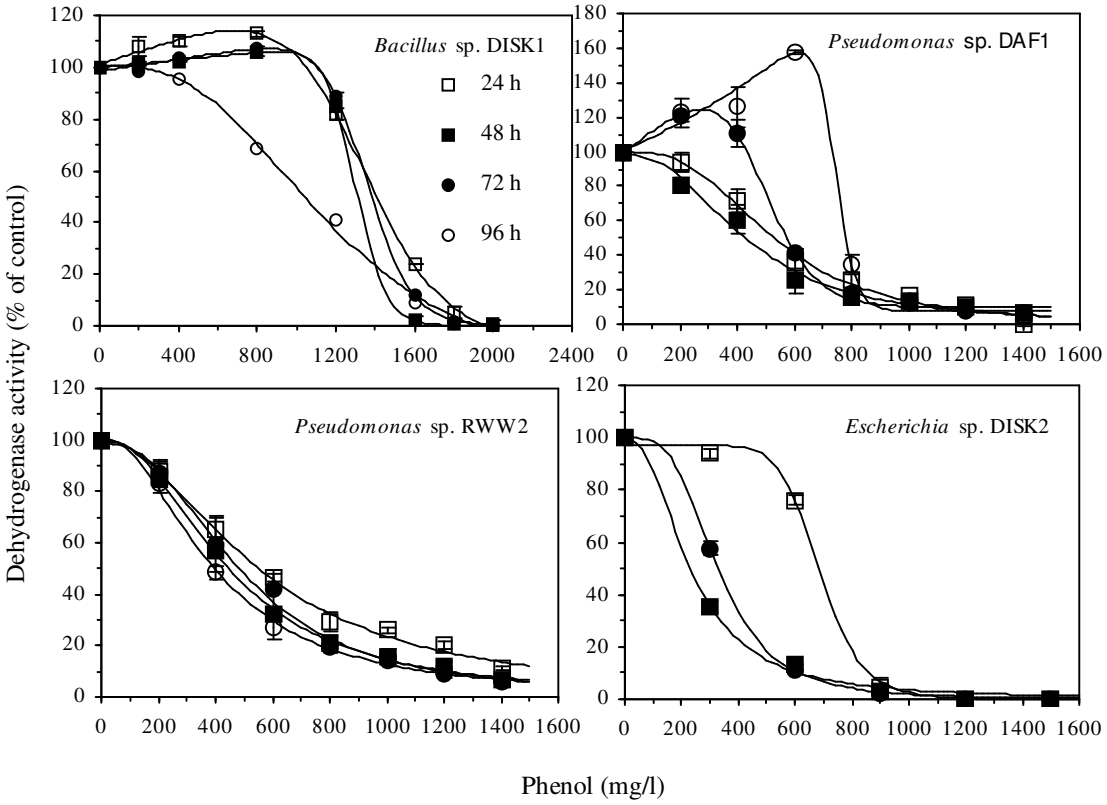

Figure 4. Relative effects of phenol on dehydrogenase activity in pure cultures of bacteria at different exposure time.

among each bacterial strain and microbial community at different incubation times was determined using student's t-test. The results are shown in Table 1. In most cases, the toxicity thresholds vary significantly within each bacterial pure culture and microbial community. Within 24 hours of incubation, the median inhibitory concentrations of phenol (IC_{50}) ranged from 251.390 ± 29.324 mg/l in the microbial community to 1400.203 ± 15.468 mg/l in *Bacillus* sp. DISK1. A 75min IC_{50} of 6309.57mg/l has been reported for phenol via dehydrogenase activity (Gül and Öztürk, 1998). Median inhibitory concentration of phenol against dehydrogenase activity of *Pseudomonas fluorescens* strain ATCC 13525 was reported to be 210 mg/l (Abbondanzi et al., 2003). Cenci et al. (1987) reported an IC_{50} of 6.76 mM (≈ 636 mg/l) for phenol against dehydrogenase activity of *Escherichia coli*. In a similar assay, a 5-day IC_{50} of 487 mg/l phenol have been reported for mixed bacterial culture based on oxygen consumption during biodegradation of peptone (Tišler and Zagorc-Končan, 1995). These reported toxicity thresholds are in most cases comparable with the IC_{50} reported for the pure cultures of bacteria and microbial community in this study. However, *Bacillus* sp. DISK1 with IC_{50} ranging from 1282.510 to 1415.671 mg/l phenol appears to have high tolerance to phenol.

The incubation time seem not to affect the response of *Pseudomonas* sp. RWW2 much like other bacteria. Also IC_5 of *Bacillus* sp. DISK2 did not vary significantly at 24, 48 and 72 h of incubation. This is attributed to insignificant changes in the response of the bacterium to phenol toxicity at low concentrations within these periods of incubation. In the microbial community, the toxicity thresholds at 72 and 96 not vary significantly. Although there was acclimation of the organisms to phenol, it is important to note that for *Pseudomonas* and *Escherichia* species, concentrations higher than 800 mg/l were still inhibitory to dehydrogenase activity of the organisms even with increase in exposure time. Similar inhibitory effect at higher phenol concentrations was observed in the microbial community. Similarly, *Bacillus* sp. DISK1 could not overcome the toxicity of phenol at concentrations higher than 1600 mg/l. These concentrations indicate the tolerance limit of the organisms to phenol and it has bearing upon phenol loading rate design in phenol wastewater treatment system.

The progressive increase in the toxicity thresholds and triphenyl formazan production with microbial community indicates that microbial community adapted better to toxicity of phenol with time. Better degradation of phenol by mixed microbial cultures have been reported (Oboiren et al., 2005). Inhibitory problems associated with pure cultures are solved by the synergy and and co-meta bolism in mixed culture (Singleton, 1994). Similar acclimation to phenol was observed with *Pseudomonas* sp.

Table 1. Effects of exposure time on toxicity thresholds of phenol against pure cultures of bacteria and microbial community.

Culture exposure/time(h)	Toxicity thresholds (mg/l)				
	IC_5	IC_{10}	IC_{20}	IC_{50}	IC_{80}
***Pseudomonas* sp. DAF1**					
24	196.266 ±41.303 b,c,d	250.957 ±44.479 b,c,d	329.491 ± 48.950 c,d	527.881 ± 56.462 d	848.299 ± 58.103 c
48	76.429 ± 28.733 a,c,d	146.632 ± 37.781 a,c,d	242.928 ± 45.501 c,d	442.786 ± 54.284 c,d	704.121 ± 72.456 **
72	447.377 ±20.795 a,b,d	461.778 ± 19.269 a,b,d	489.138 ± 16.983 a,b,d	574.106± 13.222 b,d	726.113± 17.644 a,d
96	762.253 ±25.755 a,b,c	765.057 ± 23.517 a,b,c	770.683 ± 19.013	789.595 ± 3.813 a,b,c	824.540 ± 26.478 c
***Pseudomonas* sp. RWW 2**					
24	122.301 ± 29.899 **	179.471 ± 27.233 **	271.252 ± 27.537 d	549.455 ± 34.050 b,d	1113.467 ± 45.815 b,c,d
48	118.908 ± 0.567 d	165.113 ± 4.004 d	237.563 ± 8.941 d	445.792 ± 23.341 a	839.175 ± 53.419 a
72	135.663 ± 40.470 **	192.486 ± 53.401 **	272.342 ± 65.100 **	481.618 ± 77.860 **	847.260 ± 68.083 a
96	102.752 ± 2.326 b	141.827 ± 4.206 b	205.607 ± 7.945 a,b	396.827 ± 22.039 a	773.432 ± 56.521 a
***Bacillus* sp. DISK 1**					
24	1101.122 ± 8.251 d	1142.669 ± 7.666 c,d	1214.443 ± 8.380 c,d	1400.203 ± 15.468 b,d	1626.063 ± 19.435 b,c,d
48	1141.015 ± 25.416 d	1176.049 ± 24.424 d	1224.053 ± 26.599 d	1320.547 ± 38.037 a,d	1417.873 ± 56.254 a,c
72	1152.769 ± 33.738 d	1191.514 ± 25.700 a,d	1249.869 ± 17.993 a,d	1380.018 ± 10.631 d	1530.059 ± 8.852 a,b,d
96	412.544 ± 28.607 a,b,c	522.061 ± 24.456 a,b,c	678.000 ± 24.942 a,b,c	1038.879 ± 33.137 a,b,c	1450.999 ± 36.348 a,c
***Escherichia* sp. DISK 2**					
24	455.901 ± 30.675 b,c	525.216 ± 13.205 b,c	583.487 ± 8.330 b,c	681.742 ± 4.789 b,c	790.245 ± 2.310 b,c
48	62.325 ± 5.925 a,c	62.325 ± 5.925 a,c	124.783 ± 8.853 a,c	231.057 ± 11.407 a,c	427.749 ± 11.868 a,c
72	143.157 ± 1.842 a,b	177.608 ± 2.851 a,b	224.279 ± 4.310 a,b	333.979 ± 8.133 a,b	497.204 ± 14.607 a,b
Microbial community					
24	4.289 ± 1.506 b,c,d	14.016 ± 4.034 b,c,d	46.805 ± 10.266 b,c,d	251.390 ± 29.324 b,c,d	649.816 ± 34.871 c,d
48	129.472 ± 42.905 a,c,d	172.689 ± 45.851 a,c,d	235.293 ± 48.529 a,c,d	396.592 ± 47.267 a,c,d	651.264 ± 34.487 c,d
72	320.464 ± 90.854 a,b	371.921 ± 77.617 a,b	457.722 ± 63.121 a,b	694.626 ± 41.326 a,b	1032.654 ± 36.102 a,b
96	370.600 ± 67.050 a,b	425.356 ± 62.170 a,b	515.546 ± 56.980 a,b	758.367 ± 48.022 a,b	1113.483 ± 40.360 a,b

ND: Not determined
At $P < 0.05$ (within each threshold and bacterial pure culture or microbial community)
a = significantly different from 24th h threshold, b = significantly different from 48th h threshold, c = significantly different from 72nd h threshold, d = significantly different from 96th h threshold, ** = not significantly different from other thresholds.

DAF1 at 200 – 600 mg/l phenol when the culture was incubated for 72 and 96 h.

Generally, the dehydrogenase activity increases steadily up to 48 h of incubation. In *Pseudomonas* sp.

DAF1, the dehydrogenase activity at 72 and 96 h

did not follow the normal dose-response pattern that can fit to simple logistic or sigmoid models. It is thus suggested that for acute TTC-dehydrogenase assay involving bacteria, reliable and reproducible result would be best achieved within 48 h.

The *in vitro* study of acute toxicity of phenol to pure cultures of bacteria and microbial community of petroleum refinery wastewater indicates that phenol is potentially toxic. However, these organisms have acclimation potential in phenol-containing medium. This property can be exploited for treatment of phenolic wastewater.

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