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## ***In vitro* effects of petroleum refinery wastewater on dehydrogenase activity in marine bacterial strains** (doi:10.4136/ambi-agua.133)

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### **ABSTRACT**

Toxicity of oil refinery effluent on four bacteria strains isolated from refinery effluent impacted river water sample was assessed via dehydrogenase assay. Pure cultures of the bacterial strains were exposed to various effluent concentrations [12.5 – 100% (v/v)] in a nutrient broth amended with glucose and TTC. The response of the bacterial strains to refinery effluent is concentration-dependent. At 12.5% (v/v), the effluent stimulated dehydrogenase activity in *Streptococcus* sp. RW3 and *Pseudomonas* sp. RW4. In all strains, dehydrogenase activity was progressively inhibited at concentrations greater than 12.5% (v/v). The IC<sub>50</sub> ranges from 25.46 ± 4.75 to 31.30 ± 2.63% (v/v). The result of the *in vitro* study indicated that the bacterial strains are sensitive to oil refinery raw wastewater stress. Therefore, the improperly treated effluent when discharged would pose serious threat to the metabolism of the bacterial strains in natural environments.

**Keywords:** Refinery effluent; toxicity; marine bacteria; dehydrogenase.

## **Efeitos *in vitro* de efluentes de refinaria de petróleo em atividade de desidrogenase de cepas de bactérias marinhas**

### **RESUMO**

A toxicidade de efluentes de refinaria de petróleo em quatro linhagens de bactérias isoladas de amostras de água que sofreram influência de efluente de rio refinaria foi avaliada por meio de ensaio de desidrogenase. Culturas puras das estirpes bacterianas foram expostas a diferentes concentrações de efluentes [12,5-100% (v/v) em uma amostra de nutrientes alterada com glicose e TTC. A resposta das cepas bacterianas a efluentes de refinaria é dependente da concentração. A atividade de desidrogenase de efluentes com concentração de 12,5% (v/v) foi estimulada em culturas de *Streptococcus* sp. RW3 e *Pseudomonas* sp. RW4. Em todas as estirpes, a atividade de desidrogenase foi progressivamente inibida em concentrações superiores a 12,5% (v/v). O IC<sub>50</sub> variou de 25,46 ± 4,75-31,30 ± 2,63% (v/v). O resultado do estudo *in vitro* indicou que as bactérias são sensíveis ao stress de efluente bruto de refinaria de petróleo. Portanto, a descarga de efluentes com tratamento inadequado representa uma ameaça grave para o metabolismo das bactérias em ambientes naturais.

**Palavras-chave:** Toxicidade de efluente de refinaria; bactérias marinhas; desidrogenase.

### **1. INTRODUCTION**

Water as resource for life on earth, has several unique properties that help make it such a necessary part of the environment. For example, the entire essential functions within living cells are maintained by water. Water ecosystems are as varied as their individual sites because

they are influenced not only by characteristic local climate, soil, resident communities but also by the surrounding terrestrial ecosystem. As man advances in technology and industry, large amounts of water are used for industrial activities and consequently significant volumes of wastewaters are generated. Based on the type of industry, various levels of pollutants are deliberately released and discharged into the environment directly. Among these industries that discharge their effluents into the aquatic environments are the petroleum oil refineries. As not all refineries have the same processes, the effluents that are produced will have different chemical compositions depending on the type of treatment they received (Wake, 2005; Hernandez et al., 1998; Lehtinen, 1986). Wastewaters released by oil refineries contain large amounts of toxic derivatives such as oil and grease, phenols, sulphides, cyanides, suspended solids, nitrogen compounds as well as heavy metals such as iron, nickel, copper, selenium, zinc, molybdenum, etc. (Burks, 1982). Due to the ineffectiveness of purification systems, wastewaters from the refineries may become seriously dangerous, leading to the accumulation of toxic products in the receiving water bodies with potentially serious consequences on the ecosystem (Otokunefor and Obiukwu, 2005). Thus the discharge of these effluents containing persistent chemicals into a receiving waterbody may result in the long term effects to aquatic biota (Tisler et al., 1999). The toxicity of oil refinery effluents to aquatic organisms has being reported in many literatures. Toxicity of petroleum refinery depends on a number of factors which include quantity, volume and variability of discharge. The different components of the effluents may have varying effects and toxicity (Saha and Konar, 1985). Aruldoss and Viraraghavan (1998) reported the toxicity of refinery wastewater to luminescent bacteria (*Photobacterium phosphoreum*) using microtox in the bioassay. This is based on monitoring changes in natural light emissions from the organism. Toxicity and end point was measured as the effective concentration of a test sample that can cause 50% decrease in light out ( $IC^{50}$ ) after 30min of contact time.

Microorganisms are vital for the efficient functioning of any ecosystem; hence factors that affect their metabolism, composition and abundance are of great concern. Monitoring microbial responses has been recommended as an early warning indicator of ecosystem stress as microbes respond promptly to environmental perturbations (Nweke et al., 2007; Griffiths, 1983). Measurement of microbial enzyme activity is used in the assessment of ecotoxicological impacts of environmental substrates. In this regard, dehydrogenase activity has been widely used. The dehydrogenase assay is an effective primary test for assessing the potential toxicity of chemicals to microbial activities (Ghaly and Mahmoud, 2006; Griebe et al., 1997). In this assessment, dehydrogenase activity (DHA) is measured using the reduction of 2, 3, 5-triphenyltetrazolium chloride (TTC) to triphenylformazan (TPF). Determination of their ability to reduce TTC to the formazan product after exposure to test compounds, compared to the control situation, enables the relative toxicity of the chemicals to be assessed.

This study was aimed at assessing the *in vitro* effects of petroleum refinery wastewater on the dehydrogenase activity in bacterial species isolated from refinery effluent impacted Okrika River water in Port Harcourt, Nigeria.

## 2. MATERIALS AND METHODS

### 2.1. Characterization of petroleum oil refinery wastewater

Composite mechanically (gravity separation that include API separators and tank separation) and physicochemically (addition of additives, flocculation, sedimentation and filtration) pretreated petroleum oil refinery wastewater was collected at the inlet to the biological treatment unit (Rotary biodisk) at the Port Harcourt oil refinery complex using 5 litres polyethylene containers. The containers were rinsed several times with the effluent sample at the point of collection. The samples were taken to the laboratory in icebox within

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6h of collection. Phenol concentration, pH, COD, BOD, THC, cations ( $\text{Pb}^{3+}$  and  $\text{Cu}^{2+}$ ) and anions ( $\text{PO}_4^{2-}$  and  $\text{Cl}^-$ ) in wastewater samples were determined according to standard methods (APHA, 1998).

## 2.2. Bacterial strains and culture conditions

The bacterial strains used in these studies were isolated from water samples collected from oil refinery effluent-impacted Okrika river water samples located in Port Harcourt, Nigeria.

The isolates - *Citrobacter* sp. RW1, *Staphylococcus* sp. RW2, *Streptococcus* sp. RW3, *Pseudomonas* sp. RW4 were purified on nutrient agar (Fluka) plates and characterizations were done using standard microbiological methods. Identifications to the genus level followed the schemes of Holt et al. (1994). The isolates were maintained and sub-cultured once in every month in a basal minimal medium containing (per liter): ammonium chloride 10 g, ammonium nitrate 4.0 g,  $\text{K}_2\text{HPO}_4$  0.2 g,  $\text{KH}_2\text{PO}_4$  0.8 g,  $\text{MgSO}_4$  0.1 g; phenol as the sole carbon and energy source. The pH was adjusted to 8.0 using phosphate buffers.

The bacterial strains were grown to mid exponential phase in nutrient broth (Lab M) on a rotary incubator (Stuart, ST150SA, UK, 150 rpm) at room temperature ( $28 \pm 2^\circ\text{C}$ ). The cells were harvested by centrifugation at 8000 rpm for 10 min. Harvested cells were washed twice in deionised distilled water and re-suspended in the same deionized distilled water. The re-suspended cells were standardized in a spectrophotometer to an optical density of 0.90 at 540 nm. The dry weight of the standardized cells was determined by drying 15 ml of the cell suspension to constant weight in an oven (Gallenkamp, England) at  $110^\circ\text{C}$ . The standardized cell suspensions were used as inoculum in the dehydrogenase activity assay.

## 2.3. Dehydrogenase activity assay

Dehydrogenase assay method as described by Nweke et al. (2007) was employed with little modification. Briefly, dehydrogenase activity was determined using TTC (BDH England) as the artificial electron acceptor, which was reduced to the red-coloured triphenyl formazan (TPF). The assay was done in 3.5 ml volumes of nutrient broth-glucose-TTC medium supplemented with varying concentrations [0 – 100 % (v/v)] of oil refinery wastewater in separate 20 ml screw-capped test tubes. Aliquots (0.2 ml) of the bacterial suspensions were inoculated into triplicate glass tubes containing 2.5 ml of nutrient broth-glucose medium amended with graded concentrations of oil refinery wastewater that was diluted with 0.7ml deionized distilled water and preincubated on a rotary incubator (150 rpm) at room temperature ( $28 \pm 2^\circ\text{C}$ ) for 60 min. Thereafter, 0.1 ml of 0.1 % (v/v) TTC in deionised distilled water were added to each tube to obtain final effluent concentrations of 0, 12.5, 25, 50, 75, 100% (v/v) in different test tubes. The final concentrations of nutrient broth, glucose and TTC in the medium were 2, 2 and 0.267 mg/ml, respectively. The controls consisted of the isolates and the media without wastewater. The reaction mixtures were further incubated statically at room temperature ( $28 \pm 2^\circ\text{C}$ ) for 6h. The TPF produced was extracted in 4 ml of amyl alcohol and determined spectrophotometrically at 445 nm ( $\lambda_{\text{max}}$ ). The amount of formazan produced was determined from a standard dose-response curve [0 - 20  $\mu\text{g/ml}$  TPF (Sigma) in amyl alcohol;  $y = 0.0484x$ ;  $R^2 = 0.9958$ ]. Dehydrogenase activity was expressed as milligrams of TPF formed per mg dry weight of cell biomass per hour. Inhibition of dehydrogenase activity in the isolates by wastewater was calculated relative to the control. The percentage inhibitions for bacterial strains were linearized against the concentrations of the refinery wastewater using gamma parameter ( $\Gamma$ ) [ $\Gamma = \% \text{Inhibition} / (100 - \% \text{Inhibition})$ ] (Tarkpea et al., 1986). The toxicity threshold concentrations ( $\text{IC}^{50}$ ) which is an inhibitory concentration of toxicant required to reduce 50% of the dehydrogenase activity were then determined from the linear regression plots

## 2.4. Statistical analysis

Data obtained from the study were analyzed by the use of two-way analysis of variance (ANOVA) and values for  $P < 0.05$  were considered statistically significant.

## 3. RESULTS AND DISCUSSION

The results of the chemical characterization of the oil refinery wastewater sample are shown in Table 1. The Wastewater sample had elevated levels of organic compounds as indicated by the concentrations of COD BOD, THC and phenol as all measured values exceed the FEPA permissible limit values (Table 1). The bacterial strains were able to reduce TTC to its formazan and so were used to assess toxicity of refinery wastewater through the dehydrogenase assay.

The dehydrogenase activity (DHA) varied among the bacterial strains (Table 2). The Gram-negative strains of *Pseudomonas* sp. and *Citrobacter* sp. have higher rate of dehydrogenase

**Table 1.** Characteristics of petroleum oil refinery wastewater.

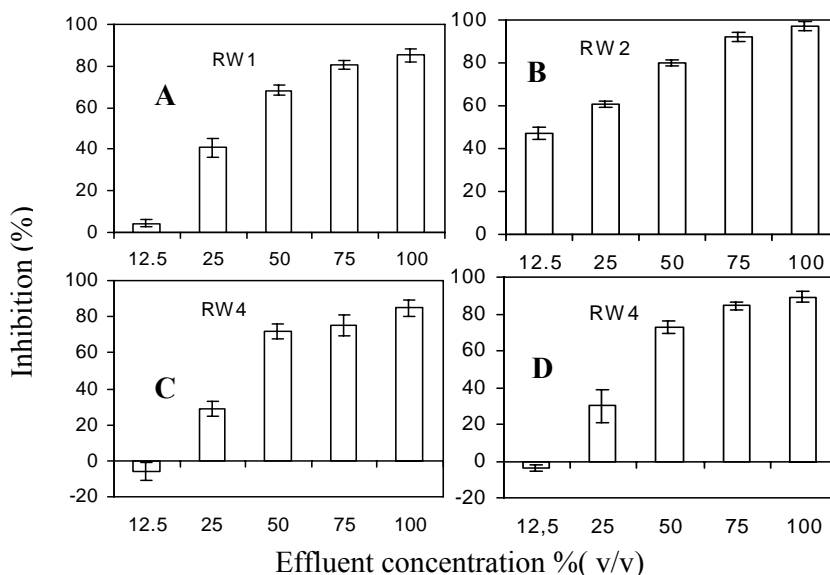
Parameter/unit	Refinery wastewater	FEPA wastewater limitations Guideline (1991)
pH	7.64	6.0 – 9.0
THC (mg/l)	17.5	10.0
TDS (mg/l)	950	2000.0
BOD (mg/l)	32.0	10.0
COD (mg/l)	112	40.0
Phenol (mg/l)	71.2	0.05
Phosphate (mg/l)	0.22	5.0
Chloride (mg/l)	44.0	600.0
Lead (mg/l)	<0.01	0.5
Copper (mg/l)	<0.01	1.0

**Table 2.** Uninhibited dehydrogenase activities in the isolates.

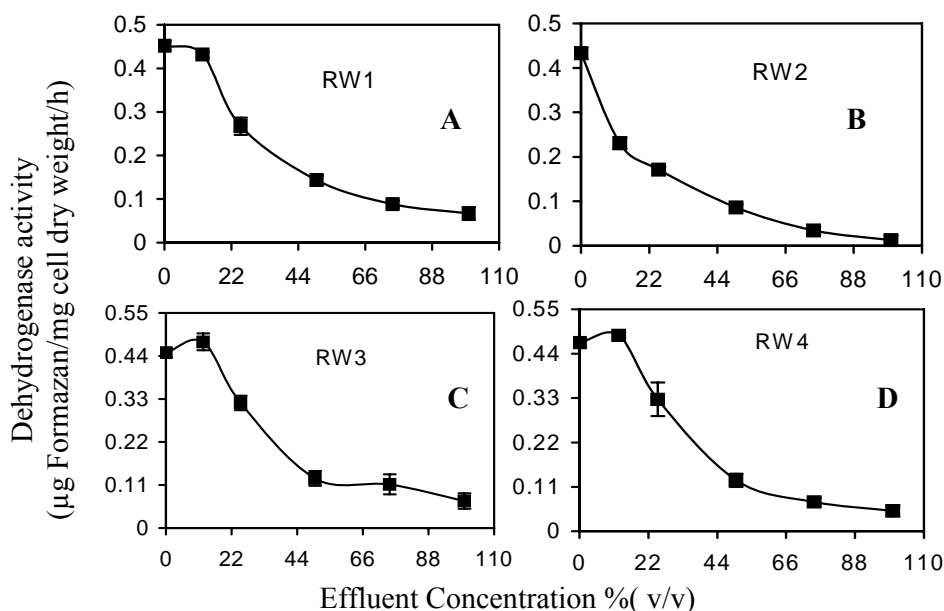
Bacterial Strain	Dehydrogenase activity ( $\mu\text{g}$ Formazan/mg cell dry weight/h)
<i>Citrobacter</i> sp. RW1	$0.452 \pm 0.011$
<i>Staphylococcus</i> sp. RW2	$0.433 \pm 0.012$
<i>Streptococcus</i> sp. RW3	$0.448 \pm 0.012$
<i>Pseudomonas</i> sp. RW4	$0.467 \pm 0.011$

activity than the Gram-positive strains of *Staphylococcus* sp. and *Streptococcus* sp. This is in agreement with Nweke et al. (2007) and Nwogu et al. (2007) in which the Gram-negative organisms (*Pseudomonas* species isolated from the environment and human sources respectively) had higher dehydrogenase activity than gram-positive organisms. Earlier report (Alisi et al., 2008) is however at variance with this observation. These variations may be due to differences in bacterial physiology, including cell wall components or dehydrogenase systems, since different microorganisms have been reported to have different dehydrogenase systems (Praveen-Kumar, 2003).

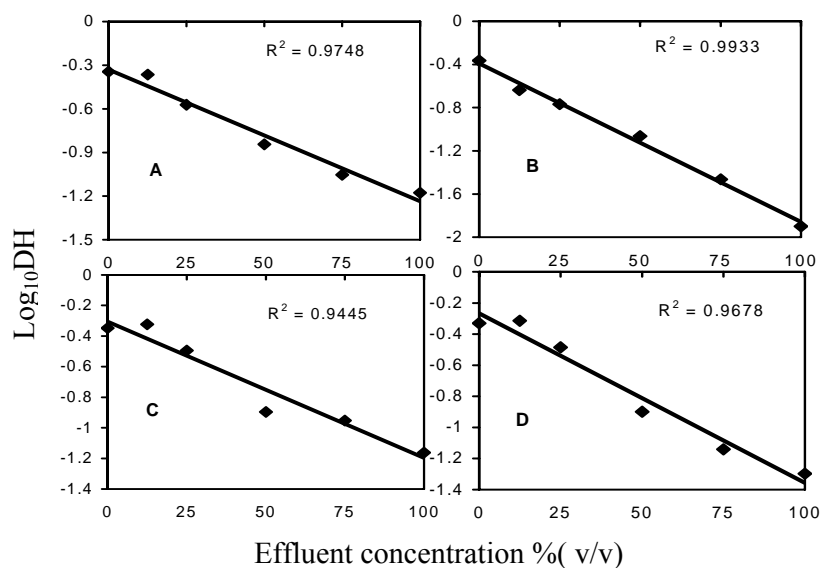
The effects of the different concentrations of the effluent on the bacterial isolates with respect to inhibition and dehydrogenase activity are shown in Figures 1 and 2. The responses of the bacterial dehydrogenase activities to the refinery effluent are concentration-dependent and vary among the organisms. For *Citrobacter* sp. RW1 and *Staphylococcus* sp. RW2,



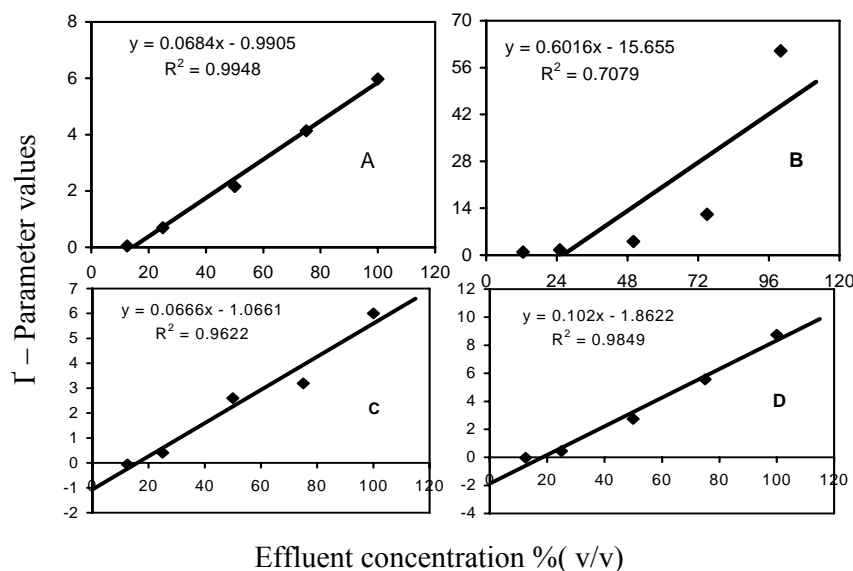
**Figure 1.** Effluent inhibition of dehydrogenase activity in *Citrobacter* sp. RW1 (A), *Staphylococcus* sp. RW2 (B), *Streptococcus* sp. RW3(C), *Pseudomonas* sp. RW4 (D). (> 0% = Inhibition; < 0% = Stimulation).



**Figure 2.** TTC reduction activity in response to different concentration of the refinery effluent by *Citrobacter* sp. RW1 (A), *Staphylococcus* sp. RW2 (B), *Streptococcus* sp. RW3 (C), *Pseudomonas* sp. RW4 (D). Mean  $\pm$  standard deviation (n=3) are indicated by bars. Some standard deviations are within data point.



**Figure 3.** Correlation of effluent concentrations with dehydrogenase activity (DHA) in response to effluent toxicity by *Citrobacter* sp. RW1 (A), *Staphylococcus* sp. RW2 (B), *Streptococcus* sp. RW3 (C), *Pseudomonas* sp. RW4 (D).



**Figure 4.** Gamma ( $\Gamma$ ) parameter values of *Citrobacter* sp. RW1 (A), *Staphylococcus* sp. RW2 (B), *Streptococcus* sp. RW3 (C), *Pseudomonas* sp. RW4 (D) in response to refinery effluent [0 – 100%(v/v)].

dehydrogenase activity reduced with increasing concentrations of effluent (Figure 2). On the contrary, for *Streptococcus* sp. RW3 and *Pseudomonas* sp. RW4, dehydrogenase activities were stimulated at 12.5% (v/v) and thereafter progressive inhibition was also observed at concentrations above 12.5% (v/v). The stimulation of *Streptococcus* sp. RW3 and *Pseudomonas* sp. RW4 observed at lower concentration of effluent is attributable to the use of phenols and other inorganic pollutants within the effluents by these bacteria. The inhibition of dehydrogenase activities observed in this study is consistent with the reported toxic effects of



industrial effluents at high concentrations (Tisler et al., 1999; Fountoulakis et al., 2002). Results presented in Figure 1 showed that at lower effluent concentrations [ $\leq 25.0\%$  (v/v)], *Streptococcus* sp. RW3 had higher percentage inhibition than other organisms. This implies that *Streptococcus* sp. RW3 was more sensitive to refinery effluent stress than the other bacterial strains studied. Comparatively, at higher concentrations [ $\geq 50.0\%$  (v/v)], *Pseudomonas* sp. RW4 was more tolerant to the refinery effluent than the other bacteria. Different sensitivities of microbes to refinery effluent toxicity could be related to the long-term exposure to toxic components of the effluent by the organism.

**Table 3.** Threshold inhibitory concentrations of petroleum refinery wastewater against the bacterial strains.

Bacterial strain	Inhibitory concentration (IC <sub>50</sub> ) % (v/v)
<i>Citrobacter</i> sp. RW1	29.25 ± 1.13
<i>Staphylococcus</i> sp. RW2	25.46 ± 4.75
<i>Streptococcus</i> sp. RW3	31.30 ± 2.63
<i>Pseudomonas</i> sp. RW4	31.30 ± 1.71

**Table 4.** R<sup>2</sup> values of linear regression plots of transformation data of dehydrogenase activity of the bacterial strains.

Regression plot	R <sup>2</sup> - values		
	Log dehydrogenase	Inhibition (%)	Γ - parameter
<i>Citrobacter</i> sp. RW1	0.9748	0.955	0.9948
<i>Staphylococcus</i> sp. RW2	0.9933	0.940	0.7079
<i>Streptococcus</i> sp. RW3	0.9445	0.8025	0.9622
<i>Pseudomonas</i> sp. RW4	0.9678	0.9630	0.9498

The gram-negative *Pseudomonas* sp. RW4 seems to tolerate the toxicity of the effluent more than the gram-positive *Staphylococcus* sp. RW2. The evidence is seen from the threshold inhibitory concentration data (Table 3). Dehydrogenase activity correlates with effluent concentration as shown in Figure 3. The higher R<sup>2</sup> values ( $0.9445 \leq R^2 \leq 0.9933$ ) indicate that the concentration was a strong determinant of dehydrogenase activity in the isolates. Thus, the effluent at high concentration exerted serious stress on the organisms. The logarithmic plot of the dehydrogenase activity (Figure 3) and the gamma parameter model (Figure 4) gave good linearization of the dose response data for the bacterial isolates. The high R<sup>2</sup> values for the linear regression plots of Log DHA and gamma parameter (Table 4) lays credence to the linear relationship. Gamma parameter models had higher R<sup>2</sup> values than the % inhibition plots (Table 4) and hence the linear regression models were used to assess the threshold inhibitory concentration of the effluent on the organism.

The 2-way analysis of variance shows that the dehydrogenase activity and its percentage inhibition varied significantly ( $P < 0.05$ ) with bacteria strain and effluent concentration. The result of this in vitro study indicated that oil refinery effluent exerts toxic effect against the tested organisms and Gram-positive *Staphylococcus* sp. was more responsive than the Gram-negative *Pseudomonas* sp.



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## 4. CONCLUSIONS

In all strains, dehydrogenase activity was progressively inhibited at concentrations greater than 12.5% (v/v). The  $IC_{50}$  ranges from  $25.46 \pm 4.75$  to  $31.30 \pm 2.63\%$  (v/v). The result of the *in vitro* study indicated that the bacterial strains are sensitive to oil refinery raw wastewater stress. Therefore, the improperly treated effluent when discharged would pose serious threat to the metabolism of the bacterial strains in natural environments.

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