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Growth responses of petroleum refinery effluent bacteria to phenol

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ABSTRACT:

Studies of the fungal and bacterial population of petroleum refinery effluent samples were carried out by microbial enumeration and determination of growth responses of bacterial isolates in increasing doses (0 - 15mM) of phenol in mineral salt -phenol agar. The total aerobic heterotrophic bacterial count ranged from (2.05 ± 0.4) x 10^7 to (2.52 ± 0.5) x 10^8 CFU/ml, total phenol-utilizing bacteria ranged from (1.18 ± 0.3) x 10^6 to (1.02 ± 0.3) x 10^7 CFU/ml and the total fungal count ranged from (3.1 ± 1.3) x 10^3 to (3.9 ± 0.5) x 10^4 CFU/ml in the effluent samples. *Bacillus* sp. RWW, *Aeromonas* sp. RBD, *Escherichia coli* OPWW and *Staphylococcus* sp. DP were isolated from the samples. Growth responses of the isolates on increasing doses of phenol in mineral salt-phenol agar showed that *Bacillus* sp. RWW and *Escherichia coli* OPWW had highest growth on 0.5mM (≈ 47.06mg/l) while *Aeromonas* sp. RBD and *Staphylococcus* sp. DP had their highest growth on 1.0mM (≈ 94.11mg/l). *Bacillus* sp. RWW and *Escherichia coli* OPWW showed least growth on 15.0mM (≈1,412mg/ml) of phenol. *Escherichia coli* OPWW exhibited highest growth in mineral salt broth containing 11.0mM of phenol with OD_{540nm} of 0.324 in 144 h resulting in the fastest utilization of phenol for growth. The highest specific growth rate of 0.013 h^{-1} at 11 mM (≈1,035mg/l) of phenol was obtained for *Bacillus* sp. RWW and *Escherichia coli*. Staphylococcus sp. DP had the lowest specific growth rate of 0.011 h^{-1} at 11 mM of phenol. These bacterial strains could be considered phenol-resistant and are potentially applicable in the removal of phenolic compounds from contaminated environmental media.

Keywords:
Growth responses, phenol-utilizing bacteria, refinery effluent.

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INTRODUCTION

The utilization of chemicals as carbon or energy sources by living cells is basic to all forms of life. Adaptation of living cells over the centuries to consume the natural biochemicals found on earth is the generally accepted narrative, but the significantly different organic species which are produced by man have led to environmental problems due to the resistance or complete recalcitrance to mineralization by any living species (Hill *et al*, 1996; Khleifat, 2006).

Phenol and its derivatives are common constituents of industrial effluents such as pharmaceutical, industrial resin manufacturing, pharmaceutical industries, plastic, coke oven plants, petrochemical and petroleum refineries (Charest *et al*., 1999; Ruiz-Ordaz *et al*., 2001; Jindrova *et al*., 2002; Rigo and Alegre, 2004). Phenol is toxic to all forms of life even at low concentrations and causes taste and odour problems in drinking water (Boszczyk-Maleszak *et al*., 2002; Monkiedje *et al*., 2004). The toxic action of phenol is always associated with the loss of cytoplasmic membrane integrity. As a result of phenol membrane-damaging properties on microorganisms, cytoplasmic membrane integrity is lost which in turn results in the disruption of energy transduction, disturbance of membrane barrier function, inhibition of membrane protein function and subsequent cell death (Kewelo *et al*., 1990; Heipieper *et al*., 1991; Yap *et al*., 1999). The high-volume use of phenols world wide and their potential toxicity has led to the inclusion of phenols on the list of priority pollutants (Veeresh *et al*., 2005). Owing to the importance of microbial activities in biogeochemical cycling, several biological parameters have been used to evaluate the toxic effect of phenol on bacterial population which includes changes in cell numbers, biomass measurement, specific and non specific enzymatic activities (Kuo and Genthner, 1996; Nwanyanwu and Abu, 2010, Nweke and Okpokwasili, 2010a,b; 2011).

Industrial effluent discharges have led to the pollution of aquatic and terrestrial environments over the years. Many mesophilic microbial species have been implicated in the degradation of phenol leading to a successful bioremediation of phenol-contaminated environments. Improvement of microbial efficiency has been directed towards the utilization of microbial seeding as a means of controlling industrial effluent discharges (Idise *et al*., 2010). Biodegradation enables organic pollutants to be converted from harmful organic substances into harmless inorganic substances. The ability of microorganisms to degrade phenol and the rate of phenol degradation is governed by certain factors such as oxygen tension, temperature, pH, and microbial abundance as well as nutrient availability (Zhao and Zhu, 1997). The type and number of microbial species involved in degradation may influence the rate and extent of the process (Atlas, 1981; Leahy and Colwell, 1990). Despite the presence of microorganisms capable of degrading the aromatic compounds, the concentration and type of the pollutant limit the biodegradation of the inherent compounds. If the organic pollutant is not available to the microorganisms and the appropriate enzymes do not come in contact with the substrate in the proper way, degradation will not occur (Atlas, 1981). Biodegradation of phenol and other pollutants in the natural ecosystem though complex has greatly enhanced the removal of harmful ions and solids from the environment by transformation into readily usable and less/non toxic materials by microorganisms (Tanaka *et al*., 1993). This research evaluated the growth responses of bacteria isolated from petroleum refinery wastewaters to various concentrations of phenol.

MATERIALS AND METHODS

Sample collection and characterization

Physicochemically treated raw effluent (addition of additives, flocculation, sedimentation and filtration) (RWW), biologically treated effluent [Rotary BioDisk,

(RBD)], observation pond treated effluent [Oxidation pond, (OPWW)] and Discharge Pipe effluent (DP) samples of Port Harcourt petroleum refinery were collected in sterile polyethylene containers. The containers were rinsed thrice with the effluent samples at the point of collection. To avoid deterioration, the samples were taken to the laboratory in icebox within six hours of collection for the determination of pH, Total Hydrocarbon (THC), electrical conductivity, Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Phosphate (PO_4), Sulphate (SO_4), phenol (C_6H_5OH), Lead (Pb), Zinc (Zn), and Copper (Cu) following standard procedures (APHA, 1985).

Enumeration of microorganisms

Aerobic heterotrophic bacteria, phenol-utilizing bacteria and fungal populations in the samples were enumerated on nutrient agar, mineral salt-phenol agar (Hill and Robinson, 1975) and potato dextrose agar plates respectively. In each medium, 0.1 ml aliquot of sample dilutions were spread-inoculated onto the agar surface and incubated at room temperature (28 ± 2^{o}C) for 24, 72 and 120 h for aerobic heterotrophic bacteria, phenol-utilizing bacteria and fungi respectively. The discrete bacterial colonies that grew profusely on the mineral salt-phenol agar representing the preponderant morphotypes in their respective sources were purified on freshly prepared nutrient agar (Lab M) and stored in nutrient agar slant at 4^{o}C. Characterizations of phenol-utilizing bacteria were done using standard microbiological methods. Identification to the genus level followed the schemes of Holt *et al* (1994). The isolates were designated according to their sources (RWW for Raw Waste Water, RBD for Rotary BioDisk, OPWW for Observation Waste Water and DP for Discharge Pipe).

Preparation of inoculum and culture condition

The phenol-utilizing bacterial strains used for the assay were grown in 100 ml of sterile nutrient broth media (HIMEDIA) contained in 250 ml Erlenmeyer flasks. The flasks were incubated on a rotary shaker incubator operating at 150 rpm for 24 h at room temperature (28 ± 2^{o}C). 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 medium. The cell suspensions were standardized in a spectrophotometer to an optical density of 0.5 at 540 nm.

Assay for the effect of phenol on the growth of bacteria in BH agar

The phenol-utilizing bacterial isolates were used for this assay. Aliquots of stock solution of phenol (20mM) were diluted with Bushnell and Haas (BH) broth medium (containing per litre: K_2HPO_4, 1.0g; KH_2PO_4, 1.0g; NH_4NO_3, 1.0g; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.02; $FeCl_3.6H_2O$, 0.085g) in 250ml Erlenmeyer flasks to obtain different concentrations of phenol (0-15 mM). This was followed by the addition of 2% agar into the flasks. The flasks were sterilized by autoclaving at 121^{o}C for 15 min after which the contents were poured into sterile plates set up in triplicates. The plates were allowed to solidify at room temperature and were, then dried in the oven at a temperature of 80^{0}C. Control flasks that received the same treatment except phenol was set up. An aliquot (0.1ml) of the standardized inoculum decimally diluted in physiological saline (0.85 % w/v NaCl) were spread on the agar surface. All inoculated, plates were incubated aerobically at 28 ± 2^{0}C for 72 h, after which colonies were counted.

Growth and phenol utilization assay

Liquid cultures were conducted in order to examine more parameters such as phenol concentration and growth of the bacterial strains. Hundred millilitre (100 ml) of BH medium was placed in a duplicate set of 250ml Erlenmeyer flasks. The flasks were supplemented with aliquot of phenol (20 mM) to bring the final phenol concentrations in the flasks to 2, 5, 8 and 11 mM. The flasks were sterilized by autoclaving, inoculated with the test organisms upon cooling and incubated at 30 ^{o}C in an

incubator. At specific time, samples were withdrawn to determine cell growth and remaining phenol. Controls included cells in BH medium without phenol and the medium supplemented with phenol but without cells. This is to assess for endogenous respiration of the cultures as well as to measure abiotic loss of phenol during the test period. At specific intervals of time samples were collected and measured for cell growth and phenol degradation. The cell growth was determined by measuring the Optical Density (OD) at 540 nm using spectrophotometer. The concentration of phenol in the medium was determined by the method of Folsom *et al*. (1990). For each initial concentration of phenol, specific growth rate (μ, h^{-1}) was estimated using **equation 1.**

$$\mu = \frac{In(X_2/X_1)}{(t_2 - t_1)} \quad (1)$$

Where X_1 and X_2 are the cell biomass (cell density) obtained at time t_1 and t_2.

Phenol degradation rate (Qs, mg/l.h) was determined from the maximum slope in plot of phenol concentration (mg/l) versus time of incubation as described by Afzal *et al*. (2007).

Statistical analysis

The statistical analysis was achieved using Microsoft Excel 2003. Biodegradation rate of phenol for each bacterium was compared pairwise using student's t-test with 5 % significance level

RESULTS AND DISCUSSION

The physicochemical properties and the microbial counts of the petroleum refinery effluent samples are shown in **Tables 1 and 2** respectively. The samples are slightly alkaline (pH 7.64 – 8.87). The microbiological analysis of oil refinery effluents indicated that the effluents are a good habitat for bacteria and fungi. The isolation of various bacterial genera from the effluents that are able to utilize phenol as carbon and energy sources showed that they are potential phenol degraders present in such specific habitats (Kopytko and Jacome, 2008). Total viable counts of aerobic heterotrophic population were highest in RBD and lowest in RWW with microbial load of (2.52 ± 0.5) x 10^8 and (2.05 ± 0.4) x 10^7 CFU/ml respectively. The difference in microbial load of the effluents may be attributed to differences in phenolic content. The utilization of phenol in the rotary biodisk may have resulted in the increased number of bacterial cells. The nutrient content of the effluent samples measured as Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), phosphate and nitrate may also be a contributing factor in the microbial population obtained (Ganapathiselvam *et al*., 2011). Total phenol-utilizing bacteria were found to be highest in DP and lowest in OPWW with total load of (1.02 ± 0.3) x 10^7 and (1.18 ± 0.3) x 10^6 CFU/ml respectively while total fungal load was found to be highest in RBD and lowest in DP with total load of (3.9 ± 0.5) x 10^4 and (3.1 ± 1.3) x 10^3 CFU/ml respectively.

The bacterial isolates from the petroleum refinery effluent samples were identified and designated as *Bacillus* sp. RWW, *Aeromonas* sp. RBD, *Escherichia coli* OPWW and *Staphylococcus* sp. DP comprising two

Table 1: Physicochemical properties of petroleum refinery effluent samples

Parameter/unit	Sample source[a]			
	RWW	RBD	OPWW	DP
pH	7.64	8.18	7.45	8.87
Temperature ^oC	26.4	26.1	26.8	26.7
Elect. Conduc. (μscm^{-1})	845	443	926	643
THC (mg/l)	17.5	15.0	21.0	16.0
BOD (mg/l)	32.0	8.0	12.8	12.8
COD (mg/l)	112.0	76.0	114.0	84.0
PO_4 (mg/l)	0.22	0.14	0.13	0.12
NO_3 (mg/l)	2.60	1.20	1.80	1.20
Phenol (mg/l)	71.2	13.6	10.1	9,4
Pb (mg/l)	<0.01	<0.01	<0.01	<0.01
Zn (mg/l)	0.13	0.02	0.06	0.08
Cu (mg/l)	<0.01	<0.01	0.01	0.01

[a] RWW =Raw Wastewater, RBD = Rotary Biodisk, OPWW = Observation Pond Wastewater, DP = Discharge Pipe

Table 2: Microbial load of petroleum refinery effluent samples

Sample source[a]	Count (CFU/ml)		
	Total aerobic heterotrophic bacteria	Total phenol-utilizing bacteria	Total fungi
RWW	$(2.05 \pm 0.4) \times 10^7$	$(1.24 \pm 0.4) \times 10^6$	$(4.1 \pm 1.2) \times 10^3$
RBD	$(2.52 \pm 0.5) \times 10^8$	$(1.31 \pm 0.3) \times 10^6$	$(3.9 \pm 0.5) \times 10^4$
OPWW	$(2.76 \pm 0.3) \times 10^7$	$(1.18 \pm 0.3) \times 10^6$	$(3.6 \pm 0.5) \times 10^3$
DP	$(2.50 \pm 1.2) \times 10^8$	$(1.02 \pm 0.3) \times 10^7$	$(3.1 \pm 1.3) \times 10^3$

[a] RWW =Raw Wastewater, RBD = Rotary Biodisk, OPWW = Observation Pond Wastewater, DP = Discharge Pipe

each of Gram negative and Gram positive organisms that exhibit a great deal of metabolic versatility. The toxicity of phenol to the isolate was assessed using population growth on phenol-amended mineral salt medium. **Figure 1** showed the effect of phenol on the population of the test isolates growing on increasing doses of phenol ranging from 0.05 to 15.0mM (≈ 4.71 -1,411.7 mg/l). At 0.5 mM (≈ 47.06mg/l) phenol, *Bacillus* sp. RWW and *Escherichia coli* OPWW have their highest growth with total viable counts of $(7.1 \pm 0.5) \times 10^6$ and $(7.9 \pm 0.2) \times 10^6$ CFU/ml respectively while *Aeromonas* sp. RBD and *Staphylococcus* sp. DP have their highest growth at phenol concentration of 1.0mM (≈ 94.11mg/l) with a total viable counts of $(7.5 \pm 0.3) \times 10^6$ and $(7.3 \pm 0.4) \times 10^6$ CFU/ml respectively. This implies a dose-dependent population growth response of the isolates to the concentrations of phenol in the growth medium as compared to the controls with growth population values of $(2.1 \pm 0.4) \times 10^5$, $(5.7 \pm 0.2) \times 10^5$, $(3.3 \pm 0.3) \times 10^5$ and $(3.3 \pm 0.5) \times 10^5$ CFU/ml for *Bacillus* sp. RWW, *Aeromonas* sp. RBD, *Escherichia coli*. OPWW and *Staphylococcus* sp. DP respectively. The growth of bacteria in the control medium (without phenol) may be as a result of organic impurities present in the medium

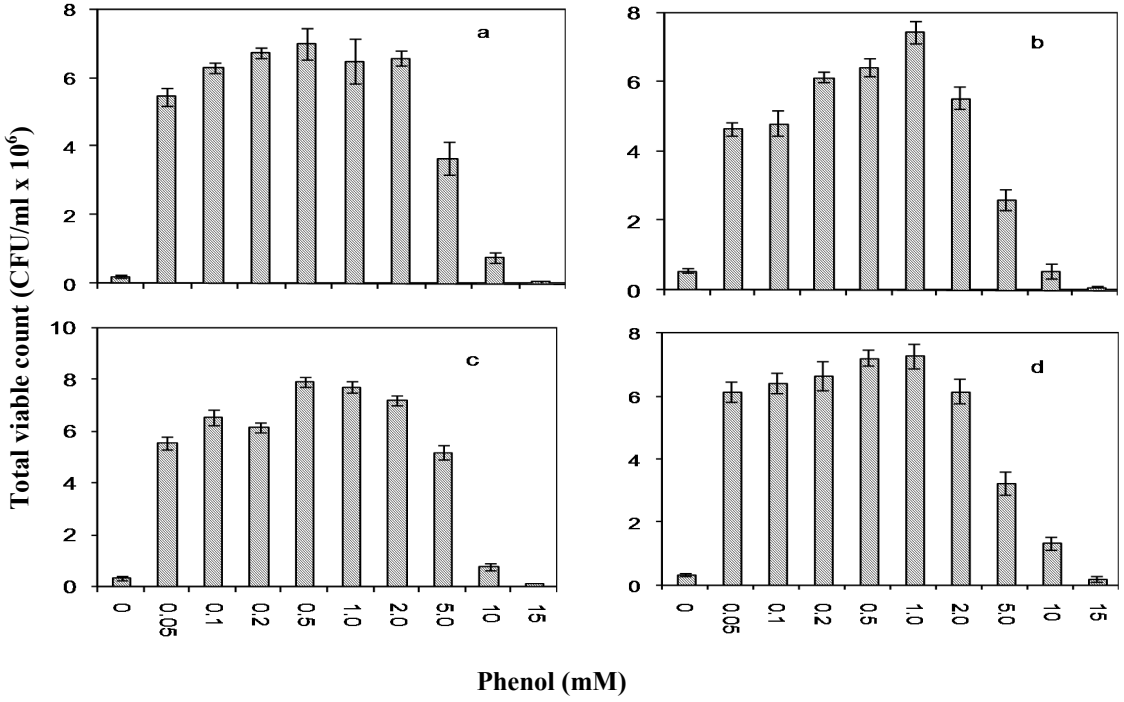

Figure 1: Growth of *Bacillus* sp. RWW (a); *Aeromonas* sp. RBD (b); *Escherichia coli*. OPWW (c) and *Staphylococcus* sp. DP (d) on Bushnell-Haas agar medium amended with increasing doses of phenol.

components, which are utilized by the organisms as carbon and energy source. The total viable counts of the organisms progressively decreased as the phenol concentration increases. At 15.0mM (≈ 1,411.7 mg/l) *Bacillus* sp. RWW and *Escherichia coli* OPWW were highly inhibited while *Aeromonas* sp. RBD and *Staphylococcus* sp. DP showed little population growth of (1.0 ± 0.01) x 10^5 and (2.0 ± 0.1) x 10^5 CFU/ml respectively. The inhibitory nature of phenol at high concentrations is well known, and has been reported by many authors (Kotturi *et al*., 1991; Margesin *et al*., 2004; Khleifat, 2006; Okpokwasili and Nweke, 2006).

Although phenol was toxic to the organisms, they could utilize it as source of carbon and energy at low concentrations. The growth patterns of the phenol-utilizing bacteria in BH medium containing different concentrations of phenol presents an interesting observation. The results conform to the work of Marrot *et al*. (2008) that reported microbial responses to phenol at lower concentrations, depicting an increase in microbial biomass. Depression of growth of *Bacillus* sp. RWW and *Escherichia coli* OPWW at 15mM (≈ 1,411.7 mg/l) may be due to inactivation of enzymes by phenol toxicity at such high concentration leading to incomplete utilization of phenol in the medium (Acuña-Argüelles *et al*., 2003; Ruiz-Ordaz *et al*., 1998). Also, studies have shown that lower doses of phenol are more readily utilized than higher doses. This corroborate with the high growth responses at low doses (0.5 and 1.0 mM respectively) of phenol. Stimulation of dehydrogenase activity at low dose of phenol was reported for *Bacillus* and *Pseudomonas* species isolated from petroleum refinery wastewater (Nweke and Okpokwasili, 2010a, b). Stimulation of dehydrogenase activity in some bacteria by phenol could indicate the use of phenol as a growth substrate. Similar stimulation and inhibition of dehydrogenase activity in soil *Acinetobacter* species by phenol derivatives at low and high concentrations respectively was reported by Okolo *et al*. (2007). The progressive inhibition of dehydrogenase activity with increasing concentration of phenols is in line with the well documented inhibitory nature of phenols at high concentrations even for organisms which can use phenols as growth substrates (Acuña-Argüelles *et al*., 2003; Ruiz-Ordaz *et al*., 1998).

Growth of the organisms in BH medium

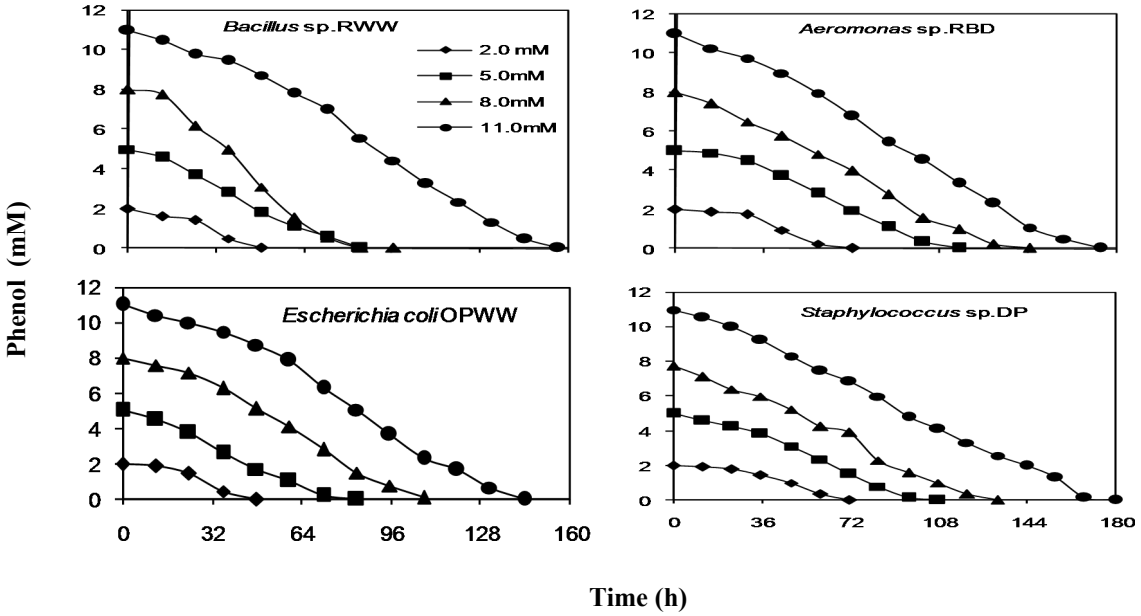

Figure 2: Biodegradation of different concentrations of phenol in mineral salt medium by the bacterial strains. Values are mean of duplicate determination.

supplemented with different concentrations of phenol was studied. Phenol utilization was well correlated with growth. Utilization and growth of the bacterial strains in different concentrations of phenol are shown in **Figures 2 and 3** respectively. Phenol was completely utilized by the isolates within 180 h. Two millimolar [(2.0 mM (≈188.2 mg/l)] of phenol was utilized completely within 60 h by *Bacillus* sp. RWW and *Escherichia coli*. OPWW while same concentration of phenol was utilized completely within 72 h by *Aeromonas* sp. RBD and *Staphylococcus* sp. DP. *Staphylococcus* sp. DP completely degraded 11.0 mM (≈ 1,035.2 mg/l) in 180 h **(Figure 2).** Time-dependent degradation of organic compounds has been reported to be linked with concentration of the organic compound as observed by many authors (Colwell and Walker, 1977; Kotresha and Vidyasagar, 2008). This longer period of utilization of phenol by the organisms may be attributed to non-acclimation of the bacterial strains to increasing doses of phenol or substrate inhibition effect of phenol at high concentrations. This corroborates the observation of Satsangee and Ghosh (1990) that non acclimated organisms take longer period than acclimated ones to degrade organic compounds. Also, Joseph and Joseph (1999) reported that phenol toxicity depends on the sensitivity as well as pre-exposure of organism to the toxicant. At high concentrations, phenol has been observed to inhibit microbial growth even to organisms that utilize it as sole source of carbon and energy. This is in line with the reports of Collins and Daugulis (1997) and Nwanyanwu and Abu (2011) who observed the toxic effect of phenol at the membrane level, thereby disrupting the activity of enzymes in phenol-utilizing bacteria.

The biomass concentration profile of the pure cultures expressed as optical density (OD_{540nm}) at different initial concentrations of phenol is shown in **Figure 3.** It was observed that the lag phases of growth of the bacterial isolates were below 12 h. Highest cell densities (OD_{540nm}) recorded were 0.341 at 11 mM and 0.182 at 2 mM phenol for *Escherichia coli* OPWW. This resulted in the faster conversion rates of phenol for the

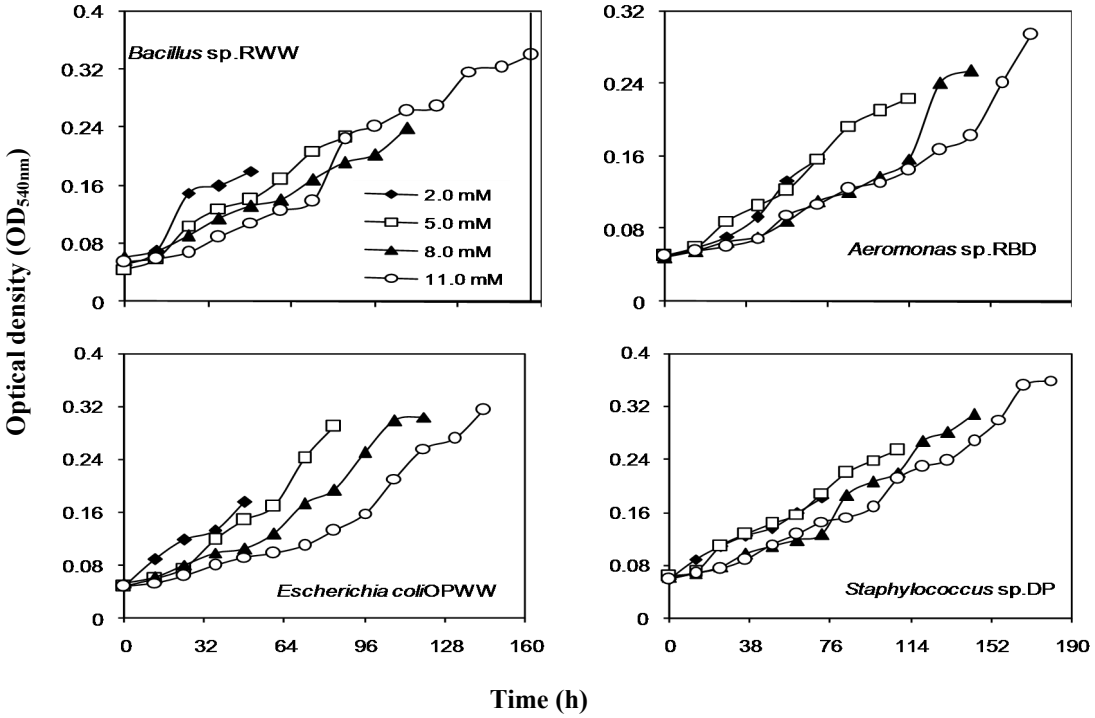

Figure 3: Growth of the bacterial strains on different concentrations of phenol in mineral salt medium. Values are mean of duplicate determination.

bacterial growth compared to other pure cultures. Though, the other pure cultures showed robust absorbance of cell growth in all phenol concentrations indicating that the cultures are capable of utilizing phenol much efficiently. Specific growth rates (μ) for each initial phenol concentration (S_o) was calculated from the plot of ln (X) versus time in logarithmic phase. The slope of the linear logarithmic plots of optical density during the exponential phase gives the specific growth rate. Specific growth rate of all the bacterial strains increased rapidly at phenol concentration of 2.0 mM (≈ 188.22mg/l) and then progressively decreases with increase in substrate concentration suggesting phenol toxicity to the organisms **(Figure 4).** The highest specific growth rates obtained at phenol concentration of 11.0 mM (≈ 1035.21 mg/l) was 0.013 h^{-1} for *Bacillus* sp. RWW and *Escherichia coli* OPWW while the lowest specific growth rate of 0.011 h^{-1} at 11.0mM was observed in *Staphylococcus* sp. DP. The *Escherichia coli*, *Aeromonas*, *Bacillus* and *Staphylococcus* species grew relatively at slow rate. The mean maximum specific growth of *Acinetobacter* species in phenol was reported to be 0.83 h^{-1} (Hao *et al*., 2002). *Ewingella americana* degrading 300mg/l phenol grew at maximum specific growth rate of 0.32 and 0.29 h^{-1} for starved and non-starved cells respectively (Khleifat, 2006). A phenol-degrading *Alcaligenes faecalis* grew at maximum specific growth rate of 0.15 h^{-1} (Bai *et al*., 2007). Similarly, mixed microbial consortium grew at maximum specific growth of 0.37h^{-1} (Saravanan *et al*., 2008), 0.143 h^{-1} (Nuhoglu and Yalcin, 2005) and 0.31 h^{-1} (Bajaj *et al*., 2009). However, the growth rates of *Escherichia coli*, *Aeromonas*, *Bacillus* and *Staphylococcus* species are similar to that of *Pseudomonas aeruginosa* and *Pseudomonas pseudomallei* degrading phenol in saline solutions (Afzal *et al*., 2007). The rate of phenol degradation of the organisms during their growth in phenol medium are shown in **Table 3,** The highest rate of phenol degradation (9.16 mg/l.h) at 11 mM (1035 mg/l) was observed with *Escherichia coli* OPWW. The degradation rates at 11 mM were higher than the degradation rates at 2 mM. This indicates strong potential of these organisms to degrade phenol. The slow rate of growth and biodegradation may be attributed to the lack of acclimation or decrease in the effective reactivity of the enzyme system within the cell. Similar report had been observed by Yap *et al*., (1999) in their work using *Comamonas testosteroni* strain P15 to eliminate phenol. They reported that the low degradation rate of phenol by the organism was in order to counteract the adverse effects of phenol inhibition. The t-test showed that the

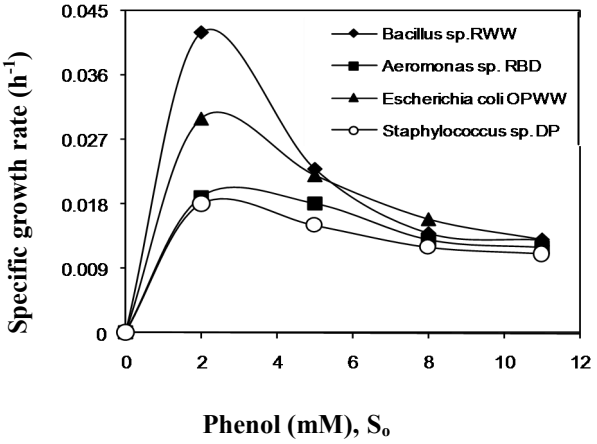

Figure 4: Specific growth rate of the bacteria under different initial phenol concentrations

Table 3: Biodegradation rate (Qs) of refinery effluent bactria under different initial phenol concentrations

Bacteria	Biodegradation rate (mg/l.h)			
	Phenol concentration (mg/l)[a]			
	188(2)	471(5)	753(8)	1035(11)
Bacillus sp. RWW	4.49	6.18	11.63	8.22
Aeromonas sp. RBD	5.88	6.23	7.30	8.47
Escherichia coli OPWW	5.25	6.88	8.32	9.16
Staphylococcus sp. DP	3.61	5.63	6.06	6.33

values in parenthesis represent phenol concentration in mM

biodegradation rate varied significantly ($p < 0.05$) among the test organisms. However there was no significant difference in the biodegradation rates of *Aeromonas* sp. RBD and *Staphylococcus* sp. DP as well as *Escherichia* sp OPWW and *Staphylococcus* sp. DP.

Although, they grew at low rate, the organisms demonstrated strong potential to utilize and degrade phenol at high phenol concentrations of 11.0 mM (≈1,035.2 mg/l). This indicated that these strains have remarkable potential for application in the treatment of phenolic wastewater and in the bioremediation of phenol -contaminated media.

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