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RESEARCH ARTICLE

UTILIZATION OF PHENOL BY MICROORGANISMS ISOLATED FROM A PETROLEUM POLLUTED SOIL IN PORT HARCOURT, RIVERS STATE

***Williams, Janet Olufunmilayo and Wilcox, Ibifuro Mabel**

Dept. of Microbiology, Rivers State University, Port Harcourt, Nigeria

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ABSTRACT

An investigation on the ability of microorganisms to utilize phenol as a carbon source for growth was carried out using standard microbiological techniques. The sample used in this investigation was a petroleum polluted soil which was serially diluted and plated out on sabouraud dextrose, nutrient and cetrimide agar. The Total Heterotrophic bacteria and fungi that grew on petroleum polluted soil were: *Staphylococcus* spp, *Bacillus* spp, *Lactobacillus* spp, *Clostridium* spp, *Micrococcus* spp and *Pseudomonas* spp for the bacterial species and the fungal species were, *Aspergillus niger, Penicillium* spp, *Rhizopus* spp, *Aspergillus fumigatus and Trichophyton spp*. A control sample was used to check for the occurrence of these organisms found in the polluted soil. The bacterial species isolated were: *Staphylococcus* spp, *Bacillus* spp, *Lactobacillus* spp, *Clostridium* spp, *Micrococcus* spp and the fungal species found were; *Aspergillus niger, Penicillium spp, Rhizopus* spp, *Aspergillus fumigatus and Trichophyton spp.* This showed that these organisms can exist also in the control sample. These organisms were tested for their ability to utilize phenol as a sole carbon source by spreading 0.1ml of the isolated organism on mineral salt agar, and adding varying concentrations of phenol, 100ppm, 200ppm, 500ppm and 1000ppm as sole carbon source. It was deduced that *Staphylococcus* spp, *Bacillus* spp, *Pseudomonas* spp and *Aspergillus niger* were the best fitted organisms that utilize phenol. Statistical analysis using ANOVA showed that in terms of phenol concentration, 100ppm, 20pp, 500ppm and 1000ppm; there was a significant difference in the growth rate of the organisms in comparison with the different days (2,3,4,5,6and 7) observed during this study.

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INTRODUCTION

Petroleum is a diverse compound which consists of saturated hydrocarbons. Petroleum due to its diversity have several components are and inbetween separated into different fractions based on their boiling points and they include; natural gas, hydrocarbons; benzene, phenol, naphthalene, camphor, diesel, kerosene, fuel etc. (Dubey, 2009). Alkenes, Alkanes, phenols, polycyclic aromatic hydrocarbon, several mechanical and chemical methods have been employed in removing hydrocarbons from affected contaminated sites. The use of microorganisms have proven to be more efficient, since these microorganisms utilize these hydrocarbons, using them as a source of carbon, hence enhancing their growth and cleaning up the polluted areas (Agbogidi *et al*., 2005).Phenol which is known by different names like phenylic acid, carbolic acid etc. is the primary structure for a wide range of synthetic organic compounds. Phenol is obtained from both natural and man-made source. The natural being from plant materials as a result of their metabolite, coal tar or distillation.

Corresponding author: **Williams, Janet Olufunmilayo,**
Dept. of Microbiology, Rivers State University, Port Harcourt, Nigeria

The man-made source being from industries like the plastic, pharmaceutical, oil refinery, pesticide industries and many others (Nair *et al*., 2008). Phenol is a major pollutant of the environment and it is toxic at very low concentrations, how it adversely affects microbial cells has been determined. Continuous exposure to phenol leads to central nervous disorders, damage in blood, weak pulse, cardiac depression and a reduced blood pressure. A report has shown that ingestion of 1g of phenol can cause death in humans, hypothemia, myocardinal infarction, burning effect on the skin, cancer, eye irritation and gastrointestinal disorders (Tziotzois *et al*., 2005). Phenol is toxic to the soil environment even in trace amount. The presence of phenol in the soil leads to a decrease in nitrogen mineralization, pH reduction, calcium and phosphate reduction as well as decreased enzyme activity. A reduction in all these components will lead to nutrients being limited, a reduction in microbial activity which will affect the rate of decomposition. Certain microorganisms have been found to use phenol as carbon source. Microorganisms like *Staphylococcus* spp, *Bacillus* spp, *Pseudomonas* spp and *Aspergillus niger* utilize phenol and grow, hence these organisms can be used for further studies in bioremediation processes.

Table 1. Physicochemical parameters

PARAMETERS	Control	Sample
pH	6.88	6.30
Nitrogen(mg/kg)	484	120
Total Hydrocarbon Content (THC) (mg/kg)	91.35	235
Phosphorus(mg/kg)	34.03	14.25
Potassium(mg/kg)	50.00	1.68

Table 2. Biochemical characterisation of total heterotrophic bacteria

BIOCHEMICAL REACTION							SUGAR FERMENTATION							PROBABLE ORGANISM
ISOLATES	GRAM'S REACTION	CATALASE TEST	INDOLE TEST	METHYL RED TEST	VOGE PROSKAUER TEST	MOTILITY TEST	COAGULASE TEST	OXIDASE TEST	STARCH HYDROLYSIS TEST	UREASE TEST	GLUCOSE	MALTOSE	MANNITOL	LACTOSE	
A	+ Cocci	+	-	+	-	+	+	-	+	-	A	A+G	A+G	A+G	*Staphylococccus* spp
B	+ Rod	+	-	+	-	-	-	-	-	-	A+G	A	A+G	A+G	*Bacillus* spp
C	+ Rod	+	-	+	-	+	+	-	+	-	AG	A	A	A+G	*Lactobacillus* spp
D	+Rod	+	+	+	-	+	-	-	+	-	A+G	A+G	A+G	A+G	*Clostridium* spp
E	+ Rod	+	-	+	-	-	+	-	-	-	-	-	-	-	*Micrococcus* spp
F	+ Rod	+	-	+	-	-	-	+	+	-	A+G	A+G	A+G	A+G	*Bacillus* spp
G	-Rod	+	-	+	-	+	+	+	-	-	A+G	A+G	A+G	A+G	*Pseudomonas* spp

KEYS: + =Positive, - =Negative, A = Acid production, G = Gas production A+G = Acid and Gas production

MATERIALS AND METHODS

Source of sample collection: Polluted hydrocarbon soil was aseptically collected from Nembe Waterside, Creek Road, Port Harcourt, Rivers State and Control soil sample was taken from 25 Hospital Road, Port Harcourt, Rivers State. The polluted and Control soil were taken from a depth of 5-10cm, put in sterile plastic bags and taken to the Microbiology laboratory, Rivers State University, Port Harcourt for analysis.

Determination of physiochemical properties of soil samples: Methods for the determination of physiochemical properties of petroleum polluted and control soil samples were used as outlined by POCEMA (2018). The pH meter used was pocket-sized HANA pHep + H198108 with automatic temperature compensation. Nitrogen content was determined using distillation process. Phosphorus content was determined by filtration and bray's reagent. Potassium content was determined using kjeldahl digestion method. Total hydrocarbon content was determined using the UV spectrophotometer.

Bacterial and Fungal counts: The samples were microbiologically analysed using spread plate method. About 1g of the soil samples were serially diluted. An aliquot was plated on nutrient agar and incubated at 37^{o}C for 24hrs and sabouraud dextrose agar at 27^{o}C for 72-120 hrs. The bacterial and fungal counts were thereafter enumerated.

Isolation and characterization of isolates: Pure isolates of bacteria were obtained by streak plate on nutrient agar and stored (4^{o}C) using agar slants. Pure isolates of fungi were transferred with an inoculating needle on sabouraud dextrose agar and stored (4^{o}C) in peptone and glucose. Bacterial colonies were identified by morphological and biochemical techniques using Bergey's Manual of Determinative Bacteriology (2006), fungal colonies were determined macroscopically and microscopically (Dhawale and LaMaster, 2003). The isolated bacteria were; *Staphylococcus* spp, *Bacillus* spp, *Lactobacillus* spp, *Clostridium* spp, *Micrococcus* spp and *Pseudomonas* spp,. The fungal isolates were *Aspergillus niger*, *Penicillium* spp, *Rhizopus* spp, *Aspergillus fumigatus* and *Trichophyton* spp.

Enumeration of phenol utilizing microorganisms: The mineral salt medium (MSM) was compounded based on Mills description. The composition (g/l) of the MSM is as follows: $MgSO_4$. $7H_20$, 0.42g; KH_2PO_4, 0.83g; NaCl, 10.0g; KCl, 0.29g; Na2HPO4 1.25g; NaNo3, 0.42g; Agar, 15g; Distilled water, 1L. The medium was enriched with phenol as a sole carbon source at different concentrations of 100, 200, 500and 1000ppm. The medium was incubated at room temperature for 7 days. Phenol utilizing organisms were observed for each of these days and recorded.

RESULTS

Table 1 shows the physiochemical parameters carried out on the control and polluted soil samples. The parameters include: pH, nitrogen level, total hydrocarbon content, phosphorus and potassium levels. Table 2 below shows the biochemical characteristics of Total Heterotrophic Bacteria. The probable organisms were *Staphylococcus* spp, *Bacillus* spp, *Lactobacillus* spp, *Clostridium* spp, *Micrococcus* spp and *Pseudomonas* spp. Table 3 shows the different morphology of fungi isolated. The probable organisms were *Aspergillus niger*, *Penicillium* spp, *Rhizopus* spp, *Aspergillus fumigatus, Trichophyton* spp. Figure 1 is a comparison of the frequency of occurrence of each bacteria spp isolated in the polluted and control soil samples, Figure 2 is the summary of the log form (cfu/ml) on each day (hours) at the different phenol concentrations; 100ppm, 200ppm, 500ppm and 1000ppm. Figure 3 shows the growth rate of *Staphylococcus* spp at different phenol concentration; 100ppm, 200ppm, 500ppm, 1000ppm on day 2(48hrs), day 3(72hrs), day 4(96hrs), day 5 (120hrs), day 6 (144hrs) and day 7 (168hrs). Figure 4 shows the growth rate of *Bacillus* spp at different phenol concentration; 100ppm, 200ppm, 500ppm, 1000ppm on day 2(48hrs), day 3(72hrs), day 4(96hrs), day 5 (120hrs), day 6

Table 3. Morphological identification of fungi

ISOLATES	STRUCTURAL/ COLONIAL MORPHOLOGY	MICROSCOPIC MORPHOLOGY	IDENTIFICATION
A	Dark brown colonies, rapid growth on SDA and covers the plate.	Septate hyphae and radiate conidium	*Aspergillus niger*
B	Blue-green colonies with yellowish-white reverse side, powdery, cottony.	Septate hyphae, branched conidiophore, phialides grouped in brush lke clusters.	*Pennicillium* spp
C	Whitish and fluffy, turns brown with age, pale white on reverse side.	Branchiing hyphae, non septate hyphae	*Rhizopus*spp
D	Initially white and turns green with time, cottony texture.	Septate hyphae, unbranched conidiophore and enlarged at the tip. Presence of phialides which produce conidia	*Aspergillus fumigatus*
E	Colony is flat to slightly raised, cream colour with wine red reverse side/ pale yellow.	Septate hyphae, macroconidia is smooth, thin walled	*Trichophyton* spp

Table 4. Observation of phenol utilizing fungi

PHENOL(PPM)	ORGANISMS	DAYS
	Aspergillus niger	
100	-	3
200	-	3
500	-	3
100	+	6
200	+	6
500	+	6
	Penicillium spp	
100	-	3
200	-	3
500	-	3
100	-	6
200	-	6
500	-	6
	Rhizopus spp	
100	-	3
200	-	3
500	-	3
100	-	6
200	-	6
500	-	6
	Aspergillus fumigatus	
100	-	3
200	-	3
500	-	3
100	-	6
200	-	6
500	-	6
	Trichophyton spp	
100	-	3
200	-	3
500	-	3
100	-	6
200	-	6
500	-	6

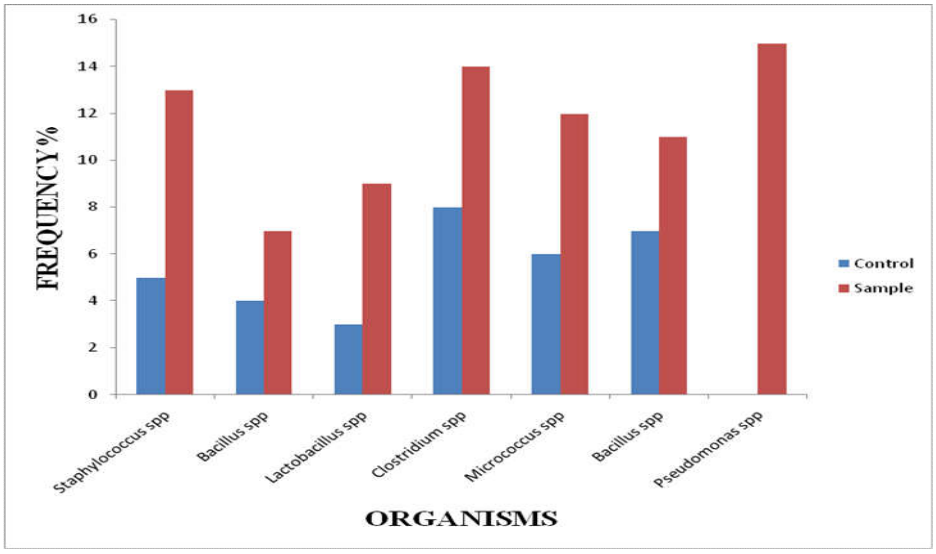

Figure 1. Frequency of Occurrence for Total Heterotrophic Bacteria

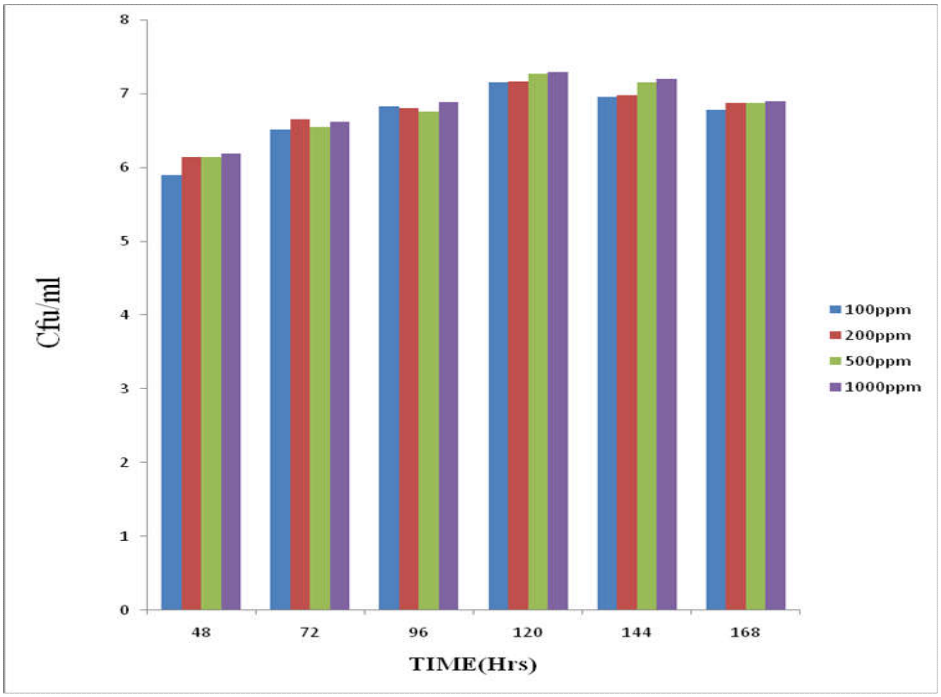

Figure 2. Colony forming unit formed in the different phenol concentrations for bacteria on different days (hours)

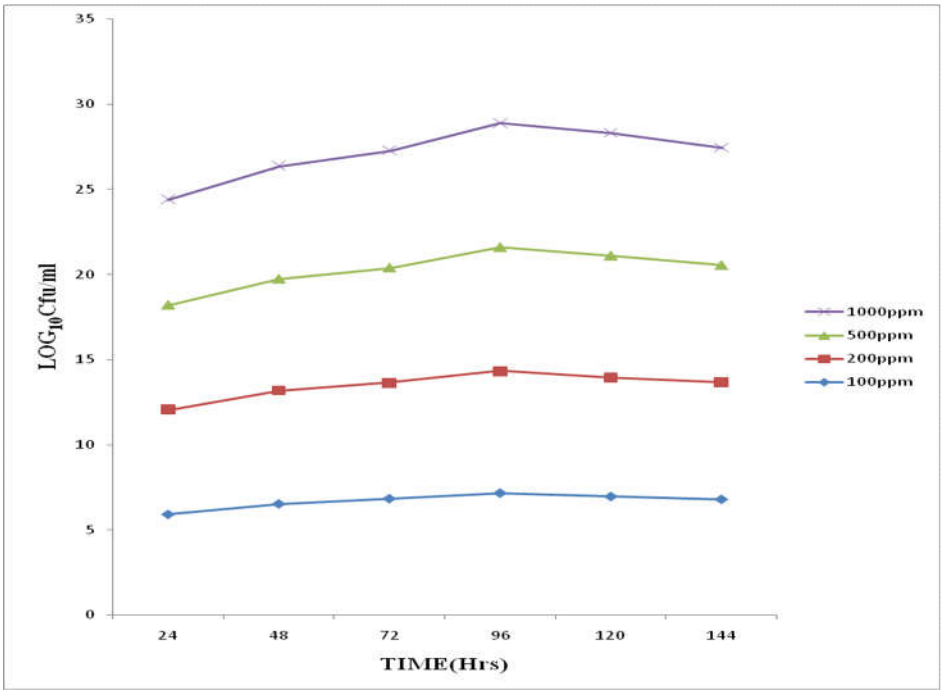

Figure 2a. Log of the colonies formed in the different phenol concentration, at different days (hours)

(144hrs) and day 7 (168hrs). Figure 5 shows the growth rate of *Lactobacillus* spp at different phenol concentration; 100ppm, 200ppm, 500ppm, 1000ppm on day2(48hrs), day3(72hrs), day 4(96hrs), day 5 (120hrs), day6 (144hrs) and day 7 (168hrs). Figure 6 shows the growth rate of *Bacillus* spp at different phenol concentration; 100ppm, 200ppm, 500ppm, 1000ppm on day 2(48hrs), day3(72hrs), day4(96hrs), day 5 (120hrs), day 6 (144hrs) and day 7 (168hrs). Figure 7 shows the growth rate of *Pseudomonas* spp at different phenol concentration; 100ppm, 200ppm, 500ppm, 1000ppm on day 2(48hrs), day 3(72hrs), day 4(96hrs), day5 (120hrs), day 6 (144hrs) and day 7 (168hrs). Figure 8 is a comparison of the frequency of occurrence of the fungi spp isolated from the polluted and control soil samples.

DISCUSSION

This study is primarily aimed at determining the species of microorganisms that is best fitted to utilize phenol as a carbon source. Phenol being a major component of petroleum is very toxic and even at very low concentrations, it is detrimental to soil nutrients, habitat, hence, needs to be checked and eliminated to increase the soil nutrient and its habitat leading to an increase in crop production, provision of healthy foods for man and a reduction in the health risk associated with phenol. Hydrocarbons being a component of petroleum; if phenol as a hydrocarbon contaminates soil and gets removed, up to 80% of the petroleum product is eliminated. The phenol concentrations in soil have reduced essential nutrients found in soil such as nitrogen, phosphate and potassium which are highly essential for plant growth.

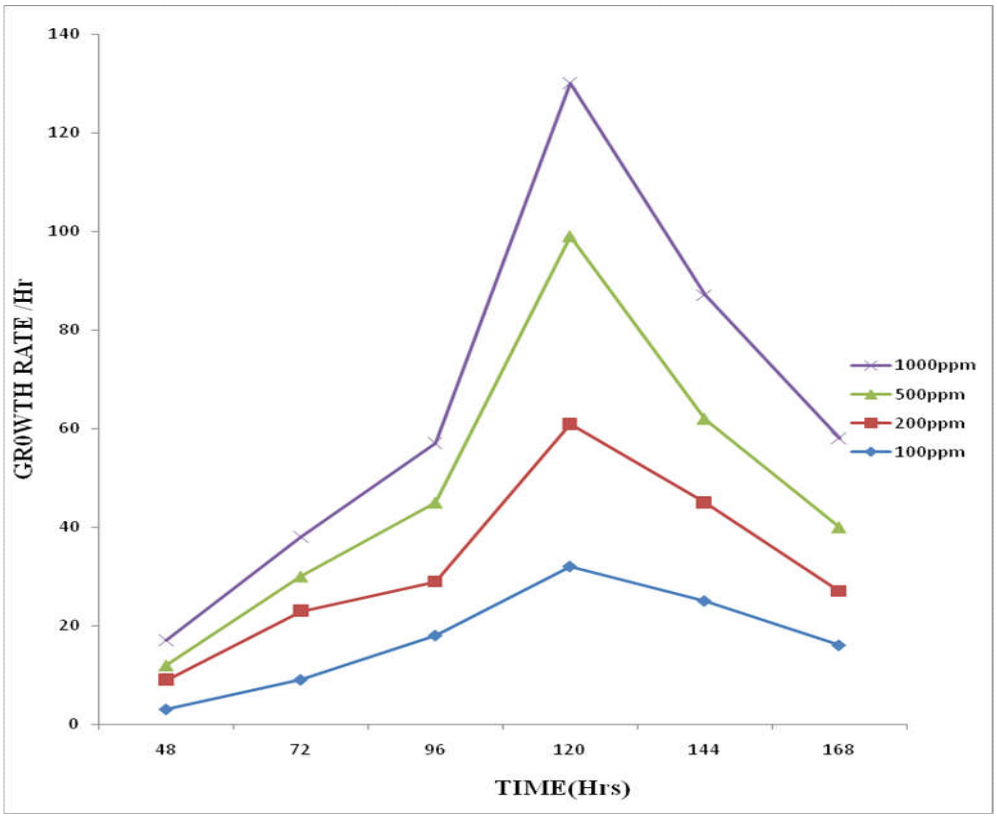

Figure 3. Growth rate of *staphylococcus* spp at different phenol concentrations

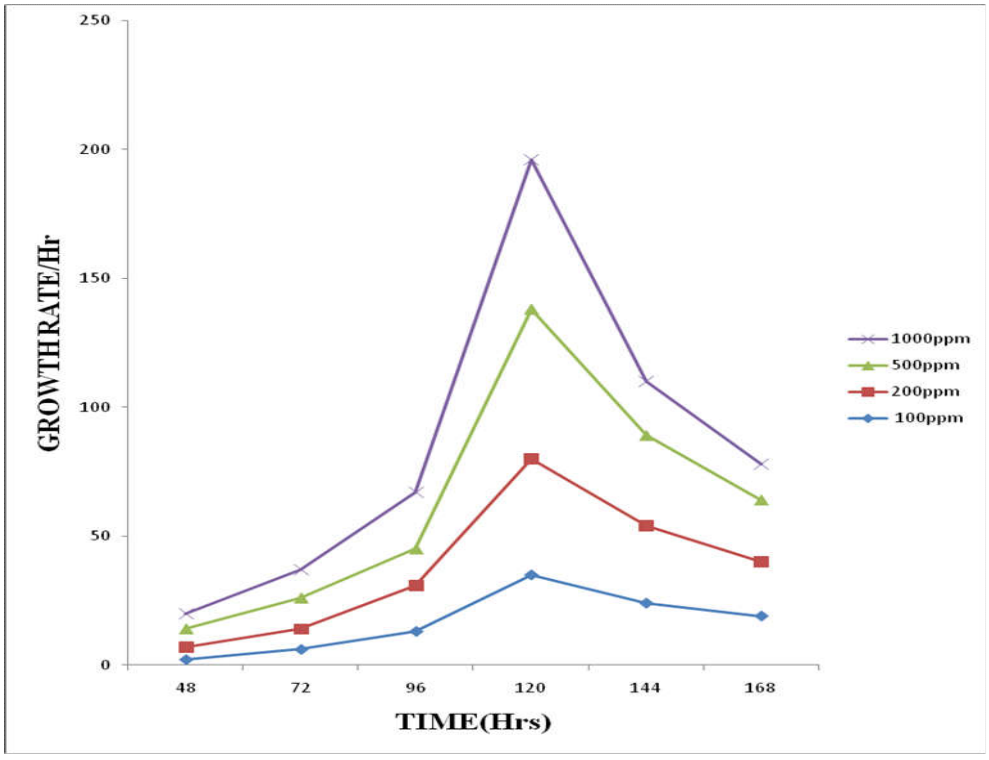

Figure 4. Growth rate of *Bacillus* spp at different phenol concentrations

It has led to a reduction in the yield of plants, enzymatic activity which is necessary for nutrient cycling, the breakdown of soil organic matter and mineral components as stated by Acosta *et al*. (2000). Phenol has also led to a reduction in microbial activity which helps in our biogeochemical cycle, nutrient cycling and in the decomposition of organic matter as stated by Kraus *et al.* (2004). When phenol gets into the human body, it acts as a carcinogen, reducing blood pressure and central nervous disorders (Agary*et al*., 2008). The presence of total heterotrophic bacteria and fungiindicates that microorganisms can grow in such areas. However, the ability to grow in these polluted areas does not indicate their ability to utilize the components of the petroleum and phenol is a major constituent. The bacteria and fungi determined were plated by means of spread plate technique for fungi and transferred to mineral salt agar which is used to grow acidic organisms.

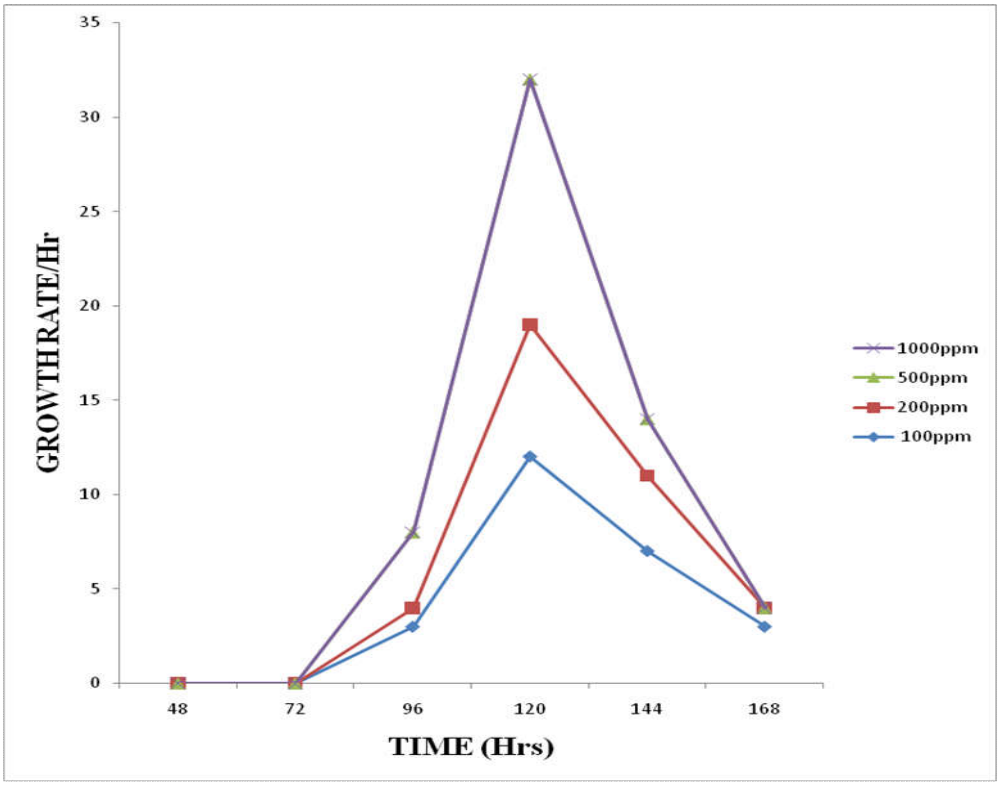

Figure 5. Growth rate of *Lactobacillus* spp at different phenol concentrations

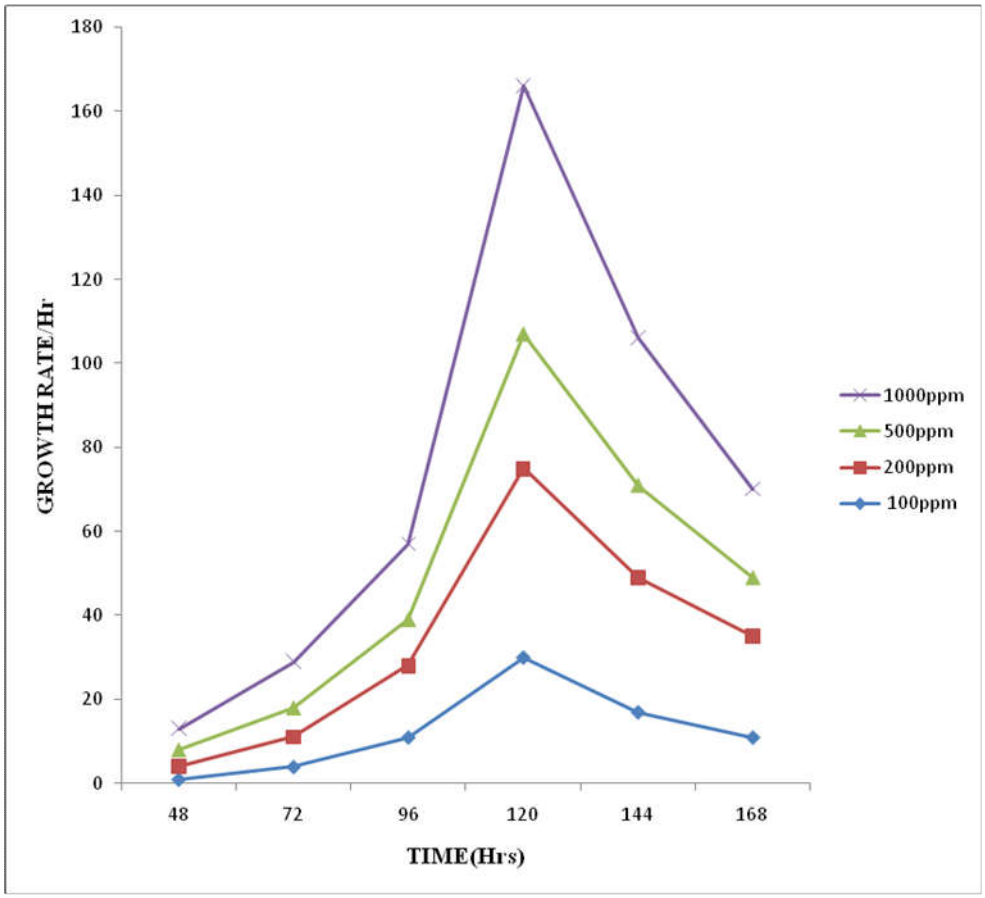

Figure 6. Growth rate of *Bacillus* spp at different phenol concentrations

Different concentrations of phenol were added to the mineral salt agar ranging from 100ppm (0.1ml), 200ppm (0.2ml), 500ppm (0.5ml) and 1000ppm(1ml) as carbon source. A similar approach was done by Sataya (2012).

Results showed that not all the isolated organisms from the polluted petroleum soil could use phenol as a carbon source to grow and observed at different days, this is illustrated in Tables 5-10 and in Figures 3-8.

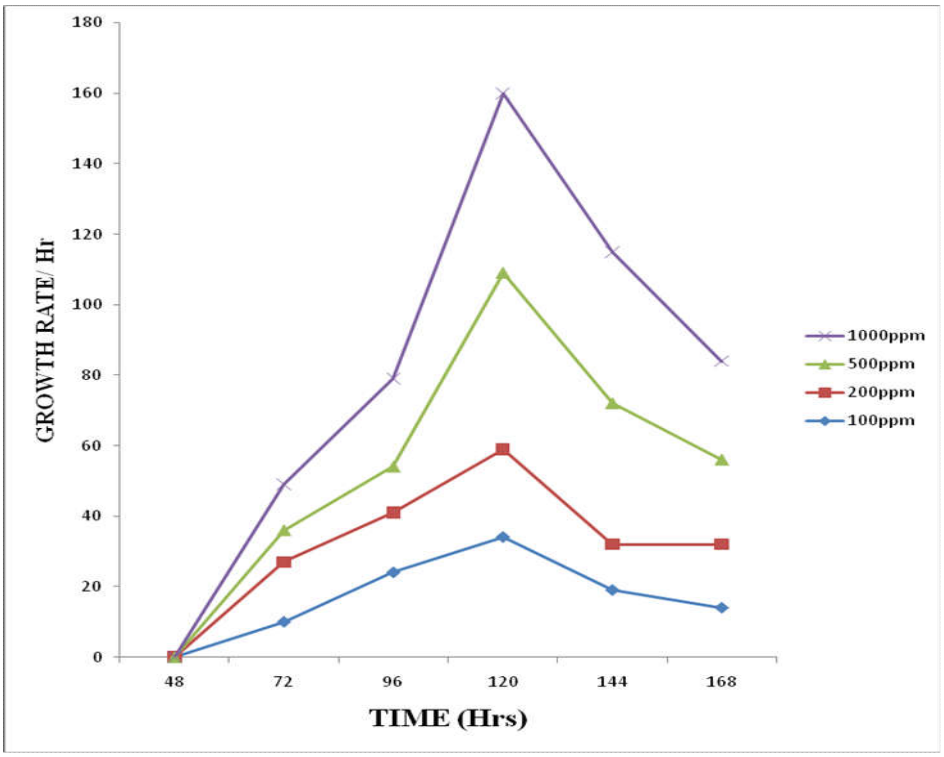

Figure 7. Growth Rate of *Pseudomonas* spp at different phenol concentrations

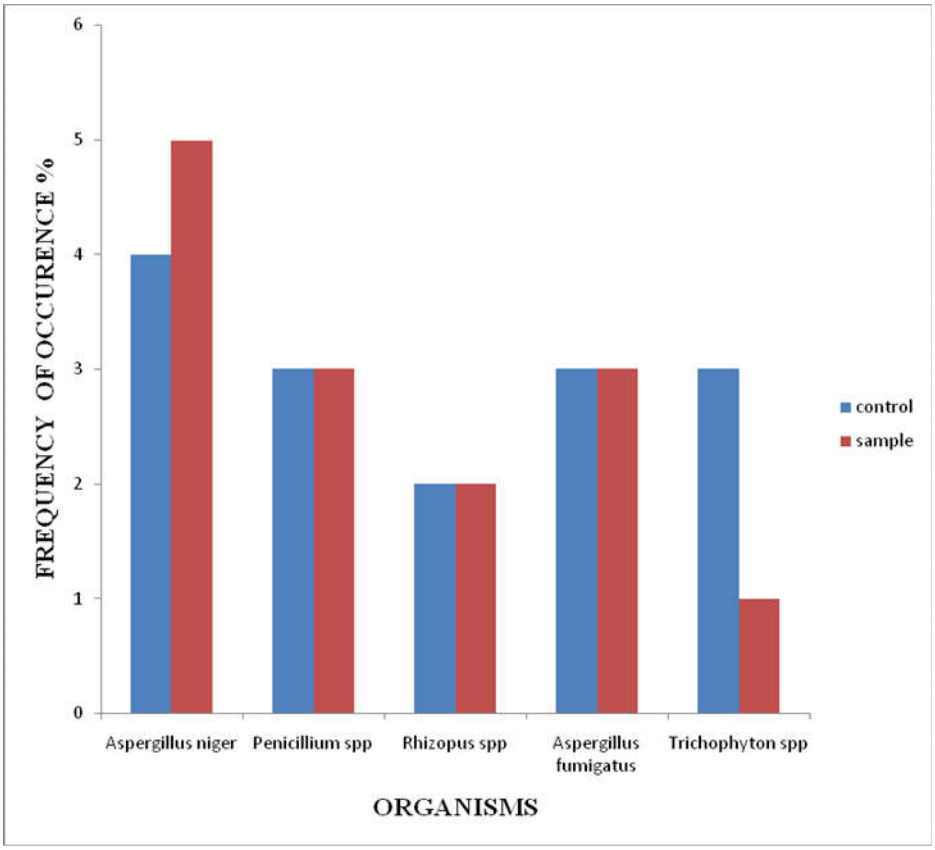

Figure 8. %Frequency of Total Heterotrophic Fungi

This observation was also made by Sataya (2012). The growth rate of each organism was monitored and it can be summarized as follows: There was no much growth on Days 2 and 3 which is illustrated in Figures 3 and 4. There was a rapid increase in the growth rate on Day 5, which is illustrated in Figure 5. There was a decline in growth on Day 6 and 7, which is illustrated in Figure 7 and 8.

The organisms isolated were *Staphylococcus* spp, *Bacillus* spp, *Clostridium* spp, *Micrococcus* spp and *Pseudomonas* spp, which are mostly soil organisms. This is similar to the results obtained by Agary*et al.,* (2008), Boboye *et al* (2010). Results showed that *Staphylococcus* spp, *Bacillus* spp and *Pseudomonas* spputilized phenol as a carbon source for growth which is illustrated in Figures 3, 4, 7 and 8.

The fungal isolates had negative growth rate. Only *Aspergillus niger* was able to utilize phenol for growth on Day 6 as illustrated in Table 4. It is evident from this study that some petroleum hydrocarbon organisms are phenol utilizing organisms, which can be used extensively in other research works and can be applied in bioremediation processes.

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