Isolation of 2,4-Dichlorophenol Tolerant Bacteria in Wastewater Effluent from University of Nigeria Sewage Treatment Plant Site.

Godwin E. Oyiwona. 'Chukwudi Uzoma AnyanwuJ Lewis Ezeogu

Department of Microbiology, Faculty of Biological Sciences, University of Nigeria, Nsukka, Nigeria

chukwudi.anyanwu@unn.edu.ng (Chukwudi E. Anyanwu)lewis.ezeogu@unn.edu.ng(Lewis Ezeogu

Tel.:+234-8061507594

Abstract: Phenol tolerant bacteria were isolated from sewage treatment site. Sixteen bacterial colonies were isolated and tested in 100-500 mg/L of 2,4-Dichlorophenol (2,4-DCP). After screening, two isolates which showed high phenol resistances were selected. Both isolates, XC, and N3 were able to tolerate 200 mg/L of 2,4-DCP but isolate N3 tolerated 400 mg/L of 2,4-DCP. Isolate N3 was identified as *Bacillus pasteurii* while isolate XC was identified as *Aeromonas hyrophila. Bacillus pasteurii* was selected for further studies and was subjected to plasmid curing experiment which confirmed that 2,4-DCP resistant trait was mediated by a single plasmid of approximately 22kb. Amendment with organic nitrogen sources, to enhance the tolerance of 2,4-DCP by *Bacillus pasteurii* showed that the maximum 2,4-DCP tolerance level of 300mg/L at an optical density(OD) of 0.35 which was 133% higher than that obtained for control was achieved at 0.5mg/L of peptone. Similarly, a maximum 2.4-DCP tolerance level of 300mg/L at an GD of 0.24 which was 60% higher than that obtained for control was achieved at 0.5 mg/L of yeast extract. A maximum 2,4-DCP tolerance level of 300mg/L at an optical density of 0.19 which was 26% higher than that obtained for control was achieved at 0.5mg/L of tryptone. In all experiments, it was observed that the decrease in inhibition capability of 2,4-DCP and the increase in the tolerance capacity of the strains are synonymous. Among the nitrogen sources tested, peptone showed a significantly ($P < 0.05$, Student's t-Test) higher level of enhancement of 2,4-DCP tolerance by *Bacillus pasteurii.*

Key words: 2,4-dichlorophenol, phenol tolerant, organic nitrogen sources, plasmid DNA, *Bacillus pasteurii*

Introduction

Chlorophenols are xenobiotic contaminants, which form a significant part of all organic chemicals either produced or used by many industries such as petrochemicals, oil refineries, plastics, insulation materials, pesticides, biocides, pulp and wood preservers. Due to their high toxicity, strong odor emission, persistence in the environment and suspected carcinogenicity, chlorophenols pose critical ecological issues (Kusic *et al.* 2011).Most of the chlorophenols have been included in the US Environmental Protection Agency EPA list of priority pollutants (Basak *et al.* 2013).The environmental regulations in many countries specify the maximum allowable concentration of phenols in the industrial effluents to be less than 1 mg L^{-1} (Kusic *et al.,* 2011). However, higher concentrations were frequently found in contaminated environments, with reported levels of chlorophenols ranging from 0.15 to 200 mg L^{-1} and even more (Angelini *et al.* 2011).The removal of chlorophenolic compounds from wastewater is a necessary task to conserve the quality of natural resources (Ursal and Turkman, 2005). Therefore, to save the soils and aqueous ecosystems, pretreatment of industrial wastewater has been mandatory worldwide. Phenolic compounds have been reported to be highly stable due to the difficulty of cleaving the benzene ring (Annadurai et al. 2008).

*Corresponding author:

godwinloveth1@gmail.com ***Godwin E. Oyiwona***

The conventional methods of removing phenols from wastewater are very expensive. Degrading phenols and phenolic compounds by microorganisms is an alternative technology. A variety of microorganisms are known to utilize phenol as the sole carbon or energy source. Exploring the ability of microorganisms to metabolize phenols has received much attention due to their environmental persistence and their toxicity (Weta and Dhandayuthapani, 2013).

As for chlorophenols, they are even more resistant to biodegradation as the enzyme activity needed for ring cleavage could be suppressed by the chloride atom (Wang *et al.* 2007a).Biological treatment of phenols has emerged as an increasingly important method in pollution management (El-Naas *et al.* 2010a). Compared with physico-chemical methods, the biodegradation method of phenols removal is widely preferred as a more environmental friendly and cost effective approach, due to the possibility of complete mineralization of phenol or its derivative inorganic mineral constituents with harmless end products and minimum secondary metabolites (Sridevi *et al.* 2011; Liu *et al.* 2009; Wang *et al.* 2000; Nair *et al.* 2008). Novel bioremediation strategies are rising as a favorable and most promising green technology for the treatment of wastewaters (Menale *et al.* 2012).In a recent study (Bhishma and Kumar, 2016), 2,4-DCP degradation was enhanced by the optimization of experimental parameters using kinetic study. In a related recent study, Taghreed *et al.,* (2017) optimized the process parameters for the aerobic biodegradation of

2, 4 DCP, using an immobilized commercial strain *of P. putida,* by Response Surface Methodology (RSM) experiments.

Since the biodegradation of chlorophenols is highly dependent on many conditions, such as pH, temperature, initial substrate concentration and others, it is critical to evaluate the effects of the key influencing parameters. Optimization of the biodegradation conditions is a prerequisite to large-scale applications of biodegradation processes (Basak et al. 2013). Optimization techniques have been employed to tackle important issues of contaminated site management (Bhattacharya et al. 2009).In order to find an effective method for treating these wastes, much attention has been directed towards an understanding of biogenic transformation of phenol, via ring-cleavage and dechlorination of chlorinated aromatic pollutants (Gallizia *et al.* 2003).

Plasmids are important in the overall physiology and survival of many bacteria. They carry genes that may be involved in virulence, degradation of toxic compounds, and antibiotic and heavy metal resistance, as well as their own transfer among species or even genera (Kunnimalaiyaan and Vary, 2005).

The objective of the present study is to evaluate the efficiency of supplementation of organic nitrogen sources in the optimization the tolerance of 2, 4 DCP by bacterial isolate *Bacillus* sp to achieve prolific utilization of 2,4-DCP as carbon source. Organic nitrogen sources were added as supplementary nutrients into medium to enhance 2,4-DCP utilization and to elucidate whether the pesticide resistance trait is plasmid-encoded or chromosomal. Thakur et al., (2002) reported the role of plasmids in pentachlophenol utilization as sole carbon and energy source by *Pseudomonas* sp. but little or no study have reported the role of plasmid in 2,4-DCP utilization as sole carbon and energy source by bacteria. The different types of organic nitrogen sources were meant to elucidate which of them would enhance more tolerance of 2,4-DCP by the isolate.

Materials and methods

Collection and pretreatment of soil samples

Soil samples (about 20g) were collected from the University of Nigeria sewage treatment plant site at Nsukka as source of inoculum. In order to enrich for bacteria, soils were air-dried in the sun for 48 to 72 hours at 28°C to 45°C. The air-dried soils are sieved through a 3mm screen to remove large aggregates, particulate matters and un-decomposed residues.

Enrichment, screening and isolation of 2, 4-dichlorophenol tolerant bacteria

The bacterial species were isolated by using an enrichment medium known as the mineral salt medium (MSM) comprising (g/l distilled water): K_2HPO_4, 0.65; KH_2PO_4, 0.17; $(NH_4)_2SO_4$, 0.1; $FeSO_4$, 0.14; $MgSO_4.7H_2O$, 0.1; Na_2HPO_4, 7; $CaCl_2.2H_2O$, 0.1; Trace elements solution containing: $NaMoO_4.2H_2O$, 0.05 and $ZnSO_4.7H_2O$, 0.05.

One point five grams of the sun-dried soil was added to a pre-sterilized flasks containing 50 ml of enrichment culture medium supplemented with 100 mg/L of 2,4-Dichlorophenol [dissolved in dimethylsulphooxide (DMSO) and added after autoclaving the MSM] as the sole carbon and energy source. It was incubated at 120 rpm at 30°c in a Gallenkamp orbital shaker (1H-460, England) for five (5) days. Growth was observed by the monitoring the turbidity of the flask. One milliliter of samples were taken from the flask and streaked repeatedly on solid agar media containing the MSM and 15.0 g/L pure agar supplemented with yeast extract (0.02g) for the isolation of discrete colonies. Enrichments were carried out by inoculating 100 ml Erlenmeyer flasks containing 50 ml of MSM with different concentrations of 2,4-DCP (100-500 mg/L) after autoclaving.

Cultures were maintained on MSM containing 100-500 mg/L of 2, 4-DCP. Inocula were prepared by transferring loopfuls of colonies grown on agar plates into100ml Erlenmeyer flasks containing 50ml of the MSM and 2,4-DCP. The flasks were again incubated on orbital shaker at 30°c at 120rpm. Colonies were purified by repeated sub culturing on fresh MSM agar plates previously sterilized at 121°C for 15 min in an autoclave. By this procedure, sixteen (16) different distinct isolates were obtained.

Characterization of isolates

Biochemical and microbiological characterization of the bacterial colonies was carried out according to Cowan and Steel's Manual for *Identification of Medical bacteria (Colman,* 1993). Individual colonies were characterized on the basis of colony morphology (shape, size, texture and colour) and gram staining. Streak plate method was used to obtain single colonies. The isolated colony was streaked on nutrient agar slants and incubated at 37°C for 24 h to obtain optimum growth. The preliminary characterization was based on colony morphology on nutrient agar plates after 2 - 3 days at 32°C of incubation. Out of the sixteen isolates obtained, only two which gave higher optical density (OD) reading than others (using Spectronic 20D spectrophotometer Cole-Parmer Instr. Comp., Vernon Hills, USA) were selected for further study. They were identified using conventional biochemical procedures as *Aeromonas* sp and *Bacillus* sp

Effect of supplementation of organic nitrogen sources on 2,4-DCP tolerance by isolates

The influence of some organic nitrogen sources namely; yeast extract, peptone and tryptone on the toxicity of 2,4DCP to the isolates were studied. Fifty milliliter volumes of MSM were dispensed into eight 250ml Erlenmeyer flasks in triplicates. The same amount of the nitrogen sources were incorporated

(1mg/L). After sterilizing in an autoclave at 121°C for 15minutes, different concentrations of 2, 4-DCP were inoculated with the isolates and incubated with shaking for 72 hours. At 12 hourly intervals, 5.0ml volumes were withdrawn and assayed for OD at A_{600nm}). The pH of the medium was maintained in the range of 6.8-7.0 using phosphate buffer comprising (g/L): K_2HPO_4, 5.1 and KH_2PO_4 2.9 and adjusted with 1N NaOH.

Plasmid DNA isolation

Plasmid DNA was isolated from *Bacillus* sp, which had the peak OD value of 0.09 at highest 2,4-DCP concentration of 350 mg/L. *Aeromonas* sp had an OD value of 0.06 at the peak concentration of 200mg/L. The protocol of Kado and Liu (1981), was used with slight modifications. The isolate was grown in a 3ml double strength nutrient broth for 24 hours at 37°C. The overnight culture was spun at 4,500 x g for 10 minutes. The supernatant was decanted, pellets vortexed at a high speed for 5 minutes. Thereafter, 300ml of Tris-EDTA (TE) buffer and 150 ml of 3.0M aqueous Sodium acetate was added at pH 5.2 and vortexed for 5 min and a further 2 min in a micro centrifuge (Biofuge sepatech Co. Ltd, USA). The pellets were transferred to a fresh tube, mixed well with 0.9 ml of 90% ethanol that was pre-cooled to -20°C.

It was centrifuged again for 2 minutes and supernatant decanted. The pellet was rinsed twice with 1ml of 70% ethanol and was dried under vacuum for 3 min. Thereafter, it was re-suspended in 20 µl of TE buffer and analysed by agarose gel electrophoresis (AGE) on 0.8% (w/v) agarose gels stained with ethidium bromide and visualized under UV light

Plasmid curing

Plasmid curing was performed to investigate if the 2, 4-DCP resistance trait was plasmid or chromosomally encoded. The isolate *Bacillus* was subjected to plasmid curing at 40°C to check the loss of 2,4-DCP resistance trait following Deb Mendal et al., (2005) with slight modifications. The isolate was grown in a 3ml nutrient broth for 24 hrs. The curing agent incorporated into the medium was 0.003mg/ml acridine orange dye. The overnight culture was centrifuged at 4,500 x g for 10 minutes. The supernatants decanted and the cell pellets washed twice with 300ml TE buffer. The washed cells were plated on MSM agar plates containing 100-200 mg/L 2,4-DCP and nutrient agar plates containing 25 mg/L of 2,4-DCP. Positive control containing only cells (without the curing agent) and negative control containing only acridine orange were also run and all tubes were incubated. Following the spreading technique, equal volumes of inoculum from different concentrations were spread on nutrient agar plates in which growth and clear zones of hydrolysis of culture were observed.

Statistical analysis

Both students' T-test and correlation coefficients(r), to evaluate the variance effects of all treatments, were used to determine the level of significant difference between different means and $P < 0.05$ was considered statistically significant.

Results and Discussion

Characterization of isolates

Out of the 16 isolates, two strains which showed higher optical density (OD) values in the 24 to 48 hrs of incubation were selected for further studies. Biochemical characterization of the isolates as shown in Table 1 tentatively identified them as *Bacillus* sp *and Aeromonas* sp respectively. When re-streaked on MSM agar containing different concentrations of 2,4-DCP, *Bacillus* showed a maximum 2 4-DCP tolerance of 350 mg/L while *Aeromonas* showed its maximum tolerance of 200 mg/L (Table 2). Therefore, strain *Bacillus* was selected for further studies. Morphologically, *Bacillus* colonies were usually circular and glossy.

Table1. Growth and biochemical characteristics of isolates obtained from UNN waste treatment site

Tests	Reactions and appearance	
	XC	N3
Shape	Rod	rod
Motility	+	+
Gram stain	-	+
Spore	-	+
Growth tests		
Glucose	A/G	A
Maltose	A	A
Sucrose	A	-
Lactose	-	-
Starch hydrolysis	+	-
Growth at 41°C	-	+
Biochemical tests		
Oxidase	+	+
Catalase	+	+
Indole	-	-
Oxidative fermentation (O/F)	O	O
Citrate	-	+
Methy red (MR)	-	-
Voges proskeur (VP)	+	+
Nitrate reduction	-	+
Mannitol	+	+
Galactose	+	-
Plasmid	-	+

Organisms identified:
Aeromonas hydrophila (XC), *Bacillus pasteurii* (N3)
+ Positive reaction
- Negative reaction
A Acid production
G Gas production
O Oxidative

Table 2. Tolerance of bacterial isolates to concentrations of 2,4-DCP

Isolates	Concentration of 2,4-DCP (mg/L)							
	50	100	150	200	250	300	350	400
Bacillus sp (A_{620nm})	0.18	0.17	0.15	0.12	0.11	0.09	0.05	0.01
Aeromonas sp (A_{620nm})	0.1	0.09	0.08	0.06	0	0	0	0

Effect of amendment with 0.5mg/L of organic nitrogen sources on 2, 4-dichlorophenol tolerance

Figure 1 shows tolerance level of *Bacillus* sp and its cured mutantto 2,4-DCP in MSM supplemented with organic nitrogen sources. The wild strain supplemented with peptone [MSdP(O)] tolerated up to 500 mg/L of 2,4-DCP; with an OD of 0.09. However, the growth was prolific up to 300 mg/L with an OD of 0.35, showing high peaks in the presence of 200–300 mg/L. Beyond 400mg/L, the chlorophenol started showing inhibitory effects. Although its cured mutant [MSdP(C)] tolerated up to 250 mg/L of 2,4-DCP, with an OD of 0.02, 300mg/L 2,4-DCP was inhibitory.

The wild strain supplemented with yeast extract [MSdY(O)] tolerated up to 500 mg/L of 2,4-DCP; with an OD of 0.04. However, the growth was prolific up to 300 mg/L with an OD of 0.24. Beyond 400mg/L, the phenol showed inhibitory effects. Its cured mutant [MSdY(C)] tolerated 2,4-DCP up to 150 mg/L of 2,4-DCP, with an OD of 0.03, 200mg/L 2,4-DCP was inhibitory.

The wild strain supplemented with tryptone [MSdT(O)] tolerated up to 500 mg/L of 2,4-DCP; with an OD of 0.02. However, the growth was prolific up to 300 mg/L with an OD of 0.19. Beyond 300mg/L, the phenol showed inhibitory effects. Its cured mutant [MSdT(C)] tolerated 2,4-DCP up to 150 mg/L of 2,4-DCP, with an OD of 0.01, 200mg/L 2,4-DCP was inhibitory.

Growth on 2,4-DCP provided as the sole carbon source [MSd(O)] was studied in MSM. The *B. Pasteurii* was found to show capability of utilizing 2,4-DCP up to 400 mg/L in MSM (OD,0.04), with peak growth at 300mg/L (OD,0.15), after an incubation for 24 hours at 28°C. Its cured mutant [MSd(C)] tolerated 2,4-DCP up to 100 mg/L of 2,4-DCP, with an OD of 0.01, beyond 100mg/L 2,4-DCP was inhibitory. Among the nitrogen sources tested, peptone showed a significantly higher enhancement of tolerance of *Bacillus* to 2,4-DCP ($P < 0.05$, Student's t-Test) (OD 0.35 at 2,4-DCP concentration of 300mg/L) (Fig.1). This suggests that the toxicity of 2,4-DCP to *Bacillus* was reduced by supplementing the MSM with peptone. This is consistent with the report of Kotresha and Vidyasagar (2008) that the enhanced rate of phenol degradation by peptone can be attributed to the attenuation of phenol toxicity by peptone, and the increase in cell mass formed as a result of the additional nitrogen source. However, increase in tryptone concentration failed to significantly improve the rate of 2,4-DCP tolerance by the isolate.

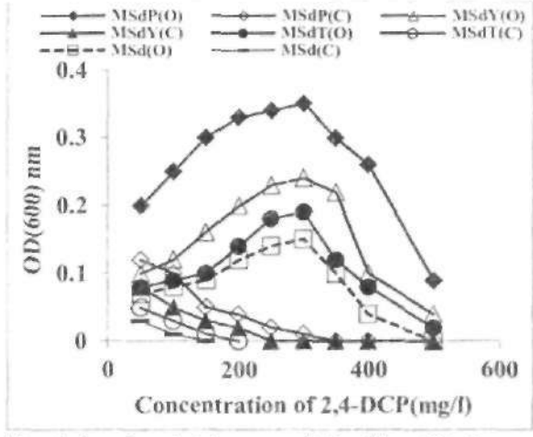

Fig. 1 Levels of tolerance of *Bacillus* (O), its cured mutant (C) to 2,4-DCP in MS Mamended with 2,4-DCP: peptone (MSdP), yeast extract (MSdY) and tryptone (MSdT),2,4-DCP only (MSd).

Plasmid profiles

Fig. 2 shows the electrophoretic profiles of the isolated plasmid DNA from *Bacillus*. In the present study, the cured strains, were unable to tolerate 2,4-DCP in MSM with or without nitrogen sources, beyond 200 mg/L as the growth of the cured mutants were completely inhibited by 2,4-DCP at concentrations > 250 mg/L. A single plasmid of approximately 22 Kb conferring phenol tolerance property was isolated from the soil bacterium *Bacillus* sp. This is consistent with the findings of Thakur et al.,(2002) that two different plasmids of molecular sizes 80 and 4 Kb, respectively, obtained from *Pseudomonas* sp, were found to be responsible for carrying genes for the utilization of pentachlophenol as sole carbon and energy source.

In this study, *Bacillus* sp tolerated 2,4-DCP up to the concentration of 300 mg/L in MS medium. The strain showed a maximum optical density of 0.15 in MS medium without amendment with organic nitrogen sources after 24 hours incubation at 28°C.

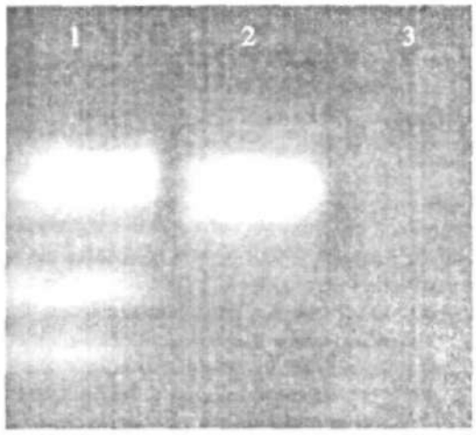

Fig. 2. Agarose gel electrophoretic profiles of isolated plasmid DNA of *Bacillus* sp.
Lane 1:23 kb ladder, lane 2: *Bacillus sp* lane 3:cured *Bacillus sp.*

Conclusion

The present study showed the peptone-enhanced tolerance and ultimately utilization of the toxic 2, 4 DCP, up to 400 mg/L as carbon and energy source by wastewater isolate *Bacillus* sp.The discovery of bacteria capable of tolerating or growing in high concentrations of phenol provides a potentially interesting avenue for treating phenolic wastes. The tolerance of high concentrations of 2,4-DCP by the *Bacillus* sp. was mediated by plasmid, of approximately 22 kb. Thus the results obtained from the present investigation permits us to conclude that the pesticide resistant *Bacillus* isolated in this study could be used as potential bioagent for bioremediation of 2,4-DCP contaminated field soil. The potential of *Bacillus* as bio-agent in cleanup of contaminated pesticide waste has been brought to the fore in this study. Although biodegradation of chlophenolic pesticides cannot occur without first carrying out tests on the growth and tolerance of the bio-agent on the pesticide as carried out in this study, further studies need to be carried out to confirm chloride release into the medium with time.

Acknowledgment

The authors thank the Microbiology Department, University of Nigeria Nsukka for providing an enabling environment for this research. We appreciate the efforts of Prof (Mrs) I.M. Ezeonu for helping out in the plasmid DNA isolation and the curing experiments.

References

Angelini, V.A., Orejas, J., Medina, M.I., Agostini, E. (2011). Scale up of 2,4-Dichlorophenol removal from aqueous solutions using *Brassica napus* hairy roots. *J Hazardous Mater*, 185: 269-274. DOI: 10.1016/j.jhazmat.2010.09.028.

Annadurai, G., Ling, L.Y.,Lee, J.F. (2008). Statistical optimization of medium components and growth conditions by response surface methodology to enhance phenol degradation by *Pseudomonas putida*. *J Hazardous Mater*, 151: 171-178. DOI: 10.1016/j.jhazmat.2007.05.061.

Bhattacharya, S.S., Karmakar, S., Banerjee, R.(2009). Optimization of Laccase mediated biodegradation of 2,4-Dichlorophenol using genetic algorithm. *Water Res*, 43: 3503-3510. DOI: 10.1016/j.watres.2009.05.012.

Basak, B., Bhunia, B., Dutta,S., Dey, A. (2013). Enhanced biodegradation of 4-chlorophenol by *Candida tropicalis* PHB5 via optimization of physicochemical parameters using Taguchi orthogonal array approach. *Int Biodeteriorat Biodegradat*, 78: 17-23. DOI: 10.1016/j.ibiod.2012.12.005.

Bhishma, P.P., Kumar, A.(2016). Biodegradation of 2,4-dichlorophenol by *Bacillus endophyticus* strain: optimization of experimental parameters using response surface methodology and kinetic study. *Desalin Wat Treat.* 57(34).15932-15940.

Colman, G., Fox, E., Gross, R.J., Holmes, B., Jenkins, P.A., Jones, D.M. (1993). *"Theory and practice of bacterial identification,"* in Cowan and Steel's Manual for the Identification of Medical Bacteria, G. I. Barrow and R. K. A. Feltham, Eds., p. 21e44, Cambridge University Press, Cambridge.

D'angelo, E.M., Reddy, K.R. (2002). Aerobic and anaerobic transformation of pentachlorophenol in wetland soil. *Soil Sci Soc Americ* J, 64:933-943.

Deb Mandal, M., Mandal, S., Pal. N.K. (2005). Plasmid-Mediated Dimethoate Degradation by *Bacillus licheniformis* Isolated From a FreshWater Fish *Labeo rohita*,*J Biomed Biotech.* 3: 280–286 DOI: 10.1155/JBB.2005.280.

El-Naas, M.H., Al-Zuhair, S., Makhlouf, S.(2010a.)Batch degradation of phenol in a spouted bed bioreactor system. *J Industr Eng Chem*, 16: 267-272. DOI: 10.1016/j.jiec.2009.09.072

Gallizia,I., Mclean, S., Ibrahim, M.B. (2003). Bacterial biodegradation of phenol and 2,4-Dichlorophenol. *J Chem Tech Biotech.* 78: 959-963.

Igbinosa, E.O., Odjadjare, E.E., Chigor,V.N., Igbinosa, I.N., Emoghene, A.O., Fredrick O. Nicholas, E., Igiehon, O., Idemudia, O.G. (2013). Toxicological profile of chlorophenols and their derivatives in the environment: The public health perspective. *The Sci World J* http://dx.doi.org/10.1155/2013/460215. 1-11.

Kado, C.I. Liu, S.T.(1981). Rapid procedure for detection and isolation of large and small plasmids. *J Bact*, 145:1365-1373.

Kotresha, D.,Vidyasagar, G.M. (2008). Isolation and characterisation of phenol-degrading *Pseudomonas aeruginosa* MTCC 4996. *World J Microbiol Biotechnol*, 24:541–547. DOI 10.1007/s11274-007-9508-2.

Kunnimalaiyaan, M.,Vary, P.S.(2005). Molecular characterization of plasmid pBM300 from *Bacillus megaterium* QM B1551.*Appl Environ Microbiol*, 71(6): 3068–3076 doi:10.1128/AEM.71.6.3068–3076.2005.

Kusic, H., Koprivanac, N., Bozic, A.L. (2011). Treatment of chlorophenols in water matrix by UV/ferrioxalate system: Part I. Key process parameter evaluation by response surface methodology. *Desalination*, 279: 258-268. DOI: 10.1016.

Liu, Y.J., Zhang, A.N., Wang, X.C. (2009). Biodegradation of phenol by using free and immobilized cells of *Acinetobacter* sp. XA05 and *Sphingomonas* sp. FG03. *Biochem Eng J*, 44: 187-192. DOI: 10.1016/j.bej.2008.12.001.

Menale, C., Nicolucci,C., Catapane, M., Rossi, S.,Bencivenga, U. et al.(2012). Optimization of operational conditions for biodegradation of chlorophenols by laccase-polyacrilonitrile beads system. *J Molecular Catalysis* B: Enzymatic, 78: 38-44. DOI: 10.1016/j.molcatb.2012.01.021.

Nair, C.I., Jayachandran, K., Shashidhar, S. (2008). Biodegradation of phenol. *Afric J. Biotechnol*. 7: 4951-4958. DOI: 10.5897/AJB08.087.

Sridevi, V., Lakshmi, M.V., Swamy, A.V., Rao, M.N. (2011). Implementation of response surface methodology for phenol degradation using *Pseudomonas putida* (NCIM 2102). *J Bioremediat Biodegradat*, DOI: 10.4172/2155-6199.1000121.

Thakur, I.S., Verma, P.K., Upadhaya, K.C. (2002). Involvement of Plasmid in Degradation of Pentachlorophenol by *Pseudomonas* sp. from a chemostat. *Biochem Biophys Res Com*, 286(1): 109-113.

Taghreed, Al-K., Muftah, H. E.N.(2017). Biodegradation of 2, 4 Dichlorophenol. *Amer J Eng Appl Sci* 10 (1): 175.191 DOI: 10.3844/ajeassp.2017.175.191.

Ursal, A., Turkman, A.(2005). Effect of biosurfactant on 2,4-Dichlorophenol biodegradation in activated sludge bioreactor. *Pro Biochem*. 40: 2745-2749.

Wang, C.C., Lee, C.M., Kuan, C.H. (2000). Removal of 2,4-Diclorophenol by suspended and immobilized *Bacillus insolitus*. *Chemosp*,41: 447-452.

Wang, S.G., Liu, X.W., Zhang, H.Y., Gong, W.X., Sun, X.F. et al. (2007a). Aerobic granulation for 2,4-dichlorophenol biodegradation in a sequencing batch reactor. *Chemosp*, 69: 769-775.DOI: 10.1016/j.chemosphere.2007.05.026.

Weta, S.H and K. Dhandayuthapani (2013).Influence of media supplements on phenol biodegradation by *Pseudomonas aeruginosa* SPD10 *Int. J. Curr. Microbiol. App. Sci.* 2(6): 64-69.