INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

www.ijabpt.com Volume-5, Issue-4, Oct-Dec-2014 Coden : IJABPT Copyrights@2014

ISSN : 0976-4550

Received: 28th July-2014 *Revised: 04th Oct-2014* *Accepted: 05th Oct-2014*

Research article

BIODEGRADATION OF PHENOL AND EMULSIFICATION PROPERTIES OF NATIVE MICROORGANISMS FROM COAL CARBONIZATION PLANT

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ABSTRACT: Phenols (aromatic organic compounds) are commonly occurring organic pollutants in the environment. Some of the toxic effects of phenol in humans are corrosive to eyes, skin and respiratory track, in addition also causes lethal effects in animal and plant community when discharged even in low concentrations. The problem associated with phenol in the environment, is in need to removal such hazardous compound. Considering the importance, the present investigation attempts for degradation of phenol by native bacterial strains isolated from coal carbonization plant wastewater. About 36 bacterial isolates were found to exhibit phenol degrading activity. Among them, higher phenol degrading strains were identified tentatively as CA_1 – *Rhodococcus* sp., CA_2 – *Bacillus* sp., CA_3 – *Pseudomonas* sp. and CA_4 –*Citrobacter* sp. Phenol degrading ability of *Rhodococcus* sp. was found to be 60% with maximum cell dry weight. Surface active compounds were observed to be 1.31G for *Rhodococcus* sp, 0.85 G for *Bacillus* sp, 1.26 G for *Pseudomonas* sp. and 1.02 G for *Citrobacter* sp. In case of mixed cultures, 1.62 G maximum rate among the tested was observed. The Emulsification Index (E_{24}) for CA_1, CA_2, CA_3, CA_4 and mixed culture was observed as 78%, 65%, 59%, 22% and 80% respectively. The plasmid profile was additionally studied for the isolates. Significantly, the study reveals the potentials of native strain, *Rhodococcus* sp. (CA_1), in degradation of phenols.

Key words: Phenol, degradation, Biosurfactant, and Emulsification Index

INTRODUCTION

Phenols are hydroxy compounds of aromatic hydrocarbons often found in wastewaters from coal gasification, coke-oven batteries, refinery and petrochemical plants and other industries, such as synthetic chemicals, herbicides, pesticides, antioxidants, pulp and paper, photo developing chemicals, etc. (Chakraborty *et al.,* 2010). Phenols are one of the commonly occurring organic pollutants in the environment. These phenolic compounds have high toxicity to most of the microorganisms, plants, fish and animals which can cause a stern damage to the environment. The associated problem due to phenol is that when inhalation and dermal contact causes cardiovascular diseases and severe skin damage, while ingestion can cause serious gastrointestinal damage (Ho *et al.,* 2009). Therefore, the removal of such toxic compound from the environment is of great importance.

Though various treatment methods are available for the reduction of phenol content in wastewater (Arutchelvan *et al*., 2006) such as hybrid process, electrocatalytic degradation, adsorption on to different matrices, chemical oxidation, solvent extraction or irradiation, flocculation (Wei *et al*., 2008), they pose other problems both economically and environmentally *viz*., higher price and formation of hazardous byproducts. Among different methods available for the removal of phenol, biological treatment is the most promising and versatile approach that has been proved to be economical as well as eco-friendly.

Number of studies on biodegradation of phenol by means of pure and mixed cultures have been accounted (Collins *et al.,* 2005; Dursun and Tepe, 2005; Razika *et al*., 2010; Nor *et al*., 2010). In this study, we investigated (i) the isolation of phenol degrading native bacterial strains from coal carbonization plant and to test its phenol degradation efficiency (iii) to investigate the biosurfactant and emulsification properties and (iv) to observe the plasmid profile of the native isolates.

MATERIALS AND METHODS

Chemicals and Reagents

Analytical grade phenol used in the study was purchased from Merck, India. All other chemicals were also of analytical grade which were purchased from Merck, India and Hi-Media laboratories, India

Collection of sample

Soil and wastewater samples were collected from coal carbonization plant, Neyveli Lignite Corporation (NLC), Tamil Nadu. Clean, sterile sampling bags and bottles were used for collection of samples. The collected soil samples were brought to the laboratory for isolation of bacterial strains.

Enrichment and isolation of phenol degrading bacteria

Water (5 mL) and soil (3 G) samples were inoculated in sterile Mineral Salt Medium (MSM) (GL^{-1})(K_2HPO_4 - 0.4; KH_2PO_4 - 0.2; NaCl - 0.1; $MgSO_4$- 0.1; $MnSO_4.H_20$ - 0.01; $Fe_2(SO_4).H_2O$ - 0.01; $NaMoO_4.2H_2O$ - 0.01; $(NH_4)_2SO_4$ - 0.4) supplemented with filter-sterilized phenol (0.1%) as a carbon source (Maulin P. Shah, 2014; Siti *et al.,* 2011). The inoculated flasks were incubated at 30°C, 150 rpm for about 3 days. Following, serial dilutions of cultured broth were prepared and spread to the Mineral Salt agar plates supplemented with phenol (0.1 %) and again incubated at 30°C for a period 3 days in both broth cultures and slant culture. Isolates that showed distinct single colonies were further purified by repeated sub-culturing, and the pure isolates were stored on MSM agar slants at 4°C until further use.

Selection of efficient phenol degrading microorganisms

Isolated bacterial cultures were inoculated in 150 mL Mineral Salt Medium (MSM) containing 0.15 G phenol *viz*., 0.1% and incubated in a shaking incubator, 30°C at 150 rpm for 48 h. Phenol degradation was monitored after 48 h by measuring the optical density ($O.D_{600nm}$) at UV visible spectrophotometer (JASCO). The isolates exhibited highest phenol degrading activity was chosen and used for further studies.

Identification of isolates

The selective bacterial isolates were identified based on morphologically and biochemically (data not shown). Biochemical identification was performed according to the standard manual (Bergey's manual of Determinative Bacteriology) for all the four isolates (GoodFellow, 1994).

Batch experiments

The isolated four bacterial cultures both individually and combined state (consortium) (1:1:1:1) were inoculated in 100 mL of Mineral Salt Medium (MSM) supplemented with phenol at 0.1% concentration. The inoculated flasks were incubated at 30°C in a rotary orbital shaker. The samples were withdrawn at regular time (24 h) intervals, centrifuged at 10000 rpm and the supernatant was analyzed for residual phenol concentration. Each experiments were carried out for a period up to 240 h or until the residual concentration of phenol in flask was found to be saturated with respect to time (Nor *et al.,* 2010).

Biomass analysis

Cell concentration in terms of cell dry weight was determined by filtration and oven dried method (Debadatta and Rajdeep, 2012).

Analytical (4-Amino Antipyrine) method

Samples from regular intervals were centrifuged at 10,000 rpm for 3 min and the supernatant was transferred to a separating flask along with equal volume of petroleum ether and vigorously shaken. The separated phenol was collected in a clean dry test tube. The residual phenol was allowed to react with 4-Amino Antipyrine at pH 7.9 ± 0.1, in the presence of Potassium ferricyanide, which forms a colored solution. Antipyrine dye was determined by the method of Maulin P. Shah, (2014) and Yang and Humphrey (1975).

To perform the analysis, 70 µL of 0.5 N NH_4OH solution was added into the sample and pH was adjusted to 7.9 ± 0.1 with phosphate buffer (PB), 30 µL of 4-Amino Antipyrine solution was added and mixed well and 30 µL of $K_3Fe(CN)_6$ solution was added under alkaline conditions which produces a red-colored product was measured at 500 nm.

Biosurfactant extraction and determination method

The four isolated organisms were transferred to 50 mL of Mineral Salt Medium (MSM) with phenol supplementation at 1 % concentration as carbon source. The culture flasks were incubated at 30°C for 5 days in a shaking incubator at 150 rpm. At the end of incubation, cells were centrifuged at 12,000 rpm for 15 min at 4°C. The biosurfactants were extracted from the supernatant by mixing ethyl acetate on mild shaking at room temperature for overnight. The solvent was evaporated and the oily residue was dissolved in 1 mL of methanol for further screening (Vijaya *et al.,* 2013).

The biosurfactant from the isolate was estimated using orcinol assay method (Saravanan and Vijayakumar, 2012). To 100 µL of each biosurfactants, 900 µL of a solution containing 0.19% orcinol (in 53% H_2SO_4) was added. Control was prepared with distilled water. Samples were boiled for 30 min at 80ºC and then were cooled at room temperature further the optical density (OD) at 421 nm was measured and expressed as rhamnose equivalents (RE) (GL^{-1}).

Emulsification activity assay

The extracted biosurfactant was diluted to 1 $GL^{-1}H_2O$, based on its solid yield. The emulsification activity was measured by the method of Cooper and Goldenberg, (1987).

Briefly, 4 mL of biosurfactant solution was added to 6 mL of kerosene. The mixture was vortexed at 3000 rpm for 2 min. The emulsion stability was determined after 24 h and the emulsification index (E24) calculated by dividing the measured height of emulsion layer by the mixture's total height and multiplying by 100. For the control test, the 4 mL biosurfactant in the above protocol was replaced with water (Anyanwu and Chukwudi, 2010).

Plasmid profile of isolates

The isolation of plasmid DNA (data not shown) for all the isolates namely *Rhodococcus* sp., *Bacillus* sp., *Pseudomonas* sp. and *Citrobacter* sp were performed according to Birnboim (1983).

RESULTS AND DISCUSSION

Isolation and selection of efficient phenol degrading bacteria

Total 36 bacterial isolates from the soil and water samples collected at coal carbonization plant, were found to exhibit phenol degrading activity at varying degrees. The isolates that showed maximum growth in the cultivation medium (MSM) degraded the phenol to maximum whereas the isolates that showed a subtle growth in the medium was found to degrade phenol at very low level. This clearly depicts that, the percentage of degradation rate was directly proportional to the cell concentration. From these 36 isolates, only four isolates namely CA_1, CA_2, CA_3 and CA_4 were established to exhibit high phenol degrading activity. Among the four, CA_1 was found to degrade phenol at optimum level and also exhibited higher biomass content. Fig.1 indicated that the degradation of phenol by all the four chosen isolates.

Identification of the phenol degrading bacterium

The isolated colonies were characterized both morphologically and biochemically designated as CA_1 – *Rhodococcus* sp., CA_2 – *Bacillus* sp., CA_3 – *Pseudomonas* sp. and CA_4 -*Citrobacter* sp. Bacterial strains isolated from different phenol contaminated niches were reported to exhibit phenol degrading activity namely *Bacillus* sp. (Cobos-vasconcelos *et al.*, 2006; Aysha and Mumtaj, 2014), *Pseudomonas aeruginosa* (Anyanwu and Chukwudi 2010; Razika *et al.*, 2010; Maulin P. Shah *et al.*, 2014), *Pseudomonas stutzeri* (Tambekar *et al.*, 2012), *Rhodococcus* UKM-P (Nor *et al.*, 2010), *Acinetobacter* (Fumikoyamaga *et al.*, 2011; Siti *et al.*, 2011) and *Rhodococcus erythropolis* UPV-1 (Begona *et al.*, 2011).

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Efficacy of growth and phenol degradation by isolates

The degradation of phenol was associated with growth of isolates. Growth was apparent after a lag period and was exactly mirrored by phenol consumption. After an initial log phase up to 48 h there was a steady increase in growth. Maximum growth was recorded at 144 h; stationary phase was achieved at 192 h for all organisms. The growth (OD at 600nm) and cell dry weight were estimated and tabulated (Table 1). In the present study CA_1 – *Rhodococcus* sp., showed maximum cell dry weight followed by CA_3 – *Pseudomonas* sp. , CA_2 – *Bacillus* sp., and CA_4 - *Citrobacter* sp. The results showed that the lag period, the biodegradation time and the number of microorganism increased inturn simultaneously decreasing the phenol concentration.

The degradation percentage of phenol by individual isolates and mixed cultures were shown in Fig. 2. The process of degradation starts slowly at 48 h and gradually increased till 240 h. Of the four individual strains tested, *Rhodococcus* sp (CA_1) showed a maximum of 60% degradation. The mixed cultures tested observed to be 72% of phenol degradation which is about 12 % higher degrading efficiency than the individual strains. There are several reports on the mechanism of degradation of phenol by pure cultures of bacteria (Siti *et al.*, 2011; Chakraborty *et al.*, 2010; Zheng *et al*., 2009). Also very few reports stated Gram-positive soil bacteria namely *Pseudomonas* and *Rhodococcus* sp. which were isolated from various environments were found to degrade phenol (Begona *et al*., 2002; Barad *et al.*, 2010).

Biosurfactant analysis

The biosurfactant property was analyzed for all the four isolates. Among them all the individual strains tested namely *Rhodococcus* sp., *Bacillus* sp., *Pseudomonas* sp. and *Citrobacter* sp. showed maximum biosurfactant at 96 h such as 1.31 G, 0.85 G, 1.26 G and 1.02 G respectively. However, the mixed cultures showed the higher biosurfactant property such as 1.62 G at 144 h (Fig. 3). A lucid appearance had been observed during the extraction process in the separating funnel by all the strains including the mixed cultures. The biosurfactant property by the all four individual strains decreased 50 % at 240 h when compared with maximum biosurfactant yield at 96 h whereas in the case of mixed cultures it showed only 12 % of reduction in biosurfactant yield at 240 h when compared with its maximum yield obtained at 144 h. This type of biosurfactant property was also reported

earlier in *Rodococcus equi* and *Bacillus methlyotrophicus*(Lobna *et al.,* 2014); *Pseudomonas* and *Bacillus* (Vijaya *et al.,* 2013) and *Rhodococcus erythropolis* (Graziela *et al.*, 2010).

Emulsification property

The emulsification index (EI_{24}) of all the five extracted biosurfactants both individual and consortium (CA_1, CA_2, CA_3, CA_4 and mixed culture) were tested against kerosene (Fig. 4). The emulsification Index (EI_{24}) was found to be high (78 %) in *Rhodococcus* sp. (CA_1), whereas the emulsification index (EI_{24}) for other strains was found to be less. Comparable to the individual strains tested the emulsification index (EI_{24}) for the mixed culture (1:1:1:1) was observed to be maximum (80%). In control, the emulsification index (EI_{24}) was found to be nil. Similar reports on biosurfactant property and emulsification index percentage was reported in *Rhodococcus erythropolis* at the concentrations of 0.23 GL^{-1}, 0.45 $GL^{-1}$and 0.9 GL^{-1}which resulted in EI_{24} values of 27%, 40% and 60%, respectively (Graziela *et al.*, 2010). Likewise, in *Pseudomonas aeruginosa* LS1 it was reported that biosurfactants was effective in producing very good emulsification rate (85%) with glucose against kerosene, whereas no stable emulsions were formed at the 0 h (Anyanwu and Chukwudi, 2010). In control an expected activity close to zero against kerosene was documented. In the same way, biosurfactant extracted from *Rhodococcus Erythropolis* at the concentrations of 0.23 g/L, 0.45 g/L and 0.9 g/L resulted in EI_{24} values of 27%, 40% and 60%, respectively (Graziela *et al.*, 2010).

Plasmid profiling of the isolates

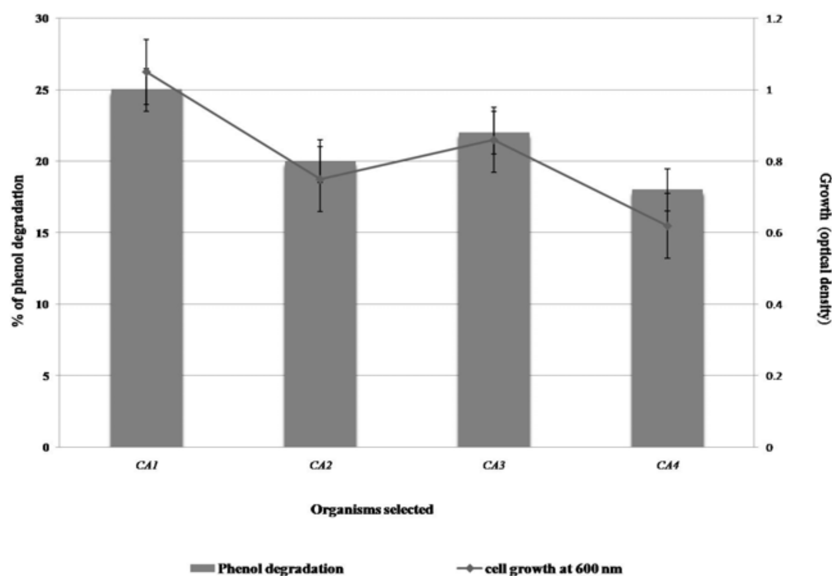
The plasmid DNA extracted from *Rhodococcus* sp., *Bacillus* sp., *Pseudomonas* sp. and *Citrobacter* sp. was almost equal to pBR322 which is used as marker. This can be stated that plasmid might be involved in efficient degradation of phenol and this investigation would further pay way for efficient mutant that degrade phenol to the maximum in future.

Though consortium showed maximum degradation ability, isolating a potential individual strain is necessary for large scale application and also for molecular approach studies. The present investigation reveals that native bacterial strain CA_1 - *Rhodococcus* sp., isolated from coal carbonization plant that produced efficient growth in phenol supplemented medium and high phenol degradation ability, can be applied to the phenol polluted environment, after suitable manipulation of the organisms. Though this preliminary attempt isolated and optimized, an efficient phenol degrader, a clear study further on the enzymatic part and metabolism for degradation is to be elucidated in future.

Table 1. Growth and cell dry weight of four strains isolated from coal carbonization plant

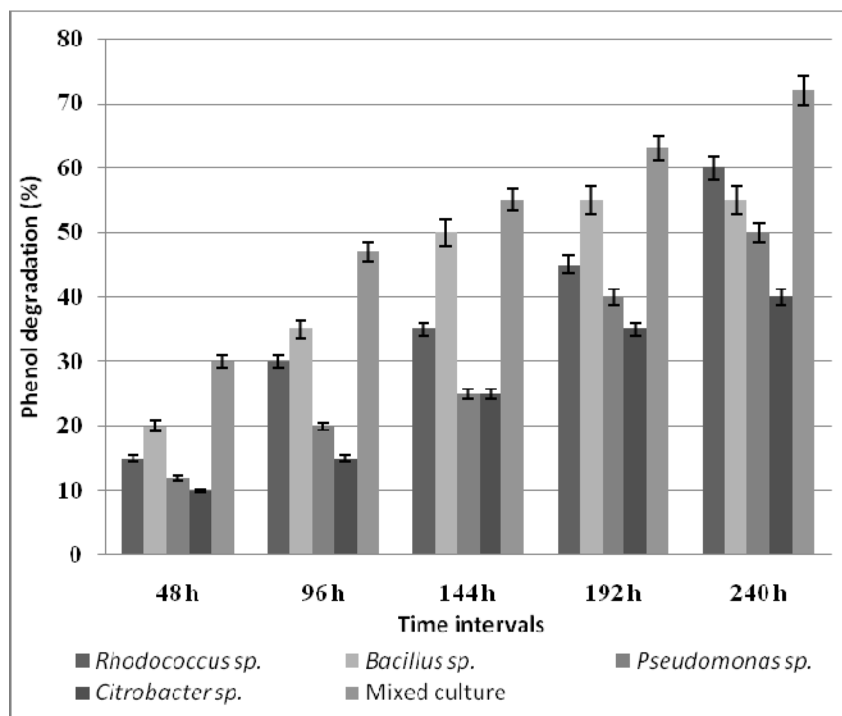
Isolates studied	Growth at different time intervals (OD at 600 nm)					Cell dry weight (mG mL^{-1})				
	48 h	96 h	144 h	192 h	240 h	48 h	96 h	144 h	192 h	240 h
***Rhodococcus* sp.**	1.103	1.116	1.120	1.008	0.802	14.38	14.96	15.02	14.08	14.42
***Bacillus* sp.**	1.052	1.102	1.116	0.952	0.720	12.20	13.10	13.50	13.08	13.00
***Pseudomonas* sp.**	0.975	1.100	1.110	0.960	0.909	10.0	12.84	12.96	12.32	11.30
***Citrobacter* sp.**	2.852	0.950	1.102	0.967	0.802	8.04	10.04	10.50	10.00	9.25
Mixed culture (1:1:1:1)	1.53	2.02	2.05	2.03	2.03	25.2	23.4	21.2	20.3	20.1

Figure 1. Growth and phenol degrading activity of isolates



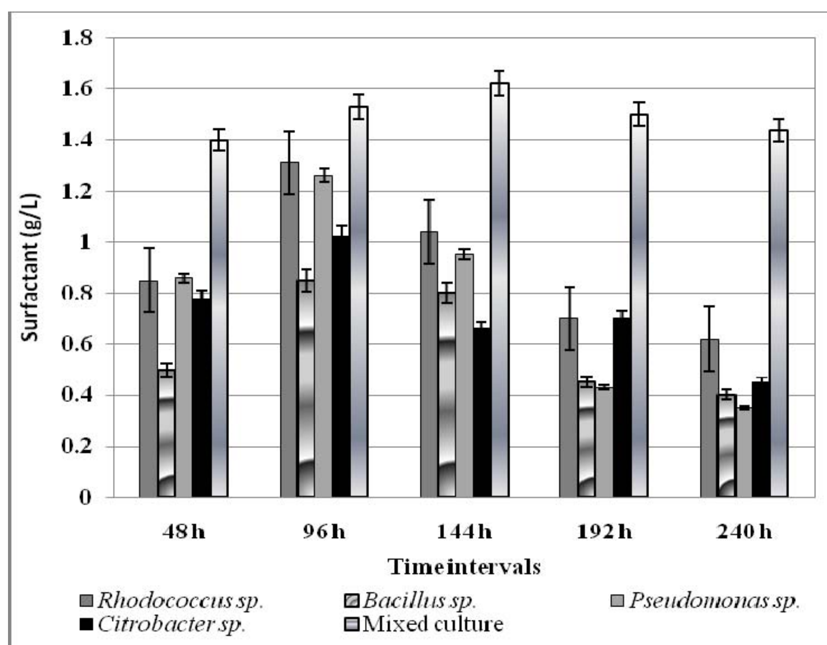
Values are mean ± SE of triplicates

Figure 2. Efficacy of phenol degradation by individual and mixed isolates



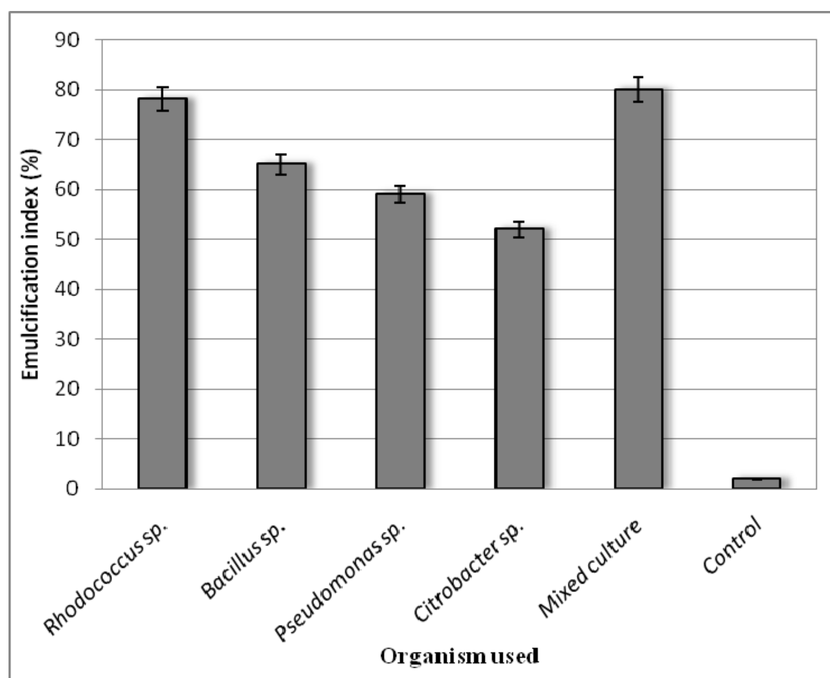
Values are mean ± SE of triplicates

Figure 3. Production profile of biosurfactant by individual and mixed isolates



Values are mean ± SE of triplicates

Figure 4. Emulsification Index of the four isolates



Values are mean ± SE of triplicates

REFERENCES

Anyanwu and Chukwudi. (2010). Surface activity of extracellular products of a *Pseudomonas aeruginosa* isolated from petroleum contaminated soil. Int. J. of Envt. Sciences. 1(2): 225 – 235.

Arutchelvan, V., Kanakasabai, V., Nagarajan, S. and V. Muralikrishnan. (2006). Kinetics of high strength phenol degradation using *Bacillus Brevis*. J. of Hazardous Materials. 129 (1-3): 216-222.

Aysha, O.S. and K. Mumtaj. (2014). Degradation of phenol by selected strains of *Bacillus* species isolated from marine water. American J. of Pharmacy and Health Science. 2(6): 41-56.

Bared Razika., Boucheta Abbes., Chaib Messaoud. and Kacimi Soufi. (2010). Phenol and benzoic acid degradation by *Pseudomonas aeruginosa.* J. of Water Res. and Prot. 2: 788-791.

Begona Prieto, M., Aurelio Hidalgo., Juan L. Serra. and Marıa J. Llama. (2002). Degradation of phenol by *Rhodococcus erythropolis* UPV-1 immobilized on biolite in a packed-bed reactor. J. of Biotech. 97:1–11.

Birnboim, H.C. (1983). A rapid alkaline extraction method for the isolated of plasmid DNA. Methods in Emzymol. 100: 243 – 255.

Chakraborty, S., Bhattacharya, T., Patel, T.N. and K.K. Tiwari. (2010). Biodegradation of phenol by native microorganisms isolated from coke processing wastewater. J.of Envt. Biology. 293-296.

Cobos-Vasconcelos, D.D.L., Santoyo-Tepole, F., Juarez-Ramírez, C., Ruiz-Ordaz, N. and C.J.J. Galindez-Mayer. (2006). Co-metabolic degradation of chlorophenols by strain of *Burkholderia* in fed-batch culture. Enzyme and Microbial Tech. 40: 57-60.

Collins, G., Clare, F., Sharon, M., Therese, M. and V.O. Flaherty. (2005). Anaerobic biological treatment of phenolic wastewater at 15-18°C. Water Res. 39: 1614 -1620.

Cooper, D.G. and B.G. Goldenberg. (1987). Surface active agents from two *Bacillus* sp. Appl. Envt. Microb. 53: 224- 229.

Debadatta, D. and K. Rajdeep. (2012). Biodegradation of phenol by various indigenous microorganisms. J. Environ. Res. Dev. 1(2A): 1009-1015.

Dursun, Y. and O. Tepe. (2005). Internal mass transfer effect on biodegradation of phenol by Ca-alginate immobilized *Ralstonia eutropha*. J. Hazardous Material. 126: 105-111.

Fumikoyamaga, Kenjiwashio and Andmasaaki Morikawa. (2010). Sustainable biodegradation of phenol by *Acinetobacter calcoaceticus* P23 isolated from the rhizosphere of duckweed *Lemna aoukikusa.* Envt. Sci. Tech. 44: 6470–6474.

GoodFellow, M. (1994). Bergey's manual of determinative bacteriology 9th edition, *Williams* and *Wilkin,* London.

Graziela Jardim Pacheco., Elisa Mara Prioli Ciapina., Edelvio de Barros Gomes and Nei Pereira Junior. (2011) Biosurfactant production by *Rhodococcus erythropolis* and its application to oil removal. Brazilian J. of Microb. 41: 685-693.

Ho, K.L., Lin, B., Chen, Y.Y. and D.J. Lee. (2009). Biodegradation of phenol using *Corynebacterium* sp. DJ1 aerobic granules. Bioresource Tech. 100 (21): 5051-5055.

Lobna, A. Moussa and Ahmed, Z. Abdel Azeiz (2013). Identification and characterization of biosurfactants produced by *Rodococcus equi* and *Bacillus methlyotrophicus*. J. Biol. Chem. Envt. Sci. 8(2):341-358.

Maulin P. Shah. (2014). Microbiological removal of phenol by an application of *Pseudomonas sp.* ETL: An innovative biotechnological approach providing answers to the problems of FETP. J. of Applied and Envi. Microb. 2(1): 6-11.

Nor Suhaila, Y., Ariff, A., Rosfarizan, M., Abdul Latif, I., Ahmad, S.A., Norazah, M.N. and M.Y.A. Shukor. (2010) Optimization of parameters for phenol degradation by *Rhodococcus* UKM-P in shake flask culture. Proceedings of the World Congress on Engineering. Vol 1, London, U.K.

Razika, B., Abbes, B., Messaoud, C. and K. Soufi. (2010). Phenol and benzoic acid degradation by *Pseudomonas aeruginosa.* J. of Water Resource and Protection. 2:788-791.

Saravanan, V and S. Vijayakumar (2012). Isolation and screening of biosurfactant producing microorganisms from oil contaminated soil. J. Acad. Indus. Res. 1(5): 264- 268.

Siti Aqlima Ahmad., Mohd Arif Syed., Noorliza Mat Arif, Mohd Yunus Abdul Shukor and Nor Aripin Shamaan. (2011). Isolation, identification and characterization of elevated phenol degrading *Acinetobacter* sp. strain AQ5NOL 1. Aust. J. of Basic and Appl. Sci. 5(8): 1035-1045.

Tambekar, D. H., Bhorse, P.S. and P.V Gadakh. (2012). Biodegradation of phenol by native microorganisms isolated from Lonar Lake in Maharashtra State (India). Int. J. of life science and pharma research. 2(4): 26 – 30.

Vijaya, B., Jayalakshmi, N.R. and K. Manjunath. (2013). Enumeration of biosurfactant producing microorganisms from oil contaminated soil in and around Bangalore (India). Int. J. Curr. Sci. 5: 86-94.

Wei, G., Yu, J., Zhu, Y., Chen, W. and L. Wang. (2008). Characterization of phenol degradation by *Rhizobium* sp CCNWTB 701 isolated from *Astragalus Chrysopteru* in mining tailing region. J. of Hazardous Materials. 151(1): 111-117.

Yang, R.D. and A.E. Humphrey. (1975). Dynamic and steady state studies of phenol biodegradation in pure and mixed cultures. Biotech. Bioengg. 17: 1211-1235.

Zheng, Y., Liu, D., Xu, H., Zhong, Y. and Y. Yuan (2009). Biodegradation of *P*-Nitrophenol by *Pseudomonas Aeruginosa* HS-D38 and analysis of metabolites with HPLC– ESI/MS. Int. Biodeterioration & Biodegradation. 63 (8): 1125-1129.