JOURNAL OF PURE AND APPLIED MICROBIOLOGY, March 2013. Vol. **7**(1), p. 683-690

Isolation of Phenol Degrading Bacteria from Industrial Waste Water and their Growth Kinetic Assay

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(Received: 01 July 2012; accepted: 10 August 2012)

Phenol is one of the organic pollutants in various industrial wastewaters especially petrochemical and oil refining. Biological treatment is one of the considerable choices for removing of phenol present in these wastewaters. Identification of effective microbial species is considered as one of the important priorities for production of the biomass in order to achieve desirable kinetics of biological reactions. Phenol and its components are extremely toxic and can easily be isolated from different industrial sewage such as oil refinery, petrochemical industry and mines, especially collier and chemical factories. Hence the presence of these compounds in the environment could cause environmental pollution, especially in water resources. In the past, physicochemical method was used for the elimination of phenol and its compounds, but today, bioremediation is preferable. The aim of this study is to isolate phenol degrading bacteria from industrial waste water of Haryana and to assay their kinetic growth. Seventy samples of waste water and sedimentation from different location of Panipat petro Refinery were collected. In order to isolate phenol degrading bacteria, samples were cultured on salt base phenol broth media. For screening of degrading bacteria, bromothymole blue indicator was added to media, which formed green colour in it. Finally, the ability of bacteria to degrade different concentrations of phenol ranging from0.2 to 1.0 g/l was measured using bacteria culture. Cultivated bacteria on the salt base phenol broth media containing indicator changed the colour of the media from green to yellow by using the phenol and decreasing the pH. These bacteria were, chiefly, gram negative and they belong to *Pseudomonaceae* and *Acinetobacteraceae* Family. *Psuodomonas* spp. are the most important phenol degrading bacteria in water bodies of Bokaro Jharkhand, which showed vast diversity in different parts of this area. Species of *Acinetobacter* and other species such as *Kelibsiella, Citrobacter* and *Shigella* were found as well. Most of the isolated bacteria showed a good ability of degradation of phenol, where *Pseudomonas* and *Acinetobacter* showed 0.8 - 0.9 g/l, and *Kelibsiella*, *Citrobacter* and *Shigella* showed 0.6 - 0.7 g/l and the rest showed 0.2 - 0.3 g/l of phenol degradation. Findings show that all different locations of Panipat has a lot of high ability phenol degrading bacteria. The most important species belong to *Psuodomonas* and *Acinetobacter*.

Key words: Phenol-Degrading Bacteria, Wastewater, *Pseudomonas Putida*, Activated sludge, *Pseudomonas*, *Acinetobacter*.

Phenol is an aromatic pollutant, which is present in the wastewater of numerous industries including oil refining, petrochemical, plastic manufacturing, dyestuff, pharmaceutical, production of resin and coke (Prpich and Daugulis, 2005; Gerrard *et al*., 2006; Juang and Tsai, 2006). Simple phenol is liquid or solid with low melting

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point, but its boiling point is high because of hydrogen bonds. Phenol is slightly soluble in water due to its ability to make hydrogen bonds with water (9 gram in 100 ml water) Today, there is a lot of anxiety regarding the existence of toxic chemical substances like nitrate, selenium, mercury, cadmium and phenol in water, which could enter the human body through consuming aquatic animal. Freshwaters is usually contaminated with factories sewages. Thus treatment of phenolic wastewaters is necessary and biological methods are the most appropriate techniques due to mineralization of toxic organic compounds and inexpensiveness (Prpich and Daugulis, 2005). Identification of effective microbial species is considered as one of the important priorities for production of the optimum conditions of biomass in order to achieve desirable kinetics of biological reactions (Noworyta *et al*., 2006). Conventional methods for complete identification of microorganisms in environmental samples are based on culture and differential biochemical and serological tests that are time-consuming and have relatively low sensitivity. Many aerobic bacteria are capable of using aromatic compounds as their sole carbon source and energy. The biological degradation is accomplished through benzene ring cleavage mediated by intracellular enzymatic reaction (Kumar *et al*., 2004). The use of pure cultures, especially adapted to metabolize the contaminant, can be envisaged as an attractive alternative. The microorganisms that are normally used in phenol degradation include *Pseudomonas* sp (Nurdan and Azmi, 2005; Gonzalez *et al*., 2001; Monteiro *et al*., 2000), *Candida tropicalis* (Chen *et al*., 2002), *Azotobacter* sp (Hughes and Bayly, 1983), *Rhodococcus* sp (Prieto *et al*., 2002), *Alcaligenes* sp (Valenzuela *et al*., 1997), and *Acinetobacter* sp (Hao *et al*., 2002). Acclimatization of the microorganisms overcomes the substrate inhibition problems that normally occurred in phenol biodegradation at high concentration (Lob and Tar, 2000). Certain intracellular enzymes are induced during acclimatization stage so that the microbes are available to take part in the reaction (Kumar *et al*., 2004).

Most of the rivers have been turbid and obscured due to entry of sewages, chemicals, oily materials and other extraneous material. Now a days, river, gulf, lake and oceans are the most contaminated waters resource, respectively. Although, developed countries are mainly responsible for river contamination from the beginning of industrial revolution till now, developing countries, in the near future, would have the main role in increasing river pollution. Around 80% of disease is related with water. At the beginning of this century, around 50% of the world population consists of urban population, in which this issue seriously threatens water resources (Fitzhugh & Richter, 2004). Phenol and its compounds are one of most important pollutant of the environment especially, water. Study on human and animal shows that phenol is effectively absorbed through inspiration and digestion. The vapor of phenol can be easily absorbed through the skin. Phenol in solution form, easily passes through the skin, and its metabolism occurs in the liver, although, it could occur in the lung and kidney too. Phenol is toxic in environment and could decrease enzymatic activity as well. Also, it is toxic to fishes and is mortal between 5 – 25 mg/l for them. Moreover, direct effect of phenol is a blocker for biological reaction. Phenolic compounds are serious pollutant for rivers (EPA, 2004) and they have harmful effects such as growth inhibition, decrease of resistance against diseases, aquatic mortality and increase in growth of weedy plants. If phenolic pollution goes to underground water, it causes serious ecological problems. Hence, permissible limit of phenol in industrial outgoing must not be more than 0.5 mg per liter. Considering the above issue, phenol elimination from the environment, especially from water and water resource, is of vital importance. Routine physico-chemical methods were used in the degradation of phenol, but this had a high expense and they produce harmful intermediate too. Today bioremediation is considered as a new tool to eliminate environment pollutions. Numerous phenol degrading micro-organisms have been isolated from different sources; those include bacteria, yeast, fungi and algae. Among them, bacteria are of specific importance. Bacteria (such as *Pseudomonas* spp., *Acinetobacter* spp.), yeast (such as *Pleurotutus ostreatus, Candida tropicalis, Trichosporon cutaneum* and *Phanerochaete chrysosporium*) and fungi (like *Fusarium flucciferum* and *Aspergillus fumigates*) can degrade phenol; although, among algae,

Ochromonas danica can degrade phenol while meta pathway (Xiangchun et al., 2004). The aim of this study is to isolate and identify the phenol degrading bacteria from water and sedimentation at different locations of water bodies of Bokaro. Also, the study aims at assaying the growth kinetic of isolated species, examination of capacity and measuring the elimination level of phenol by isolated bacteria.

MATERIALS AND METHODS

This experimental study was carried out at Environmental Lab, Department of Biotechnology, Meerut Institute of Engg. & Technology, Meerut and Central soil salinity research Institute, Karnal during three intervals of year (every 4th Month). Samples were collected different location of Panipat nearby petro Refinery and industrial waste. Sampling was done with sterile dishes thrice for each 24 sample (12 sedimentation, 12 water), amounting to a total of 72 samples. They were kept in a flask containing ice for less than 2 h and transferred to the laboratory.

Growth media

A basic mineral salts medium (MS medium) was used for the isolation of the organisms. Bacteria were grown at pH=7 on a MS medium containing (per liter) 2.75 g K2HPO4, 2.25 g KH2PO4, 1 g (NH4)2SO4, 0.2 g MgCl2.6H2O, 0.1 g NaCl, 0.02 g FeCl3.6H2O and 0.01 g CaCl2 (Watanabe *et al*., 1998).

Isolation of phenol degrading bacterial strains

Regarding sedimentation samples, isolation was done as follows: 10 g of sedimentation samples were mixed with 100 ml of phenol broth media and incubated at 30°C with aeration for one week. Then 1 ml of this media was inoculated to 100 ml of new phenol broth media and aerated in 30°C for another one week. Again, 1 ml of the second passage was inoculated into the new phenol broth media and incubated in the above mentioned situation. These passages were repeated until turbidity was obtained from bacteria growth, which was not due to mixed sedimentation with the first media. After the last passage, it was cultured on phenol agar media as an isolate and the bacterium was isolated as a colony alone. Regarding water samples, same method was applied, except that 10 ml water was mixed with 100 ml phenol broth media and all previously mentioned stages were repeated. Koutny et.al. (2003)

Assay capacity of phenol elimination capacity of bacteria

In this study, for evaluating the phenol elimination capacity of bacteria, different concentration of salt base phenol broth media were used. A total number of 8 tubes of phenol broth were devoted to each bacterium. Phenol concentration of 0.1 - 0.9 was added to the tubes, respectively. Bacteria was incubated in 30 c and regularly aerated for one week. Then, the tubes were examined for turbidity.

Assay of phenol elimination by isolated bacteria

For evaluating phenol elimination with degrading bacteria, Gibes method was used. In this method, gibes indicator or 2,4-dichloroquinone- 4-chloroimide was used, which reacted with phenol and produced a blue color compound. For assay after centrifuging media, 150 ìl of media supernatant (pH = 8) was mixed with 30 ìl of NaHCO3 (pH = 8). Then, 20 ìl of gibes indicator (1 mg/l) was added to the mixture, vortexes and kept for 15 - 45 min at room temperature with thermo mixer and finally, the mixture absorbance was read at 630 nm (Quintana et al., 1997).

Growth assessment of isolated bacteria in different concentration of phenol with optic absorption survey

In this method, best phenol degrading species were isolated from phenol degrading bacteria with an optic absorption study. About 20 ml of phenol broth media was poured into the separated Erlenmeyer flask (with different concentration of phenol). Then, 5 ml of media containing bacteria was added to each tube. For each bacteria, 8 Erlenmeyer flask (with 0.1 - 0.9 phenol) was considered. In the control media, there was just a base media and a specific species, but without phenol. Media was incubated for 24 h at 30°C and then, their absorbed mixture was read at 600 nm (Ali *et al.,* 1998).

RESULTS AND DISCUSSION

Isolated bacteria included *Providencia* spp., *Entrobacter* spp., *Serattia* spp., *Escherichia coli* spp., *Citrobacter* spp., *Shigella* spp., *Klebciella* spp., *Acinetobacter* spp. And

Pseuodomonas spp. In evaluating the growth of isolated species during one week, the capacity of bacteria in phenol elimination was evaluated. As Table 1 shows, most phenol degrading bacteria belong to *Pseuodomonas* and the least to *Providensia* spp. The growth curve of isolated species shows (Table 2 and 3) almost the same pattern of growth for all isolated species in 0.1 g of phenol. According to Table 4 which is same for all species, 0.2 and 0.05 g/l (remains) of phenol reached their maximum growth after 24 h, and after 54 h, phenol completely disappeared. Different methods have been used for de-phenolisation, but the use of bacteria can be one of the cheap, reproduction in the presence of phenol and its compound have shown extraordinary ability in de-phenolisation. So with isolation, purifying and growing of species which has high ability of de-phenolisation, can be used in areas with phenol pollution. Different bacteria of different genus have been isolated as phenol degrading. Most of them, which chiefly belong to family *Pseudomonaceae,* are gram negative. Isolated phenol degrading bacteria from Siberia soils. They found out that the permanent genus of phenol degrading bacteria in these soils is *Pseudomonas* and especially, *Pseudomonas putida.* Although, this agrees with the finding, their vast distribution in soils and the ability of phenol or phenol compounds to be eliminated was done by Williams and Sayers (1994). However, the study's result agreed with previous findings (Torres et al., 1999) Whitely and colleague in 2001 evaluated an ecological and physiological *pseudomonas* strain in dephenolisation system. They found that the observed diversity supports the possibility of a complex system and the presence of phenotypic and genotypic ability of *pseudomonas* spp. Whiteley and colleagues introduce *Pseudomonas pseudoalcaligenes* strain in their research as the main phenol degrading isolate (Whiteley and Mark, 2000). For example, increase in concentration of phenol in the controlled system can decrease the usability of degrading strains which is an implication of direct toxic effects in *P. pseudoalcaligenes.* One logical deduction is that the increase in the main pollutant causes the selection of the degrading population with less diversity or selecting groups with more tolerance

Table 1. Survey of degrading with isolated species during one week

S. No.	Name of Bacterial Strain	Phenol Conc. gr/litre
1	*providencia*	0.3
2	*entrobacter*	0.4
3	*serattia*	0.5
4	*e.coli*	0.6
5	*citrobacter*	0.6
6	*shigella*	0.7
7	*klebciella*	0.8
8	*acinetobacter*	0.8
9	*providencia*	0.9

Table 2. Growth curve of enterobacter. This bacterium shows most OD in 0.2 g/l of phenol concentration

S.No.	Phenol Conc. gm/lt	OD at 600 nm
1	0.1	0.07
2	0.2	0.19
3	0.3	0.17
4	0.4	0.08
5	0.5	0.04
6	0.6	0.03

Table 3. Growth of *Shigella*. This bacterium shows most OD in 0.2 g/l of phenol concentration

S.No.	Phenol Conc. gm/lt	OD 600 nm
1	0.1	0.16
2	0.2	0.21
3	0.3	0.17
4	0.4	0.16
5	0.5	0.12
6	0.6	0.09

Table 4. Elimination curve of phenol for species *Acinetobacter* with gibes Method during 54 h

S. No.	OD AT 600nm	Phenol Conc. gm/lt	Time
1	0.05	0.21	6
2	0.1	0.17	12
3	0.15	0.11	18
4	0.2	0.06	24
5	0.2	0.03	30
6	0.12	0.02	36
7	0.08	0.02	42
8	0.08	0.01	48
9	0.08	0.01	54

when compared with the non-degrading population. Whitely and colleagues stated that the isolated analysis in bioreactors has revealed different important features which relate to diversity, physiology and function of *pseudomonas* population that is found in industrial dephenolisation bioreactors. However, it has seen a considerable physiologic heterogeneity in the tolerance range of isolates. Beside the mentioned strain called phenol degrading, Wanger et al. (1999) report that bacterium, *Klebsiella,* from the family of entrobacteriaceae, which has a plasmid like TOL and a new code called phenol hydroxilase gene, can degrade phenol (Wagner and Schawarz, 1999). Eduardo et al. (2000) report a bacterium *Alcaligenes faecalis* and yeast *Candida tropica,* which could degrade the phenol and still had a high salt concentration tolerance (15%). In this study dephenolising bacteria, which were isolated from different water bodies of Bokaro, were mostly from genus *Pseudomonas* and *Acinetobacter,* which is in agreement with the result of other researchers. Different methods have been used by the researcher to assay phenol elimination. Wantanabe et al. (1998,1996) used calori-metric method (using dye 4-aminoanti pyrene) in measuring the rest of the phenol in media. Other researchers used this method for measuring the rest of the phenol as well. Selvartanam et al. (1997) assay phenol with high performance liquid chromatography (HPLC). In this study, gibes method (using indicator 2,4-dichloro kinon-4-chloroimid) was used for phenol

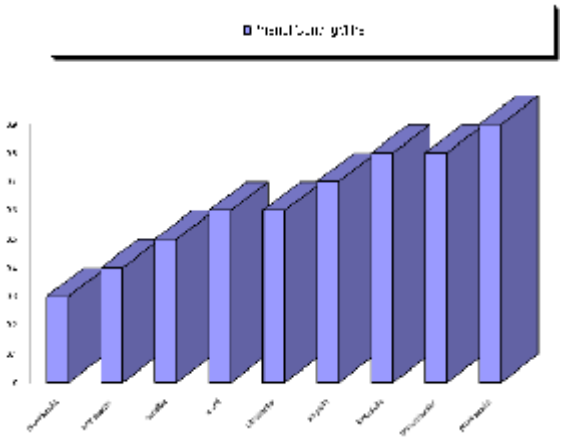

Fig. 1.

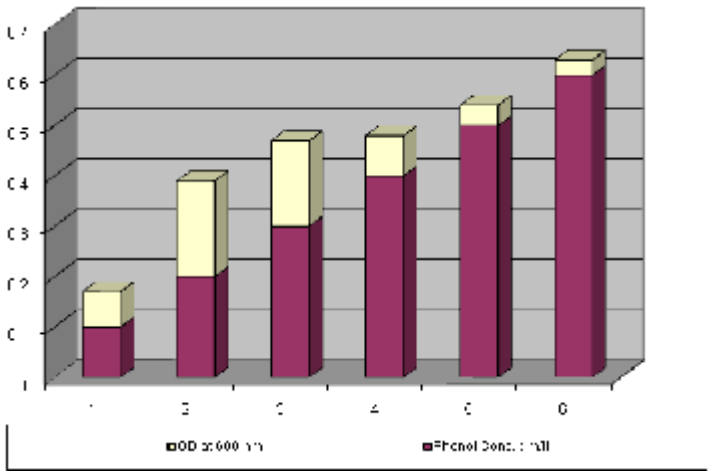

Fig. 2.

elimination assay. The result of phenol assay, using this method, is parallel with results of other researchers. Finally, the result of this study showed that phenol degrading bacteria have a vast diversity in nature, especially in biological dephenolisation plant, Bokaro. Isolated phenol degrading species mostly have a potential in phenol and phenol compound degrading. Considering the evaluated study and results of this study, it could be that phenol degrading bacteria mostly belong to the family of *Pseudomonaceae*. Species of *Acinetobacter* is next in the ranking of importance. It should be said that different species of this genus has shown different ability of degrading phenol. Finally, using new and less expensive methods (using bacteria) for elimination of phenol and its compound is preferable than older and expensive methods. For isolating and phenol tolerance determination of bacteria, liquid media is more suitable than solid media. Isolated phenol degrading species could be used in industrial sewage, which has phenolic pollution such as iron boiling and collier and petrochemical waste water. It should be considered that the local

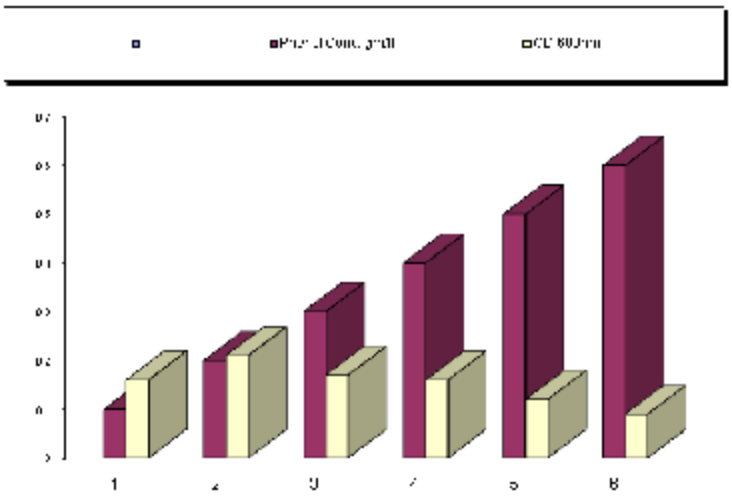

Fig. 3.

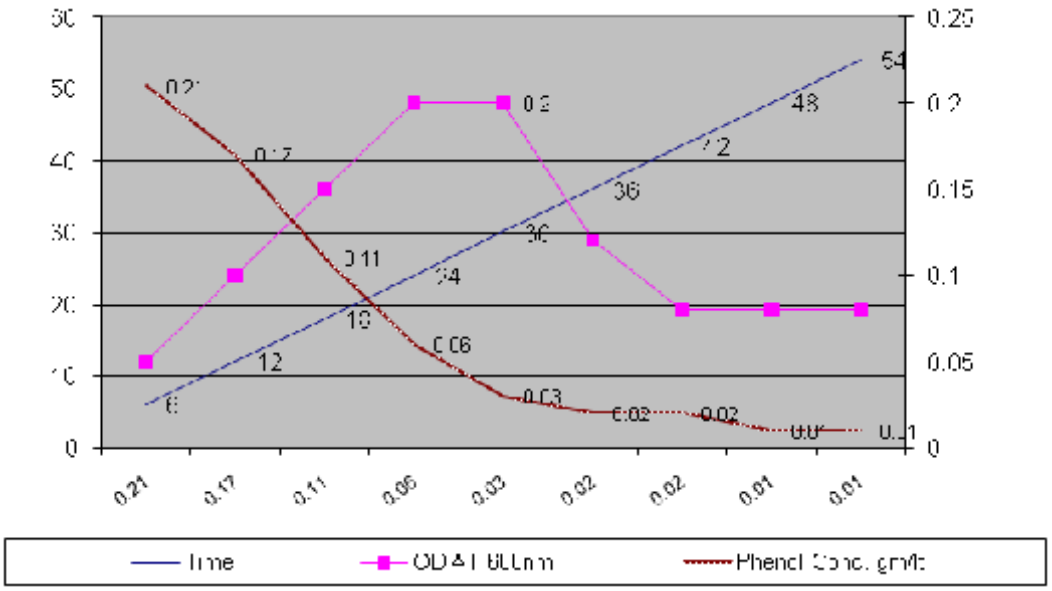

Fig. 4.

microorganism of each area is more suitable for the elimination of phenol and phenolic compound in comparison to the microorganism used by other researchers in other areas of the world. Some bacteria of fungi, yeast and algae as mentioned before, can eliminate phenolic compound, but none of them is as efficient, as a bacterium, to eliminate phenol. Conclusively, water bodies of Bokaro, with biologic variety and different species density, are a suitable bed for more research work.

CONCLUSION

Wastewater treatment plant and oil-contaminated soil are the most potential sources to isolate high performance phenol degrading microbes. Their degradation performance may be examined in a series of different phenol concentrations. Serial exposure to increasing level of phenol concentration can be used to determine acclimatizability of a particular isolates. Highly acclimatizable microbes or ones that are able to degrade phenol at highest concentration and at greatest rate will be the best phenol degrader candidates. This work has proved a useful guideline in evaluating potential phenol degraders isolated from different environment

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