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Full Length Research Paper

Isolation and identification of phenol degrading bacteria from Lake Parishan and their growth kinetic assay

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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 and identify phenol degrading bacteria from Lake Parishan and to assay their kinetic growth. Sixty samples of water and sedimentation of different area of Lake Parishan 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 color in it. Finally, the ability of bacteria to degrade different concentration of phenol was measured using culturing bacteria in different concentration of phenol from 0.2 to 0.9 g/l. Cultivated bacteria on the salt base phenol broth containing indicator changed the color 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 Lake Parishan which showed vast diversity in different parts of this lake. 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 the Lake Parishan has a lot of high ability phenol degrading bacteria. The most important species belong to *Psuodomonas* and *Acinetobacter*.

Key words: Phenol, degrading, *Pseudomonas*, *Acinetobacter*, Lake Parishan.

INTRODUCTION

Phenols are compounds with ArOH formula which are extremely toxic and found in different form or together with other elements (EPA, 2004). Simple phenol is liquid or solid with low melting point, but its boiling point is high because of hydrogen bonds. Phenol is slightly solvable in water due to its ability to make hydrogen bounds with water (9 gram in 100 ml water) (Morrison and Boyd, 1992). 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. Most of the rivers have been turbid and obscured due to entry of sewages, chemicals, oily materials and other extraneous material. Nowadays, rivers, gulf, lake and oceans are the most contaminated waters resource, respectively. Although, developed countries are mainly responsible for rivers contamination from the beginning of industrial revolution till now, developing countries, in the near future, would have the main rule at increasing river pollution. Around 80% of diseases are

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Figure 1. Satellite position of Lake Parishan which was taken from Google

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 biologic 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, allowable amount 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 (EPA, 2004). 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 (Ariana et al., 2004; Xiangchun et al., 2004; Godjevargova et al., 2003). Lake Parishan is

one of the attractive Lake of Iran which is in latitude E 51° 51’, longitude N 29° 32’ and altitude of 820 m above sea level. The basin of this lake is 266.5 sq km. Domestic, agriculture and industrial waste water continually spill to this lake so that it has considerable amount of phenol and its compounds.

The aim of this study is to isolate and identify the phenol degrading bacteria from water and sedimentation of Lake Parishan. 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 done on Lake of Parishan during three seasons of autumn, winter and spring (Figure 1). 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 6 h and transferred to a laboratory.

Method of isolating phenol degrading bacteria

Regarding sedimentation samples, isolation was done as follows:

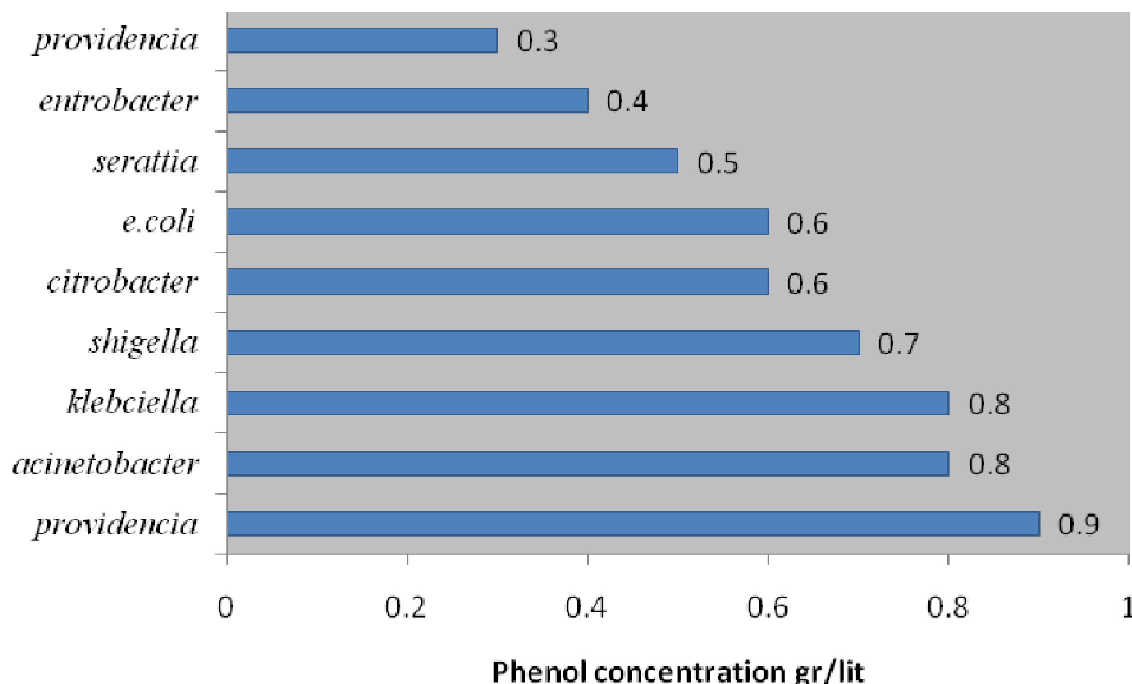

Figure 2. Survey of degrading with isolated species during one week.

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 0.2 - 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 (Koutny et al., 2003).

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-dichlo-roquinone-4-chloroimide was used, which reacted with phenol and produced a blue color compound. For assay after centrifuging media, 150 µl of media supernatant (pH = 8) was mixed with 30 µl of NAHCO3 (pH = 8). Then, 20 µl of gibes indicator (1 mg/l) was added to the mixture, vortexed and kept for 15 - 45 min at room temperature with thermo mixer and finally, the mixture adsorbance 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 0.2 - 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 in 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 *Pseudomonas* spp. In evaluating the growth of isolated species during one week, the capacity of bacteria in phenol elimination was evaluated. As Figure 2 shows, most phenol degrading bacteria belong to *Pseudomonas* and the least to *Providensia* spp. The growth curve of isolated species show (Figures 3 and 4) almost the same pattern of growth for all isolated species in 0.2 g of phenol. According to Figure 5 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 48 h, phenol completely disappeared.

Different methods have been used for the elimination of phenol, but the use of bacteria can be one of the cheap,

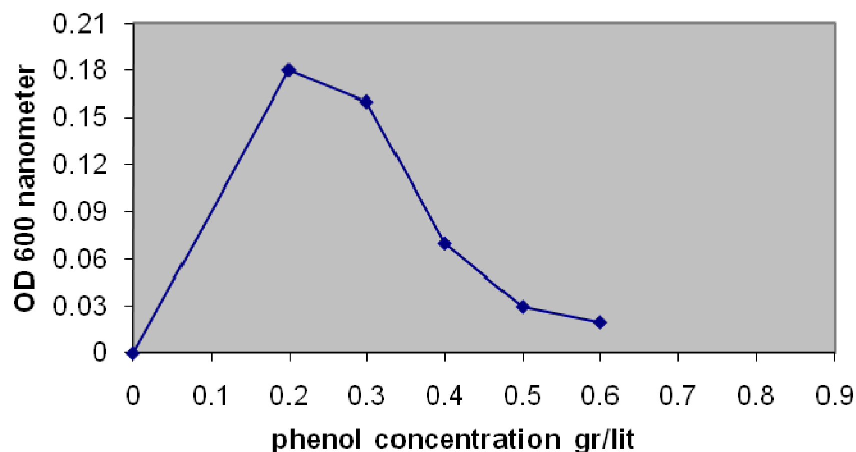

Figure 3. Growth curve of enterobacter. This bacterium shows most OD in 0.2 g/l of phenol concentration.

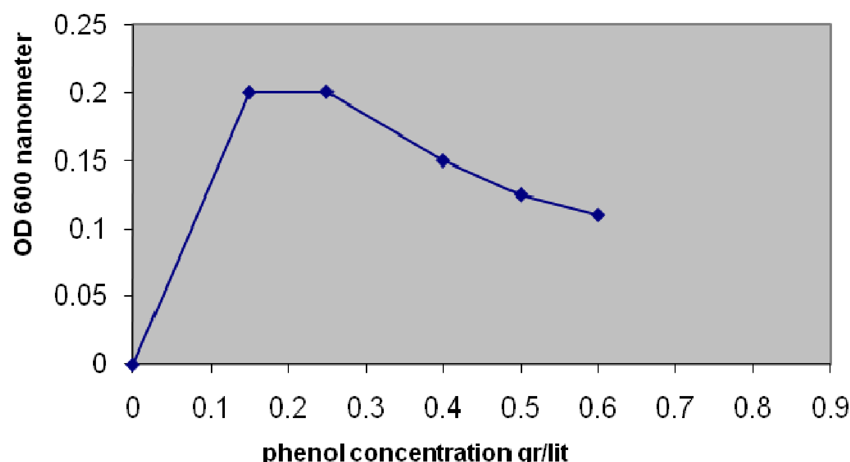

Figure 4. Growth curve of S*higella*. This bacterium shows most OD in 0.2 g/l of phenol concentration.

operative and secure methods. Bacteria with rapid reproduction in the presence of phenol and its compound have shown extraordinary ability in phenol elimination. So with isolation, purifying and growing of species which has high ability of phenol elimination, they 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. Kounty et al. (2003) isolated phenol degrading bacteria from Siberia soils. They found out that the permanent genus of phenol degrading 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) and Torres et al. (1999). However, the study's result agreed with previous findings (Torres et al., 1999; Powlowski and Shingler, 1994; Williams and Sayers, 1994). Whitely and colleague in 2001 evaluated an ecological and physiological *pseudomonas* strain in phenol elimination systems. 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 et al., 2001; 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 when compared with the non-degrading population. Whitely

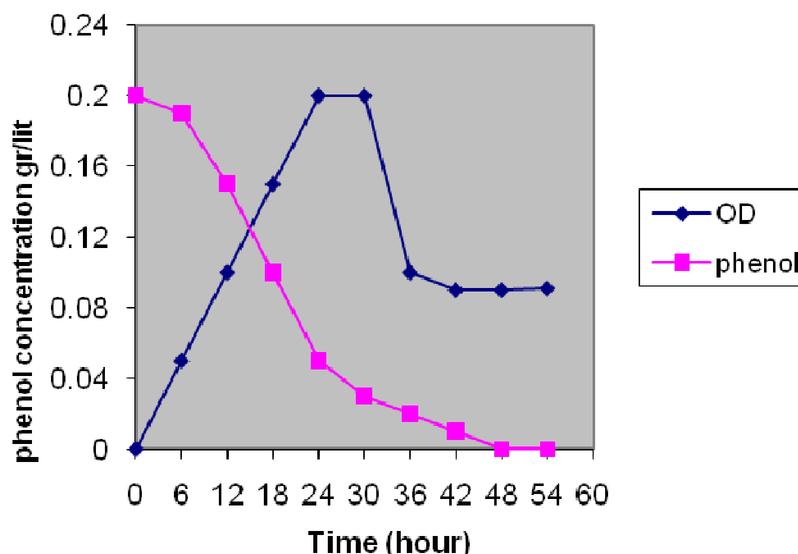

Figure 5. Elimination curve of phenol for species *Acinetobacter* with gibes method during 48 h.

and colleagues stated that the isolated analysis in bio-reactors has revealed different important features which relate to diversity, physiology and function of *pseudomonas* population that is found in industrial phenol degrading bioreactors. However, it has seen a considerable physio-logic heterogeneity in the tolerance range of isolates (Whiteley et al., 2001; Whiteley and Mark, 2000). Beside the mentioned strain called phenol degrading, Wanger et al. (1998) 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, phenol degrading bacteria, which were isolated from Lake Parishan, 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 like Whitely et al. (2001) and Kotny (2003) used this method for measuring the rest of the phenol as well. Selvartanam et al. (1997) assay phenol with high performance liquid chromatography (HPLC). Also, Heinaru (2000) and Fries (1997) also use this method (Koutny et al., 2003; Whiteley et al., 2001; Whiteley and Mark, 2000). In this study, gibes method (using indicator 2,4-dichloro kinon-4-chloroimid) was used for phenol elimi-nation assay. This method has been used by other researchers like Gohn et al. (2003) and Quintana et al. (1997). The result of phenol assay, using this method, is parallel with results of other researchers (John and White, 2003). Finally, the result of this study showed that phenol degrading bacteria have a vast diversity in nature, especially in Lake Parishan. 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 old and high expense methods. For isolating and phenol tolerance deter-mination 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 micro-organism 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 able, as a bacterium, to eliminate phenol. Conclusively, Lake Parishan, with biologic variety and different species density, is a suitable bed for more research work.

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