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ORIGINAL ARTICLE

Development of Microbial Biosensors for Environmental Monitoring

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ABSTRACT

A rapidly developing method to address global pollution issues is bioremediation. We designed a microbial biosensor based on *Pseudomonas aeruginosa* for the bioremediation approach for the removal of phenol from an aqueous solution. This bacterium was highly effective in using phenol as a carbon source and maximum degradation of phenol (750 ppm) was achieved using 10% (v/v) of 18 h old inoculum at pH 7 and 35°C in 135 h.

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INTRODUCTION

Rapid industrial growth and fast demand for advancement in environmental control for the betterment of the public need sophisticated techniques and instruments, which monitor fast assessment of a specific analyte with greater accuracy and minimum cost requirement. Generally, for precise sample analysis, they are sent to a laboratory for recognition of low analyte in the sample. These techniques are cost-effective and need more time for scrutiny of the analyte in a sample. Also, professional experts are required to perform these techniques[1].

Biosensors can be used to analyze quickly and accurately during monitoring of contaminated sites and environmental control due to benefits nowadays mostly used testing methods considering portability, on-site working with capacity of complex pollutants analysis with improved and less time-consuming sampling preparation techniques. Considering beneficial aspects of biosensors determines specific chemicals along with their effects on biological systems like cytotoxicity. The biosensor development key step involves biological constituent immobilization on the surface of the transducer, which acquires transducer and biomaterial proximity and stabilization of biomaterial. For immobilization purposes, different methods used are solid surface physical adsorption, surface covalent binding, molecules cross-linking, microcapsule or polymer membrane entrapment, and matrix surfactant. Additional procedures used for immobilization include entrapment by sol-gel, deposition through Langmuir-Blodgett (LB), electro-polymerization, self-gathered biomembranes, and bulk variation[2].

The most common metal contaminants observed in the environment are zinc, chromium, mercury, copper, cadmium, and lead[3,4]. For heavy metals, precise detections, conventional analytical methods used are cold vapor AAS, ICP, and mass spectrometry but due to limitations of high cost, trained personnel, and specific laboratory requirement[5]. For this purpose, bacterial biosensors are designed like biological receptors by using specific genes accountable for bacterial confrontation with such elements. Bacterial strains have been isolated with

biological receptors resistant to several metals like cobalt, copper, mercury, silver, tin, and zinc[6,7,8].

Phenolic compounds and their highly toxic organic derivatives pollutants like chlorophenols and nitrophenols are common in industrial effluents and distributed in the environment. These effluents are produced from industrial activities like plastics production, oil refineries, polymers, dyes, synthetic resins, drugs, pesticides, disinfectants, detergents, antioxidants, and largely from the paper and pulp industry[9]. Silva *et al*., (2011), to detect phenolic chemicals in wastewater matrices used biosensors[10].

An amperometry biosensor was developed by Degiuli and Blum (2000) to detect phenol in environmental samples. Tyrosinase, a polyphenol oxidase with a broad selectivity for phenolic compounds, was immobilized on a graphite electrode using a hydrogel[11]. Using a flow-injection chemiluminescence fiber optic biosensor to boost the chemiluminescence reaction of luminal, which is catalyzed by horseradish peroxidases, discovered chlorophenols.

Due to the antimicrobial activity of phenol, microbes are susceptible to this compound. However, some microbes are resistant to phenol and capable of degrading phenol effectively. In recent years, researchers have focused on phenol microbial degradation which resulted in new novel culture isolation, adaptation, and microorganism enrichment which can utilize phenol compound as a sole carbon and energy source. In these studies, the organism's growth kinetics determination is also considered for a better understanding microorganism's capacity for phenol degradation and selection[12].

Numerous phenol-degrading bacteria have been identified, and the biodegradation of phenolic compounds by microorganisms has been thoroughly investigated[13]. It was discovered that *Pseudomonas* species degraded the phenolic compounds the best[14]. These bacteria can proliferate on many organic substances. The procedure of cell immobilization significantly improves phenol biodegradation[15]. While phenol is biodegradable in both aerobic and anaerobic environments, it can also suppress greater levels of microbial growth in species that use phenol as a substrate[16]. The objective of this study was the development of a biosensor using *P. aeruginosa* for removal of phenol from aqueous s environment.

MATERIALS AND METHODS

Materials

Phenol, 4-amioantipyrene, potassium ferricyanide, ammonium chloride, ammonium hydroxide, nutrient agar, Nutrient broth, sodium chloride (NaCl), dipotassium phosphate (K_2HPO_4), monopotassium phosphate (KH_2PO_4), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), ammonium sulfate (NH_4SO_4), ammonium nitrate (NH_4NO_3), staining kit, peptone, citric acid, hydrogen peroxide (H_2O_2), and yeast extract were used in the study.

Sample Collection for Isolation of Phenol Degrading Microorganism

Samples including water and soil contaminated with sewage discharged from different industrial areas collected from Islamabad and Rawalpindi. The isolation process was done by mixing 1 g of soil and 5 mL of water from each said source in 100 mL distilled water and agitated for 15 min at 35˚C, and 1 mL added on nutrient agar and incubated at 35^{o}C for 24 h. The colonies appeared, selected based on different concentrations of phenol tolerance.

Screening of Phenol Tolerating Strain

Ten strains isolated from soil and water samples of different industrial areas suspended in five flasks having 100 mL minimal media along 100 mg/L phenol only as carbon source were incubated at 35˚C inside a shaking incubator at 150 rpm for 24 h. After growth time, these isolated strains were again transferred on 100 mL minimal medium having phenol 200 mg/L to obtain an enriched population of bacteria and this procedure was repeated 3 times. The bacterial culture that showed maximum growth and which is capable of growth on phenol was obtained and used for advanced studies. Based on Gram-staining and biochemical tests such as the coagulase, catalase, and indole synthesis tests, bacterial strains were identified.

Optimization of Parameters

In this experiment, the impact of various initial phenol concentrations on the percentage of phenol degradation was investigated. Specifically, initial phenol concentrations of 100 to 400 ppm were used, along with 30˚C and pH of 7, and the speed was maintained at 150 rpm for 36 hours. Every twelve hours, the samples were gathered, and the

optical density was measured at 600 nm. The final concentration of phenol degradation by bacteria was observed with the help of the phenol standard curve (10-1000 ppm, R^2 = 0.99). The best initial concentration was observed based on the maximum percentage degradation of phenol by bacteria.

A temperature range of 25 to 45°C and an initial concentration of 500 ppm were used to test the percentage degradation of phenol over a 36 h period while maintaining a speed of 150 rpm. Every twelve hours, the samples were gathered, and the optical density was measured at 600 nm. With the use of the phenol standard curve, the ultimate concentration of phenol breakdown by bacteria was discovered. For more research, the optimal temperature that supports the highest percentage of phenol decomposition was chosen.

The percent phenol degradation was studied at various pH ranging from 6, 7, and 9 with an initial concentration of 500 ppm at 35°C for 36 h by keeping the speed at 150 rpm. The samples were collected after every 12 h of interval and the optical density was observed at 600 nm. The final concentration of phenol degradation bybacteria was observed with the help of the phenol standard curve. The best pH was observed based on the maximum percentage degradation of phenol by bacteria.

The impact of varying inoculum ages on the percentage of phenol degradation was investigated through the use of inoculum after 12, 18, and 24 h, as well as a starting concentration of 500 ppm at 35 °C and pH of 7 for 36 h while maintaining a speed of 150 rpm. Every twelve hours, the samples were gathered, and the optical density was measured at 600 nm. With the use of the phenol standard curve, the ultimate concentration of phenol breakdown by bacteria was discovered. The greatest percentage of phenol breakdown by bacteria was used to determine the optimal inoculum age.

In this experiment, the effect of the inoculum size on the percentage degradationof phenol was studied by using the inoculum size of 1,5 and 10 percent, an initial concentration of 500 ppm at 35°C, and pH at 7 for 36 h by keeping the speed at 150 rpm. The samples were collected after every 12 h of interval and the optical density was observed at 600 nm. The final concentration of phenol degradation by bacteria was observed with the help of the phenol standard curve. The best inoculum size was observed based on the maximum percent degradation of phenol.

Immobilization of Microbial Strains

The isolated bacterial isolates were transferred in sterilized nutrient broth and incubated for 24 h at 150 rpm at 35°C. The cells produced from the media were used for immobilization on glass slides. After being cleaned with distilled water three times, bacterial cells were suspended in 0.2% DEI solution with a pH of 7.0 (adjusted with HCl). For two hours, the suspension was gradually stirred at room temperature. Centrifugation was used to gather the extracted cells, and distilled water was used to wash them. Bacterial cells were immobilized on microscopic slides and then submerged in ethanol 99 % for 24 h. Afterward, the slides were washed with tap water and air-dried. DEI-coated bacterial cells were then transferred over the glass slides kept horizontally ina petri dish. After 30 minutes slides were finally washed under tap water extensively. The slides coated with bacterial cells were stored vertically in water at 4°C. After incubation at 4°C, the binding was observed under a compound microscope.

RESULTS

Isolation of Phenol Degrading Microorganism

The bacterial strain *Pseudomonas aeruginosa* was isolated on agar nutrient plates. The *Pseudomonas aeruginosa* produces a distinguishing greenish color subtle on the plates and gives greenish blue, fluorescent color under glowing light (Figure **1A**) while greenish yellow, fluorescent color under shining light.

Screening of Phenol Tolerating Strain

The phenol degrading strain was identified as the culture that demonstrated growth in the mineral salt medium containing 500 ppm phenol (Figure **1B**). The phenol tolerating strain was further purified by subculturing (Figure **1C**)

Gram- Staining

Bacterial isolate identification was confirmed by the Gram-staining procedure. Finally, prepared slides were washed gently with distilled water kept for drying, and checked in the compound microscope, results indicated

Pseudomonas aeruginosa strains which were rod-shaped (Figure **1F**).

Biochemical Characteristics

Coagulase Test

Bacteria was picked by sterilized wire and added to both plasma and distilled water followed by gentle mixing. Bacterial clumping was not observed within 10 sec. indicated negative coagulase test (Figure **1D**).

Catalase Test

Bacterial strains were tested for catalase test by incubating on nutrient slants at 35°C for 24 h. After 24 of incubation, 3% hydrogen peroxide was added into the tubes and observed for gas bubbles production which indicates positive catalase activity (Figure **1E**).

Optimization of Parameters

The samples were collected every 12 hours at intervals and the optical density was observed at 600 nm. The final concentration of phenol degradation by bacteria was observed with the help of the phenol standard curve. The best initial concentration was observed based on the maximum percentage degradation of phenol by bacteria.

During the study, the degradation of phenol against different concentrations was checked. *P. aeruginosa* was tested for the degradation of different initial concentrations (100, 200, 300, 400 ppm) of phenol to get optimum phenol degradation (Figure **3**).

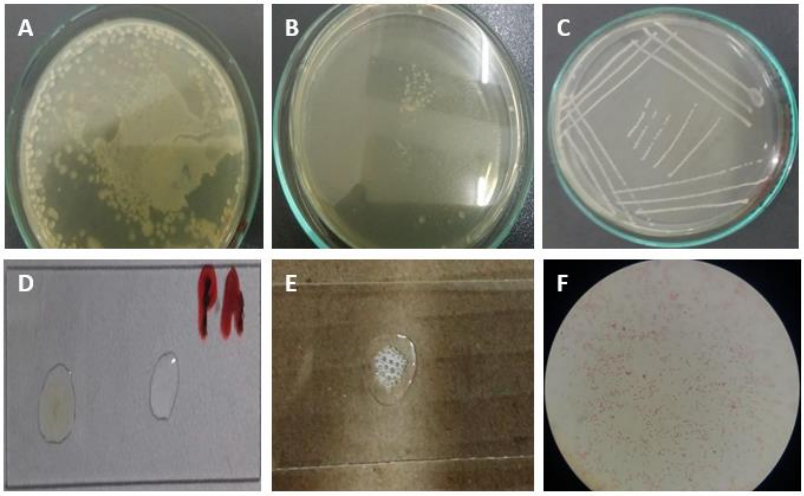

Figure 1. Appearance of *Pseudomonas aeruginosa* greenish blue on nutrient agar plates (A), screening of phenol tolerating strain (B), purification of phenol tolerating strain (C), *Pseudomonas aeruginosa* negative coagulase test (D), *Pseudomonas aeruginosa* catalase positive test (E), microscopy of strain, *Pseudomonas aeruginosa* rod shape and gram negative (F).

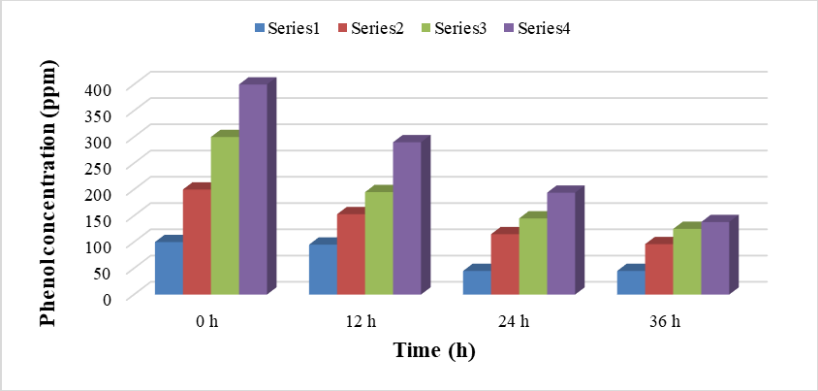

Figure 3. Phenol Degradation Concentration (ppm) values at different time intervals, series 1,2,3,4 were concentrations of phenol at 100, 200, 300, and 400 ppm respectively.

Table 1. Phenol Concentration Degradation at Various Operating Temperatures.

Temperature (°C)	Phenol concentration (ppm) at 0 h	Phenol concentration (ppm) 12 h	Phenol concentration (ppm) 24 h	Phenol concentration (ppm) 36 h
25	500	362	263	176
35	500	370	276	189
45	500	352	251	162

Table 2. Phenol Degradation of Concentrations at Different pH.

pH	Phenol Concentration at 0 h	Phenol Concentration at 12 h	Phenol Concentration at 24 h	Phenol Concentration at 36 h
6	500	370	279	145
7	500	389	293	158
9	500	352	264	127

Table 3. Effect of Different Inoculum Age on Phenol Degradation.

Inoculum age	Phenol concentration at 12 h	Phenol concentration at 24 h	Phenol concentration at 36 h
12 h	383	294	160
18 h	370	286	145
24 h	352	264	127

Table 4. Different Inoculum Size in Phenol Degradation.

Inoculum Size (%)	Phenol concentration at 12 h	Phenol concentration at 24 h	Phenol concentration at 36 h
1	304	184	95
5	342	203	125
10	322	225	140

The final concentration of phenol degradation by bacteria was observed with the help of the phenol standard curve. The best temperature supporting the maximum percentage of phenol degradation was selected for further studies as shown in Table **1**.

The best pH was observed based on the maximum percentage degradation of phenol by bacteria as shown in Table **2**.

The best inoculum age was observed based on the maximum percentage degradation of phenol by bacteria as shown in Table **3**.

The final concentration of phenol degradation by bacteria observed by inoculum size is mentioned in Table **4**.

Phenol Degradation Behavior of Immobilized Pseudomonas Aeruginosa

The degradation curve of immobilized *Pseudomonas aeruginosa* at different initial concentrations of phenol such as 100 ppm and 500 ppm at a temperature 35°C and pH, inoculum age, inoculum size of 7, 18 hours, and 10% spore suspension for 96 hours by keeping the speed at 150 rpm. On immobilization, it was observed, as seen in (Figure **4**) that *Pseudomonas aeruginosa* was able to degrade 500 ppm of phenol within 35h while it can degrade 700 ppm of phenol within 70 h.

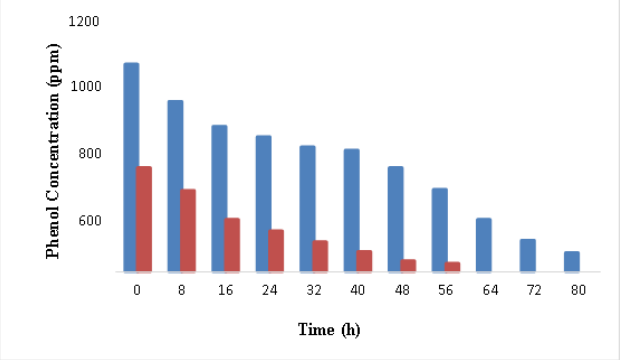

Figure 4. Degradation behavior of free and immobilized cells of *Pseudomonas aeruginosa* (Red represents free cells and blue represents immobilized cells.

As previously mentioned, *Pseudomonas aeruginosa* was unable to grow in free cell form at concentrations of phenol more than 750 ppm. However, it was discovered that *Pseudomonas aeruginosa* could withstand up to 1000 ppm of phenol upon immobilization and could break down such a high concentration of phenol in around 85 h.

DISCUSSION

Microorganisms like bacteria, algae, and yeasts, possess diverse groups of varieties from unicellular to multicellular organisms in the universe and play significant functions in several regular processes like therapeutic, curative, and manufacturing processes like bioremediation, cleaning of environment and biodegradation of biological and synthetic material. Among bacterial species, *Pseudomonas* sp. is a major group that performs a variety of extensive industrial processes. *Pseudomonas* sp. relates to a composite group known as *Pseudomonadaceae* that possesses various heterogeneous microorganisms comprising 211 species among which 56 are categorized as isolated genus[17]. Zheng *et al*., (2009) reported the morphological, biological, and biochemical features of *Pseudomonas aeruginosa* isolated industrial wastewater at SidiBel abbes, Algeria[18]. Saeed *et al*., (2005) screened *Pseudomonas aeruginosa* and further characterized based on 16S rRNA sequencing and biochemical investigation. Puert *et al*., (2006) estimated that the blue-green strain is an indication of *Pseudomonas aeruginosa* and are Gram-negative rod-shaped *Pseudomonas aeruginosa* catalase positive test indicates human contamination[19].

In the present study, *Pseudomonas aeruginosa was* obtained and further maintained on agar nutrient medium and nutrient broth. The species were identified *as Pseudomonas aeruginosa* on the base of their growth condition and morphological distinctive with greenish blue pigment. Under shining light observation, *Pseudomonas aeruginosa* gives a luminous greenish-blue color with a round dense culture. The isolated strain was as Gram-negative rod-shaped with positive catalase and oxidase test.

The *Pseudomonas aeruginosa* was experimented with different parameters, temperature 35^{o}C, pH 7, and different initial concentrations of phenol from 100 ppm to 500 ppm in minimal salt media. The phenol degradation concentration was determined at regular intervals of time using the standard 4-amino antipyrine methodology suggested by Yang *et al.,* (2007)[20]. This study represents the biodegradation behavior of the pseudomonas species with different initial concentrations of phenol. Determining *Pseudomonas aeruginosa* has the capacity to degrade 500 ppm of phenol 69 % in 36 h while it takes more time for the whole degradation of 500 ppm. Liu *et al*., (2009) where the microbe was exposed to a phenol concentration of 800 ppm and was able to degrade up to 99%, have reported the same results[21].

Pseudomonas aeruginosa was given a phenol concentration of 500 ppm as the sole source of carbon and energy in MSM. Temperature 35^{o}C, pH 7, inoculum age 18 h and size 10%. The (P.A) is degraded approximately 69 % in 36 h. Zheng *et al.,* (2010) described strain *Pseudomonas aeruginosa* HSD38 is the ability to degrade up to 500 ppm of phenol below the detection level but unable to tolerate more than 700 ppm of initial concentration of phenol[13].

Sivasubramanian, and Namasivayam, (2015) reported different concentrations of phenol degradation from 100 mgl^{-1} to 1000 mgl^{-1} that concentration was completely degraded in 48 h to 156 h. They optimize different parameters' effects on phenol degradation by bacteria[22]. They observe bacteria degraded phenol high in temperature 20 to 35^{o}C. If increase the temperature from 40^{o}C to 50^{o}C the phenol degradation is low. They optimize different pH 6 to 9 phenol degradation by bacteria. They observed if pH increases or decreases the phenol degradation low by bacteria. The neutral pH 7 is best for phenol degradation by bacteria. The current study optimizes on different temperatures, pH, inoculum ages and size correlates in these results[23,25].

Biodegradation of phenol was studied using pure bacterial cultures of *Pseudomonas aeruginosa*. However, microbial growth is repressed at higher concentrations of phenol. To overcome substrate inhibition, several methods were proposed by several researchers. In different research and industrial applications immobilized cell technology has been extensively applied[23]. The aromatic phenol complexes degradation through immobilized cells was observed. Concentrations of harmful contaminants are broken down by immobilized cells and released cells. Several studies have found that

immobilized bacteria are more resistant to phenolic chemicals than free cells[24]. A variety of support matrices, including glass slides, PAA, calcium alginate beads, and others, were utilized to immobilize the microbial cells. Sandhu *et al*., (2009) reported using immobilized cells to degrade phenol[23].

CONCLUSION

The growing urbanization and huge industrialization cause many problems of environmental pollution. The wastewater is enriched with pollutants like phenol to remove many physical and chemical methods are used. The degradation ability of *Pseudomonas aeruginosa* has been determined and the biodegradation capability of *Pseudomonas aeruginosa* has been compared with other known phenol biodegrading bacteria reported earlier. *Pseudomonas aeruginosa* was immobilized with glass slides to enhance the biodegradation potential compared to free and immobilized cells. Optimized parameters, temperature 35^{o}C, pH 7, inoculum age 18 h, and inoculum size 10% is best for phenol biodegradation by pseudomonas species pH 7.

CONFLICT OF INTEREST

None.

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None.

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