

PHENOL DEGRADATION BY AN INDIGENOUS SOIL *PSEUDOMONAS AERUGINOSA* AND *BACILLUS SUBTILIS*

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ABSTRACT

Phenol and phenolic compounds, widely used in industries are of growing concern owing to their high toxicity, and wide distribution in industrial wastes. The present study envisages microbial potential of *Psuedomonas aeruginosa* and *Bacillus subtilis* isolated from the activated sludge effluent of oil refinery, for the biodegradation of phenol. The individual isolated culture was acclimatized with different phenol concentrations, under ambient temperature in nutrient broth and in mineral medium. Growth kinetics of adapted bacterial culture was investigated. The study reveals that successive increase in phenol concentration directly increased the generation time. While using mineral salt medium supplemented with phenol, generation time increased compared to growth in nutrient broth. With the passage of time culture became adapted with the higher concentration of phenol. However, once the organisms adapted itself to phenol containing environment, the generation time decreased. This means that to expose the organism directly to higher concentrations of phenol would be lethal rather the culture should be gradually exposed to increasing concentration of phenol. Such adapted cultures have the potential to degrade phenol in relatively short time. Present findings prove that phenol adapted culture would reduce the cost of treatment of phenol containing wastewater.

Key words: Toxicity, growth kinetics, microbial potential, bioremediation

INTRODUCTION

The quantity and diversity of hazardous wastes have grown with the progression of technology. The effective disposal of such waste has become an important challenge. Sustainable development focuses on making social and economic progress to satisfy global human needs and desires without damaging the environment. Biological treatment of organic waste is an efficient and well-established technology. There is a great concern about the treatment of industrial wastewater containing organic and inorganic chemicals of different nature, which are not easily biodegradable (Iniesta *et al.*, 2001).

Phenol is a known toxic pollutant and its removal from the effluent is expensive through known methods of wastewater treatment. It is present in many industrial effluents and is currently removed by costly and inadequate physical or chemical methods. Current methods of its treatment often produce other toxic end products as well requiring other processing steps. Kobayashi and Rittman (1982). Phenol and its derivatives, which are present in many wastewater streams, must be specifically treated because of their extreme toxicity for aquatic life (Miro *et al.*, 1999). They have high water solubility as well as toxicity and they happen to be of great industrial significance as solvent in petroleum, fine chemical and pesticide industry.

Exposure to phenol may result in irritation of eyes, dermal rash, dysphasia, gastrointestinal disturbances, vomiting, weakness, weightlessness, muscle pain, hepatic tenderness and nervous disorder. (Nair *et al.*, 2008). Phenols and phenolics are introduced in the environment in the waste stream of several industrial operations, such as pulp bleaching with chlorine, water disinfection or even waste incineration and as degradation product of other chlorinated xenobiotics (Bolag *et al.*, 1986).

Other sources of phenol can be oil refineries, steel industries, wood preservative plants, chemical plants, explosives manufacturing, resin manufacturing, rubber reclamation, plastic, disinfectants, textile mills and plants, etc. frequently contain high concentrations of phenolic compounds, which represents a serious ecological problem due to their widespread use, toxicity and existence throughout environment (Ordaz *et al.*, 2001) (Haribabu *et al.*, 1993).

Several investigations have shown that many toxic compounds can enter the environment, disperse and persist to a greater extent. Their increasing release by industries causes many health related problems (Haleem, 2003) (Panday and Jain, 2002). Genetic approaches are used to optimize the enzymes. Metabolic pathways and organisms relevant for biodegradation may provide the most effective technology as it is a natural process that is essentially taking advantage of the nature's recycling abilities (Pieper and Reneke 2000). Bioremediation is emerging as most ideal technology for removing pollutants from the environment by the action of microbes or other biological systems, restoring contaminant sites and preventing further pollution (Pieper and Reneke, 2000; Caplan, 1993; Dua *et al.*, 2002). Biodegradation describes the complete mineralization of the starting compound into simpler ones

(Atlas and Bartha, 1998). According to the principle of microbial infallibility, no natural organic compound is totally resistant to biodegradation provided that the environmental conditions are favourable (Alexander, 1965).

MATERIALS AND METHODS

Sources of bacterial strains for biodegradation of xenobiotic are sludges which are contaminated by xenobiotic of interest. Bacterial strains resistant to that toxicant are capable of degrading it effectively can be found there. Sludge sample was collected from effluent of activated sludge system of Pakistan Refinery Limited; it was collected from a catchment area nearby the seaside of Karachi. A loopful sludge sample was inoculated in nutrient broth (N.B) in three tubes, they were incubated for 24 hours. Next day culture was stained by Gram staining. The concentration of phenol was gradually increased to check their tolerance, the highest concentration was 2000 mg/L phenol. Finally gram positive bacilli and gram negative short rods were selected as phenol degraders. The cultures were maintained on nutrient agar slopes at 4 C and sub cultured after every three months. During preservation purity of the cultures was checked periodically.

Identification and characterization of bacterial culture

The identification and characterization of bacterial isolates was performed using morphological, cultural and biochemical tests as described in (Collin and Lyne, 1985). The two isolates were classified as *Pseudomonas aeruginosa* and *Bacillus Subtilis*.

Growth Kinetic Studies of Bacterial isolates

Inoculum was prepared by transferring a loopful of culture from the stock nutrient agar slant, into 10 ml nutrient broth tube and incubated at 37 C for 24 hours followed by transferring 2.5 ml inoculated broth into 250 ml of nutrient broth contained in 500 ml flask for growth and biodegradation studies. The optimum concentration of phenol that supports bacterial growth was evaluated.

Preparation of the inoculum for Growth Kinetic in Mineral Medium

Inoculum was prepared for growth kinetics in mineral medium by scratching the 24 hours grown culture on nutrient agar with a wire loop, phosphate buffer saline was added in this scratched culture in a screw capped tube, it is centrifuged, the upper layer of liquid was discarded, then again phosphate buffer saline added in the screw capped tube, the liquid layer again discarded, now this suspension is matched with the McFarland index VI. (1.8×10^9 bacteria/ml)

The isolate has repeatedly been used for phenol biodegradation studies. For use in the experiments 250 ml of mineral salts medium supplemented with different concentration of phenol was inoculated with 1% culture and incubated at 35 C, rpm was kept at 125.

The mineral salts medium contained the following constituents (gm %): phenol variable; $(\text{NH}_4)_2\text{SO}_4$, 0.5; NaCl, 0.5; KH_2PO_4 , 0.15; Na_2HPO_4 , 0.25; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.005, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0015. The components of the medium except phenol were autoclaved at 121 °C. Phenol was filter sterilized separately and added to other components after autoclaving.

Growth of the bacterial isolate in the presence of added phenol was determined immediately by taking optical density in spectrophotometer at 600 nm by a suitable interval of time.

Growth curve

The selected bacterial strain was incubated in nutrient broth and was used as inoculum (2.5%) for nutrient broth and mineral medium containing phenol. Flasks were then incubated at shaking water bath. Samples were collected at different time intervals and their optical density at 600 nm was noted. The generation time (g) was calculated.

Analytical methods

Phenol was determined spectrophotometrically by the modified 4-aminoantipyrin method. (Yang and Humphery, 1975). To the 50 ml of a filtered sample, 0.5 ml of 1 % (w/v) potassium ferricyanide and 2.5 ml of 1 % (w/v) 4-aminoantipyrine were added. The absorbance of the resulting mixture was read at 500 nm with a Shimadzu UV-VIS 1201 spectrophotometer. Phenol concentration was determined with a calibration curve made from known phenol standards.

Growth Kinetic Studies

This study was aimed to determine the growth kinetics of *IES.S*, *IES.B* capable of degrading phenol using nutrient broth alone, broth supplemented with phenol 100, 500, 1000 mg/L phenol and mineral medium containing 1, 2, 3, 10, 20, 40, 80, and 150 mg/L phenol in shaking water bath.

24 hours grown culture was inoculated in 500 ml flask containing 250 ml nutrient broth in shaking water bath at 35 C and 125 rpm. Generation time was calculated by measuring the absorbance at 600 nm at 1 hour time interval till stationary phase of growth was achieved. The results are shown in the following tables followed by the figures.

The results clearly show that with the increase in time the growth was increased and stationary phase was achieved after a certain time.

Statistical Analysis

For statistical analysis standard error of mean was calculated, t- test was performed which showed the difference between *IES.S* and *IES.B* growth is non significant.

RESULTS AND DISCUSSION

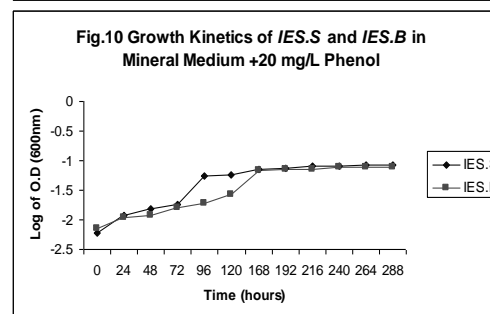
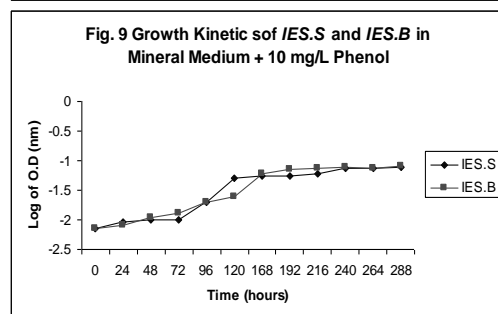
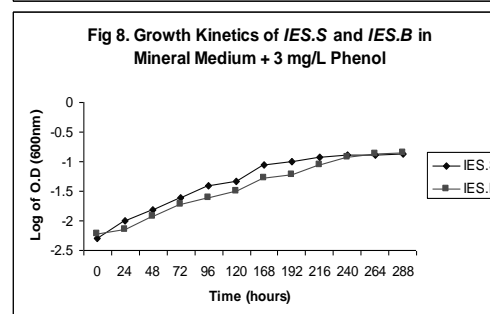
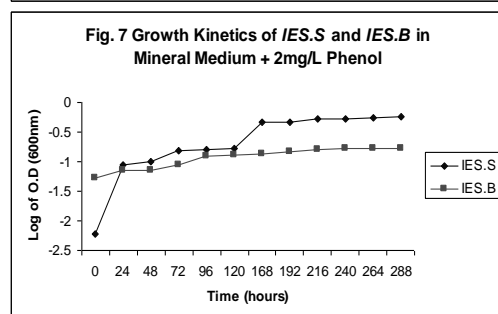
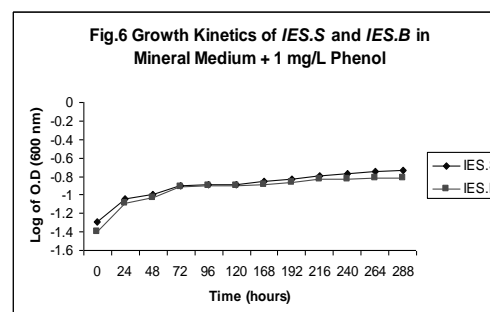
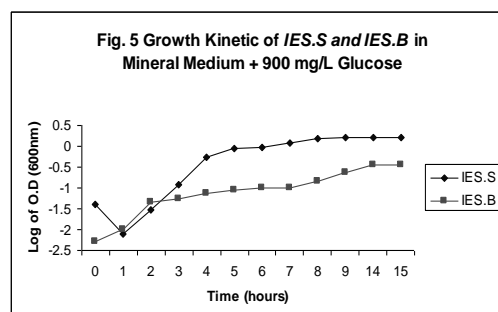
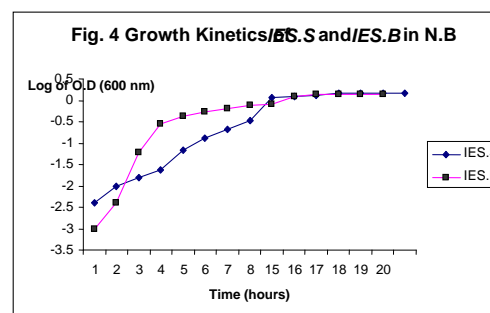
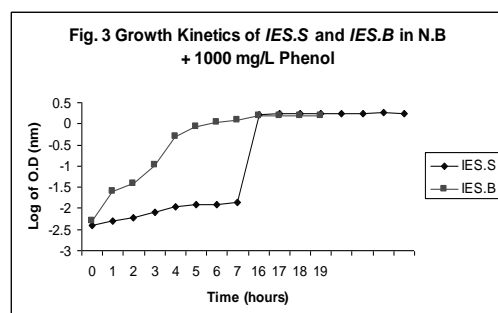
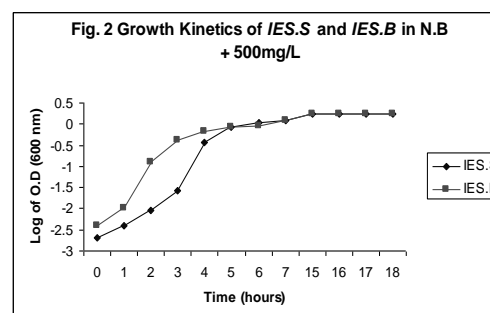
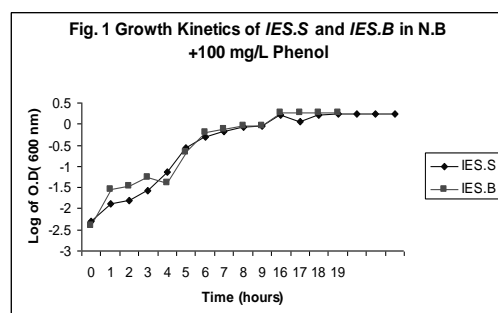
Biodegradation is as old as life itself, where microbes use their potential to remove pollutants from the environment. As it is a natural way of reducing toxic organic materials to harmless carbon dioxide, water and various types of salts, it is nowadays a preferred technology to remove toxic pollutants from the environment than the traditional methods that involve caustic, solvent based cleaners or hauling. Caustic chemicals often create even more environmental problems. Rapid industrial and economic development and human exploitation of fossil fuels increase production of synthetic compounds have introduced many compounds like phenolics which are not naturally occurring, they are toxic to living systems and their presence in atmospheric, aquatic and terrestrial environment poses serious ecological problems (Mathur *et al.*, 2008).

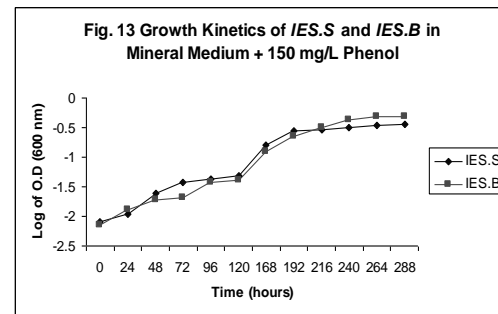
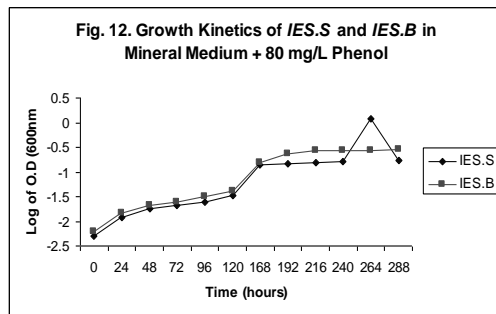
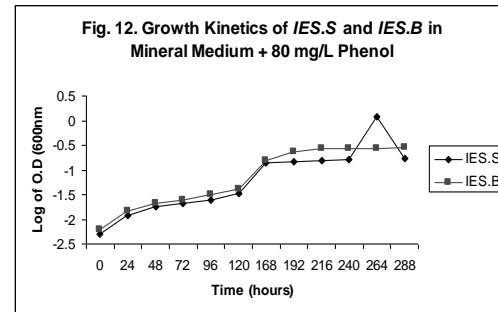
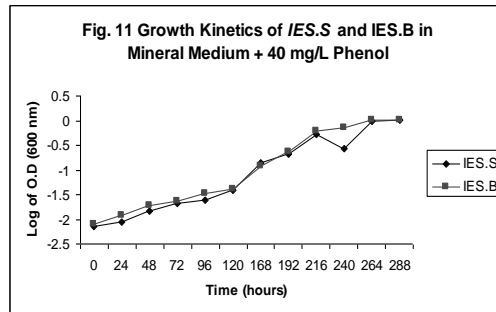
Microbes existing naturally, in all soil, water, produce enzymes that break down hydrocarbons into smaller, less toxic chemicals. Bioremediation swiftly reduces, neutralizes or eliminates the toxicants through mineralization. The extensive use of benzene, phenol and their halogenated derivatives in industrial processes has led to wide spread pollution of the environment by these toxic compounds. Therefore, information on their biodegradation is of great interest. In the recent years, research has been focused on the use of the ability of microbial strains to completely degrade these chemicals. A large number of microbes are known to degrade a variety of these toxic hydrocarbons. (Zaistav *et al.*, 1995. Keeping this view in mind, the present study was aimed to study the degradation kinetics of phenol degraders along with their growth kinetics. Apart from these, identification of cultures degrading phenols was also performed in laboratory. Studies on the toxicity of phenol to sediment bacteria can adapt to ambient phenol concentrations, but increasing phenol concentrations appear to decrease overall phenol biodegradation. Brantner *et al.*, 1996).

Phenol degrading cultures were identified as *Pseudomonas*, *Bacillus subtilis*. *Pseudomonas* and *Bacillus subtilis* were isolated from the activated sludge effluent of oil refinery. *Pseudomonas* is a champion degrader as it degraded other toxic compounds also.

The results of growth kinetics in nutrient broth, of *Pseudomonas*, *Bacillus subtilis* are shown in figures 1, 2, 3 and 4. These show that the generation time in nutrient broth is fairly low as compared to the broth supplemented with phenol. And the organisms have potential to grow in environment having high concentration of phenol. Although the organisms are under stress but the exponential phase could be accelerated once the organism adapted itself to such an extreme environment. Figures 1, 2, 3 show that the organism attained the stationary phase after 11, 9 and 10 hours in 100, 500 and 1000 mg/L of phenol in nutrient broth. With the passage of time culture became adapted with the higher concentration of phenol.

Pseudomonas gained the stationary phase after 9, 7 and 10 hours in 100, 500 and 1000 mg/L phenol in nutrient broth. The Generation time is quite high in mineral salt medium as compared with that in nutrient broth, which could satisfy the nutritional requirements of the organisms. However, once the organisms adapted it self to phenol containing environment, the generation time decreased. This means that to expose the organism directly to higher concentrations of phenol would be lethal rather the culture should be gradually exposed to increasing concentration of phenol. Such adapted cultures have the potential to degrade phenol in relatively short time. Moreover, such culture in addition would reduce the cost of treatment of phenol containing waste water. Figure 15 shows that *IES.B* has higher potential of degradation of phenol than *IES.S*. In mineral medium least degradation has shown by both cultures in 20 mg/L and highest degradation shown in 40 and 80 mg/L Phenol.



Table 1 Growth Kinetic Studies of Culture *IES.S* in Nutrient Broth supplemented with phenol.

Growth Kinetics	Nutrient broth	Nutrient broth supplemented with phenol		
		100 mg/L	500 mg/L	1000 mg/L
Generation Time (min)	100	117	151	112
Specific growth rate (min^{-1})	0.01	0.0085	0.0066	0.0089

Table 2. Growth Kinetic Studies of *IES.B* in nutrient broth supplemented with phenol.

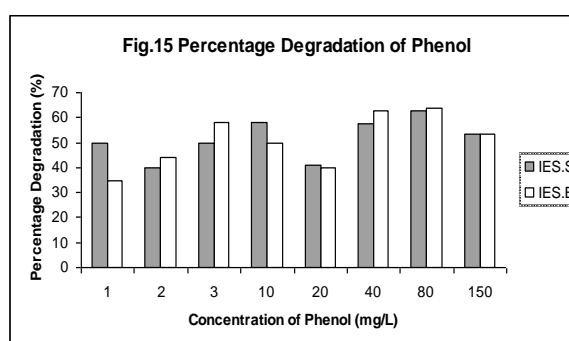
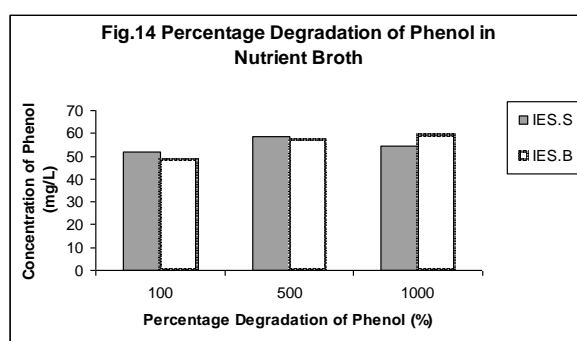
Growth Kinetics	Nutrient broth	Nutrient broth supplemented with phenol		
		100 mg/L	500 mg/L	1000 mg/L
Generation Time (min)	97	108	92	162
Specific growth rate (min^{-1})	0.010	0.0092	0.010	0.0156

Table .3 Growth Kinetic Studies of Culture *IES.S* in Mineral Medium supplemented with phenol.

Growth Kinetics	Mineral Medium supplemented with phenol								
	Glucose 900 mg/L	1 mg/L	2 mg/L	3mg/L	10 mg/L	20 mg/L	40 mg/L	80 mg/L	150mg/L
Generation Time (min)	155	3113	1614	2428	2508	1788	2317	2104	2242
Specific growth rate (min^{-1})	0.006	0.00032	0.0006	0.0004	0.0003	0.0005	0.0004	0.0004	0.0004

Table 4. Growth Kinetic Studies of *IES.B* in Mineral Medium supplemented with phenol.

Growth Kinetics	Mineral Medium supplemented with phenol								
	Glucose 900 mg/L	1 mg/L	2 mg/L	3mg/L	10 mg/L	20 mg/L	40 mg/L	80 mg/L	150mg/L
Generation Time (min)	724	6916	7400	3230	3230	3101	2575	2165	2458
Specific growth rate(min^{-1})	1.38	0.0004	0.0001	0.0003	0.0003	0.003	0.0003	0.0004	0.0004



Conclusion

Adaptation of *IES.S* and *IES.B* to higher concentration of phenol indicates that they can be successfully exploited to treat waste water and other effluents with high concentration of phenol. It is suggested that isolated organisms can be used to transform phenol in aqueous environment.

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