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BIODEGRADATION OF PHENOL BY FREE AND IMMOBILIZED CELLS OF A NOVEL *Pseudomonas* sp. NBM11

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Abstract - In the present study, a pure culture of bacterium (*Pseudomonas sp.* Strain NBM11) was isolated from the soil sample from a site contaminated with medical wastes and wastewater. The isolated strain can degrade up to 1000 mg/L of phenol completely. It was observed that temperature, pH and initial concentration of phenol play key roles in determining the rate of phenol degradation. The isolated strain exhibited the maximal degradation of the substrate within a range of pH 6.8 to 7.2 and an incubation temperature between 30 ºC and 32 ºC. It was found that by increasing the concentration of phenol, the lag phase gets extended due to the inhibitory nature of phenol. The kinetic parameters such as μ_{max} (maximum specific growth rate), K_s (half-saturation coefficient) and K_i (substrate inhibition constant) were estimated as 0.184 1/h, 7.79 mg/L and 319.24 mg/L, respectively, by fitting the growth kinetics data to the Haldane model of substrate inhibition. The bacterial strain was immobilized in alginate beads and its phenol degradation efficiency was observed to increase many fold. The immobilized cells were found to be used efficiently for seven cycles consecutively without any decrease in their efficiency.
Keywords: *Pseudomonas sp.*; Immobilization; Calcium alginate; *Acinetobacter calcoaceticus*; Phenol Biodegradation.

INTRODUCTION

Phenol is the basic structural unit of a wide variety of synthetic organics (Agarry and Solomon 2008) (Agarry *et al*., 2008). It is a listed priority pollutant by the U.S. Environmental Protection Agency and Agency for Toxic Substances and Disease Registry (Services 2003). Phenolic pollutants are generated from several sources, like the partial degradation of phenoxy herbicides, the use of wood preservatives and the generation of wastes by petroleum-related industries such as petroleum refineries, gas and coke oven industries, pharmaceuticals, explosive manufacture, phenol-formaldehyde resin manufacture, plastic and varnish industries and related metallurgical operations, etc. (Arutchelvan *et al*., 2005; Bandhyopadhyay *et al*., 2001; Kumar *et al*., 2005; Kumaran and Paruchuri, 1997). Acute exposure to phenol by the oral route leads to damage to blood, liver, kidney and cardiac toxicity, including weak pulse, cardiac depression, and reduced blood pressure. Various physicochemical methods like ionization, adsorption, reverse osmosis, electrolytic oxidation, H_2O_2, photocatalysis, etc. have been used for the elimination of phenol from contaminated waters. Phenol removal by biological methods is preferred to physicochemical methods because of its eco-friendliness and cost effective nature and the possibility of

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complete mineralization of the substrate (Kobayashi and Rittmann, 1982; Prpich and Daugulis, 2005). Biodegradation of phenol using pure and mixed cultures of suspended bacteria has been studied in detail. Nevertheless, owing to the inhibitory effects of phenol on the microbes, biological treatment of phenol-containing effluent has been facing challenges (Liu *et al*., 2011; Jiang *et al*., 2007). Therefore, to remove higher concentration of phenol from effluents, it is peculiarly significant to isolate appropriate microorganism that can endure as well as effectively degrade phenol at relatively high concentration. Phenol-degrading microbes have shown substrate inhibition at higher concentration of phenol, and dynamics of microbial growth have been reported with different substrate inhibition models. The growth kinetic parameters such as maximum specific cell growth rate (μ_{max}), substrate affinity constant (K_S), and substrate inhibition constant (K_i) specify the efficiency of the biodegradation process and vary over a wide range depending upon the microorganism and culture conditions (Banerjee and Ghoshal 2010).

Microbial cell remediation efficiency for xenobiotic pollutants remains a major challenge to microbial ecologists and process engineers. Hence, some strategies have been proposed to overcome the issue. Use of microbial immobilization technique is one of them. Nevertheless, the application of free microbial cells for treatment of wastewater in activated sludge processes creates issues such as solid waste disposal. Immobilized microorganisms have proved to be effective to address phenol-containing wastewater with little sludge yield and have been receiving increasing attention (González *et al*., 2001; Annadurai *et al*., 2007; Pazarlioğlu and Telefoncu, 2005). Immobilized cell technology has been widely applied in a variety of research and industrial applications.

The purpose of the present investigation has been studying the biodegradation of phenol using free and immobilized *Pseudomonas* sp. strain NBM11. Factors affecting the degradation process and stability of immobilized cells were also looked into.

MATERIALS AND METHODS

Microorganism and Growth

A phenol-degrading *Pseudomonas* sp. strain NBM11 (Gene Bank accession number JQ889810) was isolated from a site contaminated with sewage and waste water discharged from infirmary origin. It is capable of using phenol as the sole source of carbon and energy. The microbe was grown on mineral salt medium which contains K_2HPO_4: 500 mg; KH_2PO_4: 250 mg; NaCl: 0.5 g; NH_4SO_4: 230 mg; $CaCl_2.2H_2O$: 7.5 mg; $MgSO_4.7H_2O$: 100 mg; $MnSO_4.7H_2O$: 100 mg; $FeCl_3$: 1 mg; Double Distilled Water: 1000 mL at pH = 7. The bacteria *Acinetobacter calcoaceticus* (NCIM 2286), *Pseudomonas putida* (NCIM 2650) and *Pseudomonas resinovorans* (NCIM 2599) were procured from the National Collection of Industrial Microbiology (NCIM), National Chemical Laboratory, Pune, in lyophilized form. These cultures were revived in a solid agar Petri dish and in the liquid medium as per the instructions provided by the suppliers. Stock cultures were then obtained by standard spread plate microbial techniques and used for degradation study. Analytical grade phenol was used as a sole source of carbon, and the sterilized phenol solution was added directly to Mineral salt Medium (MSM). The medium was sterilized by autoclaving, and the phenol was used after filter sterilization. Growth was in 250 mL Erlenmeyer flasks containing 50 mL culture medium at 30 °C on a rotary shaker at 150 RPM.

Molecular Analysis of the Isolate

Approximately 100 mg (wet) of the microbial cells was used for extraction of DNA as per the method described by Sinha *et al*. (2008). The 16S rRNA gene was amplified using the extracted DNA by universal primer 16s - 27F (5′ to 3′AGAGTTTGATCMTGGCTCAG, M = A or C) and 16s-1492R (5′ to 3′ACGGCTACCTTGTTACGA) (Bangalore Genei, India). Polymerase Chain Reaction was performed in 50 µL volumes containing 2 mM $MgCl_2$, 2.5 Unit per reaction of Taq polymerase (Bangalore Genei, India), 100 µM of each dNTP, 0.2 µM of each primer and 3 µL template DNA. The PCR program used was an initial denaturation at 96 °C for 5 min followed by 30 cycles of 95 °C for 15 s, 49 °C for 30 s and 72 °C for 1 min and a final extension at 72 °C for 1 min. Amplified DNAs were purified by the Bangalore Genei™ gel extraction kit following manufacturer's instructions. Finally, the amplicon obtained was sequenced along with the primers used to get a consensus sequence for the isolate.

Identification of the Isolated Bacterial Strain

Sequence data obtained was examined for sequence homology with the archived 16s rDNA sequences of GeneBank, NCBI using BLAST algorithm. Multiple alignments of sequences were performed

with the Clustal X (1.83) program (Saitou and Nei, 1987). A phylogenetic tree was constructed using the neighbor-joining DNA distance algorithm (Thompson *et al*., 1997) using MEGA 6.0.5. The partial sequences of the 16s rDNA gene of the isolate were submitted to GeneBank, NCBI.

Optimization Studies

The present study was undertaken to optimize the physiological parameters such as pH, incubation temperature and initial substrate concentration of the isolate for maximum degradation of phenol. The effect of a change in incubation temperature on phenol degradation was studied with various initial concentrations of phenol (200 mg/L to 1000 mg/L) at neutral pH in the MSM. Similarly, pH was varied between 6 and 8 at a different initial concentration of phenol at a constant temperature. The residual phenol concentration was measured at regular time intervals.

Growth Kinetics of the Isolated Strain for Phenol Biodegradation

Various substrate utilization and inhibitory models have been extensively studied for growth kinetics of microbes on phenol (Sahoo *et al*., 2011). Out of these models, the Haldane growth model is simple and widely accepted for representing growth kinetics of inhibitory compounds. Hence, the Haldane growth model was used for the study of growth kinetics of the isolated strain in phenol degradation over the concentration range. We have assumed that the aeration provided maintains the oxygen levels constant and does not limit the microbial growth. Hence, the influence of oxygen was not considered and it was presumed that the growth and phenol degradation rate of the isolated strain was only inhibited by substrate concentration at a given initial pH and temperature (Wang *et al*., 2010).

Kinetics of cell growth in a batch reactor may be described by Equation (1),

$$\frac{dx}{dt} = \mu X - K_d X \tag{1}$$

where μ is specific growth rate (1/h), X is biomass concentration (mg/L), K_d is the endogenous coefficient (1/h) and t is time (h). During exponential growth, K_d can be assumed to be negligible. Hence, the above equation can be written as:

$$\frac{dx}{dt} = \mu X \tag{2}$$

The Haldane model equation that relates microbial specific growth rate (μ) and limiting substrate concentration (S) is as follows:

$$\mu = \frac{\mu_{\max} S}{K_S + S\dfrac{S^2}{K_i}} \tag{3}$$

where $\mu_{\max}$ is the maximum specific growth rate (1/*h*), K_s is the half-saturation coefficient (mg/L) and K_i is the substrate inhibition constant (mg/L). The biokinetic parameters $\mu_{\max}$, K_i and K_s for the isolate have been estimated by fitting their respective experimental growth data to the Haldane kinetic model. This model was solved by the use of a non-linear regression method using computer software (MATLAB V 7.11).

Immobilization of the Microorganisms

Liquid cultures were centrifuged in a 50 mL plastic centrifuge tube (2500 g) at room temperature for 10 min, and the supernatant was discarded. The pellet was suspended in a previously autoclaved solution of sodium alginate to a final concentration of 40 g/L and 100 mL/L bacterial biomass. The alginate-bacterial mixture was added dropwise with a sterile syringe (20 mL) fitted with a wide bore needle (1 mm diameter) from a height of about 20 cm into an autoclaved solution of calcium chloride (30 g/L, adjusted to pH = 7.0), where beads formed immediately. The beads were left in this hardening solution overnight at 4 °C before being harvested by filtration.

Storage Stability and Reusability of Immobilized Cells

Free and immobilized cells were stored for (0, 10, 20 and 30) d at 4 °C and then the cells were tested for phenol degradation rate. Cells were grown at 30 °C for 48 h in minimal media (pH = 7.2) consisting of phenol. To test the reusability of immobilized cells, the immobilized cells were repeatedly used in several consecutive phenol degradation processes, and the phenol degradation rate was detected. The immobilized cells were incubated in MSM for 24 h as one cycle.

Analytical Methods

Phenol was colorimetrically estimated at 520 nm according to the method described by Yang and

Humphrey (1975) based on the principle of rapid condensation of phenol with 4-amino antipyrene followed by oxidation with alkaline potassium ferricyanide.

RESULTS

All experimental results reported in this section were based on average results of repeated experimental runs (duplicates).

Isolation, Characterization and Identification

Initially, about thirty strains were isolated from the enrichment cultures of the contaminated soil sample, which were individually subjected to phenol amended medium. Out of these isolates, only six bacterial strains demonstrated more than 80% phenol degradation. Based on its ability to survive the increasing concentration of phenol the isolate NBM11 was selected for further study. The morphological & biochemical characteristics of the isolate were given in Table 1.

The 16S rRNA gene sequence determined in this study has been deposited in the GenBank database under the accession number JQ889810. A BLAST search revealed that the obtained nucleotide sequence showed a high degree of sequence similarity with *Pseudomonas sp*. A phylogenetic tree was constructed based on 16s rRNA gene sequences available in the GenBank database by the Neighbor-joining method using MEGA software (Figure 1). The isolate was designated as *Pseudomonas* sp. NBM11.

Table 1: Biochemical characteristics of isolate *Pseudomonas* sp. NBM11.

CHARACTERISTICS	ISOLATE
Gram staining	Gram Negative
Cell shape	Rod shaped Bacilli
Motility	Motile
Colony shape	Circular, smooth, wet, convex
Oxygen requirement	Aerobic
Catalase	+
Nitrate Reduction	+
Indole Production	-
Oxidase	+
Gelatin Liquefaction	+
Dextrose Fermentation	+
Sucrose Fermentation	+
Lactose Fermentation	-
Citrate Utilization	+
Methyl Red	-
VP	-

Figure 1: Phylogenetic tree showing the strain NBM 11 in the *Pseudomonas* tree based on 16S rRNA partial gene sequence analysis. Numbers at nodes are bootstrap values (%) based on neighbour-joining analysis. The scale bar indicates the number of nucleotide substitutions per site.

Effect of Incubation Temperature

The residual phenol estimation data revealed that maximum degradation occurred at 30 ºC as shown in Figure 2. Significant degradation also observed at 35 ºC even if it is less than at 30 ºC. Degradation was inhibited both at low as well as high temperatures. These results substantiate with previous studies carried out by Cordova-Rosa *et al*. (Cordova-Rosa *et al*. 2009), on phenol degradation by soil Pseudomonad. Similar results have been reported on the *Pseudomonas pictorum* at 30 ºC (Annadurai *et al*. 2007).

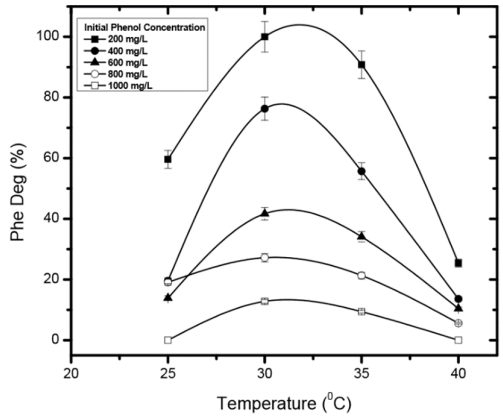

Figure 2: Effect of temperature on phenol degradation by the isolate *Pseudomonas* sp. NBM11 at various initial concentrations of phenol.

Effect of pH

Biodegradation of phenol in an aqueous solution is highly dependent on pH of the solution, which affects the surface charge of the absorbent and the degree of ionization (Annadurai *et al*. 2000). Increasing the pH of the media from 6 to 8 at 30 ºC increases the rate of degradation of phenol (Figure 3). Further increasing the pH had the reverse effect on the phenol removal potentiality of the isolate. Both acidic and alkaline pH caused a marked inhibition of the phenol removal efficiency. Similar results were obtained by Karigar *et al*. (2006) for *Arthrobacter citreus*. This may be due to the effect of pH on the ionization, which in turn affects the metabolic pathway of the organism. Changes in pH even cause denaturing of proteins that might affect the microorganism negatively.

Effect of Concentration of Phenol

A study of the effect of varying initial concentration of phenol on the phenol degradation efficiency of the microbe is shown in Figure 4. With the increase in the initial phenol concentration, a decrease in the percentage of phenol degradation is observed. Since the organisms were kept at higher phenol concentration for 48 h, the death of cells results in an increase in degradation time. Similar observations have been reported by Hinteregger *et al*. (1992) for *P. putida* Ek II at 1100 g/L and 1200 g/L phenol concentration.

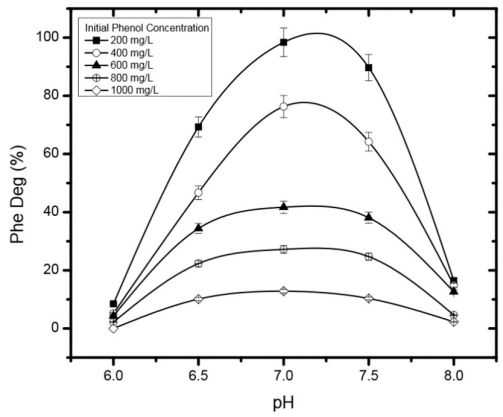

Figure 3: Effect of pH on phenol degradation by the isolate *Pseudomonas* sp. NBM11 at various initial concentrations of phenol.

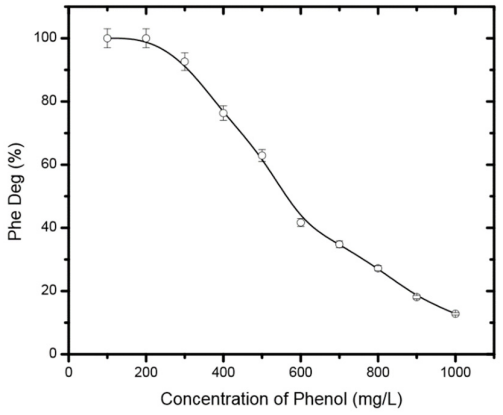

Figure 4: Effect of initial concentration of phenol on degradation by the isolate *Pseudomonas* sp. NBM11 at various initial concentrations of phenol.

Biodegradation of Phenol by Free and Immobilized Cells of Strain NBM11

The batch biodegradation study of the strain was carried out in mineral salt media containing phenol as the sole source of carbon. The microbe was subjected to varied initial phenol concentration of 250 mg/L to 1250 mg/L. The study was carried out in the optimum physiological conditions of an incubation temperature of 30 °C and pH 7 in an orbital

shaker incubator at 150 RPM. Figures 5 and 6 depict the growth profile and phenol degradation data of the isolate *Pseudomonas* sp. NBM11 at various initial concentrations of phenol. In the present study, the microbial strain was immobilized in Ca-alginate beads and the biodegradation potential of the microbes were observed. Figure 7 reveals the degradation potential of the microbe on being immobilized in the alginate beads. A significant increase in the degradation potential of the microbial strain can be observed from the obtained results.

Immobilized Cells Comparative Analysis

The immobilized cells of the strain *Pseudomonas sp*. NBM11 along with the immobilized microorganisms, *Acinetobacter calcoaceticus* (NCIM 2286) and *Pseudomonas resinovorans* (NCIM 2599) and *Pseudomonas putida* (NCIM 2650) were studied in the same experimental condition. Figure 8 depicts that, when the initial concentration of phenol in the medium is 500 mg/L, the isolate in its immobilized form degrades the hydrocarbon faster than immobilized *Pseudomonas putida*. On the contrary, the immobilized cells of *Acinetobacter calcoaceticus* degrade phenol faster than the immobilized isolate even if both exhibit almost the same degradation profile in their respective freely suspended forms. When the initial concentration of phenol is 1000 mg/L, it can be observed that the microbe in its immobilized state not only shows a better degradation profile than the other two microorganisms in their immobilized state, but also is able to degrade the xenobiotic easily within 48 h, while the other microbes *Acinetobacter calcoaceticus* and *Pseudomonas putida* can degrade phenol completely in 60 h.

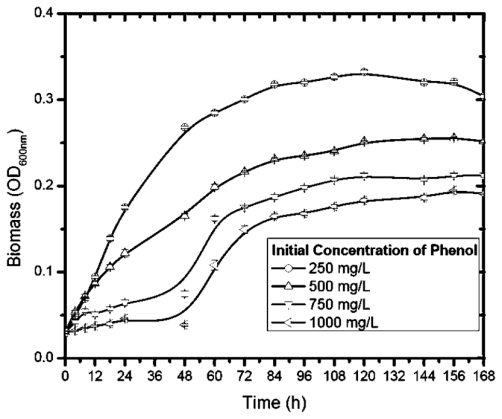

Figure 5: Growth profile of the isolate *Pseudomonas* sp. NBM11 at different initial concentrations of phenol.

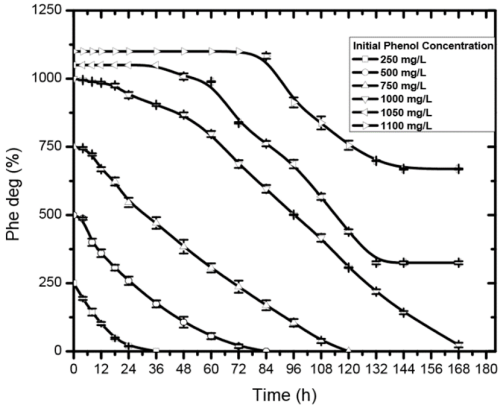

Figure 6: Degradation profile of the isolate *Pseudomonas* sp. NBM11 at different initial concentrations of phenol.

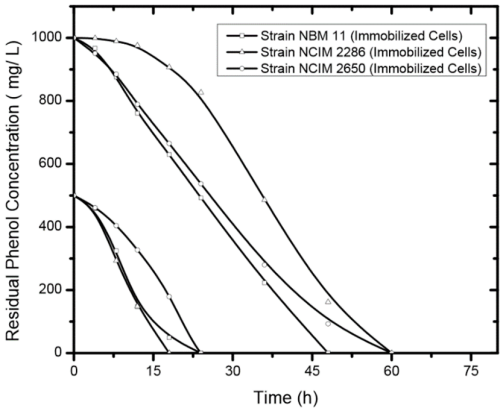

Figure 7: Degradation profile of the immobilized cells of the isolate *Pseudomonas* sp. NBM11 at different initial concentrations of phenol.

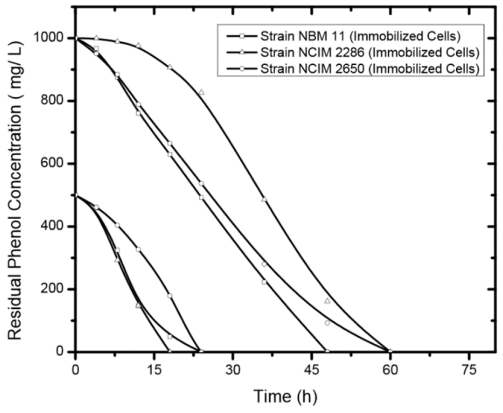

Figure 8: Comparative analysis of the degradation profile of the various microorganisms in their immobilized state at various initial concentrations of phenol.

Repeated Use of Immobilized Cells

The long-term storage stability is a crucial factor for the application of the immobilized cell system.

On the extension of storage time, immobilized cells adapt a stable phenol degradation rate. The immobilized cells can degrade up to 95% phenol after being stored at 4 °C, while the rate of degradation by the free cells decreases after a prolonged storage. This suggests that immobilized cells feature stronger storage stability than that of free cells. Furthermore, immobilized cells were tested in several consecutive phenol degradation processes to determine if there was deactivation of cells after repeated use. Immobilized cells were consecutively used for phenol degradation and remained active without loss of any activity over seven cycles of reuse. This indicates the suitability of using the immobilized cells for several batches, as reported earlier.

Modelling the Growth Kinetics of the Strain NBM11 in the Presence of Phenol

The observation of the phenol inhibition of the growth of the culture can be modelled using a suitable substrate inhibition model described in the literature. Microbial growth is usually represented by the simple Monod equation:

$$\mu = \frac{m_{\max} S}{S + K_S} \tag{4}$$

But this equation becomes unsatisfactory, for explaining the inhibitory growth of the microorganism at higher substrate concentration. In such cases, the Haldane or Hans-Levenspiel model has been used to represent the growth kinetics of the culture in the presence of inhibitory substrates. The kinetic parameters for phenol degradation by isolated strain *Pseudomonas* sp. NBM11 were obtained by fitting their respective experimental growth data to the Haldane model equation (Equation (3)). These parameters are listed in Table 2. The coefficient of correlation (R^2) found to be 0.9815, indicating that the model well described the growth behaviour of the isolated strain NBM11 in media containing phenol. Figure 9 establishes that the specific growth rate increases with the increment in the initial phenol concentration up to a certain phenol concentration, after which it starts decreasing with the increase in phenol concentration. This suggests the inhibitory effect of phenol at higher concentrations. The inhibitory effect of high phenol concentrations on microbes such as *Pseudomonas putida* as well as on the mixed microbial consortium has been described by using the Haldane model (Saravanan *et al*., 2008; Bakhshi *et al*., 2011). Thus, in the present study, the Haldane model explained well the inhibition effect of phenol at high concentrations.

Table 2: The kinetic parameters for phenol degradation by the isolated strain.

Strains	Haldane Model		
	μ_{max} (1/h)	K_s (mg/L)	K_i (mg/L)
Pseudomonas sp. NBM11	0.184	7.79	319.24

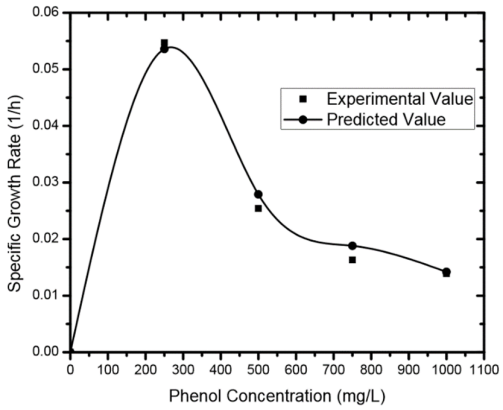

Figure 9: Haldane growth kinetic model fitted to experimental batch growth data of the strain *Pseudomonas* sp. NBM11.

DISCUSSION

One of the most alarming situations in today's world is the generation of a huge amount of waste water contaminated with the toxic organic substances like phenolics from the industrial sector. Phenol is highly water soluble, and its presence in the water imparts a carbolic odor to the receiving water bodies and can have harmful effects on aquatic as well as terrestrial flora and fauna, including human beings (Agarry *et al*., 2008). Hence, removal of phenol from the discharged sewage and effluent is highly necessary. Conventional methods for treatment of phenol have their set of disadvantages, hence the biological method is the current choice. Table 3 shows some of the reported bacterial strains that are capable of degradation of phenol available in the literature. The biodegradation potency of the microbial strains at a different initial concentration of phenol was compared with reference microorganisms like *Acinetobacter calcoaceticus* (NCIM 2286) and *Pseudomonas putida* (NCIM 2650) procured from commercial laboratories. Each microbe exhibits a different degradation profile of phenol. The result suggests that, with an increase in the initial concentration of phenol, an

Table 3: Summary of maximum tolerance of phenol exhibited by various microorganisms obtained in various studies.

Microorganism	Source	Maximum Phenol Concentration Tolerated (mg/L)	Reference
1. *Acinetobacter* sp.	Wastewater treatment plant	500	Abd-el-haleem *et al*. (2003)
2. *Acinetobacter calcoaceticus*	Industrial coal wastewater treatment plant	1200	Cordova-Rosa *et al*. (2009)
3. *Pseudomonas putida*	MTCC	1000	Kumaran and Paruchuri (1997)
4. *Pseudomonas putida*	MTCC	1000	Bandhyopadhyay *et al*. (1999)
5. *Pseudomonas putida*	Wastewater	800	Vázquez-Rodríguez *et al*. (2006)
6. *Mixed consortia*	Sewage treatment plant	800	Saravanan *et al*. (2008)
7. *Pseudomonas resinovorans*	PCP contaminated Site	500	Yang and Lee (2007)
8. *Pseudomonas fluorescence*	Oil-polluted Area	500	Agarry and Solomon (2008)
9. *Streptococcus epidermis*	Oil Spill industry	200	Mohite *et al*. (2010)
10. *Acinetobacter calcoaceticus*	NCIM	1000	Present study
11. *Pseudomonas putida*	NCIM	1000	Present study
12. *Pseudomonas* sp. NBM11	Soil contaminated with hospital sewage and medical waste	1100	Present study
13. *Pseudomonas resinovorans*	NCIM	750	Present Study

increase in the lag phases is observed, even though well-acclimatized inoculums were used during the experiments. The lag-phase of one week had been reported by Hill and Robinson (1975) during phenol degradation at 700 mg/L of initial concentrations using well-acclimatized *P. putida*. The study concluded that not only inhibitory effect of the substrate, but also the inoculum size might affect the duration of the lag phase. Thus, to minimize the lag-phase the amount of inoculums should be increased. It was observed that, with an increase in the concentration of the substrate, the degradation potential of the isolate decreases and is completely inhibited at a concentration of 1150 mg/L of phenol. The microbe was able to degrade the substrate completely up to 1000 mg/L of phenol in the medium. Bandhyopadhyay *et al*. (1999) reported the inhibiting effect of phenol as substrate above the concentration of 600 mg/L.

CONCLUSION

In the present study a novel *Pseudomonas sp.* was reported to utilize phenol as the sole source of carbon and energy. Temperature, pH, and initial substrate concentration play key roles in determining the rate of phenol degradation. Immobilization of the microbes in a solid matrix is an advantage over the freely suspended ones since they can repeatedly be used, giving the treatment process an enhanced cost effective rate of degradation and tolerance to the toxic substrate. In the future scope of the study, biodegradation of phenol by the mixed bacterial strains may diminish the substrate inhibition effect of the compound. The present study evidently suggests that the *Pseudomonas* sp. NBM11 is an efficient strain for the bioremediation of a variety of phenol contaminated effluents. Though many reports are available in the literature about phenol degradation by various *Pseudomonas* groups, this is the first report of such fast phenol degradation by a *Pseudomonas sp*.

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