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Microbial Degradation of Phenol by an Application of *Pseudomonas mendocina*

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Abstract

The present investigation was undertaken to isolate and assess the microbial degradation of phenol by bioaugmentation of *Pseudomonas mendocina* isolated from activated sludge of Common Effluent Treatment Plant (CETP) based at Ankleshwar (Gujarat, India). The strain was isolated and designated as *Pseudomonas cepacia* ETL 2413 after examined for colony morphology, gram stain characteristics and various biochemical tests. *Pseudomonas cepacia* ETL 2413 was found to be highly effectual for the removal of phenol which was used as sole carbon and energy source. From an initial concentration of 200mg/L, it degraded to 76.43 ± 1.23 mg/L. In turn the effect of temperature (25 to 500C), pH (5.5 - 10.5) and glucose concentration (0, 0.25 and 0.5%) on the rate of phenol degradation was investigated. Observations revealed that the rate of phenol biodegradation was affected by pH, temperature of incubation and glucose concentration. The optimal conditions for phenol removal were found to be pH of 7.5 (82.63% removal), temperature 300C (78.69% removal) and 0.25% supplemented glucose level (98.28% removal). The significance of the study is the utilization of native bacterial strains isolated from the waste water itself having potential for environmental bioremediation in the activated sludge process of a CETP Plant.

Keywords: Pseudomonas; Phenol; Environmental bioremediation; CETP

Introduction

During the last few decades, an array of foreign compounds has been introduced into the environment due to the revolution of industrial field. The accumulation of these compounds has resulted in environmental contamination and contributed too many deleterious effects on living systems. The need to remove these contaminations has led to the development of new technologies with different removal performance and cost levels. Traditionally waste waters were treated by Physico Chemical methods, but recently Microbial Degradation has been widely studied and used as a low-cost alternative and offering the possibility of complete mineralization of organic compounds [1]. Studies on microbial means of treating Phenolic effluents date back at least two decades. The toxicity of Phenol has been widely documented and their disastrous effect towards human and environment is greatly concerned. Phenolics constitute 11 of the 126 chemicals that have been designated as priority by the United States Environmental Protection Agency [2]. Phenol in water and wastewater has been the major organic chemicals [3]. and is associated with pulp mills, coal mines, gasoline, petrochemicals, wood preservation plants, pesticides, insecticides, herbicides, detergents, solvents, polymeric resin production, plastic rubber proofing, disinfectants, pharmaceuticals, metallurgical, explosives, textiles, dyes, the coffee industry, domestic waste, agricultural run-off, and chemical spills [4-7]. The maximum Permissible limit of Phenolic compounds in leachates for safe disposal to inland surface water is 1mg/L. Phenol is currently removed by methods such as precipitation/coagulation, osmosis, ion-exchange, ultra filtration, electro dialysis, electrochemical degradation, floatation, etc., which are costly and inefficient. These current treatment methods often produce other toxic end products, requiring further processing

Steps [6,8]. In future technologies, for bioremediation microbial systems might be the potential tool to deal with the Environmental Pollutants [9]. Microbial degradation of Phenol has been actively studied and these studies have shown that Phenol can be aerobically degraded by wide variety of fungi and bacteria cultures such as Candida tropicalis [10], *Acinetobacter calcoaceticus* [11], Alcaligensm eutrophus [12,13]. *Pseudomonas putida* [14,15].

Phenol biodegradation has been chosen as a method to remediate environments contaminated by Phenol, which is massively discharged from uncontrolled industrial waste disposal. Phenol has traditionally been removed from industrial effluents by costly Physico chemical methods, but biodegradation method has been studied recently as an alternative [16,17] on accounts of its lower cost associated with this as well as the possibility of complete mineralization of xenobiotics.

Materials and Methods

Chemicals and reagents

Phenol used in the study was of analytical grade and purchased from Merck, India. All other chemicals were also of analytical grade which were purchased from Merck and Hi-Media laboratories, India.

Isolation of bacterial strain by enrichment method

The activated sludge sample was collected from Common Effluent Treatment Plant (CETP) of Ankleshwar, Gujarat, India. A quantity of one gram of activated sludge sample was suspended in 100 ml of Minimal Salt Medium (MSM) containing Na_2HPO_4 (6g), KH_2PO_4

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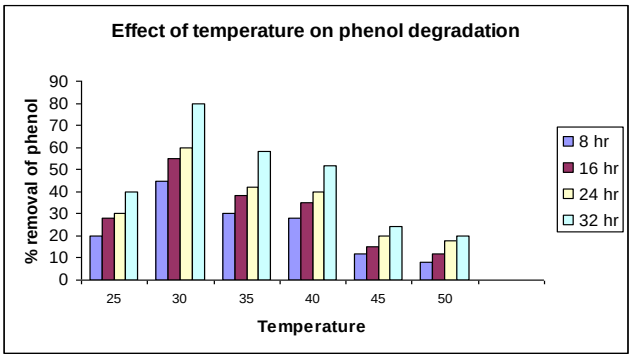

Figure 1: Effect of temperature on phenol degradation.

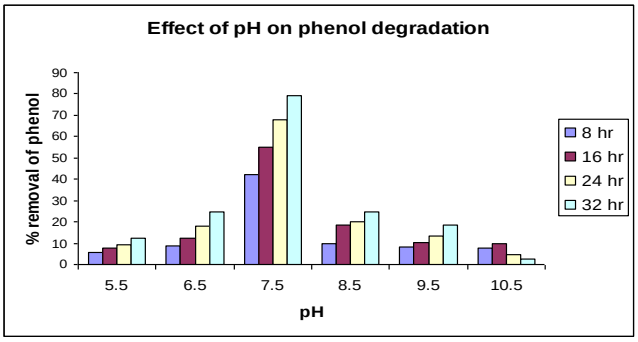

Figure 2: Effect of pH on phenol degradation.

(3g), NaCl (0.8g), NH_4Cl (0.5g), $CaCl_2.2H_2O$ (1M) and $MgSO_4.7H_2O$ (1M) in 1000 ml of distilled water. 10mg/L of phenol was used as sole source of carbon and then incubated in 250ml flask at 37± 2°C on rotary shaker incubator (REMI, India) at 130rpm for a week [18].

A volume of 5 ml of enriched media was transferred into freshly prepared media on each week supplemented with 10mg phenol and then incubated at 30°C. The isolated single colonies were streaked on Nutrient Agar Plates, incubated at 30°C overnight. Pure isolates were stored on LB agar slants supplemented with phenol as sole source of carbon at 4°C until further use.

Identification of isolates

The isolate was identified based on morphological observations and biochemical characterization (Table 1). The tests involved were Gram staining, Amylase and Gelatinize production, Citrate utilization, Indole test etc., [19]. Bergey's manual of determinative bacteriology was used as a reference to identify the isolates [20].

Strain selection based on phenol acclimatization

The isolate coded as ETL 2413 was inoculated into MSM (Mineral Salt Medium) containing 10mg/L phenol as carbon source for 72hrs shaking at 130rpm. After 24hrs, the growth of cells was determined by turbidity measurement at 600nm using spectrophotometer (Shimadzu, 1800). The concentration of phenol was increased from 10mg/L to 250mg/L subsequently.

Phenol degradation studies

Bacterial isolate strain coded ETL 2413 was grown in the Nutrient Broth by incubating overnight at 37°C on shaker at 130rpm. This

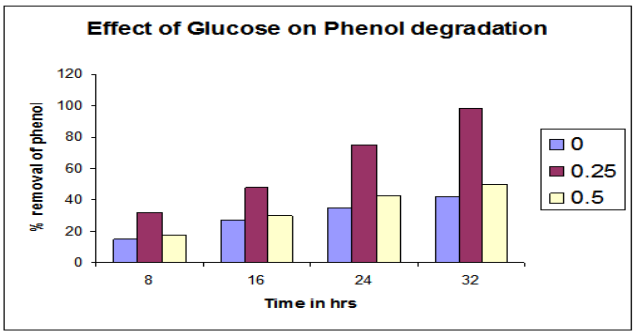

Figure 3: Effect of Glucose on phenol degradation.

24hrs old culture was inoculated into MSM medium with phenol as sole carbon source. Preliminary degradation studies were carried out with addition of bacteria on media containing 10mg/L of phenol and cultivated in submerged conditions at 37°C at 120rpm for 120hrs. The reaction mixture containing all components but devoid of bacterial inoculums were used as control. Then same procedure was followed by increasing concentration of phenol from 10mg/L to 250mg/L.

The phenol concentration was determined by analyzing samples at each 8 hours interval by using UV spectrophotometer 1800 SHIMADZU, JAPAN. The residual amount of phenolic compounds present in the sample at different inoculation period were measured by colorimetric assay 4-Amino Antipyrine method (APHA, 1992) (Figure 1).

Experimental procedure

To study the optimum functional pH, temp., and carbon source for maximum degradation, variation in incubation temperature (25 to 50°C) with constant initial concentration of Phenol (200mg/L) and neutral pH in absence of carbon was carried out. Similarly, other Parameters were kept constant, and pH was varied between 4.5 - 10.5. For optimization of glucose as carbon source, keeping the cultures at pH 7 and 30°C, three different glucose status *viz.* without glucose, with 0.25% glucose and 0.50% of glucose were chosen in the media containing bacterial suspension and Phenol. The residual Phenol concentration was measured at time slots of 8, 16, 24, 32hrs. All the results were given as a mean with Standard Deviation (±SD). The experimental results confirmed that aeration and mixing do not cause Phenol volatilization. All experiments were carried out in triplicates and the mean value considered.

Results and Discussion

Bacterial isolation & identification

The present study was aimed to degrade aromatic organic compound Phenol using microbes isolated from activated sludge of Common Effluent Treatment Plant of Ankleshwar, Gujarat, India. Many different isolates were obtained from the Activated Sludge of Common Effluent Treatment Plant (CETP), but one major colony was taken and identified based on morphological, cultural and biochemical characteristics. Up to 45 days, sample was enriched in sterile MSM medium using phenol as sole carbon source. The sample was further treated with phenol to ensure that only phenol resistant strain would be selected.

Table 1: Biochemical characterization of microbes.

(A) MORPHOLOGICAL PROPERTIES:	
1. Gram's Staining	Gram negative short rods
2. Motility	Positive
(B) CULTURAL CHARACTARISTICS	
1. On nutrient Agar	Translucent bluish green
2. pigmented	Diffused colonies

Table 2: Biochemical properties.

S. No.	Test	Result
1	Catalase production	+
2	Oxidase production	+
3	Indole production	-
4	Methyl Red	-
5	Voges Prouskaur	-
6	Nitrate reduction	+
7	Citrate utilization	+
8	Urease production	-

The bacterial isolate coded as ETL-2413 had the best potential for phenol biodegradation based on high resistance of this xenobiotic compound. The bacterial isolate was morphologically and biochemical characterized as listed in (Table 2). According to Bergey's manual of determinative of Bacteriology 95% of results showed the similarity in characteristics with Pseudomonas cepacia.

Effect of Incubation Temperature on Phenol degradation studies

Microbial degradation of phenol was observed over a wide temperature range (250C - 500C) with an optimum of 300C. Temperature might play an equivalent or larger role than nutrient availability in the degradation of organic pollutants [21]. In this work batch culture of *Pseudomonas mendocina* was studied in media containing just phenol as a sole carbon and energy source. As a result biodegradation is limited by phenol concentration only. The most important factor that can affect negatively on biodegradation process is phenol inhibition which is stronger at high phenol concentration more than 200mg/L the process. As the subjective of this study is to evaluate performance of phenol removal process, the studied phenol concentrations were 10, 20, 50, 100, 200mg/L, while in 250mg/L the inhibitory effect of phenol could stop the growth and phenol biodegradation, so which means the bacteria could not tolerate substrate toxicity in this case, therefore studied concentration is limited to 200mg/L .According to Pakula, et al. [22], phenol biodegradation was significantly inhibited at 300C. However, most laboratory studies on phenol degradation have been carried out at an optimum temperature of 300C [23,24]. Annadurai, et al. [23]. And Chitra [25]. Described that when the temperature increased to beyond 300C or 350C, no or less phenol degradation was observed due to cell decay, which is a temperature-dependent parameter. At the end of 32 hrs 80% of phenol was degraded by ETL - 2413 at 30°C and 60% at 40°C, while at extreme temperatures of 25°C and 50°C it was only 40% and 20% respectively. This corroborates with previous studies by Polymenakau and Stephanous [26]. On phenol degradation by soil Pseudomonad. They recorded maximum degradation rates for phenol to be at 30°C. However temperature of 35°C also showed considerable degradation but level of degradation was lower than 30°C (Table 3).

Table 3: Residual phenol concentration in mgl^{-1} after 8,16,24,32 hrs.

Culture	after 8 hr	after 16 hr	after 24 hr	after 32 hr
Pseudomonas Mendonia	175 ± 2.05	148 ± 1.98	9 ± 1.58	76.43 ± 1.23

Table 4: Carbohydrate fermentation.

S. No.	Test	Result
1	Glucose	+
2	Lactose	-
3	Mannitol	-
4	Maltose	+
5	Xylose	+
6	Sucrose	+
7	Rhamnose	+

Effect of pH on phenol degradation

The pH range from 4.5 to 10.5 with an optimum of 7.5 was found suitable for the degradation of phenol. The internal environment of all living cell is believed to be approximately neutral. Most organisms cannot tolerate pH values below 4.0 or above 9.0 [27]. At low (4.0) or high (9.0) pH values, acids or bases can penetrate into cells more easily, because they tend to exist in un dissociated from under these conditions and electrostatic force cannot prevent them from entering cells [23,25,27]. Increasing the pH of media at 30°C increased the rate of phenol degradation (Figure 2) from 4.5 to 7.5. On increasing the pH further it had reserved effect on ETL-2413 phenol removal potentially. In 8 hrs 40% phenol was removed at pH 7.5, while rest of the pH conditions could not degrade phenol more than 10%. Both acidic and highly alkaline pH had marked inhibitory growth on phenol removal efficiency. After 8, 18, 24 &32 hrs also analogous result was seen with only 82.63% removal till end at pH 7.5 at 30°C. These results were found to be corresponding with work by Karigar [28]. On Arthrobacter citrus.

Effect of glucose on phenol degradation

Phenol removal efficiency was determined at different glucose concentration at a neutral pH of 7.5 and 30°C temperature for ETL - 2413. The data collected after 36 hrs showed that maximum phenol removal efficiency of 98.28% was accessible at 0.25% of glucose concentration (Figure 3). It was found that it was decreased to 48.35% with increasing glucose concentration to 0.75% and also in the absence of glucose media devoid of glucose, at the end of the 36hrs phenol removal was about 88%. Previously Kar, et al. [29]. Showed the effect of glucose on phenol degradation and the results indicated that when a mixed substrate (Phenol and glucose) was used, phenol acclimatized population showed initial preference for phenol to glucose concentration. A glucose concentration of 0.50% repressed the induction of phenol oxidation though glucose did not fully repressed utilization of phenol. Alike results were obtained by Khaled [30]. In studies (Table 4).

Conclusion

Considering the present situation of Environment, long term

strategy for the permanent solution of phenol removal in waste water is permanently required. Industrial waste water treatment is now emerging as a challenging task for greener and sustainable environment. Hence it was quite important to adopt a technology which was eco friendly as well as economically viable with this motto and vision; we have initiated a novel research work for the benefit of upcoming environmental scenario. Contamination of the environment with hazardous and toxic chemicals is one of the major problems faced by industrialized nations today.

Therefore it can be concluded that *Pseudomonas mendocina* isolated from common effluent treatment Plant of Ankleshwar (Gujrat, India) can be a promising phenol degrader at an optimum pH of 7.5 and an incubation temperature of 30°C. Glucose addition up to a specific low concentration could improve the degradation rate, but

Impeded the degradation process at higher concentrations. Hence, this strain has remarkable potential for application in bioremediation and wastewater treatment, especially in detoxification of phenolic waste. The present study mainly focused on *Pseudomonas mendocina* for its dynamics not only on phenol degradation but also removal of toxic pollutants using cost effective process as a part of developing an innovative Microbial Technology for cheaper and effective treatment of Phenol degradation.

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