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Microbiological Removal of Phenol by an Application of *Pseudomonas spp.* ETL-: An Innovative Biotechnological Approach Providing Answers to the Problems of FETP

Maulin P Shah*

Industrial Waste Water Research Laboratory, Division of Applied & Environmental Microbiology Lab, Enviro Technology Limited (CETP), Ankleshwar, Gujarat, India
*Corresponding author: shahmp@uniphos.com

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Abstract The present investigation was undertaken to assess the phenol biodegradation by bioaugmentation of *Pseudomonas spp. ETL-2412.* The strain was isolated and designated as *Pseudomonas spp. ETL 2412* after examined for colony morphology, gram stain characteristics and various biochemical tests. *Pseudomonas spp. ETL 2412* 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 200 mg l-1 it degraded to 76.43 ± 1.23 mg l-1. In turn the effect of temperature (25 to 50°C), 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 and glucose concentration. The optimal conditions for phenol removal were found at pH 7.5 (82.63%), temperature 30°C (78.69%) and 0.25% supplemented glucose level (98.28%). It can be concluded that this strain has remarkable potential for application in bioremediation and wastewater treatment, especially in detoxification of phenolic waste. The significance & impact 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 FETP Plant.

Keywords: *Pseudomonas, bioremediation, FETP, bioaugmentation*

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1. Introduction

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 [9]. Phenolic constitute 11 of the 126 chemicals that have been designated as priority by the United States Environmental Protection Agency [6]. Phenol in water and wastewater has been the major organic chemicals [24], 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 [1,5,13,23,40]. The maximum Permissible limit of Phenolic compounds in leachates for safe disposal to inland surface water is 1 mg/l. It is greatly concerned pollutant and included in list of EPA (1979). 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 [13,21,33]. On the other hand, biodegradation has been studied as an alternative approach due to the low costs associated with this option, as well as the possibility of complete mineralization of xenobiotic [39]. In future technologies, for bioremediation microbial systems might be the potential tool to deal with the Environmental Pollutants [27]. 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 bacterial cultures such as *Candida tropicalis* [7,36,37], *Acinetobacter calcoaceticus* [30], *Alcaligensm eutrophus* [16,22], *Pseudomonas putida* [15,28]. 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 has been studied recently as an alternative [10,11] on account of its lower cost associated with this as well as the possibility of complete mineralization of the xenobiotics.

2. Materials and Methods

2.1. Chemicals and Reagents

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

2.2. Isolation of Bacterial Strain by Enrichment Method

The activated sample was collected from Final Effluent Treatment Plant (FETP) 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 (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. 10 mg/L of phenol was used as sole source of carbon and then incubated in 250 ml flask at 37± 2°C on rotary shaker incubator (REMI, India) at 130rpm for a week [26]. A volume of 5 ml of enriched media was transferred into freshly prepared media on each week supplemented with 10 mg phenol and then incubated at 30°C. The isolated single colonies were streaked on Nutrient Agar Plates, incubated at 30°C overnight and then the pure isolates were stored on LB agar slants supplemented with phenol as sole source of carbon at 4°C until further use.

2.3. Identification of Isolates

The isolate was identified based on morphological observations and biochemical characterization. The tests involved were Gram staining, Amylase and Gelatinase production, Citrate utilization, Indole test etc., [4,41]. Bergey's manual of determinative bacteriology was used as a reference to identify the isolates [12].

2.4. Strain Selection Based on Phenol Acclimatization

The isolate coded as ETL 2412 was inoculated into MSM (Mineral Salt Medium) containing 10mg/L phenol as carbon source for 72 hrs shaking at 130 rpm. After 24 hrs, the growth of cells was determined by turbidity measurement at 600 nm. The concentration of phenol was increased from 10 mg/L to 250 mg/L subsequently.

2.5. Phenol Degrading Studies

Bacterial isolate strain coded ETL 2412 was grown in the Nutrient Broth by incubating overnight at 37°C on shaker at 130 rpm. This 24 hrs 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 10 mg/L of phenol and cultivated in submerged conditions at 37°C at 130 rpm for 120 hrs. 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 10 mg/L to 250 mg/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).

2.5.1. The 4-Amino Antipyrine Method

When phenol reacts with 4-Amino Antipyrine at pH 7.9±0.1 in the presence of Potassium ferricyanide forms colored Antipyrine dye this dye is kept in aqueous solution and the absorbance is measured at 500 nm. 70µl of 0.5 N NH_4OH solution was added into the sample and pH is adjusted to 7.9 ± 0.1 with phosphate buffer, 30 µl of 4-Amino Antipyrine solution was added and mixed well and 30 µl of $K_3Fe(CN)_6$ solution is added . After 15 minutes it was transferred to cells and absence of sample was monitored against the blank at 500 nm. It is noted that after the addition of various concentrations of phenol (10, 20, 50, 100, 200 mg/l). The initial sample was collected and readings were monitored by 4-Amino Antipyrine method and kept as standard optical density values.

2.5.2. 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 (200 mg/L) and neutral pH in absence of carbon was carried out. Similarly, other Parameters were kept constant, and pH was varied between 4.5 and 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, 32 hrs. 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.

2.5.3. Phylogenetic Analysis

Almost the full length of 16S rRNA genes of bacteria was amplified by PCR with following sets of primers 5¢-GAGTTTGATCCTGGCTCAG-3¢ and 5¢-AAGGAGGTGATCCA GCC-3¢ corresponding to the positions 9 to 27 and 1525 to 1541, respectively, in the 16S rRNA gene sequence of Escherichia coli [42]. PCR products were sequenced directly using ABI PRISM Big Dye Terminator Cycle Sequencing Kit on an ABI 3100 DNA sequencer following the manufacturer's instruction. Multiple alignments of the sequences were performed, and a neighbor joining phylogenetic tree [43,44] was constructed using the latest version (ver. 1.8) of the CLUSTAL W program [45]. Similarity values of the sequences were calculated by using the GENETYX computer program.

3. Results and Discussion

3.1. Bacterial Isolation & Identification

The present study was aimed to degrade aromatic organic compound Phenol using microbes isolate from activated sludge of Final Effluent Treatment Plant of Ankleshwar, Gujarat, India. Many different isolates were obtained from the Activated Sludge of Final Effluent Treatment Plant (FETP), 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. The bacterial isolate coded as ETL-2412 have the best potential for phenol biodegradation based on high resistance of this xenobiotic compound. The bacterial isolate was morphologically and biochemical characterized & properties were listed in Table 1. According to Bergey's manual of determinative of Bacteriology, 95% of results showed the similarity in characteristics with *Pseudomonas spp.*

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

Sr. 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	-

Table 3. Carbohydrate Fermentation

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

Table 4. Residual phenol concentration in mgl^{-1} after 8,16,24,32 hr

Culture	after 8 hr	after 16 hr	after 24 hr	after 32 hr
Pseudomonas	175± 2.05	148± 1.98	98±1.58	76.43±1.23
Spp. ETL-2412				

3.1.1. Identification by Molecular Approach

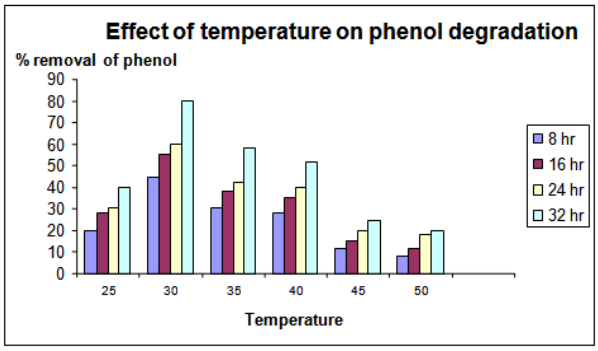

Figure 1. Effect of temperature on phenol degradation

The results of 16S rDNA sequence alignment and phylogenetic tree analysis revealed that 16S rDNA sequence of *pseudomonas* spp. ETL was 100% identical to that of Figure 4. The DNA–DNA hybridization between *pseudomonas* spp. ETL and a reference strain *P. aeruginosa* JCM 5962T was 96%. The taxonomic characteristics of *pseudomonas* spp. ETL were mostly the same as those of *P. aeruginosa* JCM 5962T, that is, tests for production of catalase and oxidase, reduction of NO3 to NO2, and hydrolysis of casein and gelatin are positive, but o-nitrophenyl-b-D-galactopyranoside (ONPG) test and hydrolysis of starch were negative for the both strains. However, *pseudomonas* spp. ETL was able to hydrolyze neither lipids (supplied as tributyrin), maltose nor D-mannose, all of which were hydrolyzed by *P. aeruginosa* JCM 5962T.

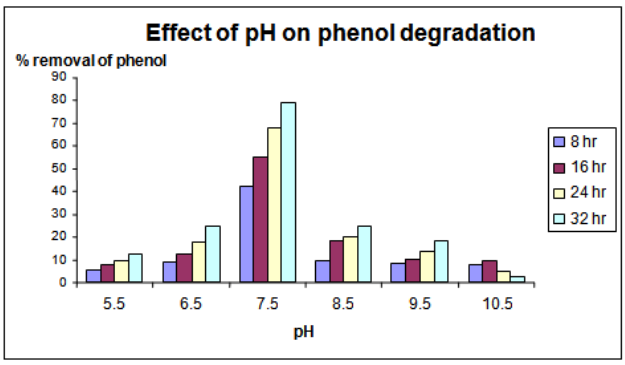

Figure 2. Effect of pH on phenol degradation

3.2. Effect of Incubation Temperature on Phenol Degradation Studies

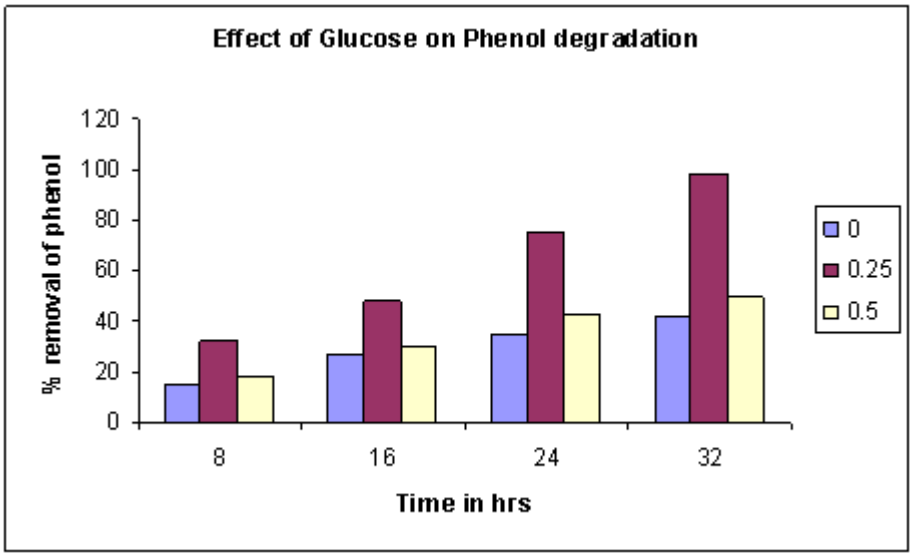

Figure 3. Effect of Glucose on phenol degradation

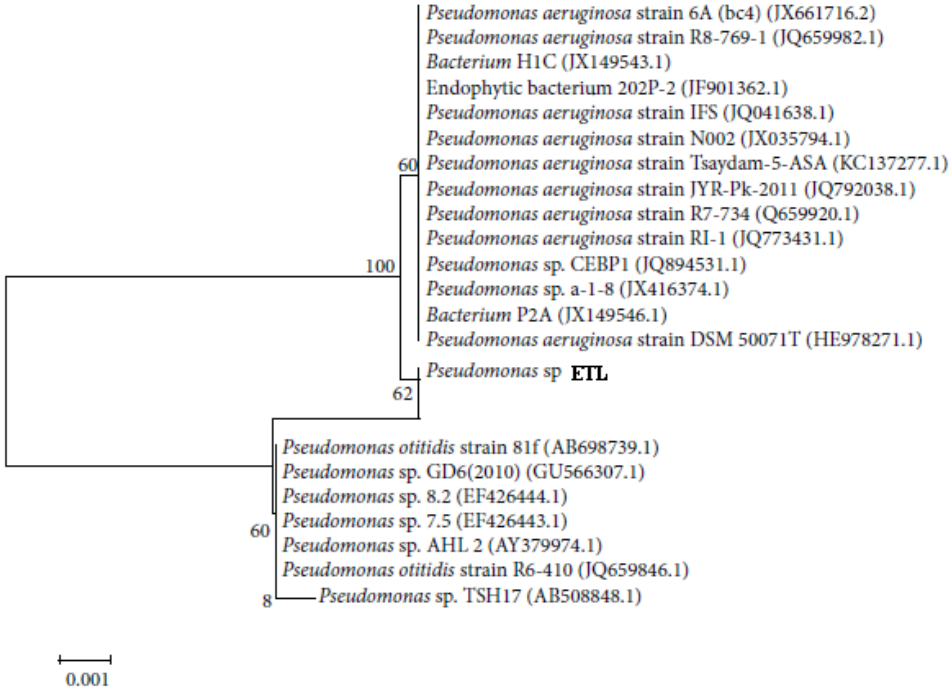

Figure 4. Phylogram (neighbor-joining method) showing genetic relationship between *pseudomonas* spp. ETL and other related reference microorganisms based on the 16S rRNA gene sequence analysis

Microbial degradation of phenol was observed over a wide temperature range (25°C – 50°C) with an optimum of 30°C. Temperature might play an equivalent or larger role than nutrient availability in the degradation of organic pollutants [25]. In this work batch culture of *Pseudomonas spp.* ETL-2412 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 200 mg/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, 200 mg/L, while in 250 mg/L the inhibitory effect of phenol could stop the growth and phenol biodegradation, which means the bacteria could not tolerate substrate toxicity in this case, therefore studied concentration is limited to 200 mg/L and higher concentrations are not examined. In order to evaluate the possibility of phenol removal by aeration or mixing control experiments were conducted in the same condition without the bacterium. According to Pakula *et al.*, (1999), phenol biodegradation was significantly inhibited at 30°C. However, most laboratory studies on phenol degradation have been carried out at an optimum temperature of 30°C [2,31]. Annadurai *et al.*, (1999) and Chitra (1995) described that when the temperature increased to beyond 30°C or 35°C, 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-2412 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 (2005) and Rosa *et al.,* (2004) 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. Similar results have been reported on the *Pseudomonas piclorum* at 30°C [14].

3.3. 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 [20]. 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 undissociated from under these conditions and electrostatic force cannot prevent them from entering cells [3,8,20,34]. 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-2412 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 sustainable with work by Karigar *et al.,* [18] on *Arthrobacter citrus*. This may be due to the effect of pH on the ionization and therefore binding and interaction of a myriad of molecular process, which in turn affect the metabolic Pathway. It could even causes denaturing of proteins which might result in lethal toxicity.

3.4. 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-2412. The data collected after 36 hrs showed that maximum phenol removal efficiency of 98.28 % was accessible at 0.25% of glucose concentration. This might be due to the fact that glucose acts as a growth factor in presence of phenol in the waste water due to its simple structure as compared to phenol. But noticeably 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 36 hrs phenol removal was about 88%. Previously Kar *et Al.,* (1996) 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 through glucose did not fully repressed utilization of phenol. Alike results were obtained by Santos *et al.,* (2003) and Khaled (2006) in their respective studies.

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 ecofriendly 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 spp*. ETL-2412 isolated from final effluent treatment Plant of Ankleshwar (Gujarat, India) can be a promising phenol degraders 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 spp*. ETL-2412 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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