Biodegradation of P-nitro phenol using a novel bacterium Achromobacter denitrifacians isolated from industrial effluent water

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ABSTRACT

In the present investigation, Achromobacter denitrifacians was isolated from industrial wastewater and used in the degradation of para nitrophenol. Experiments were made as a function of different carbon sources, organic and inorganic nitrogen sources and metal ions to analyse the removal efficiency of para nitro-phenol present in the industrial wastewater sources. Observations revealed that the rate of phenol biodegradation was significantly affected by pH, temperature of incubation, glucose, peptone and metal ion concentration. The optimal conditions for phenol removal was found to be pH of 7.5, temperature, 35 °C and 0.25 g L⁻¹ supplemented glucose level, 0.25 g L⁻¹ supplemented peptone level, and 0.01 g L⁻¹ zinc ion. The key importance of the present study is the utilization of native bacterial strain isolated from the industrial effluent water itself having an impending role in the bioremediation process of phenol.

Key words: Achromobacter denitrifacians, bacterial strain, glucose, peptone, pH, phenol degradation, temperature, zinc

HIGHLIGHTS

• Achromobacter denitrifacians was isolated from industrial wastewater.
• Experiments were made as a function of different carbon sources, organic and inorganic nitrogen sources and metal ions.
• Observations revealed that the rate of phenol biodegradation was significantly affected by pH, temperature of incubation.

INTRODUCTION

In the past few decades, due to the rapid increase in the population and industrialization development results in environmental pollutants in air, soil and water. Recently, various chemical industries are developed and during the processing of dyes, pharmaceuticals resins, varnishes, plastics, and pesticides (Sun et al. 2015). According to the environmental protection agency, if the phenol concentrations is greater than the 1.0 ppb level lead to toxic to all kind of living organisms (Kazemi et al. 2014). Nowadays agro-chemical industries play a major role over the world which releases phenolic compounds and creates serious environmental pollution (Mohammadi et al. 2015). Generally, phenol compounds are classified into several types, among the various types, nitro-substituted phenol is the most serious pollutant contaminant compounds in the industrial wastewater which causes serious ecological impact in the environment. P-nitro phenol has been listed in priority pollutants by US EPA which recommends the concentration of phenol in natural water should be < 10 mg/L. The exposure of P-nitro phenol could leads to skin and eyes irritation, kidney damage, liver malfunction, systematic poisoning (Kulkarni & Kaware 2013). The microbial resistance enhances with the nitro group of PNP which favors Bio-degradation. However several methods are used for the removal of phenol present in the water and soil sources, bio-remediation is more cost effective and economical (Mukherjee et al. 2013). In the past decades, several studies have done on bioremediation of Phenol using strains like Arthrobacter, Rhodobacter, Bacillus, Burkholdoria, Pseudomonous, Rhodobacter etc. has the ability to utilize P-nitro phenol as carbon and nitrogen source (Yanase et al. 1992; Koutny et al. 2003; Bergauer et al. 2005; Rehfuss Urban 2005;
Khazaali et al. 2014; Deepanraj et al. 2016), but so far there is no much report has been done on achromobacter species. The P-nitro phenol degradation pathway involves the formation of benzentroid contains aromatic ring cleavage results in biodegradation of P-nitro phenol. The p-nitro phenol degradation pathway using Arthrobacter strain is based on the BT as intermediate and then converted into benzoquinone (BQ) and HQ (Kadyala and Spain 1998). However, phenol containing wastewater is difficult to treat because of substrate inhibition, whereby microbial growth and contaminant can occur in biodegradation of phenol are delayed by the toxicity employed by high concentrations of the substrate itself. Numerous nitro-phenol-degrading bacteria have been isolated, and their degradation pathways were studied (Zhang et al. 2012). The major degradation pathways of nitro-phenol is converted to maleylacetate via hydroquinone desirable pathway for gram-negative bacteria such as Burkholderia spp. and Moraxella species (Prakash et al. 1996; Suresh et al. 2002; Vasanharaj et al. 2017). The degradation pathway involved 4-nitro-phenol is converted via 4-nitrocatechol and hydroxyquinol was favorably found in gram positive bacteria such as Bacillus spp. and Arthrobacter species (Jain et al. 1994). Consequently, several studies were done on 4-nitro phenol degradation have been reported. In association with the hydroquinone pathway, the 4-nitro phenol degradation genes were cloned from Pseudomonas sp. strain and Pseudomonas putida. In the case of the hydroxyquinol pathway, no 4-nitro phenol catabolic gene has been reported. The individual nucleotide sequence of 4-nitrophenol degradation genes (npha1A2) were reported using Rhodococcus sp. strain PN1 (Zylstra et al. 2000). In the present study, isolated and identified a bacterial strain from industrial effluent water. The bacterial strain Achromobacter denitrifacians has the capability to degrade the para-nitro phenol compound. In this study, the degradation of para nitro phenol by a naturally occurring bacterial strain present in the industrial effluent wastewater under different growth conditions, includes pH, incubation temperature, additional different carbon sources, nitrogen sources and metal ions were studied using experimental and statistical analysis.

MATERIALS AND METHODS

Collection of samples

Chemical industrial effluent water samples were taken from ten randomly selected areas of Thirumalai and Maladi pharmaceutical industries located in Ranipet District, Tamilnadu. All samples were placed in separate sterile bottle and stored in a refrigerator at 4 °C till use.

Isolation of microorganism

The bacterial species were isolated from the industrial wastewater samples. The samples were diluted from 10⁻¹ to 10⁻⁵ and the diluted water samples were spread on sterile Nutrient agar plates. The inoculated plates were incubated at 37 °C for about 24 hours. The mixed cultures obtained after incubating on agar plates and then single colony was isolated from the mixed culture. The sub-cultured in nutrient broth was stored at 37 °C for about 24 hours and then streaking plate method was used to get single colonies out of pure culture.

Characterization of the isolated pure culture using 16S rRNA

The most potential phenol degrading strain was identified and characterized by 16S rRNA sequencing to identify the isolate up to species level. Based on the molecular characterization study, the isolated strain was identified as Achromobacter denitrifacians.

Process optimization parameters on PNP degradation by Achromobacter denitrifacians

The isolated bacterial strain was grown in the minimal salt medium containing 500 mg/L phenol. The liquid mineral salt medium (MSM) consisted of (g/l): K₂HPO₄, 0.4; KH₂PO₄, 0.2; NaCl, 1; MgSO₄ 0.1; MnSO₄ 0.01; FeSO₄·H₂O, 0.01; Na₂MoO₄·2H₂O, 0.01; (NH₄)₂SO₄, 0.4; and phenol, 0.5 was used in all cultivation and phenol degradation experiments. The initial pH value of the medium is adjusted to 7.5 using 30% NaOH prior to autoclaving. The phenol was sterilized separately by filtration using 0.2 μm regenerated cellulose membrane filter and added to the sterilized medium after cooled down to room temperature. The medium (100 mL) in 250 mL shake flask was inoculated with 10% (v/v) inoculum to initiate the cultivation and degradation of phenol. The flask was incubated at 30 °C on a rotary shaker, agitated at 160 rpm. The variables were varied for the cultivation experiments include pH, temperature, different carbon sources, different organic and inorganic nitrogen sources, metals. During the cultivation, 10 mL of culture samples were withdrawn at different time intervals for analysis.
Effect of pH
To determine the effect of pH on growth and phenol degradation, the isolate was grown in different pH (5–8.5) at a constant temperature of 37 °C under static condition for 48 hrs. The sample was drawn every 4 hours and various parameters like growth and phenol degradation were measured.

Effect of temperature
Similarly, to study the influence of temperature, the isolate was grown in varying temperatures (30 °C) at a constant pH 7.5 and the above parameters were measured.

Effect of carbon sources
To determine the influence of carbon source on the growth of microbes and percentage of phenol degradation using different carbon sources like glucose, fructose, sucrose, maltose, and galactose. These carbon sources were added to the basal medium at different concentration 0.5–2.0 g/l. The flasks were incubated at 37 °C under static condition. The growth and phenol degradation was measured after 24 hours incubation.

Effect of organic and inorganic nitrogen sources
Influence of nitrogen on the growth of microbes and phenol degradation was determined by the addition of various organic nitrogen sources like peptone, yeast extract, urea and tryptone (0.5, 1, 1.5, 2 and 2.5 g/l). Similarly, inorganic nitrogen sources like sodium dihydrogen phosphate, ammonium sulphate, potassium carbonate, sodium nitrate, (0.5, 1, 1.0 and 2.0 g/l) were added as a sole source of nitrogen to the basal medium and incubated at 37 °C in static condition. The growth and phenol degradation were measured after 24 hours incubation.

Effect of metals
To check the effect of metal ions on the growth of microbes and phenol degradation used various metal salts like zinc, copper, cobalt and lead were added to the basal medium at different concentration (0.01, 0.02, 0.03 and 0.04 g/l). The variation in growth and phenol degradation by the addition of metal salts to the growth medium were monitored.

Phenol estimation
The concentration of phenol under graded in the solution was determined by a UV-vis spectrophotometer using 4-amino anti-pyrine as a colour reagent. The samples were centrifuged at approximately 6,000 rpm for 10 min. The supernatant was used for phenol determination. The residue was suspended in distilled water and optical density of this suspension was measured against distilled water as reference at 550 nm using UV-Visible double beam spectrophotometer. All experiments were performed in triplicates and the average of the three independent experiments was taken as the result.

Design of experiment
To optimize the range of experiments the $2^3$ full factorial central composite design was applied. The variables like pH, Glucose, Peptone and Zinc were selected in CCD analysis. Each variable was examined at two levels: ($-1$) for the low level and ($+1$) for the high level. Each row represents different experiment and each column represents different variables. The range and the levels of the process variables were served as a critical variables A, B, C and D respectively as shown in Table 1.

| Variables     | Coded levels | $-1$ | 0  | $+1$ |
|---------------|--------------|------|----|------|
| pH (A)        | 6.5          | 7.5  | 8.5|
| Glucose (g/L) (B) | 0.5          | 1.0  | 1.5|
| Peptone (g/L) (C) | 1.0          | 1.5  | 2.0|
| Zinc (g/L) (D) | 0.01         | 0.02 | 0.03|
RESULTS AND DISCUSSION

The following optimization parameters were used to study the effects of different parameters on phenol degradation. The various parameters used for the optimisation of phenol degradation was pH, temperature, carbon source, nitrogen source, metal ions and organic solvents, etc. The optimum values for phenol degradation were found out by plotting graphs.

**Effect of pH**

The maximum phenol degradation was observed at pH 7.5, which showed 90% phenol degradation. To identify the optimum pH level for the degradation of phenol, different pH readings were taken and their absorbance values were measured at 550 nm in UV-Visible spectrophotometer. The percentage phenol degradation was calculated using the standard formula. A graph was plotted with different pH readings versus percentage phenol degradation as shown in the Figure 1.

**Effect of temperature**

The maximum phenol degradation was observed at 35 °C, which showed 89% phenol degradation. To study the influence of temperature, the isolate was grown in varying temperatures from 25 °C to 50 °C. To identify the optimum temperature level for the degradation of phenol, different temperature readings were taken and their absorbance values were measured at 550 nm in UV-Visible spectrophotometer. The percentage phenol degradation was calculated using the standard formula. A graph was plotted with different temperature readings versus percentage phenol degradation as shown in the Figure 2.

**Effect of carbon sources**

For the growth of microbes, generally microorganisms utilize electrons, energy and other nutrients from their surrounding environment. Phenol is a toxic compound liberated from the industrial effluents. In this study, *Achromobacter* species isolated from wastewater used as a source material for the degradation of phenol. Different carbon sources were used to check the growth profile of *Achromobacter denitrificans* and percentage removal of phenol degradation. Totally five different carbon sources like glucose, fructose, sucrose, maltose, and galactose were used in the experiment. The results had shown that maximum phenol degradation of 91% was achieved using glucose as carbon source at a concentration of 1 g/l as shown in the Figure 3.

**Effect of organic and inorganic nitrogen sources**

Influence of nitrogen on the growth of microbes and phenol degradation was determined phenol degradation was determined by the addition of various organic nitrogen sources like peptone, yeast extract, urea and tryptone (0.5, 1, 1.5, 2 and 2.5 g/l). Similarly, inorganic nitrogen sources like sodium dihydrogen phosphate, ammonium sulphate, potassium carbonate, sodium nitrate, (0.5, 1, 1.0 and 2.0 g/l) were added as a sole source of nitrogen source. The results had shown that, peptone as organic nitrogen source achieved highest phenol degradation of 90%, whereas in the case of inorganic nitrogen source, ammonium sulphate had shown highest phenol degradation 89% as shown in the Figures 4 and 5.

**Figure 1** | Effect of pH.
Effect of Metals

To determine the effect of metal ions on the growth of microbes and phenol degradation. The analytical solution of 0.1% of various metallic salts like Na\(^{2+}\), K\(^+\), Hg\(^{2+}\), Ca\(^{2+}\), Mg\(^{2+}\), Cd\(^{2+}\), Fe\(^{2+}\), Cu\(^{2+}\) and Zn\(^{2+}\) were prepared and these metal salt solutions were added to the basal medium. After addition of the different metal salt solutions determined the highest phenol degradation efficiency. The results had shown that, zinc achieved optimum phenol degradation of 92% as shown in Figure 6.

Effect of pH, Temperature and phenol concentration on growth of *Achromobacter denitrifacians*:

Growth and biodegradation of any microorganism depends on various physiochemical parameters. The aim of the project is to optimize the parameters like temperature of incubator, pH of the medium and concentration of phenol which is used as a sole of carbon source and energy. Study on growth of bacteria at different temperature, pH and concentration of phenol were carried out and optimize conditions was found out. As biomass increases the optical density also increases (or) with increase in cell growth optical density increases. Increase in cell growth indicates increase in degradation rate. From the graph

![Figure 2](image1.png)  
*Figure 2* | Effect of temperature.

![Figure 3](image2.png)  
*Figure 3* | Effect of different carbon sources.

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maximum degradation was observed at 30 °C. In case of 25 and 35 °C growth is reduced which in turn reduced the rate of degradation as shown in Figure 7.

From the graph maximum growth was observed at pH 8. In case of pH 6 and pH 7 growths is less which ultimately gives rise to less degradation as shown in Figure 8.

From the graph maximum degradation was observed at 200 ppm. In case of 100 and 400 ppm the cell growth was less than that in 200 ppm, which in turn gives rise to less degradation as shown in Figure 9.

**Evaluation of experimental results with CCD**

The design of experiments were carried out for the analysis using design expert software 12. The phenol degradation was analyzed using different concentration of glucose, peptone and zinc based salt solution at different pH levels. The CCD
containing a total of 30 experiments with four variables like pH (A), glucose (B), peptone (C) and zinc ions (D), to estimate the experimental error was employed to analyze the response function (para-nitro phenol removal %). The mathematical relationship between the response function (Y) and independent variables (x) was generated to fit a general second degree polynomial model that was selected as the most appropriate equation to represent the experimental data using response surface regression.

Phenol Degradation (%) = 95.3208 + 1.58A + 0.48B + 1.11C + 0.78D + 0.11AB + 0.3AC + 0.25AD
- 0.05BC - 0.48BD + 0.3CD - 0.15A^2 - 0.1B^2 - 0.7C^2 - 0.14D^2
The effects of the glucose and peptone concentration and pH concentration on the percentage phenol degradation shown in Figures 10 and 11. An increase in the pH with glucose concentration up to the saturated point improved the percentage phenol degradation to a maximum level and an additional rise in the pH with glucose concentration results in reversed process. Similarly, the response was observed for the glucose concentration at various level of the metal ion concentration (Zn\(^{2+}\)) an increase in the glucose concentration with metal ion concentration (Zn\(^{2+}\)) up to saturated point improved the percentage phenol degradation to a maximum level and an further increase in the glucose concentration with metal ion concentration (Zn\(^{2+}\)) decreased the phenol degradation is shown in Figure 12. Consequently, the effect of peptone concentration with pH and glucose concentration increased the percentage phenol degradation to maximum level and a further increase in the concentration decreased the phenol degradation as shown in Figures 13–15. The results of the second order response surface model fitting in the form of Analysis of Variance (ANOVA) were given in Table 2. It is required to test the significance and competence of the model. The ANOVA of the regression model proves that the model is highly significant. The R\(^2\) value provides a measure the variability in the observed response values can be explained by the experimental variables.
and their interactions. The R² value is always between 0 and 1. The closer the R² value is to 1, the stronger the model is and the better it predicts the response. In this case, the value of the determination coefficient (R² = 0.97753) indicates that 97.53% of the variability in the response could be explained by the model. In addition, the value of the adjusted determination coefficient (Adj R² = 0.95655) also very high to advocate for a high significance of the model.

**Figure 10** | Response and contour plot of pH vs glucose concentration on phenol degradation (%).

**Figure 11** | Response and contour plot of pH vs peptone concentration on phenol degradation (%).

**Figure 12** | Response and contour plot of Zinc concentration vs glucose concentration on phenol degradation (%).
CONCLUSION

Industrial Pollution has become a major concern which is seriously reducing the quality of environment and creating health effects to humans and other living beings. The waste water or Effluent generated by industries has enriched with various pollutants which causing adverse effects to the humans and environment. The waste water which often mixes with water bodies makes them unusable. Phenol and Phenolic compounds is one of the major pollutants that are being discharged from waste water. So, the treatment is necessary to prevent its adverse effects on environment and living beings. As many methods are available now days to treat the waste water but in which most of it are not preferable in terms of cost, Efficiency and in danger of production of hazardous by products. After the several studies are conducted worldwide on Bio remediation, it has found that Bio remediation is the preferable method in terms of cost and efficiency. It also has advantage that it does not produce hazardous by products and it has a possibility of complete mineralization. Several research efforts have been made to analyze the biodegradation of phenol by microbes isolated from industrial effluents. The study has carried out on effect of parameters such as pH, Temperature, Initial Phenol concentration, Carbon sources and nitrogen sources on the Achromobacter denitrificans in the phenol degradation process. The results shown that pH of 7.5 are optimum for phenol degradation at which microbes has achieved 90% of phenol degradation. The optimum temperature has found as 35°C at which microbes has achieved 89% of phenol degradation. The various carbon sources such as glucose, Fructose, Sucrose etc., used to find optimum carbon source for the phenol degradation by Achromobacter Aegrifecians. It has found that glucose as an optimum carbon source at which microbes has attained 91% of phenol degradation. The Peptone is found as optimum nitrogen source at which microbes achieved 92% of phenol degradation. In the metal ions, It has found that zinc as an optimum nitrogen source at which microbes achieved 90% of phenol degradation. The designed produced may be used for the treatment plant for phenol waste effluents where collection can be achieved on a large scale.

Figure 13 | Response and contour plot of pH vs Zinc concentration on phenol degradation (%).

Figure 14 | Response and contour plot of Glucose concentration vs peptone concentration on phenol degradation (%).
Table 2 | Analysis of variance (ANOVA)

| Source                  | Sum of | df | Mean   | F-value | p-value | p-value |
|-------------------------|--------|----|--------|---------|---------|---------|
| Model                   | 122.6276 | 14 | 8.759115 | 46.61033 | 8.79E-10 | significant |
| A-pH                    | 42.18176 | 1  | 42.18176 | 224.4639 | 1.97E-10 |
| B-Glucose Concentration | 3.703101 | 1  | 3.703101 | 19.7055  | 0.000478 |
| C-Peptone Concentration | 29.89729 | 1  | 29.89729 | 159.094  | 2.18E-09 |
| D-Zinc Concentration    | 13.34363 | 1  | 13.34363 | 71.00614 | 4.52E-07 |
| AB                      | 0.126883 | 1  | 0.126883 | 0.67519  | 0.424117 |
| AC                      | 1.542892 | 1  | 1.542892 | 8.210269 | 0.011794 |
| AD                      | 1.02111  | 1  | 1.02111  | 5.435686 | 0.034112 |
| BC                      | 0.027019 | 1  | 0.027019 | 0.143776 | 0.70987 |
| BD                      | 2.415905 | 1  | 2.415905 | 12.85588 | 0.002705 |
| CD                      | 1.542453 | 1  | 1.542453 | 8.207934 | 0.011804 |
| A²                      | 0.620693 | 1  | 0.620693 | 3.02924  | 0.089186 |
| B²                      | 0.239851 | 1  | 0.239851 | 1.276332 | 0.276325 |
| C²                      | 10.62944 | 1   | 10.62944 | 56.56297 | 1.83E-06 |
| D²                      | 0.51338  | 1    | 0.51338  | 2.731874 | 0.119136 |
| Residual                | 2.818833 | 15  | 0.187922 |          |          |
| Lack of Fit             | 2.432302 | 10  | 0.24323  | 3.14632  | 0.10885  |
| Pure Error              | 0.386531 | 5   | 0.077306 |          |          |
| Cor Total               | 125.4464 | 29  |          |          |          |
| R²                      | 0.97753  |      |          |          |          |
| Adjusted R²             | 0.956557 |      |          |          |          |

Figure 15 | Response and contour plot of Zinc concentration vs peptone concentration on phenol degradation (%).

**DATA AVAILABILITY STATEMENT**

All relevant data are included in the paper or its Supplementary Information.

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