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Studies on Biodegradation of P-Nitrophenol by *Arthrobacter chlorophenolicus* and Its Metabolites

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ABSTRACT

The biodegaradation of p-nitrophenol was studied experimentally at different initial p-nitrophenol concentrations under different environmental and operational conditions (like incubation temperature, pH, inoculum size etc.) by using microbial strain *Arthrobacter chlorophenolicus* A6. The rate of degradation of p-nitrophenol was observed maximum at incubation temperature 30˚C and pH 7 ± 0.2, inoculum size OD_{600} = 0.2, incubation rotor speed 150 rpm, pH in the range 5-8 and temperature 25-35 ˚C. In experiments on kinetic studies the initial concentration of biomass is fixed OD_{600}=0.2 and variation in biomass concentration has been experimentally observed with time at p- nitrophenol concentrations (10-200 mg/l). The value of maximum specific growth rate is found to be equal to 2.370 h^{-1} and it is achieved at initial p-nitrophenol concentration of about 70 mg/l. The p-nitrophenol is inhibitory type substrate and the inhibition effect of p-nitrophenol becomes predominant above 70 mg/l. The various growth kinetic models are available in literature to represent the growth kinetics data of an inhibitory compound. The results illustrate that most of the experimental data points close to the prediction by Haldane model and lie within the permissible range of ±10%. It is therefore concluded that Haldane model is the most suitable kinetic model for biodegradation of p-nitrophenol on *Arthrobacter chlorophenolicus* A6 for the substrate concentration ranging from 10-200 mg/l. Haldane kinetic model is used for further studies. For the present study on p-nitrophenol using *Arthrobacter chlorophenolicus* A6 the value of max specific growth rate, saturation constant and inhibition constant are 2.372 h^{-1}, 860.681 mg/l and 6.180 mg/l respectively upto 200mg/l concentration of p-nitrophenol, i.e. $S_m = (Ks \times Ki)^{1/2}$ = 71.8. In view of the above studies it can be concluded that *Arthrobacter chlorophenolicus* can be successfully used to treat waste water containing phenolic compounds such as p-nitrophenol.

Keywords: *Arthrobacter chlorophenolicus*, p-nitrophenol, inhibition kinetic model, biodegradation rate, max specific growth rate

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INTRODUCTION

p-nitophenol is a potential environmental contaminant of water due to its high solubility into water. It readily breaks down in surface water but persists longer in deep soil and in groundwater and due to its toxicity causes deadly effects on the ecosystem. PNP irritates the eyes, skin, and respiratory tract. It may also cause inflammation of those parts. Acute exposure of PNP may lead to liver and kidney damage, anaemia, skin and eye irritation, and systemic poisoning [1]. The U.S. environmental protection agency (EPA) has listed p-nitrophenol (PNP) as a priority pollutant and recommended its concentrations in natural waters and drinking waters to below 10 ng/l [2,3] whereas, monthly average industrial effluent concentrations of PNP should not exceed 162µg/l [4]. The major sources of wastes that discharge PNP are the industries mainly involved in the management of explosives, drugs, dyes, phosphoorganic insecticides (methyl parathion), pesticides and leather.

PNP is extremely harmful for the environment and human health it calls for a highly efficient treatment of wastewaters contaminated with PNP prior to their discharge into the environment. There are several techniques such as volatilization, photo-decomposition, physical adsorption, solvent extraction, chemical oxidation and electrochemical methods for the removal of PNP and other phenolic compounds from wastewaters [5] but these cannot be used for a long term purpose with large amount of contaminated water because of the limiting factors like high cost, low efficiency and generation of toxic by-products. And that is why the eco-friendly biodegradation process is gaining utmost attention due to its advantages over the traditional physico-chemical methods.

However, the presence of nitro groups enhances the resistance of the aromatic ring against biodegradation by many microorganisms [6,7,8], and hence only selective species of bacteria belonging to *Flavobacterium, Alcaligenes, Pseudomonas, Rhodococcus Ochrobactrum sp Ralstonia eutropha* and *Arthrobacter*, have shown ability to degrade PNP [7-9]. Among these microbial species, actinomycetes secrete both extracellular as well as intracellular enzymes and have thus revealed good potential in degrading PNP more effectively. *Arthrobacter chlorophenolicus* is aerobic actinomycetes that has been demonstrated to degrade wide different types of toxic substituted phenols in batch shake flask and is also reported to be one of the most efficient strains that completely mineralize 4-chlorophenol (4-CP) even at 300 mgl^{-1} within 24 h of culture [10].

The present study investigated the batch studies to investigate the biodegradation of p-nitrophenol as single substrate using *Arthrobacter chlorophenolicus* and have to develop the kinetic model for specific growth rate and substrate utilization rate.

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MATERIAL AND METHODS

Materials

All the chemicals and reagents used were either of analytical reagent (AR) grade or laboratory reagent (LR) grade. AR grade p-nitrophenol (PNP) was procured from Himedia (India).

Microorganism and cultivation medium

The media used for the growth of *Arthrobacter chlorophenolicus* containing PNP as the sole carbon source was MSM (Mineral Salt Media). This media includes the basal salts having composition of K_2HPO_4: 2.75 g/l, KH_2PO_4: 2.25 g/l, $(NH_4)_2SO_4$: 1 g/l, $MgCl_2.6H_2O$: 0.2 g/l, NaCl: 0.1 g/l, $FeCl_3$: 0.01 g/l, $CaCl_2$: 0.01 g/l and prepared in distilled water. The salts were added and mixed one by one in distilled water to avoid precipitation and the pH value of the medium was maintained at 7.0.

Acclimatization of culture

The revived cultures were first grown in MSM with glucose as sole carbon source. The cultures were acclimatized to PNP by exposing the cultures in a series of shake flasks (250 ml) wherein the content of glucose, initially 2%, was decreased and that of PNP increased gradually. All the batch experiments were performed at 30˚C.

Biodegradation of PNP by *Arthrobacter chlorophenolicus*

Once the acclimatization was over and certain enzymes were induced in the bacteria to participate in the metabolism in the presence of toxic PNP, experiments were set for the degradation of PNP by *Arthrobacter chlorophenolicus* A6 at different initial concentrations of PNP.

Analytical methods

Biomass concentration in samples was determined by measuring optical density at wavelength 600 nm (OD_{600}) using a UV– visible spectrophotometer. The absorbance values were expressed as dry cell weight using a calibration curve of optical density (OD_{600}) versus mixed liquor suspended solids (MLSS) of the sample. One unit of absorbance was found equivalent to 235 mg/l of MLSS. Biomass concentration on the support material, PUF, was measured by following Bradford method of protein assay [11]. Residual PNP concentration in biomass free samples was also determined by measuring optical density at 398 nm using a UV–visible spectrophotometer and MSM as control. To determine the parameters of Haldane's Kinetics model from the obtained data of biomass concentration and residual PNP concentration in the experimental culture flasks, the software Curve Expert 1.3 was used.

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RESULTS AND DISCUSSIONS

Acclimatization of PNP degrading bacteria

PNP degrading bacteria are required to be adapted to the new environment especially in the case of toxic compounds so that the enzymes that will degrade the compound can be expressed and induced in the bacteria. In the present study bacteria *Arthrobacter chlorophenolicus* A6 was grown in mineral salt media with initial 2% glucose concentration which was gradually reduced to zero accompanied with parallel increase in PNP. When the process of acclimatization was over as full growth of bacteria was found in media with PNP as sole source of carbon and energy, it was stored and incubated on agar plates for future experiments. To develop the seed culture for the experimental flasks these acclimatized cultures were grown in the nutrient broth media with a loop full of inoculum from the freshly grown culture plates. Hazy appearance was found after around 16 hours of incubation at 30˚C which demonstrated the growth of bacteria. This culture was then processed to prepare the inoculum.

Biomass growth and PNP degradation

The time profile of PNP degradation by the *A. chlorophenolicus* A6 is shown in figure 5 at its various initial concentrations in the medium. It can be deduced from the figure that the time taken by the microorganism to degrade the compound was dependent upon its initial concentration. For the degradation of lower concentrations like 10, 20 or 30 it takes less time but for higher concentration from 80 to 200 mg/l it took much higher time for complete degradation of the compound started delaying after PNP concentration around 50mg/l. The maximum PNP concentration studied so far in the literature for its degradation in a bioreactor system is only 320 mg/l [12], whereas in batch shake flasks it is 400 mg/l. The biomass production followed a similar pattern to the time taken by the culture to degrade PNP at its various initial concentrations, for its growth. This is illustrated in figure 6 where biomass growth (OD_{600} nm) of the culture is plotted against time for various initial PNP concentrations in the media. It could be seen from Fig. 5 that PNP concentration between 10 and 50 mg/l did not show any significant repression on the biomass output without any lag phase, but at concentrations greater than 50 mg/l, a lag phase in its growth was evident as it began from 60mg/l and correlated well with the delay in the utilization of PNP by the culture. Thus, it could be inferred that PNP at concentration above 50 mg/l exerted toxic effect on the culture growth. Moreover, at high initial PNP concentration in the media the culture took more time for complete utilization of the compound. The samples were extracted at three different intervals depending on the time taken for the degradation of PNP by the microorganism. It was one hour initially for lower concentrations but as the duration to complete the degradation increased the samples were extracted at an interval of 3 hours and then 5 hours for higher concentration as short interval removal of samples reduces the working solution which changes the rate of metabolism and as a result the overall kinetics of the culture. Thus to avoid any variation in the PNP degradation rates and the microbial growth rate three different intervals were used.

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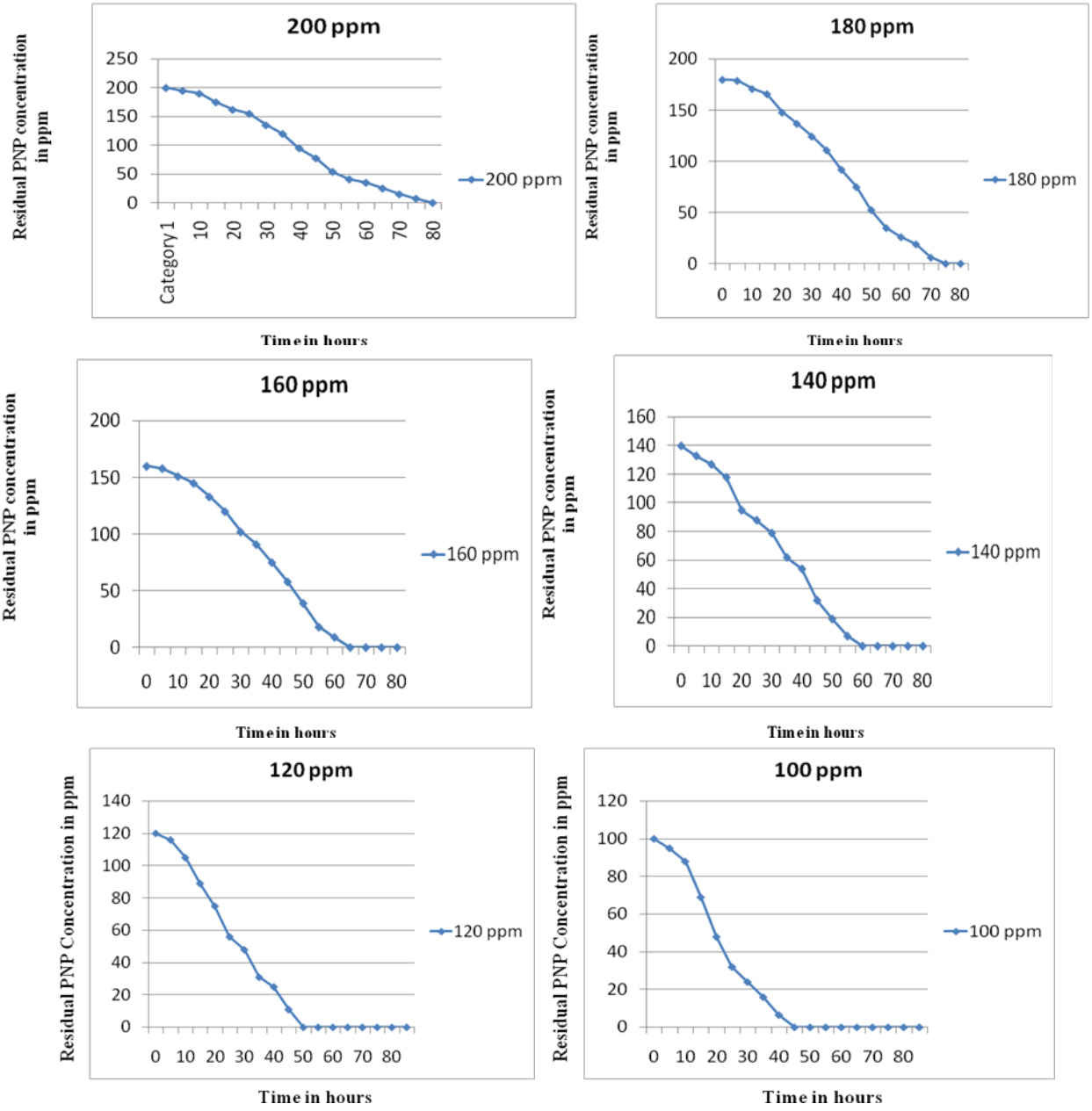
The OD_{398} values were then used against this plot to determine the residual PNP concentrations after regular intervals which are given under. The OD values (398 nm) of samples with different initial PNP concentrations are as under (Figure-1):



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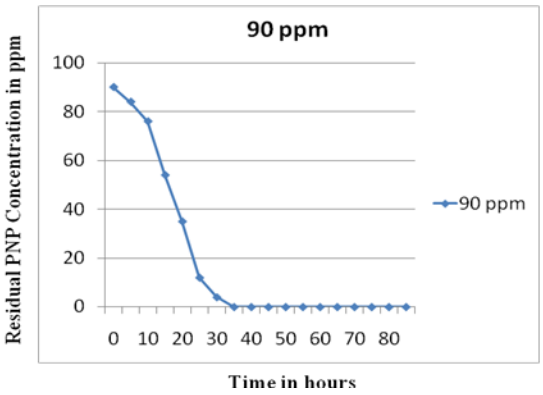

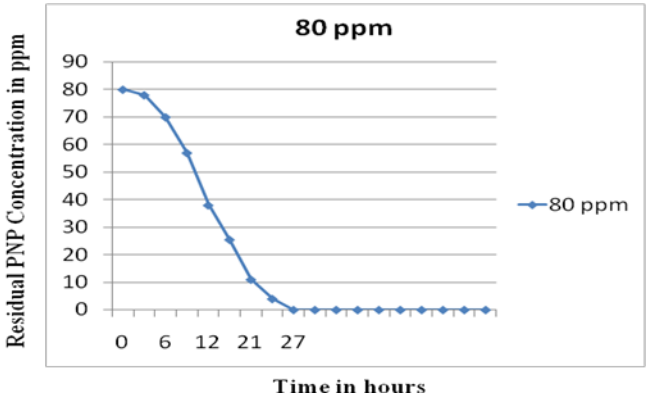

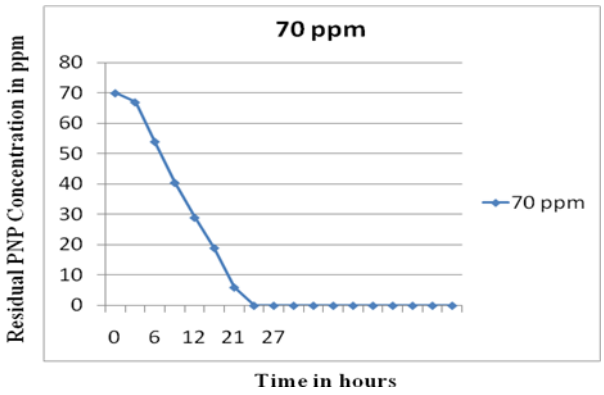

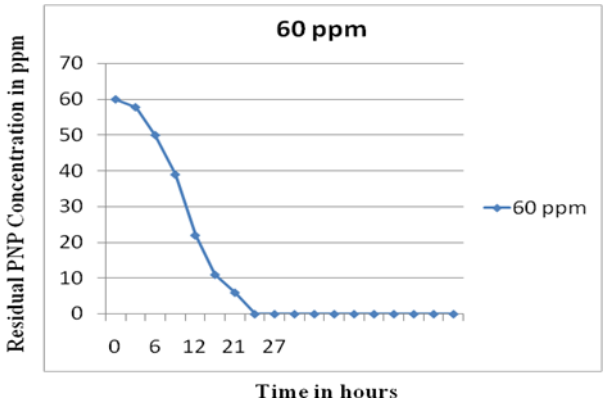

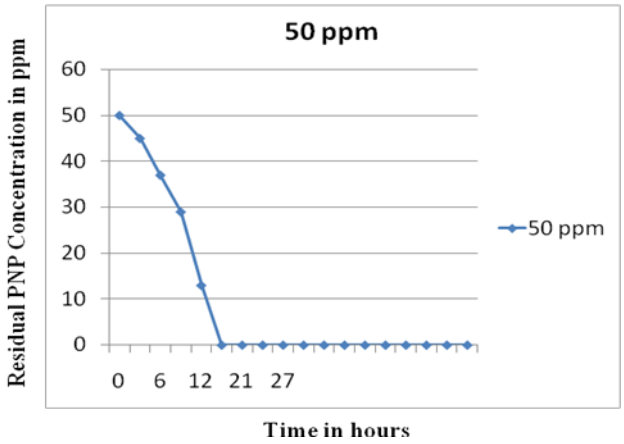

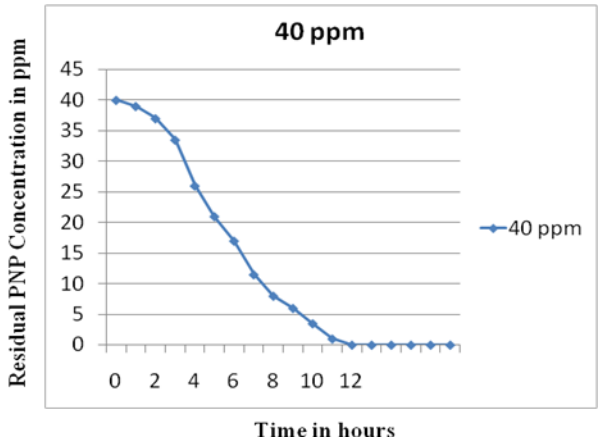

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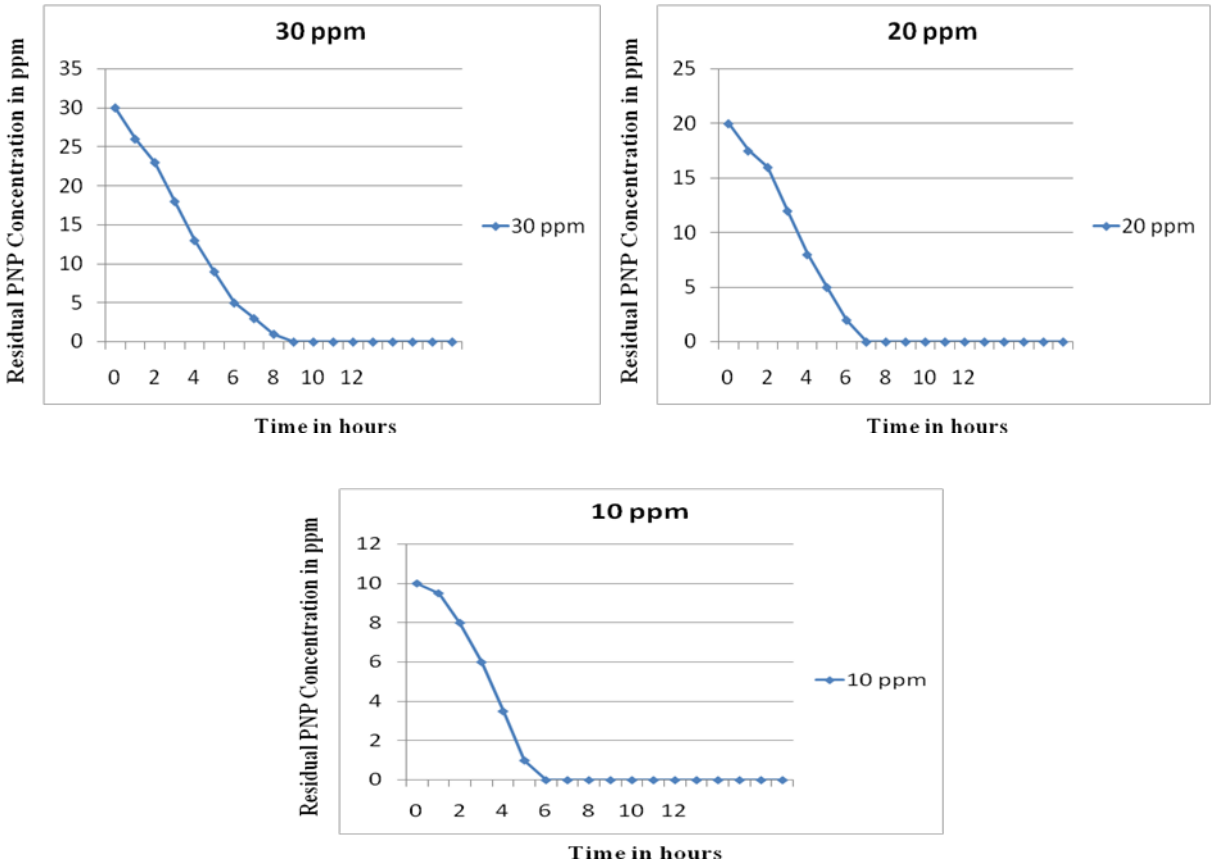
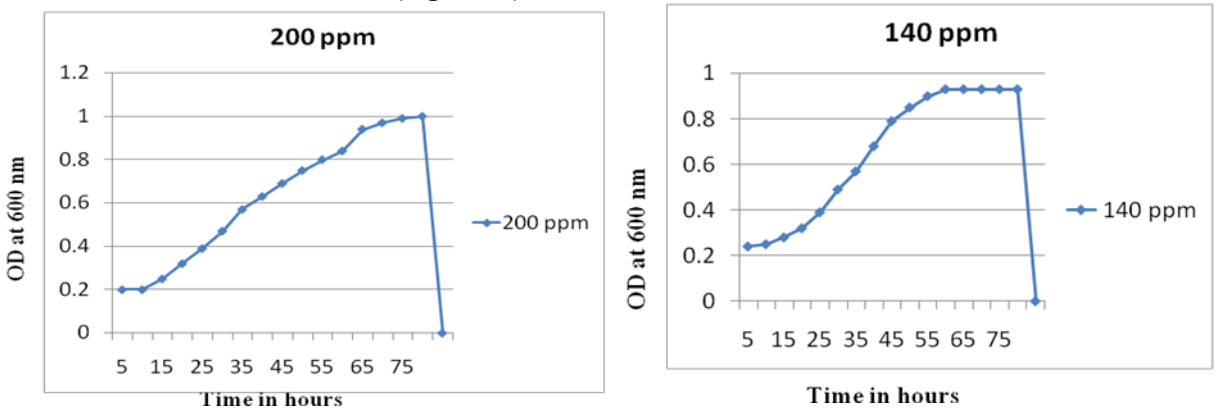


Figure-1: Graphical Reperesenttion of Residual PNP concentrations at different initial concentrations

To analyze the biomass growth at different initial PNP concentrations the OD at 600 nm were taken which are as under (Figure-2):



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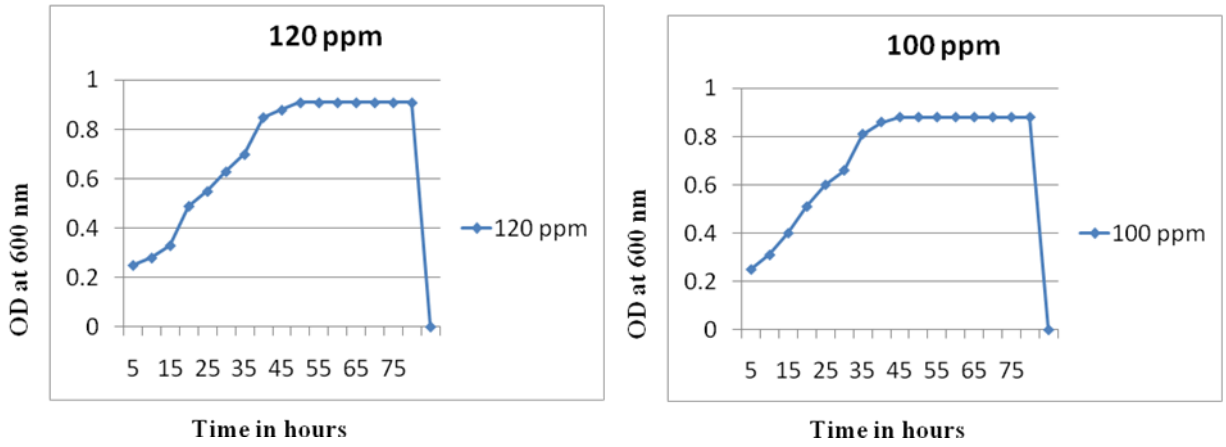

Figure-2: Graphical Representation of biomass growth at different initial PNP concentrations

The results of the study show that higher the concentration of the compound, the more time it takes to be consumed fully by the microorganism. At high concentrations lag phase were observed though the well acclimatized inoculums was used. Hill and Robinson [13] concluded that not only inhibitory effect of the substrate but the size of inoculums also affect the duration of the lag phase. Therefore to avoid large lag phase the inoculums of this size was used. At each of the initial concentrations there was a period of exponential growth when the substrate was being consumed at a faster rate. It was observed that towards the end of the substrate consumption curve, there is a region of relatively less rate of substrate removal. The possible explanations to this activity are the deficiency of oxygen as flasks were cotton plugged or the fall in pH of the solution. The fall in the pH of the solution had previously been reported during the degradation of phenol by mixed cultures of Pseudomonadaceae, vibrionaceae etc [14]. Low values of both oxygen and pH may affect the kinetics of substrate consumption adversely [15]. A typical growth curve proceeds with a decline in cell population once the substrate is completely consumed. During this declining phase, some part of the cell population becomes food for the rest of the cell population. This part of the growth curve in a batch reactor is modeled by the decay constant (k_d) whose value correlates with biomass growth and is also independent of initial substrate concentration.

Microbial Growth Kinetics

In order to evaluate growth kinetics, the biomass growth data from different PNP batch experiments was plotted on a semi logarithmic graph. After a short lag phase, linear plots were obtained at all initial concentrations, which indicated that the PNP was the limiting substrate in this region and the culture was growing exponentially. Such plots had earlier been used for the determination of specific growth rates at the initial phenol concentration [16]. In the present work also these plots were used to calculate specific growth rate μ for that particular initial PNP concentration. The experimental specific growth rates were plotted against the initial PNP concentrations. The graph so obtained shows atypical trend that the value of specific growth rate increases with the increase in initial PNP concentration up to a certain concentration level and then it decreases with further increase in the initial concentration (see in figure-3). This

suggests that PNP is an inhibitory type of substrate. Various kinetic models are available to represent the growth kinetics of inhibitory compounds for example Webb model, Edward model, Aiba model, Yano model, Andrews Model, Haldane Model etc. Here in this study we used Haldane Equation model on the premise that this has less number of parameters and lends itself to be used easily in model Equations representing continuous biological reactors.

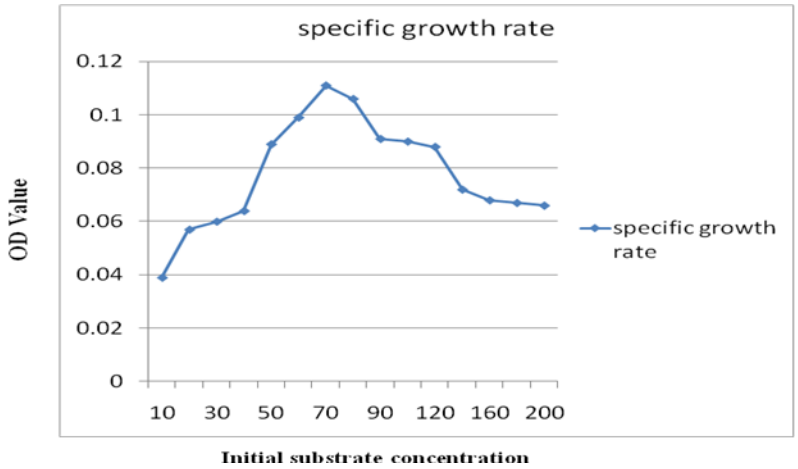

Figure-3: Specific growth rates with respect to initial PNP concentration

The three parameter model of Haldane was fitted to the experimental data using Curve Expert 1.3 software. The kinetic parameters estimated from Haldane model are given below

μ_{max} = 2.371608 h^{-1}
K_s = 860.68145 mg/l
K_i = 6.1802764 mg/l
Standard error = .009

The high value of μ_{max} obtained in the study indicates that the substrate is readily utilized by *A. chlorophenolicus* for its growth. The larger value may also be due to high initial inoculums size and initial PNP concentrations used in the experiments. Half saturation constant (K_s) is a measure of affinity between biomass and substrate [16] and the value obtained in the study shows that high affinity between the two. The value inhibitions constant (K_i) signifies the degree of resistance of the microorganism to the toxic effects of PNP and in general high K_i values reveal that the biomass is highly resistant to inhibition by its substrate.

CONCLUSIONS

The bacterial strain *Arthrobacter chlorophenolicus* could be acclimatized to high concentrations of PNP and so the wastewater containing PNP could be treated effectively using *A. chlorophenolicus* with complete degradation of the compound within an acceptable duration of time. Although it is inhibitory above 70 mg/l concentration of PNP the results obtained from the growth kinetics explains its utility and significance for the biological remediation. PNP

exhibited inhibitory behavior and its growth kinetics could be correlated well by the simple Haldane's inhibitory growth kinetics model. The concentration of the stimulant and that of the compound to be degraded would, therefore, be of prime importance while designing an effective remediation strategy.

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