Degradation of Phenol, an Innovative Biological Approach

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Abstract

A bacterial strain capable of utilizing phenol as a sole carbon source was isolated from the soil samples, collected at a vicinity of industrial wastewater treatment plant of phenol manufacturing unit. Based on the biochemical tests and 16S rRNA sequencing results the organism was identified as Serratia marcescens. The organism is capable of degrading the highly toxic phenolic compound to an optimum concentration of 2500 mgL^{-1} in 120 h at approx. a neutral pH of 8. The organism was also efficient with wide pH and temperature tolerance with an extremely stipulated lag phase in the case of higher influent concentrations. Amid two different models incorporated to justify the growth kinetics of the organism the Haldane's model fits (R2=0.930) very satisfactorily with kinetic constants in the range of μ_{max} = 0.05 - 0.095 h^{-1}; Ks = 8.49 - 16.1 mgL-1; Kj - 1154.75 - 1700.68 mgL^{-1}. Despite limitations in terms of inhibition of microbial growth subjected to an extremely elevated concentration of phenol the study claims the feasibility of Serratia marcescens to an utmost concentration of 2500 mg/L and suggests the method as best possible alternative to the existing chemical treatment procedures.

Keywords: Biodegradation; Phenol; Serratia marcescens; Kinetic modeling; Phenolic compounds

Introduction

Phenol and its higher homologues are aromatic molecules containing hydroxyl group attached to the benzene ring. Phenolic compounds are widely distributed in the environment from various industrial as well as natural sources [1-3]. Phenol pollution is associated with petroleum refineries, pharmaceuticals, pulp mills, gas and coke oven industries, explosive manufacture, phenol- formaldehyde resin manufacture, plastic and varnish industries, wood processing industries etc [4-7].

Natural sources of phenol include forest fire, natural run-off from urban area where asphalt is used as the binding material and natural decay of ligno-cellulosic materials. The presence of phenol in water imparts carbolic odor to receiving water bodies and can cause toxic effects on aquatic flora and fauna [8]. Phenolic compounds are hazardous to human health and resistant to biodegradation [9,10]. It acts as a substrate inhibitor in the biotransformation [11]. Phenol is also a priority pollutant and is included in the list of EPA [12].

Several physico-chemical treatments have been suggested in the last two decades to remove phenol efficiently: adsorption with bone char or zeolites, stripping with air or stream [13] wet air oxidation etc. [14]. On the other hand, biological treatments with pure or mixed cultures of microorganisms have also been used due to its eco-friendly, cost effective and complete bio-mineralization

[15]. Bacteria, yeast and fungi are capable of utilizing Phenolic compounds. Although phenol is a toxic and substrate inhibitory, it is also a carbon and energy source for several bacterial strains belonging to the species of Pseudomonas, Bacilli, Klebsiella, *Ochrobactrum*, *Rhodococcus*, *Aureobasidium* etc [16-21]. In the present investigation, a novel high strength phenol degrading bacterial strain was isolated, identified and its phenol utilization was studied under varied experimental conditions like the effect of pH, initial phenol concentration and inoculum size. Also, the results were validated in Monod and Haldane's models.

Materials and methods

All fine chemicals used were of analytical grade from Merck, India. The chemicals used for media preparations were of Bacteriological grade from Hi Media, India. Glass wares were sterilized in hot air oven at 180 °C for 3 h. All growth media, water blanks were steam sterilized m autoclave at 121 °C, 15 lb/inch for 20 min. pH of the growth media was adjusted to 8.01 using phosphate buffers.

Collection of samples

The soil samples rich in phenol were collected from six different points from the vicinity of the effluent treatment plant of phenol-formaldehyde resin manufacturing industry, Chennai,

India and stored in plastic containers at 4°C as per the standard procedure [22]. The industry is discharging phenol concentration of about 4000 mgL⁻¹ in the effluent [23].

Isolation of bacteria

The medium used for isolation of bacteria contains glucose 5.0 gL⁻¹, peptone 5.0 gL⁻¹, NaCl 5.0 gL⁻¹, beef extract 3.0 gL⁻¹, phenol 100 m gL⁻¹ and 10 gL⁻¹ of soil sample. The broth was inoculated with soil and kept for 48 h at 25 °C under aerobic conditions in a mechanical shaker at 100 rpm. Aliquot from the enrichment flask was plated on an agar plate containing glucose 5.0 g L⁻¹, peptone 5.0 gL⁻¹, NaCl 5.0 gL⁻¹, beef extract 3.0 gL⁻¹, agar 20 gL⁻¹ and phenol 200 mgL⁻¹. The plates were incubated at 25 °C for 36 h under aerobic condition. Colonies developed were carefully observed for the uniformity and differences. Four morphologically distinct colonies were obtained. The colonies were transferred to new plates and further screened by sub-culturing in a basal minimal medium containing (per liter): NH₄Cl 10 g, NH₄NO₃ 4 g, K₂HPO₄ 0.2 g, KH₂PO₄ 0.8 g, MgSO₄.7H₂O 0.2 g and 200 mgL⁻¹ of phenol as the sole carbon and energy source.

The morphological, physiological, biochemical tests were carried out to identify the selected efficient isolate as per Bergy's Manual of systematic Bacteriology [24]. The bacterium was further confirmed by 16S rRNA sequencing from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India.

Phenol degradation

The experimental studies were performed in the shake flask as batch reactor. Each flask contains sample of 100 ml basal minimal medium with varying concentrations of phenol as sole carbon source. The shake flasks were maintained at 34 °C±0.1°C at 150 rpm. The samples were regularly analyzed for phenol concentration and cell growth.

Growth of the organism was recorded by monitoring the Optical Density (OD) of the culture in a Hitachi-U-2001 UV-vis Spectrophotometer at 600 nm. Phenol was estimated spectrophotometricaly using 4-aminoantipyrine as per standard procedure [22].

Results and Discussion

Isolation and Identification

The soil samples were collected from the different locations of effluent treatment plant of phenol-formaldehyde resin manufacturing industry. The samples were inoculated in the basal minimal medium containing phenol as a sole carbon source. Eighteen morphologically different colonies were developed, out of which four were distinct and one of the isolate exhibits more growth in high phenol concentration.

The morphological, physiological, biochemical and carbon source utilization characteristic of the isolate is given. The biochemical test results expressed the organism was a Gram-negative with brown pigment producing, having a proficient growth between 10 and 37°C, pH tolerance to a wide range of 5.0

- 11.0 and growth on NaCl upto 7%. The metabolic activity of the isolate was dynamic both in aerobic and anaerobic conditions, showing the organism as a facultative. The organism has positive results on MacConkey agar and Voges Proskauer test. It utilizes citrate, hydrolyze casein and reduces nitrate. It produces acid from the tested carbohydrates viz. adonitol, arabinose, dextrose, melibiose, salicin and xylose. Based on these characteristics and 16S rRNA sequencing results, the isolate has been identified as *Serratia marcescens*.

Influence of Initial concentration

Shake flask experiments were conducted to examine the effect of various initial concentrations of phenol on the degradation behavior of *S. marcescens* at 34±0.1°C. The results of batch studies for high strength phenol degradation in minimal media by *S. marcescens* are given. The organism degrades phenol with an initial concentration of 1000, 1500, 2000 and 2500 mgL⁻¹ at about 98.5, 99.1, 98.9 and 98.2 % in 72, 96, 108 and 120 h respectively. At higher the initial concentration of phenol more time required for the organism to degrade.

The growth of organism increases with increase in phenol concentration as, but the lag phase of the organism was extended at higher concentration of phenol. A positive correlation between cell biomass and phenol degradation was noted. In the batch studies, the biomass concentration of *S. marcescens* increases after 24 h of inoculation. During initial stages of biodegradation, the corresponding biomass was less, subsequently in stages the biodegradation was much pronounced with less increase in biomass and this result was in conformity with the low conversion efficiency to biomass with high removal of phenol [25]. This organism more effectively degrades 2500 mgL⁻¹ of phenol in 120 h. The results of cell biomass concentration versus time indicate that the lag phase increase with increase in phenol concentration. This organism has a very short lag phase and thus overall degrading period was reduced. The short lag phase character of the organism may also help to optimize the design of bioreactor.

During the exponential growth phase of the organism the phenol degradation was maximum. Further, the substrate removal rate was relatively slow towards the end of the substrate consumption. This may be due to deficit in availability of oxygen as well as the substrate for the whole biomass, which in turn the fall in pH of the solution during the degradation period being noticed. The fall in the oxygen and pH of the solution during degradation has been reported when the phenol was metabolized by mixed culture composed Pseudomonadaceae, Vibrionaceae, etc. [26-33].

Inoculum size

The inoculum concentration in the shake flasks were varied from 2 to 6 % (v/v). Six percent (v/v) of biomass concentration provided the best degradation in short duration and 5 % (v/v) concentrations also have an efficiency of degradation equivalent as 6 % concentration. Since the results show that the lag phase for all concentrations of phenol is almost same, the optimum size of inoculum (5%) can be adopted for *S. marcescens*. Hill and Robinson (1975) concluded that not only the inhibitory effect of

the substrate but also the size of the inoculum might affect the duration of lag phase.

Growth Kinetics of the isolated organism in presence of phenol

After isolation and identification of organism, determination of growth and degradation kinetics of the same organism has also been considered in this study. The study of growth kinetics is vital to know more about the capacities of the organisms in degradation and to design an appropriate treatment unit. The observation made on phenol inhibition on the growth of culture can be modeled using proper substrate inhibition model described in literature [7,10,28].

By considering phenol as non-inhibitory compound for an adapted population, the microbial growth can be represented by a simple Monod's non-inhibitory kinetic Eq. [29]

$$\mu = \mu_{max} S / \left(Ks + S \right) \qquad (1)$$

Where, p is the specific growth rate (h^{-1}), S is the limiting substrate concentration (mg L^{-1}), μ_{max} is the maximum specific growth rate (h^{-1}), Ks is the half saturation co-efficient (mg L^{-1}). However, this Eq. becomes unsatisfactory for explaining inhibitory growth of microorganism at higher substrate concentrations. In such a case to represent the growth kinetics of inhibitory compounds, several kinetic models were fitted to the experimental data for selecting the most suitable models. Amongst Haldane's model was adopted due to its mathematically simplicity and wide acceptance for representing the growth kinetics of inhibitory substrates. The Haldane or Andrews's model has the same form (Kennes and Veiga, 2001) as:

$$\mu = \mu_{max} S/Ks + S + \left(S^2/Ki \right) \qquad (2)$$

Ki is the inhibition coefficient (mgL-1). At higher substrate concentrations, S » Ks, the above Eq. reduce as follows:

$$\mu = \mu max \, S / S + \left(S2 / Ki \right) \, or \, 1/\mu = 1/\mu_{max} + S / \left(Ki\mu_{max} \right) \quad (3)$$

This is the linearized Haldane's Eq.

The experimental data on specific growth rate (μ) obtained at various initial phenol concentrations (So) were used for estimating the kinetic parameters from the above two models. the experimental specific growth rate and model predicted ones. From the fig., it could be evident that Haldane model was found to fit the data well. the values of the growth kinetic parameters for various species obtained from different researchers for phenol and were found to be higher compared to the literature report. The Haldane model is more appropriate to explain the substrate inhibition on growth of a microbial culture. Therefore, Haldane's model fitted the experimental data obtained in the study reasonably well. The inhibition constant estimated by Haldane model was high as compared to the values reported in literature [7,30,31].

Endogenous or decay coefficient

The endogenous growth phase curve in a batch reactor has been modeled using the

Eq. $dx \, / \, xy \, = \, - \, k_d X \qquad (4)$

Where, K$_d$ is the first-order death rate constant

And X is cell mass concentration

In order to determine the value of kd, the growth of culture was continued, and the cell mass concentration was observed for few days even after the complete consumption of phenol. The selection of the particular growth run was arbitrary, assuming that the kd is not dependent on initial concentration. The values of the decay rate coefficients obtained were in the range of 0.002 - 0.042 h^{-1} for phenol. Kumaran and Paruchuri [2] have reported the value of decay coefficient as 0.005 h^{-1} for phenol degradation by a mixed culture. The values obtained in this study are comparable with these values of decay coefficients. The decay coefficient affects the growth kinetics. The growth rate will be reduced by as much the value of decay coefficient so that the wash out condition would occur at lower dilution rates.

Yield coefficient

The values of the yield coefficient for various phenol concentration ranges from 0.243 to 1.157 mg VSS/mg bs COD. These values are comparably smaller than the values obtained by other researchers, this may be due to high substrate concentrations.

Conclusion

Biodegradation of highly toxic inorganic compounds provoked the idea of isolation and utilization of a micro-organic pure strain. In this context, a pure culture of *S. marcescens* was isolated from the soil samples collected near the effluent treatment plant of phenol-formaldehyde resin manufacturing industry. The same found to be extremely effective in absolute degradation of high strength phenolic compounds, concentration ranging from 1000 to 2500 mg L^{-1}. The duration taken up by the species to degrade the maximum concentration of 2500 mg L^{-1} was 120 h. This favorable degradation was obtained with pH 8.0, temperature 34±0.1°C and biomass concentration 5% (v/v). The organism has a very short lag phase at high substrate concentration. The substrate inhibition due to phenol and the growth kinetics of the culture was explained using the Monod non- inhibitory model and Haldane's substrate inhibitory model. Between the two models, the Haldane model gave a better fit (R^2=0.930) to the experimental data. The bio-kinetic constants estimated shows the organism is efficient in phenol degradation.

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