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Media Optimization for Biodegradation of Phenol by *Nocardia hydrocarbonoxydans* NCIM 2386

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ABSTRACT

In the present scenario the widespread pollution due to the release of phenolic substances from industry into the environment has been escalating. Among various techniques available for removal of phenols, biodegradation is an environment friendly and cost effective method. The present study is aimed at enhancing the rate of phenol biodegradation by Nocardia hydrocarbonoxydans through screening of nitrogen sources in the media followed by media optimization with respect to pH and nitrogen source concentration. Nocardia hydrocarbonoxydans NCIM 2386, an actinomycete can grow using phenol as the only carbon source and has the ability to degrade phenol. The maximum tolerable level of phenol in the media for N. hydrocarbonoxydans was found to be upto the concentration of 1300ppm and phenol concentration of 1400ppm was found to be toxic to the organism. Six different nitrogen sources namely ammonium chloride, ammonium nitrate, sodium nitrate, urea, peptone and tryptone were screened and peptone was found to be the best nitrogen source with an optimum concentration of 0.25g/L. Degradation of phenol was studied at three different pH conditions of which acidic pH(5.0) and neutral pH(7.0) showed almost similar and higher rates of degradation when compared to basic pH(9.0) condition. Maximum phenol degradation of 99.5% was achieved by Nocardia hydrocarbonoxydans within 8hrs when peptone was used as the nitrogen source at a concentration of 0.25g/L at pH 7.0. It has been observed that the rate of phenol degradation is enhanced significantly with the nitrogen source optimized media as compared to the unoptimized media, thus proving the importance of optimization in process development.

Keywords *– Actinomycete, Biodegradation,Nitrogen source, Nocardia hydrocarbonoxydans, Phenol.*

INTRODUCTION

Environmental pollution caused due to industrial effluents is a threat to mankind. Soil and water of lakes, rivers and sea are highly contaminated with different toxic compounds which include organicsand can be dreadfully hazardous to human health and aquatic life.Phenolic compounds are hazardous pollutants that are toxic at relatively low concentration. Phenols are widely distributed aromatic compounds that are characteristic pollutants in effluents from chemicals, petrochemicals, pharmaceuticals, textiles and oil refineries[1]. Currently, extensive contamination of surface water, ground water and soil by the phenol released with industrial effluents has been a major environmental concern. Different methods have been applied to remove phenolic compounds from industrial effluents and among all,biodegradation process offers an advantage of completely destroying the pollutants or at least transforming them to innocuous substances. Many microorganisms use aerobic and/or anaerobic conditions to metabolize or mineralize phenol [2]. Biodegradation is based on the fact that the microorganisms need a source of carbon and energy to carry out basic life process such as maintenance and growth. The majority of organic chemicals can be utilized as a carbon and energy source for living organisms (yeasts, bacteria or fungi). The most efficient microbe is the one which can withstand the toxic environment of phenol and utilize that organic compound as the sole carbon source to proliferate [3]. Many bacterial strains like *Acinetobacter* sp. [4], *Alcaligenes faecalis* [5], *Moraxella* sp. [6], *Pseudomonas aerogenosa* [7]-[9], *Pseudomonas fluorescens* [2],[10],*Pseudomonas putida*[11]-[14] and some yeasts such as *Candida tropicalis* [15], [5], *Aspergillus awamori* [16], [17]etc. has been studied for the degradation of phenol. But little attention was given to species of actinomycetes, even though certain species of actinomycetes were known to utilize and degrade many complex chemical substances such as C6-C8 hydrocarbons including phenol. Actinomycetes such as *Streptomyces setonii* [18], *Nocardia coralline* [19], *Nocardia hydrocarbonoxydans* [20],[3] were found to utilize phenol. The rate of biodegradation of pollutants in natural environment

may be affected by a variety of factors, including acclimatization of the microbial community to a particular chemical. *Nocardia hydrocarbonoxydans* were acclimatized to phenol, by exposing them repeatedly to a particular concentration and also to gradually increasing phenol concentrations [20]. *Nocardia hydrocarbonoxydans* has been found to degrade phenol to catechol by meta-cleavage pathway[21] following subsequent mineralization to simpler compounds. The choice of a good medium is virtually as important to the success of biological process as is the selection of an organism to carry out the intended bioprocess. Phenol is used as the sole source of carbon and energy for phenol degradation[3],[20]. The type of nitrogen source influences the efficiency of phenol degradation as reported by [8]in their studies on phenol degradation by *Pseudomonas aeruginosa* NCIM 2074.pH ofthe medium also plays an important role in phenol biodegradation[14]. Batch biodegradation studies have shown that around 100 mg/L of phenol is completely degraded in around 36hours by *Nocardia hydrocarbonoxydans*[3] with a minimal media containing ammonium nitrate and ammonium sulphate as the nitrogen source and at initial pH of 7.However, there arises a need to enhance the rate of degradation as it is essential for economic operation of scaled up processes. Thus, the current paper presents the mineral media optimization in terms of nitrogen sources and initial pHfor phenol biodegradation by *Nocardia hydrocarbonoxydans*.

MATERIALS AND METHODS

A. Microorganism and the growth media

Nocardia hydrocarbonoxydans NCIM 2386 chosen for the present study was obtained from National Chemical Laboratory, Pune, India. The stock cultures of *Nocardia hydrocarbonoxydans* NCIM 2386 were maintained in nutrient agar plates and the freshly, well-grown subcultured organisms were used for preparing the primary acclimatized culture. In the present investigation, an identical mineral medium, as suggested for the similar selected genera of microorganisms used by [20] was used, with phenol as the sole carbon source. The mineral media for growth and acclimatization contained:1.0 g/l of NH_4NO_3; 0.5 g/l of KH2PO4;1.5 g/l of K2HPO4; 0.5 g/l of (NH4)2SO_4; 0.5 g/l of NaCl; 0.002 g/l of FeSO4; 0.5 g/l of MnSO4 and 0.01 g/l of CaCl2dissolved in distilled water.

B. Acclimatization of the microorganism to phenol and determination of its maximum tolerable concentration

Organisms were grown on phenol as the sole carbon and energy source in the defined medium.Both time based and concentration based acclimatization procedure followed by [20]and [22]for acclimatization of *Nocardia hydrocarbonoxydans* to phenol, can ensure complete adaptation to phenol. Hence, this acclimatization procedure was adopted and the organism was acclimatized to 200ppm phenol. The organisms were first acclimatized four times to 100 ppm phenol followed by subsequent acclimatization forfour times to 200 ppm phenol. The acclimatization was carried out by shake flask experiments using 100 mL of mineral media in distilled water containing required concentration of phenol.

A loopfull of test organism from the freshly subcultured plate was inoculated into the medium containing 100 ppm phenol and the culture flask was shaken at 120 rpm in a rotary shaker for 3 days at room temperature of 30±2°C.This is the primary acclimatized culture.1 mL of primaryacclimatized culture was then transferred to media containing 100 ppm phenol and was incubated for 48 h in a shaker to obtain the secondary acclimatized culture. This procedure was continued for the third and fourth acclimatization by using inoculums from second and third acclimatized cultures respectively and with incubation for 48 h. 100ppm acclimatized organism was thus obtained and was used as inoculums for acclimatization to 200 ppm phenol in the similar manner.100ppm acclimatized organism thus obtained, was further acclimatized gradually to 200ppm, 300ppm, 400ppm, 500ppm, 600ppm,700 ppm,800 ppm and 900 ppm using the above procedure.The inoculums were transferred from a flask containing the organisms acclimatized four times at lower concentration to successive flask after 48 hour upto the concentration of 900 ppm. However, at each concentration acclimatization was carried out four times. When the organism was transferred to flasks with 1000 ppm from 900 ppm phenol,no significant growth was observed after 48th hour, but sufficient growth was shown after 72 hour. So the experiments were further continued with acclimatization to higher phenol concentrations by transferring inoculum after 72 hour of incubation for phenol concentrations of 1000 ppm and above. Maximum tolerable phenol concentration was determined by noting the visible growth of the microorganisms.

C. *Phenol degradation studies*

1% (v/v) of 200ppm acclimatized, active, bacterial culture was inoculated into 100mL mineral media at a desired pH containing the required nitrogen source at the desired concentration and 150 ppm of phenol. The culture flasks were kept in a shaking condition at a temperature of30 ± 2°C at 120rpm. Samples were withdrawn from the flask at fixed time interval and analyzed for biomass concentration. Further the

samples were centrifuged at 10000 rpm for 10 min at 4ºC and analysed for phenol concentration. Percentage degradation of phenol was calculated using Eq. (1).

$$\text{Percentage degradation} = \frac{(C_0 - C) \times 100}{C_0} \text{------(Eq. 1)}$$

where C_0 is the initial concentration of phenol

C is the concentration of phenol at any time, t

D. Screening of nitrogen sources in the mineral media

Three inorganic nitrogen sources namely ammonium chloride, ammonium nitrate, sodium nitrate and three organic nitrogen sources namely urea, peptone and tryptone were selected for the phenol reduction study. For screening, the nitrogen source used for the initial study i.e., ammonium nitrate was replaced by other nitrogen sources keeping the concentration of the nitrogen source at 1g/L and other components being the same as given in section A. The media with the nitrogen source showing highest phenol degradation at the end of 8h was selected for further studies.

E. Effect of initial pH on phenol degradation

Phenol degradation studies were conducted at three different levels of Initial pH of the mediumi.e., at acidic (pH of 5), neutral (pH of 7) and basic level (pH of 9). The same procedure as described in section Cwas carried out for three different pH by adjusting it using H_2SO_4/NaOH. pH at which the organisms showed maximumdegradation of phenol was chosen for further studies

F. Optimization of nitrogen source concentration

With the selected nitrogen source and pH based on the above studies, the effect of nitrogen source concentration on phenol reduction were studied. Different concentrations of the selected nitrogen source were 0.25g/L, 0.5g/L, 0.75g/L, 1g/L and 1.25g/L. Experiments were performed as mentioned in section C in 100mL of growth media containing 150mg/L of phenol.The results were analyzed to obtain optimum value of nitrogen source concentration at which the organism *Nocardia hydrocarbonoxydans* degrades maximum amount of phenol.

G. Biomass and phenol analysis

Biomass was analysed spectrophotometrically at 610nm using UV- visible spectrophotometer (Labomed, UVWin 5.0). Samples from the flasks were withdrawn at regular time intervalsand centrifuged at 10000 rpm for 10 min at 4ºC. Cell-free supernatant were used for finding residual phenol concentration by absorbance measurement at 510nm using pre-calibrated UV-visible spectrophotometerafter rapid condensation with 4-amino antipyrine, followed by oxidation with potassium ferricyanide under alkaline conditions to form red coloured antipyrine dye [23].

RESULTS AND DISCUSSION

A. Determination of maximum tolerable concentration of phenol byN. hydrocarbonoxydans

The maximum tolerable concentration of phenol was determined during the acclimatization of phenol as described in Section 2.2 by observing the visible growth of phenol during the growth of *N. hydrocarbonoxydans*. Significant growth at 48 h of incubation was observed in the media containing upto 900 ppm phenol. However, at concentrations of 1000 ppm to 1300 ppm significant growth was observed only after 72 h of incubation. It indicated that the growth rate was inhibited at concentrations above 900 ppm. The organism grew upto the concentration of 1300ppm phenol. Further, when the organisms were transferred to flask containing 1400ppm of phenol it failed to survive which was confirmed by the decrease in the optical density of the media at 610nm as shown in Fig 1. It indicates that phenol concentration of 1000ppm and above inhibits the growth of *N.hydrocarbonoxydans* and concentration of 1400ppm phenol is toxic to the microorganism. So 1300ppm phenol may be considered as the maximum tolerable concentration of phenol by*N.hydrocarbonoxydans*.

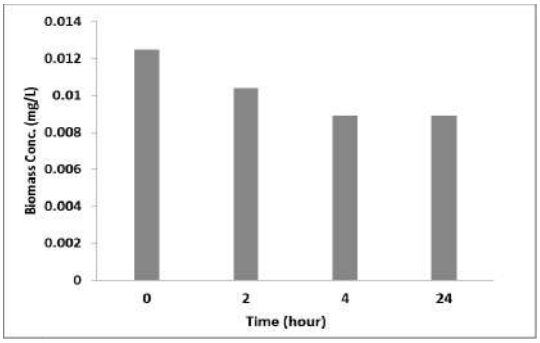

Fig. 1Biomass concentration w.r.t. time at 1400ppm phenol concentration

B.Screeningof nitrogen source

Among the six nitrogen sources i.e., ammonium chloride, ammonium nitrate, sodium nitrate (inorganic nitrogen sources), urea, peptone and tryptone (organic nitrogen sources)selected for the screening, all in the concentration of 1g/L and initial pH of 7.0,peptone showed the maximum degradation of phenol within 8hrs as seen in Fig. 2. Around 98.5% degradation of phenol could be achieved in 8h with peptone as the nitrogen source. However, similar magnitude of percentage degradation was exhibited at 24 h with other nitrogen sources. Urea showed lesser degradation than peptone but better than other nitrogen sources.

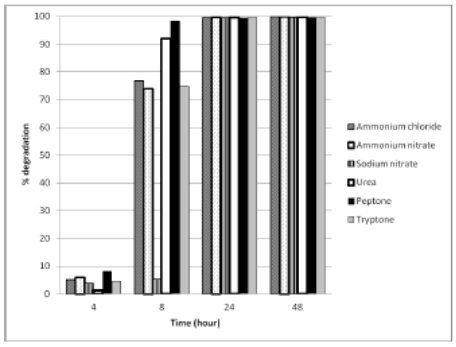

Fig. 2 Percentage degradation of phenol with respect to time using different nitrogen sources

All the media containing different nitrogen sources showed more than 99% degradation in 24h of incubation and further increase in time of incubation did not exhibit any further change in percentage degradation. Peptone contains fats, metals, salts, vitamins and many other biological compounds like proteins and amino acids which may trigger growth and enzyme secretion, hence leading to highest biodegradation when compared to other nitrogen sources.Therefore peptone is considered as the best nitrogen source for higher degradation of phenol in shorter period, i.e. maximum rate of degradation.

C.Selection of optimum pH

It is observed from Fig. 3, that the initial rate of degradation was faster in acidic and neutral media when compared to basic media with peptone as the nitrogen source (1g/L). By the end of 48th hour, 99.89% degradation could be achieved in all the three cases. Considering degradation upto 8th hour, higher initial rate of degradation could be achieved with pH 5.0 and 7.0, as compared to that with pH of 9 which is similar to the results reported by [14] for *Pseudomonas putida* where the pH of the medium was varied from the acidic (pH 5.0) to alkaline (pH 9.0). The phenol degradation achieved at acidic pH and at neutral pH were almost similar.However, operation at acidic pH is not favourable considering the effluent discharge standards or material of construction aspects, thus pH of 7.0 was selected as the optimum operational pH.

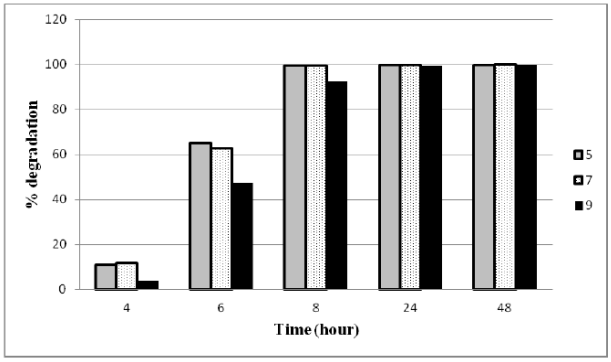

Fig. 3 Percentage degradation of phenol with respect to time at different pH

D.Optimization of nitrogen source concentration

Based on screening studiespeptone was selected as the best nitrogen source and pH of 7.0 was considered as the favorable pH for phenol biodegradation. The concentration of peptone in the media was varied (0.25g/L, 0.5g/L, 0.75g/L, 1g/L and 1.25g/L)and phenol degradation experiments were conducted.

It is observed that nearly 100% phenol degradation could be achieved in 8h at the peptone concentrations studied. However, the rate of phenol degradation was found to be the maximum at 0.25 g/L of peptone as indicated by maximum degradation of around 82% phenol in 6h of incubation. Maximum degradation of phenol was obtained at lowest concentration of peptone (i.e., 0.25g/L)in 6 h and it can also be observed that the degradation decreased with increase in concentration of peptone (Fig. 4). By the end of 8th hour all flasks showed almost complete degradation irrespective of peptone concentration. Though the reason behind this trend cannot be explained clearly, this observation is in good agreement with that of [24]-[25], [8].The reasons may be peptone being the organic nitrogen source provides carbon for *N. hydrocarbonoxydans.*

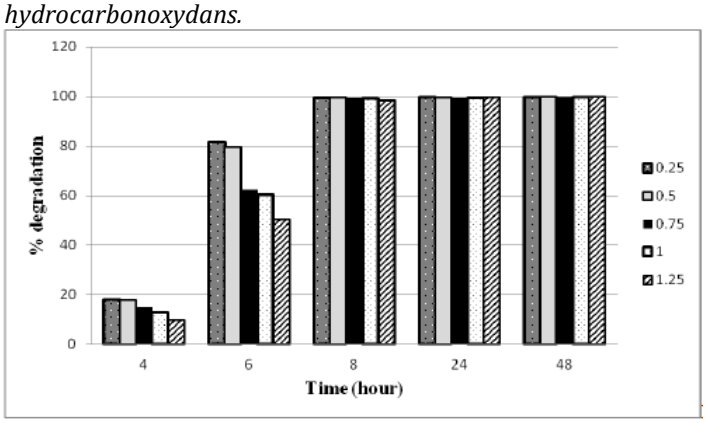

Fig. 4Percentage degradation of phenol w.r.t. time at varying peptone concentrations

Peptone enhances growth and increases the biomass but hinders phenol uptake by the organisms since microorganisms utilize peptone as carbon source till peptone is available in the growth medium,thus showing lower phenol degradation at higher concentrations of peptone. However, once the organic carbon in peptone is utilized completely, the phenol degradation rate enhances owing to higher biomass concentrations. Thus, 0.25 g/L peptone concentration is the optimum for maximum phenol degradation. Screening of nitrogen source followed by optimization of its concentration and pH was found to enhance the rate of phenol degradation. Under un-optimized conditions around 36 h was required for complete degradation of 100 ppm phenol [3], whereas 200 ppm of phenol could be degraded almost completely in 8h. Thus, optimization has improved the rate of phenol degradation.

CONCLUSIONS

Nocardia hydrocarbonoxydans NCIM 2386 acclimatized to phenol by time and concentration course acclimatization procedure was found to be inhibited at phenol concentrations of greater than900 ppm. Phenol concentration of 1300 ppm is considered as the maximum tolerable concentration for *Nocardia hydrocarbonoxydans*and 1400ppm phenol is found to be toxic to the organism. Out of the six different nitrogen sources screened namely, ammonium chloride, ammonium nitrate, sodium nitrate, urea, peptone and tryptone, maximum phenol removal of 98.4% was observed within 8th hr when peptone was used as the nitrogen source with optimum concentration of 0.25g/L. Although acidic (5.0) and neutral (7.0) pH showed similar and higher rates of degradation when compared to basic (9.0) pH with peptone as the nitrogen source, neutral pH of 7 was selected as the optimum operational pHby considering the effluent discharge standards or material of construction aspects.Screening of nitrogen source followed by optimization of its concentration and pH was found to enhance the rate of phenol degradation. Hence *Nocardia hydrocarbonoxydans* can be used for achieving maximum phenol degradation when optimized media containing peptone as the nitrogen source with concentration 0.25g/L and initial pH of 7.0 was used. The higher rate thus obtained by optimization may prove beneficial by reducing the bioreactor size for continuous wastewater treatment thus decreasing the fixed cost of the process.

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