Optimization of *o*-Cresol Degrading *Pseudomonas monteilii* CR13 and Kinetics of Degradation

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ABSTRACT

In the present study, Pseudomonas monteilii *CR13 isolated from petroleum contaminated soil demonstrated the highest specific* o-*cresol degradation rate at all tested* o-*cresol concentrations and also was not disturbed by the starting substrate concentration used (*o-*cresol-500 mg/L). After a serial transfer of the isolate into a series of increasing* o-*cresol level, the organism demonstrated significant improvement on degradation ability up to 3000 mg/L. The optimum condition for the cell mass increase and biodegradation of* o-*cresol by* Pseudomonas monteilii *was in the minimal mineral medium of 3 at a pH of 6.5 and temperature 30 °C, stirring velocity of 160 rpm, and the substrate concentration of 500 mg/L. The biodegradation kinetic study was carried out by bacteria in different initial substrate concentrations (500–3000 mg/L). In the present test the* μ_{max}, K_s *and the* μ *were found 0.332* h^{-1}, *0.166 mg/L and 0.0282 mg/L for 500 mg/L of* o-*cresol, respectively. The organism is highly promising and could be used to remove high concentrations of* o-*cresol from highly polluted aquatic and soil regions. The cells could be immobilized on a suitable matrix and the efficiency of degradation could be effectively improved.*

Keywords: *biodegradation;* o-*cresol;* Pseudomonas monteilii*; petroleum contaminated soil*

ABSTRAK

Dalam penelitian ini, Pseudomonas monteilii *CR13 yang diisolasi dari tanah yang terkontaminasi minyak bumi menunjukkan tingkat degradasi* o-*cresol spesifik tertinggi pada semua konsentrasi* o-*cresol yang diuji dan juga tidak terpengaruh oleh konsentrasi substrat awal yang digunakan (*o-*cresol-500 mg/L). Setelah transfer serial isolat menjadi serangkaian peningkatan level* o-*cresol, organisme menunjukkan peningkatan yang signifikan pada kemampuan degradasi hingga 3000 mg/L. Kondisi optimum untuk peningkatan massa sel dan biodegradasi* o-*cresol oleh* Pseudomonas monteilii *berada di medium mineral minimal 3 pada pH 6,5 dan suhu 30 °C, kecepatan pengadukan 160 rpm, dan konsentrasi substrat 500 mg/L. Studi kinetik biodegradasi dilakukan oleh bakteri dalam konsentrasi substrat awal yang berbeda (500–3000 mg/L). Dalam tes ini* μ_{max}, K_s *dan* μ *ditemukan masing-masing 0,332* h^{-1}, *0,166 dan 0,0282 mg/L untuk 500 mg/L* o-*cresol. Organisme ini sangat menjanjikan dan dapat digunakan untuk menghilangkan konsentrasi tinggi* o-*cresol dari daerah air dan tanah yang sangat tercemar. Sel-sel dapat diimobilisasikan pada matriks yang sesuai dan efisiensi degradasi dapat ditingkatkan secara efektif.*

Kata Kunci: *biodegradasi;* o-*cresol;* Pseudomonas monteilii*; tanah terkontaminasi minyak bumi*

INTRODUCTION

A huge amount of chemical compounds is released into the environment by different industries, of which cresols are highly toxic. *O*-cresol is an isomeric phenol with methyl substituent in the ortho position relative to the hydroxyl group. United States Environmental Protection Agency (USEPA) [1] and the Central Pollution Control Board of India (CPCB) have included cresols in the list of priori ty pollutants.

Cresol is an important commercial mixture of chemicals used as a starting material to make other chemicals including pesticides, fragrances, antioxidants, and resins. Cresol also is used as a solvent for industrial processes and certain paints and as a wood preservative. Ortho-Cresol is used as a solvent and starting material for making various resins, antioxidants, herbicides, and pesticides and they cause damage to the environment.

The in vitro toxic effect of cresol on kidney has been extensively s tudied [2] and the presence of cresols have shown to reduce the leaching capacity of the soil. [3].The huge amount of the chemical released from various industries and the extreme toxicity of o-cresol to the flora and fauna make the removal of the compound from the environment an important task.

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Bioremediation is a process that utilizes biological agents as much as possible for the elimination of environmental pollutants. Biological treatment methods are generally considered more effective due to its cheapness and ability for mineralization [4-5] and it has rapidly become the most effective way for complete mineralization of organic pollutants. In a natural environment, several microorganisms like fungi and bacteria make use of *o*-cresol even though it is highly toxic [6-9]. Although the biodegradation process of many phenolic compounds have been extensively studied it is very difficult to obtain the data where o-cresol is used as a single carbon source. The ability to biodegrade any chemical highly depend on different physical and nutrient factors [10]. It is very important to understand the optimum conditions required by the organism for the complete Mineralization of the chemical compounds so that the efficiency of their degradation capacity could be improved.

In the present study, a bacterial strain was isolated from the contaminated soil which could be used to degrade high concentrations of *o*-cresol. Out of many bacteria isolated, only one species was able to degrade high concentration of *o*-cresol. This bacteria was identified based on the methods described in Bergey's manual of determinative bacteriology and diagnostic microbiology based on its morphological, cultural and biochemical characteristics and further confirmed based on the 16S rRNA sequence analysis and BLAST identification as *Pseudomonas monteilii* CR13 [11] in our laboratory. The culture was maintained by weekly subculturing with 500 mg/L of *o*-cresol and kept in 4 °C.

Given wide distribution, growth rate and efficiency of *Pseudomonas* species and its diverse biodegradative capabilities, the conditions for the biodegradation of *o*-cresol have been optimized and applicability of the Monad model for *o*-cresol biodegradation has been analyzed.

EXPERIMENTAL SECTION

Culture Medium Used

Pseudomonas monteilii CR13 were cultivated aerobically at 30 °C in a rotary shaker (200 rpm) in minimal medium [12] where *o*-cresol, was provided as a sole carbon source.

Acclimation of Bacterial Strains for o-cresol Biodegradation (500–3000 mg/L)

Bacterial strains were cultivated in a mineral salt medium having increasing concentrations of *o*-cresol and 0.2% of glucose (500–3000 mg/L) in batch mode. The culture was acclimatized to *o*-cresol by exposing the culture in a 250 mL conical flask in which the concentration of *o*-cresol was gradually increasing. After growing cells in 3000 mg/L *o*-cresol cells, harvested by centrifugation at 8000 rpm for 10 min. The cell suspensions obtained were used as inoculum in biodegradation studies.

Optimization of the Growth Conditions of the Organism

A set of experiments were conducted to examine the effects of the mineral medium compositions, temperature, and pH on cresol removal and growth of the organism. The concentration of the substrates (500 mg/L) were kept constant. The initial pH varied in the various media components that were tested. Their effects on cresol biodegradation and specific growth rate were examined [13].

Effect of pH

One of the main physical factors that influence the increase in cell mass of microorganism is the pH of the medium. The growth rate was observed spectrophotometrically after 24 h at OD 550 nm to analyze the biodegradation of *o*-cresol and organisms grown in particular pH. The temperature and stirring speed were kept constant at 30 °C and 200 rpm, respectively.

Effect of Temperature

The effect of temperature on the removal of *o*-cresol was carried out at different temperatures at 200 rpm in an incubator shaker. Mineral salt medium maintained at pH 7 was used to check the effect of varying temperatures (20, 25, 30, 35, 40, 45, and 50 °C). Cell growth was analyzed spectrophotometrically after 24 h to estimate the rate of degradation of *o*-cresol and growth of microbes at respective temperature. The temperature at which the growth and utilization of *o*-cresol were maximum was used for the further studies.

Effect of Stirring on the Removal of *o*-Cresol

A set of experiments were conducted to study the effect of stirring on the biodegradation of *o*-cresol at 30 °C and at pH 7. Stirring velocity used were 80, 100, 120, 140, 160, 180 and at 200 rpm. Samples were analyzed spectrophotometrically after 24 hours to estimate *o*-cresol concentration and biomass increase.

Optimization of Media Composition

Different media used for the study were basal salt medium, synthetic mineral salt medium, minimal salt

medium 1 minimal salt medium 2, minimal mineral medium 3 and M3 liquid medium [14-17].

Biomass Increase and Biodegradation Kinetics under Optimized Conditions

To study growth and biodegradation kinetics of isolated strains, pure cultures of strain were inoculated into a 500 mL Erlenmeyer flasks having 100 mL MMM3 medium containing 500 to 3000 mg/L of *o*-cresol. Flasks were incubated at 30 °C and pH 6.5 in a shaking incubator at 160 rpm. Samples were drawn from the flask at an interval of 3 h. Growth kinetics was monitored by taking the optical densities at 550 nm and degradation kinetics were studied by analyzing the *o*-cresol in the medium.

Study of Growth Kinetics

The growth kinetics was analyzed using the various initial amount of o-cresol which varied between 500 to 3000 mg/L of o-cresol.

The Monod equation is:

$\mu = \mu_{max} \times (S / (K_S \times S))$

μ = specific growth rate,
μ_{max} = maximum specific growth rate,
S = substrate concentration,
K_S = substrate saturation constant

Analytical Methods

The residual o-cresol determination in the medium

The residual concentration of *o*-cresol was determined by using 4-aminoantipyrine (4-AAP) as a coloring agent. The reaction of 4-APP and o-cresol at alkaline pH produces a colored antipyrine dye which was analyzed at 506 nm. Each concentration of *o*-cresol level for the microbes were repeated twice or thrice to get the best result [18-20].

Measuring cell growth

The cell growth was estimated by spectrophotometry in 1 cm cuvettes at a wavelength of 550 nm [21-22] using UV spectrophotometer (Shimadzu). They were rotated for 10 minutes at 10,000 rpm to separate the biomass from the supernatant. A standard calibration curve was prepared with the concentration of *o*-cresol in the range of 0.1–1 g/L.

RESULT AND DISCUSSION

Acclimatization of Pseudomonas monteilii CR13 to *o*-Cresol (500-3000 Mg/L)

To acclimatize the microbes to *o*-cresol very little amount of glucose was mixed to the media for commencing cell mass increase of the microorganisms. As soon as glucose was depleted from the medium, organism started to use o-cresol as a carbon source. The importance of our study is the adaptation of *Pseudomonas monteilii* CR13 above 1500 mg/L of *o*-cresol, which is the highest concentration of *o*-cresol reported so far. Many previous scholars have reported a degradation rate of 99% in 2.5 months, [23] whereas we could reach the same rate in10 to14 h. The cell growth and residual *o*-cresol at the different initial concentrations of *o*-cresol is given in Fig. 1.

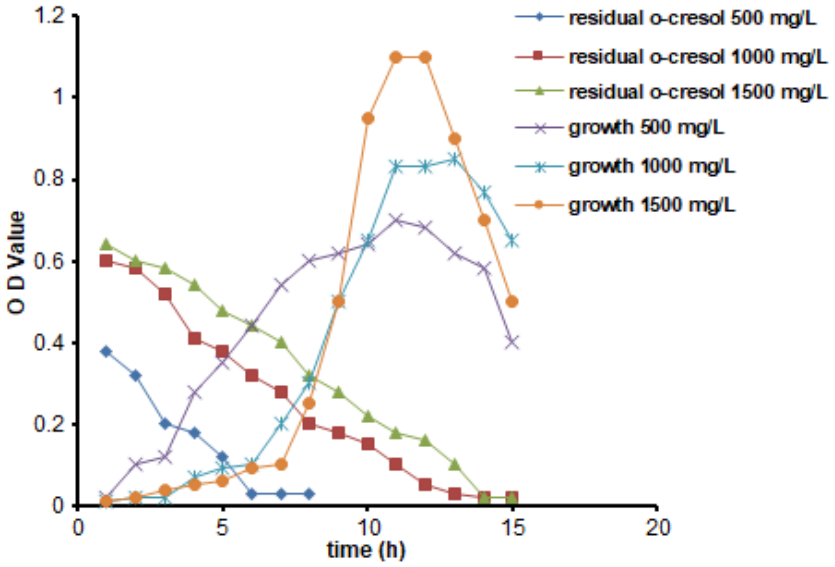

Fig 1. Acclimatized cell growth and residual *o*-cresol of *Pseudomonas monteilii* CR13 at various concentration of *o*-cresol

Optimization of Cultural Conditions

Optimization of pH

The growth profile and residual *o*-cresol activity were analyzed by taking OD at 550 nm (Fig. 2). From the values obtained it was inferred that *Pseudomonas monteilii* CR13 had maximum growth at pH 6.5. For further studies, the pH of 6.5 ± 0.2 was used. For conducting all studies on phenol degradation neutral pH values were used [24-25]. From the values, we can infer that the cardinal temperature for the degradation of o-cresol is 5.5, 7 and 9.

Optimization of temperature

Pseudomonas monteilii CR13 could remove *o*-cresol between temperature range from 25 to 35 °C but the highest *o*-cresol degradation was obtained at 30 °C (Fig. 3). The growth of *Pseudomonas monteilii* CR13 and *o*-cresol degradation consequently decreased after increasing the temperature from 35 °C. Temperature above 40 °C showed a negative correlation.

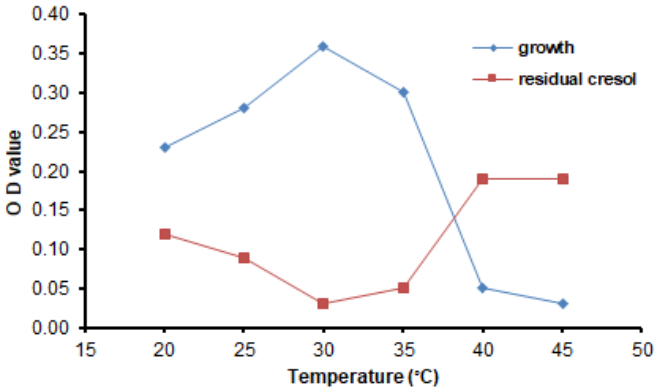

Fig 3. Growth profile and residual o-cresol of *Pseudomonas monteilii* CR13 at different temperature

Effect of stirring on the removal of o*-cresol*

Shaking speed is a prominent factor in deciding the aerobic growth of the microorganism and its ability to degrade *o*-cresol. The stirring effect on the *o*-cresol degradation of *Pseudomonas monteilii* CR13 was investigated by using various stirring speeds. Maximum

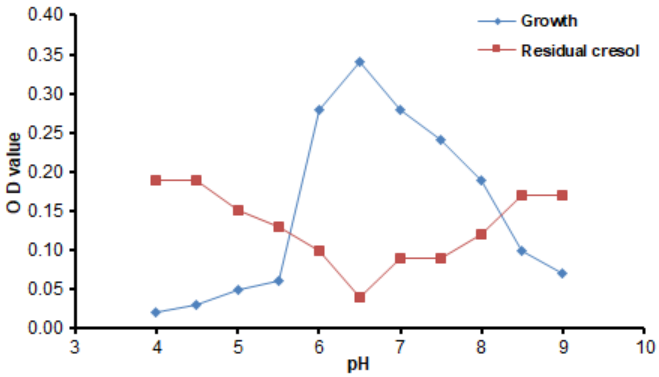

Fig 2. Growth profile and residual o-cresol of *Pseudomonas monteilii* CR13 at different pH

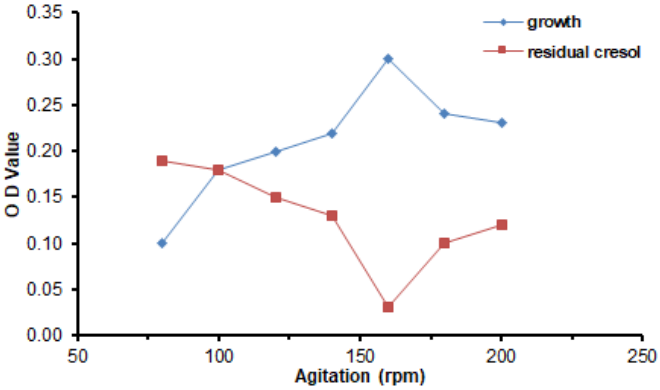

Fig 4. Growth profile and residual o-cresol of *Pseudomonas monteilii* CR13 at different agitation

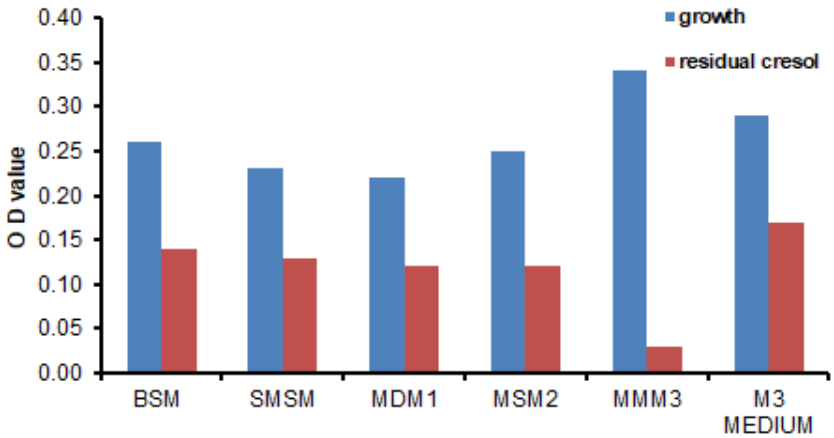

Fig 5. Growth and residual cresol concentration of *Pseudomonas monteilii* CR13 in a different medium

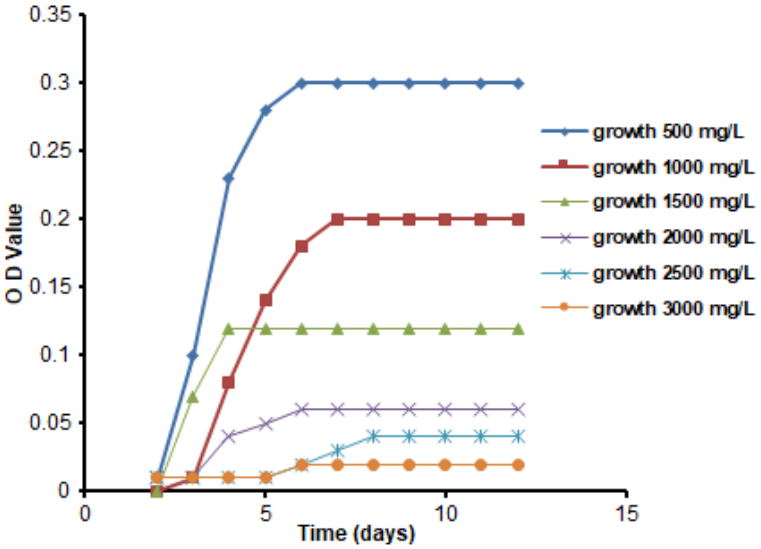

Fig 6. Growth of *Pseudomonas monteilii* CR13 in MMM3 medium with o-cresol as a carbon source

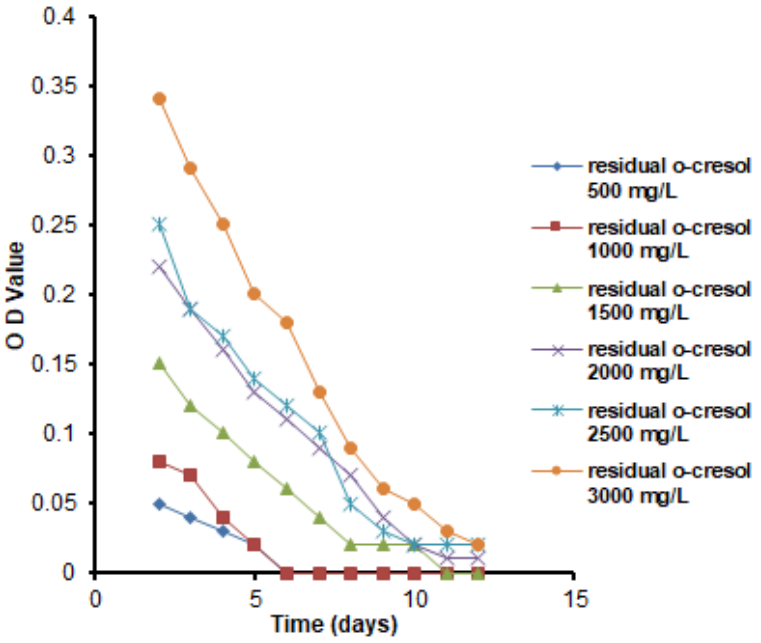

Fig 7. Residual *o*-cresol reduction by *Pseudomonas monteilii* CR13

degradation rate was observed at 160 rpm. Shaking speed of 80 and 140 rpm showed a very little reduction in *o*-cresol. (Fig. 4) higher stirring speed may give extra stress to the cells which obviously affected the degradation rate.

Optimization of media components

Six different media compositions were used for the study, which was selected from previous phenol degradation studies. The basal salt medium (BSM) pH 6.7, synthetic mineral salt medium pH 6.9, minimal salt medium 1 (MSM 1) pH 7.8, minimal salt medium 2 (MSM 2) pH 6.5, minimal mineral medium 3 (MMM3) pH 6.5 and M3 liquid broth pH 7.2 were selected for the study. The maximum *o*-cresol biodegradation (99.8%) was found in the MMM3 medium after 9-10 h incubation. In this experiment, the maximum cell concentration (1.48) was observed after 24 h of growth. (Fig. 5).

O-Cresol Biodegradation Kinetic Studies

Evaluation of growth kinetics was done by plotting the increase in biomass from various initial *o*-cresol biodegradation experiments on a semi-logarithmic graph.

Table 1. The growth kinetics of *Pseudomonas monteilii* CR13

compound	Concentration of o-cresol (mg/L)	μ_{max}	K_S	μ
o-cresol	0.5	0.332	0.166	0.0282

Fig. 1 reveals that the value of specific growth rate (μ) increases with the increase in initial o-cresol concentration up to a certain concentration level, then this rate starts decreasing with the increase in the concentration suggesting that *o*-cresol is an inhibitory substrate.

The specific growth rate is higher at low concentrations of *o*-cresol than at high concentrations because substrate inhibition began to appear after the *o*-cresol concentration of 800 mg/L. Many scholars have achieved similar results [26]. Table 1 shows the μ_{max}, KS, and μ of the reaction. Some experiments showed that *o*-cresol is much preferred than phenol by microorganisms. [27] The Information obtained from the kinetic studies of *o*-cresol biodegradation is used for optimizing the degrading procedures of microorganisms. The increase in cell mass residual cresol concentration in media showed the inverse proportion with each other. The decrease in cresol concentration accompanied by an increase in biomass is shown in Fig. 6 and 7. Complete degradation of various concentrations of *o*-cresol was carried out in within 5–11 days depending on the initial concentration provided.

CONCLUSION

Several works are being done to isolate new and efficient microorganism that has the ability to degrade phenolic compounds. A high potential exists in microorganisms in catabolizing aromatic compounds as carbon sources and *Pseudomonas monteilii* CR13 isolated from petroleum contaminated soil can degrade 3000 mg/L of *o*-cresol after acclimatization of the organism to the substrate. The degrading capacity of the organism could be improved when the conditions were optimized. The organism is highly promising and can be effectively used to detoxify the soil polluted with methylated phenols.

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