

## Aerobic Batch Degradation of Cresol by Newly Isolated *Pseudomonas monteilii* Cr13

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Microorganisms have broad spectrum of applications varying from metabolite production to breakdown of complex carbon sources. Cresols are one such complex that has drawn attention due to its disposal in un-degraded or partially degraded form that enters into air, water and soil polluting the environment; its extremities extend to surrounding ecosystem and destroying the aquatic and human life forms. Cresol possesses special affection towards blood plasma leading to kidney, liver and heart disorders. Many have reported biological degradation of hazardous chemical using different organisms with fine degradation efficiency. The current investigation adds another strain *Pseudomonas monteilii* which was found to exhibit the potential to utilize cresol as its major carbon source for growth and proliferation. Further, the Cresol inhibitory concentration was checked by varying concentration from 100 to 1500 ppm. The remnant concentration over the time period was analyzed by aminoantipyrine assay. The strain was capable of >99% removal at neutral pH, 150 rpm and 30p C in 24h. The phylogeny of the strain was analyzed post 16S rRNA sequencing using in silico tools. The strain can be optimized for degrading higher cresol dose without compromising on the biodegradation efficiency.

**Keywords:** Cresol degrading bacteria, 16S rRNA Gene Sequencing, *Pseudomonas monteilii*.

The methylphenols are the aromatic organic compounds that have variable melting points decided by the surrounding temperatures. These methylphenols have various applications in manufacturing of pesticides, petroleum products, dyes and also in a few pharmaceutical products and are generally known as cresols<sup>1,2,3,4,5</sup>. Cresols are generally found dumped in regions with petroleum or dye dumping sites. They are severely toxic for humans if inhaled or ingested, even at a very low concentration that often leads to consequences varying from irritation of eyes, mouth, throat and skin, vomiting, liver and heart damage, paralysis,

coma and death<sup>1,6,7</sup>. In human, cresol finds more affinity towards the ligand binding protein, Human Serum Albumin (HSA). HSA contributes as carrier proteins for various steroids, fatty acids and helps in maintaining the antioxidants in the body. Cresol binds to albumin reducing the carrier profile of protein resulting in kidney and blood disorders<sup>8,9,10,11</sup>. It is not only toxic for human life but equally deleterious for the aquatic forms, thus, direct disposal in water, air and soil is strictly prohibited<sup>12,13,14</sup>.

The environment exposure to cresols has been observed in terms of petroleum leakage, sludge from dye, pharmaceutical or other industries using derivatives of cresols as raw material<sup>1</sup>. There have been a lot of investigation on degradation and sequestration of the hazardous compound, where

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the microorganisms including, *Pseudomonas*<sup>15</sup>, *Acinetobacter*<sup>16</sup>, *Ralstonia*<sup>17</sup> etc. have been predominantly isolated from the contaminated sites. These microorganisms possess a strong specific mechanism to metabolize these phenols to a usable carbon source for their growth and proliferation. The enzymes such as monooxygenases, hydroxylases and dehydrogenases play a major role in formation of cresol intermediates and further, direct to Tri-carboxylic acid cycle (TCA). The end product thus, remains carbon dioxide and ATP, energy providing molecules<sup>18,19</sup>.

In the current study, a novel strain of *Pseudomonas* has been isolated from petroleum contaminated site, which has been found quite suitable for cresol degradation. The isolate has thus, been phylogenetically analyzed.

**Table 1.** Microscopic features of CR-13

S. no.	Features	Isolates
1	Gram's staining	Gram positive
2	Motility	Motile
3	Endospore	present
4	Capsule	Absent

**Table 2.** Colony characteristics of CR-13

S. no.	Colony morphology	
1	Size	Pin point
2	Shape	Circular
3	Margin	Smooth, Entire
4	Elevation	Raised and Convex
5	Optical features	Wet and Translucent
6	Pigmentation	NIL

## MATERIAL AND METHODS

### Isolation and Screening of Cresol Degrading Bacteria

The soil samples were collected from the various localities in and around Calicut which were contaminated with petroleum, including, petrol pumps, automobile workshops, Petroleum transfer areas to the reservoir, etc. The 30 different soil types were inoculated in to Minimal mineral medium without carbon source as a negative control, whereas, for the test samples, various isomers of cresol were fed as carbon source in minimal mineral medium. The concentration of cresol isomers were maintained as low as 100 ppm in 1L of the medium. 1% of each soil sample was inoculated into the control and test samples and was incubated for 24h at 30°C with 150 rpm. Depending on the microbial growth the concentration of cresol variants was gradually increased to 500 ppm for the consortium showing a good affinity towards the cresol minimal medium.<sup>20</sup>

**Table 3.** Biochemical characterization of CR-13

S. no.	Biochemical tests	Result
1	Indole production test	Negative (-ve)
2	Methyl Red	Negative (-ve)
3	Voges Proskauer test	Negative (-ve)
4	Citrate Utilization	Positive (+ve)
5	Oxygen requirement	Aerobic
6	catalase	Positive (+ve)
7	oxidase	Positive (+ve)
8	Dextrose Fermentation	Positive (+ve)
9	Sucrose Fermentation	Positive (+ve)
10	Lactose Fermentation	Negative (-ve)



A



B

**Fig. 1.** (A) and (B) Gram staining of CR-13 and (B) wet, circular and smooth colonies of CR-13 on minimal mineral medium supplemented with 500 ppm of *o*-cresol

The cresol acclimatization by the grown consortium was tested at higher concentration ranging from 500 ppm to 1200 ppm. Based on the responses of each bacterial culture, the strain with best cresol tolerance and efficiency of survival on the cresol medium was isolated for phenotypic characterization.<sup>21</sup>

#### Morphological and Phenotypic Characterization

The isolated strain was undergone a series of biochemical and morphological identification. The strain was Gram stained and observed under the microscope to understand morphological characteristics. Further, the pigment producing efficiency, colony shape, aerobic requirements and other sugar utilizing efficiencies were tested.<sup>22</sup>

The study of phenotypical characterization was initiated with DNA isolation. The obtained sample was analyzed for purity and integrity on 2% agarose gel prepared in 1X TE buffer. PCR was run to amplify the product. Amplified product was sequenced using 16S rRNA sequencing.<sup>23</sup> Phenotypical characterization was done in the Department of Life Sciences, Kristu Jayanti College, Bangalore. Phylogenetic analysis was performed using clustalW.

#### Cresol Degradation Study

The cresol degradation was checked by evaluating the remnant cresol concentration in the medium after the optimum incubation period.

The evaluation of cresol content was performed by a colorimetric assay using 4-amino antipyrine method.<sup>24</sup>

Cresol was treated with 4-aminoantipyrine at alkaline pH around 10.00 in presence of an oxidizing agent, potassium ferricyanide that results in the formation of stable reddish-brown colored antipyrine dye. The dye can be estimated by taking O.D. at 460 nm. The intensity of color or the optical density is directly proportional to the concentration of cresol. The standard graph for the assay was plotted with known concentrations of cresol between 10 to 50µg/L. So, the O.D. values obtained for known concentration of cresol were extrapolated or interpolated on the standard curve to the quantitative cresol concentration.<sup>25,26,27</sup>

## RESULTS AND DISCUSSION

#### Isolation and Screening of Cresol Degrading Bacteria

The thirty different soil types were inoculated in to minimal medium with and without cresol variants to grow the bacterial consortium possessed by each. The grown culture was found to have a good population of bacteria that was screened further based on the tolerance at high cresol concentration. Among the cresol variants used, *o*-Cresol was found to have better results in

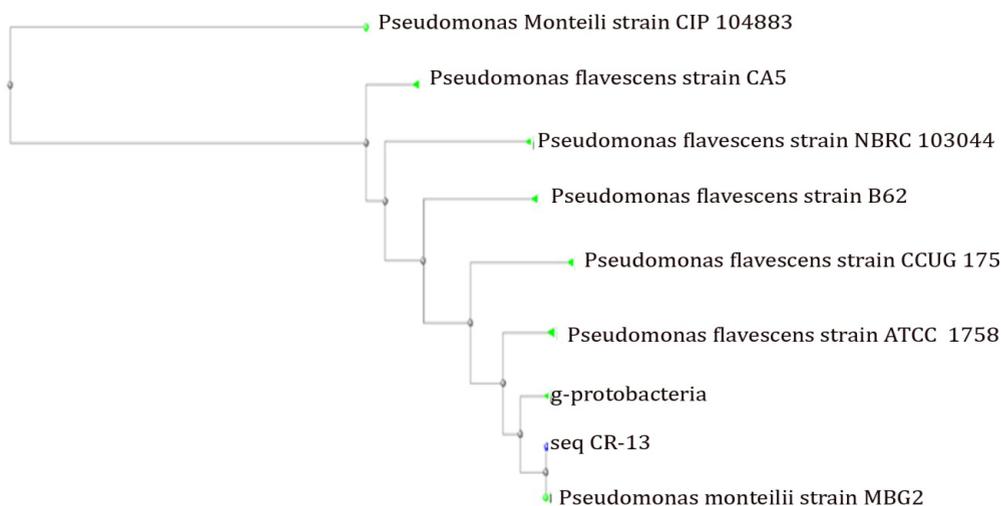


Fig. 2. Phylogenetic analysis of seq. CR-13 using CLUSTAL-W

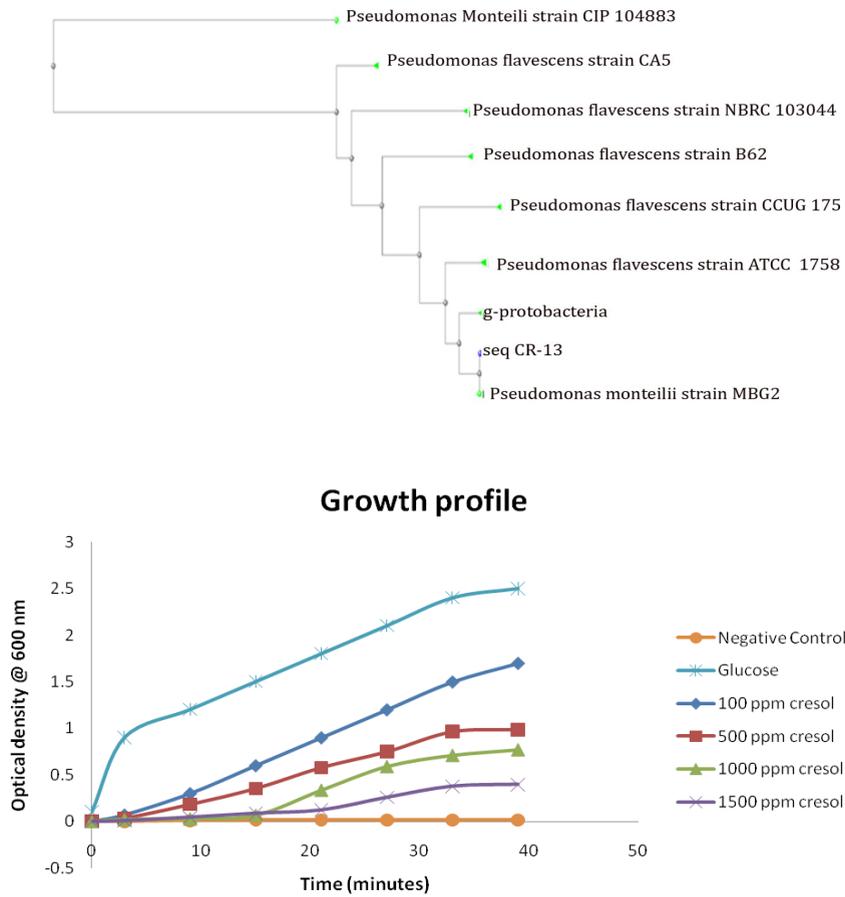


Fig. 3. Growth profile of *Pseudomonas monteilii* CR 13 at the different initial concentrations of *o*-cresol

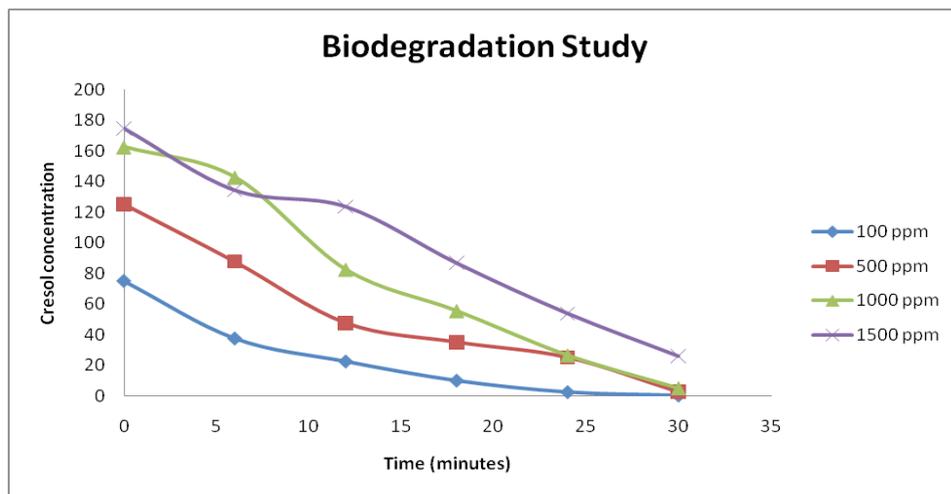


Fig. 4. Initial concentration of cresol influences the degradation efficiency of *pseudomonas monteilii* CR-13

terms of microbial affinity. *o*-Cresol concentration was increased from 100ppm to 500ppm that resulted into survival of only 15 bacteria, namely CR-01 to CR-15. The *o*-Cresol tolerance was checked by varying concentration from 500ppm to 1200ppm. Most of the bacteria were found dead after 700ppm, with CR-13 showing exceedingly high tolerance for *o*-Cresol that survived even at 1200ppm. A similar study has been performed by Fatiha Lassouane *et. al.* on *Pseudomonas aeruginosa* strain (strain S8) where, the strain has a high *o*-Cresol biodegradation potential, it could degrade completely 1250 mg/L of *o*-Cresol within 85 h.<sup>28</sup>

Thereafter, CR-13 was characterized with various microscopic features, (Table 1) colony characters (Table 2) and biochemical tests (Table 3). The strain was found to be Gram negative, rod-shaped motile organism, without spore and capsule. [Figure 1 (A)]. It possessed circular colonies with smooth and wet texture, [Figure 1 (B)]. This aerobic bacterium was found catalase and oxidase positive.

#### Phylogenetic Characterization

The sequence, received from the sequencer was converted into FASTA file using bioinformatics software. The sequence then used for Blast search against NCBI and phylogenetic analysis was performed using clustalW. The phylogenetic analysis predicted that the sequence, obtained was found to have 99% similarity with *Pseudomonas monteilii* strain MBG2 16S ribosomal RNA gene. (Figure 2)

The growth profile of *Pseudomonas monteilii* CR-13 at different concentration of *o*-Cresol was studied in the presence and absence of standard carbon source, cresol and without any carbon source. The growth of the cells was studied using UV-Vis spectrophotometer at 600nm. (Figure 3). All the varied initial concentrations of *o*-Cresol were consumed by *P. monteilii* CR-13. The *P. monteilii* CR-13 could survive and utilize upto 1500ppm of *o*-Cresol with a low growth profile than the standard carbon source (glucose). Similar studies have reported by Ahamad, P.Y.A. *et al.*<sup>29</sup>, and Mamma D<sup>30</sup> where acclimatized *Pseudomonas putida* cells could overcome the inhibitory effect of phenol by the addition of glucose, a conventional carbon source.

#### Degradation Efficiency of *P. monteilii* CR13

The biodegradation study of cresol by *P. monteilii* CR13 was performed by 4-aminoantipyrine assay. The biodegradation study in terms of residual concentration of *o*-Cresol was performed and the unknown concentration was calculated based on the optical density of the standard curve for 4-aminoantipyrine assay. The maximum degradation was affected by the initial cresol concentration in the medium. The amount of remnant cresol concentration was determined by interpolating the O.D. values obtained after 4-amino antipyrine assay. The values of O.D. at different time intervals between 0 to 30 h have been plotted over the standard curve to determine the unknown concentrations. So, by the end of each experimental set, the concentrations calculated was 0.25, 2.5, 2.75 and 5 ppm, respectively with initial cresol dose of 100, 500, 1000 and 1500 ppm. The degradation efficiencies achieved are 99.75, 99.5, 99.35 and 98.28, respectively for 100 ppm, 500 ppm, 1000 ppm, and 1500 ppm respectively. (Figure 4)

#### CONCLUSION

The isolated *Pseudomonas monteilii* CR-13 is an efficient bacterial strain to biodegrade *o*-Cresol. The *P. monteilii* CR-13 is efficiently degrading 1500 ppm of *o*-Cresol and shows good growth profile. Further investigations are recommended for immobilization and optimizing the growth conditions for enhancing the degrading capacity of *P. monteilii* CR-13. In the light of results that were gathered from our batch culture, the organism is showing high promise in degradation of *o*-Cresol.

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