

Biodegradation of Phenol by the Yeast *Candida tropicalis*: An Investigation by NMR Spectroscopy

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Abstract The process of phenol biodegradation by the yeast *Candida tropicalis* NCIM 3556 in aqueous medium was studied by ^1H , ^{13}C , and DOSY NMR techniques. Samples at regular intervals were centrifuged to separate the cells, and ^1H spectral data were collected at 400 MHz. Though a gradual decrease in the concentration of phenol was observed, after an incubation period of ~8 h, formation of any intermediate products could not be detected. Experiments carried out with uniformly ^{13}C -labeled phenol also failed to detect formation of any carboxylic acid intermediates during degradation. The studies indicated that the phenol was completely degraded to carbon dioxide and water in approximately 20 h. Self-diffusion coefficient measurements showed that the lifetime of phenol in the bound form is too small to impart any change in its diffusion behavior and the intermediates formed are converted to carbon dioxide and water at a very fast rate.

Keywords Enzyme · NMR spectroscopy · Carboxylic acid · Biocatalysis · Diffusion · Phenol

Introduction

Phenol and its derivatives are major pollutants present in the wastewater from oil refineries, cooking plants, coal conversion processes, and the steel industry. These toxic compounds are hazardous to plant and aquatic life, and their removal from these wastewaters before discharging into the environment is crucial [1]. There are many techniques employed for

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the removal of phenol from the wastewater. Though several chemical methods have been reported for the degradation of phenols, biological processes are eco-friendly and therefore preferred for treatment of industrial effluent [2, 3]. A number of yeast, bacterial, and filamentous fungal strains have phenol-degrading capacity; *Candida tropicalis* NCIM 3556 has been identified to degrade phenol and its derivatives at relatively high phenol concentration [4].

NMR has been used as a tool for studying biological reaction mixtures by several research groups [5–7]. Biotransformation reactions are mostly carried out in aqueous medium at low concentrations. Consequently, product isolation and purification for analysis is often fraught with difficulties. A direct analysis of the whole reaction mixture would give quick results of the reaction progress and the products formed.

We were interested in studying the biodegradation of phenol by spectroscopic techniques with the idea of getting deeper insight into the pathways involved. Chemically, the reaction could be followed by disappearance of phenol using colorimetric method [8], but product information was not possible. Product isolation proved to be elusive, indicating the possibility of formation of volatile compounds. To comprehend the reaction process and products, we performed ^1H NMR spectroscopy for the whole reaction mixture to achieve qualitative and quantitative data. ^{13}C -labeled phenol was used in order to check the formation of intermediate products such as oxalic acid or any other di- or monocarboxylic acids during the process of degradation. Degradation of 2,6-dimethylphenol was also studied using the same method. Self-diffusion coefficient measurements by pulsed field gradient spin echo (PFGSE) experiments were carried out to get information about the nature of interaction between the cells (catalyst) and phenol (substrate). In situ studies were also performed to check the effect of area of contact between cells and substrate.

Materials and Methods

Chemicals

Phenol used for biodegradation was purchased from SD Fine Chemicals, Mumbai, India. ^{13}C -labeled phenol was purchased from CortecNet, Voisins-le-Bretonneux, France. Media components used for growth were purchased from HiMedia, Mumbai.

Biomass

The culture *C. tropicalis* NCIM 3556 was acquired from NCIM culture collection (National Chemical Laboratory, Pune, India). Whole cells of *C. tropicalis* NCIM 3556 were grown in MGY medium (malt extract 3 g, glucose 10 g, yeast extract 3 g, and peptone 5 g l^{-1}) under a condition of 220 rpm and 28 °C for 24 h. The age of inoculums was 24 h, and the volume was 10 %. Cells were separated by centrifugation and used for study.

Biodegradation of Phenol

A series of tubes containing 0.5 g of cells, 5 ml of water, and 10 mg of phenol (one batch with ordinary phenol and another with ^{13}C -labeled phenol (2,000 mg l^{-1})) were kept on a shaker at 220 rpm and 28 °C. At regular time interval, 1 ml of sample was withdrawn from one of the tubes, centrifuged at 8,000 rpm for 5 min to separate the cells. The supernatant was used for analysis.

NMR Study

Approximately 0.6 ml of the supernatant was transferred into a standard 5-mm NMR tube, and ^1H NMR measurements were carried out using an external D_2O capillary for field-frequency locking. All ^1H NMR measurements were done under identical conditions using the same experimental parameters. The NMR parameters used for the measurements are the following: 30° flip angle, 64 scans for normal spectra, and 256 scans for spectra with water suppression by presaturation and water suppression by gradient-tailored excitation (WATERGATE) pulse sequence with a 0.844-s acquisition time, 5-s relaxation delay, 4,850-Hz spectral width, and 32,768 data points. The raw data were processed with 0.3-Hz line broadening, and the baseline of the spectra was corrected in an identical manner using an automatic baseline correction routine.

For quantitative estimation, the area of the signals under an identical region has been used. The area of the signals of phenol (7.8 to 6.5 ppm) for the sample at 0 h was assigned as 100, and the concentration of phenol present in the samples taken at later intervals was estimated with respect to this. The procedure was repeated for cell blank and substrate blank, i.e., the reaction mixture that did not contain phenol and cells, respectively. ^1H NMR spectra were recorded on a Bruker AV 400 NMR spectrometer equipped with a 9.4-T ultrashield magnet and a standard 5-mm BBO Z gradient probe at ambient temperature (296 K).

In order to get good sensitivity for aliquot samples with uniformly labeled phenol, ^{13}C measurements were conducted at 125 MHz on a Bruker AV 500 spectrometer using a 10-mm dedicated probe. Samples for ^{13}C NMR were withdrawn from another batch containing ^{13}C -labeled phenol in the same way as described before. For these samples, ^1H and one-dimensional (1D) ^1H - ^{13}C heteronuclear single-quantum coherence (HSQC) measurements also were conducted at 400 MHz using a BBFO probe. The cells separated at 12, 14, and 16 h were ruptured under sonication and then centrifuged. The supernatant of this was analyzed by ^1H NMR for the presence of any occluded phenol.

NMR diffusion coefficients were measured using a standard Bruker pulse sequence (ledbpgp2s) with longitudinal eddy current delay [9]. Data were acquired with a 50-ms diffusion delay, 1.8-ms bipolar gradient pulses, 1-ms spoil gradient pulse, and 5.00-ms eddy current delay. The bipolar pulse gradient strength was varied incrementally from 2 to 95 %. The diffusion coefficients were determined by on line processing of the data using the Bruker T1/T2 analytical tool. The data were fitted to Eq. 1:

$$I = I_0 \exp \left[-D (G \delta \gamma)^2 (\Delta - \delta/3) \right] \quad (1)$$

In Eq. 1, I is the observed intensity, I_0 is the signal intensity or integral in the absence of gradient pulse, D is the diffusion coefficient, γ is the gyromagnetic ratio of proton, G is the magnetic gradient pulse amplitude, δ is the length of the gradient pulse, and Δ is the diffusion delay time [10].

Results and Discussion

The 400-MHz ^1H spectrum of the substrate, phenol, (Fig. 1) used for the degradation studies indicated that the proton signals of phenol are practically invisible unless the signals are amplified considerably (~256 times). Since the concentration of phenol is very low, the signal arising from the water protons dominates the ^1H spectrum. The estimated ratio of phenol to water, from the spectrum, was 1:3,380 (~16.4 mM solution). The phenol signals become clearly

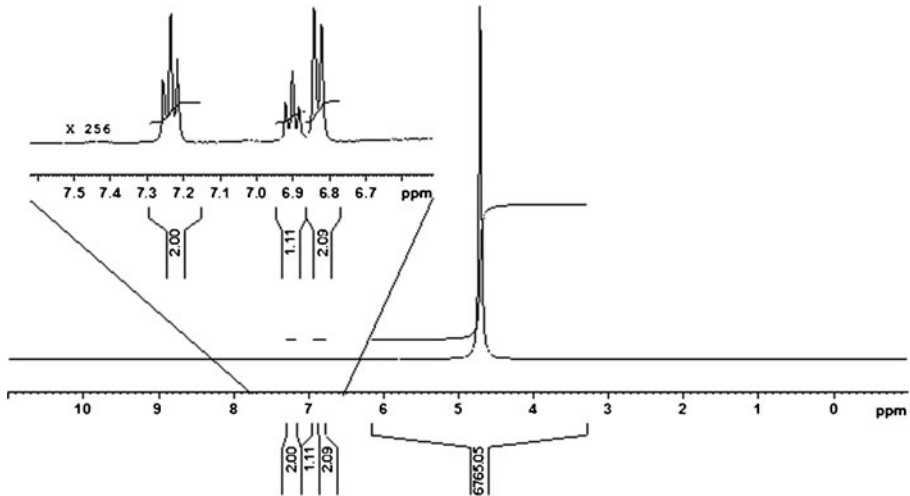


Fig. 1 The 400-MHz ^1H NMR spectrum of the substrate, phenol, in the medium of reaction (H_2O). The spectrum was obtained by a one-pulse experiment

visible when water suppression technique by a WATERGATE or presaturation pulse sequence is used for the data collection. Spectra of samples drawn from three sets of experiments, viz. substrate blank, cell blank, and reaction mixture, are given in Fig. 2. The signal intensities for the substrate blank (Fig. 2a) remained unaltered even after 20 h, indicating that no biodegradation takes place in the absence of the biocatalyst. It is evident from Fig. 2c that degradation of phenol begins only after 8 h, and a sudden decrease in the phenol proton signals after that indicated faster disintegration of the substrate by the cells; at the end of 16 h, the process of biodegradation was nearly complete. Apart from the signals of phenol, some additional signals can also be seen in the aromatic and aliphatic regions. These signals are actually arising from the soluble metabolites from the biocatalyst. This is evident from the comparison of the ^1H spectrum of the cell blank (Fig. 2b) and the one with biocatalyst and phenol at each time interval.

The aromatic and aliphatic regions of the spectra showed no additional signals with noticeable intensities, indicating the absence of any aromatic or aliphatic degradation product/s of the substrate. Since the degradation was under aerobic conditions, formation of oxidation products can only be envisaged. Accordingly, one would expect initial formation of the dihydroxylated product, catechol or hydroquinone (quinone), or other polyhydroxy aromatic compounds [11].

These compounds may further be oxidized to dicarboxylic acids such as maleic or fumaric acid and ultimately to carbon dioxide and water as shown in Scheme 1 [5]. The absence of any detectable amounts of any aromatic hydroxylated product indicates that they are not formed, or if formed, the lifetime is too small to be detected, i.e., they undergo further oxidation instantaneously. Interestingly, our measurements failed to detect any other dicarboxylic acids such as maleic or fumaric acid. The detection of carboxylic acids is hampered by the aromatic cell metabolites present in the solution. To overcome this, we have monitored the degradation using uniformly ^{13}C -labeled phenol. The degradation was followed by ^{13}C and 1D ^1H - ^{13}C HSQC spectroscopies. ^{13}C and 1D HSQC (Supporting information Figs. S1 and S2) allow preferential detection of the signals arising from the labeled material and thus provide a handle to monitor the nature of phenol during degradation. Thus, the formation of intermediate products containing labeled ^{13}C should be picked up specifically in the NMR spectra. These experiments also failed to detect any hydroxylated phenols and carbonyl peak of carboxylic acids. ^{13}C spectra

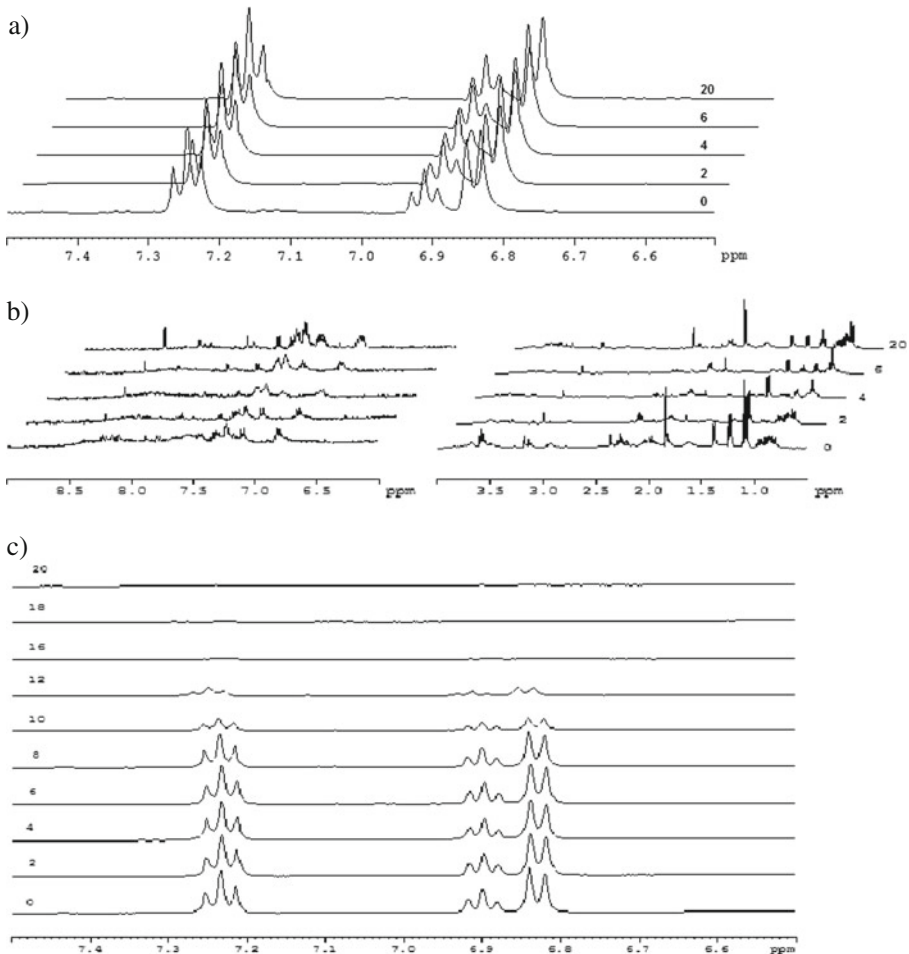


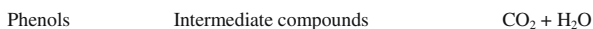
Fig. 2 The 400-MHz ^1H NMR spectra of phenol degradation at different time intervals. **a** Substrate blank. **b** Cell blank. **c** Reaction mixture

and NMR spectra did not show any additional signals in the region expected. These observations can only be rationalized by considering a complete oxidation of phenol to carbon dioxide and water after its binding to the biocatalyst.

Attempts were also made for an in situ study of phenol degradation in the NMR tube itself. Here, the biocatalyst with the phenol was transferred into a 5-mm NMR tube, and ^1H

Degradation pathway

The most likely pathway of phenol degradation can be the following:



Scheme 1 Proposed degradation pathway. The expected intermediate compounds are dihydroxylated product, catechol or hydroquinone (quinone), or other polyhydroxy aromatic compounds and dicarboxylic acids such as maleic, oxalic, or fumaric acid, which are not detected

signals were monitored as a function of time. The “in situ” measurements showed a slower rate of degradation. For example, even after 24 h, only ~50 % degradation was observed (Fig. 3a). This retardation can be attributed to the lack of agitation of the heterogeneous system in the NMR tube as the surface area available for the interaction (contact) is small. The phenol-to-cell concentration ratio used in this study was found to be optimum as higher concentrations of phenol could not be degraded with the same quantity of cells.

Biodegradation of 2,6-Dimethylphenol

An identical procedure was also applied for the study of biodegradation of the substituted phenol 2,6-dimethylphenol (DMP) by the same microorganism. A comparison of the ^1H spectrum of the reaction mixture at 0, 24, and 48 h with substrate blank in Fig. 3b showed a degradation of ~15 and ~40 %, respectively, indicating that the degradation of DMP is much slower than that of phenol. As in the case of phenol degradation, here formation of any intermediate organic compound was not detected also. Hence, the mode of degradation can be assumed to be similar to that of phenol (Scheme 1). The water-to-2,6-dimethoxyphenol ratio in the ^1H spectrum for the sample studied was 1:5,620, corresponding to a concentration of ~10 mM.

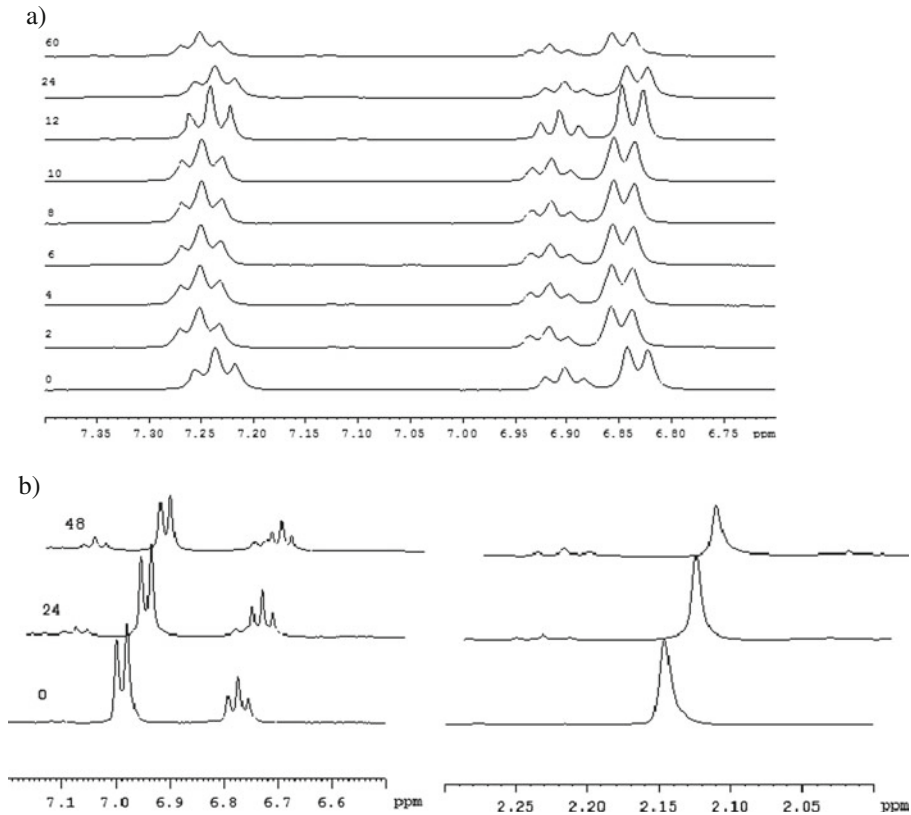


Fig. 3 a In situ monitoring of the degradation of phenol by 400-MHz ^1H NMR. The time intervals are mentioned in the figure. b The 400-MHz ^1H NMR spectra of 2,6-dimethylphenol degradation at different time intervals. Only 40 % is degraded in 48 h

PFGSE NMR Studies

The interaction between the catalyst and substrate during the reaction process was comprehended by measurement of self-diffusion coefficients by PFGSE NMR. The diffusion pattern of phenol in the system at different stages of biodegradation was studied by comparing the self-diffusion coefficient D at 298 K, which is presented in Table 1. The systems compared were phenol in water, i.e., before addition of the cells, phenol–enzyme complex immediately after mixing, phenol in this complex during the biodegradation at a representative stage of 40 % degradation, and phenol present in the supernatant solution. The phenol diffusion coefficient decreases marginally in the presence of cells which is likely to be due to its interaction with the cells. We have not observed any appreciable changes on the self-diffusion coefficient of phenol in the complex at various stages of the degradation as shown in Table 1 for a representative case at ~40 % degradation. The diffusion coefficient of the supernatant obtained during the degradation is nearly the same as that for the dilute phenol solutions given in Table 1. These observations can be rationalized in the following way.

On complexation with the cells, the diffusion of the substrate, phenol, becomes slower, leading to the observed reduction in the self-diffusion coefficient. The extent of the decrease depends on the lifetime of the substrate in the bound form which is usually in equilibrium with the free ligand (substrate). The fact that the self-diffusion coefficient of phenol in the complex does not show appreciable changes during biodegradation (Fig. 4) means that the phenol in the cell complex, which may further interact with the active enzyme, has only a short lifetime in the enzyme-bound form. Such a short lifetime will be too small to impart any appreciable change in its diffusional behavior. The phenol, which is bound to the active enzyme only for a short period, degrades to carbon dioxide and water instantaneously.

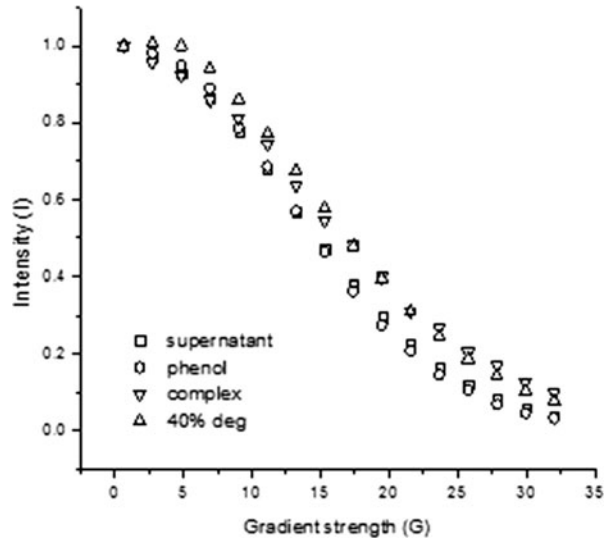
Kinetics of Biodegradation

Quantitative estimation of the unreacted phenol in the reaction mixture was made from the ^1H NMR spectrum of the supernatant obtained by centrifuging the reaction mixture withdrawn at regular intervals. The NMR spectra were collected under identical conditions, and the area of the phenol protons (7.8 to 6.5 ppm) was monitored with respect to the sample at 0 h. The rate of the reaction was determined by plotting the relative concentration of the unreacted phenol as a function of

Table 1 Comparison of diffusion coefficient of phenol at various stages of biodegradation

Serial No.	Status of phenol	Diffusion coefficient ($10^{-9}\text{m}^2\text{s}^{-1}$)
1	Phenol in water ~10 mM	1.07
2	Phenol in water ~20 mM	1.07
3	Phenol–enzyme complex	0.75
4	Phenol–enzyme complex at 40.0 % degradation	0.78
5	Phenol–enzyme complex supernatant obtained at 40.0 % degradation	1.07

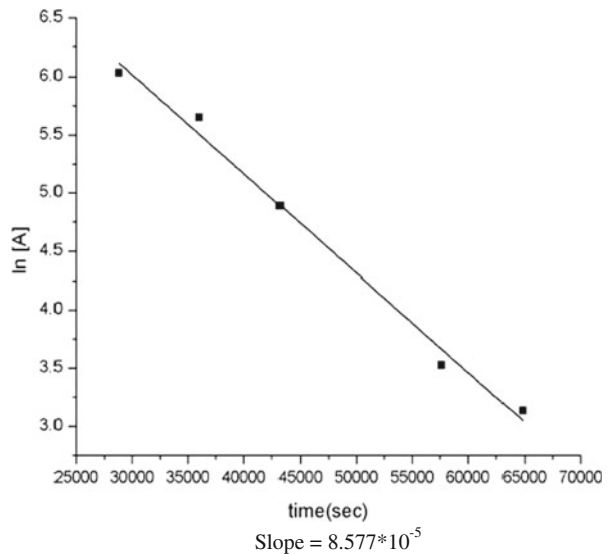
Fig. 4 The plot of signal intensity vs. gradient strength in the BPLED experiment for determination of self-diffusion coefficient by PFGNMR of phenol in the system at different stages of biodegradation. The signal intensity plotted is for the *ortho* protons of phenol



degradation time. The analysis showed that the biodegradation follows a first-order pathway with a rate constant of 0.868 s^{-1} (Fig. 5).

To rule out the possibility of occluded phenol inside the cells, which were removed by centrifuging the same and taking NMR of the supernatant solution, cells were sonicated, and after rupturing of the cells, the supernatant was tested for residual phenol, if any (Supporting information Fig. S3).

Fig. 5 Kinetics of the reaction followed by NMR. Natural logarithm of ^1H signal intensity of phenol present in supernatant ($\ln[A]$) is plotted against degradation time. Only the intensity values obtained from 8 to 18 h are considered for the plot



Conclusion

Biodegradation of phenol by *C. tropicalis* NCIM 3556 has been monitored by ^1H NMR spectroscopy. The study suggests that there is an incubation period in the reaction of about 8 h, after which phenol consumption by the cells begins and the degradation is nearly complete in 20 h. The study indicated that phenol is degraded to carbon dioxide and water by *C. tropicalis* NCIM 3556. The self-diffusion coefficient of phenol was found to remain practically the same over the entire period of measurement, indicating that the lifetime of phenol in the bound form is too small to impart any change in its diffusion behavior. The intermediates formed during the reaction are converted to carbon dioxide and water at a very fast rate. The reaction was of first order with a rate constant of 0.868 s^{-1} . *C. tropicalis* NCIM 3556 also degrades 2,6-dimethylphenol in a similar fashion but at a slower rate.

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