Analysis of the Pathway of Phenol Biodegradation by *Alcaligenes* sp. *d2*

25

Merlin Antony, Indu C. Nair, and K. Jayachandran

Abstract

Alcaligenes sp. *d2*, an aerobic bacterium, was proved earlier to be a promising strain for the biodegradation of phenol. The rate-limiting step in the aerobic degradation of phenol by *Alcaligenes* sp. *d2* is the formation of catechol from phenol by the action of the enzyme phenol hydroxylase. The presence of phenol hydroxylase in the culture supernatant strongly supports the fact that *Alcaligenes* sp. *d2* starts the degradation of phenol by converting it to catechol. The optimum condition for the production of phenol hydroxylase by the organism was done by statistical modeling with Design-Expert software 7.1.4 – Plackett-Burman. The optimum conditions for the production of the enzyme were found to be temperature, 40°C; pH, 9.8; incubation period, 16 h; phenol, 200 µl (5%); catechol, 1 mg ml^{-1}; $MgSO_4$, 5 mg ml^{-1}; and KH_2PO_4, 100 mg ml^{-1}. The organism degrades phenol completely to Krebs cycle intermediates through the meta pathway of degradation. This fact is supported by the data obtained from the FT-IR analysis of the samples taken at different time intervals. FT-IR bands obtained from samples taken at 16 h (1,725 cm^{-1}), 24 h (2,960 cm^{-1}, 1,705 cm^{-1}), 32 h (1,703 cm^{-1}), and 40 h (1,729 cm^{-1}) add on to the fact that *Alcaligenes* sp. *d2* follows the meta pathway o f biodegradation.

Keywords

Phenol biodegradation • *Alcaligenes* sp. *d2* • Phenol hydroxylase • Meta pathway

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Introduction

Phenol and its derivatives are the basic structural unit in a wide variety of synthetic organic compounds [1]. Phenol is more commonly produced artificially from industrial activities. Currently, the largest use of phenol is as an intermediate in the production of phenolic resins, which are used in the plywood, adhesive, construction, automotive, and appliance industries. Phenol is also used in the production of synthetic fibers such as nylon and for epoxy resin precursors such as bisphenol A. Phenol is water soluble and highly mobile [2], and, hence, waste waters generated from industrial activities contain high concentrations of phenolic compounds [3]. This may eventually reach down to streams, rivers, lakes, and soil representing a serious ecological problem [4]. Phenol is a listed priority pollutant by the US Environmental Protection Agency [5] and is considered to be a toxic compound by the Agency for Toxic Substances and Disease Registry [6].

Pollution due to phenolic compounds has been drawing attention of the research community for the past few years. Many technologies have been investigated for removing phenolic compounds from environment. They include adsorption [7], biodegradation [8, 9], UV/Fe^{+3} [10], extraction by liquid membrane [11], chemical oxidation [12–15], etc. Powdered animal charcoal treatment is one such strategy to eliminate phenol from industrial effluents. But these methods are incompetent as far as the complete removal of the pollutant from environment is concerned. They also have serious drawbacks such as high cost and formation of hazardous by-products.

Because of the widespread occurrence of phenol in the environment, many microorganisms have started utilizing phenol as the sole carbon and energy source. Biodegradation of phenol follows both aerobic and anaerobic pathways. Aerobic biodegradation of phenol has been studied in the early nineteenth century. The common first step in the aerobic degradation of phenols is its hydroxylation to catechol. Phenol hydroxylase (PH) enzyme catalyze the hydroxylation of phenol to catechol. This reaction is considered to be the first and rate-limiting step in the aerobic phenol-degrading pathway [16].

In aerobic degradation of phenol, the enzyme phenol hydroxylase adds a hydroxyl group to phenol utilizing molecular oxygen and a molecule of $NADH_2$ to form catechol (1,2-dihydroxybenzene). Catechol is then degraded via two alternative pathways: ortho pathway or β-ketoadipate pathway and meta pathway. It is further metabolized to Krebs cycle intermediates. In meta pathway, enzyme catechol 2,3-dioxygenase transforms catechol to 2-hydroxymuconic semialdehyde. This compound is metabolized further to intermediates of Krebs cycle.

We have been doing research in phenol biodegradation for the last few years. The organism we used was *Alcaligenes* sp. d_2, isolated from soil through soil enrichment culture technique.

Alcaligenes sp. d_2 is an efficient phenol-degrading microorganism and is capable of withstanding high concentrations of phenol. Its phenol-degrading property and the conditions required for degradation were reported in soil science [17]. We could use this organism for the better treatment of phenolic paper factory effluent [9]. Later we could also establish the fact that the organism while removing phenol was capable of progressively accumulating PHB, the mostly recommended biopolymer [18]. The present work mainly focuses on establishment of the role of phenol hydroxylase and elucidation of the pathway of phenol biodegradation by *Alcaligenes* sp. d_2.

Experimental

Strain and Source

The bacterium *Alcaligenes* sp. d_2 was used in the present study. The strain was collected from the culture collection of School of Biosciences, Mahatma Gandhi University Campus, Kottayam.

Estimation of Phenol

Phenol was estimated as per the protocol by [19].

Biodegradation

Hundred milliliter of mineral salt phenol medium with pH 6.5 and 200 µl of 5% phenol substrate were prepared in different flasks and inoculated with 3% inocula of the strain. The culture was then incubated for different time intervals in a 150 rpm shaker at room temperature. After every 8-h time interval, the media was centrifuged at 10,000 rpm for 15 min, supernatants was collected and assayed for the presence of phenol.

Enzymatic Assay of Phenol Hydroxylase

Phenol hydroxylase was estimated as per the protocol by Gurujeyalakshmi et al. [20]. One unit of phenol hydroxylase activity was defined as 1 µmol of phenol conversion per minute under the assay conditions.

Optimization of the Conditions for Phenol Hydroxylase Production

Hundred milliliter of mineral salt phenol medium at pH 6.5 and 200 µl of 5% phenol as substrate was inoculated with 3% inoculum. The broth was incubated for 24 h at room temperature in a shaker at 150 rpm for enzyme production. After incubation, the broth was centrifuged at 10,000 rpm for 15 min. Supernatant was collected and assayed for the presence of phenol hydroxylase enzyme. The conditions favoring the maximum secretion of the enzyme was found to be the optimum condition. Optimization of the process conditions for the production of phenol hydroxylase was done with Design-Expert software 7.1.4 – Plackett-Burman, downloaded from the website www.statease.com. Eleven parameters with 2 options were given to the software which gave 12 trials to be performed.

Analysis of Degradation Products by GC-MS and FT-IR

The supernatants collected after biodegradation at 8-h time interval were extracted with diethyl ether in a separating funnel. Separating funnel was filled with equal volumes of supernatant and diethyl ether. The mixture was shaken well and kept undisturbed for half an hour. The ether portion was collected in a beaker and kept in water bath at 50 °C for removing ether by evaporation. A control (mineral salt phenol medium without inoculum) was also extracted with diethyl ether. The extracts were collected and subjected to GC-MS and FT-IR analysis. Both control and samples were extracted at different time intervals of 8 h and were subjected to GC-MS and FT-IR analysis. Spectroscopic analysis was done at National Institute of Interdisciplinary Science and Technology (NIIST), Trivandrum.

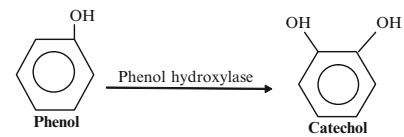
Results and Discussion

The present research work was carried out to evaluate the pathway of phenol degradation followed by *Alcaligenes* sp. d_2. The medium used was mineral salt phenol medium (MSPM), and the process was carried out under optimized laboratory conditions [17].

Biodegradation of phenol follows both aerobic and anaerobic pathways. *Alcaligenes* sp. d_2 is an aerobic organism. The aerobic biodegradation of phenol occur either through ortho pathway or by meta pathway. In both cases, the first step is the conversion of phenol to catechol by the enzyme phenol hydroxylase.

Phenol hydroxylase is a flavoprotein enzyme, and its action (conversion of phenol to catechol) is considered to be the rate-limiting step in the whole process of phenol biodegradation by aerobic pathway. The most important function of phenol hydroxylase is the hydroxylation of phenol, i.e., addition of one more –OH group to phenol.

This step is very important as it gives room for the subsequent enzymes to act either in ortho



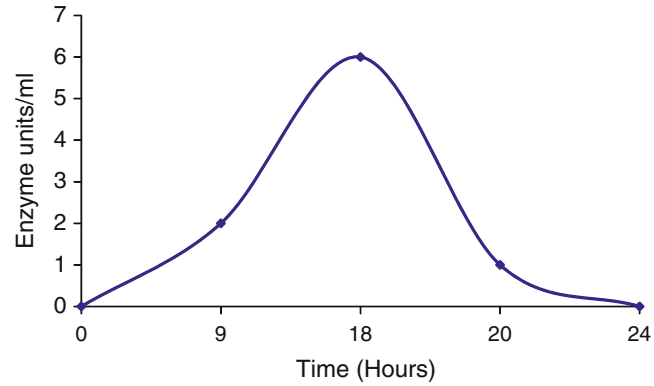


Fig. 25.1 Production of phenol hydroxylase enzyme at different incubation period

pathway or in meta pathway to cleave the benzene ring. Our first objective was to establish the presence of phenol hydroxylase in the culture supernatant. Phenol hydroxylase activity was evaluated in the culture supernatants at various time intervals and was plotted with activity against time (Fig. 25.1).

The strain produced phenol hydroxylase enzyme during the initial stages of phenol degradation, and its rate was found to decrease gradually. Production of enzyme was found at the maximum during 18 h of incubation. The presence of phenol hydroxylase in the culture supernatant indicated that the biodegradation of phenol by *Alcaligenes* sp. d_2 is aerobic in nature.

Phenol hydroxylase is an inducible enzyme and hence may be influenced by the presence of phenol in the medium. In addition to this, several other factors such as temperature, pH, incubation time, salt concentration, buffers, and nitrogen sources can also influence the production of phenol hydroxylase. A high level of phenol hydroxylase production can enhance the rate of phenol degradation, and, hence, it is appropriate to look into the optimum production of phenol hydroxylase enzyme.

Optimization of the production conditions of enzyme can be done by the conventional one at a time approach. This is a time-consuming experiment and requires intensive lab work. More than that, the final optimized conditions may not be contributing to the expected results. This is because in many of the conventional optimization strategies, the individual trials are found to have better yield than the finally optimized conditions. It follows that it is not the individual parameter which is very important, what matters is how the variables interact with each other in combination. A suitable combination of the various factors may be contributing better yield than other conditions.

It is with this objective that scientists all over the world are shifting their experimental strategies of media optimization to statistical modeling. A number of software are currently available for the media optimization. In the present project, we have made an attempt to optimize the conditions of phenol hydroxylase production using one such software. The software used was Design-Expert software 7.1.4 – Plackett-Burman, downloaded from the website www.statease.com.

We have analyzed the optimum production strategies using the first-level application of Design-Expert system. The first-level analysis helped us to find out the most important impact making parameters from the selected variables. The Plackett-Burman statistical model of the Design-Expert software proposes the conduct of "n" trials for $n-1$ variables. The first task before initiating the analysis is to fix up the number and nature of the variables. In this experiment, we have opted 11 variables comprising of temperature, pH, incubation period, phenol concentration, NaCl, KH_2PO_4, $MgSO_4$, $(NH_4)_2SO_4$, $CaCl_2$, and catechol. Temperature, pH, and incubation period have been incorporated as a measure to optimize the external conditions affecting the enzyme production. Phenol has been incorporated to

Table 25.1 Selected parameters and their value applied in the Plackett-Burman model for process optimization

		Values	
Sl. no.	Parameter	Highest	Lowest
1	Temperature	40 °C	30 °C
2	pH	9.8	6.8
3	Incubation time	48 h	16 h
4	5% Phenol	200 µl	0
5	NaCl	5 mg/100 ml	1 mg/100 ml
6	KH_2PO_4	100 mg/100 ml	10 mg/100 ml
7	$MgSO_4$	50 mg/100 ml	5 mg/100 ml
8	$(NH_4)_2SO_4$	100 mg/100 ml	0
9	$CaCl_2$	1 mg/100 ml	0
10	Catechol	1 mg/100 ml	0
11	Distilled water (dummy variable)	B1	B2

evaluate how far it can act as an inducer for the production of phenol hydroxylase enzyme.

In each of these parameters, two values were taken, one lower and the other higher (Table 25.1). Totally 12 trials have been conducted as per the data given by the software (Table 25.2). Finally on analyzing the impact factor contributed by the software analysis, it became evident that the most striking impact making parameters were phenol (1.14) and $(NH_4)_2SO_4$ (1.03) along with KH_2PO_4 (1.31) (Table 25.3).

The results truly agreed with the theoretical expectation that phenol hydroxylase was an inducible enzyme and get induced at a high concentration of phenol (200 µl of 5% phenol). Among the 12 trials conducted, the second trial gave the highest response of 17.70, and the second highest response obtained was 3.18, which is only 18% to the highest response (Table 25.2). Hence, it follows that the parameters fixed for the second trial yielding the highest performance was ideally suited for maximal production of the enzyme. The parameters selected were temperature, 40°C; pH, 9.8; incubation time, 16 h; phenol, 200 µl (5% phenol); NaCl, 5 mg ml^{-1}; KH_2PO_4, 100 mg ml^{-1}; $MgSO_4$, 5 mg ml^{-1}; and catechol, 1 mg ml^{-1}. $(NH_4)_2SO_4$ and $CaCl_2$ were completely absent in the medium.

The presence of phenol hydroxylase enzyme in the initial stages of phenol biodegradation established the fact that phenol biodegradation by *Alcaligenes* sp. d_2 was aerobic and that it followed either ortho pathway or meta pathway. Hence, the second objective was to find whether the organism follows ortho pathway or meta pathway. Ortho pathway has characteristic intermediates like catechol, cis,cis-muconic acid, mycolactone, β-oxoadipate, acetyl CoA, and succinyl CoA, whereas in meta pathway the intermediates are catechol, 2-hydroxymuconic semialdehyde, 2-oxopent-4-enoate, oxaloacetate, acetaldehyde, and pyruvate.

The identification of the nature of degradative pathway followed by *Alcaligenes* sp. d_2 was done by establishing the presence of any one class of the intermediates during the degradation of phenol. The experimental strategy we took for the above objective was GC-MS and FT-IR analysis.

GC analysis was unique in representing the number of structurally different components in the sample. MS was indicative of the mass fractionation of the component peaks obtained from the GC spectrum. GC analysis of the control (non-biodegraded phenol) gave a major peak at 7.008 min, and the MS analysis confirmed that the peak represented phenol with molecular weight 94 (Figs. 25.2 and 25.3).

GC analysis of the samples at varied incubation period gave ample evidence showing the degradation of phenol and generation of new intermediates. The peak of phenol indicated at 7.008 RT in the control gets progressively reduced resulting in the formation of many other peaks in the subsequent samples (Fig. 25.4).

Table 25.2 Yield obtained for phenol hydroxylase as per the trials conducted by the software analysis

Sl. no.	Temperature	pH	Incubation time	Phenol 5% µl	NaCl	KH_2PO_4	$MgSO_4$	$(NH_4)_2SO_4$	$CaCl_2$	Catechol	Distilled water	Response
1	30	6.8	16	200	1	100	50	0	1	1	B2	3.18
2	40	9.8	16	200	5	100	5	0	0	1	B1	17.70
3	30	6.8	16	0	1	10	5	0	0	0	B1	.84
4	40	9.8	16	0	1	100	5	100	1	0	B2	.35
5	30	9.8	48	0	5	100	50	0	0	0	B2	1.20
6	30	9.8	48	200	1	10	5	100	0	1	B2	0
7	40	9.8	48	0	1	10	50	0	1	1	B1	1.13
8	40	6.8	16	0	5	10	50	100	0	1	B2	.212
9	40	6.8	48	200	1	100	50	100	0	0	B1	2.3
10	30	6.8	48	0	5	100	5	100	1	1	B1	1.4
11	30	9.8	16	200	5	10	50	100	1	0	B1	1.4
12	40	6.8	48	200	5	10	5	0	1	0	B2	.84

Table 25.3 Analysis of yields and evaluation of the impact of the variables

Sl. no.	Variables	Temperature	pH	Incubation time	Phenol 5%	NaCl	KH_2PO_4	$MgSO_4$	$(NH_4)_2SO_4$	$CaCl_2$	Catechol	Distilled water
1	ΣH	22.53	21.78	6.87	25.42	22.75	26.13	9.42	5.66	8.3	23.62	24.77
2	ΣL	8.02	8.77	23.68	5.13	7.8	4.42	21.13	24.89	22.25	6.93	5.78
3	Difference ($\Sigma H - \Sigma L$)	14.51	13.01	−16.81	20.29	14.95	21.71	−11.71	−19.23	−13.95	16.69	18.99
4	Effect $\frac{(\Sigma H - \Sigma L)}{6}$	2.42	2.17	−2.80	3.38	2.5	3.62	−1.95	−3.20	−2.32	2.78	3.16
5	Mean square $\frac{(\Sigma H - \Sigma L)^2}{6}$	.488	.392	.653	.952	.520	1.092	.316	.853	.448	.644	–
6	Error Square	–	–	–	–	–	–	–	–	–	–	.832
7	$\frac{\text{Mean Square}}{\text{Error Square}}$ (Impact Factor)	.59	.47	.78	**1.14**	.63	1.31	.38	1.03	.54	.77	–

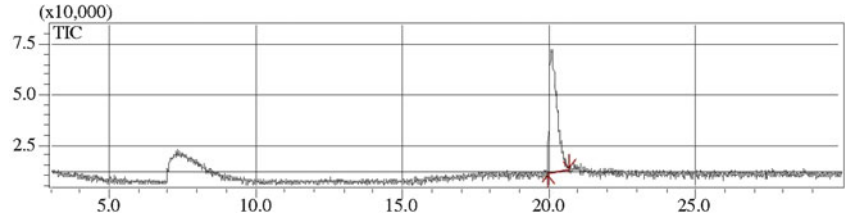


Fig. 25.2 Gas chromatogram of control: S1 (non-biodegraded phenol)

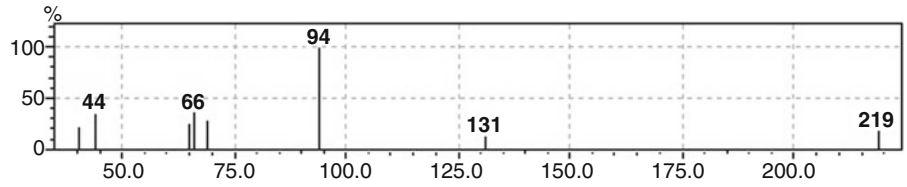


Fig. 25.3 Mass spectrogram of the peaks obtained at 7.008 in the gas chromatogram of the control (non-biodegraded phenol). RT = 7.008

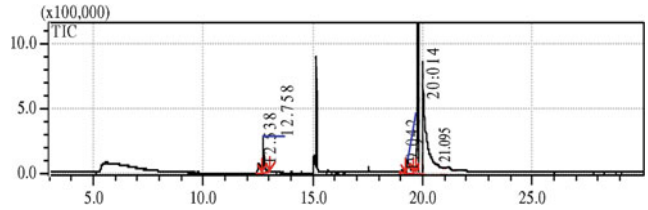


Fig. 25.4 Gas chromatogram of biodegraded phenol after 40 h of incubation: S6

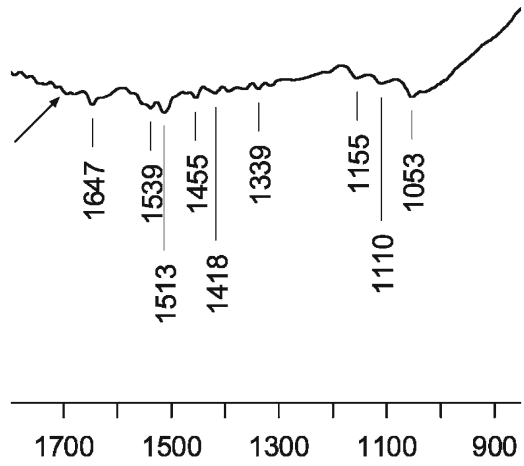


Fig. 25.5 FT-IR of control: S1 (non-biodegraded phenol)

We could analyze the sample extracts at different time intervals using FT-IR. The FT-IR of the control (Fig. 25.5) gave a definite indication of the presence of phenol in the non-biodegraded sample. The characteristic bands were observed at a wave number of 3,731, 1,647, 1,455, and at 1,155 cm^{-1}. There were no bands in the range of 1,700–1,750 cm^{-1}.

The FT-IR analysis of the samples taken at 8 h (Fig. 25.6), 16 h (Fig. 25.7), 24 h (Fig. 25.8),

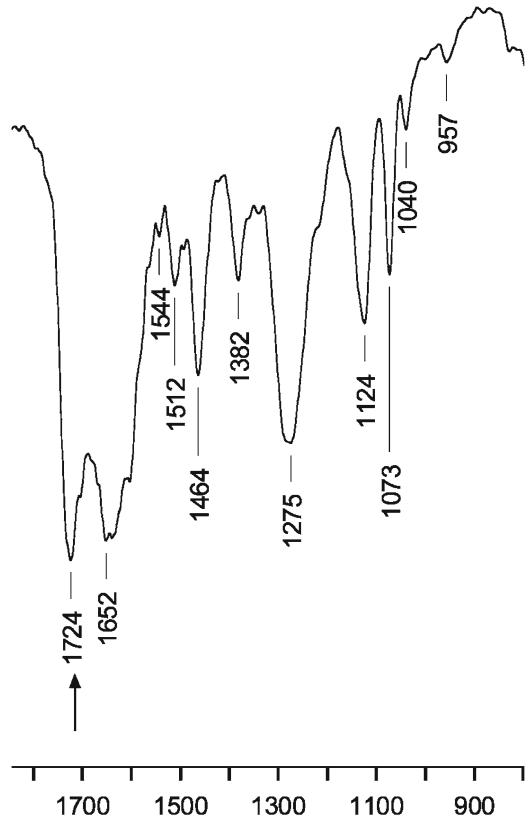


Fig. 25.6 FT-IR of biodegraded phenol after 8 h of incubation

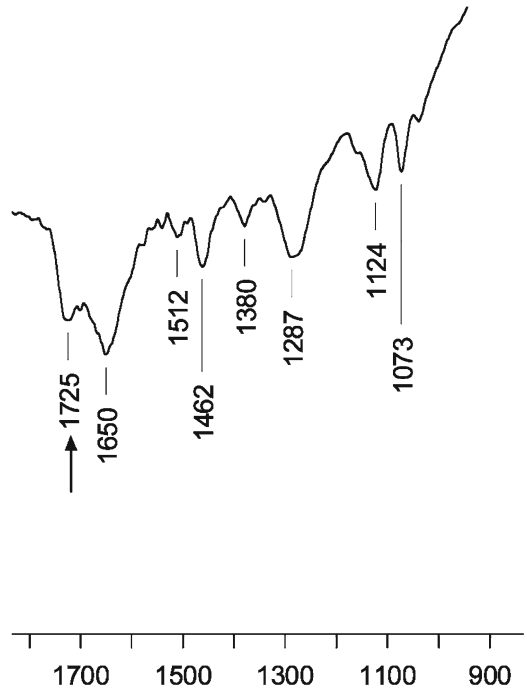


Fig. 25.7 FT-IR of biodegraded phenol after 16 h of incubation

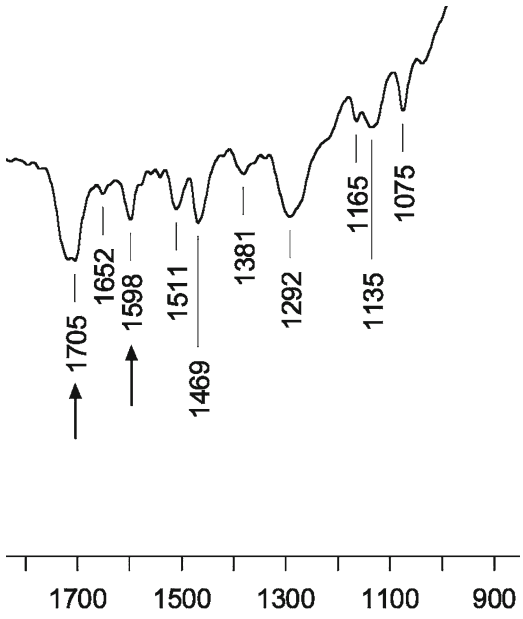


Fig. 25.8 FT-IR of biodegraded phenol after 24 h of incubation

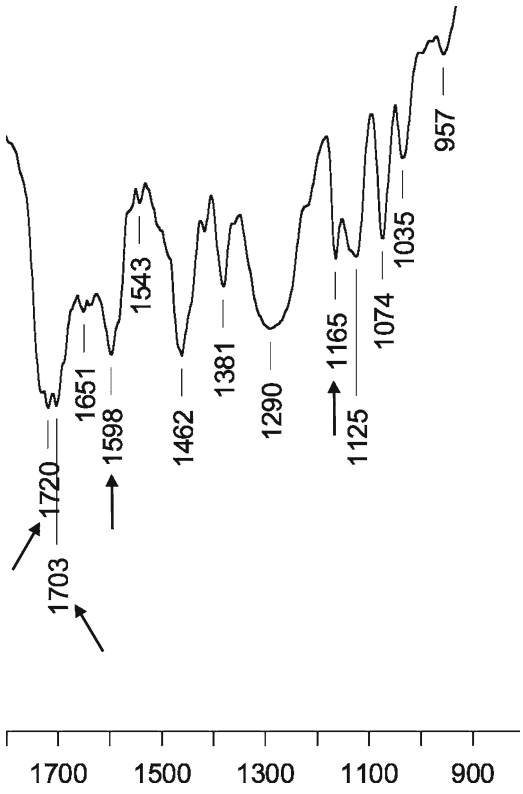


Fig. 25.9 FT-IR of biodegraded phenol after 32 h of incubation

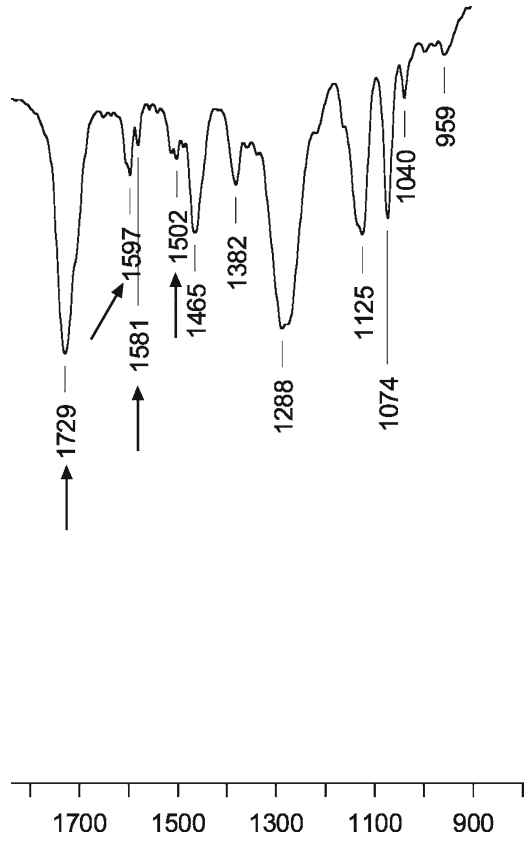
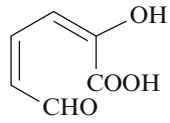


Fig. 25.10 FT-IR of biodegraded phenol after 40 h of incubation

32 h (Fig. 25.9), and 40 h (Fig. 25.10) gave strong characteristic indication of aerobic breakdown of the aromatic ring with new bands appearing in the range 1,750–1,000 cm^{-1}. Many of the bands appeared in this range were characteristic of meta pathway.

The FT-IR analysis of the sample after 8 h (Fig. 25.6) brought new bands at 2,929 and 2,862 cm^{-1}, which were characteristic of acid: hydrogen bond (O-H) stretching. Together with this, there was a characteristic band at 1,724 cm^{-1} which gives the strong indication of the presence of C═O group.

FT-IR analysis of the sample after 16 h (Fig. 25.7) gave strong representation of band at 1,725 cm^{-1}, characteristic of the aldehyde, 2-hydroxymuconic semialdehyde.



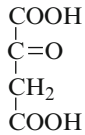
2-hydroxymuconic semialdehyde

The presence of aldehyde can be accepted as a strong indication toward meta pathway, as the ortho pathway does not contain any aldehyde intermediates in the beginning.

In the FT-IR spectrum of sample at 24 h (Fig. 25.8), there was a band at 2,960 cm^{-1}, indicating the presence of aliphatic group, which again confirmed the cleavage of aromatic ring inducing aliphatic ring generation. The shifting of the band toward 1,705 cm^{-1} also indicated the oxidation of aldehyde to ketone, which took place in meta pathway during the conversion of 2-hydroxymuconic semialdehyde to 2-oxopent-4-enoate.

The presence of 1,703 cm^{-1} band in the FT-IR of the sample after 32 h (Fig. 25.9) clearly indicated the presence of two carboxylic acids, which took part in the formation of oxaloacetate.

Oxaloacetate



Oxaloacetate

Finally, in the FT-IR spectrum of sample taken after 40 h (Fig. 25.10), there was strong indication of CH_3—C—CH_3 and aldehyde. CH_3 was represented by the reappearance of the band at 2,960 cm^{-1}. There was a strong band at 1,729 cm^{-1} indicating the presence of aldehyde. Finally, the spectrum represented the presence of alkyl aldehyde in the sample. This was mostly indicative of acetaldehyde (CH_3—CHO) formed in the last stage of meta pathway. From the above data, we could state that *Alcaligenes* sp. d_2 followed meta pathway for the biodegradation of phenol.

The present study has proved that *Alcaligenes* sp. d_2 is a promising strain for the biodegradation of phenol. It could degrade phenol even at high concentrations. The industrial effluents before being released into the environment can be treated with a suspension of this strain to remove phenol, which reduces its hazardous effect on environment. The strain can also be recombinantly modified to increase its potency in degradation. The intermediary metabolites PHB and catechol, produced by *Alcaligenes* sp. d_2, are important intermediates in the synthesis of biopolymers, pharmaceuticals, agrochemicals, flavors, antioxidants, etc. If utilized effectively, the strain will be a boon to mankind in the fields of food industry, pollution control, pharmaceuticals, biopolymer production, etc.

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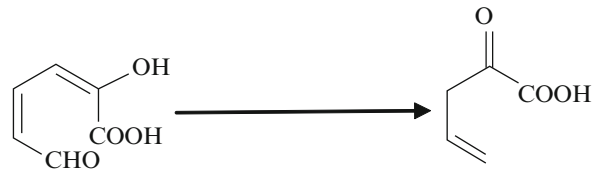

2-hydroxymuconic semialdehyde **2-oxopent-4-enoate**

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