OXIDATION OF PHENOLS BY YEAST

II. OXIDATION OF CRESOLS BY *CANDIDA TROPICALIS*

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Candida tropicalis utilized phenol, catechol, and 3- and 4-methylcatechols for growth as sole carbon source, but did not utilize *p*-, *m*-, or *o*-cresols, or *p*-, *m*-, and *o*-hydroxybenzoates. The yeast cells, however, rapidly oxidized cresols without a lag period after incubation with phenol.

In a medium containing phenol, two major metabolites were accumulated, *i.e.*, one, catechol and the other, the ring-cleaved metabolite of catechol, identified as $C_6H_6O_4$. Therefore, it appears that the first step of the phenol oxidation by the yeast is monohydroxylation of phenol.

The metabolite from *p*- or *m*-cresol by phenol-adapted cells was isolated and identified as 5-formyl-2-hydroxy-4-methyl-2,4-pentadienoic acid. The same compound was obtained from 4-methylcatechol used as substrate. It was suggested that *p*- and *m*-cresols were hydroxylated to form 4-methylcatechol, followed by the cleavage with extra-diol type ring fission. The oxidation of *o*-cresol at slower rate was also demonstrated. The metabolite from *o*-cresol gave different UV absorption spectrum from that formed from 3-methylcatechol.

DAGLEY *et al.* (1) and BAYLEY *et al.* (2) showed that when, cresols were metabolized by certain species of *Pseudomonas*, the benzene ring was cleaved by extra-diol type fission after cresols were oxidized to methylcatechols. Furthermore, FEIST and HEGEMAN (3) showed that the bacteria grown on phenol contained an enzyme which catalyzed the benzene ring cleavage by an extra-diol type fission, and that the bacteria grown on benzoate contained an enzyme which catalyzed the cleavage by an intra-diol type fission.

CAIN *et al.* (4) studied the oxidation of phenols that were related to lignin by various strains of fungi and yeasts using whole cells and cell-free extracts. They showed that a strain of fungi possessing the ability to grow on catechol forms *cis*, *cis*-muconic acid from catechol and this result suggests that the strain has catechol 1,2-oxygenase. NEUJAHN and VARGA (5, 6) demonstrated by use of *Trichosporon cutaneum* that the cells grown on phenol

hydroxylated various phenols, which were further cleaved to *cis, cis*-muconic acid by catechol 1,2-oxygenase.

The present author (7) has shown that the cells of a yeast strain isolated from the soil can oxidize *p*-cresol after the cells had been incubated with phenol, although the yeast still could not grow on *p*-cresol as the sole carbon source. Furthermore, the established chemical structure of the metabolite of *p*-cresol oxidation suggested that *p*-cresol was hydroxylated to 4-methylcatechol and the benzene ring of 4-methylcatechol was oxidatively cleaved by extra-diol type fission.

The present report describes the result of studies on the metabolism of cresols by the phenol-adapted cells of *C. tropicalis* which can grow on phenol as sole carbon source but not on cresols. The enzymic reaction sequence in phenol and cresol oxidation is discussed.

MATERIALS AND METHODS

Culture of C. tropicalis. Yeast strain used in the present study was *Candida tropicalis* IFO 1070 (ATCC 7349). All of the media were prepared from the following mineral base: 0.25% $(\text{NH}_4)_2\text{HPO}_4$, 0.1% KH_2PO_4 , 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.5% NaCl . The final pH of the mineral base medium was 7.2. Complex medium was prepared from these mineral bases with the addition of 1.0% Bactopeptone (Difco) and 0.5% yeast extract (Difco). Phenol medium was prepared from this mineral base with the addition of 0.1% phenol as sole carbon source. Cultures of the organism were shaken at 30° in an incubator.

The ability to use as sole carbon source was examined with the following chemicals: Phenol, catechol, *p*-cresol, *m*-cresol, *o*-cresol, *p*-hydroxybenzoate, *m*-hydroxybenzoate, salicylate, protocatechuate, 4-methylcatechol, 3-methylcatechol, and glucose. The growth culture was carried out with 200 ml of the medium containing 0.5 mM or 5.0 mM of the carbon source in a 500-ml shake flask at 30° in a shaking incubator, after the cells of *C. tropicalis* were incubated. The cell growth was measured by the absorbance at 660 nm with a Hitachi Photoelectric Spectrophotometer Model EPU-2A. The values of absorbance at 660 nm were converted to the number of cells per ml by a standard curve previously drawn between absorbance value and cell number of *C. tropicalis*.

The yeast cells incubated with phenol were prepared by the following procedure: The cells were incubated in a 3,000-ml shake flask containing 1,000 ml of the complex culture medium in a shaking incubator for 48 hr. The cells grown in the medium were harvested by centrifugation, and re-incubated for 72 hr in a 3,000-ml shake flask containing 1,000 ml of the phenol medium.

To examine the ability of the cells incubated with phenol to grow on *p*-cresol as the sole carbon source, a small amount of the cells incubated with

phenol was washed with distilled water and then incubated in a medium containing *p*-cresol.

Preparation of the oxidation reaction mixture. Cell suspension was prepared as follows: Yeast cells incubated with phenol were washed three times with 1/15 M phosphate buffer (pH 7.2) and suspended in the same buffer to make cell concentration of *ca.* 30 mg dry weight/ml. The reaction was started by mixing an equal volume of the cell suspension and the substrate solution (10 mM in 1/15 M phosphate buffer, pH 7.2).

Manometric experiments. Oxygen uptake and carbon dioxide evolution were measured by the direct method in a Warburg manometer (8). Each Warburg flask contained 1.0 ml of the substrate solution in the main chamber, 1.0 ml of cell suspension in the side arm, and 0.2 ml of 20% KOH solution or 0.2 ml of distilled water in the center well.

To follow the disappearance of substrate in a reaction mixture, UV absorption spectrum of the supernatant was measured at the time of the measurement of oxygen uptake. The supernatants were obtained by centrifugation of the reaction mixture at three different periods; just before manometric experiment was started, when oxygen was actively consumed, and when oxygen uptake stopped.

Isolation of metabolite. The reaction mixture, containing 1,000 ml of 5 mM substrate in a 3,000-ml shake flask, was incubated at 30° for 60 or 300 min in a reciprocal shaking incubator. The metabolite was extracted with ether from the reaction mixture adjusted to pH 2 with 2 N HCl. The metabolite in the ether extracts was isolated by the method described in a previous paper (7).

Thin-layer chromatography. Thin-layer chromatography was carried out on the plate of silica gel (Silica gel G, Merck) or cellulose (Cellulose microcrystalline, Merck), and the metabolite was developed by the following solvent systems: (Solvent a), ethanol : conc. NH₄OH : water (8 : 1 : 1, v/v) (9); (solvent b), butanol : acetic acid : water (25 : 5 : 1, v/v) (9); (solvent c), benzene : tetrahydrofuran : acetic acid (15 : 9 : 1, v/v) (2); (solvent d), benzene : dioxane : acetic acid (90 : 25 : 4, v/v). Aliphatic and aromatic compounds in the metabolite were detected by the spot test with the aqueous solution of 3% FeCl₃, or diazotized *p*-nitroaniline.

Spectrophotometric determinations. Ultraviolet (UV) absorption spectra were obtained with a Beckman Model DK-2 Ratio Recording Spectrophotometer and infrared (IR) absorption spectra with a Parkin-Elmer Model 21 Infrared Spectrophotometer.

RESULTS

Growth of C. tropicalis on phenols and catechols

Candida tropicalis, known to grow on phenol as the sole carbon source (4, 10), was examined for the ability to grow on 12 different substrates in-

Table 1. Growth of *C. tropicalis* on various phenols and catechols.

Each shake flask contained 500 ml of the medium. Flasks were incubated in a shaking incubator at 30°. Cells were incubated for 6 days. Growth of the cells was measured by absorbance at 660 nm.

Carbon source	Growth of yeast cell			
	Concn. mM	Days required for max growth	Absorbance at 660 nm	No. of cells × 10 ⁷ /ml
Phenol	0.5	2	0.043	0.15
	5.0	2	0.282	0.94
Catechol	0.5	6	0.069	0.23
	5.0	6	0.550	1.84
<i>p</i> -Cresol	0.5	6	0.005	(—)
	5.0	6	0.013	(—)
<i>m</i> -Cresol	0.5	6	0.006	(—)
	5.0	6	0.002	(—)
<i>o</i> -Cresol	0.5	6	0.005	(—)
	5.0	6	0.000	(—)
<i>p</i> -Hydroxybenzoate	0.5	6	0.003	(—)
	5.0	6	0.003	(—)
<i>m</i> -Hydroxybenzoate	0.5	6	0.009	(—)
	5.0	6	0.006	(—)
Salicylate	0.5	6	0.007	(—)
	5.0	6	0.007	(—)
Protocatechuate	5.0	6	0.031	0.11
4-Methylcatechol	5.0	6	0.265	0.88
3-Methylcatechol	5.0	6	0.320	1.07
Glucose	0.5	4	0.043	0.15
	5.0	4	0.230	0.77
Complex medium		2	1.200	6.92

cluding phenol, three isomers of cresol, three isomers of hydroxybenzoate, catechol, two isomers of methylcatechol, protocatechuate, and glucose, as the sole carbon source. Table 1 shows the maximum absorbance value by the cell growth during the period of 6 days' incubation.

Growth of *C. tropicalis* in the phenol medium was rapid and reached the maximum value after only 2 days' incubation. Similarly, catechol, and 3- and 4-methylcatechol were utilized easily for the growth. In contrast, none of the three isomers of cresol and the three isomers of hydroxybenzoate could be utilized during the period of 6 days' incubation. Moreover, utilization of protocatechuate for growth was negligible.

Table 2. Growth on phenol and *p*-cresol of *C. tropicalis* incubated with phenol.

Each shake flask contained 500 ml of the medium (0.5 mM or 5.0 mM phenol or *p*-cresol as carbon source). The small amount of washed cells of *C. tropicalis* incubated with phenol was inoculated in the medium. Each flask was incubated in a shaking incubator at 30°. Measurement of the cell growth is the same as described in Table 1.

Carbon source	Growth of yeast cell					
		at 0 day	at 2 days		at 6 days	
	Concn. mM	Absorbance at 660 nm	Absorbance at 660 nm	No. of cells $\times 10^7$ /ml	Absorbance at 660 nm	No. of cells $\times 10^7$ /ml
Phenol	5.0	0.029	0.390	1.26	0.371	1.23
<i>p</i> -Cresol	0.5	0.020	0.019	(—)	0.060	0.21
<i>p</i> -Cresol	5.0	0.027	0.022	(—)	0.035	0.12

These results coincide with the observation by HARRIS and RICKETTS (10) that some *Candida* strain was able to utilize phenol for growth but the strain was unable to utilize *p*-, *m*-, and *o*-cresols, *p*- and *m*-hydroxybenzoates, and salicylate. Moreover, the utilization of catechols was in accord with the observation of CAIN *et al.* (4) that *C. tropicalis* grew on the isomers of dihydroxyphenol, but did not on other phenols including those that contain a carboxyl group in the molecule.

The cells of *C. tropicalis* incubated with phenol were carefully washed with distilled water and then inoculated in the medium containing *p*-cresol as the sole carbon source. The growth on *p*-cresol was compared with the growth on phenol by the cells (Table 2). Cells of *C. tropicalis* incubated with phenol showed greater growth on phenol compared with the growth of native cells shown in Table 1, but the growth of the cells on *p*-cresol was very slight.

Oxidation of various phenols by C. tropicalis

Cresols were not utilized for growth by the native cells of *C. tropicalis*, although methylcatechols were utilized. Results of the respirometric experiments revealed that the native cells of *C. tropicalis* could not oxidize the three isomers of cresol, although the cells oxidized phenol and catechol. In the present experiment, oxidation of 11 different substrates by the cells incubated with phenol was examined by measuring oxygen uptake and carbon dioxide evolution. Table 3 shows the values of Q_{O_2} and Q_{CO_2} during the first 60 min of oxidation, and also shows the total values of oxygen taken up and carbon dioxide absorbed.

Both phenol and catechol were rapidly oxidized by the cells without a lag period. Values of gas exchange in the oxidation of phenol were 5.3 mole oxygen and 4.7 mole carbon dioxide per mole of the substrate.

Table 3. Oxidation of phenols and catechols by the cells of *C. tropicalis*.

Each Warburg flask contained 1.0 ml of cell suspension (*ca.* 30 mg dry wt. of the cells per ml of 1/15 M phosphate buffer, pH 7.2) and 1.0 ml of substrate solution (10.0 mM substrate in 1/15 M phosphate buffer, pH 7.2). The center well contained 0.2 ml of 20% KOH solution or distilled water. QO_2 values are calculated from the oxidation during the first 60 min, and the total amount of O_2 taken up or CO_2 evolved are also listed. Endogenous values were subtracted.

Substrate 10 μ M concn.	O ₂ Uptake			CO ₂ Evolution		
	QO_2 (60 min)	μ l	mole/mole substrate	QCO_2 (60 min)	μ l	mole/mole substrate
Phenol	9.7	1182	5.3	7.4	1056	4.7
Catechol	8.9	987	4.4	6.5	966	4.3
<i>p</i> -Cresol	7.4	718	3.2	2.9	506	2.3
<i>m</i> -Cresol	7.3	844	3.8	4.7	753	3.4
<i>o</i> -Cresol	4.7	322	1.4	3.8	307	1.4
<i>p</i> -Hydroxybenzoate	1.2	85	0.4	1.8	130	0.6
<i>m</i> -Hydroxybenzoate	1.4	62	0.3	3.2	206	0.9
Salicylate	0.4	12	0.1	1.1	105	0.5
Protocatechuate	0.5	21	0.1	0.6	34	0.2
4-Methylcatechol	11.2	480	2.1	8.0	381	1.7
3-Methylcatechol	11.3	1442	6.4	8.6	1594	7.1

Similarly, the three isomers of cresol were rapidly oxidized without a lag period, but the values of total oxygen taken up and carbon dioxide evolved were lower than those for phenol; values for the oxidation of *p*-cresol were 3.2 mole oxygen and 2.3 mole carbon dioxide. In the case of oxidation of *o*-cresol, there were lower oxygen uptake (1.4 mole) and carbon dioxide evolution (1.4 mole).

Both 3- and 4-methylcatechol were oxidized very rapidly without a lag period. Values for the oxidation of 4-methylcatechol were 2.1 mole oxygen and 1.7 mole carbon dioxide. In the case of 3-methylcatechol, the values of total oxygen taken up and carbon dioxide evolved were 6.4 mole and 7.1 mole, respectively.

A low QO_2 value and negligible oxygen uptake show that oxidation of the three isomers of hydroxybenzoate and protocatechuate was very sluggish. These results suggested that cresols were not metabolized through hydroxybenzoate and protocatechuate by the cells incubated with phenol.

The disappearance of substrate and appearance of some metabolites were also followed by the measurement of UV spectrum of the reaction mixture in parallel with manometric experiments. Supernatants of the reaction mixture were obtained from the reaction vessel at three different periods, *i.e.*, before the oxidation was started, when oxygen was actively consumed, and

Fig. 1. UV absorption spectra of the supernatant of reaction mixture containing phenol and catechol.

a : The supernatant of reaction mixture containing phenol. Supernatants were diluted 1 : 15 with 1/15 M phosphate buffer, pH 7.2. Curve 0, at 0 min; curve 1, after oxidation with 802 μ l of O₂ uptake; and curve 2, after oxidation with 1182 μ l of O₂ uptake. b : The supernatant of reaction mixture containing catechol. Supernatants were diluted 1 : 15 with 1/15 M phosphate buffer. Curve 0, at 0 min; curve 1, after oxidation with 355 μ l of O₂ uptake; and curve 2, after oxidation with 987 μ l of O₂ uptake.

after the oxidation ceased. Spectra of the reaction mixture are presented in Figs. 1, 2, and 3.

In the oxidation of phenol, absorption maximum at 269 nm of phenol disappeared as the oxidation advanced. No new absorption due to oxidation product appeared in the UV region (Fig. 1a). As for the spectra of the reaction mixture containing catechol, the absorbance of catechol at 276 nm decreased with the increase in oxygen uptake, while there was an increase in absorbance at 256 nm due to the absorption of accumulated metabolite (Fig. 1b).

All of the three substrates of *p*-cresol, *m*-cresol, and 4-methylcatechol, which have the absorption maxima at 278, 272, and 281 nm, respectively, showed a shift to the new absorption maximum at 264 nm (Figs. 2a, b, and 3a).

It is notable that change in the spectra of the reaction mixture containing *o*-cresol was slight despite the observed oxygen consumption (Fig. 2c). Ap-

Fig. 3. UV absorption spectra of the supernatant of reaction mixture containing methylcatechol.

a : The supernatant of reaction mixture containing 4-methylcatechol. Supernatants were diluted 1 : 60 with 1/15 M phosphate buffer. Curve 0, at 0 min; curve 1, after oxidation with 275 μ l of O_2 uptake; and curve 2, after oxidation with 480 μ l of O_2 uptake. b : The supernatant of reaction mixture containing 3-methylcatechol. Supernatants were diluted 1 : 15 with 1/15 M phosphate buffer. Curve 0, at 0 min; curve 1, after oxidation with 295 μ l of O_2 uptake; curve 2, after oxidation with 1142 μ l of O_2 uptake.

Fig. 2. UV absorption spectra of the supernatant of reaction mixture containing cresol.

a : The supernatant of reaction mixture containing *p*-cresol. Supernatants shown by curves 0 and 1 were diluted 1 : 15 and that shown by curve 2 was diluted 1 : 150 with 1/15 M phosphate buffer. Curve 0, at 0 min; curve 1, after oxidation with 365 μ l of O_2 uptake; and curve 2, after oxidation with 730 μ l of O_2 uptake. b : The supernatant of reaction mixture containing *m*-cresol. Supernatants shown by curves 0 and 1 were diluted 1 : 15 and that shown by curve 2 was diluted 1 : 45 with 1/15 M phosphate buffer. Curve 0, at 0 min; curve 1, after oxidation with 318 μ l of O_2 uptake; and curve 2, after oxidation with 844 μ l of O_2 uptake. c : The supernatant of reaction mixture containing *o*-cresol. Supernatants were diluted 1 : 15 with 1/15 M phosphate buffer. Curve 0, at 0 min; curve 1, after oxidation with 226 μ l of O_2 uptake; and curve 2, after oxidation with 332 μ l of O_2 uptake.

Table 4. Accumulation of metabolites in the reaction mixture.

The reaction mixture was shaken at 30°. The reaction flask (3000-ml shake flask) contained 500 ml (or 250 ml for *o*-cresol) of cell suspension and 500 ml (or 250 ml for *o*-cresol) of substrate solution. Metabolites were extracted from the mixture with ether. Recovery of metabolite was calculated as per cent weight of extracts of the weight of substrate added. Absorption maximum of metabolite in UV region was measured in methanol.

Substrate	Vol. ml	Concn. mM	Oxidation (min)	Ether extract		
				Extract mg	Recovery %	Absorption max. nm
Phenol	1000	5.0	60	67.5	14.4	270
Catechol	1000	5.0	60	225.0	40.9	258
<i>p</i> -Cresol	1000	5.0	300	271.5	50.3	264
<i>m</i> -Cresol	1000	5.0	300	290.3	53.8	264
<i>o</i> -Cresol	500	5.0	60	48.3	17.9	226, 273
	500	5.0	180	43.6	16.2	225, 273
	500	5.0	300	13.0	4.8	216, 273
4-Methylcatechol	1000	5.0	300	409.5	66.1	264
3-Methylcatechol	1000	5.0	300	322.9	52.1	276

pearance of the new absorption maximum at 256 nm was observed with 3-methylcatechol.

Oxidation and oxidation products of phenol

The reaction mixture containing 5 mM phenol as a substrate was incubated at 30° for 60 min in the shaking incubator. After removal of solvent from the ether extract *in vacuo*, a small amount of a colorless amorphous solid was obtained in 14.4% of recovery per substrate added. In the case of a metabolite from catechol, the recovery was 40.9% of the added substrate and had a maximum absorption at 258 nm (Table 4).

Metabolites extracted from the reaction mixture containing phenol and catechol were chromatographed on a thin-layer plate. The two clear spots were detected at *R_f* 0.53 and 0.31 from the metabolite of phenol on the chromatogram developed with the solvent mixture d (benzene : dioxane : acetic acid, 90 : 25 : 4). In the case of the catechol metabolite, two clear spots were detected at *R_f* 0.53 and 0.31 with solvent d. The *R_f* value of the authentic catechol was 0.53.

The chloroform- and water-soluble substance isolated from the metabolite of phenol was recrystallized from water, and colorless needles were obtained in a small amount. The crystal showed an absorption maximum at 267 nm in methanol, the same as that of an authentic catechol, and its IR spectrum was completely identical with that of an authentic catechol. These UV and

Fig. 4. IR spectrum (KBr disc) of the catechol metabolite of amorphous yellow solid.

IR spectra confirmed that catechol was accumulated during the oxidation of phenol.

From the catechol oxidation products, a yellow amorphous substance was obtained which was soluble in methanol and chloroform, and insoluble in cold water. The substance melted at 183–184° and showed an absorption maximum at 258 nm in pH 7.0, 258 nm in 0.1 N NaOH, and 258 nm in methanol. Anal. Found: C, 50.58; H, 4.35%. Calcd. for $C_6H_6O_4$: C, 50.71; H, 4.26%. Its IR spectrum showed that the metabolite has a carbonyl group (1700 cm^{-1}) and a conjugated double bond (1650 and 1600 cm^{-1}) in the molecule (Fig. 4). This result indicates that the benzene ring of catechol was cleaved.

Oxidation and oxidation products of p- and m-cresols

The reaction mixture containing 5 mM *p*- or *m*-cresol as a substrate was incubated at 30° for 300 min. The value of dry weight and absorption maximum of the metabolites obtained from *p*- and *m*-cresols are shown in Table 4. The recoveries of the metabolite of *p*- and *m*-cresols oxidation were 50.3% and 53.8% in weight of added substrate, respectively. The metabolite extracted from the reaction mixture containing *p*- or *m*-cresol was a colorless solid. The absorption maxima of both metabolites in methanol solution was identical, *i.e.*, 264 nm.

The metabolite obtained from *p*-cresol oxidation was purified from a solvent mixture containing methanol and chloroform to a colorless solid. The purified metabolite had the UV absorption maximum at 264 nm in pH 7.2. The same UV absorption maximum (264 nm) was also obtained in 0.1 N NaOH and in methanol. Figure 5 shows the IR spectrum of the metabolite in KBr disc, with bands at 3400, 1690, 1630, 1600, 1410, and 1390 cm^{-1} . Ultraviolet

Fig. 5. IR spectrum (KBr disc) of the *p*-cresol metabolite.

—, *p*-Cresol metabolite formed by the cells of *C. tropicalis*; ----, 5-Formyl-2-hydroxy-4-methyl-2,4-pentadienoic acid.

and IR spectra of the metabolite suggest the presence of a hydroxyl group, a carbonyl group, a conjugated double bond, and a methyl group in the molecule. Benzene ring was not detected in the IR spectrum. Ultraviolet absorption maximum at 264 nm in methanol and the IR spectrum suggest that the compound is a derivative of aliphatic α, β -unsaturated acid (11). The same compound was obtained from *m*-cresol with the identical UV and IR spectra.

These results show that the metabolite of *p*-cresol oxidation by the cells of *C. tropicalis* is completely identical with the known ring-fission product from *p*-cresol, produced by the yeast previously isolated from the soil by the present author (7), *i.e.*, 5-formyl-2-hydroxy-4-methyl-2,4-pentadienoic acid (Fig. 5).

Oxidation and metabolite of 4-methylcatechol

The reaction mixture containing 5 mM 4-methylcatechol as a substrate was incubated for 300 min. The metabolite was extracted with ether from the reaction mixture, and the total weight and UV spectrum of the metabolite in methanol were measured (Table 4).

The recovery of the extracted metabolite from 4-methylcatechol oxidation was 66.1% in weight of the added substrate. The amount of the metabolite was greater than that from the extract of *p*-cresol which was obtained in 50.3% recovery. The metabolite gave UV absorption maximum at 264 nm in pH 7.2, and showed completely the same IR spectrum as the metabolite from *p*-cresol. It seems from the above results that *p*- and *m*-cresols were metabolized through 4-methylcatechol.

Fig. 6. UV absorption spectra of the extracts from the reaction mixture containing *o*-cresol.

The extracts were dissolved in methanol. Curve 1, extract from 60-min oxidation; curve 2, extract from 180-min oxidation; and curve 3, extract from 300-min oxidation.

Oxidation and oxidation products of o-cresol

The reaction mixture containing 5 mM *o*-cresol was used to obtain the metabolic products of *o*-cresol. A 500-ml reaction mixture was incubated for each oxidation period of 60, 180, and 300 min at 30°. The maximum absorption of the supernatant of each reaction mixture was found at 270 nm. The product from *o*-cresol oxidation was extracted with ether. The weight of extracts and the UV absorption maximum are shown in Table 4. The amounts of the extracts recovered, with respect to the weight of added substrate, were 17.9% for 60 min, 16.2% for 180 min, and 4.8% for 300 min. The UV absorption spectra of the extracted materials are shown in Fig. 6.

R_f values of the extracted materials in thin-layer chromatography and the result of ferric chloride test are shown in Tables 5 and 6, respectively.

The results of ferric chloride test of the extracts show that *R_f* value 0.81 of the yellow spot in the case of solvent c is identical with the *R_f* value 0.81

Table 5. *Rf* value of the components in metabolite of *o*-cresol on thin-layer chromatogram.

The metabolites extracted from each oxidation period are the same as shown in in Table 4. Solvent a: Ethanol: conc. NH_4OH : water (8:1:1, v/v), solvent b: butanol: acetic acid: water (25:5:1, v/v), solvent d: benzene: dioxane: acetic acid (90:25:4, v/v).

Materials	<i>Rf</i> value		
	Solvent a	Solvent b	Solvent d
<i>o</i> -Cresol metabolite			
from 60-min oxidation	0.61, 0.88	0.86	Not detected
from 180-min oxidation	0.61, 0.88	0.88	0.58, 0.68
from 300-min oxidation	0.62, 0.87	0.86	0.57, 0.69
<i>o</i> -Cresol	Not detected	0.93	0.71
Salicylate	0.73	0.92	Not detected
Catechol	Not detected	0.85	0.53
3-Methylcatechol	0.55	0.85	0.59

Table 6. Ferric chloride test of *o*-cresol metabolite on thin-layer chromatogram.

The metabolites from each oxidation period are the same as shown in Table 4. Solvent c: benzene: tetrahydrofuran: acetic acid (15:9:1, v/v).

Materials	Solvent c	
	<i>Rf</i>	Color
<i>o</i> -Cresol metabolite		
from 60-min oxidation	0.81	Yellow
from 180-min oxidation	0.81	Yellow
from 300-min oxidation	0.81	Yellow
<i>o</i> -Cresol	0.81	Yellow
Salicylate	0.71	Red
Catechol	0.70	Black
3-Methylcatechol	0.69	Black

of *o*-cresol. The reaction products which have the following *Rf* values were also obtained: *Rf* 0.61 and 0.88 in solvent a, *Rf* 0.86 in solvent b, and *Rf* 0.58 and 0.68 in solvent d. These result suggests that one or two aliphatic compounds are contained in the extracts from the reaction mixture.

The supernatant of the reaction mixture of 3-methylcatechol had the absorption maximum at 256 nm. The oxidation products were obtained with

52.1% recovery of the substrate added. The extract was a dark brown oily substance and a metabolite could not be purified from this extract.

DISCUSSION

Oxidation of phenol by C. tropicalis. HARRIS and RICKETTS (10) and CAIN *et al.* (4) reported that the utilization of phenol for growth by some yeast strains occurred only after the cultivation of 1 to 2 weeks. In the present experiment, the growth of *C. tropicalis* on phenol was excellent compared with the growth on other carbon sources including catechol and methylcatechols, and reached a maximum value after 2 days' incubation. The cells of *C. tropicalis* that had been incubated with phenol showed a greater growth on phenol compared with the growth of native cells. Result of the respirometric experiment by the phenol-adapted cells showed the easy and rapid oxidation of phenol. The value of oxygen uptake was approximately 76% of the theoretical value of complete oxidation.

It is well known that the first step of the metabolism of phenol hydroxylase which brings about monohydroxylation of the benzene ring (12-15). It was confirmed that the cells of *C. tropicalis* incubated with phenol oxidized phenol to form catechol; in the metabolite extracted from the reaction mixture with phenol, catechol was isolated and identified from UV and IR spectra. The metabolite extracted from catechol oxidation was isolated and identified by chemical and physical analyses as a ring-fission product of catechol, corresponding to $C_6H_6O_4$.

Oxidation of cresols by phenol-adapted cells. The metabolic pathway of cresol oxidation by bacteria has been clearly elucidated. The present author reported that the cells of a yeast strain isolated from the soil could not utilize *p*-cresol for growth but they could oxidize *p*-cresol after the cells were incubated with phenol (7).

Result of the respirometric experiment showed that the phenol-adapted cells of *C. tropicalis* oxidize *p*-cresol rapidly. If *p*-cresol was oxidized completely, the consumption of 1.5 mole more of oxygen per mole of *p*-cresol than per mole of phenol could be expected. The result obtained showed, however, that oxygen uptake per mole of *p*-cresol was 3.2 mole while per mole of phenol was 5.3 mole. This phenomenon appears to show that the intermediate of *p*-cresol was accumulated in the reaction mixture. Such a conclusion on the accumulation was also supported by the fact that the metabolite was extracted in an amount of 50% weight per added substrate.

In the case of 4-methylcatechol oxidation, this substrate took up 2.1 mole of oxygen per mole, and gave the same oxidation products as from *p*-cresol. If 4-methylcatechol was an intermediate in the *p*-cresol oxidation, the amount of oxygen uptake must be less in the oxidation of 4-methylcatechol than in the oxidation of *p*-cresol. In the experimental result, 1 mole less oxygen was

consumed in 4-methylcatechol than in *p*-cresol oxidation. This result shows that *p*-cresol was oxidized *via* 4-methylcatechol.

Fission of benzene ring in cresols. The metabolite from *p*-cresol was analyzed from UV and IR spectra and identified as 5-formyl-2-hydroxy-4-methyl-2,4-pentadienoic acid. The metabolite from *m*-cresol oxidation was identified as the same metabolite as from *p*-cresol. The same metabolite was obtained in a larger amount from 4-methylcatechol than from *p*- and *m*-cresol. Although there are two possibilities of monohydroxylated intermediates in the oxidation of *m*-cresol, namely, 3- and 4-methylcatechols, the above results showed that *m*-cresol was metabolized *via* 4-methylcatechol dominantly.

These results support the idea that, in the case of *p*- and *m*-cresol oxidation, the formation of 4-methylcatechol by the monohydroxylation is dominant or exclusive. The ring-fission of 4-methylcatechol occurred by the extra-diol type cleavage, as has been demonstrated in some bacterial species, since the chemical structure of the accumulated metabolite supported the occurrence of fission between carbons at 1 and 6.

In the oxidation of *o*-cresol, the total oxygen uptake and the Q_{O_2} value were comparatively small, and the accumulation of the metabolite was very small. Moreover, the absorption spectrum of the reaction mixture was different from that of *p*- and *m*-cresol oxidation. These results suggest that the metabolic pathway of *o*-cresol is different from that of *p*- and *m*-cresol. BAYLEY *et al.* (2) and RIBBONS *et al.* (15, 16), by the use of *Pseudomonas* species, showed that the oxidation of *o*-cresol occurred through the formation of 3-methylcatechol. In the present experiment, the UV absorption spectrum of the reaction mixture and ether extracts from *o*-cresol showed quite a different pattern from the UV spectrum of 3-methylcatechol. The results shown in the present paper did not support the oxidation of *o*-cresol through 3-methylcatechol.

Nature of phenol-adapted cells of C. tropicalis. Recently, the oxidation of phenols by yeasts has been studied with *Oospora*, *Saccharomyces*, *Candida*, *Rhodotorula*, *Pullularia*, *Trichosporon*, and some unidentified species (4-7, 10, 17). In *Candida* species, utilization of phenol as growth substrate has been reported already (10, 17, 18).

Candida tropicalis can hardly utilize the three isomers of cresol and three isomers of hydroxybenzoate as carbon source. Although the cells grew on 4-methylcatechol as sole carbon source, the cells could not grow on protocatechuate. The native cells of *C. tropicalis* may have catechol oxygenase as a constituent, as judged from its ability to utilize catechol, and 3- and 4-methylcatechols for growth. It is well known that catechol oxygenase has the ability to attack catechol, and 3- and 4-methylcatechols (10). If *p*-cresol is converted to 4-methylcatechol by phenol hydroxylase, the latter will be metabolized by the cells, as is well known with many bacterial species (2, 12, 16). In contrast, the result of the growth test in the present experiment suggests that

the phenol hydroxylase in the three isomers of cresol may not be a constituent of the yeast cell.

Since the cells were capable of oxidizing cresols after the cells were incubated with phenol, whereas the native cells could not oxidize cresols, the pathways of cresol oxidation in the cells are probably initiated by the formation of phenol hydroxylase induced by phenol.

It appears that the enzyme sequence of cresol metabolism is formed within the cells of *C. tropicalis* by incubation with phenol, and the cells oxidize *p*- and *m*-cresols to 4-methylcatechol, and then the cleavage of the benzene ring between carbons 1 and 6 of 4-methylcatechol takes place. This conclusion agrees with the result of *p*-cresol oxidation by yeast cells isolated from the soil (7).

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