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Constitutive Synthesis, Purification, and Characterization of Catechol 1,2-Dioxygenase from the Aniline-Assimilating Bacterium *Rhodococcus* sp. AN-22

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Abbreviations: CD, catechol 1,2-dioxygenase; PMY, a medium consisting of 10 g of Polypepton and 5 g each of meat extract, yeast extract, and NaCl in 1 *l* (pH 6.8).

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A catechol 1,2-dioxygenase (CD) was found, which was synthesized constitutively in the aniline-assimilating bacterium *Rhodococcus* sp. AN-22 grown on a medium without aniline, as well as on aniline medium. The bacterium synthesized CD in its cells grown on all the 21 non-aromatic substrates examined, including four natural media such as meat and yeast extracts, one sugar, six organic acids, and 10 amino acids as carbon, energy, and nitrogen sources. When the bacterium was incubated on a medium with D-glucose, L-malate, isoleucine, leucine, etc., it synthesized more CD than that in cells grown on aniline. Two CDs, which were prepared from cells grown on aniline and L-malate, were purified separately to homogeneity and characterized. The two enzymes were apparently identical in molecular and catalytic properties including molecular mass, optimal pH, stability to heating, and substrate specificity for catechol analogues. However, they differed in the substrate specificity and resistance to sulfhydryl and chelating agents from the inducible CDs produced by other aniline-assimilating bacteria reported previously.

Aniline is a major industrial raw material and an intermediate used for

the production of dyes, plastics, herbicides, etc. Studies of the biodegradation of aniline as an environmental pollutant were performed by Lyons *et al.* (1). We have isolated many bacteria (2-4) from soil that grew on aniline as the sole carbon, energy, and nitrogen source, and have investigated their metabolic pathways (5) and enzyme systems (6-8). It was revealed that all the strains isolated on aniline medium degraded aniline via catechol.

Through these investigations, we found that two catechol 1,2-dioxygenase (CD; EC 1.13.11.1) isozymes are present in a high proportion of aniline-assimilating bacteria isolated, which degrade aniline via an *ortho*-cleavage pathway (9). These aniline-assimilating bacteria are divided into three groups on the basis of isozyme patterns: those with two isozymes of equal enzymatic activity; those with two isozymes of differing enzymatic activity; and those with only one enzyme. The aniline-assimilating bacteria belonging to the former two groups synthesize the dioxygenases inducibly only when they are incubated with aniline. However, we recently observed that a bacterium belonging to the third group and producing only one CD synthesized the enzyme constitutively in its cells grown on a medium without aniline, as well as on aniline medium.

CD is a key enzyme in the pathway of the *ortho*-cleavage of the aromatic compounds by microorganisms. This enzyme catalyzes the ring fission of catechol

with the consumption of one mol of O_2 per mol of substrate and produces *cis*, *cis*-muconate. A variety of CDs are distributed to a large extent in microorganisms metabolizing aromatic compounds through the *ortho*-cleavage pathway. Almost all the enzymes reported before are inducible. There have been some reports that suggest the existence of a constitutive CD in the 3-aminophenol-assimilating bacterium *Arthrobacter* sp. mA3 (10) and the *p*-nitrophenol-assimilating phototrophic bacterium *Rhodobacter capsulatus* (11). However, the level of constitutive synthesis of the CDs was low and the constitutive enzyme production was not characterized. In addition, the CDs from these bacteria were not purified or characterized.

Our aim in this study was to characterize the constitutive CD from an aniline-assimilating bacterium, *Rhodococcus* sp. AN-22. For this purpose, we prepared two enzymes from cells grown on aniline and on the non-aromatic substrate L-malate and purified them separately to homogeneity. Comparisons between the two enzymes in terms of molecular, chemical, and catalytic properties are also described in this article.

MATERIALS AND METHODS

Chemicals Aniline hydrochloride, catechol, and meat extract were purchased from Wako Pure Chemical (Osaka); 3-methylcatechol, 3-fluorocatechol,

3-chlorocatechol, and 4-chlorocatechol from Tokyo Kasei Kogyo (Tokyo); 4-methylcatechol was from Nacalai Tesque (Kyoto); DE52 cellulose was from Whatman Chemical Separation (Clifton, N. J., USA); Polypepton and yeast extract were from Nihon Seiyaku (Tokyo); the electrophoresis calibration kit LMW was from Amersham Biosciences (Uppsala, Sweden); and DEAE-Toyopearl 650S, Phenyl Toyopearl 650S, and Toyopearl HW-55S were from Tosoh Corporation (Tokyo).

Strain and culture conditions Strain AN-22, isolated on a medium with aniline as the sole carbon, energy and nitrogen source (9) and identified as a *Rhodococcus* species, was used throughout this work. For the preparation of enzymes, the bacterium was incubated at 30°C for 2 d with shaking in 20 ml of aniline (2.2 g/*l*) or L-malate (9.0 g/*l* sodium L-malate) medium in three tubes (1.8 x 18 cm) and then transferred into 450 ml of the medium in a 3-*l* shaking flask. It was cultured at 30°C on a reciprocating shaker until the growth reached the late exponential phase. Cells were harvested by centrifugation at 7700 x *g* for 10 min at 4°C and washed twice with 20 mM Tris-HCl (pH 8.0) (buffer A). The washed cells were stored at -20°C.

Constitutive synthesis of enzyme The constitutive synthesis of CD was examined using cells grown on non-aromatic substrates including one sugar, six organic acids, 10 amino acids, and four natural media such as Polypepton,

yeast and meat extracts, and PMY (see Abbreviation section). For the preparation of growth media, 2.2 g/*l* aniline was omitted from the aniline medium (12), to which sugar, organic acids, amino acids, or natural media as carbon and energy sources and 0.4 g/*l* $(NH_4)_2SO_4$ as a nitrogen source were added. The amount of carbon and energy sources used was calculated on the basis of the number (or amount) of carbon atoms contained in the substrates. The twofold amount of each substrate of six carbon atoms in the aniline molecule was used as the carbon and energy source. One percent (w/v) each of Polypepton and yeast and meat extracts was also used. For media containing natural media and amino acids, $(NH_4)_2SO_4$ was omitted. Strain AN-22 was cultured at 30°C in a tube with shaking. Cells were harvested at the early exponential phase and used for enzyme assay.

Enzyme assay The activity of CD was assayed spectrophotometrically by measuring of the absorbance at 260 nm (6). One unit of enzyme activity was defined as the amount of enzyme that formed 1 μmol of *cis,cis*-muconic acid per min. Specific activity was defined as units per mg of protein. Protein concentrations were measured by the method of Lowry *et al*. (13).

Purification of CD from cells grown on aniline All operations for the purification were carried out at 0-4°C. All centrifugations were at 20,000 x *g* and 4°C for 20 min.

Preparation of cell extracts Frozen cells (17 g, wet wt) were thawed,

suspended in 40 ml of buffer A, and disrupted with a Dyno-Mill (Willy A. Bachofen, Basel, Switzerland) for 10 min under cooling with tap water; glass beads of 0.l-0.2-mm diameter were used. The used beads were removed and washed twice with buffer A by decantation. The original and washing solutions were combined. Intact cells and cell debris in the combined solution were removed by centrifugation. The supernatant, designated as the cell extract, was collected (fraction 1; 255 ml).

Streptomycin sulfate treatment Streptomycin sulfate (20%, w/v) was added to fraction 1 with stirring to a final concentration of 1%. The solution was stirred for 30 min and centrifuged. The supernatant (270 ml) was recovered (fraction 2), and the precipitate was discarded.

$(NH_4)_2SO_4$ fractionation Fraction 2 was brought to 50% saturation with $(NH_4)_2SO_4$. The mixture was stirred for 30 min and centrifuged; the supernatant was collected and the precipitate was discarded. $(NH_4)_2SO_4$ was added to the supernatant to 75% saturation. After stirring for 30 min, the precipitate was collected by centrifugation and dissolved in buffer A. The solution was dialyzed against buffer A with two changes of buffer. The final volume of the dialyzed solution (fraction 3) was 60 ml.

Chromatography on DE52 cellulose Fraction 3 was applied to a column (2.2 x 27 cm) of DE52 cellulose equilibrated with buffer A. Proteins were eluted

with a linear gradient (0 to 0.4 M) of NaCl in 1.4 l of buffer A; the protein concentration and enzyme activity of the fractions were assayed. Fractions with a high specific activity were pooled to yield fraction 4 (84 ml).

Chromatography on DEAE-Toyopearl 650S Fraction 4 was dialyzed against buffer A and the dialyzed solution was applied to a column (2 x 13 cm) of DEAE-Toyopearl 650S equilibrated with buffer A. Proteins were eluted with a linear gradient (0 to 0.3 M) of NaCl in 1200 ml of buffer A; the protein concentration and enzyme activity of the fractions were assayed. Fractions with a high specific activity were pooled to yield fraction 5 (57 ml).

Chromatography on Toyopearl HW-55S Fraction 5 was concentrated to 0.5 ml with a collodion bag (Sartorius, Goettingen, Germany). The concentrated sample was placed on the top of a column (3 x 60 cm) of Toyopearl HW-55S equilibrated with buffer A containing 0.2 M NaCl. Proteins were eluted with buffer A containing 0.2 M NaCl. After estimating the protein concentration and enzyme activity, fractions with a high specific activity were pooled to yield fraction 6 (12 ml).

Chromatography on Phenyl-Toyopearl 650S To fraction 6, $(NH_4)_2SO_4$ was added to make a 15% (w/w) solution. The enzyme solution was applied to a column (1.7 x 13) of Phenyl-Toyopearl 650S equilibrated with buffer A containing 15% (w/w) $(NH_4)_2SO_4$. Proteins were eluted with a linear gradient (15 to 0, w/w) of

$(NH_4)_2SO_4$ in 400 ml of buffer A; the protein concentration and enzyme activity of the fractions were assayed. The purity of the enzyme in each fraction was determined by PAGE. Fractions showing a single protein band on a gel were pooled (fraction 7, 9 ml).

Purification of CD from cells grown on L-malate Frozen cells (12 g, wet wt) were thawed and suspended in 40 ml of buffer A. The preparations of cell extracts (fraction 1, 260 ml), streptomycin sulfate treatment (fraction 2, 240 ml), $(NH_4)_2SO_4$ fractionation (fraction 3, 50 ml), chromatographies on DE52 cellulose (fraction 4, 47 ml), DEAE-Toyopearl 650S (fraction 5, 24 ml), Toyopearl HW-55S (fraction 6, 24 ml), and Phenyl-Toyopearl 650S (fraction 7, 24 ml) were carried out essentially by the same methods as used for the purification of CD from aniline-grown cells. Fraction 7 was re-chromatographed on DEAE-Toyopearl 650S. Fractions showing a single protein band on the gel were pooled (fraction 8, 14 ml).

Determination of molecular mass The molecular masses of native CDs were measured by gel filtration on Toyopearl HW-55S. The molecular mass of the enzyme subunit(s) was measured by SDS-PAGE (14). The size markers used for gel filtration were cytochrome *c* (12.4 kDa), myoglobin (17.8 kDa), ovalbumin (43 kDa), and bovine serum albumin (67 kDa). The electrophoresis calibration kit LMW was used as size markers for SDS-PAGE.

Substrate specificity The intradiol cleavage activities for 3- and

4-methylcatechol, protocatechuic acid, and pyrogallol and the extradiol cleavage activities for 3-methylcatechol and 3-chlorocatechol were assayed as reported previously (6). The intradiol cleavage activities for 3- and 4-chlorocatechol and 3-fluorocatechol were assayed spectrophotometrically as above by evaluating initial rates from the data for 60 s. Molar extinction coefficients of 18,300 at 267 nm for 2-chloromuconic acid, 12,500 at 259 nm for 3-chloromuconic acid, and 15,000 at 261 nm for 2-fluoromuconic acid were used (15).

Analysis of inner amino acid sequence The N-terminal amino acids of the two CDs could not be determined by automated Edman degradation with a Shimadzu protein sequencer PPSQ-10 (Shimadzu, Kyoto), because of the possible presence of blocking groups. The inner amino acid sequences of the CDs were determined with the sequencer after fractionation of BrCN-digested peptides on a SDS-polyacrylamide gel.

Nucleotide sequence accession number The nucleotide sequences of a16S rRNA gene from strain AN-22 reported in this paper were deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases with accession no. AB087282.

RESULTS

Taxonomy of strain AN-22 Strain AN-22 was aerobic, gram-positive, non-motile, non-spore forming, not acid-fast, and pleomorphic.

Growth of rod-shaped or coccoid cells began by elongation. The mycelium formed was straight or slightly curved. Fragmentation began 10 to 13 h after inoculation in the center of most mycelia. Divided hyphal ends grew in parallel to each other. The fragmentation was repeated and mycelia multiplied. Later, most mycelia divided into rod-shaped or coccoid daughter cells. The strain showed catalase activity, but not oxidase, urease, DNase, cellulase, or caseinase activity.

Strain AN-22 had cell walls of the glycolyl-type including *meso*-diaminopimelic acid as a principal amino acid. The major isoprenoid of cells was dihydrogenated menaquinone with eight isoprene units (MK-8(H_2)). The 16S rRNA gene of the strain was 97.5 and 97.4% identical to the genes of *Rhodococcus ruber* M2 (accession number, AY247275) and *Rhodococcus coprophilus* JCM 320 (U93340), respectively. On the basis of these results, together with the description in Bergey's manual (16), we identified strain AN-22 as a *Rhodococcus* species.

Constitutive synthesis of CD Table 1 shows that *Rhodococcus* sp. AN-22 synthesized CD in cells grown on all the substrates examined, as well as on aniline. The cells grown on natural media, D-glucose, L-malate, isoleucine, aspartate, leucine, etc. produced higher specific activity of the enzyme than those grown on aniline. When the bacterium was incubated with acetate or glyoxylate,

which is a metabolic member of the glyoxylate cycle, the enzyme activity decreased, although satisfactory cell growth was observed. The specific activity of CD was maintained at almost equal levels during cultivation on the aniline and L-malate media (Fig. 1).

Other aniline-assimilating bacteria, *Rhodococcus erythropolis* AN-13 (6, 17) and *Frateuria* sp. ANA-18 (7, 18, 19), which were reported to synthesize CD inducibly, did not produce the enzyme in the absence of aniline under these culture conditions (Table 2). In addition, the benzamide-assimilating *Arthrobacter* sp. BA-7-15 (20) also did not synthesize CD on a medium lacking benzamide.

Purification of the enzymes Table 3 is a summary of a typical enzyme purification for CD from cells of *Rhodococcus* sp. AN-22 grown on aniline. The specific activity of the final preparation of CD was 35 units per mg with an overall recovery of 11%. The final enzyme preparation had a 41-fold increase in the specific activity and showed a single protein band on a polyacrylamide gel (Fig. 2A).

Table 4 shows a summary of the enzyme purification for CD from L-malate-grown cells. The specific activity of the final preparation was 34 units per mg with an overall recovery of 8.4%. The final enzyme preparation had a 36-fold increase in the specific activity and showed a single protein band on a

polyacrylamide gel (Fig. 1B).

Molecular properties of the enzymes The final preparations of the two CDs showed single protein bands on SDS-polyacrylamide gels and the molecular masses were determined to be 32 kDa (Table 5). The molecular masses of the enzymes were 33 kDa by gel filtration. These findings indicated that the two CDs were monomers.

Properties of the purified enzymes The basic properties of the purified CDs, including optimal pH, pH stability, and thermostability are listed in Table 5 and were almost identical. In addition, BrCN-digested peptides from the two enzymes showed the same patterns on a SDS-polyacrylamide gel. The inner amino acid sequences of certain corresponding peptides were identical (Table 5).

Inhibition The effects of metal salts and chelating and sulfhydryl agents on the activity of the CDs were tested (Table 6). $AgNO_3$ and $HgCl_2$ inhibited both the CDs, with $HgCl_2$ in particular inhibiting the CDs almost completely. Other metal salts and chelating and sulfhydryl agents tested did not affect the CD activity evidently.

Substrate specificity of CDs The two CDs of *Rhodococcus* sp. AN-22 showed substantial activities for the intradiol cleavage of 3- and 4-methylcatechol (Table 7). The enzyme purified from L-malate-grown cells

exhibited slight activities for 3- and 4-substituted chlorocatechols. The two CDs oxidized pyrogallol. They did not catalyze the extradiol cleavage of 3-methylcatechol or 3-chlorocatechol nor did they oxidize 3-fluorocatechol or protocatechuic acid.

DISCUSSION

In this work, the CD synthesized constitutively in cells of aniline-assimilating *Rhodococcus* sp. AN-22 was purified and characterized for the first time. The enzyme was synthesized on media with all 21 non-aromatic substrates examined; the bacterium produced the enzyme with higher specific activity on medium containing 11 substrates than that on aniline medium (Table 1). Aniline-assimilating bacteria with the *ortho*-cleavage pathway were reported to be divided into three groups on the basis of CD isozyme patterns (see Introduction section) (9); those of the third group including *Rhodococcus* sp. AN-22 have only one CD. We demonstrated here that the aniline-assimilating organism belonging to the third group synthesizes CD constitutively, although other bacteria of the first and second groups do so inducibly.

We previously analyzed the regulatory system of *cat* gene clusters of the aniline-assimilating *Frateuria* sp. ANA-18 and reported that the CD (CatA) production is induced with *cis,cis*-muconic acid, which is a product derived from

the substrate catechol (19). Although *Rhodococcus* sp. AN-22 synthesizes CD constitutively, a possible regulatory system for the production of CD is assumed to exist in its cells. We observed a low level of the enzyme activity in cells cultured with acetate or glyoxylate that is metabolized through the glyoxylate cycle. On the other hand, the bacterium produced much more CD with organic acids belonging to the TCA cycle than that produced with acetate and glyoxylate. Since the bacterium can grow satisfactorily on acetate and glyoxylate, regulation to repress the CD synthesis may function in the glyoxylate cycle, in competition with the TCA cycle.

The two CDs of *Rhodococcus* sp. AN-22 purified here were more resistant to the sulfhydryl agents PCMB and the chelating agents *o*-phenanthroline than those of other aniline-assimilating bacteria, which synthesize CDs inducibly (6, 7). They also differed from the inducible CDs in their substrate specificity for catechol analogues (6, 7, 20). On the other hand, a *K*m value of the CD for catechol from L-malate-grown cells was 1.6 μM, which is similar to those of the CDs from *Pseudomonas arvilla* C-1 (21) and *Rhodococcus rhodochrous* NCIMB 13259 (22).

The two CDs purified from aniline- and L-malate-grown cells of *Rhodococcus* sp. AN-22 showed essentially the same characteristics for the specific activity, stability to pH and heating, and molecular and catalytic properties, although minor differences in the substrate specificity and inhibition

with some reagents were observed. Judging from the data obtained, it can be assumed that the two enzymes are apparently identical with respect to their protein chemistry.

Because the CD obtained from L-malate-grown cells is synthesized without aromatic substrates such as aniline and catechol, it can be called a constitutive enzyme. However, the CD from aniline-grown cells may be an inducible and/or constitutive enzyme(s). If *Rhodococcus* sp. AN-22 produces an inducible CD in addition to a constitutive one, the two CDs would have been separated by several column chromatographic techniques. We, in fact, observed only one peak of the enzyme activity on all the chromatograms examined. In addition, the two CDs purified from aniline- and L-malate-grown cells were apparently identical, as mentioned above. For these reasons, we made two assumptions. The bacterium synthesizes either a constitutive or inducible CD in the aniline-grown cells; or the bacterium synthesizes both constitutive and inducible CDs, which are identical or have a high degree of similarity to each other.

Most aniline-assimilating bacteria with the *ortho*-cleavage pathway form an operon (*cat* gene luster) consisting of *catA*, *catB*, and *catC* encoding catechol 1,2-dioxygenase, muconate cycloisomerase, and muconolactone isomerase, respectively (17, 18). The analysis of the *cat* gene cluster of

Rhodococcus sp. AN-22 will confirm one of the assumptions mentioned above. In addition, it is necessary to determine whether *catB* and *catC* are expressed constitutively, in addition to *catA*. Genetic and enzymatic analyses concerning the aniline degradation of this bacterium are in progress.

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Figure legends

FIG. 1. Production of CDs of *Rhodococcus* sp. AN-22 on aniline (A) and L-malate (B) media. The bacterium was incubated at 30°C with shaking. At appropriate intervals, cell growth (closed circles) and enzyme activity (closed squares) were assayed.

FIG. 2. Native PAGE of CDs from aniline-grown (A) and L-malate-grown (B) cells of *Rhodococcus* sp. AN-22. The purified enzymes (4 μg each) were run on 7.5% (w/v) gels of pH 8.9 at 2 mA/tube for 2.5 h in a running buffer (pH 8.3) of Tris-glycine. The gels were stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 in a solvent of ethanol-acetic acid-H_2O (9 : 2 : 9, v/v).

TABLE 1. Constitutive synthesis of catechol 1,2-dioxygenase

Growth condition	Specific activity Growth (units/mg) (OD_{660})	
Aniline	0.80	1.6
PMY	1.6	6.0
Yeast extract	1.2	5.0
Meat extract	1.5	1.5
Polypepton	1.5	0.50
D-Glucose	1.5	0.39
L-Malate	1.7	1.0
Succinate	0.60	0.80
Citrate	0.69	0.36
Oxaloacetate	0.61	0.19
Acetate	0.16	0.60
Glyoxylate	0.12	0.36
Isoleucine	1.6	0.50
Aspartic acid	1.2	0.50
Methionine	1.0	0.38
Histidine	0.87	0.30
Glutamic acid	0.68	0.80
Asparagine	0.41	0.40
Leucine	1.5	1.1
Proline	0.71	0.35
Arginine	0.70	0.40
Glutamine	0.67	0.50

Rhodococcus sp. AN-13 was incubated at 30°C with shaking and then the enzyme activity in cells was measured. See the text for details.

TABLE 2. Constitutive and inducible synthesis of CDs by aniline- and benzamide-assimilating bacteria

Strain	Growth medium	Specific activity (units/mg)
Rhodococcus sp. AN-22	PMY	1.6
	L-Malate	1.7
	Aniline	0.80
R. erythropolis AN-13	PMY	0
	L-Malate	0
	Aniline	0.47
Frateuria sp. ANA-18	PMY	0
	L-Malate	0
	Aniline	0.64
Arthrobacter sp. BA-7-15	PMY	0
	L-Malate	0
	Benzamide	0.12

TABLE 3. Purification of CD from aniline-grown cells of *Rhodococcus* sp. AN-22

Fraction	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)
Cell extract	1100	1300	0.85	100
Streptomycin sulfate	950	1300	0.73	86
Ammonium sulfate	980	160	6.1	89
DE52	630	42	15	57
DEAE-Toyopearl 650S	390	17	23	35
HW55 SF	310	14	22	28
Phenyl-Toyopearl 650S	120	3.3	35	11

TABLE 4. Purification of CD from L-malate-grown cells of *Rhodococcus* sp. AN-22

Fraction	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)
Cell extract	610	640	0.95	100
Streptomycin sulfate	460	640	0.72	75
Ammonium sulfate	370	52	7.1	61
DE52	260	18	14	43
DEAE-Toyopearl 650S	190	12	16	31
HW55 SF	130	8.0	16	21
Phenyl-Toyopearl 650S	100	4.9	20	16
DEAE-Toyopearl 650S	51	1.5	34	8.4

TABLE 5. Characterization of CDs from *Rhodococcus* sp. AN-22

	CD from aniline-grown cells	CD from L-malate-grown cells
molecular mass (kDa)		
SDS-PAGE	32	32
Gel filtration	33	33
Optimal pH	8.5	8.0
pH stability [a]	7.5-11.6	7.5-11.6
Thermostability [b]	45°C	45°C
Internal amino acid sequence	REKDKVA	REKDKVA

[a] pH range on which the enzymes maintained more than 90% activity.
[b] Maximum temperature at which the enzymes maintained more than 80% activity.

TABLE 6. Effects of various compounds on enzyme activity

Compound	Concentration (mM)	Remaining activity (%)	
		CD from aniline-grown cells	CD from L-malate-grown cells
None	-	100	100
$FeSO_4 \cdot 7H_2O$	0.1	96	98
$FeCl_3 \cdot 6H_2O$	0.1	95	93
$AgNO_3$	0.1	12	33
$HgCl_2$	0.1	0	2
$CuSO_4 \cdot 5H_2O$	0.1	89	87
CH_2ICOOH	1.0	91	102
PCMB [a]	1.0	80	90
DTNB [b]	0.1	90	96
α,α-Dipyridyl	0.1	90	103
N-Ethylmaleimide	1.0	100	95
o-Phenanthroline	0.05	100	105
Tiron	0.1	96	94
EDTA	1.0	95	98
Na N_3	1.0	94	95

[a] PCMB, *p*-chloromercuribenzoic acid.

[b] DTNB, 5,5'-dithiobis(nitrobenzoic acid).

The enzymes were incubated with each compound at 30°C for 10 min and then remaining activities were assayed.

TABLE 7. Intradiol cleavage activities of the CDs of *Rhodococcus* sp. AN-22 for catechols

Substrate	Relative activity (%) CD from	
	aniline-grown cells	L-malate-grown cells
Catechol	100	100
3-Methylcatechol	74	71
4-Methylcatechol	86	87
3-Chlorocatechol	0	0.7
4-Chlorocatechol	0	1.0
3-Fluorocatechol	0	0
Pyrogallol	+[a]	+[a]
Protocatechuic acid	0	0

[a] A value was not calculated because the molar extinction coefficient of the product derived from this substrate was not obtained.

FIG. 1. , Matsumura et. al

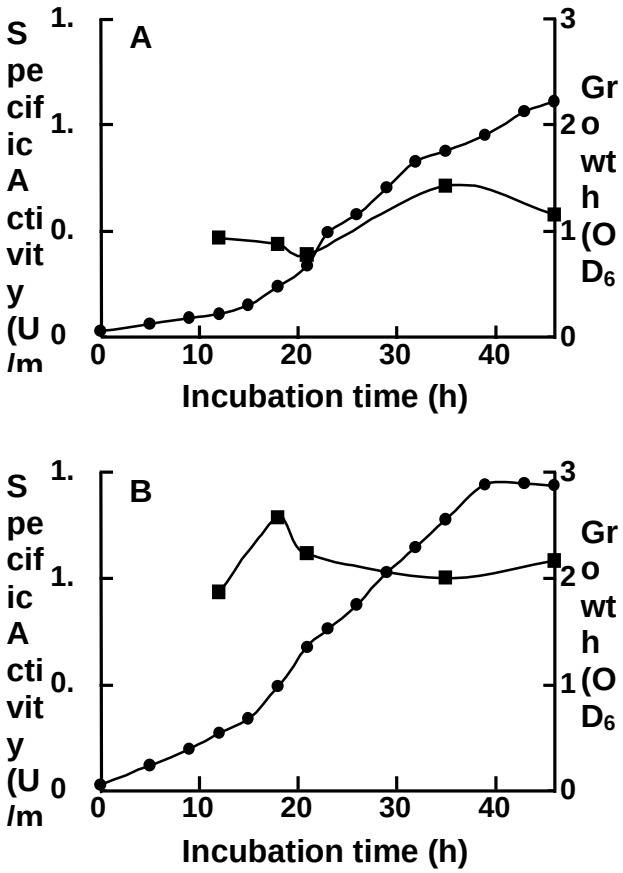

FIG. 2. , Matsumura et al.