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The Metabolism of Aromatic Compounds by Thermophilic *Bacilli*

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ABSTRACT

A range of thermophilic *Bacilli* were screened for the ability to grow on aromatic compounds. Five out of ten of those studied were able to utilize aromatic acids as a sole carbon source. Other *Bacilli* were purified after enrichment on aromatic compounds. One of these isolates, a strain of *Bacillus stearothermophilus*, degraded both phenol and benzoic acid. Phenol degradation proceeded via catechol, and thereafter by oxidative and nonoxidative *meta*-cleavage routes. The catalytic properties of cell-free extracts displaying the activities of the initial oxygenases have been described.

Index Entries: *Bacillus stearothermophilus*; thermophile; aromatic; degradation; oxygenase.

INTRODUCTION

The metabolism of aromatic compounds in *Bacillus* species has not been extensively studied. However, Crawford (*1*), showed that 10% of soil microbes were aerobic spore formers, and that 1% of these could grow on a minimal medium with *m*-hydroxybenzoate as the sole carbon source.

There have been reports on the metabolism of aromatic compounds by *Bacilli*. For example, in one strain, nicotinic acid appeared to be degraded via 2,3,6-trihydroxypyridine, to maleamic acid and fumarate (*2*). Also a species capable of metabolizing alkylbenzene sulfonate detergents has been studied (*3–5*). These compounds were metabolized to either homoprotocatechuate via *p*-hydroxyphenylacetic acid, or protocatechuate via *p*-hydroxybenzoic acid. The homoprotocatechuate and protocate-

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chuate intermediates were further degraded by *ortho* and *meta* fission, respectively.

Crawford (*6*) isolated three *Bacillus* strains that grew on *p*-hydroxybenzoate by different pathways. *B. brevis* PHB2 utilized the *ortho* fission of protocatechuate, *B. circulans* oxidized protocatechuate by a novel *meta*-2,3 fission reaction, and *B. laterosporus* PHB7a used the nonglutathione-dependent gentisate pathway. Another *Bacillus* species was isolated that would grow on the lignin related compound vanillate. It was tentatively proposed that this was metabolized via guaiacol and catechol (*7*).

The versatility of this genera was further demonstrated by the isolation of a *B. brevis* strain that would utilize 5-chlorosalicylate as a sole carbon source (*8*). This strain possessed a novel 5-chlorosalicylate 1,2-dioxygenase, which would also attack other 5-halogenated salicylate compounds, but not gentisate, protocatechuate, or catechol.

Thermophilic *Bacilli* capable of growing on aromatic compounds have also been reported. Eleven out of twenty-six strains of *Bacillus stearothermophilus* that were isolated in geothermal zones, heated by phenol containing gases, were able to metabolize the aromatic (*9*). Buswell (*10,11*) studied the metabolism of phenol and the cresols in a strain of *B. stearothermophilus* that utilized oxidative and hydrolytic cleavage routes for catechol and methylcatechol degradation. In another thermophilic *Bacillus* (*12*), *p*-hydroxybenzoate was degraded via gentisate.

In the present work we show that the ability to degrade aromatic compounds is widespread in thermophilic *Bacilli*.

METHODS

Bacterial Strains

The following strains were a gift from Dr. Richard Sharp, PHLS Centre for Applied Microbiology Research, Porton Down, Salisbury, Wiltshire, UK: *Bacillus stearothermophilus* strains ATCC 8005, ATCC 10149, and NCTC10003; *B. coagulans* ATCC 8038; *B. thermodenitrificans* DSM 465 and the *Bacillus* strains RS20, RS173, and RS53. *B. stearothermophilus* NCA1503 was obtained from Sam Amarty of this department.

Enrichment and Isolation of Strains Utilizing Aromatic Compounds

The following strains were isolated from soil samples (1 g of soil in 4 mL distilled water) that had been pasteurized for 10 min at 80°C. Strains IC1 and IC2 were enriched and isolated on the media shown below with benzoate (5 m*M*) as the main carbon and energy sources. Strain IC3 was enriched on *m*-cresol (2 m*M*) but finally isolated using phenol (2 m*M*). Strain IC2 was identified as a thermophilic *Bacillus*, and IC3 as *Bacillus stearothermophilus* atypical in growing at pH 5.7, by the National Collec-

tions of Industrial and Marine Bacteria Ltd., Torry Research Station, PO Box 31, 135 Abbey Road, Aberdeen, Scotland.

Storage Conditions

All strains were stored at −20°C in 12.5% glycerol.

Media

The medium of Buswell and Clark (*12*, Table 1) was used for the growth of *Bacillus stearothermophilus* ATCC 10149 and *B. thermodenitrificans* DSM 465. The same medium, with no casamino acids, supported the growth of *Bacillus stearothermophilus* NCTC 10003, *B. coagulans* ATCC 8038, and the *Bacilli* strains RS20, RS173, RS53, and IC2. Another variation on the medium of Buswell and Clark was developed for *B. stearothermophilus* IC3, 40 m*M* tris buffer was added, the yeast extract concentration was lowered to 0.0375 g/L, the casamino acids were omitted, and 0.0375 g/L brain heart infusion was added. The medium of Jamaluddin (*13*) was employed for growth of *B. stearothermophilus* NCA 1503 and the unidentified strain IC1. No medium supported growth without an added carbon source.

Following the report of Rowe et al. (*14*) that growth of *Bacillus stearothermophilus* NCA 1503 on solid media was inhibited by phosphate

Table 1
Components of Medium
Used by Buswell and Clark

Salts[a,b]	g/L
K_2HPO_4	0.5
NH_4Cl	1.0
$MgSO_4 \cdot 2H_2O$	0.02
Yeast extract	0.2
Casamino acids	0.1
Trace elements	
NaCl	10.0
$CaCl_2 \cdot 2H_2O$	6.5
$MnSO_4 \cdot 4H_2O$	0.4
$ZnSO_4 \cdot 7H_2O$	0.01
$CuSO_4 \cdot 5H_2O$	0.04
$CoCl_2 \cdot 6H_2O$	0.04
$NaMoO_4 \cdot 2H_2O$	0.05
$FeSO_4 \cdot 4H_2O$	0.4

[a]The salts were adjusted to pH 7.2, and used at the concentration shown. 1 mL of the trace element solution was added per L of salts.

[b]The salts and trace elements were autoclaved separately.

concentrations greater than 5 m*M* when any of the above media were required in solid form, the phosphate content was lowered to 5 m*M* and 40 m*M* tris was added. The yeast extract and brain heart infusion concentrations in the media were halved for *B. stearothermophilus* IC3. All media were solidified with 0.75% w/v Oxoid Bacterial Agar No. 1.

Carbon sources, unless indicated otherwise, were used at the following concentrations; aromatics and glucose 5 m*M*, and ethanol 25 m*M*.

Pseudomonas putida mt-2 was grown in R medium (*15*) at 30°C with 2.5 m*M* *p*-toluate as the sole carbon source.

Growth Conditions

Liquid cultures were incubated at 55°C in a shaking incubator, rotating at 200 rpm. 40, 200, and 800 mL of media were used in 100 mL, 200 mL, and 2L conical flasks, respectively.

Solid media were also incubated at 55°C, the Petri dishes being wrapped in plastic bags to reduce evaporation.

Chemicals

Chemicals were obtained from major manufacturers and phenolics were purified twice by resublimation if solid, or a double vacuum distillation if liquid. NADase was obtained from the Sigma Chemical Company Ltd., Poole, Dorset, UK.

Whole Cell Experiments

Washed samples of whole cells were prepared by harvesting at the mid-log growth phase and washing twice in 100 m*M* Tris buffer, pH 7.5 (at room temperature), preheated to 55°C. Cells were spun down at 20,000g for 2 min, resuspended, and held at 55°C awaiting use. A suitable dilution of cells was then added to a Clark oxygen electrode chamber (Yellow Springs Instruments), heated to 55°C for amperometric assays. All whole-cell O_2 uptakes shown are the average of three determinations that have been corrected for an endogenous oxygen uptake of 120 nmol of oxygen/mg of cells/min.

Preparation of Cell-Free Extracts

An overnight culture (200 mL) was used to inoculate 800 mL of media, the cells were grown, and then harvested at the mid-log growth phase. These were washed twice in 50 m*M* phosphate buffer, pH 7.0, at room temperature. For extracts where catechol 2,3-dioxygenase activity was required, the cells were resuspended in 50 m*M* phosphate buffer, pH 7.0, at 4°C containing 10 mg/mL bovine serum albumin, 10% acetone, and 100 μM phenylmethylsulfonyl fluoride. When phenol hydroxylase activity was required, the cells were resuspended in the above without the acetone, and with 1 m*M* dithiothreitol added.

The resuspended cells were then sonicated for 15 s, with intermittent cooling to 4°C, for a total of 3 min. The extracts were subsequently clarified by centrifugation at 35,000*g* for 40 min at 4°C. The cleared extracts were stored at 4°C. Under these conditions the catechol 2,3-dioxygenase and phenol hydroxylase activities had half-lives of 15 and 19 d, respectively.

NADase treated cell-free extracts were produced by adding 0.05 U of NADase per mL of extract and incubating at 37°C for 30 min.

Enzyme Assays

All enzyme rates shown are the averages of at least two determinations. When an oxygen electrode was not used, enzymes were assayed in the following manner using a Shimadzu UV240 recording spectrophotometer at 55°C. Accurate temperature measurements in cuvets were obtained using a thermocouple wire.

Phenol hydroxylase was assayed by following the rate of oxidation of 200μM NADH at 340 nm in the presence of 1 m*M* phenol, and subtracting the rate of the same mixture before the addition of phenol.

Catechol 2,3-dioxygenase was assayed by following the production of its ring fission products 2-hydroxymuconic semialdehyde (2 HMS), 2-hydroxy-6-oxohepta-2,4-dienoic acid and 2-hydroxy-5-methylmuconic semialdehyde at 375, 386, and 382 nm, respectively. Experiments with heat-treated cell-free extracts of *Pseudomonas putida* mt-2 at pH 7.0, produced by the methods of Bayly et al. (*16*), were used to deduce spectrophotometric data for the ring fission products at this pH. With these extracts the following coefficients were calculated: 2 HMS, 27,900; 2-hydroxy-6-oxohepta-2,4-dienoic acid, 7600; and 2-hydroxy-5-methylmuconic semialdehyde, 20,200. The wavelengths of maximum absorbance were unchanged.

HPLC analysis was performed with a Spherisorb 10 C18 column, using the mobile phase of Schmidt and Knackmuss (*17*). The effluent, which had a flow rate of 1 mL/min, was monitored at a wavelength of 210 nm. Under these conditions NAD and NADH, catechol and phenol had retention times of 2.70, 3.98, and 4.95 min, respectively.

The cold cell-free extract was incubated with the salt (1 m*M*) for 5 min before use to determine the effect of metal salts on the activity in enzyme essays.

Michaelis Constants

Apparent Michaelis constants for aromatic compounds were obtained by measuring the initial reaction rates at various aromatic concentrations, and at a temperature of 55°C. The constants for oxygen were measured by adding 1 m*M* aromatic to an extract sample in an oxygen electrode. The tangent of the curve obtained at various oxygen concentrations was then used for the appropriate plot. For phenol hydroxylase,

a control was performed with NADH only, and the reaction rates at similar oxygen concentrations subtracted from the rates in the presence of phenol. The constants for oxygen were an average of three plots: a Lineweaver-Burke plot ($1/v$ against $1/S$), a Haynes plot (S/v against S), and an Eadie-Hofstee plot (v against v/S). The catechol constants were derived from plots of S/v against S.

RESULTS

Aromatic Carbon Sources for Thermophilic Bacilli

The results of experiments to determine the ability of thermophilic *Bacilli* to grow in semidefined media, with aromatic compounds (2 and 5 m*M*) as the main carbon and energy sources, are summarized in Table 2. Additional carbon sources on which no strains grew were; *o*-, *m*- and *p*-toluate, *o*-, *m*-, and *p*-cresol, *o*-, and *m*-hydroxybenzoate, orcinol, resorcinol, *p*-cumate, D-phenylalanine, D- or L-tyrosine, and D- or L-tryptophan. Catechol, its monomethyl and monocarboxy substituted derivatives, and gentisate were all unstable at 55°C, and were not used in the above experiments.

No growth was observed with any of the following strains in the presence of any aromatic compound; *Bacillus stearothermophilus* strains NCA 1503, NCTC 10003, ATCC 10149, *Bacillus coagulans* ATCC 8038, and *Bacillus thermodenitrificans* DSM 465.

Although *Bacillus stearothermophilus* IC3 failed to grow on any of the cresol isomers in initial experiments, further studies have shown that all

Table 2
Aromatic Carbon Sources for Thermophilic Bacilli

Carbon source	*B. stearothermophilus* ATCC 8005	*B. caldovelox*	*Bacillus* strain RS20	*Bacillus* strain RS173	*Bacillus* strain RS53	Strain IC1	*Bacillus* strain IC2	*B. sterarothermophilus* IC3
Benzoate	−	−	−	−	+	+	+	+
p-Hydroxybenzoate	−	−	−	−	+	+	+	−
Phenylacetate	+	+	+	+	−	+	+	−
p-Hydroxyphenylacetate	+	−	+	−	+	+	+	−
L-Phenylalanine	−	+	−	+	−	+	+	−
Phenol	−	−	−	−	−	−	−	+

(+), indicates growth.
(−), indicates no growth.

three isomers were completely metabolized when 25 m*M* ethanol was added to the media. Ethanol does not appear to induce the initial enzymes required for phenol/cresol metabolism.

Delineation of the Degradative Pathways for Aromatic Compounds in Bacillus Stearothermophilus IC3

Whole Cell Experiments

The results of experiments showing the stimulation of oxygen uptake by washed whole cells of *B. stearothermophilus* IC3 are summarized in Table 3. No stimulation of oxygen uptake was detected subsequent to the addition of any of the following compounds; *o*-, or *m*-hydroxybenzoate, phenylacetate, *p*-hydroxyphenylacetate, orcinol, resorcinol, D- or L-phenylalanine, D- or L-tyrosine, D- or L-tryptophan, protocatechuate, 2,3-dihydroxybenzoate and gentisate.

Phenol grown cells used 2.02 mols oxygen/mol phenol added, and 1.03 mols of oxygen per mole of catechol added.

Phenol Metabolism by Cell Free Extracts

Further evidence for the conversion of phenol to catechol was provided by cell-free extracts. HPLC analysis of cell-free extracts incubated with 1 m*M* phenol and 1 m*M* NADH at 55°C showed the appearance of a new peak that had the same retention time as catechol.

The phenol hydroxylase enzyme is specific for NADH as a cofactor, no phenol-induced oxygen uptake was observed in its absence, or in the presence of NADPH. The background NADH oxidase activity was too

Table 3
Oxygen Uptake[a] by Whole Cells of Strain IC3

	Growth substrate		
Aromatic added[b]	Phenol	Benzoate	Glucose
Phenol	391	0	0
Benzoate	0	296	0
o-Cresol	368	218	0
m-Cresol	326	203	0
p-Cresol	272	169	0
o-Toluate	0	323	ND[c]
m-Toluate	0	155	ND
p-Toluate	0	137	ND
p-Hydroxybenzoate	0	82	ND
p-Cumate	0	35	ND
Catechol	964	803	0
3-Methylcatechol	784	799	0
4-Methylcatechol	1028	865	0

[a]Oxygen uptakes are expressed as nanomoles of oxygen per milligram of cells per minute.
[b]Aromatics added to a final concentration of 1 m*M*.
[c]ND = Not determined.

high to allow measure of the stoichiometry of NADH utilization. However, from the whole cell results, oxygen would appear to be used in a ratio of 2:1 with phenol and 1:1 with catechol. The phenol is therefore converted to catechol by a hydroxylase enzyme.

The results in Table 4 show the rate of production of ring fission products from catechol and 3- and 4-methylcatechol. The ratio of oxygen: catechol used by these extracts was 0.95:1, with 99% recovery of 2-hydroxymuconic semialdehyde from catechol.

The spectrophotometric data shown in Table 4 are consistent with the ring fission products of catechol, 3-methylcatechol, and 4-methylcatechol being 2-hydroxymuconic semialdehyde, 2-hydroxy-6-oxohepta-2,4-dienoic acid, and 2-hydroxy-5-methylmuconic semialdehyde, respectively. These results indicate that a catechol 2:3-dioxygenase enzyme is present.

The rates of degradation of the ring fission products, and the effect of NAD upon these rates, are summarized in Table 5.

Properties of Phenol Hydroxylase in Cell-Free Extracts

The effects of temperature on the phenol hydroxylase activity are shown in Fig. 1. The enzyme is most active at the growth temperatures of the strain. This activity is optimal over the range of 50–62°C. The activity is markedly lower on either side of this range, and undetectable below 30° or above 70°C.

The apparent Michaelis constants of phenol hydroxylase are 42μm for oxygen, and 2.0 μm for phenol.

The results in Table 6 show the effects of various inhibitors on phenol hydroxylase, PCMB inhibits at concentrations as low as 0.01 m*M*, despite the presence of equal amounts of DTT in the extracts.

In view of the inhibition of the phenol hydroxylase enzyme by various metal chelators, a range of metal salts was incubated with the crude extracts, and the enzyme reassayed (Table 7). In the presence of $MnSO_4$, a near twofold stimulation of activity was seen.

Table 4
Rates of Production and Yield of Ring Fission Products in Cell-Free Extracts

	Substrate		
	Catechol	3-Methyl catechol	4-Methyl catechol
Wavelength of maximum absorbance, nm	375	388	382
Rate of peak appearance[a]	67	39	44
Yield, %[b]	99	113	101

[a]Reaction mixtures contained 50 μL of cell-free extract in 1 mL of phosphate buffer. Rates of appearance are expressed as μmol/min/mL.

[b]Yield represents % recovery of ring fission product from substrate.

Table 5
Rates of Disappearance of Ring Fission Products

Ring fission product	Cell-free extract[a]	NAD-ase-treated Extracts	
		In absence of NAD	In presence of NAD[b]
2-HMS	1.25	0.98	19.9
2-Hydroxy-6-oxohepta-2,4-dienoic acid	1.38	1.19	1.26
2-Hydroxy-5-methyl muconic semialdehyde	0.36	0.30	17.0

[a]Reaction mixtures contained 100 μL of cell-free extract and 0.9 mL of phosphate buffer. Rates are expressed as nmols/min/mL.
[b]Where indicated, 1 μmol of NAD was added to 1 mL of reaction mixture.

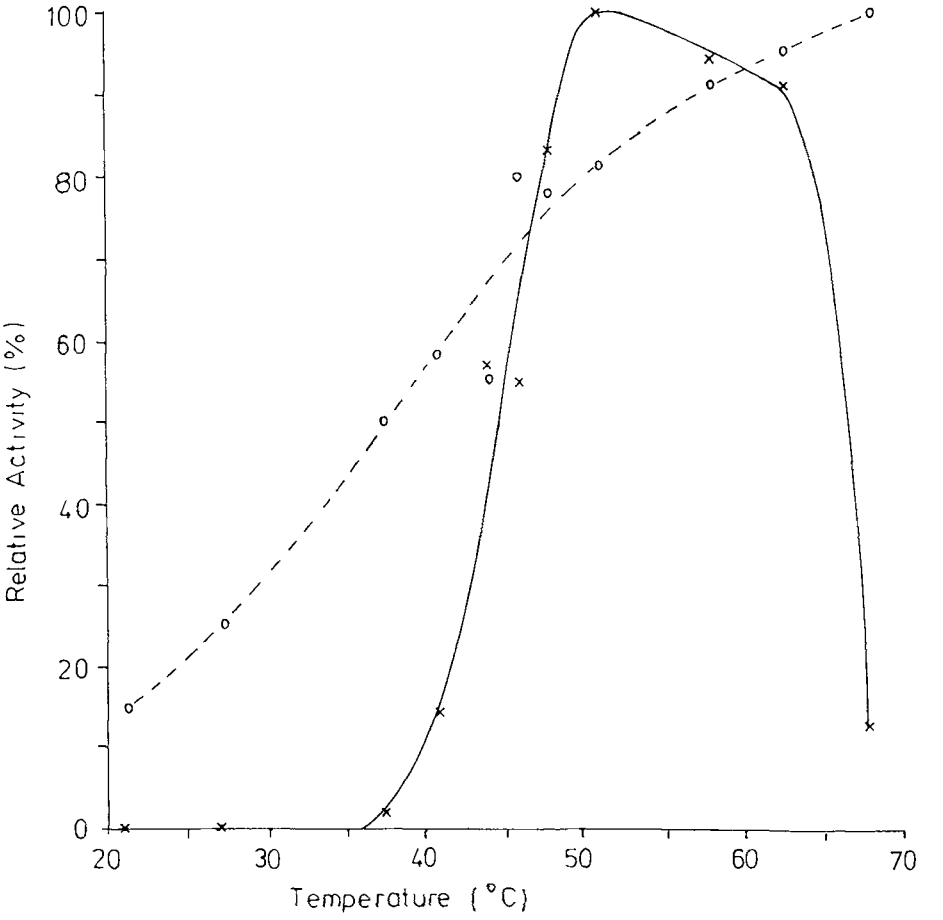

Fig. 1. Effect of temperature on relative activities of phenol hydroxylase (X) and NADH oxidase (O).

Table 6
Effect of Inhibitors on Phenol Hydroxylase and NADH Oxidase

Inhibitor	Concentration, m*M*	NADH oxidase activity,[b] %	Phenol Hydroxylase activity, %
None	—	100	100
PCMB[a]	0.01	91	57
o-Phenanthroline	1	146	15
DDCA[a]	1	123	0
EDTA	1	135	87
Na azide	1	88	73
KCN	1	48	119
Rotenone	1	5	210
2,2'Bipyridine	1	119	52

[a]Abbreviations: PCMB, = *p*-chloromercuribenzoate; DDCA = diethyldiaminocarbamic acid.

[b]The background NADH activity was three times that of the phenol hydroxylase activity.

[c]Reaction rates were measured spectrophotometrically.

The results in Table 8 show that the disappearance of NADH is stimulated by a wide range of substituted phenols, and the simultaneous disappearance of the aromatic substrate was established by HPLC analysis. Therefore, a complete uncoupling of NADH oxidation from hydroxylation, as observed with other flavoprotein hydroxylases (*18–20*), was not occurring. However, partial uncoupling cannot be ruled out. Attempts to isolate the products of hydroxylation, with the exception of catechol, have not been successful.

Properties of Catechol 2,3-Dioxygenase in Cell-Free Extracts

The stability of catechol 2,3-dioxygenase, and its activity at different temperatures, are shown in Fig. 2 and Table 9. Also shown in Table 9 are the stabilities of catechol 2,3-dioxygenase from *Pseudomonas putida* (*21*).

Table 7
Reaction Rates[a] of Phenol Hydroxylase and NADH Oxidase in the Presence of Metal Ions

Salt added	NADH oxidase activity, %	Phenol hydroxylase activity, %
None	100	100
$CuSO_4$	169	0
$FeSO_4$	69	119
$Fe(NO_3)_3$	79	93
$MnSO_4$	105	181

[a]Reaction rates were measured spectrophotometrically.

Table 8
Reaction Rates of Phenol Hydroxylase with Substituted Phenols[a,b]

Aromatic added	Phenol hydroxylase activity, %
Phenol	100
o-Cresol	136
m-Cresol	143
p-Cresol	169
o-Fluorophenol	76
m-Fluorophenol	65
p-Fluorophenol	72
o-Chlorophenol	84
m-Chlorophenol	78
p-Chlorophenol	74
o-Methoxyphenol	211
m-Methoxyphenol	160
p-Methoxyphenol	175

[a]Substrates were added to 1 m*M* final concentrations.

[b]Reaction rates were measured spectrophotometrically.

From these results it is apparent that the enzyme is active at the growth temperature (55°C). In fact, the maximum activity is seen at 60°C, slightly higher than the growth temperature, although the enzyme is more stable at the lower of the two.

The apparent Michaelis constants of catechol 2,3-dioxygenase from strain IC3, for oxygen and catechol, are 38 and 0.32 μ*M*, respectively.

The inhibitors *p*-chloromercuribenzoate, at a concentration of 0.01 m*M*, and *o*-phenanthroline, EDTA, azide, cyanide and 2,2'bipyridine at a concentration of 1 m*M*, had no significant effect on catechol 2,3-dioxygenase activity. A slight increase in activity (13%) was observed with the copper chelator diethyldiaminocarbamic acid (1 m*M*), which may have resulted from the chelating of interfering metal ions.

DISCUSSION

Thermophilic *Bacillus* species that grow on aromatic compounds appear to be numerous. Of ten organisms screened that were not specifically enriched on aromatic media, half would utilize aromatic acids as main carbon and energy sources. Strains metabolizing aromatic compounds were also readily isolated from soil samples. One of these isolates, *B. stearothermophilus* IC3, was the only organism screened that was able to use phenolics.

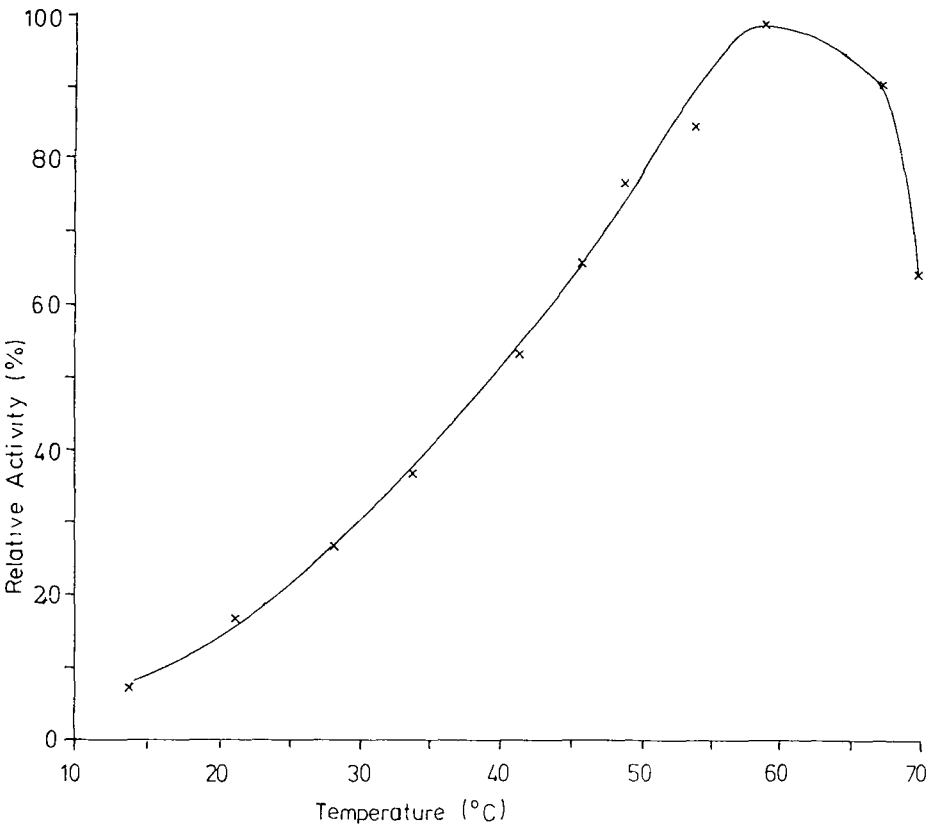

Fig. 2. Effect of temperature on relative activity of catechol 2,3-dioxygenase.

Phenol Metabolism by B. stearothermophilus IC3

The results in Table 3 indicate that phenol and benzoate are metabolized via catechol, the uptake of oxygen in the presence of cresols probably being a result of the relaxed substrate specificity of the initial hydroxylase. Subsequently, a NADH-dependent phenol hydroxylase activity has been demonstrated in cell-free extracts. Although the metabolism of

Table 9
Half Lives of Catechol 2,3-Dioxygenases

B. stearothermophilus		*P. putida*	
Temperature, °C	Half life, min	Temperature, °C	Half life, min
55	16.0	55	73.8
60	9.5	63	21.1
65	6.0	65	18.0
70	2.3	68	14.7
75	2.2	71	6.1

benzoate has not been extensively studied, a catechol 2,3-dioxygenase activity has been observed in cell-free extracts produced from benzoate grown cells, with a 100% yield of 2-hydroxymuconic semialdehyde from catechol.

Phenol hydroxylase from *B. stearothermophilus* IC3 has an apparent Michaelis constant for oxygen of 42 μ*M*, which is comparable to the approximate constant of 53 μ*M* for the phenol hydroxylase from *Trichosporon cutaneum* (*22*). However, the apparent Michaelis constant for phenol oxidation in the *Bacillus* was an order of magnitude lower than that of the yeast, 2.0 μ*M* compared to 18 μ*M*.

The results in Table 6 show that the phenol hydroxylase is sensitive to a number of inhibitors. *p*-Chloromercuribenzoate inhibits the phenol hydroxylase at a concentration as low as 0.01 m*M* despite the presence of equal amounts of DTT in the extracts. Inhibition by PMCB was also observed for the yeast enzyme, but metal chelators had no effect. The results in Table 7 show that both cupric ions, and the copper chelator diethyldiaminocarbamic acid, completely inhibited the enzyme. This may have been a result of nonspecific binding by the chelator.

Crude extracts of another phenol hydroxylase from a *B. stearothermophilus* strain were studied by Buswell (*11*). This was also inhibited by PCMB and *o*-phenanthroline, and slightly by diethyldiaminocarbamic acid. The *T. cutaneum* phenol hydroxylase was also inhibited by the cupric ion chelator.

The results in Table 8 show that a range of substituted phenols stimulated NADH uptake in the crude extracts. A wide substrate range was also observed in the phenol hydroxylase from *T. cutaneum* (*22*), and *Brevibacterium fuscum* (*23*).

It is interesting to note that the heat stability of the *Pseudomonas putida* mt-2 catechol 2,3-dioxygenase activity (*21*) is greater than that observed for strain IC3. The heat stability of this enzyme, even in mesophiles, is well known (*24*).

The apparent Michaelis constants for catechol 2,3-dioxygenase from strain IC3, for oxygen and catechol, are 38 μ*M* and 0.32 μ*M*, respectively. The constant for oxygen is considerably higher than that of 7.0 μ*M* for the purified enzymes from *P. putida* mt-2 and *P. arvilla* (*25,26*). The Michaelis constant for the aromatic compound is an order of magnitude lower in the thermophile, 0.32 μ*M* compared with 3.0 μ*M*.

The resistance of catechol 2,3-dioxygenase to inhibitors is in marked contrast to the results obtained with the same enzyme from *P. arvilla*. This enzyme was completely inhibited by *o*-phenanthroline and 2,2′-bipyridine.

The results in Table 5 indicate that strain IC3 possesses both oxidative and nonoxidative routes for the further metabolism of ring fission products. The oxidative enzyme has activities towards the fission products that are similar to the 2-hydroxymuconic semialdehyde dehydrogenases of *B. stearothermophilus* PH24 (*10*), Azotobacter (*27*), and phenol

grown *Pseudomonas putida* (*28*). The nonoxidative enzyme of strain IC3 shows less selectivity toward 2-hydroxy-6-oxohepta-2,4-dienoic acid than the nonoxidative activity in strain PH24 (*10*) and the 2-hydroxy-6-oxohepta-2,4-dienoate hydrolases of *Pseudomonas putida* mt-2 (*29*) and *Alcaligenes eutrophus* strain 345 (*30*).

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