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Metabolism of dimethylterephthalate by *Aspergillus niger*

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Abstract

Aspergillus niger (AG-1) metabolized dimethylterephthalate through monomethylterephthalate, terephthalate and protocatechuate. Degradation of dimethylterephthalate was followed by extraction of residual dimethylterephthalate from the spent medium. The quantitative UV analysis showed that 58% of the dimethylterephthalate supplement was taken up in 144 h. The metabolites were isolated from resting cell cultures. Thin layer chromatography analysis of the extract revealed the presence of two intermediates, monomethylterephthalate and terephthalate. Use of an inhibitor in resting cell culture experiment demonstrated the accumulation of protocatechuate. The time course of protocatechuate acccumulation was also studied. Metabolites were identified by employing various physicochemical methods. Enzyme studies using cell-free extracts exhibited dimethylterephthalate esterase and protocatechuate dioxygenase activities. Protocatechuate was oxidized by the *meta* cleavage pathway. A tentative pathway for the degradation of DMTP has been proposed in *A. niger*.

Abbreviations: A. niger – Aspergillus niger (AG1), DMSO – dimethyl sulfoxide, DMTP – dimethylterephthalate, MMTP – monomethylterephthalate, MS – mass spectra, NMR – nuclear magnetic resonance spectra, PCA – protocatechuate, TLC – thin layer chromatography, TP – terephthalate, UV – ultra violet spectra

Introduction

Phthalate esters have found enormous application in the plastic and textile industries. Their residues enter the environment in the form of industrial sewage and effluents. Hence these compounds have been labelled as environmental pollutants (Keith & Telliard 1979; Lawrence & Tuell 1979). The toxic nature of phthalate esters has been investigated and they are considered to be teratogenic to man and animals (Shiota & Nishimura 1982). Bacterial degradation of phthalate esters has been investigated by several workers (Keyser et al. 1976; Engelhardt et al. 1975, 1977; Karegoudar & Pujar 1984). Among the phthalate esters, dimethylterephthalate has received considerable interest since it is extensively used in the textile industry (Slizen et al. 1989; Sivamurthy & Pujar 1989). Fungal studies on phthalate esters recently have been reported by Sivamurthy et al. (1991). These investigators have observed the transformation of DMTP to MMTP and TP by the fungus, *Sclerotium rolfsii*. However, this strain did not show complete degradation of DMTP. The present study was undertaken to show the mode of degradation of DMTP in *Aspergillus niger*.

Materials and methods

Organism and growth conditions

Aspergillus niger (AG-1) maintained in our laboratory culture collection was used for the degradation studies. The organism was grown on a synthetic medium (Byrde et al. 1956) supplemented with 0.1% DMTP. This medium was distributed in 500 ml Erlenmeyer flasks (100 ml per flask) and sterilized by autoclaving at 120° C for 15 min. Stock cultures were maintained on the slants of the same medium solidified with 2%

agar. The flasks were inoculated with a heavy suspension of spores in sterile water, obtained from 72 h old agar slants. The flasks were incubated on rotary shaker at 100 rpm at 35 ± 2° C for 72 h. The mycelia were separated by filtering through sterile cheese cloth. The mycelia so obtained were used for the preparation of cell-free extracts and in resting cell culture experiments.

Resting cell culture of A. niger

Mycelial pellets of *A. niger* at various phases of growth, were harvested and suspended in 100 ml of phosphate buffer (0.025 M) pH 5.5 containing 0.1% DMTP. For the isolation of the dihydroxy compound, α,α-dipyridyl (5 mM/ml) was used with 0.1% TP. The flasks were incubated on rotary shaker at 100 rpm at 35 ± 2° C for 12 h. The metabolites formed were analysed at regular intervals.

Isolation of metabolites

Metabolites of the degradative pathway were isolated from the resting cell culture of *A. niger*. Mycelial pellets of *A. niger* were removed by filtration. The filtrate was acidified to pH 2 with 4 N HCl. The metabolites were extracted three times with ethylacetate. The extracts were dried over anhydrous sodium sulphate, concentrated in vacuo on a rotary evaporator and subjected to TLC analysis.

Characterisation of metabolites

The metabolites were purified and identified by TLC using following solvent systems: (A) benzene:ethylacetate:acetic acid (20:40:1 v/v) and (B) benzene:DMSO:acetic acid (80:5:2 v/v). The aromatic compounds were detected on the chromatogram by UV light. Phenolic compounds were identified by spraying with 1:1 mixture of 1% potassium ferricyanide and 2% ferric chloride solution. Aromatic acids were recognised by spraying with 1% bromocresol green in ethylalcohol. The compounds from the chromatogram were eluted with ethylalcohol and were identified by recording their UV spectrum using a Hitachi UV-VIS spectrophotometer model 150-20. The NMR spectra were recorded using a NMR spectrometer model WH-270 and mass spectra were recorded using a JEOL model D-300 mass spectrometer.

Estimation of DMTP utilization by A. niger

The degradation of DMTP by *A. niger* was followed by extracting the residual DMTP from 100 ml of culture medium, which initially contained 0.1% DMTP. The medium was collected at different incubation periods and fungi were removed by filtration. The filterate was acidified to pH 2 with 4 N HCl and extracted with ethylacetate. Neutral and acidic fractions from the extracts were separated using 5% $NaHCO_3$ solution. TLC of neutral fraction showed the presence of DMTP alone which was estimated by quantitative UV analysis at 240 nm (λ_{max} of DMTP). A similar experiment in the absence of *A. niger* cultures was carried out to assess the loss of DMTP due to evaporation.

Time course for accumulation of protocatechuate

The time course for the accumulation of PCA as a transient intermediate was studied. The mycelia (72 h old) were inoculated into 100 ml synthetic medium supplemented with 0.1% TP and incubated on rotary shaker at 100 rpm at 35 ± 2° C. The concentration of PCA formed during various phases of growth was determined by aseptically withdrawing 1.0 ml of medium at different time intervals and extracting with diethylether. The PCA formed was estimated by the method of Nair & Vaidyanathan (1964).

Mode of ring cleavage of PCA

Ring cleavage of PCA was determined by the method described by Stanier et al. (1966) using mycelia of *A. niger*.

Preparation of cell-free extracts of A. niger

Mycelial pellets of *A. niger* (wet weight 5 g) grown on DMTP, TP, PCA and glucose were homogenized in a mortar with an equal weight of glass powder and extracted with 0.05 M phosphate buffer, pH 7.0. The resulting slurry was centrifuged at 13,200 × g for 20 min at 5° C. The supernatant was used as the crude enzyme preparation. Protein content of the cell-free extracts was determined by the method of Lowry et al. (1951).

Enzyme assays

PCA dioxygenase activity was determined spectrophotometrically by monitoring the decrease in absorbance

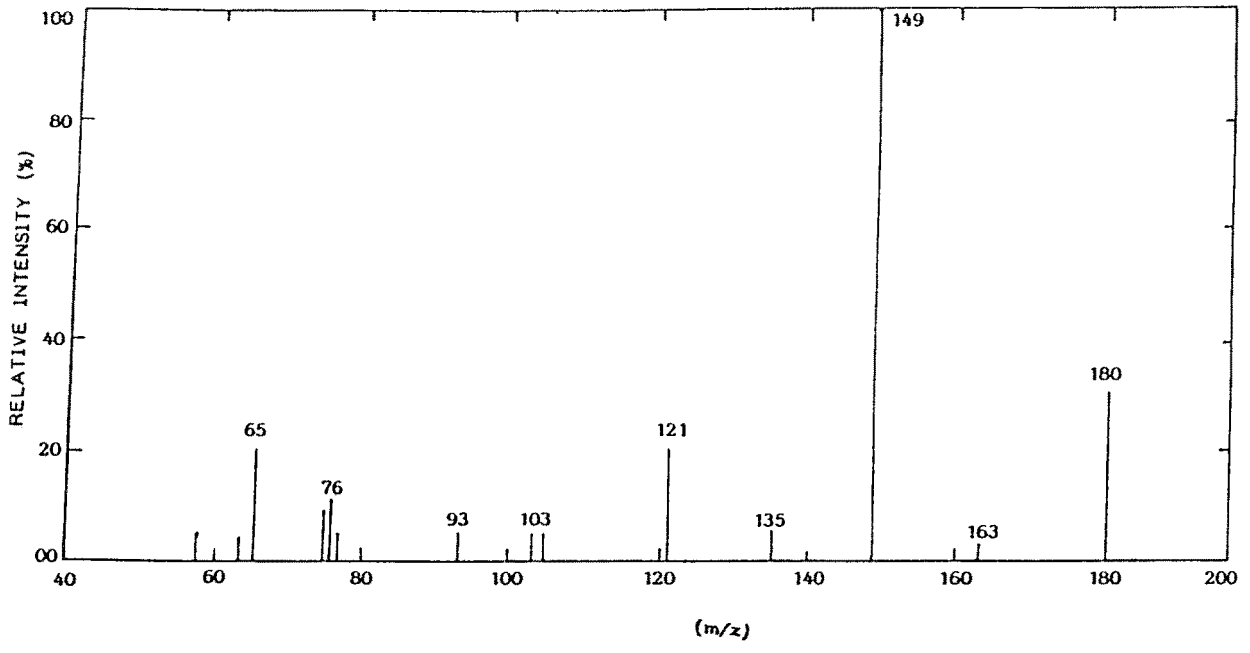

Fig. 1. Mass spectra of isolated monomethylterephthalate.

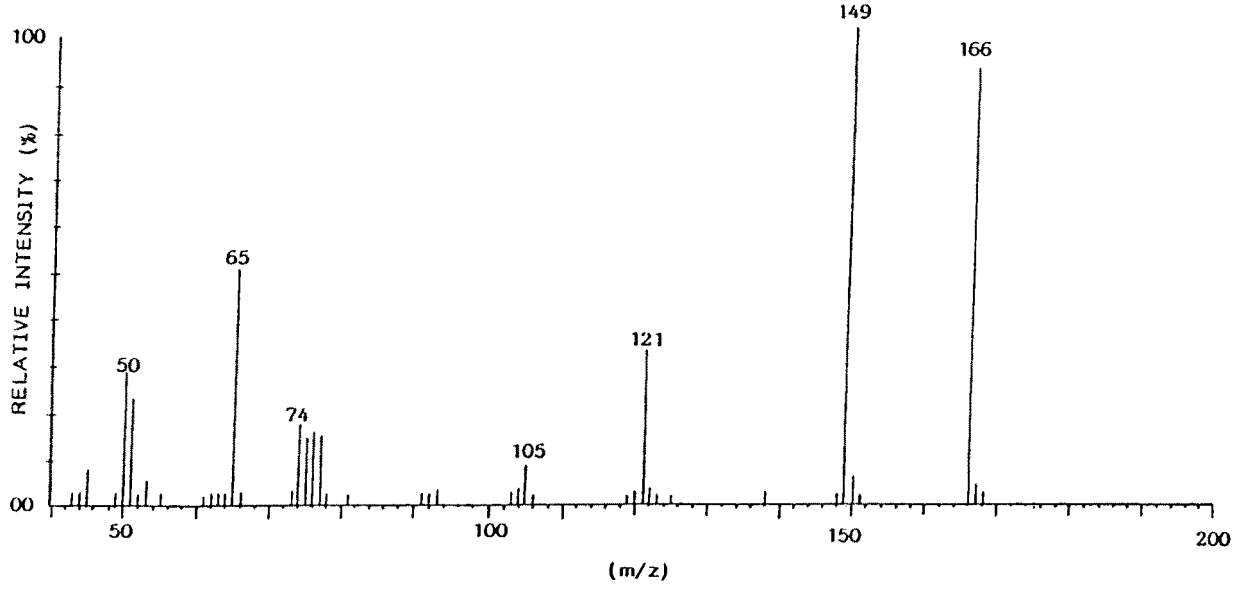

Fig. 2. Mass spectra of isolated terephralic acid.

at 290 nm due to the disappearance of PCA (MacDonald et al. 1954). The assay system in 3.0 ml contained PCA (2.0 μmol), phosphate buffer (pH 7.0; 200 μmol), and a suitable amount of crude enzyme in a cuvette of 1 cm light path at 30°C. DMTP esterase was assayed using ρ- nitrobenzylester of TP as substrate (Sivamurthy et al. 1991). The assay mixture (3 ml) contained ρ-nitrobenzylester of TP (5 μmol), phosphate buffer (pH 7.5; 50 mM), and suitable amount of crude enzyme in a 1 cm cuvette. The enzyme activity was measured

Table 1. Utilization of dimethylterephthalate by *Aspergillus niger*.

Period of growth (h)	Concentration of DMTP utilized (mg/ml)*
0	0.000
24	0.000
48	0.056
72	0.157
96	0.467
120	0.534
144	0.582

* Variable factor ± 0.002%.

Table 2. The R_f values, λ_{max} and melting point (M.P.) of the isolated and authentic compounds.

Compound	R_f values in solvent systems		λ_{max}	M.P.
	A	B	nm	° C
MMTP	a 0.58	1.00	239.6	230
	b 0.57	1.00	239.6	231
TP	a 0.37	0.36	238.0	300
	b 0.37	0.36	238.0	302
PCA	a 0.76	0.12	290.0	202
	b 0.76	0.12	290.0	201

a – authentic; b – isolated.

by monitoring the increase in absorbance at 276 nm due to the formation of ρ-nitrobenzylalcohol. Catechol dioxygenase activity was determined spectrophotometrically by monitoring the increase in absorbance at 375 nm due to the formation of 2-hydroxymuconic semialdehyde (Farr & Cain 1968). The assay mixture (3.0 ml) contained catechol (0.5 μmol), Tris-HCl buffer (pH 7.2; 300 μmol) and crude enzyme in a 1 cm cuvette. Protocatechuate non-oxidative decarboxylase activity was determined by the method of Kamath et al. (1987) using protocatechuate as substrate. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the transformation of one μmol of the substrate or the formation of one μmol of the product per minute under the standard assay conditions.

Results

Studies on degradation of DMTP by *A. niger* indicated that the utilization of DMTP commenced after about 48 h incubation and reached a maximum after 144 h

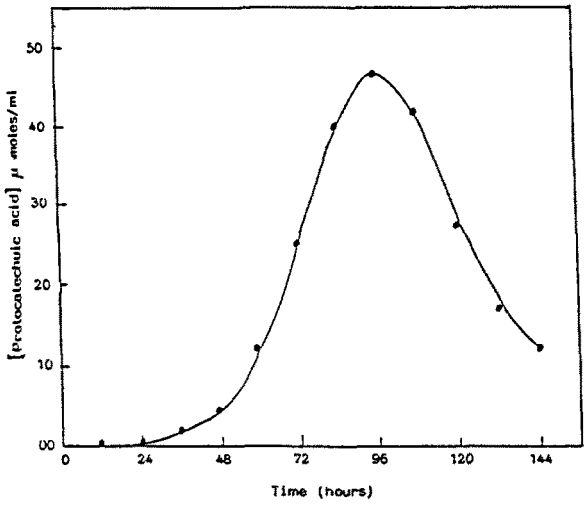

Fig. 3. Time course of accumulation of protocatechuate from TP by *Aspergillus niger*.

Table 3. Specific activities of enzymes.

Enzymes	Specific actitiby (U/mg of protein)			
	DMTP grown	TP grown	PCA grown	Glucose grown
DMTP esterase	35.65	ND	ND	ND
PCA dioxygenase	9.78	11.36	18.21	ND

ND – Not determined.

incubation. About 58% of the added DMTP was taken up by *A. niger* within 144 h incubation (Table 1).

A TLC examination of the metabolites isolated from the resting cell culture (0.1% DMTP as substrate), showed accumulation of two compounds (I & II). The R_f value and the λ_{max} of these two compounds corresponded with those of authentic MMTP and TP respectively (Table 2). The NMR spectrum of compound I showed the presence of carboxymethyl protons at 3,88 δ as a singlet. Four aromatic protons appeared as a singlet at 8.06 δ, and carboxyl protons displayed at 13.4 δ. Mass spectral analysis of this compound showed the parent ion peak at m/z 180 (Fig. 1) which is in good agreement with empirical formula $C_9H_8O_4$. The fragmentation pattern showed ion peaks at m/z: 163 base peak (M-OH), 149 (M-OCH_3), 135 (M-COOH) and 121 (M-$COOH_3$). The NMR and MS data of compound I corresponded with authentic MMTP. The NMR spectrum of compound II revealed the presence of four aromatic protons at 8.05 δ as a singlet and carboxyl protons at a higher field value. MS analysis showed

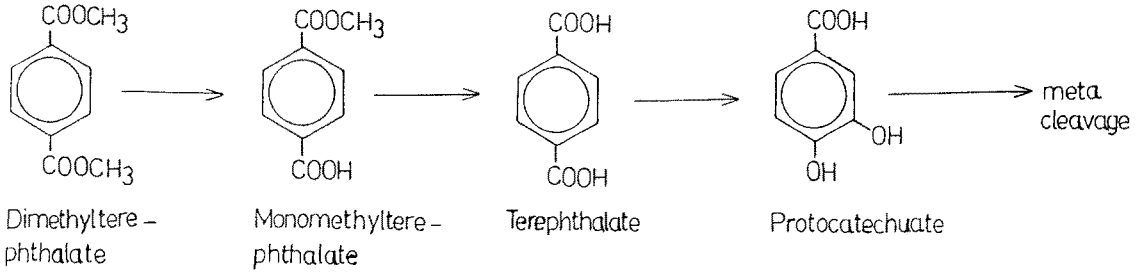

Fig. 4. Proposed pathway for the degradation of dimethylterephthalate by *Aspergillus niger*.

the parent ion peak at m/z 166 (Fig. 2) which is in good agreement with empirical formula $C_8H_6O_4$. The fragmentation pattern showed the ion peaks at m/z: 149 base peak (M-OH), 121 (M-COOH). The NMR and MS data of compound II were also found to be identical with authentic TP.

In order to accumulate the dihydroxy intermediate, 0.1% TP was used as a substrate with α,α-dipyridyl (5 mM/ml) as a dioxygenase inhibitor in a resting cell experiment. The results showed the accumulation of dihydroxy compound (III). The TLC analysis and UV spectrum of this compound III agreed with authentic PCA (Table 2).

A time course of the transient accumulation of PCA was carried out by estimating the PCA formed from TP at different time intervals. It was found that maximum accumulation of PCA occurred at 96 h incubation. Beyond 96 h PCA was rapidly degraded (Fig. 3).

The enzymes of DMTP degradative pathway are strictly inducible. DMTP esterase and PCA dioxygenase activity were not detected in the extracts of the mycelium grown on glucose alone. Also the cell-free extracts of the mycelium grown on TP and PCA did not exhibit DMTP esterase activity. DMTP esterase activity was detected only in cell-free extracts of *A. niger* grown on DMTP. PCA decarboxylase and catechol dioxygenase activity were not observed in *A. niger* (Table 3).

Studies on ring cleavage of PCA showed the formation of yellow product after incubation for 5 h at 37° C. The λ_{max} of this yellow product was found to be 410 nm at pH 9, which corresponded to λ_{max} of γ-carboxy-α-hydroxymuconic semialdehyde (Crawford 1975). The formation of γ-carboxy-α-hydroxymuconic semialdehyde indicated distal *meta* cleavage of PCA.

Discussion

This is the first report on the complete degradation of DMTP in fungal systems. The results provide evidence that DMTP is metabolized via, MMTP, TP and PCA in *A. niger*. The hydrolysis of DMTP to TP is catalyzed by DMTP esterase. Such a carboxyl esterase has been reported by several authors (Sivamurthy et al. 1991; Farkkinen 1980). The free acid formed is further degraded through PCA, which has been isolated as one of the intermediate. In certain other fungi, PCA is converted to catechol by the action of PCA decarboxylase (Cain et al. 1968).

In the present study *A. niger*, which was grown on DMTP, oxidized PCA but failed to oxidize catechol. The lack of catchol dioxygenase and PCA decarboxylase activities in *A. niger* precludes the possibility of conversion of PCA to catechol. Thus PCA is formed as terminal aromatic metabolite. Further ring cleavage studies indicated the operation of distal *meta* cleavage of PCA. A strain of *Penicillium* sp. converted PCA to pyruvate, suggesting the existence of *meta* type of ring cleavage among fungi (Cain et al. 1968).

The evidence presented in the foregoing discussion justifies the metabolic scheme represented (Fig. 4).

Acknowledgements

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