J. Microbiol. Biotechnol. (2000), **10**(4), 518–521

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Metabolism of Dimethylphthalate by *Aspergillus niger*

PRADEEPKUMAR, SHARANAGOUDA AND T. B. KAREGOUDAR*

Department of Biochemistry, Gulbarga University, Gulbarga-585 106, India

Received: April 27, 2000
Accepted: July 12, 2000

Abstract *Aspergillus niger* is capable of metabolizing dimethylphthalate. The maximum weight of mycelium was observed after about 6–8 days of incubation. A TLC analysis revealed the accumulation of metabolites in the resting cell culture. Monomethylphthalate, phthalate, and protocatechuate were shown to be the intermediates by thin layer chromatographic and spectrophotometric analyses. The fungus metabolized dimethylphthalate through monomethylphthalate, phthalate, and protocatechuate as evidenced by the oxygen uptake and an enzymatic analysis. The terminal aromatic metabolite, protocatechuate, is metabolized via the *ortho*-cleavage pathway.

Key words: *Aspergillus niger*, dimethylphthalate, monomethylphthalate, protocatechuate, metabolism

Phthalate esters are extensively used in the manufacture of plastics, textiles, and pesticides. Their residues enter into the environment in the form of industrial sewage and effluents. Hence, these compounds are frequently reported to be environmental pollutants [11, 13]. The toxic nature of phthalate esters has been investigated and is considered to be teratogenic to man and animals [21]. A considerable amount of information are available on the bacterial degradation of phthalate esters [2-6, 10, 12, 22]. However, little information is known about the metabolism of these compounds in a fungal system. Engelhardt *et al.* [6] reported on the metabolism of di-*n*-butylphthalate and related dialkylphthalates by *Penicillium lilacinum*. Sivamurthy *et al.* [23] studied the transformation of dimethylterephthalate and terephthalate by *Sclerotium rolfsii*. These investigators observed the transformation of dimethylterephthalate to monomethylterephthalate and terephthalate by the fungus. Ganji *et al.* [7] studied the metabolism of dimethylterephthalate by *Aspergillus niger* and reported the complete metabolism of dimethylterephthalate to protocatechuate. This communication reports on results of studies on the metabolism of dimethylphthalate by *A. niger*.

*Corresponding author
Phone: 91-08472-45781; Fax: 91-08472-45632;
E-mail: goudartbk@usa.net

Materials and Methods

Microorganism and Culture Conditions

The fungus, *A. niger* [20, 26], maintained in the authors' laboratory culture collection, was used for the metabolism studies. The organism was grown on a synthetic medium [1] supplemented with 0.1% dimethylphthalate. This medium was then distributed into 500-ml Erlenmeyer flasks (100 ml per flask) and sterilized by autoclaving at 120°C for 15 min. The flasks were inoculated with heavy spore suspensions of *A. niger* in sterile water obtained from 72-h-old agar slants. Thereafter, the flasks were incubated on a rotary shaker at 120 rpm at room temperature (35±2°C) for 4 days. Stock cultures were maintained on the slants of the same medium solidified with 2% agar or potato dextrose agar slants.

The dimethylphthalate was purchased from Merck Schuchardt (Germany). The protocatechuate, phthalic acid, and catechol were all procured from Aldrich Chemical Co. (U.S.A.). The monomethylphthalate was synthesized by heating equimolar amounts of phthalic anhydride with methanol, and then purified by preparative thin layer chromatography. All other chemicals used in this study were of analytical grade.

Mycelial Wet Weight Determination

The mycelial wet weight was determined by inoculating the mineral salts medium containing 0.1% dimethylphthalate with a heavy spore suspension in sterilized distilled water. The flasks were incubated on a rotary shaker at room temperature. The mycelia were harvested at different incubation periods and the mycelial pellet was weighed.

Isolation and Identification of Metabolites

The metabolites were isolated from the resting cell culture of *A. niger*. The organism was first grown in the mineral salts

medium supplemented with 0.1% dimethylphthalate. After 4 days of incubation, the mycelium was harvested and subjected to replacement culture in a phosphate buffer (0.5 M, pH 7) with 0.15% dimethylphthalate. After 4 days of incubation, the mycelial pellets were removed by filtration. The filtrate was acidified with 6 M HCl to pH 2 and extracted with equal volumes of ether. The ether extracts were dried over anhydrous Na_2SO_4 and ether was removed under a vacuum. The residue was dissolved in methanol and analyzed for metabolic products by thin layer chromatography on silica gel plates. The metabolites [10] were purified by preparative TLC using the following solvent systems: (a) benzene-ethylacetate-acetic acid (75:02:02), and (b) benzene-dioxan-acetic acid (20:40:01). The metabolites on the TLC plates were visualized with a UV-lamp (λ_{max} 254 nm). The phenolic compound (protocatechuate) was detected by spraying with Folin-Ciocalteau's phenol or Gibb's reagent. The acidic compounds (monomethylphthalate and phthalic acid) were detected with bromocresol green (0.2% w/v in alcohol). The metabolites were eluted with methanol and determined by UV-analysis (Shimadzu model 160A, UV-Spectrophotometer) with comparison to standard solutions.

Time-Course Accumulation of Protocatechuate

The mycelium obtained from the resting culture study was added into each conical flask containing a phosphate buffer (0.025 M, pH 5.5) supplemented with 0.2 ml of dimethylphthalate and incubated on a rotary shaker at room temperature. The concentration of protocatechuate formed during the various phases of growth was determined by withdrawing the culture medium at different time intervals and extracting with diethyl ether after acidification with 6 M HCl to pH 2. The protocatechuate formed was estimated using the Nair and Vaidyanathan method [17].

Oxygen Uptake Studies

The oxygen uptake [25] by the mycelium grown on the mineral salts medium containing dimethylphthalate as well as that grown on glucose alone was measured in a Warburg's constant volume apparatus at 30°C. The mycelial pellets were harvested during the logarithmic growth phase by filtration. The mycelial pellets were washed with distilled water and then with a phosphate buffer (0.05 M, pH 7) and resuspended in the same buffer. The total incubation mixture (3.2 ml) contained a phosphate buffer (0.05 M, pH 7), the mycelial suspension (2.5 mg dry weight), and 10 µM of the substrates. The central well contained 0.2 ml of a freshly prepared 20% KOH solution. The oxygen uptake was measured by tipping the substrate from a side arm. All values were corrected for endogenous oxygen consumption.

Preparation of Cell-Free Extract of *A. niger*

The cell-free extracts were prepared from mycelial pellets of *A. niger* (5.0 g) grown on dimethylphthalate. The mycelial pellets were thoroughly ground in a mortar at 4°C with the addition of a phosphate buffer (pH 7). The ground mass at 4°C was subjected to sonication (Vibra cell, U.S.A. model 375) at a nominal power of 70 W for six half minute periods, and each period of disruption was followed by 1 min during which the mycelia and sonicator probe were cooled in ice. Any cell debris was removed by centrifugation at 10,000 rpm for 15 min at 4°C. The clear supernatent thus obtained was then used as the enzyme source for all the assays. The protein content of the cell free extract was determined using Bovine serum albumin as the standard [14].

Enzyme Assays

The dimethylphthalate esterase was assayed using *p*-nitrobenzyl ester of phthalate as the substrate [15]. The assay mixture (3 ml) contained 5 mM *p*-nitrobenzyl ester of phthalate, a 50 mM phosphate buffer at pH 7, and a suitable amount of crude enzyme in a cuvette with a 1 cm light path. The enzyme activity was measured spectrophotometrically by monitoring the increase in absorbance at 276 nm due to the formation of *p*-nitrobenzyl alcohol [23]. The protocatechuate-3,4-dioxygenase activity was assayed by measuring the decrease in absorbance at 290 nm. The assay system (3 ml) contained a 200 µM phosphate buffer at pH 7, protocatechuate (1.2 µM), and a suitable amount of the cell-free extract. The catechol-1,2-dioxygenase assay [8] system (3 ml) contained a 20 µM phosphate buffer at pH 7, 10 µM EDTA, 1 µM catechol, and the cell-free extract. The activity was measured spectrophotometrically by an increase in absorbance at 260 nm due to the formation of *cis, cis*-muconic acid. The specific activity of the enzyme was defined as nmol of substrate converted or the product formed per min per mg protein.

Mode of Ring Cleavage of Protocatechuate

The mode of ring cleavage of protocatechuate was determined according to the method as described by Stanier *et al.* [24] using mycelia and by the method of Eaton and Ribbons [3] using the cell-free extract.

RESULTS AND DISCUSSION

The metabolism of dimethylphthalate by *Aspergillus niger* was indicated by a visible increase in the mycelial mass with time. The growth curve was constructed by determining the increase in the wet weight of the mycelium at different intervals during the incubation period (Fig. 1). The growth was concomitant with the disappearance of dimethylphthalate from the medium, thereby demonstrating the ability of this fungus to metabolize dimethylphthalate. The maximum weight of the mycelium increased with an increase in the incubation period.

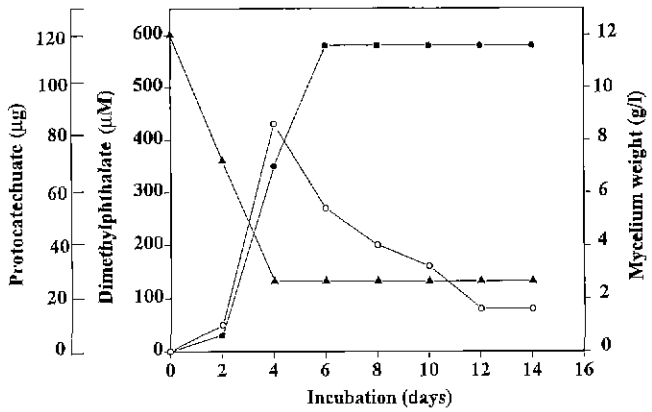

Fig. 1. Utilization of dimethylphthalate and transient accumulation of protocatechuate during the growth of *A. niger*.
(●) growth, (▲) residual dimethylphthalate, and (○) accumulation of protocatechuate.

Table 1. R_f and λ_{max} values of authentic and isolated compounds.

Compounds	R_f values in solvent systems					
	A		B		λ_{max} in methanol	
	a	b	a	b	a	b
Monomethylphthalate	0.46	0.45	0.72	0.71	275	275
Phthalic acid	0.05	0.05	0.62	0.62	282	283
Protocatechuate	0.11	0.10	0.66	0.64	290	290

Solvent systems - A and B as described in the text.
a-authentic; b-isolated.

The TLC analysis of the consumed medium from the resting cell culture showed the accumulation of three metabolites (1, 2, and 3). The R_f and λ_{max} values of these three metabolites corresponded with those of authentic monomethylphthalate, phthalate, and protocatechuate, respectively (Table 1). The melting points of metabolites 1 and 2 were identical to those of authentic monomethylphthalate and phthalic acid, respectively. The infrared spectrum of the purified metabolite 3 (νC=O, 1.660 cm^{-1}, three hydroxyls appeared at 3,350 cm^{-1}, 3.500 cm^{-1}, and 3,600 cm^{-1}) was found to be identical to that of authentic protocatechuate. The protocatechuate was accumulated as a transient intermediate in the culture medium. The maximum accumulation of protocatechuate occurred at 96 h of incubation (Fig. 1). The formed protocatechuate has been metabolized further by the fungus.

The oxygen uptake studies with the mycelium of *A. niger* grown on dimethylphthalate showed that monomethylphthalate, phthalate, and protocatechuate were all readily oxidized when compared to catechol. The cells grown in glucose oxidized glucose readily, whereas monomethylphthalate, phthalate, and protocatechuate were oxidized at lower rates (Table 2).

The enzymatic investigation carried out with the mycelial cell-free extracts of *A. niger* grown on dimethylphthalate showed high dimethylphthalate esterase (0.050 U/mg protein) and protocatechuate-3,4-dioxygenase activities (0.047 U/mg protein), and did not exhibit any catechol-1,2-dioxygenase activity. The cell-free extract of *A. niger* grown on glucose did not show any of the above enzyme activities. The studies on the ring cleavage of protocatechuate did not show formation of a yellow coloration, thereby indicating that the dimethylphthalate- grown mycelium of *A. niger* cleaved the protocatechuate by *ortho*-cleavage as evidenced by Rothera's test [19].

Table 2. Oxygen uptake of compounds by mycelial suspension of *A. niger*.

Compounds	Oxygen uptake* (nmol/min/mg dry wt.)	
	Dimethylphthalate grown cells	Glucose grown cells
Dimethylphthalate	3.00	2.10
Monomethylphthalate	2.50	1.20
Phthalic acid	2.00	1.00
Protocatechuate	1.65	0.57

*The values are corrected for endogenous consumption.
Results are representative of the average of two duplicate experiments.

The above results provide evidence that dimethylphthalate is metabolized via monomethylphthalate, phthalate, and protocatechuate in this organism. In addition, the hydrolysis of dimethylphthalate to free phthalic acid is accomplished with the intermediate accumulation of monomethylphthalate in the culture medium. Phthalic acid is metabolized *via* protocatechuate by the protocatechuate-3,4-dioxygenase mechanism. The results also showed that the formation of monoester is the most common reaction in phthalate ester metabolism. The hydrolysis of dimethylphthalate to phthalic acid is catabolized by dimethylphthalate esterase. Such esterases of microbial origin have been studied by several investigators [9, 18]. The protocatechuate is formed as a terminal aromatic metabolite which is further cleaved by the *ortho*-cleavage pathway.

Acknowledgments

The authors would like to thank AICTE, New Delhi, for their financial assistance.

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