CHAPTER V

DEGRADATION
OF
DIMETHYLTerepHTHALATE
BY
ASPERGILLUS NIGER
V. RESULTS

V.1 Growth conditions

Aspergillus niger was screened for its ability to degrade dimethylterephthalate. It was grown on agar slants containing synthetic medium. The spores from the slants were used to inoculate 100 ml of synthetic medium in 500 ml Erlenmeyer flask. The flask was incubated on a rotary shaker at 100 rpm at 35±2°C for 48 hrs, to which 100 mg of dimethylterephthalate was added and incubated further for 108 hrs (at 35±2°C) on a rotary shaker. One control was prepared by incubating the organism in the synthetic medium without substrate. Another substrate control was prepared by incubating the substrate in the medium without the organism.
V.2 Metabolite characterisation

In order to trace the degradative pattern of dimethyl terephthalate in A. niger, the metabolites of the degradative pathway were isolated.

A 5 liter synthetic medium supplemented with 0.1% dimethyl terephthalate was inoculated with thick suspension of spores of A. niger. The flasks were incubated on a rotary shaker at 100 rpm at 35±2°C for 72 h. The mycelial pellets were separated by filtering through cheesecloth and washed with distilled water. These mycelial pellets were used for replacement culture experiments.

Replacement culture experiment: The mycelial pellets were resuspended in 0.025M phosphate buffer (pH 5.5) containing 0.1% dimethyl terephthalate. This replacement culture was incubated on rotary shaker at 35±2°C for 8 to 12 h. The products formed were analysed. The mycelial pellet was separated by filtering through cheesecloth and the spent medium was acidified with 4 N HCl to pH 2 and extracted with ethylacetate. The acidic and neutral fractions were separated by treating ethylacetate layer with 5% NaHCO₃ solution. The aqueous layer was neutralised with acid and extracted with ethylacetate. The ethylacetate containing acidic fraction was dried over anhydrous sodium sulphate, concentrated and subjected to thin layer
chromatography. Thin layer chromatography was performed using solvent systems D and E. The chromatographic analysis of the extract revealed the presence of two acidic compounds (compound 1 and 2). Rf values of which corresponded to authentic samples of monomethylterephthalate and terephthalate respectively. These two compounds were separated quantitatively by preparative TLC and further purified by recrystallisation. The melting point of these two compounds (1 and 2) corresponded well with the authentic monomethylterephthalate and terephthalate. To identify these compounds, they were subjected to UV analysis. The PMR spectrum and Mass spectrum of the isolated compound (1 and 2) agreed well with monomethylterephthalate and terephthalate respectively.

An attempt was made to isolate dihydroxy compounds (terminal aromatic metabolites). Replacement culture experiment was carried out using inhibitors. The mycelial pellets grown in a synthetic medium containing 0.1% terephthalate were transferred to 25 mM phosphate buffer (pH 5.5) supplemented with 0.1% terephthalate and 5 mM/dl of dioxygenase inhibitor α,α'-dipyridyl. These cells were incubated on a rotary shaker. The product formed was analysed at different incubation period. The spent medium after 4 h of incubation was acidified with 4N HCl to pH 2 and extracted with peroxide free diethylether and dried over anhydrous Na$_2$SO$_4$ and evaporated in vacuo. Thin layer chromatographic analysis
of the extract using solvent systems D and E revealed the occurrence of a dihydroxy compound (compound 3). The \( R_f \) value of which corresponded to authentic sample of protocatechuic acid. The compound 3 was separated quantitatively by preparative thin layer chromatography. Further this compound was subjected to UV and PMR spectral analysis. The UV and PMR spectrum of compound 3 corresponded well with authentic protocatechuic acid.

The \( R_f \) values, \( \lambda_{\text{max}} \) and MP* of the metabolites (unknown samples) and those of references (authentic samples) in solvent systems D and E are given in Table V.1.

V.3 Studies on utilisation of dimethylterephthalate by A. niger

Studies on the utilisation of dimethylterephthalate by A. niger were carried out. Thick suspension of spores of A. niger were inoculated to 100 ml of synthetic medium containing 100 mg of dimethylterephthalate in a 500 ml Erlenmeyer flask. This was incubated on rotary shaker. The concentration of residual dimethylterephthalate at various phases of growth was determined by extracting the spent medium with diethylether at different incubation periods. The acidic and neutral compounds were separated by treating the ether layer with 5% NaHCO_3 solution. The neutral fraction contained only dimethylterephthalate as seen by thin layer chromatography. It was estimated spectrophotometrically by measuring absorbance at 240 nm (\( \lambda_{\text{max}} \) of dimethylterephthalate).

\* Melting Point
TABLE - V.1

The $R_f$ values, $\lambda_{\text{max}}$, and melting points of the isolated and authentic compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R_f$ values in solvent systems</th>
<th>$\lambda_{\text{max}}$ (nm) methanol</th>
<th>M.P. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D</td>
<td>E</td>
<td></td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td>a</td>
</tr>
<tr>
<td>Monomethylterephthalate</td>
<td>0.58</td>
<td>0.57</td>
<td>1.0</td>
</tr>
<tr>
<td>Terephthalate</td>
<td>0.37</td>
<td>0.37</td>
<td>0.36</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>0.76</td>
<td>0.76</td>
<td>0.12</td>
</tr>
</tbody>
</table>

*a* - authentic;  *b* - isolated.
The results are given in Table V.2 which indicated that only 58% of the added dimethylterephthalate was taken up by A. niger after incubation for about 144 h.

V.4 Time course of accumulation of protocatechuic acid

The time course for the accumulation of protocatechuic acid as transient intermediate was studied. The spores of A. niger were inoculated to 100 ml of synthetic medium supplemented with 0.1% terephthalic acid and incubated on rotary shaker. The concentration of protocatechuic acid formed during various phases of growth was determined by withdrawing, aseptically, 1 ml of culture medium at different intervals. The culture medium was extracted with diethyl ether. The protocatechuic acid formed was estimated by the method of Nair and Vaidyanathan (1964). The results of accumulation of protocatechuic acid are depicted graphically (Fig.V.1).

V.5 Enzyme Assays

The enzyme activities of esterase, protocatechuate dioxygenase, catechol dioxygenase and gentisate dioxygenase were estimated using cell free extracts (protein content 2.3 mg/ml). Esterase and protocatechuate dioxygenase activities were detected in the cell-free extract of A. niger. The results of spectrophotometric assay for protocatechuate dioxygenase and esterase are given in Table V.3. One unit of esterase activity is defined as the number of μ moles of p-nitrobenzylalcohol formed/mg of protein/min.
<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Concentration of dimethylterephthalate utilised (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>24</td>
<td>0.00</td>
</tr>
<tr>
<td>48</td>
<td>0.056</td>
</tr>
<tr>
<td>72</td>
<td>0.157</td>
</tr>
<tr>
<td>96</td>
<td>0.467</td>
</tr>
<tr>
<td>120</td>
<td>0.534</td>
</tr>
<tr>
<td>144</td>
<td>0.582</td>
</tr>
</tbody>
</table>

**TABLE - V.2**

Utilisation of Dimethylterephthalate by *A. niger* at different incubation periods.
Fig. V.1 Time course of accumulation of Protocatechuic acid by *Aspergillus niger* on terephthalate.
### TABLE - V.3

Enzyme activities of *A. niger* grown on Dimethylterephthalate

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Specific activity U/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esterase activity</td>
<td>35.65</td>
</tr>
<tr>
<td>Protocatechuate dioxygenase</td>
<td>9.78</td>
</tr>
</tbody>
</table>
The specific activity of protocatechuate dioxygenase was defined as the number of μ moles of protocatechuate utilised/mg of protein/min (U/mg of protein).

V.6 Mode of Ring fission

The degradative pathway of dimethylterephthalate by A. niger was further inferred from the mode of ring cleavage of the terminal aromatic metabolite. The 43 h old mycelial pellets grown on dimethylterephthalate were suspended in 0.05M phosphate buffer (pH 5.5) containing 0.05% of protocatechuic acid and were incubated on rotary shaker. Yellow coloured product started accumulating after 5 min of incubation. This yellow product indicated meta cleavage pathway, and was isolated by extracting the medium with peroxide free diethylether. The diethylether was dried over anhydrous Na₂SO₄ and the solvent was evaporated. The extract was dissolved in 0.05M phosphate buffer (pH 9.0). The ring cleavage product was identified spectrophotometrically by measuring the absorbance at pH 9. The λₘₐₓ of yellow product at pH 9 was found to be 410 nm, which corresponded to λₘₐₓ of γ-carboxy-δ-hydroxymuconic semialdehyde (Crawford, 1975). Formation of γ-carboxy-δ-hydroxymuconic semialdehyde indicated the operation of distal meta cleavage pathway in the degradation of dimethylterephthalate by A. niger (Fig.V.10).
V.7 Discussion

Reports on the fungal degradation of synthetic and natural compounds have appeared in the last several years, but there are hardly reports on the biodegradation of phthalate compounds by the fungal system. There was one report on the conversion of dimethylterephthalate to terephthalic acid by the mould Sclerotium rolfsii (Sivamurthy et al. 1991). This strain did not utilise it completely. We carried out investigation on the biodegradation of dimethylterephthalate by Aspergillus niger. With a view to know whether this fungal strain degrades dimethylterephthalate completely or not.

It is evident from our results on metabolite characterisation that monomethylterephthalate, terephthalate and protocatechuate were accumulated as key intermediates in degradation of dimethylterephthalate by Aspergillus niger. TLC analysis showed the presence of two major compounds. The compound 1 was identified as monomethylterephthalate with the help of authentic sample. The melting point, UV spectrum and Rf value agreed with the authentic monomethylterephthalate. PMR spectrum of isolated compound 1 revealed the presence of carboxymethyl protons at 3.88 ppm as a singlet, four aromatic protons as a singlet at 8.06 ppm and carboxyl protons at 13.4 ppm. Mass spectral analysis of this compound showed the parent ion peak at m/z 180 which is in good agreement with the empirical formula C_9H_8O_4 (Fig.V.2 to V.4). The fragmentation
Fig. V.2 Ultraviolet spectrum of authentic and isolated sample.

Fig. V.5 Ultraviolet spectrum of authentic and isolated sample.

WAVELENGTH (nm)
Fig. V. 3 PMR spectrum of monomethylterephthalate.
Fig. V.4 Mass spectrum of monomethylterephthalic acid.
pattern showed ion peaks at m/z: 163 base peak (M-OH), 149
(M-OCH₃), 135 (M-COOH), 121 (M-COOCH₃). Analysis of PMR and
Mass spectra of compound 1 showed that they corresponded well
with that of monomethylterephthalic acid. PMR spectrum of
compound 2 revealed the presence of 4 aromatic protons at 8.05 δ as
a singlet and carboxyl protons at higher field. Mass spectral
analysis showed the parent ion peak at m/z 166, which is in good
agreement with empirical formula C₈H₆O₄, the fragmentation pattern
showed the ion peaks at m/z : 149 base peak (M-OH), 121 (M-COOH).
The PMR and Mass spectra of compound 2 were found to be identical
acid with terephthalic acid [Fig. V.5 to V.7].

Isolation of metabolites other than free acid and monomethyl-
ester was not successful during the routine course of fermentation.

Probably it may be due to either high solubility of
intermediate metabolites or high activities of the enzymes operating
in the pathway. However inhibitor method was used to accumulate
possible intermediate. The use of dioxygenase inhibitor, α,α-
dipyridyl in the replacement culture technique, accumulated
a dihydroxy compound (compound 3). TLC analysis of compound 3
revealed the presence of protocatechuic acid. The melting point
and UV spectrum agreed with authentic protocatechuic acid. The
PMR spectrum of this compound revealed the presence of C₂ and
C₆ aromatic protons at 7.32 δ as a doublet and C₅ proton at
6.76 δ as a singlet. Phenolic protons at C₃ and C₄ positions
appeared at 9.3 δ and 9.6 δ respectively, and carboxyl proton
Fig. V.7 Mass spectrum of terephthalic acid.
Fig. V.10 Ultraviolet spectrum of ring cleavage product.

Fig. V.8 Ultraviolet spectrum of isolated sample.

Protocatechuic acid (PCA) (in methanol)

Ring cleavage product (from PCA)
Fig. V.9a PMR spectrum of Protocatechuic acid (authentic)
Fig. V.9 b PMR spectrum of Protocatechuic acid (isolated)
The hydrolysis of dimethylterephthalate to monomethylterephthalate is catalysed by dimethylterephthalate esterase. The results confirmed the presence of activity of this enzyme in the cell free extract of *Aspergillus niger*. Such carboxyl esterases of microbial origin have been studied by several groups (Mastunaga et al. 1974; Parkkinen E, 1980; Iwai et al. 1983; Sivamurthy et al. 1991). In mammalian metabolism also, only a single hydrolysis product, monoester of phthalate was found to accumulate (Albro et al. 1973). It shows that formation of monoester is the most common reaction in the mineralisation of phthalate esters. The formation of monomethylterephthalate is confirmed by its accumulation in replacement cultures.

The monomethylterephthalate was hydrolysed to free terephthalic acid. The formation of terephthalic acid was also confirmed by isolation of terephthalic acid from replacement cultures. Consequently, terephthalic acid may be degraded via p-hydroxybenzoic acid, protocatechuic acid, benzoic acid, catechol or gentisic acid. The different enzymes were tested from the crude extract. Oxidative decarboxylase was not detected in the cell free extract of *A. niger*. Therefore formation of p-hydroxybenzoic acid may possibly be excluded from the metabolic pathway. Also the activity of catechol
and gentisate dioxygenase were absent. However high activity of protocatechuate dioxygenase was detected in the cell free extract (specific activity, 9.78). Apart from this, protocatechuate was isolated from the replacement culture using α,α'-dipyridyl as inhibitor. This provides evidence for the presence of protocatechuate in the pathway. So, an earlier step in the degradation of terephthalate appears to be the formation of terephthalate dihydrodiol which may be transformed to protocatechuic acid. Such a mechanism has been proposed by several authors (Jamaluddin et al., 1970; Karegoudar and Pujar, 1985).

Based on the results presented we propose that dimethylterephthalate is hydrolysed to free terephthalic acid. Such type of hydrolysis of phthalate esters are known in bacterial and animal system (Engelhardt et al., 1979; Harada and Kohwa, 1977; Engelhardt 1975; Albright et al., 1973). Terephthalate is oxidised to protocatechuate. Protocatechuate is cleaved by protocatechuate dioxygenase by distal meta cleavage mechanism to γ-carboxy-α-hydroxymuconic semialdehyde (Dennis et al., 1973) which is further oxidised to pyruvate and acetoacetate through the formation of keto compound (Fig.V.10). Meta cleavage of protocatechuate in fungal system has also been reported by Cain et al. (1968).

Thus the fungus Aspergillus niger plays a pivotal role in detoxification of dimethylterephthalate which is extensively used in textile industries.
Fig. V. 10 Proposed pathway for the degradation of dimethylterephthalate by Aspergillus niger.