CHAPTER VI

RESULTS AND DISCUSSION
Degradation of orcinol in *A. niger*
The ability of organisms to use resorcinol and related compounds, as the sole source of carbon and energy is not surprising, considering that large amounts of these are present in gas liquors and are removed by biological purification before discharge into rivers (Pankhurst, 1959).

Utilization of resorcinol has been reported in Azotobacter vinelandii (Claus and Hempel, 1970; Grosedose and Ribbons, 1981). Trichosporon cutaneum, Candida tropicalis (Chapman and Ribbons, 1976), and a strain of Pseudomonas (Larway and Evans, 1965).

Aspergillus niger degrades resorcinol via 1,2,4-benzene-triol and P-ketoacid (Shailubhai et al., 1983). It was therefore interesting to check for the degradation of 5-methyl resorcinol (orcinol) in this organism. Such a study also enables one to understand the mode in which a -CH₃ substituent on the aromatic ring is attacked by A. niger. In the present study, the primary steps involved in the catabolism of orcinol by A. niger were investigated.

A. niger was capable of utilizing orcinol as the sole source of carbon and energy. The culture attained stationary phase in about 48 hours (Fig. 1). Thin layer chromatography (TLC) of the compounds extracted from the culture filtrate of cells grown on orcinol at various time intervals, revealed that the ideal time for harvesting the cells for obtaining metabolites was 36 hours. An attempt was made to standardize a solvent system which gave the best possible resolution of the suspected metabolites of orcinol degradation. Benzene : ether : acetic acid (6:3:1) was
FIGURE-1

GROWTH PATTERN OF A. niger ON ORCINOL AS CARBON SOURCE

SCALE: X-Axis - 2 cm = 12 hrs.
Y-Axis - 4 cm = 0.01 gm.
Y'-Axis - 2 cm = pH

GROWTH

TIME (HOURS)

DRY WEIGHT (in gms.)

PH

pH

0 12 24 36 48 60 72 84 96

0 12 24 36 48 60 72 84 96
Considerable variation is observed in the pathways used by different microorganisms when they are confronted by phenols and other benzenoid compounds containing alkyl substituents. The methyl substituent of meta- and para-cresols may undergo oxidation to a carboxyl group by hydroxylation of the \(-\text{CH}_2\) substituent to \(-\text{CH}_2\text{OH}\), followed by formation of \(-\text{CHO}\); and finally \(-\text{COOH}\) (Dagley and Patel, 1957). The resulting substituted benzoic acids become substrates for hydroxylases, yielding protocatechuate or related compounds which are substrates for ring fission. In some organisms, conversion of the \(-\text{CH}_2\) substituent does not take place, i.e., ortho-cresol may be converted to 3-methylcatechol by direct hydroxylation. This also occurs in the case of bacterial metabolism of meta- and para-cresols (Ribbons, 1966; Bayly et al., 1966).

Methyl group substituents, when suitably placed on substrates, are tolerated by enzymes of the gentisate or catechol meta fission pathways of \textit{Pseudomonas} species. Methylated catechols are known to be degraded by meta fission pathways, by hydrolytic or oxidative routes (Dagley, 1985). Methylated derivatives can not be degraded by ortho fission pathways because a 'dead end lactone' is formed, having the methyl group so placed, so as to prevent delactonization (Catelani et al., 1971). However, the actinomycete \textit{Gordona rubra} can degrade 4-methylcatechol to completion, forming a methyl substituted lactone of different structure which is subject to hydrolysis (Miller, 1981). The soil yeast...
# Table 1: Separation of metabolites in various solvent systems.

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>*Rf values</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>α-resorcylic acid</strong></td>
<td><strong>Orcinol</strong></td>
</tr>
<tr>
<td>Benzene:ethyl acetate: acetic acid (6:3:1)</td>
<td>0.6</td>
</tr>
<tr>
<td>Chloroform:ether:acetic acid (6:3:1)</td>
<td>0.95</td>
</tr>
<tr>
<td>Benzene:methanol:acetic acid (2:2:5)</td>
<td>0.32</td>
</tr>
<tr>
<td>Pet.ether: acetic acid (7:3)</td>
<td>0.79</td>
</tr>
<tr>
<td>Benzene:toluene:acetic acid (2:2:1)</td>
<td>0.04</td>
</tr>
<tr>
<td>Benzene:ether:acetic acid (6:3:1)</td>
<td>0.6</td>
</tr>
</tbody>
</table>

* Compounds were detected by spraying solution containing 0.1% (w/v) K$_3$Fe(CN)$_6$ and 0.1% (w/v) FeCl$_3$. 

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Trichosporon cutaneum can grow at the expense of m- and p-cresols using reactions that involve methyl-substituted catabolites of β-ketoadipate pathway (Powlowski, 1983). A modified β-ketoadipate pathway also operates during the degradation of methylated aromatic compounds by Rhodococcus rhodochrous (Bruce and Cain, 1986).

In a strain of Aspergillus niger and Aspergillus fumigatus, phenylacetic acid is degraded via the formation of homogentisate (Sugumaran and Vaidyanathan, 1978; Ueno et al., 1973). There have been no reports on the degradation of any methylated aromatic compound by A. niger. We first determined whether the -CH$_3$ group in orcinol was being oxidized to form 1,3-dihydroxy benzoate, which may be metabolised via resorcinol, 1,2,4-benzenetriol and β-ketoadipate. The TLC experiments indicated the presence of resorcinol, 1,2,4-benzenetriol and β-ketoadipate (data not presented).

Orcinol, resorcinol and glucose-grown cells were allowed to respire in the presence of resorcinol, orcinol, 1,2,4-benzenetriol, 3,5-dihydroxybenzoate or catechol as substrates. The results obtained are depicted in Fig. 2, 3 and 4. The orcinol-grown cells showed a respirometric activity similar to that of resorcinol grown cells, for all the substrates checked. Orcinol and resorcinol-grown cells showed considerable respiration in response to catechol. It has been reported earlier that the yeast Trichosporon cutaneum, when grown on benzoate was fully adapted to catechol, protocatechuate, and gentisate, and, for
FIG.-2: RESPIRATORY ACTIVITIES OF ORCINOL GROWN A. niger CELLS ON VARIOUS SUBSTRATES
RESPIRATION OF RESORCINOL GROWN CELLS ON

○○○ GLUCOSE
△△△ RESORCINOL
□□□ ORCINOL
●●● 1,2,4-BENZENETRIOL
▲▲▲ α-RESORCYLIC ACID
■■■ CATECHOL

FIG. 3: RESPIRATORY ACTIVITY OF RESORCINOL GROWN A.niger CELLS ON VARIOUS SUBSTRATES

OXYGEN CONSUMPTION (µL/O. mg. cell dry wt.)

MINUTES
FIG. - 4: RESPIRATORY ACTIVITIES OF GLUCOSE GROWN A. niger CELLS ON VARIOUS SUBSTRATES

RESPIRATION OF GLUCOSE GROWN CELLS ON

- O GLUCOSE
- △△ RESORCINOL
- □□ ORCINOL
- ●●● 1,2,4 - BENZENETRIOL
- ■■■ α- RESORCYLIC ACID
- ▲▲ CATECHOL

OXYGEN CONSUMPTION (mL/mg. cell dry wt.)

MINUTES

0 5 10 15 20 25 30 35

FIG. - 4: RESPIRATORY ACTIVITIES OF GLUCOSE GROWN A. niger CELLS ON VARIOUS SUBSTRATES
good measure, also to 2-, 3- and 4-hydroxybenzoates (Dagley, 1985). Broad specificity of catabolic enzymes has been well documented for *A. niger* (Shailubhai *et al.*, 1984; Sahasrabudhe and Modi, 1987). Glucose-grown cells, as expected, did not show very high respiration rates when confronted with hydrocarbons. Similarly, resorcinol- and orcinol-grown cells respired less when glucose was provided as substrate. The higher respiration of cells grown in resorcinol in the presence of orcinol and 3,5-dihydroxybenzoate can also be explained on the basis of non-specificity of the enzymes catabolizing 1,3-dihydroxylated benzenoids like orcinol and resorcinol in *A. niger*. The respiration in presence of 1,2,4-benzenetriol could not be monitored properly due to rapid autooxidation. The respirometric data also indicates that orcinol-grown cells respired at higher rates in response to resorcinol and 3,5-dihydroxybenzoate than the cells grown on glucose, indicating the possibility of these compounds being involved as intermediates in the pathway for orcinol degradation in *A. niger*.

It was at this stage necessary to ascertain the identity of the suspected intermediates. Attempts to detect either 3,5-dihydroxybenzoate or 1,2,4-benzenetriol in the organic fraction of the incubation mixture containing orcinol and the cell-free extract of the orcinol-grown cells, met with no success. Furthermore, the compound which had an Rf similar to that of 1,2,4-benzenetriol, when purified by preparative TLC, failed to show an overlapping UV-visible spectrum with that of authentic 1,2,4-benzenetriol (data not presented).
Finally, the methyl derivatives of partially purified metabolites (obtained from silica gel column chromatography), prepared using diazomethane did not show any peaks corresponding to derivatized authentic resorcinol, 1,2,4-benzenetriol or 3,5-dihydroxybenzoate upon gas chromatographic analysis (Fig. 5). β-ketoadipate could also not be detected from the broth of cells grown on orcinol (Table 2).

Table 2: Detection of β-ketoadipate by Rothera's reaction.

<table>
<thead>
<tr>
<th>Cells grown in</th>
<th>β-ketoadipate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>Absent</td>
</tr>
<tr>
<td>Resorcinol</td>
<td>Present</td>
</tr>
<tr>
<td>Orcinol</td>
<td>Absent</td>
</tr>
</tbody>
</table>

TLC and respirometric data, therefore, did not depict the true picture of the degradative pathway. It was also possible that the methyl group remained intact while hydroxylation occurred, as has been reported in the case of Rhodococcus rhodochrous (Bruce and Cain, 1986). Gordona rubra (Miller, 1981) and Trichosporon cutaneum (Powlowski, 1983). A. niger is known to hydroxylate phenylacetic acid in 2 and 5 positions to produce homogentisic acid, leaving the -CH₂COOH side chain intact (Sugumaran and Vaidyanathan, 1978). Similar results have been obtained in the case of Piricularia oryzae and Polyporous versicolor, where enzyme extracts oxidize 1,3-dihydroxy compounds, particularly orcinol and resorcinol (Chapman and Ribbons, 1976). A strain of Pseudomonas putida
STANDARD COMPOUNDS (Retention time)
(a) = Resorcinol
(b) = 1,2,4-benzenetriol
(c) = \( \alpha \)-Resorcylic acid

FIG. 5: GAS CHROMATOGRAPH OF CRUDE EXTRACT
is known to degrade orcinol as shown: $$\text{orcinol} \rightarrow 2,3,5$$-trihydroxytoluene $$\rightarrow 2,4,6$$-trioxoheptanoate $$\rightarrow$$ acetate + acetyl pyruvate (refer to the Fig. 5 in Chapter I). Therefore, there was a possibility of orcinol being transformed to 2,3,5-trihydroxytoluene by introduction of a hydroxyl group at C-2. 2,3,5-trihydroxytoluene is expected to have an Rf value similar to that of 1,2,4-benzenetriol which would explain the previous TLC results. Also, in the aforementioned gas chromatograph, a peak with a retention time slightly higher than that of authentic orcinol suggested the presence of a compound more polar than orcinol, such as 2,3,5-trihydroxytoluene.

Since authentic 2,3,5-trihydroxytoluene was not available, a few general characteristics as observed by Chapman and Ribbons (1976) have been compared for the identification of 2,3,5-trihydroxytoluene. The colour of the medium used for growth of A. niger turned brick red during the initial stages of growth, but this colour later disappeared. The colour may be attributed to a quinone, because upon adding sodium dithionite the colour was discharged. The medium showed maximum absorption at 485 nm (Fig. 6).

It is pertinent to note that 2-hydroxy-6-methyl-1,4-benzoquinone displays its $\lambda_{\text{max}}$ at 485 nm and forms a brick red colour that is discharged by sodium dithionite (Chapman and Ribbons, 1976). 2-hydroxy-6-methyl-1,4-benzoquinone is the oxidized form of 2,3,5-trihydroxytoluene.
For the chemical characterization of 2,3,5-trihydroxytoluene, the crude extract was derivatized by using dimethyl sulfate. This reaction was monitored by TLC. On completion of the reaction, the end product was checked once again by NMR spectroscopy. However, the presence of undegraded orcinol in the broth hindered interpretation of results (data not presented). The unmethylated crude dry extract was then treated with hexamethyldisilizane (in pyridine) and the silyl derivative formed was subjected to a mass spectral analysis (Fig. 7). A small molecular ion peak at m/z 356 corresponding to the trimethylsilyl derivative of 2,3,5-trihydroxytoluene indicated its presence as an intermediate during the degradation of orcinol by A. niger. Another peak at m/z 268 corresponded to trimethylsilyl derivative of orcinol. Fragmentation peaks (M$^+$ - 15) were detected at m/z 341 and m/z 253 for the 2,3,5-trihydroxytoluene and orcinol derivatives respectively. Thus, these results indicated that the crude extract contained 2,3,5-trihydroxytoluene, besides other unidentified products. One such peak at m/z 622 can be assigned to a dimer of trimethylsilyl derivative of 2,3,5-trihydroxytoluene and orcinol (Fig. 7). Such dimers can be formed by oxidative coupling of phenols (Kieslich, 1976).

An attempt was made to isolate the compound that exhibited properties similar to 2,3,5-trihydroxytoluene. This compound was isolated by preparative TLC. NMR spectra of the relevant fractions were obtained. The purpose of examining the NMR
FIG. 7: MASS SPECTRUM OF THE TMS DERIVATIVE OF THE CRUDE EXTRACT.
spectra of the various fractions was to check if there is a decrease in the ratio of aromatic protons to methyl protons, which is unity for orcinol but should decrease in case of nuclear hydroxylation. NMR spectrum of one of these fractions (Fig. 8b) has the ratio reduced to 0.7 (expected ratio for 2,3,5-trihydroxytoluene = 0.66). Fig. 8a exhibits the spectrum of a compound corresponding to authentic orcinol.

From the data presented above, it is clear that 2,3,5-trihydroxytoluene is indeed present, though in minor amounts, in the spent broth of A. niger cells grown on orcinol.

Enzyme activities were monitored to confirm these results. Orcinol hydroxylase, which brings about the formation of 2,3,5-trihydroxytoluene, has been purified to homogeneity and crystallized in Pseudomonas putida 01 (Ohta and Ribbons, 1970; Ohta et al., 1975). The enzyme catalyzes the hydroxylation of orcinol (with equimolar consumption of NADH or NADPH) to 2,3,5-trihydroxytoluene which is then non-enzymatically converted to a quinone. The enzyme has a molecular weight of 63,000 - 68,000 daltons and contains FAD as a prosthetic group. When orcinol was added to the cell-free extract of orcinol-grown A. niger cells, NADPH oxidation is observed (Table 3). Furthermore, the thin layer chromatogram of this reaction mixture indicated gradual formation of a compound, having a Rf similar to the fraction that yielded chemical characteristics akin to that of 2,3,5-trihydroxytoluene. Orcinol hydroxylase activity was observed only in response to NADPH and FAD. NADH could not act as an electron donor for the hydroxylation reaction. In the case of
FIG. 8a: NMR SPECTRUM OF THE COMPOUND CORRESPONDING TO ORCINOL.
FIG. 8b: NMR SPECTRUM INDICATING A SUBSTITUTION ON THE AROMATIC RING
Pseudomonas putida 01, resorcinol was hydroxylated to 1,2,4-benzenetriol by this enzyme indicating a certain degree of non-specificity (Ohta et al., 1975). The cell-free extracts of A. niger cells grown on orcinol brought about the oxidation of resorcinol (Table 3a), although at lower rates, to form 1,2,4-benzenetriol. This further substantiates the broad substrate specificity of hydroxylases in A. niger (Sahasrabudhe and Modi, 1985).

In order to check for the enzyme involved in the ring cleavage of 2,3,5-trihydroxytoluene it was necessary to assay their ortho or meta fission activities in the cell-free extracts of A. niger cells grown on orcinol. Higher activity of catechol 1,2-dioxygenase and undetectable levels of catechol 2,3-dioxygenase indicated the operation of ortho fission pathway. A. niger follows ortho pathway for all aromatic compounds studied to date (Fewson, 1981; Shailubhai, 1983). Moreover, the activity of orcinol hydroxylase and ring fission enzyme was higher in cells grown on orcinol than in those grown on glucose, indicating the possibility of these enzymes being inducible in nature. The maximum activity of these enzymes was obtained at the end of 48 hours of growth. The ratio of hydroxylase activity using resorcinol or orcinol as substrate remained constant in the cell-free extracts of A. niger cells grown for 24, 48 and 72 hours (Table 3b).

As a result of the ortho mode of ring fission, acetyl CoA is produced, and in fungi there is generally an increase in the levels of glyoxylate bypass enzymes in response to a surplus acetyl CoA pool (Cioni et al., 1981; Maxwell et al., 1975).
Table 3a: Specific activities of various enzymes at different time intervals.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Specific activity at Control (Glucose)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h.</td>
</tr>
<tr>
<td>Catechol 1,2-dioxygenase</td>
<td>2.1</td>
</tr>
<tr>
<td>Catechol 2,3-dioxygenase</td>
<td>N.D.</td>
</tr>
<tr>
<td>Isocitrate lyase</td>
<td>54.38</td>
</tr>
<tr>
<td>Orcinol hydroxylase</td>
<td>1.40</td>
</tr>
<tr>
<td>Resorcinol hydroxylase</td>
<td>0.53</td>
</tr>
</tbody>
</table>

N.D. = Not detected.

Table 3b: Ratio of specific activities of orcinol hydroxylase and resorcinol hydroxylase at different time intervals.

<table>
<thead>
<tr>
<th>Enzyme extract obtained from</th>
<th>Substrates for hydroxylase reaction</th>
<th>Ratio a/b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Orcinol (a) sp.act.</td>
<td>Resorcinol (b) sp.act.</td>
</tr>
<tr>
<td>Control (Glucose-grown cells)</td>
<td>0.12</td>
<td>N.D.</td>
</tr>
<tr>
<td>24 hrs. orcinol-grown cells</td>
<td>1.40</td>
<td>0.53</td>
</tr>
<tr>
<td>48 hrs. orcinol-grown cells</td>
<td>1.43</td>
<td>0.63</td>
</tr>
<tr>
<td>72 hrs. orcinol-grown cells</td>
<td>0.33</td>
<td>0.14</td>
</tr>
</tbody>
</table>
The cell-free extracts of cells grown on orcinol showed higher isocitrate lyase activity than the cells grown on glucose.

In the mycelial pellets of *A. niger*, induction of all the enzymes involved in the pathway for the degradation of benzoate, salicylate and resorcinol takes about 10-12 hours (Shailubhai, 1983). *A. niger* cells grown on glucose were transferred to medium containing acetate, glucose or orcinol as the sole source of carbon and energy. At the end of 12 hours, the cells were harvested and isocitrate lyase activity was checked. Cells transferred to orcinol exhibited a higher activity compared to those transferred to glucose (Table 4).

Table 4: Relative specific activity of isocitrate lyase.

<table>
<thead>
<tr>
<th>Glucose-grown cells transferred to</th>
<th>% activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>100</td>
</tr>
<tr>
<td>Orcinol</td>
<td>43.02</td>
</tr>
<tr>
<td>Glucose</td>
<td>23.64</td>
</tr>
</tbody>
</table>

Accumulation of acetate in the extracellular medium and inside the cells, was also confirmed by gas chromatographic analysis (Fig. 9). Further evidence for the presence of acetate in the culture filtrate is seen in the form of a characteristic molecular ion peak at m/z 132 for the trimethyl silyl derivative of acetic acid (Fig. 7). Also, the amount of intracellular acetate per mg. of protein was found to be higher in orcinol-grown cells than in glucose-grown cells.
FIG. 9: GAS CHROMATOGRAPH INDICATING THE PRESENCE OF ACETATE IN CULTURE FILTRATE AND CELL FREE EXTRACT.
The postulated primary steps involved in the degradation of orcinol are presented in Fig. 10. We conclude that *A. niger* is incapable of oxidizing a methyl group on the aromatic ring, at least in the case of orcinol.
Fig. 10: Primary steps involved in the catabolism of orcinol by *A. niger*.

a) orcinol; b) 2,3,5-trihydroxytoluene; c) oxidized form of 2,3,5-trihydroxytoluene; d) acetate; e) dimer of orcinol & 2,3,5-trihydroxytoluene.