CHAPTER IV

BIODEGRADATION OF 2-NITROTOLUENE

BY MICROCOCCUS SP. STRAIN SMN-1
BIODEGRADATION OF 2-NITROTOLUENE BY MICROCOCCUS SP. STRAIN SMN-1

This chapter describes results and discussions on the pathway for the degradation of 2-nitrotoluene by Micrococcus sp. strain SMN-1.

RESULTS

Growth behaviour of the Micrococcus sp. strain SMN-1

The isolated organism, Micrococcus sp. strain SMN-1 was grown in mineral salts medium supplemented with 15 mM 2-nitrotoluene as sole source of carbon and energy for its growth. Growth of the organism was determined by turbidometrically at 600 nm. The utilization of 2-nitrotoluene during the growth of the organism is shown in Fig IV.1. It is evident that the growth of the organism reached stationary phase after 6 days. The utilization of 2-nitrotoluene during growth of the organism was measured at different intervals of incubation period by extraction with diethylether. The residual 2-nitrotoluene was determined spectrophotometrically by measurement of decrease in UV absorbance at 255 nm. There was complete utilization of 2-nitrotoluene at 15 mM by the organism within 6 days of growth. There was release of nitrite (4.10 mM) during growth of organism on 2-nitrotoluene (Fig IV.1).

Effect of substrate concentration on growth

The effect of initial concentration of 2-nitrotoluene on growth of the organism is shown in the Table IV.1. When the initial concentration reached to a level of 15 mM, the growth rate of the organism reached maximum and after that the growth decreased with the increase in concentration of substrate. Hence, the increasing concentrations
Fig. IV.1. Utilization of 2-nitrotoluene (○-○) during growth (■-■) of *Micrococcus* sp. strain SMN-1 and the release of nitrite (▲-▲) in the culture supernatant. Uninoculated controls (+-+) in the mineral salts medium containing 15 mM 2-nitrotoluene. Data values represent averages of three replicate determinations.
Table IV.1. Effect of initial concentration of 2-nitrotoluene on the growth of *Micrococcus* sp. strain SMN-1.

<table>
<thead>
<tr>
<th>Initial concentration of 2-nitrotoluene (mM)</th>
<th>Growth (Absorbance at 600 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.03</td>
</tr>
<tr>
<td>3</td>
<td>0.11</td>
</tr>
<tr>
<td>6</td>
<td>0.25</td>
</tr>
<tr>
<td>9</td>
<td>0.42</td>
</tr>
<tr>
<td>12</td>
<td>0.55</td>
</tr>
<tr>
<td>15</td>
<td>0.72</td>
</tr>
<tr>
<td>18</td>
<td>0.60</td>
</tr>
<tr>
<td>21</td>
<td>0.44</td>
</tr>
</tbody>
</table>
were found to be toxic to the organism. Therefore, an initial concentration of 15 mM of 2-nitrotoluene was used for further studies.

**Identification of metabolites of 2-nitrotoluene**

The metabolites were isolated from the culture filtrates of *Micrococcus* sp. strain SMN-1 grown on mineral salts medium supplemented with 15 mM 2-nitrotoluene as described in materials and methods (Fig.II.1). Uninoculated controls were used to determine any transformation of 2-nitrotoluene affected by physical factors. Thin layer chromatographic (TLC) analysis of phenolic fraction of the culture filtrate of *Micrococcus* sp. strain SMN-1 grown on 2-nitrotoluene revealed the presence of a single compound, whose \( R_f \) values in different solvent systems corresponded well with that of authentic 3-methylcatechol run simultaneously. The TLC \( R_f \) values, HPLC retention time and UV \( \lambda_{max} \) of the isolated metabolite were corresponded well with that of authentic 3-methylcatechol (Table.IV.2). The acidic and neutral fraction of culture filtrate did not contained any metabolites. The UV spectrum of metabolite showed absorption maximum at 270 nm, which was superimposable with that of authentic 3-methylcatechol as shown in the Fig.IV.2.

The infrared (IR) spectrum of the metabolite was superimposable with that of authentic 3-methylcatechol as shown in the Fig.IV.3. The IR spectrum showed characteristic absorption bands of \(-\mathrm{OH}\) stretching at 3049 cm\(^{-1}\), \(\mathrm{C=O}\) stretching at 1674 cm\(^{-1}\), aromatic CH stretching at 2923 cm\(^{-1}\), \(\mathrm{C-O}\) stretching at 1281 cm\(^{-1}\) and \(\mathrm{C=C}\) stretching at 1625 cm\(^{-1}\).

The proton magnetic resonance spectrum of the metabolite was superimposable with that of authentic 3-methylcatechol as shown in the Fig.IV.4. The proton magnetic resonance spectrum of the isolated metabolite showed aromatic protons appeared as
multiple ranging from δ 6.4 to δ 6.8 ppm, phenolic hydroxyl protons appeared at δ 5.2 & δ 5.1 ppm and methyl protons appeared as multiple ranging from δ 2.0 to δ 2.4 ppm.

The mass spectrum of isolated metabolite (Fig. IV.5) showed molecular peak $M^+$ at $m/z$ 124, is in good agreement with empirical formula C$_7$H$_8$O$_2$. The main mass fragments observed at $m/z$ 51, 78, 106 and 124 were in good agreement with that of authentic 3-methylcatechol.

**Release of nitrite**

In order to know whether degradation of 2-nitrotoluene occurs by oxidative mechanism, the release of nitrite ions was determined in the culture filtrates. The release of nitrite ions during growth on 2-nitrotoluene is shown in Table V.3. There was release of about 4.1 mM nitrite ions after 6 days of incubation.
Table IV.2. Chromatographic and spectral characteristics of a metabolite of 2-nitrotoluene degradation by *Micrococcus* sp. strain SMN-1.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Isolated compound</th>
<th>Authentic 3-Methylcatechol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TLC: <em>R</em>(_f) values in different solvent systems:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A) Chloroform: acetic acid (95:5, v/v)</td>
<td>0.49</td>
<td>0.49</td>
</tr>
<tr>
<td>B) Toluene-ethylacetate-acetic acid (60:30:5, v/v)</td>
<td>0.74</td>
<td>0.75</td>
</tr>
<tr>
<td>C) Benzene-methanol-acetic acid (45:8:4, v/v)</td>
<td>0.78</td>
<td>0.77</td>
</tr>
<tr>
<td>D) Benzene-dioxan-acetic acid (74:2:2, v/v)</td>
<td>0.56</td>
<td>0.57</td>
</tr>
<tr>
<td><strong>HPLC (retention time in min)</strong></td>
<td>11.40</td>
<td>11.40</td>
</tr>
<tr>
<td><strong>UV absorption <em>λ</em>(_{max}) in methanol (nm)</strong></td>
<td>270</td>
<td>270</td>
</tr>
<tr>
<td><strong>GC-MS molecular peak M(^+) m/z</strong></td>
<td>51, 78,106,124</td>
<td>51, 78,106,124</td>
</tr>
</tbody>
</table>

*HPLC*, High-performance liquid chromatography.
Fig. IV.2. Ultraviolet spectra of isolated metabolite (-----) and the authentic 3-methylcatechol (------).
Fig. IV.3. Infrared spectra of isolated metabolite (-----) and the authentic 3-methylcatechol (----------).
Fig. IV.4. NMR spectrum of isolated metabolite of 2-nitrotoluene degradation by *Micrococcus* sp. strain SMN-1.
Fig. IV.5. Mass spectrum of isolated metabolite of 2-nitrotoluene degradation by Micrococcus sp. strain SMN-1.
Table IV.3. Release of nitrite ions in the culture supernatant during the degradation of 2-nitrotoluene by *Micrococcus* sp. strain SMN-1.

<table>
<thead>
<tr>
<th>Growth (days)</th>
<th>NO$_2^-$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>2.97</td>
</tr>
<tr>
<td>5</td>
<td>3.62</td>
</tr>
<tr>
<td>6</td>
<td>4.10</td>
</tr>
<tr>
<td>7</td>
<td>3.96</td>
</tr>
</tbody>
</table>
Growth studies

The growth of the Micrococcus sp. strain SMN-1 on various probable intermediates and related compounds are shown in Table.IV.4. The organism grown on 2-nitrotoluene was able to grow without any lag period on 2-nitrotoluene, 3-methylcatechol and catechol. However, 2-aminobenzoic acid, 2-hydroxybenzoic acid and protocatechuic acid could not support the growth of Micrococcus sp. strain SMN-1 or required lag period for the growth of organism. Whereas glucose-grown cells failed to utilize any of these compounds. These results suggest that 3-methylcatechol may be the metabolite of 2-nitrotoluene degradation.

Enzyme activities in the cell-free extracts

In order to get further insight into pathway for degradation of 2-nitrotoluene by Micrococcus sp. strain SMN-1, attempts were made to identify various enzymes involved in the degradative mechanism. The activities of different enzymes involved in the degradation of 2-nitrotoluene are given in the Table.IV.5. The cell-free extracts of Micrococcus sp. strain SMN-1 grown on 2-nitrotoluene contained the activity of catechol 2,3-dioxygenase. Catechol 1,2-dioxygenase activity was not found in the 2-nitrotoluene grown cells. The presence of high activity of catechol 2,3-dioxygenase in the cell-free extract of Micrococcus sp. strain SMN-1 grown on 2-nitrotoluene suggests that the metabolite 3-methylcatechol was further degraded by meta-cleavage pathway. The ring-cleavage product of 3-methylcatechol showed $\lambda_{\text{max}}$ at 375 nm, identical with that of 2-hydroxy-6-oxohepta-2,4-dienoate. The cell-free extracts of glucose-grown cells did not contain any of these enzyme activities. These results suggest that the enzymes involved in the degradation of 2-nitrotoluene were induced by growth of the organism on 2-nitrotoluene.
Table IV.4. Growth of *Micrococcus* sp. strain SMN-1 in mineral salts medium containing probable intermediates and related compounds.

<table>
<thead>
<tr>
<th>Substrate (15 mM)</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Nitrotoluene</td>
<td>+</td>
</tr>
<tr>
<td>2-Aminobenzoic acid</td>
<td>-</td>
</tr>
<tr>
<td>2-Hydroxybenzoic acid</td>
<td>-</td>
</tr>
<tr>
<td>Catechol</td>
<td>+</td>
</tr>
<tr>
<td>3-Methylcatechol</td>
<td>+</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>-</td>
</tr>
</tbody>
</table>
Table IV.5. Specific activities of enzymes in the cell-free extract of *Micrococcus* sp. grown on 2-Nitrotoluene.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity* (Units/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechol 1,2-dioxygenase</td>
<td></td>
</tr>
<tr>
<td>Catechol</td>
<td>0.000 ± 0.002</td>
</tr>
<tr>
<td>3-Methylcatechol</td>
<td>0.000 ± 0.002</td>
</tr>
<tr>
<td>Catechol-2,3-dioxygenase</td>
<td></td>
</tr>
<tr>
<td>Catechol</td>
<td>0.386 ± 0.002</td>
</tr>
<tr>
<td>3-Methylcatechol</td>
<td>0.459 ± 0.004</td>
</tr>
</tbody>
</table>

* One unit is defined as the formation or consumption of 1 µmole of the product or substrate respectively per min.

No activities were found in glucose-grown cells.

Values are the mean ± standard deviation (SD) of triplicates.
DISCUSSION

It is evident from the above results that the culture filtrates of *Micrococcus* sp. strain SMN-1 grown on 2-nitrotoluene contained 3-methylcatechol as metabolite with the release of nitrite. The initial formation of a dihydroxynitro intermediate from 2-nitrotoluene could be as reported for the other nitroaromatic compounds (Haigler et al., 1994; Nishino and Spain 1995; Nishino et al., 2000). This unstable intermediate would undergo rearomatization with loss of nitrite to yield 3-methylcatechol. Ammonia was not detected in the culture filtrates of organism grown on 2-nitrotoluene. Thus the organism degraded 2-nitrotoluene by oxidative instead of reductive mechanism. Growth and enzymatic studies have confirmed that 2-nitrotoluene was degraded through 3-methylcatechol by *Micrococcus* sp. strain SMN-1. The cells of *Micrococcus* sp. strain SMN-1 grown on 2-nitrotoluene utilized 3-methylcatechol as carbon source. The presence of high activities of catechol 2,3-dioxygenase in the cell-free extract of *Micrococcus* sp. strain SMN-1 grown on 2-nitrotoluene suggested that 3-methylcatechol was further oxidized through *meta*-cleavage pathway as shown in Fig.IV.6. The pathway for degradation of 2-nitrotoluene in *Micrococcus* sp. strain SMN-1 appears to be similar to that described in *Pseudomonas* sp. strain JS42 (Haigler et al., 1994) but differs from that described in *Pseudomonas putida* strain OU83, which involves reduction of 2-nitrotoluene to 2-aminotoluene (Walia et al., 2003).

This is the first report on the degradation of 2-nitrotoluene by a Gram-positive bacteria, *Micrococcus* sp. strain SMN-1. Moreover, this organism could utilize 2-nitrotoluene at higher concentration (15 mM) as compared to *Pseudomonas* sp. strain JS42 (Haigler et al., 1994) which utilized 1.1 mM 2-nitrotoluene and *Pseudomonas putida* strain OU83 (Walia et al., 2003) utilized only 0.34 mM of 2-nitrotoluene.
Fig. IV.6. Proposed pathway for the degradation of 2-nitrotoluene by *Micrococcus* sp. strain SMN-1.
Microbial degradation of other two isomers, 3-nitrotoluene reported to occur either by oxidative or reductive mechanism and 4-nitrotoluene was degraded by reductive mechanism. *Comamonas* sp. strain JS765 used oxidative mechanism to convert 3-nitrotoluene to 4-methylcatechol (Lessner et al., 2002). Whereas *Pseudomonas putida* OU3 converted 3-nitrotoluene to 3-nitrobenzylalcohol, which was further degraded stepwise into 3-nitrobenzaldehyde, 3-nitrobenzoic acid and finally 3-nitrophenol (Walia et al., 2003).

*Mycobacterium* sp. stain HL 4-NT-1 degraded 4-nitrotoluene to 4-hydroxylaminotoluene with the reduction of nitro group, followed by transformation into 6-amino-m-cresol (Spiess et al., 1998). Whereas *Pseudomonas* sp. strain 4-NT and *Pseudomonas putida* TW3 degraded 4-nitrotoluene to 4-nitrobenzoic acid by sequential oxidation of the methyl group, which was further oxidized to protocatechuic acid (Haigler and Spain 1993; Rhys-Williams et al., 1993). In contrast to 3-nitrotoluene and 4-nitrotoluene, which were degraded by reductive mechanism, 2-nitrotoluene was exclusively degraded by oxidative mechanism by *Micrococcus* sp. strain SMN-1 and also by *Pseudomonas* sp. strain JS42 (Haigler et al., 1994).

Thus the present studies have revealed that the isolated bacterial strain was versatile in degrading 2-nitrotoluene. Such bacterial strain could be useful in the bioremediation of soils and industrial effluents contaminated with toxic nitroaromatic compounds.