The present study was undertaken to isolate, characterize and determine the ability of the bacterial strains to metabolize and degrade the toxic insecticide endosulfan. Technical grade endosulfan consists of an α-isomer and a β-isomer in an approximately 7:3 ratio. Initially using three different media where endosulfan was supplied either as the co-substrate, the sulfur source or the carbon source, eight bacterial isolates were isolated from agricultural soils. However, due to the low degradation ability of these bacterial isolates, another soil sample from pesticide dumping site near a pesticide manufacturing industry was used to isolate microbial strains that could degrade endosulfan. A total of 26 bacterial strains and 1 fungal strain were isolated from the soil collected from the pesticide dumping site. Detailed quantitative analysis of both the sets of microbial strains including intermediate metabolites arising from the microbial degradation is presented in this chapter. The selected strains that displayed higher endosulfan degrading capacity were characterized using molecular tools and their growth pattern on endosulfan was studied. Based on the degradation rates the best degrading strain was selected for optimization of cultural parameters to enhance degradation. The growth and intermediate formation by the selected strain was further examined under optimized conditions. As the molecular basis of endosulfan degradation is not very well studied, an attempt was made to screen for monooxygenase genes reported for endosulfan degradation in selected strains. Details of the same are described in this chapter.

**Characterisation of agricultural soil samples**

Soils contaminated with endosulfan residues were considered as an appropriate source of harboring bacteria with the ability to tolerate and degrade endosulfan. Therefore initially in the study, various agricultural sites that had been exposed to endosulfan use were chosen for collection of soil samples. These sites were present in the north and north eastern regions of India (Figure 3.1). The agricultural soil samples A1, A2 and A3 were collected from different agricultural fields i.e. tea plantation in Assam (North-east India), egg plant crop
in Haryana (North India) and tomato crop in New Delhi (North India) that had reported the use of endosulfan for various periods of time (Table 4.1). The period of exposure to endosulfan in the soil samples collected from agricultural sites ranged from two to five years (Table 4.1). The soil samples collected from the tea plantation (A1) were acidic whereas the soil samples from vegetable fields (A2 and A3) displayed neutral pH (Table 4.1).

The residual concentration of the endosulfan in agricultural soil samples were analysed by GC methods as per the protocols described earlier and the representative analysis report for the agricultural soil sample A1 is shown in Figure 4.1. The A3 soil sample from the tomato field was observed to possess relatively lower residual endosulfan concentrations than the A1 and A2 soil samples (Table 4.1). On the other hand the soil from the tea plantation (A1) that reported the longest duration for exposure to endosulfan amongst the agricultural soil samples was observed to have higher residual concentrations of endosulfan (Table 4.1). Detailed characteristics of the agricultural soil samples collected from this study are mentioned in Table 4.2.

Table 4.1. Details of the agricultural soil samples collected for the enrichment and isolation of endosulfan degrading microbial strains.

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Sample description</th>
<th>Application details and contamination history</th>
<th>Residual content of endosulfan in soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>Tea plantation, Assam, Eastern India</td>
<td>Repeated application for 5 years</td>
<td>1.2 ± 0.06 mg/kg</td>
</tr>
<tr>
<td>A2</td>
<td>Egg plant growing field, Haryana, Northern India</td>
<td>Repeated application for 2 – 3 years</td>
<td>0.9 ± 0.02 mg/kg</td>
</tr>
<tr>
<td>A3</td>
<td>Tomato growing field, New Delhi, North India</td>
<td>Intermittent application for 2 years</td>
<td>0.20 ± 0.03 mg/kg</td>
</tr>
</tbody>
</table>

Table 4.2. Detail characteristics of the agricultural soil samples collected for the enrichment and isolation of endosulfan degrading microbial strains.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Soil samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A1</td>
</tr>
<tr>
<td>Texture</td>
<td>Clay</td>
</tr>
<tr>
<td>Type</td>
<td>Entisol</td>
</tr>
<tr>
<td>Organic carbon (%)</td>
<td>1.25</td>
</tr>
<tr>
<td>Available Nitrogen (as N) Kg/hectare</td>
<td>28</td>
</tr>
<tr>
<td>Available phosphorus (as P2O5) Kg/hectare</td>
<td>80.5</td>
</tr>
<tr>
<td>Available potassium (as K2O) Kg/hectare</td>
<td>321.1</td>
</tr>
<tr>
<td>Moisture content (%)</td>
<td>31.2%</td>
</tr>
<tr>
<td>Electrical conductivity (dS/m)</td>
<td>0.527</td>
</tr>
<tr>
<td>pH value (at 25 °C)</td>
<td>5.8</td>
</tr>
</tbody>
</table>
Figure 4.1. Gas chromatogram depicting residual endosulfan concentration in the agricultural soil sample A1.

**Enrichment of endosulfan degrading microbial consortia from agricultural soil samples**

A total of three soil samples (A1, A2 and A3) were collected from different agricultural fields as described in Table 4.1 in the previous section. These three agricultural soil samples were used for isolation of bacterial strains that can degrade endosulfan. The three different media used for the purpose of enrichment are as follows and the detailed composition of the same are mentioned in Annexure:

- Rich Medium (RM), which was rich in nutrients and included mannitol as the carbon source and MgSO4 as the sulfur source along with technical grade endosulfan as a co-substrate.
- Sulfur Free Medium (SFM), that contained technical grade endosulfan as the sulfur source
- Minimal Salt Medium (MSM), with technical grade endosulfan as the carbon source
Enrichment of agricultural (A1, A2 and A3) soil samples using Rich Medium (RM)

The enrichments were initiated with the agricultural soil samples (A1, A2 and A3) using Rich Medium as described in the previous chapter. Two concentration of endosulfan (4 mg/l or 10 mg/l endosulfan) were used during enrichment and a total of six different enrichments were set up. However after four enrichment cycles (each enrichment cycle of 20 days) bacterial growth, reduction in the concentration of technical endosulfan as well as intermediate formation, was observed only in two enrichment flasks (Table 4.3). The consortia from these enrichment flasks were designated as Consortium 1 and Consortium 2 (Table 4.3). Consortium 1 was developed from the enrichment cycle that used the A1 soil sample whereas Consortium 2 was developed from the A2 soil sample. Both Consortium 1 and Consortium 2 were developed in media that contained the endosulfan concentration of 4 mg/l during enrichment (Table 4.3). In contrast, enrichments initiated with the A3 soil sample and 4 mg/l endosulfan failed to develop viable endosulfan degrading consortia. In this regard it was noted that A3 soil sample that were taken for enrichment had the least residual concentration of endosulfan amongst the other collected soil samples (Table 4.1). Additionally no bacterial growth was observed in the enrichments initiated with 10 mg/l endosulfan with A1, A2 or A3 soil samples.

The utilization of endosulfan during the end of the enrichment cycle was determined by GC analysis. The details of the two consortia (Consortium 1 and Consortium 2) that showed growth and decease in concentration endosulfan are described further in this section.

Table 4.3. Enrichment of agricultural soil samples A1 and A2 in Rich Medium with 4 mg/l technical grade endosulfan

<table>
<thead>
<tr>
<th>Soil sample</th>
<th>Endosulfan concentration in enrichment</th>
<th>Growth (OD 660 nm)</th>
<th>Reduction in endosulfan concentration (mg/l)</th>
<th>Consortium designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>4 mg/l growth observed</td>
<td></td>
<td>2.82</td>
<td>Consortium 1</td>
</tr>
<tr>
<td>A2</td>
<td>4 mg/l growth observed</td>
<td></td>
<td>2.18</td>
<td>Consortium 2</td>
</tr>
</tbody>
</table>

Consortium 1 displayed higher endosulfan degradation than the Consortium 2. It was observed that the concentration of technical endosulfan in the enrichment flask of Consortium 1 reduced from an initial value of 4.3 mg/l to 1.48 mg/l. This
Results

indicated that Consortium 1 was able to degrade 65.58% of technical grade endosulfan at the end of 20 days. In case of Consortium 2, degradation of technical grade endosulfan was observed to be 51.90% as the initial concentration of 4.2 mg/l was reduced to 2.02 mg/l. In both cases there was an increase in the optical density of the culture medium as indicated in Figure 4.2 and Figure 4.3.

In case of Consortium 1, rapid growth was observed from the start as the optical density of the culture reached around 1.5 within day 1. Thereafter growth was still observed to increase until day 2 of incubation after which no further increase was observed (Figure 4.2). As Rich Medium medium is nutrient rich and contains an easily available carbon source (refer Annexure) the heterotrophic bacteria present in the soil sample might have contributed to this rapid growth rate.

Though there was a rapid exponential increase in the growth as observed with the optical density readings, a significant decline in the concentration of residual endosulfan isomers in the medium was observed only from day 2 onwards (Figure 4.2). The Consortium 1 reduced the concentration of the $\alpha$-isomer from an initial 3.0 mg/l to 1.2 mg/l, indicating 60.0% degradation within 20 days. Correspondingly the concentration of the $\beta$-isomer was reduced from 1.3 mg/l to 0.28 mg/l, which revealed 78.46% degradation within the incubation period of 20 days (Figure 4.2).

Figure 4.2. Degradation of technical grade endosulfan by Consortium 1
In case of Consortium 2 a similar pattern of rapid exponential growth observed until day 1 followed by steady but less rapid growth until day 6 (Figure 4.3). The optical density of the culture was recorded to be around 0.8 on the first day of incubation and reached around a value of 1 by day 6 (Figure 4.3). Once again the concentration of the endosulfan isomers was observed to decline from day 2 onwards. It was observed that the concentration of the $\alpha$-isomer reduced from an initial value of 2.9 mg/l to 1.65 mg/l by the end of 20 day incubation. This indicated 43.1% degradation. On the other hand the concentration of $\beta$-isomer was reduced from 1.30 mg/l to 0.37 mg/l, indicating 71.53% degradation (Figure 4.3) in 20 days.

![Graph](image)

**Figure 4.3. Degradation of technical grade endosulfan by Consortium 2**

In both Consortium 1 and Consortium 2 the $\beta$-isomer was observed to be degraded to a greater extent than the $\alpha$-isomer. The GC analysis of the samples from the enrichment flasks indicated that both the consortia produced the intermediate metabolite endosulfan diol during the degradation of technical endosulfan (Figure 4.4).

The pH of both the consortia was continuously monitored. The pH of the Consortium 1 and Consortium 2 was observed to decrease from 7 to 6.7 and 6.5 respectively during the first two days (Figure 4.5). However by Day 4, the pH increased to 7.5 in the case of Consortium 1 and 7.3 in the case of the Consortium 2. At the end of the incubation period of 20 days the pH of the Consortium 1 and Consortium 2 cultures dropped slightly to 7.3 and 7.2 respectively (Figure 4.5).
Results

Figure 4.4. Gas chromatogram depicting the degradation of technical grade endosulfan and the formation of intermediate metabolites by Consortium 1.

Figure 4.5. Change in the pH profile of Consortium 1 and Consortium 2 during the incubation period.
Enrichment of agricultural (A1, A2 and A3) soil samples using Sulfur Free Medium (SFM)

In order to isolate bacterial strains that can utilize endosulfan as the source of sulfur a set of six enrichments were initiated with the agricultural soil samples (A1, A2 and A3) using Sulfur Free Medium (refer Annexure) and either 4 mg/l or and 10 mg/l endosulfan. After the end of the enrichment cycles, growth was observed through a marginal increase in the optical density in the enrichment cultures initiated with A1 and A2 soil samples with 4 mg/l endosulfan. None of the other enrichments showed any increase in the bacterial activity. However in the enrichment flasks that showed a marginal increase in the optical density did not show any significant reduction in the concentration of technical endosulfan or formation of any intermediates. Thus using the agricultural soils, the enrichment cycle initiated with Sulfur Free Medium could not yield a microbial consortium for the degradation of endosulfan.

Enrichment of agricultural (A1, A2 and A3) soil samples using Minimal Salt Medium (MSM)

To isolate bacterial strains that can utilize endosulfan as the source of carbon another set of six enrichments were initiated with the agricultural soil samples (A1, A2 and A3) using Minimal Salt Medium (refer Annexure). As described for the previous enrichments two concentrations of endosulfan (4 mg/l or and 10 mg/l endosulfan) was used during the 20 day enrichment cycles. At the end of the enrichment cycles it was observed that growth was absent in all of the six enrichment flasks as no increase in the optical density of the cultures. This indicated that the microbial community in the agricultural soil samples were not able to utilize endosulfan as the carbon source in either 4 mg/l or 10 mg/l concentration. Thus the Minimal Salt Medium enrichments initiated with agricultural soil were also not taken up for further experimentation in this study.

Characterisation of Consortium 1 and Consortium 2 developed from agricultural soil samples

Consortium 1 and Consortium 2 obtained from agricultural soil samples A1 and A2 (Table 4.3) respectively were characterized. Aliquots from these two different consortia were plated on agar plates prepared using the Rich Medium with endosulfan concentration of 4 mg/l. After incubation of the plates under conditions described in the previous chapter individual, single, isolated bacterial
colonies were selected based on the differentiation of morphological characteristics of the respective colonies.

In total eight individual bacterial colonies were isolated from the Consortium 1 and Consortium 2. The eight strains isolated from two consortia that were developed from agricultural soil samples A1 and A2 were designated with code starting from RM1 to RM8. These strains were then taken up for further studies.

Quantitative determination of the endosulfan degradation capacity of the strains isolated from agricultural soil samples

The determination of the quantitative degradation capacities of all the eight strains isolated from the agricultural soils was conducted by GC analysis. Initially the quantitative estimation of the degradation capacity was done in Rich Medium.

Endosulfan degradation capacity of the bacterial strains RM1- RM8 in Rich Medium (RM)

In order to estimate the degradation efficiency of the bacterial strains RM1 to RM8 isolated from agricultural soil samples in media that contained rich source of nutrients, the individual strains were incubated in same medium containing 4 mg/l endosulfan under conditions described in the previous chapter. Gas Chromatography analysis showed that the strains degraded technical grade endosulfan in the range of 41.4 % to 80.3%. Among the total eight bacterial strains, the maximum degradation of technical grade endosulfan was exhibited by strain RM2 while the least degradation was observed by strain RM4. The mean of three degradation experiments and this revealed that strains RM2 and RM3 degraded technical grade endosulfan by 80.3% and 65%. Bacterial strains RM1, RM5, RM6 and RM 7 degraded technical grade endosulfan by 51.7%, 55.9%, 50.1% and 60.8%, while strains RM4 and RM8 displayed less than 50% degradation respectively. Strain RM8 degraded endosulfan by 45.2% while strain RM4 degraded it by 41.4%. After 20 days of incubation in the strain RM2 culture the concentration of the α-isomer declined by 70.4 % whereas that of the β-isomer declined by 89.7%. On the other hand strain RM3 was observed to reduce the level of the α-isomer by 56.6% and the level of the β-isomer by 73.1%. However during the incubation period a significant loss of endosulfan was observed in un-inoculated control flasks. GC analysis revealed that abiotic losses accounted for around 33.67% of endosulfan degradation observed in un-
inoculated controls endosulfan. Thus in order to determine the actual degradation of endosulfan these strains the degradation efficiency was recalculated by taking into account the losses observed in the un-inoculated control flasks from day 0 until day 20. The actual degradation of endosulfan by bacterial strains RM2 and RM3 was thus observed to be 46.7% and 31.4% respectively. Similarly bacterial strains RM7, RM5, RM1 and RM6 was observed to effectively degrade technical grade endosulfan by 26.8%, 21.9%, 19.2% and 19.0% respectively. In case of strains RM8 and RM4 a marginal degradation of 11.2% and 7.45% was observed respectively. The extent of the actual degradation of endosulfan isomers by the eight isolates is depicted in Figure 4.6.

![Figure 4.6. Actual degradation of technical grade endosulfan by strains RM1- RM8 in Rich Medium.](image)

In flasks inoculated with strain RM2 at the end of the incubation period 40.1% and 54.3% actual reduction was seen in the level of the α-isomer and the β-isomer respectively (Figure 4.6). Similarly, in the strain RM3 culture 23.2% actual reduction of the α-isomer and 39.9% actual reduction of the β-isomer was observed in 20 days (Figure 4.6).

GC analysis of the contents of the inoculated flasks further revealed the formation of the metabolite endosulfan diol in significant quantities in the cultures of strains RM2 and RM3 (Figure 4.7). Endosulfan diol was also detected in minute amount in the flasks inoculated with RM5, RM7 and RM1. However endosulfan diol were also observed in control flasks at the end of incubation period of 20 days (Figure 4.7).
Figure 4.7. Gas chromatogram depicting the degradation of technical grade endosulfan and the formation of intermediate metabolites by strain RM2 and strain RM3 in Rich Medium.
The pH of the culture flasks was monitored during the course of the degradation. It was observed that the pH of culture flasks incubated with bacterial strains RM2 and RM3 initially reduced and then increased to 8.3 and 7.7 during the incubation period. Similarly, at the end of the incubation period the pH of the culture flasks of strain RM1 was 7.1 while that of strains RM7 and RM5 was 7.2. The pH of RM6, RM4 and RM8 ranged between 6.5-6.6. The pH of the control flask remained stable at 7.0.

In a separate time course experiment the loss of α- and β-isomers was observed on particular days along with the formation of endosulfan diol for strain RM2. In the case of strain RM2 the estimation of residual concentration of endosulfan suggested a loss of 1.86 mg/l after accounting for losses in the control flasks, indicating 42.2% degradation of endosulfan. The effective degradation of the α-isomer was by 1.14 mg/l or 38.0% while that of the β-isomer was by 0.72 mg/l or 51.42%.

In the control flasks, at the end of day 20 the formation of endosulfan diol appeared to be approximately 15.5% the initial concentration of the endosulfan that was added to the medium (Figure 4.8). In case of strain RM2 the endosulfan diol concentrations were observed to be at 35.2% at day 20 (Figure 4.9.). The highest concentrations of endosulfan diol in the inoculated flasks were observed to be on day 16 at 36.3%.

![Figure 4.8. Degradation of technical grade endosulfan and the formation of endosulfan diol in control flasks](image_url)
The results of these experiments indicated that a loss of endosulfan was occurring in un-inoculated control flasks. Furthermore it also suggested that the abiological transformation of endosulfan to endosulfan diol occurred at pH 7 in un-inoculated controls. Two previous studies by Martens (1976) and Sutherland et al. (2000) have reported that at pH 7 and above endosulfan are transformed to endosulfan diol due to chemical hydrolysis. However Kwon et al. (2002) reported an absence of abiotic loss between pH 7-8. Thus in order to assess if pH plays a role in the transformation of endosulfan to endosulfan diol another set experiment was designed to estimate the abiotic losses that might occur in sterile medium at different pH values under the conditions used for biodegradation in this study. The results of this experiment are described in the next section.

**Determination of abiological loss of endosulfan and formation of intermediates in sterile medium at different pH values**

The experiment to determine the influence of pH on the abiotic loss of endosulfan was conducted in Rich Medium at different pH values. As described in the previous chapter, flasks containing sterile Rich Medium were maintained individually at pH 5.9, 6.3, 7.0, 7.5 and 8.1 and for an incubation period of 20 days.

The GC analysis indicated that at pH 5.9 and 6.3 metabolite formation was absent and abiotic losses of endosulfan due to other reasons were observed to be...
around 15.8%. However at pH 7 and 7.5 there was an increase in loss of residual endosulfan in the medium. Total losses of endosulfan on day 20 amounted to 33.7% at pH 7 and 45.5% at pH 7.5. At pH 8.1 the loss of endosulfan was 62%. The decline in the concentration of endosulfan isomers at the different pH values is depicted in Figure 4.10.

Further at pH 7 and above GC analysis revealed that endosulfan appeared to undergo chemical hydrolysis to form the metabolite endosulfan diol (Figure 4.11). At day 20, in the flasks maintained at pH 7.0 and 7.5 the endosulfan diol concentration appeared to be around 17% and 30% of the original endosulfan concentration in the medium. At pH 8.1 endosulfan diol concentrations appeared to be 40% of the original concentration of endosulfan in the medium. Apart from endosulfan diol none of the other intermediates were observed during the incubation period in these flasks (Figure 4.11). These results confirmed that greater abiotic losses of endosulfan occur at pH 7 and above when compared to the losses that occur when the pH of the media is maintained at values below 7. It also revealed that endosulfan can through abiotic means, be transformed to the metabolite endosulfan diol at pH 7 and above and that the concentration of endosulfan diol increases with increasing pH. The abiological transformation of endosulfan to endosulfan diol was not observed at pH 6.3 and below.
Figure 4.11. Gas chromatogram depicting the abiological loss of technical grade endosulfan and formation of metabolites when incubated in sterile media at different pH conditions.
Endosulfan degradation capacity of the bacterial strains RM1-RM8 in Modified-Rich Medium (M-RM)

The initial pH of the Rich Medium used in the previous experiments for enrichment using agricultural soils as well as to determine the degradation of isolated strains RM1 to RM8 was adjusted to 7. As described in the previous section, the abiotic loss of endosulfan at pH 7 and above was over 33.5%. Thus in order to minimize abiotic losses and prevent the chemical hydrolysis of endosulfan an attempt was made to buffer the medium at a lower pH. Thus the original medium composition of Rich Medium was modified. The composition of this modified medium that was referred to as ‘Modified-Rich Medium’ included a higher concentration of dihydrogen phosphate (KH₂PO₄). Further the initial pH of Modified-Rich Medium was adjusted to a value of 6.3 (refer Annexure). Thereafter the ability of the eight strains (RM1-RM8) to degrade endosulfan was once again determined in this Modified-Rich Medium in attempts to negate the effect of auto-degradation.

GC analysis revealed that the abiotic losses from un-inoculated control flasks in the modified medium during the incubation period reduced to approximately 15-16% as compared to 33.5 % observed with the previous medium. Additionally GC analysis also indicated that higher residual levels of endosulfan were present in the cultures when strains were incubated with 4 mg/l endosulfan in Modified-Rich Medium than when compared they were incubated in Rich Medium (Table 4.4). The actual degradation of technical grade endosulfan by the eight bacterial strains was calculated by taking into consideration the losses in the control flasks between day 0 and day 20. As observed in Table 4.4, strain RM2 degraded technical endosulfan to the maximum extent even in the Modified-Rich Medium, however the actual degradation values significantly reduced when compared to those observed Rich Medium.

The mean of three experimental replicate revealed that the actual degradation of technical endosulfan by strains RM2 in the modified medium was 32.2% while that of strains RM3, RM5 and RM7 was 20.4%, 17.6% and 19.3% respectively. In contrast the corresponding values for the actual degradation of endosulfan by strains RM2, RM3, RM5 and RM7 in Rich Medium (where the initial pH of the medium was 7) were 46.7%, 31.4%, 21.9% and 26.8% (Table 4.4). Similarly the actual degradation values for remaining bacterial strains RM1, RM6 and RM8 that showed low degradation capacity in RM were further decreased in Modified-Rich Medium (Table 4.4). The degradation of strains RM1, RM6 and RM8 in Modified-Rich Medium was observed to be at 13.5%,
12.9% and 10.5% respectively (Table 4.4). Strain RM4 showed insignificant degradation in Modified-Rich Medium.

Table 4.4. Comparison of the endosulfan degradation capabilities of strains RM1- RM8 in Rich Medium and Modified-Rich Medium

<table>
<thead>
<tr>
<th>S.no</th>
<th>Strain designation</th>
<th>Degradation of technical endosulfan (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rich Medium (pH 7)</td>
</tr>
<tr>
<td>1</td>
<td>RM1</td>
<td>19.2</td>
</tr>
<tr>
<td>2</td>
<td>RM2</td>
<td>46.7</td>
</tr>
<tr>
<td>3</td>
<td>RM3</td>
<td>31.4</td>
</tr>
<tr>
<td>4</td>
<td>RM4</td>
<td>7.45</td>
</tr>
<tr>
<td>5</td>
<td>RM5</td>
<td>21.9</td>
</tr>
<tr>
<td>6</td>
<td>RM6</td>
<td>19</td>
</tr>
<tr>
<td>7</td>
<td>RM7</td>
<td>26.8</td>
</tr>
<tr>
<td>8</td>
<td>RM8</td>
<td>11.2</td>
</tr>
<tr>
<td>9</td>
<td>Control</td>
<td>33.5</td>
</tr>
</tbody>
</table>

In Modified-Rich Medium at the end of day 20 the concentration of the α-isomer in the culture of strain RM2 decreased to 29.1% while that of the β-isomer decreased by 35.2%. The details of the decline of endosulfan isomers by the eight bacterial strains (RM1- RM8) in Modified-Rich Medium are presented in Figure 4.12. Once again as observed in Rich Medium, the cultures of strains RM2 and RM3 showed a greater reduction in the level of β-isomer than the α-isomer. Results indicated that all the cultures of all other strains displayed nearly equal degradation of the α-isomer and the β-isomer in the Modified-Rich Medium (Figure 4.12).

Figure 4.12. Degradation of technical grade endosulfan by strains RM1-RM8 in Modified-Rich Medium.
GC analysis of the contents of the inoculated flasks further revealed the formation of the metabolite endosulfan diol in the cultures of strains RM2 and RM3. No metabolites were detected in un-inoculated control flasks or in flasks inoculated with the other strains. Representative gas chromatogram of strains RM is presented in Figure 4.13.

Regular monitoring of the pH in inoculated flasks and control flasks revealed that the pH of cultures flasks inoculated with RM2 and RM3 initially reduced to pH 6 and then increased to 7.3 and 7.0 respectively during the incubation period. It was observed that the pH of the remaining bacterial cultures RM1, RM4, RM6, RM7 and RM8 ranged between 6.1 and 6.4 during the incubation period. The pH of the flask inoculated with strain RM5 was 6.6 at the end of the incubation period, while the pH of the control flask remained stable at 6.2. As endosulfan diol was only observed in the cultures of strain RM2 and RM3 where the pH of the cultures were at and over the value of 7 it indicated that this transformation was chemically induced due to the increase in pH brought about by these strains in the medium in which they degraded endosulfan.

Figure 4.13. Gas chromatogram depicting the degradation of technical grade endosulfan and formation of intermediates by strain RM2 in Modified-Rich Medium
Determination of the endosulfan degrading capacity of strain RM2 in Sulfur Free Medium and Minimal Salt Medium

In general the degradation capacity of strains isolated from agricultural soil was observed to be low i.e. specifically below 33%. As described in the preceding paragraphs, ability of bacterial strain RM2 to degrade endosulfan was highest when compared with the remaining seven strains isolated from agricultural soil samples (Table 4.4 and Figure 4.12). On the other hand the other seven strains from agricultural soil samples were observed to degrade insignificant concentrations of technical grade endosulfan (at or below 20%) (Table 4.4). Hence none of these seven bacterial strains were taken forward for further studies.

The bacterial strain RM2 isolated from agricultural soil sample showed degradation of 32.2% endosulfan in a 20 day incubation period with the production of endosulfan diol. However results indicated that the degradation of endosulfan by this strain in part might be attributed to its chemical hydrolysis to endosulfan diol that occurred due to the increase in pH to alkaline values as a result of bacterial intervention. As the buffering capacity of soil is high, it is most likely that strain RM2 might not be able to degrade endosulfan in acidic soils where endosulfan contamination is largely prevalent in India.

Strain RM2 was isolated from Consortium 1 that was enriched in Rich Medium. Its degradation capacity was examined in Rich Medium and Modified-Rich Medium (Table 4.4). Both Rich Medium and Modified-Rich Medium contained rich source of nutrients including a carbon source (mannitol) and sulfur source (MgSO₄) (refer Annexure). However for selection of an ideal bacterial strain(s) for bioremediation, the strain(s) should ideally use the target substrate as a nutrient source- as the source of carbon or in the case of endosulfan as a source of sulfur (as endosulfan has a sulfite moiety). Thus an attempt was made to examine the capacity of strain RM2 to degrade endosulfan in Minimal Salt Medium where endosulfan was provided as a sulfur source.

Endosulfan degradation capacity of the strain RM2 in Sulfur Free Medium (SFM)

An attempt was made to determine the ability of strain RM2 to degrade endosulfan in Sulfur Free Medium where endosulfan was supplied as a sulfur source. Since strain RM2 had been isolated from consortium developed on Rich Medium using mannitol as the carbon source, mannitol was also used as the
carbon source in Sulfur Free Medium in place of glucose. To ascertain if mannitol-Sulfur Free Medium could provide an environment for the favorable growth of strain RM2, it was grown in this medium with MgSO4 as the sulfur source. Growth studies of RM2 in mannitol-Sulfur Free Medium in the presence of MgSO4 (without endosulfan) revealed that strain RM2 showed luxurious growth in this medium. Therefore mannitol-Sulfur Free Medium with 4 mg/l endosulfan as the sole sulfur source was used to determine the ability of strain RM2 to degrade the xenobiotic as a sulfur source.

An increase in optical density was observed when strain RM2 was grown in mannitol-Sulfur Free Medium that was supplemented with 4 mg/l endosulfan as the sulfur source. However when residual endosulfan concentrations in the culture flasks of strain RM2 were compared to that of un-inoculated controls, insignificant degradation of endosulfan was observed. The pH of the inoculated flasks ranged from 5.0 to 6.0. The pH of the un-inoculated controls remained stable at 6.3 ± 0.4.

**Endosulfan degradation capacity of the strain RM2 in Minimal Salts Medium (MSM)**

Experiments undertaken for strain RM2 in minimal medium revealed that the strain was able to grow on Minimal Salt Medium supplemented with 4 mg/l endosulfan. However GC analysis revealed that there was no significant reduction in the concentration of technical endosulfan as compared to that observed in un-inoculated control flasks. Therefore the increase in the optical density during the incubation of strain RM2 in Minimal Salt Medium might be attributed to the presence of Tween 80 in the medium. No growth was observed when strain RM2 was grown in Minimal Salt Medium devoid of Tween 80 in the presence of 4 mg/l endosulfan. Thus strain RM2 might have used Tween 80 that had been added to the medium at a concentration of 0.05% (v/v) as the carbon source instead of endosulfan resulting in an increase the optical density. The pH of the inoculated flasks ranged from 6.2 to 6.4 while that of the inoculated flask remained at 6.3 ± 0.9.

Overall, the estimation of the endosulfan degradation ability of bacterial isolates from agricultural soil revealed that all the eight strains degraded endosulfan ineffectively. Therefore at this juncture in the study another soil sample (P1) was procured from a dump site of a manufacturing industry that had a long history of endosulfan exposure. Thus the focus of the study was shifted to enriching and isolating strains from the P1 soil sample that was collected from the endosulfan manufacturing industry.
Characterisation of soil sample from the pesticide dumping site of a manufacturing industry (P1)

In the later part of the study, another soil sample (P1) was procured from a dumping site at a pesticide manufacturing plant, Excel Industry, Mumbai situated in the western region of India. The soil at this site was reportedly exposed to endosulfan for over 20 years and was observed to be mildly acidic (pH 6.2). The detailed characteristics of the P1 soil sample are described in Table 4.5. The residual contamination of endosulfan in the P1 soil sample was 4.5 ± 0.03 mg/kg. Therefore this soil sample (P1) was observed to have a higher residual concentration of endosulfan when compared with the other three agricultural soil samples (A1, A2 and A3) analysed initially for this study (Table 4.1). The analysis of the residual concentration of the endosulfan by GC methods of this soil sample is shown in Figure 4.14. The GC analysis of the P1 soil sample indicated the presence of residual concentration of endosulfan sulfate aside from the endosulfan isomers (Figure 4.14).

Table 4.5. Detail characteristics of the soil sample collected from the pesticide dumping site from a pesticide manufacturing industry for the enrichment and isolation of endosulfan degrading microbial strains.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Soil sample P1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Texture</td>
<td>Clay</td>
</tr>
<tr>
<td>Type</td>
<td>Aridisol</td>
</tr>
<tr>
<td>Organic carbon (%)</td>
<td>0.14</td>
</tr>
<tr>
<td>Available Nitrogen (as N) Kg/hectare</td>
<td>53</td>
</tr>
<tr>
<td>Available phosphorus (as P₂O₅) Kg/hectare</td>
<td>102</td>
</tr>
<tr>
<td>Available potassium (as K₂O) Kg/hectare</td>
<td>45</td>
</tr>
<tr>
<td>Moisture content (%)</td>
<td>15.7%</td>
</tr>
<tr>
<td>Electrical conductivity (dS/m)</td>
<td>0.16</td>
</tr>
<tr>
<td>pH value (at 25 °C)</td>
<td>6.2</td>
</tr>
</tbody>
</table>
Figure 4.14. Gas chromatogram depicting residual endosulfan concentration in industrial soil sample P1

**Enrichment of endosulfan degrading microbial consortia from industrial soil sample**

As described for the enrichment of agricultural soils, three strategies that used three different media were used to enrich for endosulfan degrading strains from the P1 soil sample. Results from studies conducted previously in sterile media had revealed that greater abiotic loss including chemical transformation of endosulfan to endosulfan diol occurred at pH 7 and above. Therefore in the first strategy where the attempt would be to enrich for endosulfan degrading bacteria in a medium rich in nutrients, Modified-Rich Medium was used as the medium for the enrichment cycles. The Modified-Rich Medium was buffered by introducing the salt KH$_2$PO$_4$ into the composition of and adjusting the initial pH to 6.3.

Along with the Modified-Rich Medium (M-RM), the industrial soil sample P1 was also enriched in Sulfur Free Medium (SFM) and Minimal Salt Medium (MSM) where endosulfan was provided as the sulfur source or carbon source respectively. Overall a total of a total of six enrichments each were initiated using either 4mg/l or 10 mg/l endosulfan. At the end of the enrichment cycles it was observed that endosulfan degrading consortia were obtained from each of the three different enrichment cultures.
initiated using Modified-Rich Medium (M-RM), Sulfur Free Medium (SFM) and Minimal Salt Medium (MSM) respectively (Table 4.6).

**Table 4.6. Enrichment of industrial soil sample (P1) in different media with 4 mg/l technical grade endosulfan**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Concentration of endosulfan in enrichment</th>
<th>Growth (OD\textsubscript{600})</th>
<th>Reduction in endosulfan concentration (mg/l)</th>
<th>Consortium Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified-Rich Medium</td>
<td>4 mg/l</td>
<td>growth observed</td>
<td>1.9</td>
<td>Consortium 3</td>
</tr>
<tr>
<td>Sulfur Free Medium</td>
<td>4 mg/l</td>
<td>growth observed</td>
<td>2.10</td>
<td>Consortium 4</td>
</tr>
<tr>
<td>Minimal Salt Medium</td>
<td>4 mg/l</td>
<td>growth observed</td>
<td>2.78</td>
<td>Consortium 5</td>
</tr>
</tbody>
</table>

**Enrichment of industrial soil sample (P1) using Modified-Rich Medium (M-RM)**

Two enrichments were initiated in Modified-Rich Medium for soil sample P1 using either 4 mg/l endosulfan or 10 mg/l endosulfan. Significant reduction of the residual endosulfan along with increase in optical density was observed only in those enrichment flasks that contained 4 mg/l technical grade endosulfan (Table 4.5). Although increase in optical density was observed in flasks containing Modified-Rich Medium supplemented with 10 mg/l endosulfan, GC analysis indicated that endosulfan degradation did not occur in these flasks. Hence this consortium was not taken further for any experiments during the study.

The consortium from the enrichment that contained initial concentration of 4 mg/l technical grade endosulfan was designated as Consortium 3. It was observed that at the end of 20 days, the Consortium 3 was able to degrade 1.90 mg/l of technical grade endosulfan corresponding to a total endosulfan degradation efficiency of 46.3% (Table 4.6). A rapid increase in bacterial biomass was observed till Day 3. After this day an increase in growth was still observed until day 6 though at a less rapid pace (Figure 4.15).

The GC analysis of the residual endosulfan in the cultures indicated that the degradation of endosulfan occurred only from day 3 onwards. Steady decline in the endosulfan concentration was observed until day 18 after which no significant change in the residual concentration of endosulfan was observed.
(Figure 4.15). The concentration of the $\alpha$-isomer in the enrichment flask was observed to decline from an initial concentration of 2.8 mg/l to 1.5 mg/l, indicating 46.4% utilization. The concentration of the $\beta$-isomer reduced from 1.30 mg/l to 0.70 mg/l, which revealed 46.1% utilization (Figure 4.15). These results indicated that the endosulfan isomers were degraded by similar extents by Consortium 3.

The analysis of the extracts of the Consortium 3 also indicated the formation of intermediates namely endosulfan diol, endosulfan lactone, endosulfan ether and endosulfan sulfate (Figure 4.18). The pH of the culture was monitored and the values ranged from 5.5 to 6.6 between day 1 and day 20 (Figure 4.19).

![Figure 4.15. Degradation of technical grade endosulfan by Consortium 3](image)

**Enrichment of industrial soil sample (P1) using Sulfur Free Medium (SFM)**

As observed with the Modified-Rich Medium in the previous section, even in the case of the Sulfur Free Medium enrichments, bacterial growth and endosulfan degradation was observed only in one enrichment culture that was supplemented with 4 mg/l technical grade endosulfan. This consortium was designated as Consortium 4. Once again though the enrichment culture with 10 mg/l concentration of endosulfan showed an increase in optical density reading at the end of the incubation period, the GC analysis indicated an absence of a significant reduction in the endosulfan concentration (Table 4.5). Thus this enrichment culture was not taken forward in the study.
In case of Consortium 4, at the end of 20 days, the initial concentration of 4.08 mg/l endosulfan was reduced to 1.98 mg/l indicating a utilization of 2.10 mg/l technical endosulfan was observed to be utilized corresponding to a total endosulfan degradation efficiency of 51.47%. A short lag phase was observed until the first 12 hours of incubation and thereafter a rapid increase in bacterial growth was observed till 24 hours or day 1 of incubation (Figure 4.16). There was no significant decline in the concentration of endosulfan during this period as observed in Figure 4.16. This might indicate that Sulfur Free Medium was not totally free of sulfur source despite an absence of a specific sulfur containing salt. It is probable that residual sulfur contamination in the salts used to prepare Sulfur Free Medium might have been utilized as the sulfur source initially and contributed to the growth of the mixed culture. After day 1 however the optical density of the culture medium was observed to increase steadily till day 12 with a concurrent decline in the concentration of residual endosulfan in the culture flasks (Figure 4.16).

At the end of the incubation period the concentration of α-isomer reduced from an initial value of 2.8 mg/l to 1.4 mg/l, indicating 50.0% utilization within the 20 day period. Correspondingly, the concentration of β-isomer was observed to reduce from the 1.30 mg/l to 0.58 mg/l signifying 55.3% utilization (Figure 4.16).
GC analysis of the culture of Consortium 4 indicated the presence of several intermediate metabolites. Endosulfan lactone, endosulfan ether, endosulfan diol and endosulfan sulfate were detected in the extracts (Figure 4.18). The change in the pH of the enrichment culture was observed in the range of 5.0 to 6.0 during the incubation period of 20 days (Figure 4.19).

**Enrichment of industrial soil sample (P1) using Minimal Salt Medium (MSM)**

The enrichment in Minimal Salt Medium was initiated to isolate bacterial strains that can use endosulfan as the sole source of carbon. As observed in the case of the previous enrichments initiated with the P1 soil sample (in M-RM and SFM), only the enrichment initiated with Minimal Salt Medium and 4 mg/l endosulfan showed bacterial growth and potential of endosulfan degradation. Thus only this enrichment culture was taken forward for the study and was designated as Consortium 5.

Consortium 5 was able to utilize 2.78 mg/l of technical grade endosulfan as a sole carbon source corresponding. Thus after the incubation period of 20 days, the endosulfan degradation efficiency of Consortium 5 was around 65.72%. The growth pattern of Consortium 5 was slightly different when compared with the pattern observed with other consortia (Figure 4.15 and 4.16). There was an initial lag phase for 24 hours before a steady increase in bacterial growth till ninth day of incubation (Figure 4.17). The decrease in the concentration of residual endosulfan in the medium was during the first nine days of incubation corresponding with an increase in bacterial growth (Figure 4.17). The degradation rate slowed down thereafter and was insignificant toward the end of the incubation period (Figure 4.17).

In case of Consortium 5, the initial concentration of the α-isomer in the culture flasks was observed to be 2.9 mg/l. This was decreased to 0.9 mg/l in 20th day of incubation, indicating utilization of 68.9% of the α-isomer. Similarly the initial concentration of the β-isomer 1.33 mg/l and this was reduced to 0.55 mg/l. This revealed 58.6% utilization of the β-isomer by Consortium 5.
The GC analysis of the residual extracts of the Consortium 5 also indicated the formation of three intermediates namely endosulfan lactone, endosulfan ether and endosulfan sulfate (Figure 4.18). The pH of the culture was monitored and the value ranged from 5.8 to 6.0 between day one and day 20 (Figure 4.19).
Figure 4.18. Gas chromatogram depicting the degradation of technical grade endosulfan and the formation of intermediate metabolites by Consortium 3, Consortium 4 and Consortium 5.
Characterisation of Consortium 3, Consortium 4 and Consortium 5 developed from industrial soil sample

A total of six different enrichment cycles (one soil sample in three media with two endosulfan concentration) were initiated with soil sample collected from the industrial site. There were three consortia developed (Consortium 3, Consortium 4 and Consortium 5) and they had the ability to degrade endosulfan. In order to characterize the consortia and to isolate the individual constituent microbial strains, these three consortia were taken up for isolation as per the protocols described in the previous chapter. The aliquots from actively grown consortia were plated on agar plates of the respective media supplemented with 4 mg/l endosulfan. The well isolated, individual colonies were selected based on the morphological characteristics. A total of 26 bacterial strains and one fungal strain were isolated from the three consortia enriched from P1 industrial soil samples. Details of the microbial strains isolated the three consortia is mentioned in Table 4.7.

A total of nine bacterial strains and a single fungal strain were isolated from Consortium 3. The bacterial strains were designated by code starting from M-RM1 to M-RM9 while the fungal strain was designated as M-RM10. When aliquots from the Consortium 4 were plated on Sulfur Free Medium endosulfan agar plates, thirteen bacterial strains were isolated. These strains were designated by the code starting from SFM1 to SFM13. In contrast to the other
enrichment cultures only four strains were isolated from the Consortium 5. They were designated by codes ES2, ES3, ES4 and ES9.

Table 4.7. Details of the microbial strains isolated from the various consortia developed using 4 mg/l endosulfan and the P1 soil sample

<table>
<thead>
<tr>
<th>Consortia</th>
<th>Medium</th>
<th>No of total isolates</th>
<th>Strain Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consortium 3</td>
<td>Modified-Rich Medium</td>
<td>10</td>
<td>M-RM1 to M-RM10</td>
</tr>
<tr>
<td>Consortium 4</td>
<td>Sulfur Free Medium</td>
<td>13</td>
<td>SFM1 to SFM13</td>
</tr>
<tr>
<td>Consortium 5</td>
<td>Minimal Salt Medium</td>
<td>4</td>
<td>ES2, ES3, ES4, ES9</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>26 bacterial strains</strong> and 1 fungal strain</td>
<td></td>
</tr>
</tbody>
</table>

Quantitative determination of the endosulfan degradation capacity of strains isolated from industrial soil sample

A total of 26 bacterial strains and 1 fungal strain were isolated from the three enrichments initiated with the P1 soil sample. The ability of each of these strains to individually degrade endosulfan was determined by the gas chromatographic method. The degradation ability of the individual strains was initially done in the respective media from where the stains were isolated. The results of the same are described below in detail.

Endosulfan degradation capacity of the strains isolated from the Consortium 3 in Modified-Rich Medium (M-RM)

The mean of three replicate experiments revealed that out of the ten isolates, five bacterial strains (M-RM1, M-RM2 and, M-RM4, M-RM5 and M-RM6) and the one fungal strain (M-RM10) degraded technical grade endosulfan significantly. As a small percentage of abiotic loss occurs during incubation all the degradation results presented henceforth are corrected for the abiotic losses occurring in control flasks between day zero and day 20.

GC analysis indicated that the strain M-RM6 degraded 42.9% technical grade endosulfan and was the most efficient degrader amongst the strains isolated in Modified-Rich Medium. Strains M-RM1, M-RM2, M-RM4 and M-RM5 degraded technical grade endosulfan by 11.3% 20.7%, 32.8%, and 25.4% respectively. Insignificant degradation was observed when strains M-RM3, M-RM7, M-RM8 and M-RM9 were grown in Modified-Rich Medium in the presence of endosulfan.
Estimation of the residual concentration of endosulfan in the culture of strain M-RM6 revealed that the α-isomer declined by 42.7% the α-isomer while the β-isomer declined by 43.6% in 20 days. Strain M-RM4 and M-RM5 reduced the concentration of the α-isomer by 29.9% and 26.2% while the β-isomer was reduced by 35.1% and 24.7% in 20 days (Figure 4.20). The extent of the reduction of endosulfan isomers in the cultures of the strains isolated from Consortium 3 is depicted in Figure 4.20. Both isomers appeared to be degraded to nearly equal extents by strain M-RM2, M-RM5 and M-RM6. Strain M-RM1 degraded the α-isomer to a greater extent than the β-isomer. On the other hand strain M-RM 4 degrade the β-isomer to a greater extent than the α-isomer (Figure 4.20).

In the case of M-RM6 at the end of the 20 day incubation period the metabolite endosulfan lactone and endosulfan ether and endosulfan diol were detected (Figure 4.21). Endosulfan diol was observed in the culture of strain M-RM4 and M-RM5. An absence of metabolites was detected during the degradation of M-RM2 and M-RM1.

The pH of the medium in the un-inoculated controls remained stable at 6.28 ± 0.07. By the end of the incubation period the pH of the strains M-RM4, M-RM5 and M-RM6 decreased to 6.0 ± 0.05, 6.1 ± 0.12 and 5.8 ± 0.06 culture. The pH of strains M-RM1 and M-RM2 were observed to be 6.23 ± 0.13 and 5.7 ± 0.04 respectively.
A fungal strain M-RM10 was isolated from the P1 soils from the Consortium 3. The degradation ability of this fungal strain was determined by incubating it in Modified-Rich Medium supplemented with 4 mg/l endosulfan for 20 days. At the end of the incubation period it was observed that the fungal strain could degrade 39.0% technical grade endosulfan. The concentration of the α-isomer decreased by 37.6% while that of the β-isomer decreased by 39.4% (Figure 4.20).

The intermediates formed were endosulfan sulfate and endosulfan lactone. The pH of the culture was observed to drop to 4.8 in the first two days of incubation and remained at 4.9 ± 0.2 for another 7 days. From the 10th day onwards, the pH was seen to slowly rise to a value of 5.5 until the 20th day of incubation.

Figure 4.21. Gas chromatogram depicting the degradation of technical grade endosulfan and the formation of intermediate metabolites by strain M-RM6
Endosulfan degradation capacity of the strains isolated from the Consortium 4 in Sulfur Free Medium (SFM)

Amongst the thirteen strains isolated from Sulfur Free Medium, GC analysis revealed that six strains (SFM3, SFM4, SFM5, SFM6, SFM9 and SFM10) degraded technical grade endosulfan to different extents. The mean of three replicates revealed that strain SFM4 showed the highest effective degradation for technical grade endosulfan at 43.7%. Strain SFM6 degraded endosulfan by 32% whereas strains SFM3 and SFM5 were observed to degrade 27.7% and 31.6% of endosulfan. Strains SFM9 and SFM10 also degraded endosulfan but by very less amounts i.e 16.4% and 11.6% respectively. Insignificant endosulfan degradation was observed in the case of strains SFM1, SFM2, SFM7, SFM8, SFM11, SFM12 and SFM13 as compared to un-inoculated controls.

In the case of strain SFM4 40.1% utilisation of the α-isomer and 47.2% utilisation of the β-isomer was observed in 20 days. Strain SFM6 reduced the concentration of the α-isomer by 34.9% and that of the β-isomer by 29.1%. The concentration of the α-isomer in the cultures of strain SFM5 and SFM3 reduced by 32.9 % and 29.1% respectively. While the concentration of the β-isomer was reduced by 29.4 % and 25.7% respectively in 20 days (Figure 4.22).

Figure 4.22. Degradation of technical grade endosulfan by six selected strains isolated from Consortium 4 in Sulfur Free Medium
Strain SFM4 preferentially utilised the β-isomer over the α-isomer while on the other hand strain SM6 utilised the α-isomer to a larger extent than the β-isomer (Figure 4.22). SFM5 and SFM3 reduced the levels of the α-isomer and the β-isomer to nearly similar extents (Figure 4.22).

The highest degradation was shown by the strain SFM4 and the GC analysis of the culture extract at the end of the 20 day incubation indicated the presence of endosulfan diol, endosulfan lactone and endosulfan ether (Figure 4.23). The same metabolites were not observed with all the degrading strains as in the case of strain SFM3 and SFM5 only endosulfan diol was observed. The toxic metabolite endosulfan sulfate was observed in the culture of strain SFM6. No metabolites detected during the degradation of endosulfan by strains SFM9 and SFM10 till day 20 of the incubation period.

![Figure 4.23. Gas chromatogram depicting the degradation of technical grade endosulfan and the formation of intermediate metabolites by strain SFM4](image-url)
The pH of the medium in the un-inoculated controls remained stable at $6.34 \pm 0.04$ whereas there a change in pH was observed in the culture flasks of the strains under study. In case strains SFM3, SFM4, and SFM5 at the end of 20 days the pH of the culture flasks decreased to $5.96 \pm 0.17$, $6.02 \pm 0.06$ and $6.02 \pm 0.11$ respectively. Whereas in case of culture flasks inoculated with strains SFM9, SFM10 and SFM6 the pH was recorded as $6.06 \pm 0.05$, $6.05 \pm 0.07$ and $6.00 \pm 0.14$ respectively at the end of the incubation period. The pH of the cultures other strains that did not show significant degradation (SFM1, SFM2, SFM7, SFM8, SFM11, SFM12 and SFM13) was observed in the range between 5.8 and 6.5.

### Endosulfan degradation capacity of the strains isolated from the Consortium 4 in Total-Sulfur Free Medium

During the growth of Consortium 4 in Sulfur Free Medium it was observed that an initial rapid increase in growth did not produce a corresponding decrease in the concentration of endosulfan despite it being supplied as the sole sulfur source. Therefore it was surmised that the Sulfur Free Medium used might contain residual sulfur contamination despite lack of a specific sulfur source. As the Sulfur Free Medium used did not appear to be totally free of sulfur an attempt was made to prepare Total-Sulfur Free Medium as described in the previous chapter. The medium Total-Sulfur Free Medium was used to determine quantitative degradation capacity of six bacterial strains (SFM3, SFM4, SFM5, SFM6, SFM9 and SFM10) that showed the maximum degrading capability in the previous medium.

When strains SFM3, SFM4, SFM5, SFM6, SFM9 and SFM10 were inoculated into Total-Sulfur Free Medium supplemented with 4 mg/l technical grade endosulfan, significant growth was observed only in strain SFM4 and SFM6. The remaining four strains (SFM3, SFM5, SFM9 and SFM10) failed to show significant growth. GC analysis revealed that the degradation capacities of strains SFM4 and SFM6 in Total-Sulfur Free Medium was drastically reduced. At the end of 20 days in total Sulfur Free Medium strains SFM4 and SFM6 degraded technical grade endosulfan by 33.0% and 19.6%. As compared to the degradation observed in Sulfur Free Medium, the degradation of endosulfan by SFM4 and SFM6 in Total-Sulfur Free Medium reduced by approximately 11% and 12.4% respectively. Strain SFM4 utilized 21.7% of the $\alpha$-isomer and 39.3% of
the β-isomer. On the other hand 22.6% of the α-isomer and 16.6% of the β-isomer were observed to be utilised by strain SFM6 (Figure 4.24).

Endosulfan degradation capacity of the strains isolated from the Consortium 5 in Minimal Salt Medium (MSM)

Four strains were isolated from the Consortium 5 where endosulfan was supplied as a carbon source. At the end of 20 days it was observed that all four isolated strains, ES2, ES3, ES4 and ES9 degraded technical grade endosulfan to different extents. The mean of three degradation experiments revealed that strain ES9 degraded technical grade endosulfan to the greatest extent followed by strain ES3. The effective endosulfan degradation of by strains ES9 and ES3 was 54.5% and 34.3%. On the other hand GC analysis revealed that the effective degradation of endosulfan by strain ES2 and strain ES4 was relatively less. The effective degradation of endosulfan by strain ES2 was 21.5% while that of strain ES4 a mere 14.5%.

Strain ES9 displayed 58.1% utilization of the α-isomer and 48.4% utilization of the β-isomer in 20 days. On the other hand strain ES3 utilized 44.5% the α-isomer and 23.5% the β-isomer in 20 days. The α-isomer was observed to be
utilized preferentially by both strains. Additionally strain ES9 displayed greater endosulfan degradation efficiency than strain ES3 in case of both isomers. The extent of the effective degradation of endosulfan by the four isolates is depicted in Figure 4.25.

Figure 4.25. Degradation of technical grade endosulfan by four strains isolated from Consortium 5 in Minimal Salt Medium

Endosulfan ether and endosulfan lactone were detected as metabolites in the cases of ES9 and strain ES3 (Figure 4.26). On the other hand endosulfan sulfate was detected as the metabolite in strain ES2. An absence of metabolites was detected during the degradation of endosulfan by strain ES4. The pH of the medium in the un-inoculated controls remained stable at 6.36 ± 0.04. The pH of the strain ES9 culture ranged from values 5.1 to 6.2 between day 1 to day 20. The pH of strain ES3 ranged from values 5.3 to 6.2 during the incubation period. The pH of flasks inoculated with strain ES2 and strain ES4 did not exceed 6.5 ± 0.02 and 6.6 ± 0.024 respectively.
Figure 4.26. Gas chromatogram depicting degradation of technical grade endosulfan and the formation of intermediate metabolites by strain ES9 and strain ES3.
Results

Identification of selected endosulfan degrading bacterial strains by sequencing of 16S rRNA gene

In this study a total of four soil samples were taken and after enrichments with four different media five consortia (Consortium 1, Consortium 2, Consortium 3, Consortium 4 and Consortium 5) capable of degrading endosulfan was obtained. Isolation of constituent bacterial strains from agricultural soil samples (Consortium 1 and Consortium 2) yielded eight bacterial strains. Among these eight bacterial strains, strain RM2 showed limited endosulfan degradation capacity however the capacity of remaining seven strains to degrade endosulfan was found to be insignificant. Thus except of strain RM2, the other strains isolated from agricultural soil samples were not taken further for identification purposes.

A total of 26 bacterial strains and 1 fungal strain were isolated for the remaining three consortia (Consortium 3, Consortium 4 and Consortium 5) obtained from enrichment of an endosulfan contaminated industrial soil samples. However from these isolates only four bacterial strains (M-RM6, SFM4, ES3 and ES9) showed significant endosulfan degradation. Detailed analyses of these strains are already reported in the previous sections and these four strains were taken for identification. Along with these four strains, two additional strains (ES2 and ES4) isolated from Consortium 5 were taken for sequencing as these strains appeared to degrade endosulfan in Minimal Salt Medium.

As described earlier the initial selection of the strains was done through morphological characterization and colony characteristics. Gram's straining was done of all the bacterial strains taken for the study and it revealed the presence of only Gram negative type of bacterial cells. This indicated that the endosulfan contaminated sites were dominated by Gram negative bacterial strains.

Microscopic analysis of the cell cultures revealed that strains M-RM6, SFM4, ES3 and ES9 isolated from P1 soil sample and RM2 isolated from agricultural soil were all observed to be short rods.

The identification of the bacterial strains was confirmed by sequencing and analysis of the 16S rRNA gene. The 16S rRNA gene of the selected strains was initially amplified and taken up for sequencing by the Microseq™ 16S rDNA-PCR module as per the methods described earlier and a representative gel indicating the 500 bp amplicon of selected strains is shown in Figure 4.27.
Alignment of 16S rDNA sequences of these strains with nucleotide-nucleotide BLAST search of NCBI, RDP and Microseq™ database gave up to 98 % to 99 % similarity to different bacterial species. The analysis of the 16S rDNA sequences with the BLAST (N) search against the Genbank, European molecular biology laboratory (EMBL), DNA databank of Japan (DDBJ) and with ribosomal database project (RDP) also gave identical results of up to 99 % 16S rDNA sequence homology. Based on the sequence homology the respective strains isolated in this study were to different bacterial strains as mentioned in Table 4.8.

Table 4.8. Identification of endosulfan degrading strains though 16S rDNA gene sequencing

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>Identification by sequencing</th>
<th>NCBI Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-RM6</td>
<td>Pseudomonas aeruginosa</td>
<td>GQ411017</td>
</tr>
<tr>
<td>SFM4</td>
<td>Pseudomonas aeruginosa</td>
<td>GQ411018</td>
</tr>
<tr>
<td>ES2</td>
<td>Pseudomonas aeruginosa</td>
<td>DQ339466</td>
</tr>
<tr>
<td>ES3</td>
<td>Ochrobactrum anthropi</td>
<td>DQ345436</td>
</tr>
<tr>
<td>ES4</td>
<td>Stenotrophomonas maltophilia</td>
<td>DQ339468</td>
</tr>
<tr>
<td>ES9</td>
<td>Achromobacter xylosoxidans</td>
<td>DQ339467</td>
</tr>
<tr>
<td>RM2</td>
<td>Serratia marcescens</td>
<td>GQ411019</td>
</tr>
</tbody>
</table>
Results

The phylogenetic affiliation of all the bacterial isolates was done and phylogenetic tree for each of the bacterial isolate was further validated by boot strapping analysis as per the methods described earlier. The phylogenetic trees of individual bacterial strains M-RM6, SFM4, ES3 and ES9 that showed the higher endosulfan degrading phenotype is described in Figures 4.28 to 4.31. The phylogenetic tree of strain RM2 is described in Figure 4.32. The phylogenetic tree constructed by using TREEVIEW program as presented in these figures illustrates the phylogenetic relatedness of the respective strains with the selected strains from obtained from GenBank (NCBI) and RDP database (Figure 4.28 to 4.32).

![Phylogenetic Tree](image)

**Figure 4.28.** 16S rDNA phylogenetic analysis of strain M-RM6 with other members of the group taken from NCBI database. The tree is constructed by TREEVIEW program after initial analysis of the sequences by PAUP analysis and by neighbor-joining analysis by SEQBOOT.
Figure 4.29. 16S rDNA phylogenetic analysis of strain SFM4 with other members of the group taken from NCBI database. The tree is constructed by TREEVIEW program after initial analysis of the sequences by PAUP analysis and by neighbor-joining analysis by SEQBOOT.

Figure 4.30. 16S rDNA phylogenetic analysis of strain ES3 with other members of the group taken from NCBI database. The tree is constructed by TREEVIEW program after initial analysis of the sequences by PAUP analysis and by neighbor-joining analysis by SEQBOOT.
In order to establish the phylogenetic relatedness of the strains isolated in this study from the Consortium 5 (where endosulfan was provided as the carbon source), with other endosulfan degrading strains also reported/ submitted in
Gene Bank by other investigators as degrading endosulfan in Minimal Salt Medium in absence of any other carbon sources, a phylogenetic tree was deduced from the sequences retrieved from NCBI GeneBank. Sequences of strains of the present study *O. anthropi* ES3 and *A. xylosoxidans* ES9 was aligned with related sequences from GeneBank that have been reported to degrade endosulfan. The sequences were aligned using CLUSTALW and analyzed by PAUP, version 3.0 and neighbor-joining analysis (SEQBOOT) programs obtained from PHYLIP package for further validation of the tree (Figure 4.33).

**Figure 4.33.** Phylogenetic tree indicating position of the bacterial strains ES2, ES3, ES4 and ES9 isolated in this study based on relatedness of 16S rRNA gene sequences. The other sequences of other strains that are reported to degrade endosulfan were derived from the NCBI GeneBank. The unpublished sequences are marked with an asterix (*). The tree was generated by the distance neighbour joining method using MEGA 4.1.
Selection of bacterial strains with higher endosulfan degrading capacity

As described previously the eight strains isolated from agricultural soil exhibited very low endosulfan degradation potential (less than 33%). Out of the 26 bacterial strains and 1 fungal strain isolated from the various consortia initiated with the P1 soil sample (collected from a pesticide dumping site) quantitative analysis revealed that five strains displayed over 33% degradation of technical grade endosulfan (Table 4.9). The degradation efficiency of all the remaining isolates was not very significant. The bacterial strains *P. aeruginosa* M-RM6, *P. aeruginosa* SFM4, *O. anthropi* ES3 and *A. xylosoxidans* ES9 showed relatively greater degradation efficiency for technical endosulfan. Therefore these four strains that displayed higher degradation of endosulfan were taken forward for further experimentations. The fungal strain M-RM10 also degraded endosulfan by 39.1%; however it produced the toxic metabolite endosulfan sulfate as the intermediate during degradation. Hence it was considered unsuitable for the prospect of bioremediation and was not taken forward in this study.

Table 4.9. List of isolated strains exhibiting relatively higher endosulfan degradation capacity

<table>
<thead>
<tr>
<th>Strain</th>
<th>Consortium</th>
<th>Medium used</th>
<th>Degradation of technical grade endosulfan (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas aeruginosa</em> M-RM6</td>
<td>Consortium 3</td>
<td>Modified-Rich Medium</td>
<td>42.8%</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> SFM4</td>
<td>Consortium 4</td>
<td>Sulfur Free Medium</td>
<td>42.4%</td>
</tr>
<tr>
<td><em>Achromobacter xylosoxidans</em> ES9</td>
<td>Consortium 5</td>
<td>Minimal Salt Medium</td>
<td>55.1%</td>
</tr>
<tr>
<td><em>Ochrobactrum anthropi</em> ES3</td>
<td>Consortium 5</td>
<td>Minimal Salt Medium</td>
<td>34.3%</td>
</tr>
</tbody>
</table>

Growth studies and determination of endosulfan degradation rates of selected bacterial strains

The four strains, *Pseudomonas aeruginosa* M-RM6, *Pseudomonas aeruginosa SFM4, Ochrobactrum anthropi ES3 and Achromobacter xylosoxidans ES9 that were isolated from industrial endosulfan contaminated soil samples and were observed to show significant endosulfan degradation capacity were chosen for detailed growth studies as well as for the assessment of degradation kinetics.
Growth and endosulfan degradation of *Pseudomonas aeruginosa* M-RM6

The strain *Pseudomonas aeruginosa* M-RM6 was grown in Modified-Rich Medium supplemented with 4 mg/l technical grade endosulfan. There was no lag phase and strain *P. aeruginosa* M-RM6 showed a steady increase in optical density till third day of incubation (Figure 4.34). The GC analysis of the residual endosulfan in the culture flasks indicated that there was no significant change in the concentration till day 3 of incubation. The growth of *P. aeruginosa* M-RM6 increased slightly until day 6 following which it appeared to enter the stationary phase. The degradation of endosulfan by this strain appeared to be carried out after day 3 and the concentration of endosulfan was observed to steadily decrease from day 3 until day 18 (Figure 4.34). No further decline in the concentration of residual endosulfan was observed after day 18.

Taking into account abiotic losses in the control it was observed that *P. aeruginosa* M-RM6 was responsible for the degradation of 1.53 mg/l endosulfan which amounted to a degradation of 42.8%. GC analysis indicated a 1.05 mg/l effective decline of the α-isomer was observed by *P. aeruginosa* M-RM6. This suggested that the α-isomer was degraded by 42.5%. In the case of the β-isomer, the residual level in culture flasks was observed to be 0.62 mg/l, which indicated a 0.48 mg/l or 43.63% decline in the levels of the isomer.

![Figure 4.34. Growth of *P. aeruginosa* M-RM6 in Modified-Rich Medium with technical grade endosulfan](image)

The degradation constants for strain M-RM6 were determined and it was observed that for α-isomer the degradation constant was 0.0408 day⁻¹ (R² =
0.963) (Figure 4.35), while for β-isomer the degradation constant was calculated as 0.0406 day\(^{-1}\) (R\(^2\) = 0.942) (Figure 4.36). This indicated that \(P.\ aeruginosa\) M-RM6 both the α-isomer and the β-isomer at nearly equal rates.

Figure 4.35. Degradation constant of α-isomer derived from the degradation of technical grade endosulfan by \(P.\ aeruginosa\) M-RM6 in Modified-Rich Medium

\[
k_1 = 0.0408 \\
R^2 = 0.9626
\]

Figure 4.36. Degradation constant of β-isomer derived from the degradation of technical grade endosulfan by \(P.\ aeruginosa\) M-RM6 in Modified-Rich Medium

\[
k_1 = 0.0406 \\
R^2 = 0.9423
\]
Growth and endosulfan degradation of strain Pseudomonas aeruginosa SFM4

When strain *Pseudomonas aeruginosa* SFM4 was grown on Sulfur Free Medium containing 4 mg/l technical grade endosulfan, a biphasic pattern of growth was observed. An initial lag phase was observed until day 1, following which exponential growth was observed until day 3 (Figure 4.37). However during this period there was no corresponding decrease in the concentration of endosulfan was observed. From day 3 onwards the increase in growth halted and the culture appeared to enter into a second lag phase. This second lag phase lasted until day 4 after which growth was once again observed to increase steadily until day 16 however at a less rapid pace than the first exponential phase. The stationary phase appeared to commence after day 16 (Figure 4.37).

Analysis of residual concentrations of endosulfan in the culture of *P. aeruginosa* SFM4 suggested that the utilization of endosulfan appeared to begin only from day 4, i.e. after the end of second lag phase. The degradation of endosulfan continued until day 16 after which no further decline in the levels of endosulfan was observed (Figure 4.37). The decline in the concentration of the endosulfan that was due to the microbial action was determined by taking into account the abiotic losses in control flasks. This revealed that 1.58 mg/l technical grade endosulfan was utilized by *P. aeruginosa* SFM4. Therefore at the end of 20 days *P. aeruginosa* SFM4 appeared to degrade 44.2% after excluding the abiological degradation of endosulfan in the control flasks. These values indicated that *P. aeruginosa* SFM4 degraded technical grade endosulfan to an extent similar to *P. aeruginosa* M-RM6.

GC analysis further that during the 20 day incubation period 0.98 mg/l of the α-isomer and 0.6 mg/l of the β-isomer was utilized by *P. aeruginosa* SFM4. This indicated that a 40.3 % decline in the concentration of the α-isomer and 48% decline in the concentration of the β-isomer (Figure 4.37). The preferential utilization of the β-isomer over the α-isomer by *P. aeruginosa* SFM4 in Sulfur Free Medium is in contrast to nearly equal degradation of endosulfan isomers observed in the case of *P. aeruginosa* M-RM6 in Modified-Rich Medium.
The degradation constants for the $\alpha$-isomer and the $\beta$-isomer were observed to be 0.0384 day$^{-1}$ ($R^2 = 0.941$) and 0.0415 day$^{-1}$ ($R^2 = 0.942$) respectively (Figure 4.38 and Figure 4.39). These results indicated that *P. aeruginosa* SFM4 degraded the $\beta$-isomer at a slightly higher rate than the $\alpha$-isomer. Further it was also observed that *P. aeruginosa* SFM4 and *P. aeruginosa* M-RM6 degraded the $\beta$-isomer at nearly similar rates in SFM and Modified-Rich Medium respectively. On the other hand *P. aeruginosa* SFM4 demonstrated a slightly lower degradation rate for the $\alpha$-isomer when compared to *P. aeruginosa* M-RM6 (Table 4.10)
Growth and endosulfan degradation by Achromobacter xylosoxidans ES9 and Ochrobactrum anthropi ES3

Among the four strains isolated from Consortium 5 that was enriched for isolates that can use endosulfan as a sole source of carbon, *Ochrobactrum anthropi* ES3 and *Achromobacter xylosoxidans* ES9 showed significant endosulfan degradation. As strains using endosulfan as sole carbon source would be an ideal candidate for designing bioremediation strategy, both these endosulfan degrading strains from this consortium were taken for further studies for a comparison of growth patterns and their degradation rates.

Growth studies indicated that when strain *A. xylosoxidans* ES9 was grown in minimal salts medium with 4 mg/l endosulfan as sole source of carbon, an initial short lag of 24 hours was observed. Subsequent to this lag phase, steady increase in growth was observed until day 16 after which the culture entered the stationary phase (Figure 4.40). GC analysis indicated that the growth of *A. xylosoxidans* ES9 was accompanied with the together concomitant utilization of technical grade endosulfan in the medium. A decline in the levels of residual endosulfan was observed from day 1 onwards until day 16 of incubation and this reduction in the levels of endosulfan appeared concurrently with the increase in optical density (Figure 4.40). Overall in 20 days *A. xylosoxidans* ES9 was able to utilize 1.83 mg/l endosulfan after accounting for losses in the control flasks signifying 54.5% degradation. As compared to the residual endosulfan values in
controls on the 6th day results of the batch experiments revealed that 36.6% degradation (indicating utilization of 1.33 mg/l) of technical endosulfan within the first 6 days of incubation. The remaining 15.5% degradation that corresponded to a 0.5 mg/l decrease in endosulfan occurred in the next 10 days i.e. from day 6 onwards until day 18. No further decrease in the residual endosulfan concentration was observed after day 18 (Figure 4.40).

Estimation of the residual concentrations of the isomers in the culture of *A. xylosoxidans* ES9 revealed that the strain was able to utilize 1.37 mg/l of the α-isomer and 0.46 mg/l of the β-isomer after accounting for the abiotic loss in controls. This indicated a degradation of 58.0% of the α-isomer and 48.4% of the β-isomer. As Figure 4.40 indicates, a rapid decrease in the concentration of the α-isomer was observed from day 0 until day 6. During this time *A. xylosoxidans* ES9 was observed to reduce the concentration of the α-isomer by 0.93 mg/l indicating 35.3% degradation of isomer in 6 days. Another decline of 0.44 mg/l α-isomer occurred in the next 12 days taking the overall degradation of the α-isomer to 57.8%. On the other hand a 0.4 mg/l decline in the concentration of the β-isomer by *A. xylosoxidans* ES9 was observed within 6 days corresponding to 40% utilization which rose to 48.2% by the end of 20 days. Altogether the preferential use of the α-isomer over that of the β-isomer by *A. xylosoxidans* ES9 was observed.

![Figure 4.40. Growth of *A. xylosoxidans* ES9 in Minimal Salt Medium with technical grade endosulfan](image-url)
In the case of strain ES9 the degradation constant for $\alpha$-isomer was 0.0569 day$^{-1}$ ($R^2 = 0.925$) while for the $\beta$-isomer it was 0.0425 day$^{-1}$ ($R^2 = 0.774$) (Figure 4.41 and 4.42). These results revealed that *A. xylosoxidans* ES9 displayed the highest degradation rate for both the $\alpha$-isomer and the $\beta$-isomer as compared to the strains *P. aeruginosa* M-RM6 and *P. aeruginosa* SFM4 (Table 4.10). Additionally *A. xylosoxidans* ES9 degraded the $\alpha$-isomer at a higher rate than the $\beta$-isomer.

![Figure 4.41. Degradation constant of $\alpha$-isomer derived from the degradation of technical grade endosulfan by *A. xylosoxidans* ES9 in Minimal Salt Medium](image1)

![Figure 4.42. Degradation constant of $\beta$-isomer derived from the degradation of 4mg/l endosulfan by *A. xylosoxidans* ES9 in Minimal Salt Medium](image2)
Growth experiments for *O. anthropi* ES3 also revealed that growth of the strain was also accompanied by the degradation of endosulfan. However unlike *A. xylosoxidans* ES9 a longer lag period of two days was observed for *O. anthropi* ES3. During this time negligible decrease in the levels of endosulfan was observed in the culture. From day 2 onwards growth began to steadily increase until Day 8 revealing a concomitant decrease in the level of endosulfan was observed during the growth of the culture during this period (Figure 4.43). However in comparison to *A. xylosoxidans* ES9, *O. anthropi* ES3 exhibited lower growth and utilization of endosulfan. After day 8 growth appeared to slow down in the cultures of *O. anthropi* ES3 although marginal increase in optical density was observed until day 14 after which the culture reached the stationary phase.

By the end of the 20 day incubation period after accommodating the abiological losses observed in the control flasks, *O. anthropi* ES3 was observed to utilize 1.16 mg/l technical endosulfan which signified 34.9% degradation of endosulfan. GC analysis revealed that the majority of endosulfan (29.7%) was utilized within 8 days of incubation. After correction for abiotic losses *O. anthropi* ES3 was seen to utilize 0.95 mg/l of the α-isomer (40.1% degradation) and 0.21 mg/l of the β-isomer (22.1% degradation).

![Figure 4.43. Growth of O. anthropi ES3 in Minimal Salt Medium with technical grade endosulfan](image-url)
The degradation constants calculated for *O. anthropi* ES3 indicated that this strain degraded α-isomer (0.0362 day\(^{-1}\)) at a higher rate and β-isomer (0.0227 day\(^{-1}\)) as also observed in the case of *A. xylosoxidans* ES9 (Figure 4.44 and Figure 4.45). However the values also revealed that *O. anthropi* ES3 degraded the endosulfan isomers at a lower rate than *A. xylosoxidans* ES9 as well as *P. aeruginosa* M-RM6 and *P. aeruginosa* SFM4 (Table 4.10).

Figure 4.44. Degradation constant of α-isomer derived from the degradation of technical grade endosulfan by *O. anthropi* ES3 in Minimal Salt Medium

Figure 4.45. Degradation constant of β-isomer derived from the degradation of technical grade endosulfan by *O. anthropi* ES3 in Minimal Salt Medium
Table 4.10. Degradation rates of the endosulfan isomers exhibited by isolated strains displaying relatively higher endosulfan degradation capacity

<table>
<thead>
<tr>
<th>Strain</th>
<th>Degradation Medium</th>
<th>Degradation constant for α-isomer (day⁻¹)</th>
<th>Degradation constant for β-isomer (day⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas aeruginosa</em> M-RM6</td>
<td>Modified-Rich Medium</td>
<td>0.0408</td>
<td>0.0406</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> SFM4</td>
<td>Sulfur Free Medium</td>
<td>0.0384</td>
<td>0.0415</td>
</tr>
<tr>
<td><em>Achromobacter xylosoxidans</em> ES9</td>
<td>Minimal Salt Medium</td>
<td>0.0569</td>
<td>0.0425</td>
</tr>
<tr>
<td><em>Ochrobactrum anthropi</em> ES3</td>
<td>Minimal Salt Medium</td>
<td>0.0362</td>
<td>0.0227</td>
</tr>
</tbody>
</table>

Optimization of parameters for the enhanced degradation of endosulfan by *Achromobacter xylosoxidans* ES9

Strain *Achromobacter xylosoxidans* ES9 demonstrated the highest degradation rates for both the α-isomer and the β-isomer amongst all the strains displaying higher degradation capacities for endosulfan. Hence experiments were undertaken to optimize the medium composition as well as other attributes such as cultural parameters to examine their effect on endosulfan degradation with the aim to enhance it.

Optimization of inoculum size

The inoculum size of the bacterial strains used for the degradation of xenobiotic compound is known to influence the degradation rate. Hence the biodegradation of endosulfan in minimal salts medium was assessed using four different sizes of inoculum (100 µl, 200 µl, 500 µl and 1000 µl) from a culture of *A. xylosoxidans* grown in LB that had a cell density of 1.4 x 10⁷ cfu/ml. As the experiment was conducted in 10 ml medium in 100ml flask the four inoculum sizes corresponded to 1% (v/v), 2% (v/v), 5% (v/v) and 10% (v/v) of the culture medium. The cells were pelleted and washed before they were inoculated in the experimental flasks.

Growth studies indicated that an increase in inoculum size between 100 µl to 500 µl led to a proportional increase in growth. Correspondingly an increase in the biodegradation was also observed (Figure 4.46). When 100 µl of inoculum was used 40.4% degradation of technical grade endosulfan was observed while 55.0% degradation was observed with the inoculum size of 200 µl. Increase in the inoculum size to 500 µl further enhanced the degradation of endosulfan by 11.4%, revealing a total degradation of 66.4 % at the end of the incubation period (Figure 4.46). No significant increase in the biodegradation of endosulfan was
observed when the inoculum size was increased beyond 500 µl. Thus inoculum size of 500 µl was used for the all experiments conducted subsequent to those that concentrated on the optimization of cultural and environmental aspects.

![Figure 4.46. Effect of different inoculum sizes on the degradation of technical grade endosulfan by A. xylosoxidans ES9](image)

**Optimization of Tween 80 concentration**

The solubility of endosulfan in water is extremely low. Hence the non ionic surfactant Tween 80 was added to Minimal Salt Medium originally in a concentration of 0.05% as reported previously by Sutherland *et al.* (2000) to increase the bioavailability of endosulfan during degradation. The optimum concentration of Tween 80 for maximum endosulfan degradation by *A. xylosoxidans* ES9 was determined using 0.01%, 0.05% and 0.1% concentrations of the surfactant. *A. xylosoxidans* ES9 was therefore grown in Minimal Salt Medium the absence of Tween 80 as well as in Minimal Salt Medium supplemented with the four different concentrations of the surfactant. The degradation of endosulfan by *A. xylosoxidans* ES9 in Minimal Salt Medium originally containing 0.05% Tween 80 was compared with the degradation observed in Minimal Salt Medium in the absence of Tween 80 as well as with 0.01%, 0.1% concentrations of the surfactant.

Growth studies indicated that *A. xylosoxidans* ES9 was not able to grow in Minimal Salt Medium containing 0.05% Tween 80 but lacking endosulfan. This confirmed that *A. xylosoxidans* ES9 was not able to use Tween 80 as a carbon source.
source. When *A. xylosoxidans* ES9 was grown in the absence of Tween 80 an extended lag phase of 3 days was observed where as this lag phase was reduced to 1 day when the strain was grown in Minimal Salt Medium containing either 0.01% or 0.05% concentration of Tween 80.

GC analysis of residual endosulfan concentration at the end of the experiment indicated that as compared to controls in the absence of Tween 80 *A. xylosoxidans* ES9 was able to degrade endosulfan by only 25%. In the presence of 0.01% and 0.05% concentrations of Tween 80 49.2% and 55.1 % degradation of endosulfan was observed (Figure 4.47). However it was observed that in the presence of 0.1% the degradation of endosulfan by *A. xylosoxidans* ES9 was reduced to 46.4% (Figure 4.47). These results clearly indicated that original 0.05% concentration of Tween 80 that was used in the preliminary degradation experiments was most the most optimum concentration for achieving appropriate endosulfan degradation by strain *A. xylosoxidans* ES9.

**Figure 4.47. Effect of different concentrations of Tween 80 on the degradation of technical grade endosulfan by *A.xylosoxidans* ES9**

*Effect of vitamin and trace metal solutions.*

The effect of vitamin solution on the degradation efficiency of *A. xylosoxidans* ES9 was determined by using x, 3x and 5x concentrations of either solution. Growth of strain *A. xylosoxidans* ES9 in Minimal Salt Medium in the presence of the x, 3x and 5x concentrations of vitamin solution revealed no enhancement in comparison to its growth in Minimal Salt Medium (in the absence of vitamin
solution). GC analysis of the residual endosulfan concentration in the flasks showed no improvement in the degradation capacity of *A. xylosoxidans* ES9 in the presence of either x, 3x or 5x vitamin solution. On the other hand a slight decrease in the degradation capacity of *A. xylosoxidans* ES9 was observed when it was grown in Minimal Salt Medium supplemented with 5x concentration of vitamin solution (Figure 4.48).

When *A. xylosoxidans* ES9 was grown in Minimal Salt Medium in the presence of x concentration of trace metal solution once again no significant increase in the growth was observed. The mean of three degradation studies also revealed a lack of increase in the degradation efficiency of *A. xylosoxidans* ES9 in the presence of x concentration of trace metal solution. On the other hand a decrease in growth of *A. xylosoxidans* ES9 was observed when it was grown in Minimal Salt Medium supplemented with 3x and 5x concentrations of trace metal solution. Correspondingly an approximate 9.5% and 15% decrease in the degradation efficiency of *A. xylosoxidans* ES9 was observed in Minimal Salt Medium supplemented with both 3x and 5x concentrations of trace metal solution. Greatest decrease in growth and degradation capacity of *A. xylosoxidans* ES9 was observed in Minimal Salt Medium in the presence of the 5x trace metal solution (Figure 4.49).

![Figure 4.48. Effect of different concentrations of vitamin solution on the degradation of technical grade endosulfan by *A.xylosoxidans* ES9](image-url)
Effect of different concentrations of trace metal solution on the degradation of technical grade endosulfan by *A. xylosoxidans* ES9

Studies have reported that an enhancement in the biodegradation of xenobiotics in the presence of yeast extract. Therefore in the present study the concentrations of 0.1 g/l, 0.5 g/l, 1 g/l and 2 g/l were used to examine the effect of yeast extract on the degradation of endosulfan by *A. xylosoxidans* ES9. The degradation of endosulfan by *A. xylosoxidans* ES9 in Minimal Salt Medium was compared to the degradation observed by the strain in Minimal Salt Medium supplemented with individually with the different concentrations of yeast extract.

When *A. xylosoxidans* ES9 was grown in the presence of yeast extract growth was observed to commence within 24 hours in case of all concentrations. During the incubation period the initial growth of *A. xylosoxidans* ES9 in Minimal Salt Medium containing the various concentrations of yeast extract was observed to be greater than that of the strain in Minimal Salt Medium without yeast extract. Growth studies revealed that increase in the concentration of yeast extract resulted in increase in growth. However in the case of Minimal Salt Medium supplemented with 1% and 2% yeast extract growth of *A. xylosoxidans* ES9 was observed to decline on day 12 and day 8 respectively. Estimation of the degradation of endosulfan in Minimal Salt Medium supplemented with different concentrations of yeast extract revealed that 0.1 g/l and 0.5 g/l yeast extract
contributed to the enhancement in degradation by *A. xylosoxidans* ES9. Endosulfan degradation was increased to 74.4% in the case of 0.5 g/l yeast extract and 68.2% in the case of 0.1 g/l. Degradation of endosulfan in the presence of 1 g/l yeast extract was observed to be 56.1%. Therefore it was only marginally greater than the endosulfan degradation observed in Minimal Salt Medium, which was 54.5%. On the other hand in the case of 2 g/l yeast extract, *A. xylosoxidans* ES9 degraded endosulfan by 42.3% (Figure 4.50).

These results indicated that 0.5 g/l was the optimal concentration of yeast extract at which the maximum degradation of endosulfan was observed. An enhancement of approximately 20% was observed in the degradation of endosulfan in Minimal Salt Medium containing 0.5 g/l yeast extract over that of Minimal Salt Medium. At this concentration of yeast extract strain *A. xylosoxidans* ES9 degraded 79.7% of the α-isomer and 68.5% of the β-isomer. Whereas in the presence of 2 g/l yeast extract endosulfan degradation by *A. xylosoxidans* ES9 was reduced by approximately 12.0% (Figure 4.50). Therefore subsequent experiments conducted after the objective of optimization of parameters included 0.5 g/l yeast extract in the medium.

![Figure 4.50. Effect of different concentrations of yeast extract on the degradation of technical grade endosulfan by *A. xylosoxidans* ES9](image)

*TERI University-PhD. Thesis, 2009*
Effect of supplementary carbon sources

Previous studies on the degradation of endosulfan by bacterial species have revealed that the presence of supplementary carbon sources can enhance endosulfan degradation, have no effect on it or even decrease the endosulfan degrading capacity of strains.

In the present study two carbon sources glucose and xylose and were examined for their effect their presence had on endosulfan degradation by A. xylosoxidans ES9. Initial studies conducted using these three carbon sources individually in Minimal Salt Medium that was devoid of endosulfan revealed that A. xylosoxidans ES9 demonstrated significant growth in presence of 0.5 g/l concentration of glucose or xylose. Hence 0.5 g/l of glucose or xylose was added to Minimal Salt Medium that contained 4 mg/l endosulfan to compare the degradation of endosulfan by A. xylosoxidans ES9 in these media supplemented with carbon sources to that in Minimal Salt Medium containing 4 mg/l endosulfan as the sole carbon source.

When A. xylosoxidans ES9 was grown in Minimal Salt Medium containing 4 mg/l endosulfan together with 0.5 g/l of the individual supplementary carbon sources, growth was observed within 12 hours of incubation in all cases in contrast to the lag phase observed when it is grown in Minimal Salt Medium with endosulfan as the sole carbon source. Growth in the presence of 4 mg/l technical endosulfan together with supplementary carbon sources was observed to be higher than that on endosulfan alone. In Minimal Salt Medium that contained endosulfan and the supplementary carbon sources, growth was observed to be highest in the presence of glucose followed by xylose. In the absence of any supplementary carbon source and taking into account the abiotic loss in controls A. xylosoxidans ES9 degraded 55.1% of the initial concentration of 4 mg/l technical grade endosulfan present in the culture. However GC analysis of the residual endosulfan concentrations at the end of the 20 day incubation period revealed that the presence of both the supplementary carbon sources reduced the degradation of endosulfan. Specifically in the presence of 0.5 g/l glucose the degradation of endosulfan was reduced by approximately 17.0% while in the presence of 0.5 g/l xylose the degradation was reduced by 14.4% (Figure 4.51).
**Figure 4.51. Effect of different concentrations of supplementary carbon sources on the degradation of technical grade endosulfan by *A. xylosoxidans* ES9**

**Growth and degradation of endosulfan by *Achromobacter xylosoxidans* ES9 under optimized conditions**

During optimization studies it was observed that the degradation of endosulfan by *A. xylosoxidans* ES9 in Minimal Salt Medium was enhanced individually by increasing the inoculum size as well as by the addition of 0.5 g/l yeast extract to the medium. Hence the growth of *A. xylosoxidans* ES9 and its capacity to degrade endosulfan was determined in medium supplemented with 0.5 g/l yeast extract and by using 500 µl of inoculum from LB culture containing with of 10⁷ cfu/ml cell density.

When strain *A. xylosoxidans* ES9 was grown using enhanced inoculum in Minimal Salt Medium containing yeast extract and 4 mg/l endosulfan no lag phase was observed. A sharp increase in growth was observed until day 4 during which time with the residual concentration of endosulfan in the culture also fell rapidly. Growth slowed day 4 onwards but was still seen to rise steadily until day 16 after which it reached a plateau (Figure 4.52). Overall during the 20 day incubation, 85.8% degradation of endosulfan was observed after exclusion of abiotic losses. A concentration of 1.74 mg/l was seen to be utilized by day 4 following which another 1.11 mg/l was utilized until day 16. No fall in the residual concentrations of endosulfan was observed after day 16. GC analysis indicated the levels of the α-isomer and the β-isomer declined by 2.13 mg/l and 0.72 mg/l respectively. Therefore by the end of the experiment the concentration
of the $\alpha$-isomer declined by 89.7% while the concentration of the $\beta$-isomer was declined by 75.3% (Figure 4.52).

The pH of strain *A. xylosoxidans* ES9 was observed to drop to 5.8 on day 1 and continued to drop further to 5.4 on day 2. It remained at this value for until day 10. Following day 10 the pH was observed to slowly rise to 6.1 by day 14 and remained so until day 20.

![Figure 4.52. Growth of *A. xylosoxidans* ES9 in Minimal Salt Medium supplemented with 0.5 g/l yeast extract and technical grade endosulfan under optimized conditions](image)

In the case of strain *A. xylosoxidans* ES9 the degradation constant for $\alpha$-isomer and the $\beta$-isomer were 0.0135 day$^{-1}$ ($R^2 = 0.953$) and 0.083 day$^{-1}$ ($R^2 = 0.893$) respectively (Figure 4.53 and Figure 4.54).

![Figure 4.53. Degradation constant of $\alpha$-isomer derived from the degradation of technical grade endosulfan by *A. xylosoxidans* ES9 in Minimal Salt Medium supplemented with 0.5 g/l yeast extract under optimized conditions](image)
Formation of intermediate metabolites on the degradation of endosulfan by *Achromobacter xylosoxidans* ES9

In an attempt to elucidate the pathway for the degradation of endosulfan in the best endosulfan degrading strain ES9 the time course study of the formation of the intermediate metabolite was undertaken. The study was undertaken in Minimal Salt Medium with 4 mg/l endosulfan. In previous experiments it was indicated that the metabolites endosulfan ether and endosulfan lactone appeared as intermediates during the degradation of endosulfan by strains *A. xylosoxidans* ES9.

Time course studies revealed that no intermediates were observed until the 1st three days of growth of strain ES9 on Minimal Salt Medium and endosulfan. From the fourth day onwards however the metabolite endosulfan lactone was observed to be formed. GC analysis of the sixth day extracts indicated the presence of metabolite endosulfan ether. The concentration of endosulfan lactone in the culture was observed to be higher on day 6 and continued to increase to day 8. The levels of endosulfan ether were also seen to increase on day 8 and on this day both endosulfan lactone and endosulfan ether were observed in the culture at nearly equal concentrations. However after day 8 the
concentration of endosulfan lactone was observed to slowly fall in the culture until day 20. On the other hand the concentration of endosulfan ether appeared to increase in the culture and on day 10 greater levels of endosulfan ether were observed in the extracts. On day 12 the concentration of endosulfan ether was reduced in the culture and continued to do so until day 14 after which it reduced only marginally until the end of the incubation period (Figure 4.55).

An attempt was made to identify the end products in the culture at day 18 by using Gas chromatography coupled with Mass spectrometry. GC-MS analysis revealed the presence of the endosulfan isomers along with two metabolites in the extracts. The intermediates were identified as endosulfan ether and endosulfan lactone by gas chromatography using the retention time analysis of standards. The EI mass spectrum m/z fragmentation patterns for the standards of endosulfan ether and endosulfan lactone as well as those of the metabolites in the inoculated flasks formed during the degradation of endosulfan are depicted in Figure 4.56, Figure 4.57, Figure 4.58 and Figure 4.59. Strain ES9 did not produce endoulfan sulfate however this recalcitrant and toxic metabolite was detected during endosulfan degradation by strain *P. aeruginosa* ES2 and by fungal strain M-RM10. Endosulfan diol was not detected in any of flasks during the degradation of endosulfan by the isolated strains. No intermediates were observed in the un-inoculated control flasks.

The production of metabolites by strain ES9 showed that it followed the hydrolytic pathway for the degradation of endosulfan rather than the oxidative pathway.
Figure 4.55. Degradation of technical grade endosulfan and the formation of intermediate metabolites by *A. xylosoxidans* ES9 during the incubation period.
Figure 4.56. GC-MS chromatogram showing spectra of the standard of endosulfan ether

Figure 4.57. GC-MS chromatogram showing spectra of the intermediate metabolite endosulfan ether formed on the degradation of endosulfan by A. xylosoxidans ES9
Figure 4.58. GC-MS chromatogram showing spectra of the standard of endosulfan lactone

Figure 4.59. GC-MS chromatogram showing spectra of the intermediate metabolite endosulfan lactone formed on the degradation of endosulfan by *A. xylosoxidans* ES9

Incidentally the formation of similar intermediates was observed when the degradation of both the individual isomers was examined by *A. xylosoxidans* ES9 (Figure 4.60 and Figure 4.61).
Figure 4.60. Gas Chromatogram depicting degradation of the α-isomer and the formation of intermediate metabolites by *A. xylosoxidans* ES9

Figure 4.61. Gas Chromatogram depicting degradation of the β-isomer and the formation of intermediate metabolites by *A. xylosoxidans* ES9
Enrichment of endosulfan sulfate degrading microbial consortia from industrial soil sample

Endosulfan sulfate is a toxic and persistent metabolite of endosulfan. As endosulfan sulfate residues have been reported from industrial soil sample P1 (Figure 4.14), enrichments were set up to develop endosulfan degrading consortia.

In the initial analysis of the soil samples, it was observed that the P1 soil samples had residual concentrations of endosulfan sulfate while the agricultural soils (A1, A2 and A3) did not. Hence the P1 soil sample was utilized to enrich for endosulfan sulfate degrading strains. Also from enrichment studies with endosulfan it was observed that greater endosulfan degrading efficiency was observed when where endosulfan was either supplied as a sulfur source or a carbon source. Hence the enrichments used to develop endosulfan sulfate degrading consortia were also initiated with either Sulfur Free Medium or Minimal Salt Medium supplemented with two concentrations of endosulfan sulfate (4 mg/l and 10 mg/l). Thus in total four different enrichments were initiated with P1 sample.

Enrichment of industrial soil sample P1 using Sulfur Free Medium and endosulfan sulfate

A lag phase was observed in the enrichment flask that was incubated with 4 mg/l endosulfan supplementation until day one. There after an increase in optical density was observed until day 3. A negligible decline in endosulfan sulfate concentrations was observed during this period. A drop in growth was observed between day 3 and day 6 which signified another lag phase in the culture. From day 6 onwards steady growth in the culture was observed until day 12. A decline in the concentration of residual endosulfan sulfate was observed from day 6 onwards until day 18. The consortium reduced the concentration of endosulfan sulfate by approximately 30% in 20 days (Figure 4.62). This Consortium was labeled Consortium 6.
The analysis of the residual extracts of the Consortium 6 also indicated the formation of three intermediates namely endosulfan ether, α-endosulfan and β-endosulfan. Another unidentified peak at approximately 13 min was also observed (Figure 4.64). The pH of the culture was observed to range from 5.4 to 6.0 from day 1 to 20 of the incubation period (Figure 4.65).

On the other hand neither growth nor degradation was observed in flasks supplemented with 10 mg/l endosulfan sulfate and containing Sulfur Free Medium.

**Enrichment of industrial soil sample P1 using Minimal Salt Medium and endosulfan sulfate**

At the end of 20 days, the enrichment culture on minimal salts medium supplemented with 4mg/l endosulfan sulfate was able to utilize 2.1 mg/l of endosulfan sulfate as a sole carbon source indicating a degradation efficiency of 40.2%. This consortium was designated as Consortium 7. A short lag phase was observed from the start of the culture until 12 hours. From 12 hours onwards an increase in growth was observed until day 6. A simultaneous decline in the concentration of residual endosulfan in the enrichment culture was observed during this period (Figure 4.63). Growth slowed after day 9 but was observed to steadily increase until day 12 after which it began to decline. A decrease in the concentration of endosulfan sulfate was observed from day 1 until day 12. The degradation rate slowed down thereafter and was insignificant in the third week.
Figure 4.63. Degradation of endosulfan sulfate by Consortium 7

The analysis of the residual extracts from Consortium 7 also indicated the formation of intermediates namely endosulfan lactone, α-endosulfan and β-endosulfan. An unidentified peak at approximately 13 min was also observed (Figure 4.64). Traces of endosulfan ether and endosulfan diol were also observed.

The pH of the culture was observed to range from 5.2 to 6.2 from day 1 to day 20 of the incubation period (Figure 4.65). Neither growth nor degradation was observed in flasks supplemented with 10 mg/l endosulfan sulfate and containing Sulfur Free Medium.
Figure 4.64. Gas chromatogram depicting degradation of endosulfan sulfate by Consortium 6 and Consortium 7

Figure 4.65. Change in the pH profile of Consortium 6 and Consortium 7 during the incubation period
Characterisation of Consortium 6 and Consortium 7 developed from industrial soil sample

To obtain isolates from the endosulfan sulfate enrichment cultures, aliquots of the various cultures in which endosulfan sulfate degradation was observed were plated on agar plates. The plates were prepared using the corresponding medium and endosulfan sulfate concentrations as present in the enrichments. The individual strains were isolated based on the morphological distinction of the respective colonies on nutrient medium. Details of the microbial strains isolated from different endosulfan sulfate enrichments initiated using the P1 soil sample taken for the study is mentioned in Table 4.11.

Colonies were isolated based on the morphological distinction of colonies on nutrient medium. When aliquots from Consortium 6 were plated on Sulfur Free Medium endosulfan sulfate agar plates, four bacterial strains (SFM-ESS1 to SFM-ESS4) were identified and isolated. On the other hand 3 strains (MSM-ESS1 to MSM-ESS3) were isolated from Consortium 7.

Table 4.11. Details of the microbial strains isolated from the various consortia developed using 4 mg/l endosulfan sulfate and the P1 soil sample

<table>
<thead>
<tr>
<th>Consortia</th>
<th>Medium</th>
<th>No of total isolates</th>
<th>Strain Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consortium 6</td>
<td>Sulfur Free Medium</td>
<td>4</td>
<td>SFM-ESS1 to SFM-ESS4</td>
</tr>
<tr>
<td>Consortium 7</td>
<td>Minimal Salt Medium</td>
<td>3</td>
<td>MSM-ESS1 to MSM-ESS3</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>7</td>
<td>7 bacteria</td>
</tr>
</tbody>
</table>

Quantitative determination of endosulfan sulfate degradation capacity of the strains isolated from Consortium 6 and Consortium 7

The degradation ability of these seven strains isolated from the endosulfan sulfate degrading consortia (Consortium 6 and Consortium 7) using the P1 soil sample was determined using gas chromatography method.

The degradation of SFM-ESS1, SFM-ESS2, SFM-ESS3 and SFM-ESS4 was determined in Sulfur Free Medium endosulfan sulfate as the sulfur source. The mean of three degradation experiments revealed that insignificant degradation of endosulfan sulfate occurred when the individual strains were incubated in Sulfur Free Medium with 4 mg/l endosulfan sulfate as the sulfur source.

The degradation capacities of MSM-ESS1, MSM-ESS2 and MSM-ESS3 were determined in Minimal Salt Medium and endosulfan sulfate as the carbon source.
source. However GC analysis revealed that once again an absence in the decline of the concentration of endosulfan sulfate was observed in the cultures of the three strains as compared to that of the controls.

As it was seen that none of the strains isolated from either of the two consortia initiated using endosulfan sulfate were individually able to degrade endosulfan sulfate, they were not taken forward in the present study.

**Screening for the presence of the putative esd monooxygenase gene in selected endosulfan degrading strains**

There are very few reports on the molecular aspect for endosulfan biodegradation. Sutherland et al. (2002b) in their study have identified, cloned and expressed an esd gene responsible for the degradation of β-endosulfan. The gene reportedly encodes for a putative monooxygenase enzyme. In this study an attempt was made to screen and detect the presence of this particular gene in the isolated strains. To initiate the study only the bacterial strains that showed significant endosulfan degradation in Minimal Salt Medium media were taken up for screening. Initially screening was performed with primers and conditions described by Sutherland et al. (2002) in their study. However no amplification was observed in any of the strains in this case. Amplification was not observed even when annealing temperature was lowered and concentrations of Mg²⁺ and Taq polymerase were increased. Therefore a new degenerate primer was designed to amplify a 900 bp fragment of the putative gene.

**Primer design for the detection of a putative esd monooxygenase gene fragment.**

As described in the previous chapter, the ten closest matching gene sequences to the monooxygenase gene sequence responsible for β-endosulfan degradation in the reported Mycobacterium sp. strain ESD (Sutherland et al., 2002b) were retrieved from GenBank for designing a degenerate primer. The conserved regions of the deduced amino acid sequences were identified as WNVVT from the 140th to 145th position and VVPELQ from the 433 to 438th amino acid. The degenerate PCR primers ESDF and ESDR intended for the amplification of the esd gene fragment in the selected bacterial strains was designed using the conserved regions in the deduced amino acid sequences. For designing of the primer sets, Primer Premier software (PREMIER Biosoft International, Palo Alto, Calif.) was used to screen potential primer and a set of following primers were deduced:
ESD F-5’ GGCA TGAAYRTIGTICA 3’
ESD R-5’ TGNARYWCIGGIACIC 3’

The software was also used to check for hairpins and ensure that primer pairs had similar annealing temperatures. The primer sets were also compared to sequences available on the GenBank database to confirm that primer sequences were unique to the target.

**Polymerase Chain Reaction based detection of the putative esd monooxygenase gene fragment in the selected strains using ESD degenerate primers**

Based on the PCR protocols described in the previous chapter, detection of the putative esd monooxygenase gene fragment was attempted in the four selected strains that showed the best endosulfan degrading phenotype. The genomic DNA of the strains *P. aeruginosa* SFM4, *P. aeruginosa* mRM6, *O. anthropi* ES3, and *A. xylosoxidans* ES9 was amplified with the degenerate primers designed for the study. As observed in Figure 4.66, the expected 900 bp amplicon was observed with strains *O. anthropi* ES3 and *A. xylosoxidans* ES9. However, along with the 900 bp, there were few ambiguous amplicons observed with strains *P. aeruginosa* M-RM6 and *P. aeruginosa* SFM4 (Figure 4.66)

![Figure 4.66. Gel photograph depicting the amplicons obtained with primer sets used for the amplification of esd gene from A. xylosoxidans ES9 (Lane 4), O. anthropi ES3 (Lane 5), P. aeruginosa SFM4 (Lane 6) and P. aeruginosa M-RM6 (Lane 7) along with 1 Kb marker in Lane 1.](image-url)
A series of standardization experiments for PCR was conducted to obtain the expected 900bp with the designed degenerate primer set (ESD F and ESD R) for bacterial strains *P. aeruginosa* M-RM6 and *P. aeruginosa* SFM4.

Initially the annealing temperature was standardized with a temperature range from 55°C to 65°C was used as described in the previous chapter. Though the gel profile indicated the presence of an amplicon at 900 bp, the multiple bands were still observed in the gel profiles (Figure 4.67).

![Figure 4.67. Gel photograph depicting the amplicons obtained with primer sets used for the amplification of *esd* gene from the experiments during temperature standardization. *P. aeruginosa* SFM4 (Lane 1, 52 °C and Lane 3, 55 °C) and *P. aeruginosa* M-RM6 (Lane 2, 52 °C and Lane 4, 55 °C) along with 1 Kb marker in Lane 5](image)

In addition to this, the concentration of Mg²⁺ was standardized and the concentration of 1.5 mM to 2.5mM of Mg²⁺ as described in the previous chapter was used. Figure 4.68 indicates the gel profiles of the strains *P. aeruginosa* SFM6 and M-RM6 with variable concentration of Mg²⁺. Though the gel profile have considerable enhanced, it was observed that along with the expected amplicon of 900 bp, there was an band at approximately 1100 bp that concurrently appeared in all the subsequent gel profiles (Figure 4.68).
When strain *S. marcesens* RM2 was screened for the presence of the putative gene fragment, no amplification was observed. As described earlier when the degenerate primers designed were used in this study a consistent amplicon at 900 bp was observed only with the *O. anthropi* ES3 and *A. xylosoxidans* ES9 strains, there (Figure 4.69).
O. anthropi ES3 and A. xylosoxidans ES 9 were enriched in Minimal Salt Medium with endosulfan as a carbon source and were isolated from soil samples that were exposed to endosulfan for over 20 years. Thus these the amplicon observed with these two strains were taken up for further characterization.

Screening of the putative esd monooxygenase gene fragment in the plasmid DNA of the selected endosulfan degrading strains

In order to establish where the esd gene was plasmid borne or was of genomic origin, a series of screening was also conducted in the plasmid DNA of the selected strains. All the strains that were selected for screening of esd gene in the previous set of experiments were taken up for the study. When aliquotes of samples obtained from P. aeruginosa M-RM6, P. aeruginosa SFM4, O. anthropi ES3 and A. xylosoxidans ES9 were run on the gel, presence of bands was observed in case of every strain (Figure 4.70). The plasmid DNA was taken as template for amplification of esd gene under the conditions in which the PCR was earlier performed.

However when plasmid DNA was taken as a template it was observed that none of the stains showed the expected amplicon of 900 bp after the amplification reactions. Though it is often reported that the degradation genes are plasmid borne, this experiment confirmed that the esd gene in the selected strains taken in this study was chromosomal borne and not plasmid borne.

![Figure 4.70. Gel photograph depicting the plasmid preparation though manual method of Strain O. anthropi ES3 (Lane 2), A. xylosoxidans ES 9 (Lane4), P. aeruginosa SFM4 (Lane 5) and P. aeruginosa M-RM6 (Lane 6) along with 1Kb marker in Lane 1 and Lane 3 is blank.](image-url)
Cloning, sequencing and phylogenetic analysis of the putative monooxygenase gene fragment of *Achromobacter xylosoxidans* ES9 and *Ochrobactrum anthropi* ES3.

The PCR based screening of the *esd* gene with the degenerate primer set indicated the presence of a single 900 bp amplicon in two bacterial strains *O. anthropi* ES3 and *A. xylosoxidans* ES9 that have high capability to degrade endosulfan. Further, strains *O. anthropi* ES3 and *A. xylosoxidans* ES9 were isolated from Minimal Salt Medium enrichment and can use endosulfan as the source of carbon. As described earlier, the strains that use endosulfan as the sole source of carbon can be used in developing bioremediation strategies for soil contaminated with endosulfan. Thus for further characterization, the *esd* gene from these two strains were taken up for cloning, sequencing and phylogenetic analysis. The purified 900 bp amplicon from *O. anthropi* ES3 and *A. xylosoxidans* ES9 were cloned to pGEMT cloning vector as per the protocols described earlier. The positive clones were checked for the insert. Figure 4.71 represents the inserts (900 bp amplicon with approximately 100 bp of SP6 and T7 restriction site) isolated from the plasmids of positive clones of *A. xylosoxidans* ES9 and *O. anthropi* ES3. The representative insert from clonal library of strain *O. anthropi* ES3 and strain *A. xylosoxidans* ES9 was subjected to sequencing as per the protocols described earlier.

![Figure 4.71. Gel photograph depicting the amplification of the insert from plasmids isolated from positive clones. Clone 3 ES9 (Lane 2), Clone 12 ES9 (Lane 3), Clone 38 ES9 (Lane4), Clone 5 ES3 (Lane 5) Clone 27 ES3 (Lane 6) Clone 41 ES3 (Lane 7) along with 1000bp marker in Lane 1 and 8](image-url)
Analysis of the sequences from showed significant homology with the reported endosulfan monooxygenase enzyme of *Mycobacterium* species (Sutherland et al., 2002b). The DNA sequence analysis of the *esd* gene fragment strain *A. xylosoxidans* ES9 revealed an 829 bp ORF. The deduced amino acid sequence of the ORF was compared with the amino acid sequences of other proteins in the SwissProt and the SpTrEMBL databases and was found to have significant similarity to the sequences of monooxygenase family. The deduced amino acid sequence indicated a homology of around 33% with the monooxygenase gene of *dszC* family that catalyses the conversion of dibenzothiophene (DBT) to DBT sulphone. The amino acid sequence also showed a high level of protein sequence identity with the corresponding regions of the *Burkholderia* monooxygenase proteins.

The deduced amino acid sequences of amplified gene fragment of strains *O. anthropi* ES3 showed a homology of around 29% with the reported monooxygenase gene of *Mycobacterium* species strain ESD (Sutherland et al., 2002b). The ORFs that were obtained with the strain *O. anthropi* ES3 fragment did not show any significant match with protein sequences. The parsimony analysis produced the phylogenetic tree is shown in Figure 4.72. This tree shows that the putative monooxygenase from strain *O. anthropi* ES3 and strain *A. xylosoxidans* ES9 forms a monophyletic cluster that does not include the reported monooxygenase enzymes.
Figure 4.72. The phylogenetic analysis of amino acid sequences of the putative monooxygenase enzyme deduced from the esd gene sequence of O. anthropi ES3 and A. xylosoxidans ES9 with sequences of related strains obtained from NCBI GeneBank. The tree was constructed with MEGA 4.1 software after aligning the deduced amino acid sequences in CLUSTALW (http://www.ebi.ac.uk/clustalw/). The scale bar represents 0.2 substitutions per nucleotide position. Numbers at nodes are bootstrap values. Bootstrap values below 25% are not shown.
Southern hybridisation studies on the putative \textit{esd} monooxygenase gene fragment from \textit{Achromobacter xylosoxidans ES9}

PCR based screening for the presence of monooxygenase gene in the \textit{A. xylosoxidans} ES9, using the degenerate primers designed indigenously, resulted in the amplification of a 900 bp gene fragment (Figure 4.69). Southern blot analysis of the \textit{A. xylosoxidans} ES9 was done to confirm the presence of the hypothesized monooxygenase gene. The sequence of the \textit{esd} gene obtained from sequencing the fragment obtained using SP6 and T7 primers with the positive clone was submitted to NEB cutter to identify sites at which the restrictions enzymes might cut. Based on the identification of the restriction enzymes that could cut within the putative \textit{esd} gene, HinDIII was chosen to restrict the genomic DNA of strain \textit{A. xylosoxidans} ES9 as it did not cut within the \textit{esd} gene. The restricted DNA from the gel was probed with the \textit{esd} gene. The results of the southern blot confirmed the presence of the putative \textit{esd} gene fragment in \textit{A. xylosoxidans} ES9 as restriction digest of the genomic DNA of \textit{A. xylosoxidans} ES9 with HinDIII gave a positive signal in the blot (Figures 4.73 a 4.73 b, 4.73 c, 4.73 d). Two bands were observed in the blot indicating that the gene might be present in 2 copies in the strain \textit{A. xylosoxidans} ES9’s genome.

![Image](image1)

**Figure 4.73.** a, b and c. Photographs of the nylon membrane used for Southern Blot analysis of the restricted DNA of \textit{A. xylosoxidans} ES9 using \textit{esd} gene as the probe. L1 and L3 display the positive signal; d Gel photograph indicating 1 Kb ladder (L1) and restriction digest of \textit{A. xylosoxidans} ES9 (L2 gel) together with nylon membrane used for Southern Blot analysis of the restricted DNA of \textit{A. xylosoxidans} ES9 using \textit{esd} gene as the probe. L2 (membrane) displays positive signal.
Estimation of bacterial diversity by culture independent based approach

It has been reported that only a fraction of the microbial population prevalent in any environmental sample can be studied with the conventional culture dependent techniques. The inherent problems of culture bias can be overcome by using the culture independent techniques. There are numerous publications advocating nucleic acid based approach that have resulted in exploration of uncultivated microbial community. In this study, initial experiments conducted based on cultivable techniques could yield strains from only five bacterial genera from three endosulfan degrading consortia initiated with the P1 soil sample. Thus to assess the microbial diversity of in soil contaminated with endosulfan, the soil sample was estimated using culture independent techniques of denaturant gradient gel electrophorosis (DGGE).

The total bacterial diversity in one of the soil samples contaminated with endosulfan was assessed by initially isolating the total community DNA as per the methods described earlier. The soil sample collected from the industrial site (P1 soil sample) was used for analysing the diversity of uncultivable bacterial flora as it was exposed to endosulfan contamination for over 20 years and also had the highest endosulfan residual contamination amongst all the soil samples collected. Secondly, this soil sample also yielded the most efficient endosulfan degrading strains. However, several studies have reported the phenomenon of enrichment bias and it is perceived that only the dominant strains come up in the subsequent isolation step. Therefore, an attempt was made to assess the diversity of the total microbial flora in the consortia developed with endosulfan and the P1 soil sample through DGGE for a comparative analysis. Hence, in addition, total soil community DNA, total community DNA was also isolated from Consortium 3, Consortium 4 and Consortium 5. These enrichments were set up with the P1 soil sample and 4 mg/l endosulfan using Modified-Rich Medium, Sulfur Free Medium and Minimal Salts Medium respectively to isolate endosulfan degrading strains. The details of the same have already been described previously in this chapter (Table 4.6).

The total community soil DNA isolated from the samples showed good concentration as observed in Figure 4.74. Though there was some problem with the PCR inhibitory agents in the DNA preparations, subsequent cleaning gave good quality DNA for the amplification reactions. The extraction of total community DNA from Consortium 3, Consortium 4 and Consortium 5 also gave good results (Figure 4.74). The soil DNA and total community DNA from the various consortia were taken as a template for amplification of total community 16S
rRNA gene with the specific primer sets as described in the previous chapter. The amplicons were visualized in the agarose gels to confirm the amplification (Figure 4.75).

For DGGE, the variable V3 region 16S rDNA was taken for analysis though DGGE gels. Thus a nested PCR with a set of as primers specified in the previous chapter was used to amplify the V3 region using of amplified community 16S rDNA as the template. The 260 bp V3 amplicon obtained from the set of five
DNA samples are described in Figure 4. 76 For proper resolution the primer set used for amplification of V3 region had a 40bp GC clamp.

![Image of gel photograph depicting the amplicon of V3 region of 16S rDNA amplified from the community 16s rDNA from soil sample P1 (Lane 1), Consortium 3 (Lane2), Consortium 4 (Lane 3) and Consortium 5 (Lane 5) along with 100bp marker in Lane 6.](image)

The amplification product was cleaned with the Montage PCR cleansing Kit as described in the previous chapter was taken up DGGE analysis. The resolution of DGGE gels was dependent on various factors like the denaturation gradient. A set of standardization runs were initially carried out to obtain maximum resolution as well as separation of individual bands in the DGGE gels. It was found that the best resolution was observed then the denaturing gradient was kept at 40% to 60%. The resolution of DGGE gels was dependent on the percentage of Acrylamide/bis, which determines the pore size of the gel. When compared with 12 % and 10 % Acrylamide/bis concentration, it was observed that the best resolution for samples taken for this study was achieved at 10 % Acrylamide/bis concentration. Thus finally all the gels for the study used a denaturing gradient of 40% to 60 % with 10 % Acrylamide/bis and the best resolution under these conditions were achieved when the electrophoresis was carried for 30 min at 50V and subsequently at 100 Volt for 5 hours and at 60°C temperature.

Initially a composite marker with V3 region of 16S rDNA gene of the following type strains were used to see the resolution and segregation of bands in the DGGE profile: Acidovorax facilis, Aquaspirillum dispar, Arthrobacter globiformis, Bacillus subtilis, Pseudomonas fluorescens, Sphingomonas capsulata, and Synorhizobium meliloti.
The densiometry results that were obtained from the intensity of the DGGE bands gave a relative measure of different 16S rDNA fragments present in the community DNA isolated from different samples. It was observed that in the DGGE gels of the total microbial flora as represented by the unique bands of the P1 soil sample lane, was higher then the number of bands observed with the total community obtained from three enrichment consortia (Figure 4.77). It was also evident that the total operational taxonomic unit richness as seen by the number of DGGE bands was higher in the sample taken from P1 soil when compared with the number bacterial strains isolated from the enrichment culture technique (Figure 4.77).

In the DGGE profile the lanes (Lane 2 and 3) depicting the soil community microflora indicates 16 unique bands that should correspond to equal number of unique taxonomic unit (Figure 4.77). The DGGE profile of the community DNA isolated from the 3 enrichments initiated with P1 soil sample using Modified-Rich Medium, Sulfur Free Medium or Minimal Salt Medium i.e. Consortium 3, Consortium 4 and Consortium 5 are represented by lane 5, lane 6 and lane 4 respectively. It is observed the number of unique taxonomic unit represented by individual bands is significantly reduced in these lanes as compared to lanes 2 and 3 which are representative of soil community DNA. This clearly indicates the bias that exists within the cultivable techniques (Figure 4.77).

Previous results indicated that Consortium 5 enriched in Minimal Salt Medium gave the maximum diversity in the cultivable technique. Initially there were a total of 4 colonies picked up based on the initial characterization and among them ES2, ES3, ES4 and ES9 were identified respectively as *Pseudomonas aeruginosa*, *Ochrobactrum anthropi*, *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans* respectively. On the other hand Consortium 3 and Consortium 4 initiated in Modified-Rich Medium and Sulfur Free Medium respectively had showed less diversity. This fact can also be seen in the DGGE profile of the community DNA extracted from the different enrichments. The soil community DNA extracted from Consortium 5 (Lane 4) shows more number of individual bands that are representative of unique taxonomic units than those in the DGGE profile of community DNA extracted from either Consortium 3 (Lane 5) or Consortium 4 (Lane6) (Figure 4.77).
Figure 4.77. DGGE gel profile of the community 16S rRNA amplified from the total soil DNA with primers specific to V3 region. L1 represents composite sample developed by merging DNA of known strains. Lane 2 and Lane 3 represent total community DNA of P1 soil. Lane 4, Lane 5 and Lane 6 represent total community DNA of Consortium 5, Consortium 3 and Consortium 4 respectively.