RESULTS

Physico-Chemical parameters of Natural Farm (NF) and Chemical Farm (CF) soil

The soil samples from NF and CF were analysed for different physico-chemical parameters. The moisture content was found to be 10.1% in NF and 9.7% in CF. The water holding capacity was recorded as 54% in NF and 33% in CF. The pH was recorded in the range of 7.0 - 7.3 and 6.5 - 6.9 in NF and CF respectively. The electrical conductivity at 25°C was recorded as 120.4µS/cm at in NF and as 96.9µS/cm in CF. The organic carbon content was about 2.7% in NF and 0.66% in CF. Nitrogen, Phosphorus and Potassium content were found to be 672kg/ha, 55kg/ha and 448.8kg/ha respectively in NF and 347kg/ha, 80kg/ha and 273.6kg/ha respectively in CF (Table 1).

The textural class of soil from NF had loamy type of soil with 43% sand, 30% silt and 27% clay and that from CF had sandy loam type soil with 55% sand, 28% silt and 17% clay (Table 2).

Analysis of Endosulfan residues from Chemical Farm soil

Endosulfan residues were analysed in the Chemical Farm soil. Residual α- and β-endosulfan were around 0.02mgkg⁻¹ of the soil and endosulfan sulfate was not detected (Table 3 and Figure 20)

Biological analysis of soil samples from NF and CF

Soil samples from both NF and CF were analysed for the microbial load, particularly bacteria and fungi. Bacterial number in NF was recorded as 95, 47, 34, 24, 4, 1 CFU between 10⁻¹ to 10⁻⁶ dilutions respectively. In CF, bacterial number was recorded as 35, 31, 5, 3, 8, 0 CFU between of 10⁻¹ to 10⁻⁶ dilutions respectively (Table 4).

Fungal populations isolated in NF was recorded as 52, 30, 11, 4, 1 CFU in dilutions of 10⁻¹ to 10⁻⁵ respectively in NF and 23, 12, 12, 9, 2 CFU in dilutions of 10⁻¹ to 10⁻⁵ respectively in CF (Table 5).
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Overall bacterial load/g of soil outnumbered fungal population among both CF and NF. Bacterial species like *Pseudomonas* sp., *Clostridium* sp., *Azotobacter* sp., *Azospirillum* sp., *Bacillus* sp., *streptomyces* sp., and *Thiobacillus* sp. that help in maintaining soil fertility were recorded to be in large number in NF when compared with CF. Fungal species like *Rhizopus* sp., and *Mucor* sp., *Trichoderma* sp. that help in maintaining soil fertility were recorded in large number in NF compared to CF. *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., were recorded higher in number in NF and Nil in CF (Table 6 & 7). The colony characteristics of the bacteria isolated from CF and NF are summarized in Table 8 & 9.

**Biodegradation of Endosulfan**

**Enrichment of microbial communities and their isolation**

Four visibly distinct colonies were isolated on enrichment media and pure cultures obtained by repeated subculturing were maintained as stock and the other set was used for further study. The bacterial cultures isolated were mainly Gram positive rods.

**Screening of bacterial isolates for endosulfan degradation**

Efficient endosulfan degraders were screened for biodegradation studies by checking the tolerance capacity of bacterial isolates to endosulfan. Out of four visibly distinct colonies of Gram positive rods, two bacterial isolates were selected based on their growth at highest concentration of endosulfan.

**Identification of Endosulfan degrading bacterial isolates**

Preliminary identification was carried out with the aid of Bergey's Manual of Determinative Bacteriology (2005). The cultural characteristics of the bacterial isolates were documented (Table 10). Staining and biochemical tests were carried out at the initial stages that helped in identification of bacterial isolates. Both the bacterial isolates were motile with presence of endospores, exhibiting growth both at 20°C and 37°C (Table 11).

Both bacterial isolates were gram positive rods. Bacterial isolates were positive for starch hydrolysis test, catalase test, Voges Proskauer test and negative for Indole test. Both the bacterial isolates showed no growth on Mac Conkey agar. Arginine dihydrolase and Citrate utilization
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test was negative for bacterial isolate-I and positive for bacterial isolate-II. Gelatin hydrolysis, Methyl red test and Tween 80 hydrolysis test were positive for bacterial isolate-I and negative for bacterial isolate-II (Table 12). Both the bacterial isolates showed red slant and yellow colored butt on Triple sugar Iron agar media (Table 12a). The bacterial isolate-I was able to bring about fermentation of mannose and xylose while bacterial isolate-II showed negative result (Table 12b).

**Bacterial growth measurement**

The growth of the bacterial isolates was measured in terms of optical density to find out the cell number (CFU/ml) that was required for endosulfan degradation. It was found that 0.2 OD corresponds to 2x10^7 CFU/ml and 2x10^6 CFU/ml on bacterial isolate-I and bacterial isolate-II respectively (Figure 6).

**Endosulfan degradation by pure and mixed cultures of bacterial isolates**

Endosulfan degradation by pure and mixed cultures of bacterial isolates were studied using 50 mg/L of technical grade endosulfan in the broth culture on the 8th and 16th day of incubation. The bacterial isolates efficiently degraded endosulfan in the range of 60 - 82% after 16 days of incubation. But the degree of degradation was significantly different between the bacterial isolates when used as pure cultures (Table 13). The statistical analysis results showed that the F value followed by one way Analysis of Variance at P<0.001 was found to be 2536 among the bacterial isolates on 8th day and 5700 among the bacterial isolates on 16th day of incubation (Figure 3).

**Biodegradation of endosulfan isomers by the bacterial isolates**

Biodegradation of α- and β-endosulfan by the bacterial isolates was investigated at different pH to find out the optimum pH for the endosulfan degradation.

**Effect of pH on degradation of α- and β-endosulfan**

The effect of pH on degradation of α- and β-endosulfan was studied by incubating the cultures spiked with 50 mg/L of endosulfan isomers separately at different pH (5.5, 7.5 and 9.5). The results (Table 14 & 15 and Figure 5 & 6) revealed that at pH 5.5, bacterial isolates showed
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minimum endosulfan degradation in the range of 53 - 60% for α-endosulfan and 59 - 63% for β-endosulfan. Control flasks showed degradation in the range of 5 - 11%. The statistical analysis results showed that the F value followed by one way Analysis of Variance at P<0.001 was found be 235.141 for α-endosulfan degradation. The least significant difference (LSD) at this condition between control and bacterial isolate-I,-II was found to be <0.001. The LSD was found to be 0.032* between bacterial isolate-I and -II for α-endosulfan degradation (Figure 5).

The statistical analysis results showed that the F value followed by one way Analysis of Variance at P<0.001 was found be 213.21 for β-endosulfan degradation at pH 5.5. The least significant difference (LSD) at this condition between control and bacterial isolate-I,-II was found to be <0.001**. The LSD was found to be 0.249 between bacterial isolate-I and -II for β-endosulfan degradation (Figure 6).

At pH 7.5, endosulfan degradation was observed in the range of 70 - 82% in case of α-endosulfan and 75 - 86% in case of β-endosulfan (Table 14 & 15) by the bacterial isolates. The uninoculated controls showed endosulfan degradation in the range of 10%. The statistical analysis results showed that the F value followed by one way Analysis of Variance at P<0.001 was found be 537.8 for α-endosulfan degradation. The least significant difference (LSD) at this condition between control and bacterial isolates-I, -II was found to be <0.001**. The LSD was found to be 0.015* between bacterial isolate-I and -II for α-endosulfan degradation (Figure 5).

The statistical analysis results showed that the F value followed by one way Analysis of Variance at P<0.001 was found be 800.00 for β-endosulfan degradation at pH 7.5 and 30°C. The least significant difference (LSD) at this condition between control and bacterial isolate-I, -II was found to be <0.001**. The LSD was found to be 0.013* between bacterial isolate-I and -II for β-endosulfan degradation (Figure 6).

At pH 9.5, bacterial isolates showed endosulfan degradation in the range of 62 - 65% with respect to α-endosulfan and 64 - 68% with respect to β-endosulfan. Control flasks showed degradation around 8% (Figure 5 & 6 and Table 14 & 15). The statistical analysis results showed that the F value followed by one way Analysis of Variance at P<0.001 was found be 334.597 for α-endosulfan degradation. The least significant difference (LSD) at this condition between
control and bacterial isolate-I,-II was found to be <0.001**. The LSD was found to be 0.544 between bacterial isolate-I and -II for α-endosulfan degradation (Figure 5).

The statistical analysis results showed that the F value followed by one way Analysis of Variance at P<0.001 was found to be 309.944 for β-endosulfan degradation at pH 9.5. The least significant difference (LSD) at this condition between control and bacterial isolate-I,-II was found to be <0.001**. The LSD was found to be 0.305 between bacterial isolate-I and -II for β-endosulfan degradation (Figure 6).

**Effect of temperature on degradation of α- and β-endosulfan**

The effect of temperature on degradation of α- and β-endosulfan was studied by incubating the cultures spiked with 50mg/L of endosulfan isomers separately, at different temperatures (20°C, 30°C and 40°C). The results (Table 18 & 19 and Figure 8 & 9) revealed that at 20°C, the bacterial isolates showed degradation in the range of 65-70% with respect to α-endosulfan and 70-76% with respect to β-endosulfan. Control flasks showed degradation in the range of 5 - 8%. The statistical analysis results showed that the F value followed by one way Analysis of Variance at P<0.001 was found be 575.88 for α-endosulfan degradation at temperature 20°C. The least significant difference (LSD) at this condition between control and bacterial isolate-I, -II was found to be <0.001**. The LSD was found to be 0.120 between bacterial isolate-I and -II for α-endosulfan degradation.

The statistical analysis results showed that the F value followed by one way Analysis of Variance at P<0.001 was found to be 579.66 for β-endosulfan degradation. The least significant difference (LSD) at this condition between control and bacterial isolate-I, -II was found to be <0.001**. The LSD was found to be 0.082 between bacterial isolate-I and -II for β-endosulfan degradation (Table 19 and Figure 9).

At 30°C, endosulfan degradation was observed in the range of 70 - 82% in case of α-endosulfan and 75- 86% in case of β-endosulfan (Table 18 & 19) by the bacterial isolates. The uninoculated controls showed endosulfan degradation in the range of 10%. The statistical analysis results showed that the F value followed by one way Analysis of Variance at P<0.001 was found to be 537.8 for α-endosulfan degradation. The least significant difference (LSD) at this condition between control and bacterial isolates-I,-II was found to be <0.001**. The LSD
was found to be 0.015* between bacterial isolate-I and -II for α-endosulfan degradation (Figure 8).

The statistical analysis results showed that the F value followed by one way Analysis of Variance at P<0.001 was found be 800.00 for β-endosulfan degradation at 30°C. The least significant difference (LSD) at this condition between control and bacterial isolate-I, -II was found to be <0.001**. The LSD was found to be 0.013* between bacterial isolate-I and -II for β-endosulfan degradation (Figure 9).

At 40°C, bacterial isolates showed degradation at around 47% with respect to α-endosulfan and 45% with respect to β-endosulfan. The control showed around 10% degradation (Table 18; Figure 9). The statistical analysis results showed that the F value followed by one way Analysis of Variance at P<0.001 was found be 184.96 for α-endosulfan degradation at temperature 40°C. The least significant difference (LSD) at this condition between control and bacterial isolate-I,-II was found to be <0.001**. The LSD was found to be 0.697 between bacterial isolate-I and -II for α-endosulfan degradation (Table 19 and Figure 8).

The statistical analysis results showed that the F value followed by one way Analysis of Variance at P<0.001 was found be 171.71 for β-endosulfan degradation at 40°C. The least significant difference (LSD) at this condition between control and bacterial isolate-I,-II was found to be <0.001**. The LSD was found to be 0.734 between bacterial isolate-I and -II for β-endosulfan degradation (Table 19 and Figure 9).

**Effect of pH on growth of bacterial isolates**

The growth of bacterial isolates-I,-II was determined by measuring optical density (OD) at 580 nm during the entire study. Effect of pH on growth of the bacterial isolates during 16 days of incubation are presented in Table 14 & 20. At pH 5.5, the growth of bacterial isolate-I and -II was found to be 0.3 OD and 0.2 OD on 16th day of incubation.

At pH 7.5, the growth of bacterial isolate-I was recorded as 0.73 OD and 0.76 OD on 8th and 16th day of incubation respectively while growth of bacterial isolate-II was recorded as 0.5 OD and 0.65 OD on the 8th day and 16th day of incubation, respectively.
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At pH 9.5, the growth of bacterial isolate-I was recorded as 0.3 OD on 8th day which increased to 0.4 on 16th day of incubation respectively while growth of bacterial isolate-II was recorded as 0.4 OD on 8th day and decreased to 0.2 OD on 16th day of incubation (Table 20 and Figure 4). Statistical analysis carried out through ANOVA showed that there was moderate significant difference in the effect of pH on growth of the bacterial isolates during endosulfan degradation at pH 5.5 followed by 7.5 when compared to pH 9.5 during 16 days of incubation.

Effect of temperature on growth of bacterial isolates

Effect of temperature on growth of the bacterial isolates during 16 days of incubation are presented in Table 17 & 21. At 20°C, the growth of bacterial isolate-I was recorded as 0.3 OD on 8th day which increased to 0.5 on 16th day, while the growth of bacterial isolate-II was recorded as 0.2 OD on 8th day which slightly increased to 0.3 OD on 16th day of incubation.

At 30°C, the growth of bacterial isolate-I was recorded as 0.73 OD and 0.76 OD on 8th and 16th day of incubation respectively while growth of bacterial isolate-II was recorded as 0.5 OD and 0.65 OD on the 8th day and 16th day of incubation, respectively.

At 40°C, the growth of bacterial isolate-I was recorded as 0.2 OD on 8th day which showed no change even on 16th day, while the growth of bacterial isolate-II was recorded as 0.2 on 8th day which decreased to 0.1 on 16th day of incubation Statistical analysis carried out through ANOVA showed that there was moderate significant difference in the effect of temperature on growth of the bacterial isolates during endosulfan degradation at 20°C when compared to 40°C and 30°C during 16 days of incubation (Table 21 and Figure 7).

Changes in pH of the nutrient media during endosulfan degradation (incubation)

Changes in the pH of the nutrient media during 16 days of incubation are presented in Table 22 & 23. The bacterial isolate-I initially grown at pH 5.5 decreased the pH of the culture medium to 4.94 by 8th day which further decreased to 4.87 after 16 days of incubation, whereas bacterial isolate-II decreased the pH to 5.42 by 8th day which further decreased to 5.16 after 16 days of incubation (Figure 10).

The bacterial isolate-I initially grown at pH 7.5 decreased the pH of the medium to 6.1 by 8th day which further decreased to 4.7 after 16 days of incubation, while bacterial isolate-II
decreased the pH to 7.13 by 8th day which further decreased to 6.15 after 16 days of incubation (Figure 11).

The bacterial isolate-I initially grown at pH 9.5 decreased the pH of the culture medium to 8.54 by 8th day which further decreased to 7.28 after 16 days of incubation, while bacterial isolate-II decreased the pH to 8.89 by 8th day which further decreased to 8.11 after 16 days of incubation. Statistical analysis carried out through ANOVA showed that there was significant changes in pH (5.5,7.5,9.5) of the media incubated at 30°C during endosulfan degradation on 8th and 16th day among control and bacterial isolates (Table 22 and Figure 12).

The bacterial isolate-I incubated at 20°C decreased the pH of the medium from 7.5 to 6.36 by 8th day which further decreased to 5.83 after 16 days of incubation, while bacterial isolate-II decreased the pH from 7.5 to 6.87 by 8th day which further decreased to 6.23 after 16 days of incubation (Figure 13).

The bacterial isolate-I initially grown at 30°C decreased the pH of the medium from 7.5 to 6.1 by 8th day which further decreased to 4.7 after 16 days of incubation, while bacterial isolate-II decreased the pH to 7.13 by 8th day which further decreased to 6.15 after 16 days of incubation (Table 23 and Figure 14).

The bacterial isolate-I incubated at 40°C decreased the pH of the medium from 7.5 to 7.43 by 8th day which further decreased to 6.9 after 16 days of incubation, while bacterial isolate-II decreased the pH to 7.47 by 8th day which further decreased to 7.15 after 16 days of incubation. Statistical analysis carried out through ANOVA showed that there was significant change observed in pH of the media incubated at 30°C and 40°C except 20°C during endosulfan degradation on 8th and 16th day among control and bacterial isolates (Table 23 and Figure 15).

**Analysis of endosulfan residues and its metabolites**

Analysis of endosulfan residues and its metabolites was carried out using Gas Chromatograph. The results showed the presence of endosulfan lactone and endosulfan ether with retention time of 35.49 min. and 34.27 min. respectively along with residues of α- and β-endosulfan with retention time of 40.94 min and 43.52 min respectively (Figure 17). Endosulfan sulfate was not detected throughout the study.
Genomic characterization of Bacterial Isolates

The genomic DNA of both the bacterial isolates were isolated. The 16S rDNA amplification through PCR was carried out (Table 24) and sequencing was also carried out (Figure 16 & 17). The amplification and sequencing were carried out at SCIGENOM labs, Kakanad, Cochin, Kerala.

The 16S rDNA fragments were compared with those available in the Gen Bank databases using the gapped BLASTN 2.0.5 programme through the National Centre for Biotecnology Information (NCBI) Server. The bacterial isolates-I and -II showed 99% similarity to Bacillus cereus and Bacillus amyloliquefaciens respectively.