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Complete Mineralization of Endosulfan Isomers By A Biosurfactant Producing Bacterial Consortium

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Abstract— Endosulfan is one among the Stockholm listed persistent organochlorine pesticide which was widely used in India against various pests of agriculture and plantation crops. Its low solubility limits its biodegradation in the water and soil. The present study aims to formulate a bacterial consortium comprising of biosurfactant producing bacteria capable to mineralize endosulfan isomers and its metabolite endosulfate. Screened bacterial isolates capable of producing biosurfactant and degrade endosulfan was grouped to produce nine bacterial consortium comprising of three bacterial strains each. These nine consortia were tested for endosulfan degradation. All the bacterial consortia (A, B, C, D, E, F, G, H and I) were able to degrade between 65% and 80% of endosulfan in twelve days. Degradation was faster in case of consortium F with a degradation of 81% of α endosulfan and β endosulfan in eight days. Further the consortium F was checked for complete mineralization of endosulfan. Analysis of intermediates revealed the presence of metabolites of endosulfan mainly endosulfate, endosulfan diol, endosulfan ether and phthalic acid. In the present study, complete mineralization of endosulfan by consortium F was confirmed based on the carbon dioxide and chloride ion released during the biodegradation of endosulfan. The study revealed that the consortium comprising of biosurfactant producing bacteria was able to enhance the degradation of endosulfan and mineralize endosulfan.

Keywords—Bioavailability, endosulfan, biodegradation, biosurfactants, Bordetella petrii, endosulfate.

I. INTRODUCTION

Endosulfan is a pesticide belonging to the organochlorine group of pesticides, under the Cyclodiene subgroup and belongs to the class of organochlorine insecticides. Introduced in the 1950 and it emerged as a leading chemical used against a broad spectrum of insects and mites in agriculture and allied sectors (Harikrishnan et al 2004). Endosulfan is a broad spectrum contact insecticide and acaricide registered for use on a wide variety of vegetables, fruits, cereal grains, and cotton, as well as ornamental shrubs, trees, vines, and ornamentals in agriculture. Commercially it is available in two different formulations- 35% EC and wettable powder in the name of endosulfan, thiodan, Thionex, Endosan, Farmoz, Nufarm etc.. Chemically endosulfan (1,2,3,4,7,7 hexachlorobicyclo- 2,2,1-heptene–2,3-bishydroxy methane-5,6-sulfite) is a mixture of stereo isomers of α and β endosulfan in the ratio 7:3. It has a distinct odour similar to turpentine. Its water solubility is 0.33 mg/L and hence its halflife is more than hundred years. This pesticide belongs to the Category Ib – Highly Hazardous classification of the US Environmental Protection Agency (EPA) and Category II – Moderately Hazardous of World Health Organization (WHO). Residues of endosulfan were detected from air, water (surface and ground water) and soil in India, water and sediments in Ghana Mensah et al, 2011, marine water and sediments in India, shallow ground water in Pakistan, river water in China (Jabbar et al, 1999), lagoons in Spain (Hongliang et al, 2012) surface and ground waters in Portugal (Ruzafa et al, 2000), ground and well waters in the Philippines (Cerejeira et al, 2003), coastal, estuarine and river sediments in Israel, water in Benin, Malawi, Nigeria and from drinking, ground, surface and marine waters in South Africa (Fatokia et al, 2004), soil in Benin, Nigeria, Sudan and Zambia, sediments in Benin and Nigeria, vegetation in Madagascar, Zambia and Ghana, Paddy fields in Mediterranea, water from remote mountain lakes in Europe and river and sea water in South East Asia etc..(Pozo et al, 2004). The fate of endosulfan released in the environment is different for the two isomers and also depends on the medium it gets deposited. Beta endosulfan is more persistent than alpha isomer. Endosulfan sulfate is the main degradation product of both isomers, which is equally toxic and is itself more persistent in the environment than its parent compounds. Endosulfan can be broken down by photolysis, hydrolysis and biodegradation. Endosulfan diol, endosulfan lactone, endosulfan ether etc. are some of the other byproducts. Although the isomers are fairly resistant to photolysis, the break down products is susceptible. On plant surfaces endosulfan rapidly degrades to its metabolites. Endosulfan is fairly immobile in soil and is highly persistent. Major products in soil are endosulfan diol, endosulfan sulfate and endosulfan lactone.

Many studies were conducted on biodegradation of endosulfan and its biochemical pathway in aqueous system, but studies on endosulfan and endosulfate degradation by using a biosurfactant producing bacterial consortium is less. Our previous studies on endosulfan degradation by biosurfactant producing single bacterial isolate revealed that the biodegradation of endosulfan can be enhanced by biosurfactant producers (Greeshma and Vasudevan 2013, 2015). Many microbes are capable of utilizing endosulfan and other pesticides in the optimized laboratory condition and showed higher efficiency. But when they are brought to field condition, there was considerable reduction in degradation. In the case of endosulfan its bioavailability is one of the major constrain in the field condition. Hence a study addressing those limitations in the field is needed for successful removal of pesticide contaminated sites. Hence the present study aims to study the endosulfan mineralization and its enhanced degradation by biosurfactant producing bacterial consortium.

II. MATERIALS AND METHODS

A. Chemicals and Bacterial Strains

All the chemicals and standards used in the present study were procured as described in Greeshma and Vasudevan, 2016. Bacterial strains used in the present study were isolated and screened for biosurfactant production as described by Greeshma and Vasudevan (2013, 2015.

B. Formulation of Endosulfan Degrading Bacterial Consortia

Biosurfactant producing endosulfan/ endosulfate degrading bacterial strains and non biosurfactant producing endosulfan/endosulfate degrading bacterial strains were grouped into a consortium of three bacterial strains in various combinations (Consortium A,B,C,D,E,F,H and I). A consortium of non biosurfactant producing endosulfan/endosulfate degrading bacterial strains was also developed with most promising endosulfan and endosulfate degrading bacterial strains (Consortium G). Single bacterial isolate which was grown in endosulfan spiked growth medium for 24 h was centrifuged and pellet was suspended in MSM in required volume to maintain the bacterial count (105 cfu/mL). Bacterial suspension so prepared was mixed in equal proportion (v/v) to make a consortium (Table 1).

Consortium	Non Biosurfactant Producer	Non Biosurfactant Producer	Biosurfactant Producer
A	ES-7	ES-45	ES-47
B	ES-34	ES-45	ES-47
C	ES-36	ES-45	ES-47
	Non Biosurfactant Producer	**Biosurfactant producer**	**Biosurfactant producer**
D	ES-7	ES-36	ES-47
E	ES-7	ES-34	ES-47
	Biosurfactant producer	**Biosurfactant producer**	**Biosurfactant producer**
F	ES-47	ES-34	ES-36
H	ES-47	ES-40	ES-36
I	ES-47	ES-40	ES-34
	Non Biosurfactant Producer	**Non Biosurfactant Producer**	**Non Biosurfactant Producer**
G	ES-7	ES-45	ES-38

Table 1: Grouping of Promising Bacterial Strains

C. Biodegradation of Endosulfan By Bacterial Consortium

About 50 µL of 24 h old cultures of the above grouped consortia were inoculated in MSM (50 mL) spiked with endosulfan (50 mg/L concentration) in a 250 mL Erlenmeyer conical flask. The study was carried out for duration of 12 days with samples withdrawn in duplicates on 0, 4, 8 and 12th days for analysis. The growth was monitored based on OD by measuring absorbance at 590 nm in UV VIS spectrophotometer and viable plate count. About 50 µL of 24 h old culture of the above grouped consortia was inoculated in MSM (50 mL) spiked with endosulfan (50 mg/L) in a 250 mL Erlenmeyer conical flask. The study was carried out for duration of 12 days and samples were withdrawn in duplicates on 0, 4, 8 and 12th day. Endosulfan degradation was accessed based on the residual endosulfan concentration in the broth which was extracted using ethyl acetate. The quantification of endosulfan was carried out using GC-ECD Greeshma and Vasudevan, 2016.

D. Mineralization of Endosulfan by Consortium F

Complete mineralization of endosulfan in MSM by consortium F was confirmed based on the carbon dioxide evolved and chloride ions released during the degradation of endosulfan. The study was conducted as explained above in 100 mL saline bottle with α and β endosulfan (10 mg/L) for duration of 8 days. The bottles were sealed completely (air tight) with aluminum stopper. Gas samples from the

headspace of the saline bottle were withdrawn in duplicates using an airtight glass cylinder on alternate days and analysed for carbon dioxide in a Gas Chromatograph with thermal conductivity detector (TCD) detector. Release of chloride ions were estimated by Mohr's Method. Endosulfan degradation and total organic carbon was also monitored.

E. Gas Chromatograph-Mass Spectrophotometric Analysis

Metabolites formed during the degradation of endosulfan were analysed in a Gas Chromatograph Mass Spectrometer Shimadzu make (GC-MS) using DB5-MS column (30 m long, 0.25 mm inside diameter and 0.25 µm film thickness). The operating conditions were as as described by Greeshma and Vasudevan, 2016.

F. Identification and Characteristics of Isolates in the Consortium F

The isolates in the consortium were characterised according to Greeshma and Vasudevan, 2015. The isolates (ES-34, ES-36, ES-47) were grown on mineral salts medium (MSM) for 24h, centrifuged and pellets were immediately resuspended in 2% glutaraldehyde with 0.05M phosphate buffer and 4% sucrose at pH 7.3. [12] Pellets were placed on aluminium foil disks, air dried, gold coated and examined under SEM (Hitachi model No S-3400N).

G. Statistical Analysis

of the triplicate data. Standard deviation which was within 5% of the mean is represented as error bars in the graph. Spearman's rank correlation was used to test the correlation between growth of bacteria and degradation in MSM. Correlation coefficient r < 0.5 was considered to be statistically significant. Linear relationship of a particular set of data is determined based on the R2 value of data plotted against X and Y axis in excel software.

III. RESULTS AND DISCUSSION

A. Utilization of Endosulfan Isomers By Various Bacterial Consortia

Growth of the nine formulated bacterial consortia on endosulfan is represented in terms of viable bacterial count (Fig.1) and OD (Fig.2). Exponential growth of all the consortia was observed from 4th day with a maximum growth till 8th day in case of consortia A, B, C, H and I whereas, for consortia D, E, F and G growth was observed phase till 12th day. Study on the viable count also showed similar growth pattern. Among the nine consortia, maximum viable count of 59×108 cfu/mL was observed in the case of consortium F.

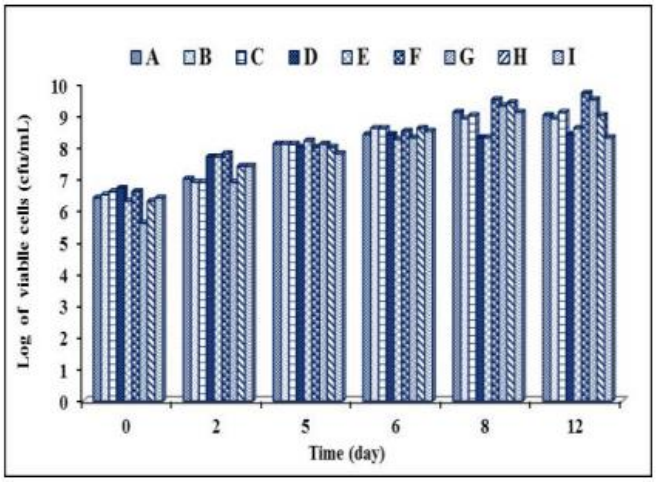

Fig.1. Growth of Various Consortia on Endosulfan

Growth of various consortia on endosulfan reveals that all the consortia were able to utilize endosulfan and utilize endosulfan as the carbon source. In most of the studies on biodegradation of endosulfan, maximum period of growth was till 14 or 16 days (Ngangbam and Dileep, 2011; Kumar and Ligy, 2006; Krishna and Rai, 2012). Krishna and Rai, 2012 reported a slow growth of Pseudomonas aeruginosa on endosulfan till 4thday.

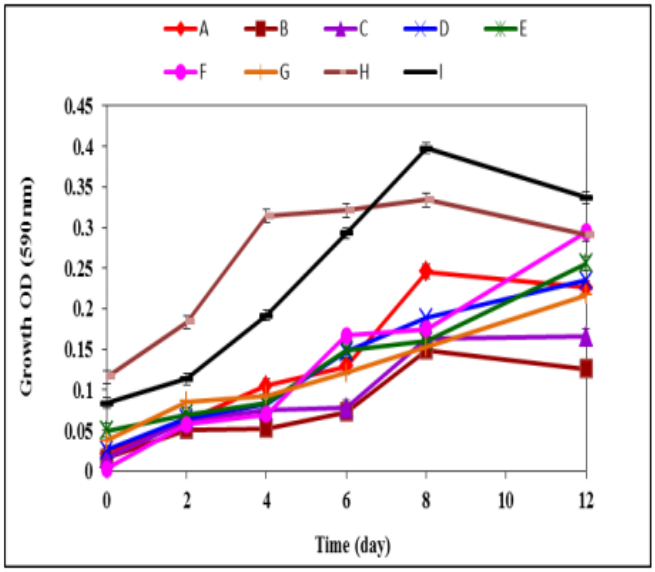

Fig.2. Growth of Selected Consortia on Endosulfan

B. Biodegradation of Endosulfan By Various Bacterial Consortia

All the bacterial consortia (A, B, C, D, E, F, G, H and I) were able to degrade between 65% and 80% of endosulfan in twelve days (Fig. 3). Percent degradation of endosulfan by all the consortia showed some variation in degradation of both the

endosulfan isomers (p0.05). Among the nine consortia, B & F were able to degrade 80% and 81% of α endosulfan, 81% and 83% of β endosulfan respectively. Minimum degradation of 67% of α endosulfan and 70% of β endosulfan was observed in consortium G which was formulated with non biosurfactant producing bacteria. From this study it was inferred that the degradation was faster in case of consortium F with a degradation of 81% of α endosulfan and β endosulfan in eight days. This may be due to the enhanced bioavailability of endosulfan by the biosurfactant produced by the consortium F which was formulated with three biosurfactant producing bacteria. Compared to single isolates in the consortium F, degradation of endosulfan was faster as a consortium and no residue of endosulfate was detected on 12th day. This may be due to the grouping of promising bacterial strains which are capable of degrading endosulfan and endosulfate.

GC MS analysis of the culture broth on eighth day revealed the presence of metabolites of endosulfan mainly endosulfate, endosulfan diol, endosulfan ether and phthalic acid. The concentration of endosulfate formed on eighth day was 8.6 µg/L and 69 µg/L for F and G consortia, respectively. Endosulfan diol and phthalic acid were the other intermediates detected in case of F whereas with G endosulfan diol and endosulfan ether were detected. In the case of F, a new intermediate phthalic acid was detected which was not previously reported by others and also formation of nonchlorinated intermediate reveals that the consortium was able to remove chlorine by dehydrohalogenation. Kumar and Philip, 2006 reported the formation of non-chlorinated endosulfan metabolite carbenium ion and ethylcarboxylate. Endosulfan was degraded to Endosulfan lactone which then was degraded to either 1,2,5,6, hexa-chloro 5-chloromethyl-3-carbenium ions and carbenium ions or C6H2Cl6 and ethylcarboxylate (Kumar and Philip, 2006). In the present study, the endosulfan lactone might have been degraded to phthalic acid. All other metabolites formed in the present study were previously reported (Kwon et al, 2005; Shetty et al, 2000). Weir et al 2006 also reported the degradation of endosulfan sulfate to endosulfan monoalcohol or endosulfan dimethylene through endosulfan hemisulfate by Arthrobacter sp. KW.

The presence of endosulfate in the culture extract reveals that the biodegradation of endosulfan followed an oxidative metabolic pathway and further the consortium was able to utilize endosulfate via hydrolysis of endosulfate to endosulfan diol. According to Sutherland et al, 2002 formation of endosulfan diol via endosulfate hydrolysis also reveals that the degradation of endosulfan followed a biotic hydrolysis pathway. Weir et al 2006 also reported that the oxidation of endosulfan or endosulfan sulfate by the monooxygenase in Arthrobacter sp. KW yielded endosulfan alcohol. Among the biosurfactant producing consortia, F showed faster degradation with dechlorination.

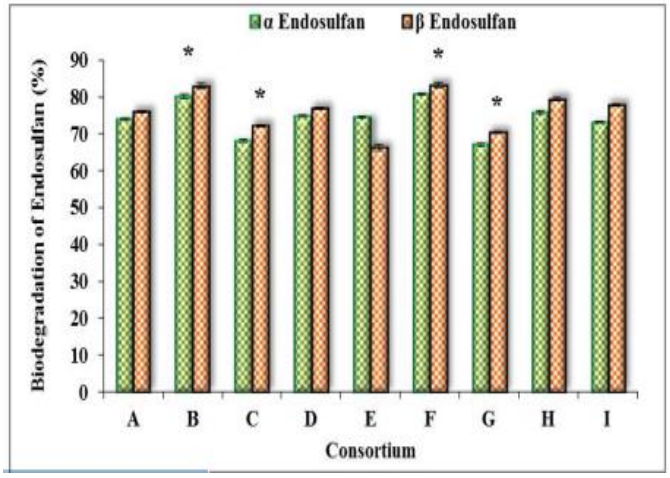

Fig.3. Utilization of Endosulfan by Various Bacterial Consortia * Represent Percent Degradation of Endosulfan Differs Significantly (P≤0.05)

C. Characteristics of Isolates ES-34 and ES-36

The isolates ES-34 and ES-36 are gram negative rodococci of size ranging 0.5-0.7 µm in diameter and 1.18 - 1.84 µm in length (Fig. 4) belonging to family Alcaligeneceae and genus Bordetella. It's a motile species with peritrichous flagella. The colonies in MSM are smooth, convex, pearly, glistening, and nearly transparent. The strains were able to survive under anaerobic condition in the presence of nitrate. Genus Bordetella is also reported for biosurfactant production. Bayoumi et al, 2010 reported biosurfactant production by Bordetella hinizi-DAFI which reduced the surface tension of water to 40 Dcm-1 . Bordetella petrii was reported as the first member of genus Bordetella which was isolated from the environment and capable of anaerobic growth (2001). In the present study also Bordetella petrii I GV 34 and Bordetella petrii II GV36 were capable of anaerobic growth and biosurfactant production. Supriya & Dileep, 2009 reported 80% and 86% degradation of α and β endosulfan in MSM in a duration18 days by a Bordetella sp. In the present study strain Bordetella petrii I GV 34 degraded 89% and 84% of α and β endosulfan and Bordetella petrii II GV 36 degraded 82% of both isomers in 14 days.

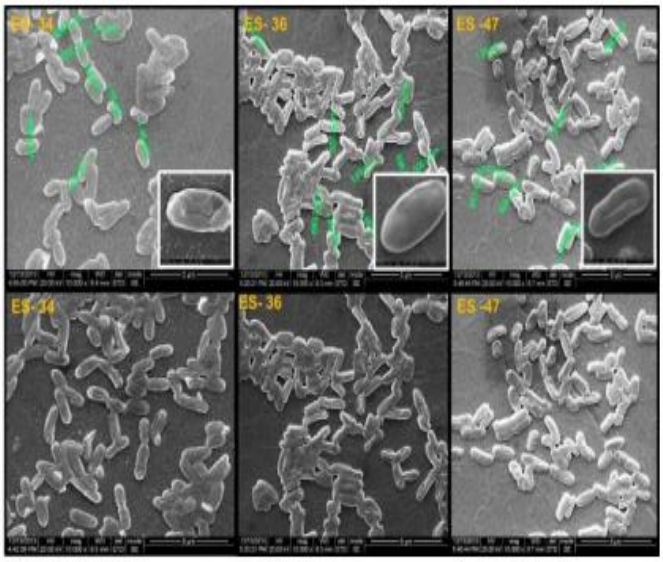

Fig.4. Scanning Electron Microscopic Images of Bacterial Isolates in the Consortium F

D. Characteristics of Isolate ES – 47

The strain ES-47 belongs to family Alcaligeneceae and genus Achromobacter. It is a gram negative rod of size ranging 0.5-0.7 µm in diameter and 1.5 - 1.84 µm in length (Fig. 4). ES-47 is a motile species with peritrichous flagella. The colonies in MSM are smooth, convex, pearly, glistening, and nearly transparent. The isolate was able to survive in anaerobic condition in the presence of nitrate. Carbohydrates are usually not utilized, but utilized D-glucose. Achromobacter sp was also reported to produce biosurfactant on hydrocarbons (Tambekar and Gadakh, 2013) and endosulfan degrader (George et al, 2005). Sarat & Dileep, 2011 reported that Achromobacter xylosoxidans strain C8B degraded 94% and 84% α and β endosulfan respectively in 20 days. In this study, the strain Achromobacter xyloxidans GV 47 utilized 89% and 99% α and β endosulfan in 9 days. The strain also utilized endosulfate formed during endosulfan degradation. The strain also produced biosurfactant in endosulfan containing medium, solubilized endosulfan with subsequent degradation.

E. 16S rRNA Sequencing of Genomic DNA of Bacterial Strains in Consortium F

Similarity search carried out using blastn tool in the NCBI blast search for ES-34 and ES-36 showed 100% coverage and 99% homology with Bordetella petrii respectively which was already reported in our study Greeshma and Vasudevan, 2013. ES-47 showed 100% coverage and 99% homology with Achromobacter xyloxidans. Even though the strain ES-34 and ES-36 showed close homology with Bordetella petrii multiple sequence alignment (Fig. 5). Thus, after blast analysis the strains ES-34, ES-36 and ES-47 were named as Bordetella petrii I GV 34, Bordetella petrii II GV 36 and Achromobacter xyloxidans GV 47, respectively. All the three sequence of the strains were submitted to Genbank (NCBI) and their respective accession numbers are KJ022624, KJ022625 and KJ022626. From a phylogenetic perspective, Bordetella, Achromobacter, and Alcaligenes are closely related. This relatedness is reflected in the high level of phenotypic similarity observed between Alcaligenes and Achromobacter species and the motile and more rapidly growing species of Bordetella. This relatedness among the three strains is also revealed in the similarity in major region of nucleotide sequence.

Phylogenetic tree analysis of the NCBI blast results showed the ES-34 and ES-36 belongs to phylum proteobacteria, class betaproteobacteria, order Burkhoderiales, family Alcaligeneceae, Genus Bordetella, Species Petrii, whereas ES-47 belongs to Genus Achromobacter and species Xylosoxidans.

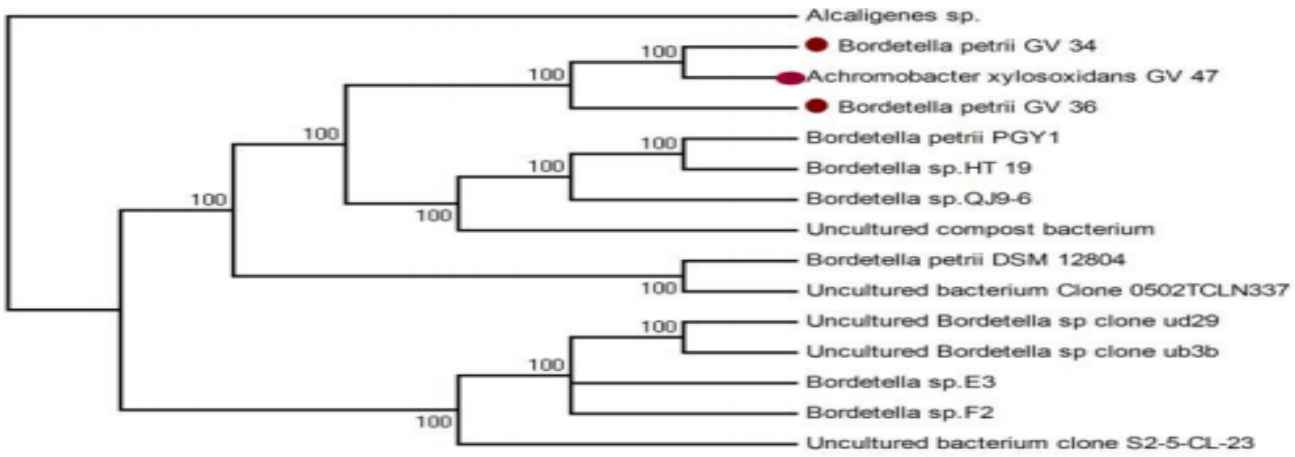

Fig.5. Phylogenetic Tree of 16S rRNA Blast Sequence of the Isolates

F. Mineralization of Endosulfan by Consortium F

Degradation of endosulfan has been reported based on the disappearance of parent compound and detection of metabolites formed during the degradation (Weir et al, 2006; Mohit et al, 2008; Niti and Bharathi, 2013; Jayashree and Vasudevan, 2007. Endosulfan diol and phthalic acid were the major metabolites. Since some of the degradation products are toxic and more persistent than the parent compound, complete mineralization of the parent compound is necessary for confirming the utilization of organic compound by the microbnde. The major metabolites produced by Achromobacter xylosoxidans is endosulfate, endosulfan diol and other lower carbon compound whereas in the case of Bordetella petrii endosulfate was not formed instead endosulfan is directly converted to endoulfan diol (Fig. 6).

In the present study complete mineralization of endosulfan by consortium F was confirmed based on the carbon dioxide and chloride ion released during the biodegradation of endosulfan. Degradation of endosulfan isomers, growth of the consortium, release of chloride ion and total organic carbon removal are represented in Fig. 7. Maximum viable count of bacteria observed was 109 cfu/mL on 8th day. Increase in degradation of endosulfan was observed with an increase in the growth of bacteria (r=0.8, p

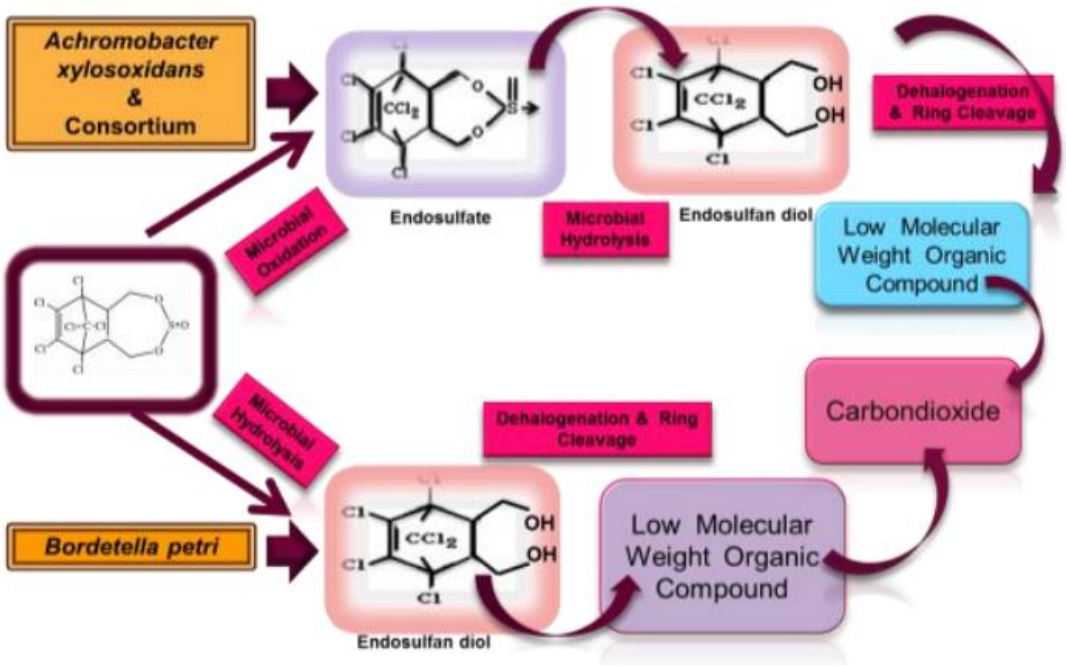

Fig.6. Biodegradation Pathway of Endosulfan by the Different Isolates in Consortium F

Degradation of endosulfan has been reported based on the disappearance of parent compound and detection of metabolites formed during the degradation (Weir et al, 2006; Mohit et al, 2008; Niti and Bharathi, 2013; Jayashree and Vasudevan, 2007. Endosulfan diol and phthalic acid were the major metabolites. Since some of the degradation products are toxic and more persistent than the parent compound, complete mineralization of the parent compound is necessary for confirming the utilization of organic compound by the microbnde. The major metabolites produced by Achromobacter xylosoxidans is endosulfate, endosulfan diol and other lower carbon compound whereas in the case of Bordetella petrii endosulfate was not formed instead endosulfan is directly converted to endoulfan diol (Fig. 7).

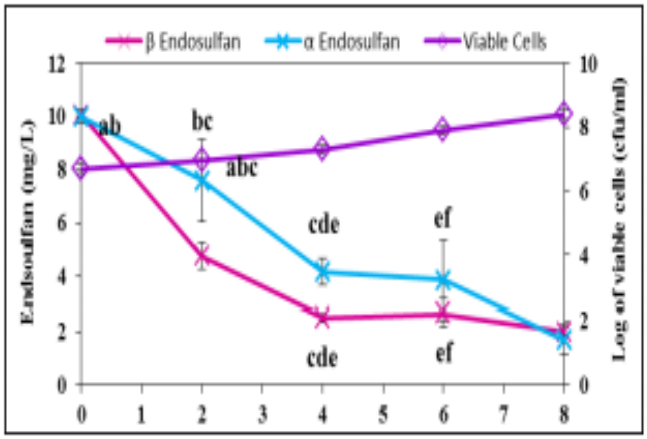

Fig.7. Chloride and Carbon Dioxide Release During the Mineralization of Endosulfan by Consortium F

IV. CONCLUSION

Complete mineralization of endosulfan in the field condition is affected by factors like its solubility, toxicity of the intermediate endosulfate and the abiotic factors in the field. Its solubility is one of the major factors limiting its degradation which can be enhanced using a biosurfactant producing bacteria. In the present study, the capability of a bacterial consortium comprising of biosurfactant producing bacterial strains in enhancing the degradation and complete mineralization of endosulfan was studied. The study revealed that the consortium comprising of three bacterial strains was able to enhance the degradation of endosulfan and was capable of completely mineralizing the endosulfan.

V. ACKNOWLEDGMENT

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