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Enrichment and isolation of endosulfan degrading microorganism from natural resource

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Abstract

The enrichment and isolation of microbes from natural resource for endosulfan degradation was carried out to search for the potential candidates for development of *in situ* bioremediation technology for endosulfan. Twelve soil samples, with history of Endosulfan application, were enriched for microorganisms that can utilize Endosulfan as carbon source in Carbon-Deficient Medium (CDM) or as sole source of sulfur in Non Sulfur Medium (NSM). Five monocultures (N1, N2, N3, N4 & N5) were obtained after Round 2 enrichment of 15 days each, which showed significantly prolific growth on NSM-agar supplemented with Endosulfan as sole sulfur source. On comparison of these monocultures for amount of pesticide degradation, microbial growth and the changes in pH of the medium, when grown in NSM with Endosulfan as sole source of sulfur, strain N2 showed maximum endosulfan degradation (92.2%), microbial growth (0.382) and reduction in media pH (52.78%). The culture conditions of strain N2 were optimized for maximum pesticide degradation. In an optimized culture condition, strain N2 degraded endosulfan upto 94.2% within 7 days, when estimated quantitatively by Gas-Chromatography Electron Capture Detection (GC-ECD) method. The study suggests that strain N2 is worth investigating for its biochemical and molecular characterization, to develop a valuable candidate for bioremediation of endosulfan.

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Introduction

After worldwide ban/restriction on organochlorine pesticides like DDT and BHC, the production and use of endosulfan increased dramatically during 1980s. Presently it is one of the most extensively used organochlorine pesticides throughout the world and now endosulfan has become a toxic environmental menace that needs a thoughtful and effective address. Endosulfan is used extensively worldwide as a contact and stomach insecticide for Colorado potato beetle, flea beetle, cabbageworm, peach tree borer, tarnished plant bug and as an acaricide on field crop like cotton, paddy, sorghum, oilseeds and coffee (Lee *et al*, 1995; Kullman and Matsumura, 1996; USEPA, 2002). In addition to agricultural use, endosulfan is also used in vector-control (tsetse fly), as a wood preservative and for the control of home garden pests (CNRC, 1975). It is a highly toxic substance and World Health Organization (WHO) classifies it as Category II (moderately hazardous) while United States Environment Protection Agency (USEPA) classifies it as a Category 1b (highly hazardous) pesticide. It has been reported to be highly toxic to aquatic fauna like fish and invertebrates (Sunderam *et al*., 1992). Also, there are reported implications in mammalian gonadal toxicity (Sinha *et al*., 1997), genotoxicity (Chaudhari *et al*., 1999), teratogenic effects (Yadav, 2003) and mutagenic effects (U.S. Department of Health & Human Services, 1990). These acute and chronic toxicity and environmental concerns have attracted scientists to search for an effective and economically viable option for endosulfan degradation.

Bioremediation has evolved as a very economical and viable process for detoxification of xenobiotics in general and pesticides in particular, as an alternative to existing methods like incineration and landfill. Technology development of bioremediation process starts with search for potential microorganisms (MOs) as a source of xenobiotic-degrading enzymes and identification and establishment of *in-situ* and *ex-situ* methods of enzymatic degradation of xenobiotics (Hussain *et al*., 2009). Therefore, the present study was designed to isolate the endosulfan degrading MOs from natural resources by enrichment culture method. The isolated strains were further studied for their comparative pesticide degradation ability to select the best biodegrader.

Materials and methods

Field site

Twelve soil samples were collected, for the study, from the agricultural-crop fields of Ratangargh (Churu), Lakshmangarh (Sikar) and Nawalgarh (Jhunjhunu) of Shekhawati region of Rajasthan, India, which have the history of endosulfan application (Table 1). The collected soil samples were stored at 4°C until they were analyzed.

Chemicals

Technical grade endosulfan (99% pure), an organochlorine insecticide with the chemical name 6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3 benzo-dioxathiepin-3-oxide; gifted by Excel India Pvt. Ltd., Ahmedabad, India, was used for the present study. The endosulfan isomers (α- and β-isomers) and endosulfan sulfate standards for Gas-Chromatography-Electron Capture Detection (GC-ECD) were purchased from Hewlett Packard Company, Wilmington, Delaware, USA. Chloroform for UV-spectroscopy, of spectroscopic grade and n-Hexane for GC-ECD, of chromatographic grade, were used. All other chemicals used for the study were of analytical grade.

Sample collection

Twelve soil samples, used for enrichment study were collected from the crop fields of Ratangarh (Churu), Lakshmangarh (Sikar) and Nawalgarh (Jhunjhunu), the three constitutive districts of Shekhawati region of Rajasthan state of India. All these fields were having the history of endosulfan application. The collected samples wee stored at 4°C until further study.

Table 1. Source for soil samples collected for enrichment studies.

Soil sample code	**Sample description**
S1	Top soil from crop field of Bidasar village, Nawalgarh, Jhunjhunu, Rajasthan.
S2	Top soil from vegetable farm of Nawalgarh, Jhunjhunu, Rajasthan.
S3	Top soil from crop field of Nawalgarh, Jhunjhunu, Rajasthan.
S4	Top soil from agricultural field of Dudwa village, Lakshmangarh, Sikar, Rajasthan.
S5	Top soil from crop field of MIER campus, Lakshmangarh, Sikar, Rajasthan.
S6	Top soil from crop field of Jajod village, Lakshmangarh, Sikar, Rajasthan.
S7	Top soil from agricultural field of Ratangarh, Churu, Rajasthan.
S8	Top soil from crop field of a village, Ratangarh, Churu, Rajasthan.
S9	Top soil from crop field of a village, Ratangarh, Churu, Rajasthan.
S10	6-inch deep soil from vegetable farm of Nawalgarh, Jhunjhunu, Rajasthan.
S11	6-inch deep soil from crop field of MIER campus, Lakshmangarh, Sikar, Rajasthan.
S12	6-inch deep soil from agricultural field of Ratangarh, Churu, Rajasthan.

Experimental design

Endosulfan is an organochlorine pesticide with sulfate moiety. Therefore, the experiments were designed to search for MOs that can use it as either carbon source or as sulfur source. This was achieved by enriching the soil samples collected from the agricultural fields for endosulfan degrading MOs.

Table 2. Composition of Carbon Deficient Medium (CDM)- pH-7.2.

S. No.	Chemical	Amount (g/lt)
1.	Sucrose	1.00
2.	NaNO3	3.00
3.	K2HPO4	1.00
4.	MgSO4	0.50
5.	KCl	0.50
6.	FeSO4	0.01

Enrichment of microbes

The whole enrichment process was carried out, in triplicates, with two parallel strategies. On one hand, the soil samples were enriched for endosulfan degrading microbial population in a medium deficient in carbon source, Carbon Deficient Medium (CDM) (Table 2), which was supplemented with endosulfan as carbon source. On other hand, enrichment was done in a culture medium without sulfur, Non Sulfur Medium (NSM) (Table 3), which was supplemented with endosulfan as sole source of sulfur. These enrichment media were designed for the study according to earlier reports with certain modifications (Siddique *et al.*, 2003).

Each soil sample (20 g) was first enriched for endosulfan degrading MOs by addition of 2mg of technical grade endosulfan in 100µL of acetone to moisten the soil, followed by incubation in dark at room temperature for 1 month. Microbial inocula for further enrichment studies were prepared by shaking each soil sample (20 g) overnight in 100 ml of respective enrichment media (*i.e.* CDM and NSM) at 30°C and 160 rpm rotatory shaking. The solid particles were allowed to settle for one hour and aliquots of the supernatant were used to inoculate the respective medium.

Table 3. Composition of Non-Sulfur Medium (NSM)- pH-7.2.

S. No.	Chemical	Amount (g/lt)
1.	K_2HPO_4	0.225
2.	KH_2PO_4	0.225
3.	NH_4Cl	0.225
4.	$MgCl_2.6H_2O$	0.845
5.	$CaCO_3$	0.005
6.	$FeCl_2.4H_2O$	0.005
7.	D-Glucose	1.000
8.	Trace Element Solution	1mg/lt

For enrichment study, Erlenmeyer flasks (50 ml) and enrichment media (CDM & NSM) were autoclaved separately for 15 minutes at 121°C and 15 lbs pressure. Each sterilized flask was spiked with 50 µL of acetone containing 0.5 mg endosulfan under laminar flow, allowing acetone to evaporate. 9ml of enrichment media containing 0.05% Tween 80, was added to each respective flask followed by inoculation with 1 ml of supernatant solution from the source (inocula) flasks. Uninoculated spiked flasks were also set up as control to check for any chemical, thermal or photo–degradation. The aerobic culture was incubated at 30°C and 160 rpm orbital shaking for two weeks (Round 1 Enrichment culture). Thereafter, 1ml of the culture was transferred to 9ml of fresh CDM and NSM containing 50mg/L (50 ppm) endosulfan and further incubated under same incubation conditions for two weeks (Round 2 Enrichment culture). The microbial growth was studied by measuring the optical density (OD) at 600 nm using spectrophotometer.

A sulfur-free medium was also designed because contaminating sulfur in the enrichment medium could promote spurious culture growth. A second soil culture was initiated for the purpose of preparing a medium free of contaminating sulfur. Sulfur-free medium was prepared by growing soil cultures overnight in enrichment medium without endosulfan and then removing the cells by centrifugation (6000 rpm for 10 minutes).The supernatant was then filtered through a 0.22 µm-pore-size filters using vaccum-filtration assembly. After inoculation of this medium with respective endosulfan-degrading cultures and *E. coli* DH5α (as negative control), no growth was observed until the addition of 50µM sodium sulfite as a source of sulfur. The microbial growth was studied by measuring the O.D. at 600 nm. The sterility of sulfur-free medium was confirmed by the absence of growth when the aliquots were incubated on nutrient agar medium plates.

Isolation of Endosulfan-Degrading Monocultures

Cultures after Round 2 enrichment were further sub-cultured 3 times at a time span of 10 days each to encourage the adaptability and degradability of the endosulfan degrading cultures. Pure cultures of single strains were obtained by centrifuging 1-ml aliquots of sub-cultured Round 2 enrichment cultures at 8000 rpm for 10 minutes with microcentrifuge. The supernatant was removed and cell residues were resuspended in 50µL of sterile NSM and CDM culture media by vortexing.

Aliquots of this suspension were placed on respective CDM-endosulfan or NSM-endosulfan agar media by streaking. The solid medium was prepared by adding 2% agar to the enrichment basal medium followed by autoclaving. Thereafter, 50-ppm endosulfan dissolved in acetone was aseptically added after cooling the molten agar to about 50°C. Agar plates were incubated under aerobic conditions at 30°C for 15 days and discrete colonies were isolated. Isolates were further purified by streaking on fresh plates of respective agarified-enrichment culture media.

Screening of isolates for endosulfan degradation

The five monocultures (renamed as N1, N2, N3, N4 and N5) obtained after enrichment were screened for their relative endosulfan degrading ability. All these isolates showed prolific growth on NSM as compared with CDM. Hence, NSM was selected as the culture medium for further studies and was supplemented with endosulfan as sole source of sulfur. This screening was done on the basis of i) Endosulfan content remaining in the culture (O.D. $_{248nm}$), ii) The decrease in pH of the culture medium during incubation and iii) Increase in microbial optical density (O.D. $_{600nm}$).

Wavelength of maximum absorbance (λ_{max}) for technical grade endosulfan was calculated, using chloroform (spectroscopic grade) as solvent, with the help of double beam UV-Visible spectrophotometer (Systronic, Type 117). A graph

was plotted for O.D. against wavelength (236nm to 260nm) to obtain the λ_{max} at 248nm and a standard curve was prepared by plotting a graph of $O.D._{(248nm)}$ against respective concentration range of endosulfan (6.25 ppm – 200 ppm).

For the screening study the same NSM medium was used as for the enrichment studies, supplemented aseptically with 50-ppm of technical-grade endosulfan dissolved in acetone. The monocultures were inoculated onto the sterilized liquid culture media (NSM) and were incubated for 15 days at 30°C and 160 rpm in orbital shaker incubator. Un-inoculated spiked flasks were also set up as control to check for any chemical degradation. All the studies were done in triplicate using control as reference or blank.

For endosulfan extraction and estimation, 5 ml of culture-broth was acidified to pH 2.0 with 6.0 M Hydrochloric Acid (HCl) and extracted three times with an equal volume of ethyl acetate as per the method given by Awasthi *et al.* (2003). The organic phase containing the pesticide was separated using separatory funnel and was passed through a 6-cm $MgSO_4$ column in a Pasteur pipette to remove any residual water (Sutherland *et al.*, 2000). The columns were pre-washed with ethyl acetate. The extracted elutes containing pesticide were gently evaporated at 50°C in oven and were dissolved in chloroform (spectroscopic grade) and stored in glass vial at 4°C for UV-spectroscopic analysis. A fortification test was also conducted to check the endosulfan recovery from culture with known concentration of endosulfan and the extraction method was found to be 98.0 $\pm$ 1.4% accurate, when done in triplicate.

Degradation and culture condition optimization of best degrading isolate

On the basis of comparative screening for endosulfan degradation, isolate N2 was selected to optimize the growth profile for maximum endosulfan degradation. The culture condition selected for optimization included media-pH, incubation-temperature and agitation, incubation days and nutritional supplementation. The degradation of endosulfan was estimated by UV-spectroscopic analysis ($O.D._{248nm}$) of residual endosulfan, extracted by the method described earlier. The microbial-growth was estimated by spectroscopic analysis ($O.D._{600nm}$) of the culture medium. Finally, the degradation profile of endosulfan was monitored using Gas-Chromatography-Electron Capture Detection (GC-ECD) method under the optimized culture conditions.

The medium used for optimization of degradation and growth conditions was NSM. Optimal pH for maximum endosulfan degradation and microbial growth was calculated by screening the medium pH in a range of 5.5-7.5. Similarly, optimal temperature was estimated by screening temperature range of 25°C - 37°C. Aeration condition was optimized by screening the agitation speed (rpm of orbital shaker incubator) in a range of 100 rpm – 200 rpm. Effect of supplementation of extra source of sulfur was also checked by providing 50 µM $NaSO_3$ to the culture medium. The incubation time and effect of sub-culturing on reduction of incubation time was calculated by estimating the degradation of endosulfan after 10th, 15th and 17th sub-culturing of isolate N2 under optimized culture conditions.

The degradation profile of isolate N2 was checked under culture conditions optimized as above. The endosulfan degradation was monitored by UV-spectroscopic analysis ($O.D._{248nm}$) of residual endosulfan and by GC-ECD method after 3rd and 7th day of incubation. For GC-ECD, the endosulfan residues in the culture medium were extracted by the method described earlier. After evaporation of organic phase from elutes, the resultant dried extracts were dissolved in 5ml of n-hexane (GC/HPLC Grade) and were kept in glass vials at 4°C in refrigerator until analysis.

GC-ECD methodology

The sample extracts of the N2-culture medium were analyzed the residues of endosulfan by GC-ECD. The analysis was carried out on a Shimadzu Model 2010 Gas Chromatograph (GC) equipped with ^{63}Ni Electron Capture Detector (ECD) and a capillary column HP ultra 2 (US 4293415) 0.52 x 25 x 0.32. The instrument was supported by Lab Solutions software for the analysis of endosulfan (α- and β-isomers) and endosulfan-sulfate. The stock standards (200-ppm) of endosulfan isomers and endosulfan sulfate were obtained from Hewlett Packard Company, USA. Stock standards of 100-ppm were prepared by diluting standard mixture in 1:1 solvent mixture of HPLC grade iso-octane and toluene. These stocks were stored under freezing conditions. Working standard of the mixture was prepared from 100-ppm stock solution. 0.5 – 1.0 ppm of this mixture of endosulfan isomers and endosulfan sulfate was used for calibrating the Gas-chromatograph for analyzing residues of endosulfan in the sample analyzed.

Statistical analysis

The experiments in the present investigation were carried out in triplicates and the mean values were taken into consideration and the standard errors of the mean were calculated for each observation. After the 2nd round of enrichment, Student's't'-test was used to analyze the significance of difference of the means of microbial growth (O.D. $_{600nm}$) in the two enrichment media namely, NSM and CDM. A null hypothesis was generated which stated that there was no significant difference between the mean values of O.D.600nm in the two media (NSM and CDM). The paired 't'-test was conducted with degree of freedom (df) value of 22 (df = N_1+N_2-2). The calculated t-value was compared with the value of't'-table at p=0.05 (5% level) to accept or reject the hypothesis.

For selecting the best growing microbial culture after enrichment, the average microbial growth (OD_{600nm}) was taken as standard value as no external standard value is reported for microbial growth in the given enrichment media. The individual OD_{600nm} values were compared with the calculated standard (average) value and cultures having growth values above the standard value were selected for further investigation.

The same statistical tool was applied when the selected five monocultures (N1, N2, N3, N4 and N5) were screened for their microbial growth (OD_{600nm}), endosulfan degradation and pH reduction of the culture medium after 15 days of incubation. The monocultures having respective values greater than the calculated standard (average) values were selected for further study.

Results

Enrichment

Two rounds of enrichment culture for 15 days each were carried out with 12 soil samples and their optical densities (O.D.$_{600nm}$) were noted. In NSM it ranged for 0.059 – 0.063 (Fig. 1). 5 (S2, S4, S5, S6 and S9) out of 12 soil samples showed substantial microbial growth as evident from their O.D.$_{(600nm)}$. Hence they were selected for further study. All other samples had negligible growth in either enrichment medium.

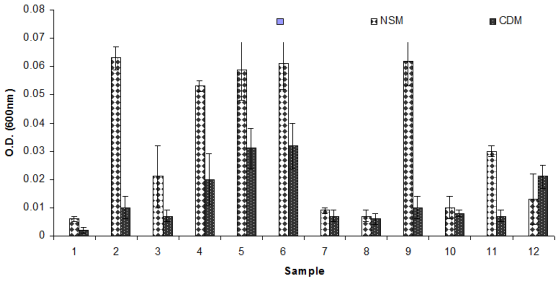

Fig. 1. O.D. (600nm) of round 2 enrichment microbial culture.

The microbial growth (O.D.$_{600nm}$) in NSM and CDM were found to have significant difference after the statistical analysis by Student's 't'-test. The difference in the mean values of microbial growth (OD_{600nm}) in the two enrichment media was found to be statistically significant. On the perusal of the microbial growth (Figure 1) in both the enrichment

media, NSM was found to promote higher growth of enriched microbes than CDM. Therefore, NSM was selected as culture medium for further study.

Endosulfan degrading monocultures

The selected microbial cultures *i.e.*, S2, S4, S5, S6 and S9, were sub-cultured thrice for 10 days each for 1st two sub-culturing and 15 days for 3rd sub-culturing. There occurred a substantial increase in the microbial densities of the respective microbial cultures, due to better adaptation in the culture medium (Fig. 2). These microbial cultures produced pure discrete colonies when streaked over solid NSM-medium and significant colony growth was observed. The respective monocultures were used for the rest of the investigations and were renamed as N1, N2, N3, N4 and N5 in further study.

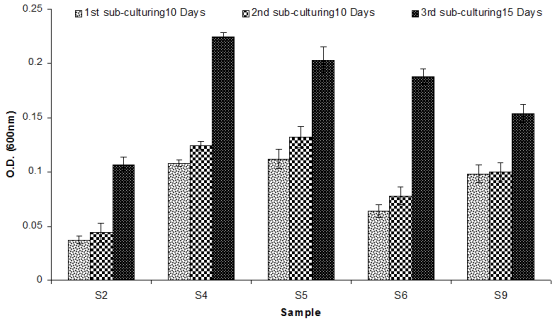

Fig. 2. O.D. (600nm) of selected monocultures after 4 days.

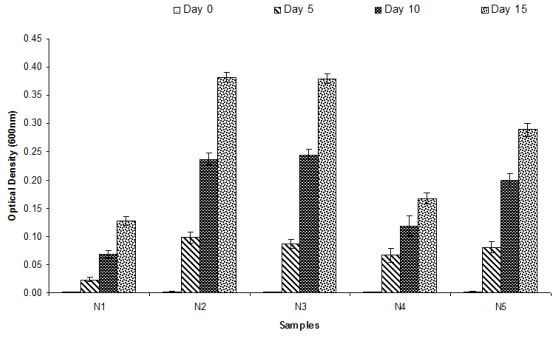

Fig. 3. O.D. (600nm) of selected monocultures.

Screening of selected isolates for endosulfan degradation

Selected monocultures (N1, N2, N3, N4 and N5) were screened for their relative ability of endosulfan degradation. The screening was based on three parameters a) The relative microbial growth of selected monocultures in NSM with endosulfan (50-ppm) as sole source of sulfur; b). Amount of endosulfan degraded; c). Decrease in medium pH after 15 days of incubation.

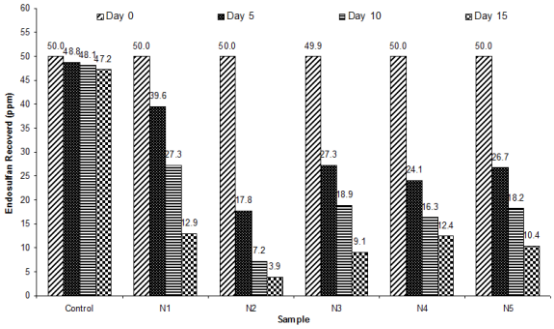

Fig. 4. Endosulfan recovered from selected monocultures.

Isolate N2 was found to have the maximum microbial density (OD_{600nm}) of 0.382 after 15 days when compared to control taken as blank (Fig. 3). Amount of endosulfan recovered from culture broth was minimum for isolate N2 and was found to be 3.9-ppm with about 92.2% degradation as observed from spectroscopic estimation at $O.D._{248nm}$ (Fig. 4). The control had the abiotic-endosulfan degradation of 5.7%. pH of the culture medium was found to reduce from 7.2 (initial) to 3.4 for the isolate N2 culture with about 52.78% pH reduction as compared to 6.9 (4.17%) of that of the control (Fig. 5).

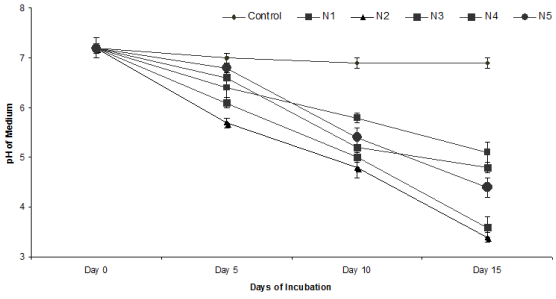

Fig. 5. pH reduction of culture medium by selected monocultures.

Looking into these comparative profiles, isolate N2 was found to be the most potential candidate for the endosulfan bioremediation and hence was selected for further investigations regarding degradation and growth optimization.

Degradation and growth profile optimization of isolate N2

The different culture and incubation parameters were optimized for maximum degradation of endosulfan and efficient microbial growth. A pH of 6.5 was found to be optimal as it elicited a maximum microbial density of 0.398 and 92.40% endosulfan degradation after 15 days of incubation. Abiotic degradation of endosulfan was 6.5% at pH 5.5, 6.0 and 6.5 while it was 19.48% and 37.66% at pH 7.0 and 7.5. Thus, pH 6.5 was found to be optimal for maximum microbial growth and biological degradation of endosulfan. Out of three incubation temperatures (25°C, 30°C and 37°C) selected for maximum microbial growth and biological degradation of endosulfan, 30°C was found to be the best promoting the maximum microbial growth (OD $_{600nm}$ = 0.397) and 92.22% endosulfan degradation.

The agitation of 130 rpm was found to show maximum microbial density (OD $_{600nm}$ = 0.414) and also the maximum endosulfan degradation of 94.8%. The stationary culture showed the lowest microbial growth of OD $_{600nm}$ of 0.093 and 53.24% endosulfan degradation. The abiotic degradation of endosulfan was found to be 3.9% for stationary phase while it was 6.5% for the agitation speeds of 100 – 130 rpm. It increased to 9.1% for the speeds of 140 – 150 rpm and was maximum of 11.68% at 200 rpm. To verify the biological degradation of endosulfan by the isolate N2, additional source of sulfur (Na_2SO_4) was supplied in the culture medium (NSM) along with endosulfan. There occurred almost no biological degradation till 5th day of incubation while after 10th and 15th day; endosulfan degradation was 11.69% and 22.08% as compared to 6.5% degradation in control. The culture medium with endosulfan as sole source of sulfur showed degradation of 87.02%, 92.22% and 94.8% after 5th, 10th and 15th day of incubation as compared to 6.5% of abiotic degradation in control (Table 4).

While observing the effect of incubation days and sub-culturing of isolate N2 on endosulfan degradation, it was found that there was 92.2% degradation of endosulfan after 15 days of incubation when inoculated with 10th sub-cultured inocula. The degradation increased to 97.42% in just 10 days for 15th sub-cultured inoculum while it remained 97.42% in 7 days for the 17th sub-cultured inoculum. The degradation profile remained at the maximum of 97.42% in 7 days, even after 20th sub-culturing.

Table 4. Microbial Growth & Endosulfan Degradation on Adding Additional Sulfur Source to Isolate N2

Sample (Days)	NSM with Endosulfan as Sole Source of Sulfur				NSM with Endosulfan (50ppm) Supplemented with Na_2SO_4 as Sources of Sulfur			
	O.D. (600nm)	O.D. (248nm)	Endosulfan (ppm)	Endosulfan Degradation	O.D. (600nm)	O.D. (248nm)	Endosulfan (ppm)	Endosulfan Degradation
Control (15)	0.000	0.036±0.005	46.75	6.5 %	0.000	0.036±0.004	46.75	6.5 %
N2 (5)	0.102±0.005	0.005±0.001	6.49	87.02 %	0.849±0.011	0.036±0.005	46.75	6.5 %
N2 (10)	0.247±0.008	0.003±0.002	3.89	92.22 %	1.513±0.013	0.034±0.004	44.16	11.69 %
N2 (15)	0.416±0.004	0.002±0.001	2.60	94.80 %	1.832±0.015	0.030±0.003	38.96	22.08 %

Values of O.D. $_{(600nm)}$ and O.D. $_{(248nm)}$ are the mean ± Standard Error of the triplicate samples

Culture condition

Temperature : 30± 1°C
pH : 6.5
Agitation : 130 ± 10 rpm
Incubation time : 15 Days

Table 5. Gas Chromatography – ECD Data of Endosulfan isomers & Endosulfan sulfate after degradation by Isolate N2.

Sample	Peak #	Retention time (min)	Area	Height	Area (%)	Compound	Concentration (ppm)	Endosulfan degradation (%)
Control (Day 3)		13.818	154053207.4	10321065.0	53.46	α–Endosulfan	24.82	18.74
	24	15.751	98608717.8	10132336.4	33.38	β-Endosulfan	15.81	
	32	17.207	6966260.7	1197855.2	2.29	Endosulfan sulfate	6.42	
	37							
Control (Day 7)		13.800	141291680.7	10335461.7	57.81	α–Endosulfan	23.93	22.58
	25	15.729	76248397.4	9776147.1	31.14	β-Endosulfan	14.78	
	32	17.205	6230901.6	1049637.3	2.56	Endosulfan sulfate	6.98	
	37							
N2 (Day 3)		13.757	98072452.1	10341409.6	69.71	α–Endosulfan	8.65	71.0
	26	15.708	2979152.8	4726282.5	21.16	β-Endosulfan	5.85	
	32	17.207	2464016.1	413903.2	1.74	Endosulfan sulfate	2.98	
	38							
N2 (Day 7)		13.709	8457622.4	1443946.9	60.10	α–Endosulfan	1.79	94.0
	27	15.697	3256538.5	562847.6	23.30	β-Endosulfan	1.21	
	32	17.207	184822.8	33147.8	1.25	Endosulfan sulfate	0.32	
	33							

Values of O.D. $_{(600nm)}$ and O.D. $_{(248nm)}$ are the mean ± Standard Error of the triplicate samples

Culture Condition:

Media : NSM with 50-ppm technical Endosulfan as sole source of Sulfur.

pH : 6.5

Temperature : 30°C

Agitation : 130 rpm

GC-ECD estimation of endosulfan degradation by isolate N2

After optimization of microbial growth the endosulfan degradation profile of the isolate N2 was verified quantitatively by GC-ECD method. After GC-separation and ECD analysis, it was found that 8.65 ppm of α-endosulfan, 5.85 ppm of β-endosulfan and 2.98 ppm of endosulfan sulfate remained in the N2-culture system, accounting for about 71.0% degradation after 3 days of incubation, as compared to 24.82 ppm of α-endosulfan, 15.81 ppm of β-endosulfan and 6.42 ppm of endosulfan sulfate detected in the control sample accounting for 18.74% abiological degradation. After 7 days of incubation, the degradation of endosulfan in N2-culture system was found to be 94.0% with 1.79 ppm of α-endosulfan, 1.21 ppm of β-endosulfan and 0.32 ppm of endosulfan sulfate detected by GC-ECD as compared to 23.93 ppm of α-endosulfan, 14.78 ppm of β-endosulfan and 6.98 ppm of endosulfan sulfate detected in the control sample that accounts for 22.58% abiological degradation (Table 5).

Discussion

The present investigation was carried out to enrich microbes from natural resources for Endosulfan degradation and to search for the potential candidates for development of *in-situ* bioremediation of Endosulfan. The observations are in accordance with several previously reported findings (Hussain *et al.*, 2007; Siddique *et al.*, 2003; Sutherland *et al.*, 2000). All these authors have reported about enrichment of microbial populations capable of utilizing endosulfan as sulfur source. This finding also support the fact that endosulfan is a poor biological energy source as it contains six potential reducing electrons and has a relatively reactive cyclic sulfite diester group (Sutherland *et al.*, 2000; Guerin, 1999; Van Woerden, 1963). On the contrary, there are findings stating that the enriched microbes have utilized endosulfan as carbon source (Siddique *et al.*, 2003; Shetty *et al*, 2000; Awasthi *et al.*, 2000; 1997).

The findings of the present investigation also suggest that the microbial growth (OD_{600nm}) in NSM

with endosulfan as sole source of sulfur, increased by 57% to 186%, when the selected microbial cultures were sub-cultured consecutively three times. This may be attributed to the better adjustment of microbial metabolism and the gene-expression system in a nutritionally stressed environment. Similar results were observed by Sutherland *et al* (2000), where it was found that after approximately six rounds of successive sub-culturing in enrichment media, there was a substantial disappearance of endosulfan with a simultaneous increase in microbial biomass.

A quick and relatively easier method of endosulfan content estimation was formulated using UV-Visible spectroscopic method. The λ_{max} for technical grade endosulfan in chloroform was calculated as 248nm. The finding is similar to the observations of Guha *et al* (1999). While screening the selected monocultures (N1, N2, N3, N4 & N5), strain N2 showed the maximum endosulfan degradation (92.2%) and microbial growth (0.382). This is in accordance with the findings of Awasthi *et al.* (1997), Siddique *et al.* (2003), Sutherland *et al.* (2000) and Hussain *et al.* (2007) where biodegradation of endosulfan was observed to be accompanied with substantial increase in microbial biomass.

While screening the monocultures for pH reduction of the culture medium, the isolate N2 showed 52.78% reduction. Earlier, Siddique *et al.* (2003) and Hussain *et al.* (2007; 2006) also reported a reduction in the pH of culture medium. This reduction in medium-pH has been attributed either to the dehalogenation of endosulfan resulting in the formation of hydrochloric acid (HCl) or organic acids produced by microorganism during their metabolic activities (Siddique *et al.*, 2003). It has also been found that the proton-transfer chemical ionization (PCI) mass spectrum of the metabolites of culture medium displayed fragment ions indicating consecutive loss of the two molecules of HCl from the molecular parent ions $[M+H]^+$ (Sutherland *et al.*, 2000). Contrary to this, Marten (1976) and Miles and Moy (1979) reported about increase in pH of the culture medium with increased endosulfan degradation. But this degradation could not be differentiated as biological or chemical degradation because the latter is favored at alkaline pH.

Isolate N2 was found to be the most efficient biodegrader of endosulfan and hence was selected for further study of optimizing the culture condition parameters for maximum endosulfan degradation. While optimizing the culture conditions, isolate N2 showed maximum growth and endosulfan degradation at a pH of 6.5. Almost similar degradation was observed at pH 7.0 but the abiotic degradation of endosulfan was found to be more (6.5%) as compared to culture at pH 6.5 (5.26%). A high abiotic degradation of 19.48% was observed at higher pH of 7.5. This observation finds its similarity with the earlier findings (Sutherland *et* al., 2000; Guerin, 1999). Sutherland *et al* (2002), in their findings, reported that endosulfan is susceptible to alkaline hydrolysis with approximately 10-fold increase in hydrolysis occurring with each increase in pH units. They suggested buffering the enrichment medium at pH 6.6 (below 7.0) to minimize non-biological hydrolysis of endosulfan. Guerin (1999) also had the same opinion regarding the endosulfan-biodegradation.

A temperature of 30°C was found to promote maximum microbial growth and endosulfan degradation (92.22%) followed by 25°C with 87.02% degradation. At incubation temperature of 37°C, higher abiotic degradation of the pesticide was noted in the control sample. This may be attributed to spontaneous thermolysis of endosulfan isomers to endosulfan sulfate. Thus, the isolate N2 may be suggested to be a potential candidate for *in-situ* bioremediation of endosulfan with an active degradation profile in the normal temperature range of 25°C – 30°C.

The rotatory speed of 130 rpm was observed to result in maximum microbial growth and endosulfan degradation (94.8%). Both the factors decreased on the either side of the rotatory speed. The degradation reduced to 63.64% at a speed of 200 rpm while it was 53.24% under stationary condition. These observations suggest that isolate N2 utilizes endosulfan as nutrient source under suitably agitated condition (130rpm) while higher agitation and aeration slows down microbial growth and endosulfan degradation.

To confirm the utilization of endosulfan as sulfur source by the isolate N2, the culture medium (NSM) with 50-ppm endosulfan was supplemented Na_2SO_4 as readily assimilable sulfur source. No biodegradation of endosulfan was observed till 5th day of incubation, while significant microbial growth was observed for the same. The biodegradation was observed after 10th day of incubation with about 22.08% endosulfan being degraded on 15th day as compared to 6.5% abiotic degradation. But when the isolate N2 was cultured in NSM with endosulfan as the sole source of sulfur, 94.8% degradation of the pesticide was observed after 15 days of incubation as compared to 6.5% of abiotic degradation. These results confirm that the isolate N2 is capable of utilizing endosulfan as the sulfur source and hence is a potential candidate for endosulfan bioremediation. The results are in accordance with the report of Weir *et al* (2006). They isolated an *Arthrobacter* sp. from soil microbial population that was enriched with continuous nutritional pressure to use endosulfan sulfate as the sole source of sulfur. They found that the organochlorine-degrading activity was absent in the presence of sodium sulfite as an alternative sulfur source. This suggested that the activity was part of the sulfur starvation response of the strain.

It was also observed that on repeated sub-culturing, the rate and extent of endosulfan biodegradation increased upto 97.42% with about 91% of net biodegradation by the isolate N2. This observation is in good accordance with the report of Sutherland *et al* (2000) who found that continuous sub-culturing in sulfur-free medium led to an increase in rate of endosulfan disappearance, with no detectable levels of pesticide remaining after 4 days by the 20th subculture as compared to 8 days after 10th sub-culturing. In the present investigation, a maximum of 97.42% endosulfan degradation was achieved in just 7 days after 17th repeated sub-culturing. No further increment in either rate or extent of pesticide degradation was observed after 17th sub-culturing.

Thus, the culture-conditions optimized for the isolate N2 for maximum microbial growth and biodegradation of endosulfan includes non-sulfur medium (NSM) with endosulfan as the sole source of sulfur, having pH maintained at 6.5. The optimal temperature, agitation and time of incubation are 30^{o}C, 130 rpm and 7 days, respectively.

Finally the endosulfan degradation by isolate N2 under optimal culture condition was quantified by Gas Chromatography with Electron Capture Detection (GC-ECD) method. The residual levels of α- and β- endosulfan were estimated to be 1.79-ppm and 1.21-ppm respectively, after 7 days of incubation with the initial combined endosulfan isomers' concentration of 50-ppm. This residual amount corresponds to about 94.0% degradation of the pesticide. The results obtained were in concomitance with the previous estimation by UV-spectroscopic method. This gives further confirmation about endosulfan degradation ability of the isolate N2. A proportionate decrease of endosulfan sulfate with both the isomers of endosulfan was observed during GC-ECD. This observation suggests that the isolate N2 is also capable of degrading endosulfan-sulfate along with its parent compound.

Conclusion

In the present work, a soil bacterium (N2) was enriched and isolated by the application of strong

selection pressure on the bacterium to release the sulfur moiety from endosulfan and use it as sole source of sulfur. The isolated bacterium showed an extensively high biotic degradation of the pesticide of about 94%. Therefore, the isolate N2 holds a strong potential to be studied further as a candidate for developing *in-situ* bioremediation process for endosulfan degradation. The strain N2 is being presently studied in our laboratory for its identification as well as biochemical and molecular characterization to develop a technically and economically viable bioremediation technology for combating endosulfan menace.

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