Int J Pharm Bio Sci 2013 Apr; 4(2): (B) 586 - 590

Research Article **Microbiology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

BIODEGRADATION OF ENDOSULFAN BY ESTUARINE HALOTOLERANT BACTERIUM AND INSINUATION OF TOXICITY TOWARDS *ARTEMIA SALINA*

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ABSTRACT

The study determines the degradation and detoxification potential of cyclodiene insecticide endosulfan by halotolerant bacterial strains. After repeated screening, *Streptococcus agalactiae* a halotolerant endosulfan degrading bacterium which tolerate upto 5000ppm of endosulfan was isolated from enriched sediment of the Vellar estuary. The bacterium shows maximum growth by 3.8 (cfu 5.2×10^8) with pH 3.5. Degradation study reveals that the endosulfan degradation efficiency of 40.77% by GCMS analysis with 80% chloride release in the medium. The optimum growth parameters of *S. agalactiae* for endosulfan degradation was found to be pH-8, 35˚C, dextrose as carbon source, salinity 30ppt, inoculum size of 100µl/ml or 10ml L^{-1} broth with 2.5×10^6 cfu/ml under shaking condition with 15days incubation respectively. The 24 hrs LC_{50} of BSLA study confirm the detoxification of endosulfan by the halotolerant bacterium. The results of the present study concluded this potential strain could be useful in reclamation of any contaminated site in the tropics.

KEYWORDS:Endosulfan, *Artemia salina*, halotolerant, toxicity assay

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INTRODUCTION

Endosulfan (6, 7, 8, 9, 10, 10- hexachloro-1, 5, 5a, 6, 9,9a hexa hydro-6, 9-methano 2, 3, 4 benzo (e) dioxathiepin -3 oxide) is an organo chlorine insecticide widely has been used in agriculture for prevention of crop damage from insects and mites infection. However the persistent of endosulfan exposure in the environment for a longer period, impact the native organisms by bioaccumulation and biomagnification. The chemical has been banned in 2011 by Stockholm Convention of Persistant Organic Pollutants due to its unacceptable neurological and reproductive risk to farm workers and wildlife. The Endosulfan is exceedingly toxic to fish and aquatic invertebrates[1] and it has been implicated gradually more in gonodal toxicity[2], genotoxicity[3] and neurotoxicity[4], while provoking chronic symptoms like testicular and prostate cancer[5], breast cancer and sexual abnormality[6] in numerous mammalian species. The accumulation of Endosulfan in living organisms leads to severe health effects such as cancer and genetic disorders. Hence the degradation of such recalcitrant pollutant in the environment is under serious concern. Surprisingly the normal microbial flora in a polluted environment genetically altered to degrade and utilize such xenobiotics as nutrient source. Hence in the present study, the highly potential endosulfan degrading bacterial strains which adapt high concentration of endosulfan was selected and optimized with physicochemical parameters for effective degradation. The degradation rate was analysed using GCMS and toxicity study.

MATERIALS AND METHODS

Chemicals

Commercial endosulfan formulation (Endosol, 35% EC) was purchased from local market, Tamil Nadu, India. Mineral salt medium[7] was prepared for endosulfan degradation study. The mineral salt medium composition includes: (in g L^{-1}) K_2HPO_4, 0.225; KH_2PO_4, 0.225; $(NH_4)_2SO_4$, 0.225; $MgSO_4 \cdot 7H_2O$, 0.05; $CaCO_3$, 0.005; $FeCl_2 \cdot 4H_2O$, 0.005 with 1 ml of trace elements solution[8] (in mg L^{-1}) $MnSO_4 \cdot H_2O$, 169; $ZnSO_4 \cdot 7H_2O$, 288; $CuSO_4 \cdot 5H_2O$, 250; $NiSO_4 \cdot 6H_2O$, 26; $CoSO_4$, 28; $Na_2MoO_4 \cdot 2H_2O$, 24 and pH 7. The endosulfan stock solution was prepared by dissolving endosulfan in acetone to enhance even dispersion in the medium. 0.1 M $AgNO_3$ and 5% K_2CrO_4 were used for chloride ion estimation. All the chemicals were analytical grade.

Enrichment and screening of endosulfan degrading bacteria

Samples were collected from the Vellar Estuary sediment nearby paddy field where endosulfan has been used for a decade. Samples (10g) were enriched with 100ml mineral salt medium with 100ppm endosulfan as the sole source for week[9]. After incubation 1 ml of the enriched culture was transferred to 100 ml of fresh sterile MSM containing 100ppm of endosulfan and incubated for another week. After incubation 1ml of the supernatant was serially diluted and plated in mineral salt agar and incorporated with endosulfan. Plates were incubated at 37ºC, for one week and individual colonies were isolated for further studies. The bacterial isolates were incubated with mineral salt medium containing varying endosulfan concentration from 500 - 5000 ppm with an interval of 500 ppm for screening the highly tolerant / degrading bacteria. The bacterial growth at different time intervals was observed with OD600nm and CFU/ml. The pH[10, 11, 12] of the medium was monitored to observe the changes of the medium after incubation. The chloride content in the culture medium was estimated calorimetrically by Mohr's method[13].

Characterization of endosulfan degrading bacteria

Cell growth was studied at different pH (3-12), temperature (0 to 50˚C), supplementary carbon source (sucrose, maltose and dextrose), inoculum size, salinity (10 - 50 ppt), shaking and static condition at different incubation period. 500ml of optimized mineral salt medium incorporated with 500ppm of endosulfan was prepared in 1000ml Erlenmeyer flask and inoculated with culture

broth. The biomass, pH and free chloride release were tested.

Degradation analysis

The extraction procedure was followed using [14] standard methods. The extracted endosulfan and its residues were analyzed and quantified using GC Clarus 500 Perkin Elmer, equipped with Turbo mass gold - Perkin Elmer detector. The Elite-5 MS (5% Diphenyl / 95% Dimethyl poly siloxane), column (30 × 0.25mm × 0.25µm df) was used. Nitrogen was used as a carrier gas at a flow rate of 1ml min^{-1}, and the injections were made in the split mode with a split ratio of 10:1. Injector temperature was maintained at 250 º C. The total GC running retention time was about 36 min.

Toxicity study using Artemia salina

Toxicological analysis of endosulfan and its formed metabolites after bacterial treatment were conducted using the *Artemia salina* (BSLA) for 24 hrs[15]. The shrimps were divided into 6 groups with 10 animals each treated with 5 pesticide concentrations (0.5 - 2.5 ppm) and 1 control, animals without endosulfan. The mortalities were recorded after 0, 6, 12, 18 and 24 hrs of exposure at 29 ± 2°C.

RESULTS AND DISCUSSION

The study is on degradation potential of endosulfan by halotolerant bacteria isolated from velar estuary, runoff from agricultural land. On the whole, 23 bacterial strains were isolated from enrichment culture (100ppm formulated endosulfan), of which 7 tolerate up to 5000 ppm of endosulfan were selected, identified and characterized for further study. The 10 different bacteria isolated from activated sludge samples which were able to grow in 300mg/l of endosulfan[12]. The optimum endosulfan concentration at which *Bordetella* sp. B9 showed the maximum growth and degradation was 100µg ml^{-1} [16]. However in the present study, the strains isolated tolerated up to 5000ppm of endosulfan formulation, the highest ever reported. Increased growth (OD value, CFU/ml), pH and chloride ion release were used as criteria for selection of potential strain.

Streptococcus agalactiae a gram negative bacterium, shows maximum growth with 3.8 OD, (5.2×10^8 CFU/ml), pH 3.5 and 80% free chloride ions release were observed at the end of 15th day incubation and selected for degradation study (Table 1). The endosulfan degradation confirmed by a *Rhodococcus* strain with increase in chloride ion (87.1%) in the growth medium, with nearly complete degradation of the insecticide[17]. The results correspond to the report, suggesting that fungal and bacterial strains significantly decrease the pH of culture media after 15 days of incubation from 7.2 to 3.2[4]. Change in pH may favour chemical transformation of endosulfan to endosulfan diols[18]. The result also confirmed the findings of previous studies[10, 11]. Initial optimization for these factors is essential before undertaking any bioremediation activity[19]. The culture pH, cells growth and residual endosulfan demonstrated that CS5 could degrade more than 24.8 mg/l α endosulfan and 10.5 mg/l β endosulfan after 8 days in aqueous medium with the formation of endosulfan diol and endosulfan ether as the major metabolites[12].

Table 1
Screening of Endosulfan degrading potential bacteria

Bacterial strains	Growth variation		pH
	OD (600nm)	CFU/ml)	
Control blank	0.02±0.0070	-	6.0
S. agalactiae	3.8	5.2×10^8	3.5
Staphylococcus sp.	3.4	4.8×10^8	5.0
Vibrio sp.	2.7	1.9×10^8	5.5
P. aeruginosa	3.3	3.2×10^8	4.0
Bacillus sp.	3.0	1.8×10^8	4.0
A. hydrophila	2.6	2.6×10^8	6.5
P. vulgaris	2.8	2.2×10^8	4.5

The bacterium showed maximum growth with optimized parameters such as pH 8, temperature 35˚C, dextrose as supplementary carbon source, inoculum size of 100µl/ml, 30 ppt salinity under shaking condition for 15 days of incubation. The addition of auxiliary carbon to the system having xenobiotic compounds increased the biodegradation potential of bacterial and fungal cultures [20] . At the end of 15th day incubation, endosulfan degradation efficiency of 40.77 % was observed. About 73% of endosulfan degradation was reported using single bacterium *JCE-4* and mixed bacterial cultures *GCE345* in 30 days treatment[21]. The optical density measurements and GC analysis confirmed substantial removal of endosulfan with simultaneous increase in bacterial mass. 50% *Artemia salina* mortality was observed in 2ppm of endosulfan with three repeated toxicological analysis after 24 hrs of incubation. Reduced death observed with bacterial broth treatment as well as cell free supernatant (Fig 1). The acute toxicity (LD 50, 24h) of endosulfan for carp was 0.075ppm previously reported [22].

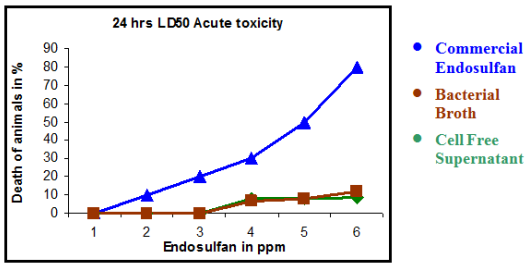

Figure 1
Acute toxicity with *Artemia salina*

Mortality of the test organisms is a sensitive end point for the assessment of the ecotoxicological risk by the environmental pollutants [23]. The 96-h LC_{50} estimates ranged from 2.1 to 3.5µg/l endosulfan sulfate tested with inland fish species reported[24].

CONCLUSION

Within two weeks around 40.77 % of endosulfan was degraded and thus the values are comparable to the other reports in literature. Interestingly the strain used in the present study tolerated up to 5000ppm of endosulfan formulation and as per the literature this seemed to be highest tolerance ever reported. Thus the present study revealed the abundance of pesticide degrading microbes in estuarine environment and their potential in degrading various pesticides in agricultural use. The study proved the bioremediation potential of microbes in pesticide contaminated sites.

ACKNOWLEDGMENTS

The author is grateful to Prof. T. Balasubramanian, Director and Dean, CASMB, Faculty of Marine Sciences, AU, TN, India, for his support throughout the study. This work is part of the M. Phil., thesis of V. Neelambari at CASMB, AU. The author is thankful to the financial support funded by UGC-RJNF fellowship.

REFERENCES

1. Sunderam RIM, Cheng DMH, Thompson GB Toxicity of Endosulfan to native and introduced fish in Australia. Environ Toxicol Chem, 11: 1469-1476, (1992)
2. Sinha N, Narayan R, Saxena DK Effect of Endosulfan on testis of growing rats. Bull Environ Contam Toxicol, 58:79-86, (1997)

3. Chaudhuri K, Selvaraj S, Pal AK Studies on the genotoxicology of endosulfan in bacterial system. Mutat Res Genet Toxicol Environ Mut, 439: 63 – 67, (1999)
4. Siddique T, Okeke BC, Arshad M, Frankenberger WTJr Biodegradation kinetics of endosulfan by *Fusarium ventricosum* and a *Pandoraea* species. J Agri Food Chem, 51: 8015-8019, (2003)
5. Saiyed H, Dewan A, Bhatnaga V, Shenoy U, Shenoy R, Rajmohan H Effect of endosulfan on male reproductive development. Environ Health Pers, 111(16): 1958-1962, (2003)
6. Arnold SF, Klotz DM, Collins BM, Vonier PM, Guillettee LJJ, McLachlan JA Synergistic activation of estrogen receptors with combination of environmental chemicals. Sci, 272:1489-1492, (1996)
7. Herman DC, Frankenberger WT Jr Bacterial reduction of perchlorate and nitrate in water. J Environ Qual, 28: 1018-1024, (1999)
8. Focht DD Microbiological procedures for biodegradation research.p.407-426. *In* R. W. Weaver *et al*. (ed.) Methods of soil analysis. Part 2. SSSA Book Ser. 5. SSSA, Madison, WI, (1994)
9. Kwon GS, Sohn HY, Shin KS, Kim E, Seo BI Biodegradation of the organochlorine insecticide, endosulfan, and the toxic metabolite, endosulfan sulfate, by *Klebsiella oxytoca* KE-8. Appl Microbiol Biotechnol, 67: 845-850, (2005)
10. Kwon GS, Kim JE, Kim TK, Sohn HY, Koh SC, Shin KS, Kim DG *Klebsiella pneumoniae* KE-1 degrades endosulfan without formation of the toxic metabolite, endosulfan sulfate. FEMS Microbiol Lett, 215: 255-259, (2002)
11. Awasthi N, Singh AK, Jain RK, Khangarot BS, Kumar A Degradation and detoxification of endosulfan isomers by a defined co-culture of two Bacillus strains. Appl Microbiol Biotechnol, 62 (2-3): 279-83, (2003)
12. Li W, Dai Y, Xue B, Li Y, Peng X, Zhang J, Yan Y Biodegradation and detoxification of endosulfan in aqueous medium and soil by *Achromobacter xylosoxidans* strain CS5.Jour Hazar Mat, 167(1-3): 209- 216, (2009)
13. Kraemer EO, Stamm AJ Mohr's method for the determination of silver and halogens in other than neutral solutions. J Am Chem Soc, 46(12): 2707-2709, (1924)
14. Kumar M, Philip L Endosulfan mineralization by bacterial isolates and possible degradation pathway identification. Biorem J, 10(4): 179-190, (2006b)
15. Dvorak P, Sucman E, Cervenakova D, Korinek K, Blechova R Utilization of bioassay with *Artemia salina* in pharmacotoxicology. *In* : Collection of abstracts from 9th conference on toxicity and biodegradability of wastes and chemicals significant in aquatic environment. Solan, Czech Republic, 13. -15. 9. 1999. *(In Czech)*, (1999)
16. Goswami S, Singh DK Biodegradation of α and β Endosulfan in broth medium and soil microcosm by *Bordetella* sp.B9. Biodeg, 20: 199-200, (2009)
17. Verma K, Agrawal N, Farooq M, Misra RB, Hans RK Endosulfan degradation by a *Rhodococcus* strain isolated from earthworm gut. Ecotoxi Environ Saf, 64: 377-381, (2006)
18. Lee SE, Kim JS, Kennedy IR, Park JW, Kwon GS, Koh SC, Kim JE Biotransformation of an organochlorine insecticide, endosulfan, by *Anabaena* species. J Agri Food Chem, 51: 1336-1340, (2003)
19. Awasthi N, Manickam N, Kumar A Biodegradation of endosulfan by a bacterial coculture. Bull Environ Contam Toxicol, 59: 928-934, (1997)
20. Kumar M, Philip L Bioremediation of Endosulfan contaminated soil and water-Optimization of operating conditions in laboratory scale reactors. Journal of Hazardous Materials, B136:354- 364, (2006a)
21. Kumar S Bioremediation of endosulfan by bacteria isolated from the agricultural fields. International Journal of Pharma and Biosciences, 2(3): 367-374,(2011)
22. Kim JM, Ghosh SR, Weiland AC, Zirkin BR Caspses- 3 and caspase activated deoxyribosenuclease are associated with testicular germ cell apoptosis in conditions of abnormal spermatogenesis. Endocrinol, 142: 3809-3816, (2001)
23. Cairns JJ, Niederlehner BR (Eds) Ecological toxicity testing. CRC, Boca Raton, Fl., USA , (1994)
24. Carriger JF, Hoang TC, Rand GM, Gardinali PR, Castro J Acute toxicity and effects analysis of endosulfan sulfate to freshwater fish species. Arch Environ Contam Toxicol, 60: 281- 289, (2011).