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STUDIES ON GROWTH KINETICS AND BIODEGRADATION POTENTIAL OF *PSEUDOMONAS* (IES-*PS*-1) USING ENDOSULFAN

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ABSTRACT

Biodegradation is one of the efficient processes for detoxification of many pesticides and their metabolites from the environment. The present study evaluates the biodegradation of endosulfan by *Pseudomonas* (IES-*Ps*-I) through growth kinetic studies for 29 h and its further monitoring through gas chromatography technique. After initial inoculation the acclimatization phase of IES-*Ps*-1 continued up to 3 h and significantly increased the optical density ($O.D_{550}$) of broth after 4 h of incubation. This increase in $O.D_{550}$ continued after 24 h, indicating that the culture was in log phase. IES-*Ps*-1 showed 50% reduction in peak amount of pesticide when grown in broth supplemented with 10mg/L and 25mg/L. Endosulfan disappearance was concomitant when this adapted culture was grown at high concentration of pesticide, i.e. 50mg/L and 100mg/L and gave more than 80% reduction at 29 h.

Keywords: Biodegradation; endosulfan; *Pseudomonas*; growth kinetics; gas chromatography

INTRODUCTION

Endosulfan, an organochlorine pesticide has been ubiquitously detected in the Environment (Sethunathan *et al*., 2002) and is one of the more toxic pesticides responsible for many fatal pesticide poisoning incidents around the world (Pesticide Action Network North America, 2006). However, due to its toxicity to humans, it has been either restricted for use or banned in many countries. Despite this it is reported as the most used pesticide products in Sindh province of Pakistan (Soomro *et al*., 2003a).

Tremendous research efforts are still required to realize the fate and effect of endosulfan residues in the agricultural environment. Early investigators were primarily concerned as to which degradation processes were important in establishing the stability of these molecules in the environment. It was realized that biological degradation is one of the important, effective and efficient processes for detoxification of many pesticides and their metabolites. Pesticides that resist degradation because of their complex structure, concentration or ambient conditions, can be degraded by the use of microbes that have been adapted in situ.

The process of bioremediation involves a breakdown of organic pollutant through microorganism to utilize it as a nutrient and energy source for their growth and metabolic processes (Tariq *et al*., 2003). Awasthi *et al*. (1997) and Sutherland *et al.* (2000) isolated microorganisms using endosulfan as a sole carbon and sulfur source, respectively for their growth and metabolic processes. Bacterial and fungal cultures transformed endosulfan to its metabolites endosulfan sulfate and endosulfan diol through oxidation with endosulfan hydroxyether and endosulfan lactone in small amount (Martens, 1977).

The products of pesticide transformations can be either more toxic with respect to microorganisms than the initial chemicals or can function as a growth substrate for one or several microorganisms (Golovleva and Golovlev, 1980). The selection of microorganisms for bioremediation entails an understanding of all the biological and biochemical aspects involved in chemical transformation so that bioremediation strategies could be developed to remediate the pollutants in the environment.

Thus the present study evaluates the biological degradation of endosulfan by IES-*Ps*-I through growth kinetic studies using shaking water bath and further monitoring the fate of endosulfan using gas chromatography technique.

MATERIAL AND METHODS

Preparation of Pesticide

The pesticide used in the study belongs to the class organochlorine. It is commercially available as Thiodan (endosulfan). Analytical grade pesticide (95-100% pure) was used as a standard for the confirmation, biodegradation and quantification of commercial grade pesticide.

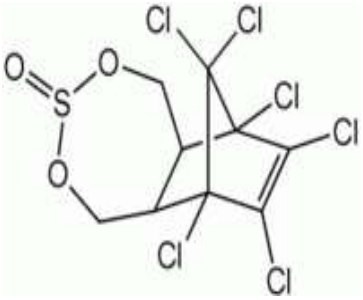

Fig. 1. ENDOSULFAN (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepine-3-oxide)

A stock solution of endosulfan (1%) was prepared in GC grade acetone. For growth kinetics and degradation studies, different concentrations of pesticide (10, 25, 50 and 100 mg/L) were used in the medium.

Identification and Characterization of IES-*Ps*-1

The identification and characterization of the IES-*Ps*-1 were accomplished using morphological, cultural and biochemical tests up to the level of the genus.

Adaptation of IES-*Ps*-1 for Endosulfan Degradation

IES-*Ps*-1 was inoculated into 100 ml nutrient broth supplemented with endosulfan (1mg/L) and incubated for 48 h at 35°C. The resulting dense culture was streaked on to nutrient agar slant and incubated for 24 h at 35°C. These tubes were stored at 4°C and adapted culture used for biodegradation studies.

Growth Kinetics Studies in Shaking Water Bath

In growth kinetic studies 2.5ml (1.8×10^9 Mcfarland's Index) of 24 h grown culture was inoculated into 250ml of nutrient broth flask and the flask containing nutrient broth supplemented with 10mg/L and 25mg/L of endosulfan. These flasks were kept in a shaking water bath at 130rpm for 29 h at ambient temperature. The optical density of nutrient broth and broth containing endosulfan was determined by spectrophotometer at 550nm. This adapted culture will be further used for the growth kinetic study at high concentration of pesticide (50 and 100 mg/L).

Biodegradation of Endosulfan by IES-*Ps*-I

The ability of IES-*Ps*-I culture for endosulfan degradation was determined in a shaking water bath under sterile conditions. The 25ml samples were collected from the flasks (on shaking water bath) at 0, 7 and 29 h respectively and processed for the extraction of endosulfan. The pH of the medium was also observed for abiotic degradation of endosulfan. The gas chromatography (GC) technique was used for determining the degradation of endosulfan.

Extraction of Metabolites for Gas Chromatography

Endosulfan in the collected samples was extracted by adding an equal volume of acetone (GC grade) and shaken for 3 h with shaking water bath at 130 rpm. The extract (1 ml) was then transferred to acetone (9 ml) and again shaken for 30 min with the same speed. The sample then dehydrated by passing it through anhydrous sodium sulphide Na_2SO_4 and concentrated with a rotary evaporator. The extracted samples were submitted to HEJ Research Institute of Chemistry, University of Karachi for gas chromatography (GC). FID detector was used for GC analysis set at 280^{o}C with INJ. Port temperature was 260^{o}C. The 30cm long column with 0.25mm diameter was used with column oven temperature of 150^{o}C.

RESULT AND DISCUSSION

Identification and Characterization of Bacterial Culture

On the basis of findings of morphological, cultural and biochemical characteristics, the IES-*Ps*-1 strain was identified as the member of the genus *Pseudomonas* according to "Bergey's Manual of Determinative Bacteriology**" (1994)**.

Pseudomonads are an enormous group of heterogeneous bacteria that are widely distributed in the soil where they are involved in the transformation and mineralization of organic matter. They have been reported to degrade aromatic hydrocarbons, oil, petroleum products and pesticides (Choudhary *et al.,* 1988). They are also known to

degrade phenolic compounds (Huges and Cooper, 1996) and therefore they can serve as the bacteria of choice for biodegradation of hazardous waste and pollution control (Hashmi, 2001).

Growth Kinetics Study of IES-*Ps*-1 (Pseudomonas)

Sterile Nutrient Broth

From Table 1 and Fig.2 it is seen that the phase of acclimatization of IES-Ps-1 continued up to almost 3 h and significantly increased in optical density ($O.D_{550nm}$) observed after 4 h of incubation period. Mean generation time and specific growth rate at 7 h were calculated as 53 min and 0.0189 respectively. The exponential increase in the O.D demonstrating that culture remained in the Lag phase for 4 h and then entered into the phase of positive acceleration (log phase). This increased in $O.D_{550}$ of broth continued after 24 h of incubation period indicating that the culture was in log phase.

Table 1. Growth Kinetics of IES-Ps-1 in sterile Nutrient Broth.

Time (h)	Experiment No. (O.D at 550nm)			Mean $O.D_{550}$
	I	II	III	
0	0.007	0.006	0.007	0.007
1	0.007	0.007	0.008	0.007
2	0.008	0.009	0.008	0.008
3	0.011	0.013	0.012	0.012
4	0.027	0.023	0.025	0.022
5	0.046	0.048	0.045	0.046
6	0.100	0.104	0.102	0.102
7	0.299	0.355	0.276	0.310
24	1.513	1.355	1.403	1.423
25	2.515	2.190	2.373	2.359

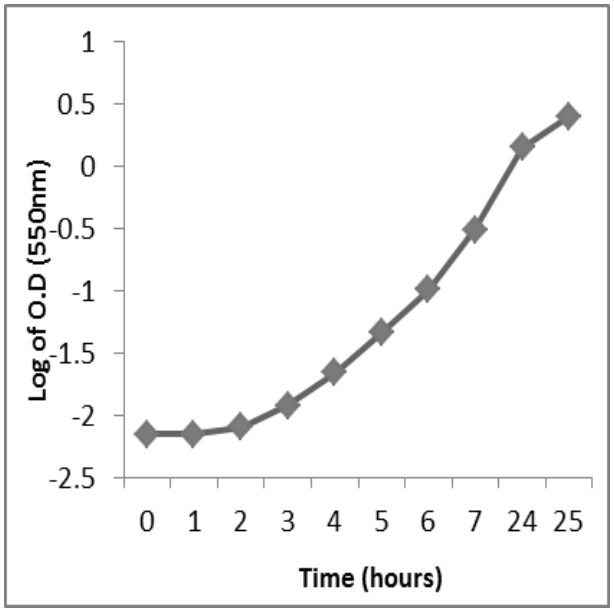

Fig. 2. Growth kinetics of IES-Ps-1 in sterile nutrient broth.

Nutrient Broth supplemented with Endosulfan.

From Fig.3 and Table 2 it is seen that IES-*Ps*-1 showed similar growth pattern in broth supplemented with different concentrations of endosulfan as compared to their respective control. After 24 h of incubation the culture was in log phase.

Table 2. Growth Kinetics of IES-Ps-1 in Nutrient Broth containing Endosulfan.

Item	Endosulfan Conc. (mg/L)	Mean Generation Type	Mean Specific Growth Rate
Control	Nil	52	0.0192
Test 1	10	52	0.0192
Test 2	25	50	0.0200
Test 3	50	52	0.0192
Test 4	100	55	0.0181

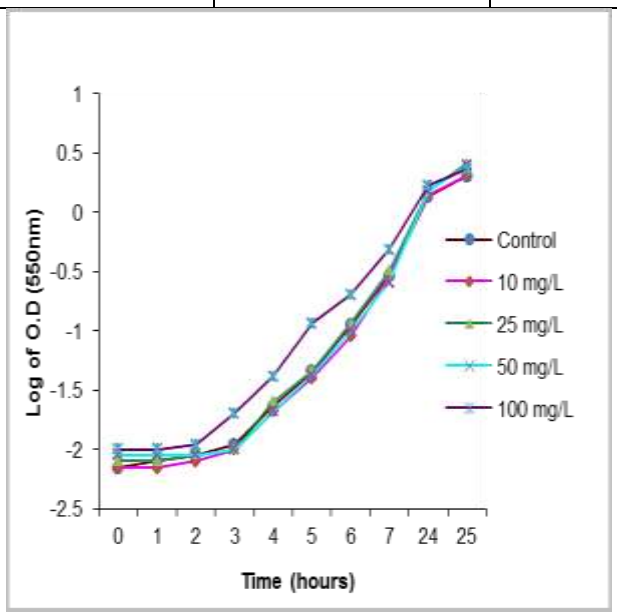

Fig. 3. Growth kinetics of IES-Ps-1 in nutrient broth supplemented with endosulfan.

Broth supplemented with 100mg/L of endosulfan gave high O.D value (Fig.2) as compared to its control. At this high concentration of endosulfan the broth becomes milky or whitish which results in high absorbance values. Hence, IES-*Ps*-1 was found to be metabolically active in the absence or presence of endosulfan and had the potential to degrade endosulfan.

Biodegradation of Endosulfan by IES-*Ps*-1 *(Pseudomonas*)

Results of endosulfan biodegradation by IES-*Ps*-1 are shown in Table 3 and Fig. 4. The bacteria belonging to *Pseudomonas* species that have reported as excellent degraders of a various xenobiotics and recalcitrant compounds (Radehaus and Schmidt, 1992), and especially in endosulfan contaminated soil, *Pseudomonas* sp. showed the maximum utilization of endosulfan (Kumar *et al.,* 2007). However, IES-*Ps*-1 was selected on the basis of its previous findings in which it showed a great potential to degrade malathion (Hashmi, 2001), cypermethrin (Seema, 2004) and also showed its promising character towards phenol degradation. This biodegradation study also revealed the effectiveness of IES-*Ps*-1 as a potential biocatalyst for bioremediation of endosulfan.

Microbial degradation was observed for 29 h in nutrient broth containing a rich culture of IES-*Ps*-1 at ambient temperature on shaking water bath. Maximum degradation of endosulfan by three bacterial strains *(P. spinosa, P. aeruginosa* and *Burkholderia cepacia)* was observed at an incubation temperature of 30°C and up to 95% with shaking condition (Sarfaraz *et al.*, 2007).

Biodegradation of endosulfan was determined by monitoring endosulfan disappearance by gas chromatography with Flame Ionization Detector (FID**).** In all chromatogram peak of endosulfan was not fully resolved. Therefore, exact quantification for the decrease in concentration was not done, while reduction in peak amount with respect to time was observed in all concentrations. Due to this reason, percentage reduction of peak amount was calculated which indicated the percentage of biodegradation of endosulfan by IES-*Ps*-1.

In broth supplemented with 10 and 25 mg/L of endosulfan, IES-*Ps*-1 showed 34.5% and 14.4% reduction in peak amount at 7 h respectively. Degradation of endosulfan increased with an increased biomass at 29 h i.e. 51%

and 55.2% respectively. This grown culture was further used for degradation study at high concentration of pesticide. Endosulfan disappearance was concomitant at 50 and 100mg/L and IES-*Ps*-1 gave a high percent reduction value of endosulfan at 7 h 55.9% and 80.4% and at 29 h 71% and 83% respectively.

This increase in percentage reduction of endosulfan with its increased concentration showed that IES-*Ps*-1 had adapted itself to the pesticide environment and was also indicative of its promising role in the rapid utilization of energy source either Carbon or Sulfur. This is most likely accredited to the fact that microbial cells require carbon in the highest amount followed by Nitrogen, Phosphorus and Sulfur for their growth and metabolic processes (Alexander, 1998). As comprises only six potential reducing electrons, endosulfan is a poor biological energy source and previous efforts to enrich microorganisms using the endosulfan as a carbon source have been unsuccessful **(**Guerin**,** 1999). IES-*Ps*-1 had been grown in nutrient broth for providing constant carbon energy source. Disappearance of endosulfan might be a result of the release of sulfite group from endosulfan by bacteria to utilize this as a source of sulfur for growth. Microorganisms degrade endosulfan at the sulfite group through both oxidation and hydrolysis to form the endosulfate and endodiol, respectively (Sutherland *et al*., 2000).

Table 3. Degradation of endosulfan in nutrient broth by IES-Ps-1 (Pseudomonas).

Endosulfan (mg/L)	Time (h)	GC data*		Percentage Reduction in Peak Area
		Retention time (min)	Peak area	
10	0	20.074	385439	-
	7	20.271	252586	34.5
	29	20.070	186687	51.6
25	0	20.289	688688	-
	7	20.392	589260	14.4
	29	20.246	308095	55.3
50	0	20.340	1827718	-
	7	19.981	805233	55.9
	29	20.224	516737	71.7
100	0	20.671	4518810	-
	7	20.466	885638	80.4
	29	20.086	528198	88.3

*****GC data are taken from the chromatograms

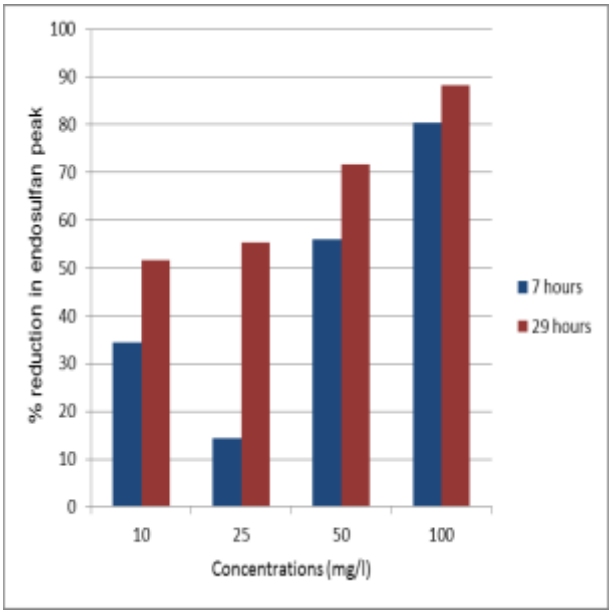

Fig. 4 Percentage reduction of endosulfan peak amount.

Alkaline hydrolysis can result in the degradation of endosulfan (Martens, 1976) with each increase in pH unit increases up to ten folds in the hydrolysis of endosulfan. Abiotic degradation resulted up to 21% of the spike amount of endosulfan (Sarfaraz *et al*, 2007).

As microbial growth also increases an alkalinity of the medium therefore it would be difficult to distinguish between chemical and biological hydrolysis of endosulfan. For this reason pH of the medium was also checked at 0, 7 and 29 h, respectively.

From Fig.5 it is determined that the pH of the broth decreases with prolific increased in the growth of IES-*Ps*-1 and progressive degradation of endosulfan. The highest decrease in pH from 7.51 – 5.01 was recorded in neutral broth supplemented with 100mg/L of endosulfan which has shown an 83% reduction in peak amount. This decrease in pH of the broth is the result of formation of acidic substances due to dehalogenation of endosulfan. This supports the previous findings that the formation of HCl or organic acids by micro-organisms resulted in a substantial reduction in pH (Sutherland *et al.*, 2002; Awasthi *et al.*, 2003; Siddique *et al*., 2003 a). However the findings of (Miles and Moy, 1979 and Martens, 1976) reported an increased in the pH of the growth media which are in contradiction to these results.

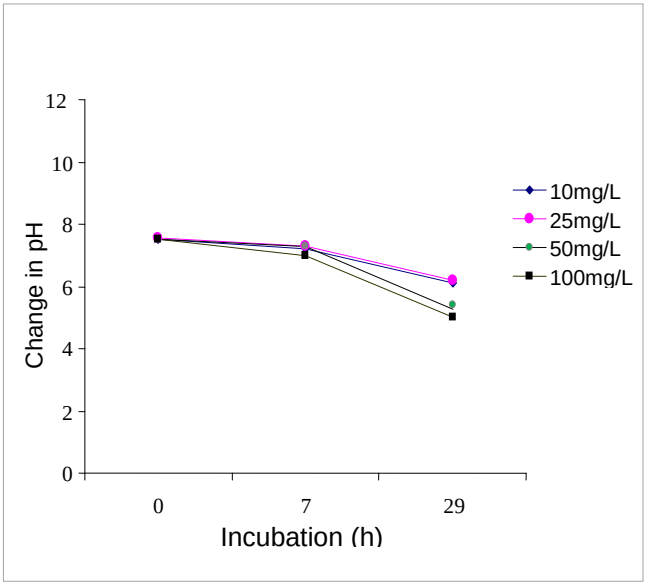

Fig.5 Change in pH of nutrient broth containing different concentrations of endosulfan.

Moreover, the release of metabolites of endosulfan from culture media or soils occurs readily by volatilization and adsorption to surfaces (Guerin and Kennedy, 1992) therefore their detection is important for the confirmation of degradation of endosulfan. The actual enzymatic metabolic degradation of endosulfan by IES-*Ps*-1 has not yet been known, but experimental evidence regarding endosulfan hydrolysis in some bacteria (*Pseudomonas aeruginosa, Berckholderia cepaeia*) results a less toxic Endosulfan diol (Kumar *et al*, 2006). This metabolite can be further converted to Endosulfan ether (Hussain *et al.*, 2007) and Endosulfan Lactone (Lee *et al*., 2003). Walse *et al*., 2003 reported that endosulfan lactone upon hydrolysis yields Endosulfan hydroxycarboxylate.

The mentioned literature suggests the hydrolytic degradation pathway. Also, existing is the oxidative pathway of endosulfan which results in toxic product endosulfan sulfate. Most of the *Pseudomonas* spp. degrade endosulfan into endosulfan diol and endosulfan sulfate (Jayashree *et al.*, 2007) describing the fact that they followed the metabolic pathway for endosulfan degradation (Kumar *et al.*, 2007). All these degradation pathways result in desulphurization of endosulfan, the removal of sulfur dramatically decreases the vertebrate toxicity of endosulfan, which results in concurrent detoxification of the insecticide. However, many aromatic organohalogens are resistance to bioremediation due to the presence of intact chlorine.

Conclusions

The present study of the growth kinetics and biodegradation potential of *Pseudomonas* IES-*Ps*-1 has been summed up with the conclusion that IES-*Ps*-1 has the potential to degrade endosulfan. Further a low cost bioremediation strategy could be framed with the findings for decontamination and detoxification of endosulfan containing soil and effluents.

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