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Bioremediation of Dimethoate by Effective Microorganisms in Water

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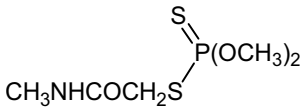
ABSTRACT

A unique approach for the degradation of organophosphorus pesticide dimethoate in aqueous media with effective, microorganisms (EM) was investigated. These microbial consortia could tolerate up to about 120 mg L^{-1} of dimethoate (technical grade). Several factors influencing dimethoate degradation were investigated. The growth rate μ (h^{-1}) of these effective microorganisms was *ca*. 0.925. The acetonitrile extracts of EM cultures were subjected to gas liquid chromatography (GLC) using two different solvent systems: hexane–chloroform–methanol and cyclohexane–acetone–chloroform. GLC analyses revealed the complete degradation and disappearance of dimethoate after 3 days. However, the precise conditions for this pesticide degradation were not fully understood. A change in pH of the culture medium to an acidic range supported the biological transformation of the compound. Optimal growth conditions were pH 8.5 and 27°C. Two isolates from these microbial consortia lost their ability to utilize dimethoate. The intermediate compounds were also metabolized, further resulting in complete mineralization of dimethoate. Thus, the present study establishes the EM degradation of dimethoate and also suggests their role in the bioremediation of other pesticide-contaminated water.

Keywords: degradation, dimethoate, organophosphorous, pesticides

INTRODUCTION

Pesticides represent the major pollutants of the aquatic environment and their presence is of great concern because of their potential toxicity towards vertebrates (Mishra *et al*. 2006). Organophosphorus pesticides (OPs) have been used intensively throughout the world since the first introduction of a synthetic insecticide, parathion in plant protection in 1944 (Saunders 1957). Dimethoate is an organophosphorus insecticide with a contact and systemic action.



O,O-dimethyl *S*-methylcarbamoylmethyl phosphorodithio-ate;
2-dimethoxyphosphinothioylthio-*N*-methylacetamide

It was introduced in 1956 and is produced in many countries for use against a broad range of insects in agriculture. Despite its high toxicity especially fish and other aquatic invertebrates, dimethoate is still extensively used all over the world for its broad spectrum of action (DebMandal *et al*. 2002). The prevalence of such materials in the environment has increased interest in the study of microbes involved in their biodegradation (Zhuang *et al*. 2003). Dimethoate is considered one of the most important pesticides in killing a wide range of insects, including aphids, thrips, planthoppers and whiteflies systemically and on contact (Hayes *et al*. 1990). This compound acts as cholinesterase inhibitor by interfering with the activities of this enzyme responsible for neurotransmission. Dimethoate released to the environment does not adsorb onto the soil and is subject to considerable leaching (Sayim and Kaya 2006). It is also lost from the soil through evaporation and biodegradation (El Beit *et al*. 1977). The half-life of dimethoate in soil ranges from 4 to 16 days (Hayes *et al*. 1990). Therefore, it is neither expected to adsorb to sediments or suspended particles, nor to bioaccumulate in aquatic organisms. In general in the environment, pesticides are exposed to various degradative forces. Biotic degradation or metabolic processes play a vital role in this respect. They contribute not only to the disappearance of the original pesticides, but also change their physicochemical properties, and thus affect their transport and distribution behavior among various compartments in the environments. Moreover, dimethoate undergoes rapid biodegradation in the environment and in sewage treatment plants (Cheminova 1991). It is subjected to significant hydrolysis, especially in alkaline water (Zhuang 2003). Being a carbamate group of organophosphate, dimethoate is little less amenable to degradation as compared to other well studied organophosphates (Zhuang 2003). The pH, temperature and the type of medium are important factors affecting the stability of dimethoate in aqueous media. The degradation of dimethoate depends mainly on the alkylation of the medium rather than the time of storage. Different pathways of OPs decomposition such as hydrolysis, photolytic oxidation, microbial transformations and other biological processes have been reported (Zhuang 2003). The earlier metabolic studies on pesticides helped to develop a new approach for the detoxification of pesticides using cell-free enzymes from adapted microorganisms to resolve problems related to whole-cell metabolism of pesticides (Liu *et al*. 2001). The first report on bacterial utilization of dimethoate was reported by Liu *et al*. (2001). They isolated the strain of *Pseudomonas stutzeri* from water that was obtained in fields following frequent application of OPs. 71.82% degradation was reported at 35°C with shaking for 72 hr. Thus, microbial degradation by fungi and or bacteria is the means of disappearance of dimethoate from water as it is used as a source of 'C' and 'energy' or as a source of 'P'. Interest in the concept of effective microorganisms (EM) allowed this EM technology, developed by Teruo Higa in the 1970's at the University of the Ryukyus, Okinawa, Japan, to be intro-

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duced. This technology includes four principle types of organisms commonly found in all ecosystems, namely acid bacteria, yeast, lactic actinomyces, and photosynthetic bacteria and other microoganisms as yeast fungi and algae. This is the first manuscript that deals with EM technology through which it may be possible to exploit its merits to remediate OPs from water. Moreover, this study evaluates the efficiency of EM on the degradation of dimethoate in aqueous media in laboratorial conditions.

MATERIALS AND METHODS

The potential degradation of dimethoate by EM aqueous media was studied in laboratorial conditions using different concentrations of the dimethoate by EM containing media. Meanwhile the biodegradative capability of these microbial consortia was studied and the results were analyzed using Gas Liquid Chromatography GLC.

Chemicals and reagents

The organophosphate insecticide dimethoate [dimethyl *S*-(*N*-methylcarbamoylmethyl) phosphorothiolothionate], Defend® (0.30%) used in this study was produced by Kafr El-Zyat Pesticides and Chemicals Co. (kz), Egypt. Samples were prepared in deionised water using ethylacetate and all other reagents were of high purity and analytical grade.

Source and enrichment of EM

EM were obtained from the Ministry of Environmental Affairs, Alexandria, Egypt. Enrichment and propagation were carried out in sterilized 250 ml Erlenmeyer flasks using mineral salt medium (MSM) (Abdel-Megeed 2004) and 5 ml/L EM liquid concentrate. Then, the culture was prepared in sterilized 20 ml flasks containing 19 ml MSM, 0.1 ml EM supplemented with serial concentrations of dimethoate: 0, 20, 40, 60, 70, 80, 90, 100, 120, and 140 mgL^{-1}. These were incubated at 25 ± 4°C on a rotary shaker at 100 rpm. The pH value of the culture solution was adjusted to 7.0 with 1 N NaOH.

Determination of the growth rate (μ) h^{-1}

The growth rate was estimated every 6 hrs. The growth rate (μ) h^{-1} was calculated according to the growth curve described by Herbert and Brlli (1997). Microbial consortia growth was determined by a spectrophotometer (Uvicon 860, Kontron, Switzerland) at 550 nm.

Determination of pH and temperature optima

Different flasks containing media were adjusted to different pHs ranging from 4 to 9. The flasks were incubated on a rotary water bath shaker at room temperature and 200 rpm. In 6-hr time intervals, a 10 μl sample was taken to determine consortia growth. Depending on the optimal pH; the temperature values were adjusted to 10, 20 or 30°C with previously mentioned procedures and conditions.

Analysis and determination of dimethoate residues by gas liquid chromatography (GLC)

Dimethoate, Defend® (0.30%) and hexane as an internal standard were used in this study. Extraction and GLC analysis of dimethoate were adapted from the Parrilla and Martinez (1997). GLC conditions were: initial temperature, 80°C; temperature increased initially by 15 min^{-1} up to 280°C; injector port temperature, 250°C; detector temperature, 250°C. Retention time for dimethoate was 20.5 min.

Evidence of dimethoate-degrading enzyme

Enzyme assay was carried out using the crude extract according to the method described by Liu *et al*. (2001). Dimethoate residues were determined by GLC as previously mentioned.

Isolation and characterization of bacterial strains

Isolation and characterization of the degrading bacteria were carried out by a streak method (Kiyohara *et al*. 1982). The isolates were preliminary identified using Gram stain and other preliminary biochemical tests. Further classification and identification was confirmed by "Deutsche Sammlung von Mikroorganismen und Zellkulturen" (DSMZ), Brauenschweig, Germany by polymerase chain reaction (PCR) using 16S rDNA sequencing according to the method described by Song *et al.* (2002).

RESULTS AND DISCUSSION

Aerobic growth of EM and dimethoate degradation

EM were well enriched and cultivated on dimethoate-containing media. The microbial consortia grew well by utilizing dimthoate as was evident from the increase in optical density (**Fig. 1**). The simultaneous loss of dimethoate from the culture was observed by GLC analyses (**Fig. 2**). It was observed that the microbial consortia could grow in dimethoate-containing medium as the only carbon source. The medium was converted to a milky emulsion as a result of the released of some of the biosurfactants and extracellular enzymes, thus assisting in degrading this compound. This result is in agreement with the findings of Abdel-Megeed (2004) who proved that some microorganisms such as *Pseudomonas frederiksbergensis* could turn the media to a milky solution (emulsion) so as to facilitate the substrate utilization and assimilation. The gradual decrease in dimethoate after 24, 48 and 72 hrs was monitored by GLC analysis (**Fig. 2**). An increase in the total number of EM after dimethoate application can be explained by assuming that EM can synergistically degrade this insecticide.

The microbial metabolism of dimethoate resulted in the formation of various intermediates before its complete mineralization. The number of peaks was seen to decrease with an increase in the incubation period and all of these peaks completely disappeared after 72 hrs. In fact, the number of peaks refers to the gradual metabolism of dimethoate. This disappearance of these peaks strongly indicates the complete degradation of dimethoate by EM and these results are in an agreement to the findings of DebMandal *et al*. (2008) who proved that the total disappearance of dimethoate occurred in the culture of *Bacillus licheniformis* and *Pseudomonas aeruginosa*s was by secretion of extracellular enzymes and biosurfactants. Unfortunately, attempts to identify the intermediates like dimethyldithiophosphate, and methylamine further to ammonia, could not be detected or identified because the intermediates were rapidly metabolized, further resulting in complete mineralization of dimethoate. The trouble was that the M^+ (main) peaks of the intermediates were quite weak and could thus not be

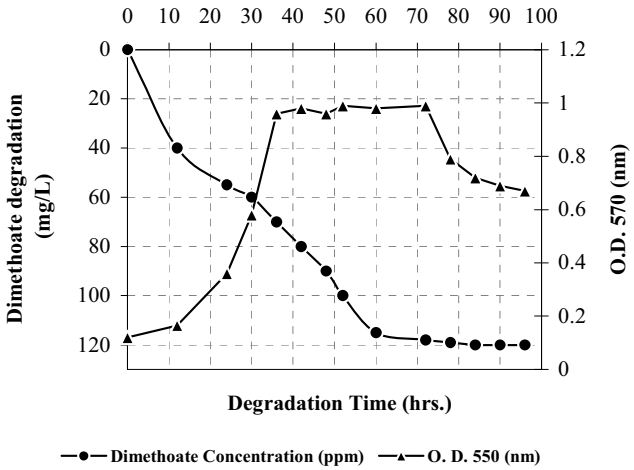

Fig. 1 Growth of the EM in dimethoate as a sole of carbon and energy source.

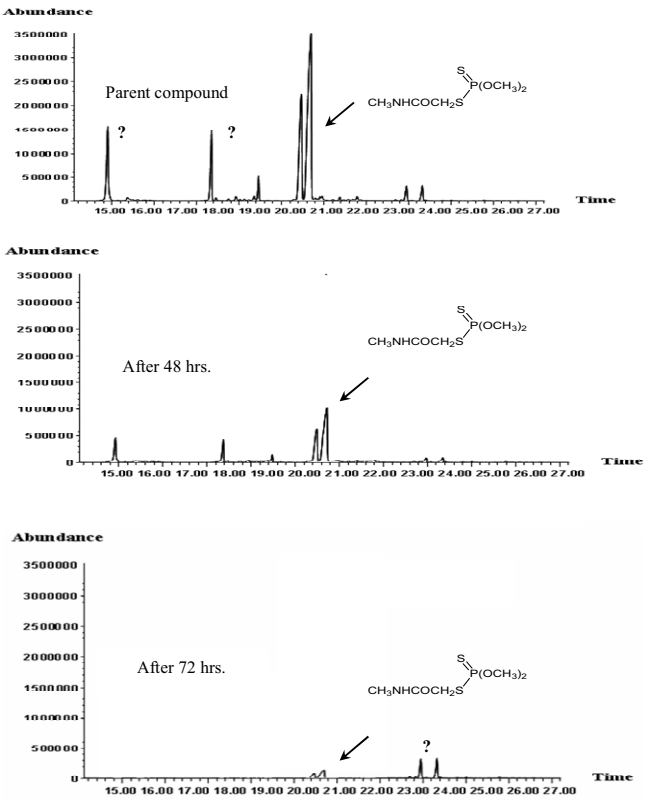

Fig. 2 The gradually degradation of dimethoate by EM analysed by GLC.

detected. In addition, these could be explained by the efficiency of these microbes to degrade this compound. The EM could metabolize the intermediates of dimethoate fast and this is due to the highly biodegradative capability of EM. Only H_2S (detected by the smell of rotten eggs) and CO_2 were detected as mineralization products.

On the other hand, cell-free extracts were used to detect enzymes. It is evident that EM releases exo- and endocellular enzymes together with biosurfactants to help degrade dimethoate. The optimal pH for growth was determined to be 8.0 with an optimal growth temperature of 27°C leading to a high growth rate μ (h^{-1}) estimated at 0.925. The addition this organophosphorus insecticide did not result in significant differences in the growth rate of EM, especially above 120 mgL^{-1}. It was clear from our results that EM exhibited a high tendency and efficiency to assimilate dimethoate. This is partially explained by the bioaccumulation of this compound in water and due to its lipophopic nature. This utilization of the compound is similar to the finding of Deshpande (2002) who observed that organochlorine compounds are degraded by emulsification due to their lipophilic nature.

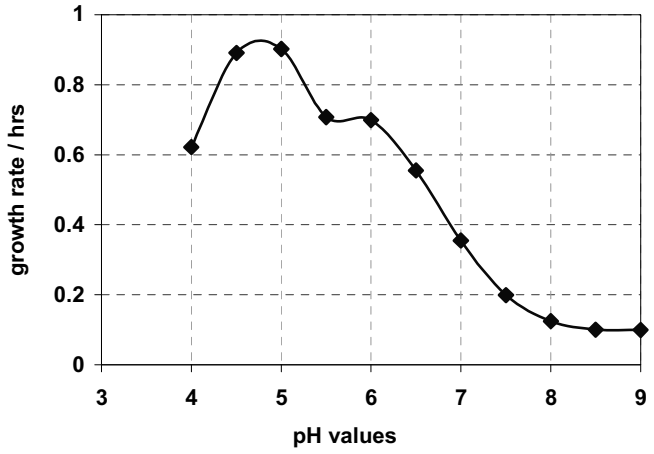

Fig. 3 pH dependence of dimethoate biodegradation by the Effective Microorganisms.

Moreover, OPs accumulation rapidly diminishes when they are removed from the medium, because of their higher bioavailability and metabolization rates (Venturino *et al*. 2001). Therefore in order to find the relationship between metabolic activities and degradation of dimethoate, the pH of culture medium was monitored (**Fig. 3**). The pH of the culture decreased drastically to acidic range due to metabolic activities with simultaneous degradation of dimethoate. These results correspond to those reported by Siddique *et al*. (2003), suggesting that fungal and bacterial strains significantly decreased the pH of culture media after 15 days of incubation from 7.2 to 3.2.

When the EM are grown in optimal temperature the transport of the substrates will be ideal through the membrane, hence the growth rate μ(h^{-1}) increased. It can be observed that growth over the maximum growth temperature, the transport of the substrates is impaired. This fact can be simply explained that near the maximal activity the intracellular and extracellular enzymes are being inactivated (Varanasi *et al*. 1981). In fact dimethoate was tested for substrate specificity of the enzyme, which was determined by measuring the decrease of substrate concentration. The results revealed that dimethoate might have been metabolized efficiently by detoxifying enzymes. Moreover, the presence of other polar metabolites stimulated the activity of other detoxifying enzymes, such as phosphotriesterases. Microorganisms degrading xenobiotic chemicals have elaborate enzyme systems. Biodegradation of organophosphates involve activities of enzymes phosphatase, esterase, hydrolase and oxygenase. Enzymatic hydrolysis of twelve commonly used organophosphorus insecticides was found to be much faster than chemical hydrolysis (Mulbry and Karns 1989). Therefore, it was concluded that the enzyme in crude extract could degrade the P-S linkage of dimethoate which is different from parathion hydrolases, which attack the P-O bond in gram negative bacterial strains and produced the metabolites of the compound (Mulbry and Karns 1989).

In addition, EM technology of degrading pesticide is crucial for enhancing our understanding of the variety of mechanisms and biodegradative pathways relating to their enhanced degradation in the environment. Dimethoate, which was previously thought to be immune to enhanced biodegradation, has now been shown to undergo enhanced biodegradation by EM. Bioremediation technologies are in the process of development for this toxic compound and related nerve agents using organophosphorus hydrolase enzyme.

In fact, the role of soil microorganisms affecting the persistence of agricultural pesticides has been the subject of two areas of study. The first is the capacity for rapid elimination of highly persistent or toxic chemicals. The second is reduced pesticide efficacy attributed to enhanced biodegradation, particularly of chemicals applied under a continuous cropping program. In one case study, a streptomycete bacterium was isolated from a field soil sample previously treated with the insecticide isofenphos and found to be capable of growing on several commercial carbamate and organophosphate insecticides (Gauger *et al*. 1986). It was also found from this study that it was possible to apply the microbial activities and/or their biocatalysts, for the remediation of natural water containing mM concentration of toxic, persistent aromatic pesticides. It is expected that pesticides will be transformed into biodegradable compounds and mineralized into H_2O and CO_2, by using these micro-organisms for an appropriate duration. These recent tools in biotechnology methods can be considered very efficient and much cleaner techniques than chemical ones for improving the quality of water and water resources and eliminating aromatic pesticide traces dissolved or dispersed in water.

Even though EM completely degraded dimthoate, the two bacterial isolates lost the ability to decompose it (**Fig. 4**).

Such loss of the ability to degrade dimethoate by iso-

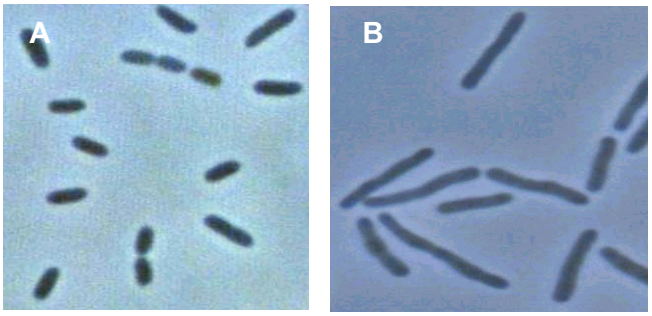

Fig. 4 *Pseudomonas aeruginosa* (**A**) and *Rhodococcus erythropolis* (**B**) isolated from EM culture on complex medium.

lated organisms has been reported by Munnecke and Hsieh (1974). Another study by Abdel-Megeed (2004) stated that mixed culture studies revealed that degradation ability of individual culture was positively affected. Mixed cultures of *Brevundimonas* sp. and *Bacillus* sp. showed 15% more dimethoate degradation than highest degradation by an individual culture (DebMandal *et al*. 2008). It has been observed in many experiments that pesticides have significant effects on microbial activities, but microorganisms recover rapidly. These effects are not drastic, but minor in nature. There is little evidence to suggest that these pesticide treatments have any prolonged deleterious effect on soil microbial activities. In general, it has been observed that pesticide treatments generally have no inhibitory effects on groups of microorganisms (Diurak and Kazanicif 2001).

CONCLUDING REMARKS

As a conclusion, microbial processes in various kinds of aerobic and anaerobic systems for treating industrial, agricultural and municipal wastes are very important because these systems represent the first point of discharge of many chemicals into the environment. The effective and stable degradation capacity of this EM technology in utilizing and degrading this compound reflected their efficacy in biotechnological application for the bioremediation of such aromatic contaminated sites. These results indicate that EM are more stable in retaining their ability to completely degr ade dimethoate than the isolated *Pseudomonas aeruginosa* and *Rhodococcus erythropolis* ones because these effective microorganisms live in symbiotic relationships and their influence on the environment is the sum of all their activities where the metabolites formed by one type of microorganism may be utilized by another. This study suggested that microorganisms endowed with the capacity to degrade toxic pollutants in the environment are a boon to mankind. Future studies on the genes responsible for enhanced biodegradation will enable us to elucidate the exact degradative pathway involved in its microbial biodegradation.

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