BIOREMEDIATION OF DIMETHOATE BY EFFECTIVE MICRO-ORGANISMS IN EGYPTIAN CONTAMINATED WATER

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ABSTRACT

A unique approach of the degradation of organophosphorus pesticide dimethoate in aqueous media with Effective Microorganisms was investigated. These microbial consortia could tolerate to about 120 mg mL^{-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 the Effective Microorganisms 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. Change in pH of culture medium to acidic range supported the biological transformation of the compound. Optimal growth conditions were 8.5 and 27°C for pH and temperature, respectively. Two isolates from these microbial consortia lost their ability to utilize the dimethoate. The intermediate compounds were also metabolized, further resulting in complete mineralization of dimethoate. These Thus, the present study establishes the EM degradation of dimethoate and also suggests their role in the bioremediation of other pesticides contaminated water.

INTRODUCTION

Pesticides constitute major pollutants of the aquatic environment and their presence is of great concern because of their potential toxicity towards animals and humans (Mishra, et al. 2006). The prevalence of such materials into the environment has an increased interest in studying microbes involved in their biodegradation (Zhuang et al. 2003). Organophosphorus pesticides (OPs) have been used in large quantities throughout the world since the first introduction of a synthetic insecticide, parathion, for use in crop protection in 1944 (Saunders, 1957). Problems of contamination resulting from OPs and wastewater from pesticide factories have become obvious. These pesticides are highly toxic to fish and other aquatic invertebrates. In the environment, pesticides are exposed to various degradative forces. Biotic degradation, or metabolic processes, are known to 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. Dimethoate one of this major group that has an insecticidal efficiency for killing a wide range of insects, including aphids, thrips, planthoppers and whiteflies systemically and on contact (Hayes et al. 1990). This compound acts by interfering with the activities of cholinesterase (an essential enzyme for the proper working of the nervous systems of both humans and insects and is possibly carcinogenic). Because dimethoate is highly soluble in water and it adsorbs only very weakly to soil particles. Therefore, it is neither expected to adsorb to

sediments or suspended particles, nor to bioaccumulate in aquatic organisms. Moreover, it undergoes rapid degradation in the environment and in sewage treatment plants (Cheminova, 1991). It is subject to significant hydrolysis, especially in alkaline waters (Zhuang, 2003). Being carbamate group of organo-phosphate, dimethoate is little less amenable to degradation as compared to other well studied organophosphates. The pH, temperature and the type of medium are important factors affecting the stability of dimethoate in water. The degradation of dimethoate depended 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 to 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 a strain of *Ps. stutzeri* from water that was obtained in fields with frequent application of OPs. 71.82% degradation was reported at 35^0C 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 source of 'C' and 'energy' or source of 'P'. The interest in the concept of Effective Microorganisms (EM) began to introduce this EM technology that was developed by Teruo Higa in 1970's at the University of Ryukyus, Okinawa, Japan. This technology includes three principles types of organisms commonly found in all ecosystem, namely (acid bacteria, yeast, lactic actinomyces, and photosynthetic bacteria) and other microoganisms as yeast fungi and algae. Thus, this is the first manuscript that deals with such technology and it may be possible to make the best use of its advantage to remediate Ops from water. Moreover, this study evaluates the efficiency of EM on the degaradation of dimethoate from contaminated water.

MATERIALS AND METHODS

Chemicals and reagents

The organophosphate insecticide dimethoate [dimethyl S- (N-methylcarbamoylmethyl) phosphorothiolothionate] 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 the Effective Microorganisms

Effective Microorganisms (EM) were got from the Ministry of Environmental Affairs, Alexandria, Egypt. The 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 estimated by 0, 20, 40, 60, 70, 80, 90, 100, 120, 140 mg/L. It was incubated at 25±4°C on rotary shaker at 100 rpm. The pH value of the culture solution was adjusted to 7.0 with 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 Herbert and Burill, (1997). Microbial consortia growth of was determined by spectrophotometer (Uvicon 860, Kontron) at 550 nm.

Determination of pH and temperature optima

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

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

The method of extraction and GLC analysis of dimethoate were adapted from the literature (Parrilla and Martinez 1997). GLC conditions were as follows: the initial temperature was 80°C; temperature increased initially by 15 $min^{-}1$ up to 280°C; injector port temperature was 250°C, detector temperature was 250°C. Retention time for dimethoate was 20.5 min.

Evidence of dimethoate-degrading enzyme

Crude extract was obtained and the enzyme assay was carried out according to the method described by Liu et al. (2001). The residues of dimethoate were determined by GLC as previously mentioned.

Isolation and characterization of the bacterial strains

Isolation and characterization of the degrading bacteria were carried out by streak method technique (Kiyohara et al. 1982). Further classification and identification was performed by Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Brauenschweig, Germany, according to the fatty acids analysis and 16s rDNA sequence.

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 an evident from the increase in the optical density (Fig. 1). The simultaneous loss of dimthoate from the culture was observed by GLC analyses (Fig. 2). It was observed that, the microbial consortia grown in dimethoate containing medium as the only carbon source secreted milky solution and it was supposed to be extracellular enzymes that help degrading this compound. The gradually decrease of the dimethoate after 24, 48 and 72 hrs were monitored by GLC analysis (Fig. 2). In fact, the biological degradation of dimethoate may be due to the secretion of extracellular microbial enzymes. The increase in the total number of EM after dimethoate application can be explained by assuming that EM can synergistically metabolize this insecticide.

The microbial metabolism of dimethoate resulted in formation of various intermediates before its complete mineralization. The number of peaks was seen to decrease with increase in incubation time and all the peaks completely disappeared after 72 hrs. These analyses strongly support the complete degradation of dimethoate by EM. These findings are in a full agreement with DebMandal et al. 2008). Unfortunately, attempts to identify the intermediates like dimethyldithiophosphate, and methylamine further to ammonia, were disappointing, but only H_2S and CO_2 were detected as mineralization products.

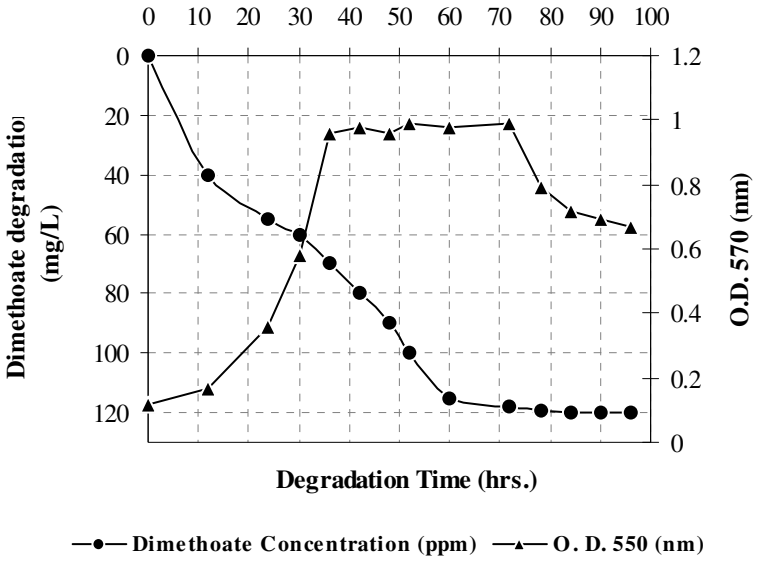


Fig. 1: Growth of the EM in dimethoate as a sole of carbon and energy source.

On the other hand, cells free extract were used for enzymes detection. It was observed that the extracellular enzymes are involved in dimethoate degradation. The optimal pH for the growth was determined at 8.0 with optimal growth temperature of 27°C and these led to high value of the growth rate μ (h^{-1}) estimated by 0.925. It was observed that the addition of this organophosphorus insecticide did not result in significant differences in the growth rate of EM especially after the concentration of 120 ppm. It was clear from the previous results that EM exhibited high tendency and efficiency to assimilate dimethoate. This is partially explained by a bioaccumulation process of those compounds from water, due to their lipophopic nature. However, this process is less significant than that observed for organochlorine compounds (Deshpande, 2002).

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.

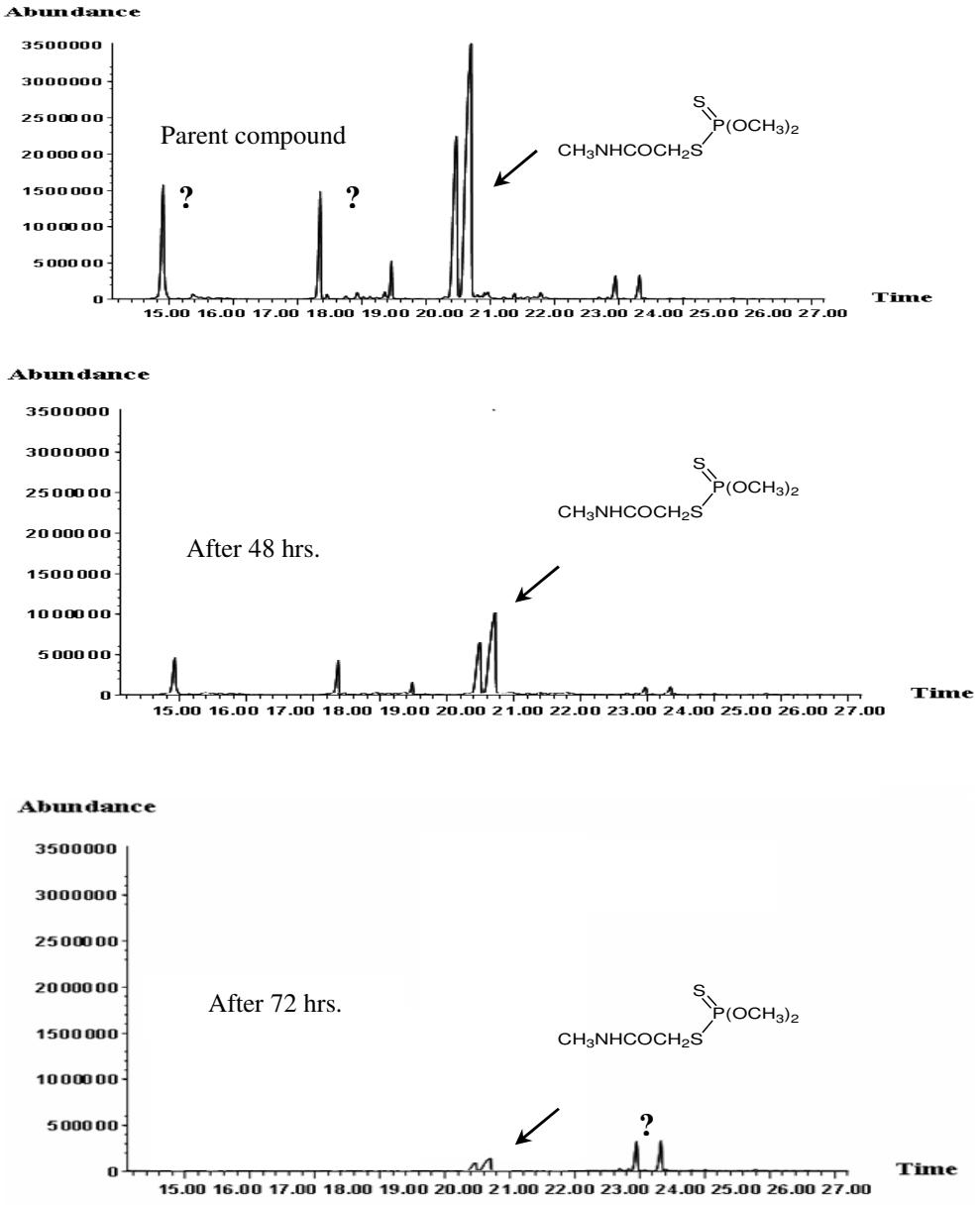


Fig. 2: The gradually degradation of dimethoate by EM analysed by GLC

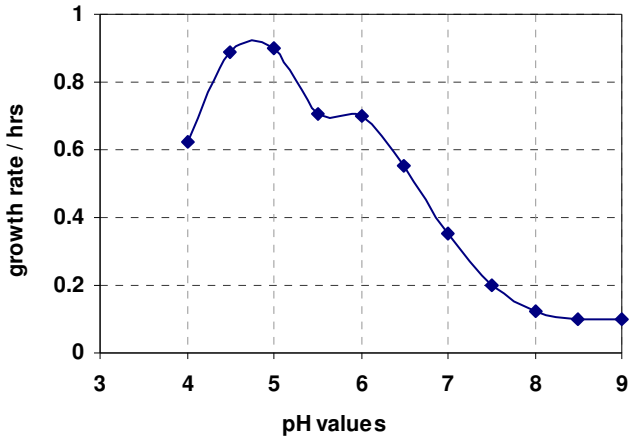


Fig. 3: pH dependence of dimethoate biodegradation by the Effective Microorganisms

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 degradaed dimthoate, the two bacterial isolates lost the ability to decompose the dimthoate (Fig. 4).

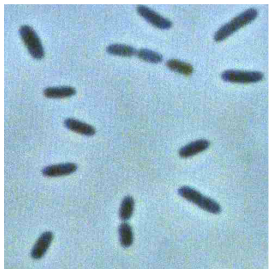
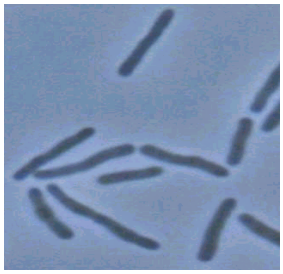
(a)   **(b)**

Fig. 4: *Pseudomonas aeruginosa* (a) and *Rhodococcus erythropolis* (b) isolated from EM culture on complex medium

Such loss of the ability to degrade dimthoate by the isolated 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 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).

As a conclusion, microbial processes in the 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 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 degrade dimethoate than the isolated ones because these effective microorganisms live in symbiotic relationships and their influence on the environment are sum of all activities of these microorganisms. Where the metabolites formed by one type of microorganisms may be utilized by other group of organisms. This study so far suggested that micro-organisms endowed with this property of degradation of toxic pollutants 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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