Türk Biyokimya Dergisi [Turkish Journal of Biochemistry–Turk J Biochem] 2008; 33 (4) ; 209–214.

Research Article [Araştırma Makalesi]

Yayın tarihi 23 Ocak, 2009 © TurkJBiochem.com

[Published online 23 January, 2009]

"Bacterial Biodegradation of Aldicarb and Determination of Bacterium Which Has The Most Biodegradative Effect"

[Aldikarbın Bakteriyel Parçalanması ve Parçalayıcı Etkisi En Fazla Olan Bakterinin Saptanması]

Turan Karayilanoglu[1,2],
Levent Kenar[1,2],
Muhittin Serdar[2],
Songul Kose[1],
Ahmet Aydın[3]

[1] Dept. of Medical CBRN Defense, Gulhane Military Medical Academy, Ankara-TURKEY
[2] Dept. of Clinical Biochemistry, Gulhane Military Medical Academy, Ankara-TURKEY
[3] Dept. of Pharmaceutical Toxicology, Gulhane Military Medical Academy, Ankara-TURKEY

Yazışma Adresi
[Correspondence Address]

Levent Kenar, MD.PhD.

Clinical Biochemist
Dept. of Medical CBRN Defense, Gulhane Military Medical Academy,
06018 Etlik / Ankara – TURKEY
E-mail : lkenar@gata.edu.tr
Telephone number: 00.90.312.3043329
Fax number: 00.90.312.3046019

[Received: 19 August 2008, Accepted: 29 December 2008]

Kayıt tarihi: 19 Ağustos 2008, Kabul tarihi: 29 Aralık 2008

ABSTRACT

Objectives: Aldicarb (2-methyl-2[methylthio] propionaldehyde-o-[methylcarbomyl] oxime), an inhibitor of acetylcholinesterase, is an extremely toxic systemic carbamate pesticide and used commonly in Turkey for crop protection against a wide variety of insects. Its agricultural usage may also cause environmental contamination leading to some harmful effects on human health. In addition, aldicarb has a potential risk that terrorist groups might use delibaretely against public as a chemical weapon due to its commercial availability on the markets and handling without any restriction. Therefore, its removal or detoxification has been increasingly gaining a great importance.

Methods: In this study, we investigated the degradation effect of soil bacteria on aldicarb which contaminates the soil following usage for a long time by using some biochemical methods including chromatograhic and spectrophotometric techniques.

Results: The results of various biochemical methods showed that some of the soil bacteria had the ability to utilize aldicarb through hydrolysis and degradation by bacterial enzyme-esterase. *Stenotrophomonas maltophilia* was also determined as having the most biodegradative effect on aldicarb.

Conclusion: Based on these data, we may expect that some soil bacteria in the form of biotechnological materials can be used in order to decontaminate / detoxify aldicarb or chemical agents similar to such pesticides.

Key Words: Aldicarb, biodegradation, HPLC, soil bacteria.

ÖZET

Amaç: Asetilkolinesteraz inhibitörü olan aldicarb (2-metil-2[metiltiyo] propiyonaldehide-o-[metilkarbomil] oksim), sistemik etkili toksik karbamat grubu pestisittir ve zararlı böcek türlerine karşı toprak uygulaması şeklinde Türkiye'de sıklıkla kullanılmaktadır. Bunun tarımsal alanda kullanımı insan sağlığı üzerindeki zararlı etkilere yol açabilen çevresel kirlenmeye de neden olabilmektedir. Ayrıca, aldikarb, herhangi bir kısıtlama olmadan kolaylıkla elde edilebilmesi ve piyasada ticari olarak satılması nedeniyle terörist gruplar tarafından kimyasal silah olarak kullanılabilme potansiyeline de sahiptir. Bu nedenle, bunun ortadan kaldırılması veya detoksifikasyonu gittikçe artan bir önem kazanmaktadır.

Yöntem: Bu çalışmada, ürünlerin korunması maksadıyla uzun süreli kullanımı sonrası toprağı kirleten aldikarb üzerinde toprak bakterilerinin parçalama etkisi çeşitli kromatografik ve spektrofotometrik biyokimyasal yöntemler aracılığıyla incelenmiştir.

Bulgular: Sonuçta, bazı toprak bakterilerinin bakeriyel enzim-esterazlar yoluyla aldikarbı kullanma yeteneklerinin bulunduğu, bunlardan da *Stenotrophomonas maltophili*'nin aldikarb üzerinde en fazla parçalama etkisine sahip olduğu çeşitli biyokimyasal yöntemlerle saptanmıştır.

Sonuç: Bu verilerden yola çıkarak, biyoteknolojik olarak bazı toprak bakterilerinin bu tür pestisitlere benzer kimyasal ajanların temizlenmesi veya detoksifiye edilmesi maksadıyla kullanımı öngörülebilmektedir.

Anahtar Kelimeler: Aldikarb, biyolojik parçalanma, HPLC, toprak bakterisi

Introduction

The direct contamination by chemical weapons (CWs) and pesticides over large land areas is primary concern of military and antiterrorism forces worldwide. The ongoing attempts of some States to use pesticides as CWs and accordingly to contaminate wide areas have emphasized the need for measures and new developments in small scale decontamination materials, although technological advances have not been properly reported in an effective use in wide area decontamination (1).

In the late of 1990s, hydrolyzing enzymes known as effective on CWs were studied as a potential means of decontaminating materials, and especially the broad spectrum organophosphorus hydrolase (OPH) which had received a particular attention (1,2). On the other hand, microbial degradation of pesticides in the soil is a natural process and one of the main mechanisms of detoxification (3). The process causing the breakdown of pesticides may contribute to the maintenance of a safe environment. It has been shown in several studies that some various types of bacteria were responsible for accelerated degradation of some pesticides (4,5).

In Turkey, aldicarb [(2-methyl-2[methylthio] propionaldehyde-o-[methyl carbomyl]oxime)], a carbamate insecticide is widely used for crop protection against mites, nematodes and aphids (Figure 1) (6). It is applied directly to the soil and then thoroughly incorporated. At the same time, questions concerning the potential accumulation of aldicarb itself and its biologically active metabolites in the soil or groundwater appeared (7). Since the primary routes of human exposure to aldicarb is the consumption of contaminated food and ingestion of drinking water from contaminated wells, it is necessary to effectively control the aldicarb contamination of environmental materials (8,9). Due to the importance of environmental pollution and toxicity in Turkey, we aimed to obtain a biotechnological method to degrade and remove it easily and cost-effectively.

The presence of aldicarb and other similar insecticides in soil samples threaten human health because of their toxicities. These compounds are persistent in several forms unless biodegradation by means of some microorganisms occur. Such microorganisms are like ubiquitous organisms found in a variety of environments including soil and fresh water sources (10,11,12). Thus, this study aimed to find out the microbial way to degrade the aldicarb, a commonly used insecticide in Turkey, and to isolate the appropriate microorganism and bacteria having the highest biodegradation capacity using some biochemical methods involving spectrophotometry and chromatography.

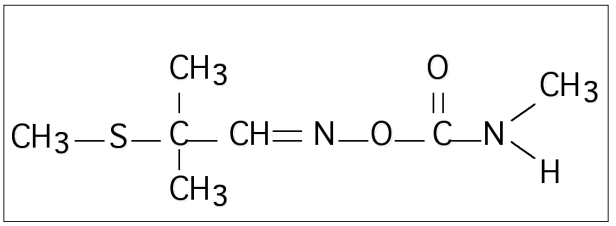

Figure 1. Chemical structure of Aldicarb, (2-methyl-2(methylthio) propanal o-[(methylamino)-carbonyl] oxime).

Materials And Methods

In this study, various types of chemicals manufactured by different chemical trade companies, a cholinesterase diagnostic kit (SIGMA), and a spectrophotometer (SHIMADZU UV-1201, UV-VIS) and High Performance Liquid Chromatography-HPLC (AGILENT) were used in addition to an API-20 E Test System to identify the bacteria with respect to morphological and biochemical properties.

A. Isolation of aldicard degrading bacteria from soil:

Isolation of Aldicarb Degrading Bacteria (ADB): Soil samples from an agricultural site in Adana province in Turkey collected from a soil surface with the depth of 10 cm were used to enrich the soil. Soil samples possibly containing soil bacteria having the ability to degrade aldicarb were isolated by using enrichment method.

1. Identification of Strains: were performed by using API-20E Test System where some morphological characteristics (size, chromogenesis, gram staining, capsule staining, motility testing and endospore staining) and some biochemical properties like glucose-lactose utilization, growth on McConkey agar, typical reaction patterns triple sugar iron agar, indole test, oxidase activity and catalase activity were determined.
2. Determination of Bacterial Growth: Identified bacteria were separated and inoculated into the aldicarb supplemented liquid basic salt / trace metal medium, and also into the medium without aldicarb. They were incubated at 37°C for 10 days. At the end of the period, 5 ml of samples were withdrawn from the liquid cultures to measure absorbance using SHIMADZU (UV-1201, UV-VIS) spectrophotometer at 540 nm.
3. Determination of Biodegradation Rate of Aldicarb: During this step, the liquid basic / trace metal cultures were centrifuged at 7000 rpm for 20 min, in order to obtain cell free extracts of aldicarb. Cell free extracts or aldicarb residues were subjected to procedures including extraction, oxidation and cleanup. In the cleanup procedure; the chromatographic column containing activated florisil and Na_2SO_4 was used. Eluted aldicarb residues were analyzed by using HPLC (equipped with a pickering post column derivatization instrument, an auto sampler and a vacume degassing unit). In this chromatographic step, HPLC analysis was

performed with an instrument equipped with a fluorescence detector (λ_{Ex} 330, λ_{Em} 465) to determine the aldicarb concentration thus indicating the biodegradation rate. Gradient seperations were performed on a reversed phase C_{18} column (diameter 2 mm; particle size 5 µm, 250 by 4.0 mm). A water/acetonitrile mixture (10:90, v/v) was supplied at a flow rate of 0.8 ml/min as a mobile phase. As a derivatizing agent, o- phytalaldehyde was used. The detection limit was 0,1-0,5 ng.

B. Bacterial lysis and determination of cytoplasmic enzyme activity:

1. Bacterial Cytoplasmic Protein Extraction: Applying the bacterial cell lysis method described by Tavares and Sellsted (13), harvested bacterial cells were washed with 62.5 mM Tris – HCl (pH: 6.8) buffer and 1 ml of the same buffer supplemented with 0.1 % (v/v) Triton x-100 was pipetted in order to solubilize the proteins associated with the cell wall. After centrifugation of the bacterial suspension at 20000 g for 5 min at 4 °C, cell wall-associated protein fractions (supernatant) were separated and pellet was resuspended in 1 ml of the same buffer for cytoplasmic protein / enzyme fraction. The last suspension including cytoplasmic proteins was extracted by sonication (Genprobe).
2. Determination of Esterase Enzymatic Activity: Bacterial cytoplasmic fraction was tested in terms of cholinesterase activity by using a SIGMA Diagnostic Kit. The method is based on kinetic determination of cholinesterase activity at 405 nm using butyrylthiocholine as a substrate to yield thiocholine which reacts with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to form a yellow colour.

Results

In this study, nine different bacteria were isolated by using the morphological tests and biochemical experiments and the identified strains of bacteria from the soil samples are listed in Table 1. In spectrophotometric analysis for determining the bacterial growth, the absorbances of identified bacteria after an incubation period in the liquid medium, were measured (Table 2).

During the degradation of aldicarb by the bacteria and /or their enzymes, the non-degraded residual aldicarb and its degradation products were monitored by photometric and chromatographic measurements. In the enzymatic degradation, we analyzed the formation of aldicarb-derived degradation products using HPLC. In this degradation process, aldicarb sulfoxide and sulphone were rapidly formed by ester hydrolysis of aldicarb. The determined aldicarb concentrations indicating the biodegradation rate using HPLC were shown in Table 3.

Table 1 : The list of isolated strains from the soil samples following the morphological and biochemical tests. The bacteria were numbered in paranthesis for practical use in figures.

Isolated and identified bacterial strains from soil
• *Stenotrophomonas maltophilia* (I)
• *Bacillus species* (II)
• *Alcaligenes denitrificans* (III)
• *Gram (+) bacillus* (IV)
• *Bacillus subtilis* (V)
• *Enterobacter gergoviae* (VII)
• *Flavimonas oryzihabitans* (VIII)
• *Flavimonas species* (IX)

Table 2: Absorbances of different bacterial strains in the medium with and without aldicarb which were obtained at 540 nm.

Type of bacteria	Medium without aldicarb Absorbance	Medium with aldicarb Absorbance
Stenotrophomonas maltophilia	0.95	2.64
Bacillus sp.	1.65	1.49
Alcaligenes denitrificans	2.58	2.63
Gram (+) bacil	2.26	1.71
Bacillus subtilis	2.36	2.36
Gram (+) bacil diphteroid	1.15	1.61
Enterobacter gergoviae	0.41	0.30
Flavimonas oryzihabitans	2.23	2.38
Flavimonas sp.	0.41	1.10

Discussion

Aldicarb, a carbamate insecticide has been known to have the potential to cause adverse effects on human health by inhibition of acetylcholinesterase activity in the neuromuscular junction (9, 14). This characteristic is common also for nerve agents, the most known toxic chemicals (15, 16). Hence, it is paramount to find a degrading agent that is effective in cleaning the environment. By this process, we targeted to decrease the possibility of some pathologies like membrane damage and inhibition of protein synthesis caused by aldicarb.

Enzymatic hydrolysis of aldicarb is brought by two kinds of enzymes called esterase and amidase. By the hydrolysis of N-metil carbamate from insecticide, its

Table 3: Aldicarb concentrations determined by HPLC in liquid medium containing different strains after 10 days incubation.

Strain Sample	Retention time (Min.)	Area	Concentration of Aldicarb (mg/L)	Dil. Fact.
1.	4.267	4905.92	628	100
2.	4.300	1315.389	1685	1000
3.	4.255	273.24	350	1000
4.	4.279	297.041	190	500
5.	4.271	2873.264	3679	1000
6.	4.273	3305.128	6355	1500
7.	4.272	194.836	373	1500
8.	4.395	3066.311	3930	1000
9.	4.270	3875.149	4967	1000
Standart	4.382	1560.13	2.0	500
Control Blank	4.489	3831.26	7367	1500

activity is demolished. Usage of such insecticides or chemicals would cause environmental contamination. In order to reduce or remove the negative effects on living health, some biotechnological developments including microbial degradation systems have been recorded (17-19). As related to this issue, nerve agent hydrolyzing enzymes have been studied as potential mean of ''environmentally friendly'' decontamination (20,21,22). Work by Edgehill and Finn suggests that some microorganisms might be useful for the removal of some chemicals from contaminated soils and waters (23). Microbial degradation of pesticides in the soil is a natural process and one of the main mechanisms of natural detoxification and it is thereby responsible for the natural removal of pesticides from the environment (12, 24).

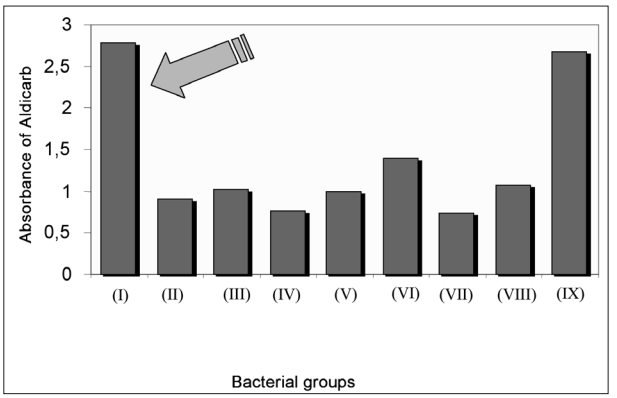

Figure 2. The illustration of the increase in absorbances of aldicarb utilization measured on spectrophotometer. The number of each bacterium is depicted in Table I.

As mentioned above, aldicarb is a pesticide used widely particularly in southern Turkey. Therefore, providing a safe environment is very important in terms of human health. In this study, firstly, it was aimed to select the aldicarb degrading bacteria with the ability to utilize aldicarb hydrocarbons as sole sources of carbon and energy. So, soil samples contaminated with aldicarb were obtained and cultured. Then, soil bacteria were isolated and identified as to determine their bacterial growth rate in the media with aldicarb. For identification, API-20E Test System was used, which indicated their morphological and biochemical characteristics. We measured the absorbances of liquid culture samples reflecting the bacterial growth rates. Bacterial growth rate shows whether the soil bacteria could utilize the aldicarb as sources of hydrocarbon and energy or not. The absorbances of bacterial strains are shown in Table 2. Taking into consideration a few bacteria with high absorbances which were grown in the medium with aldicarb, we determined the changes by measuring the absorbances based on the alterations of strains or bacterial growth rate (Figure 2). With respect to these evaluations including alterations of bacterial absorbances obtained from the culture samples with and without aldicarb, we could not select the most appropriate ADB. Accordingly, we needed to determine the biodegradation rate of aldicarb, in other words how much it was utilized by the bacteria. Therefore, we prepared the cell free aldicarb extracts from the liquid cultures by centrifugation at 7000 rpm for 20 min. Then, extracts were subjected to some experimental procedures including extraction, oxidation and clean-up recovery. After performed clean-up procedures for each cell free extract, aldicarb residues were measured by using the HPLC method. In this method, carbamate analysis column and flourescence detectors were used to run the HPLC program. Aldicarb concentrations measured spectrophotometrically in different bacteria cell free extracts are illustrated in Table 3. Determined aldicarb concentrations refered to how much aldicarb was utilized by the soil bacteria as the source of energy and carbon (25). In this regards, aldicarb residue levels may indicate the biodegradation rate of aldicarb created by

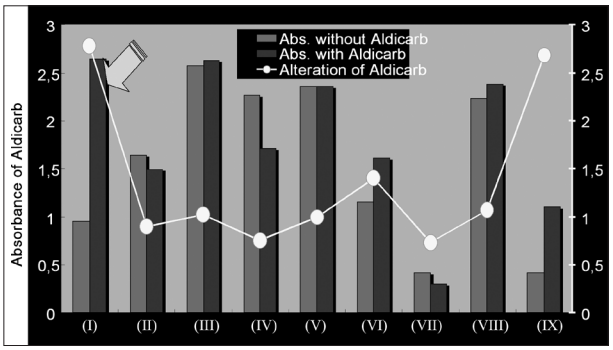

Figure 3. The relationship between the bacterial growth rate and biodegradative effect on aldicarb. The number of each bacterium is depicted in Table I.

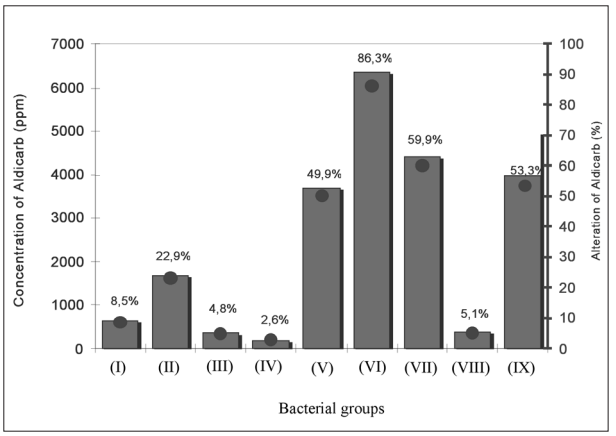

Figure 4. Aldicarb concentrations in the liquid bacterial cultures as a parameter of the biodegradation rate. The number of each bacterium is depicted in Table I.

soil bacteria or indirectly how effectively they utilized the aldicarb.

Evaluating the results obtained by the HPLC method, we determined a few candidates as having the largest biodegradation rate and compared these with the results of a bacterial growth colorimetric study. With the both studies using spectrophotometry and HPLC methods, we tried to determine two or three of the most suitable bacteria with the most biodegradative effect on aldicarb in terms of degradation and utilization as related to the biodegradation of pesticides in soil. Researchers in Israel have carried out several studies to prevent or minimize the adverse environmental effects of pesticides including aldicarb. In one of these studies, they have analyzed the aldicarb biodegradation regarding its metabolites by using a GC-MS method (9). In our study, we used the HPLC method for measuring the amount of aldicarb residues by using the carbamate analysis column and derivatization by o-Phythalaldehyde. Thus, we compared the results obtained from both spectrophotometry and HPLC methods which indicated the bacterial growth rate and biodegradation rate, respectively, and also evaluated these for determining the most suitable ADB. From the results (Figure 3), *Stenotrohomonas maltophilia* was selected as the most suitable ADB.

In this later stage, bacterial cells were lysed by chemicals and cytoplasmic protein was extracted. In order to determine whether *S. maltophilia* had a biodegradative potential on aldicarb, which is hydrolysed by esterase and amidase, we tested the cytoplasmic extract of bacteria in terms of esterase enzyme activity (Figure 4). For this, a kinetic determination method based on the reaction of thiocholine with DTNB was applied by using spectrophotometry. At the end of this spectrophotometric application, cholinesterase activity of *S. maltophilia* bacteria was measured as U/L. What was important here was to obtain the level of enzyme activity.

It should be emphasized that similar studies should also be performed in models resembling the natural circumstances since this research has been made in laboratory conditions. According to our results, we may suggest that the selected bacteria might utilize aldicarb as a source of hydrocarbon and energy through the action of esterase enzymes leading to its biodegradation.

Conclusion

Pesticides or chemicals, such as aldicarb, cause environmental pollution which affects human and animals life and health significantly. They could contaminate soil, water and the other environmental materials. In this study, we tried to determine the species of bacteria in soils in which aldicarb has been used for a long time and to find out whether they break aldicarb down and utilize it. Consequently, we can suggest that the environmental pollution in soil which is caused by pesticides an be removed by bacterial applications based on biotechnology.

References

[1] LeJeune KE, Dravis BC, Yang F, Hetro AD, Doctor BP, Russell AJ (1998) Fighting nerve agent chemical weapons with enzyme technology. Ann N Y Acad Sci. 864:153-70.

[2] Little JS, Broomfield CA, Fox-Talbot MK, Boucher LJ, MacIver B, Lenz DE (1989) Partial characterization of an enzyme that hydrolyzes sarin, soman, tabun, and diisopropyl phosphorofluoridate (DFP). Biochem Pharmacol. 38(1): 23-9.

[3] Rosenberg A, Alexander M (1979) Microbial cleavage of various organophosphorous insecticides. Appl Environ Microbiol. 37(5): 886-91.

[4] Sogorb MA, Vilanova E (2002) Enzymes involved in the detoxification of organophosphorus, carbamate and pyrethroid insecticides through hydrolysis. Toxicol Lett. 128 (1-3): 215-28.

[5] Ghosal D, You IS, Chatterjee DK, Chakrabarty AM (1985) Microbial Degradation of Halogenated Compounds. Science. 228 (4696): 135-142.

[6] Tuncbilek AS, Aysal P, Halitligil MB (1997) Loss of label during processing cotton oil grown with ^{14}C-aldicarb. Bull Environ Contam Toxicol. 58(2): 213-8.

[7] Khandaker NR, Young JC (2000) Effect of culture acclimation on the kinetics of aldicarb insecticide degradation under methanogenic conditions. J Agric Food Chem. 48(4): 1411-6.

[8] Ragoucy-Sengler C, Tracqui A, Chavonnet A, Daijardin JB, Simonetti M, Kintz P, Pileire B (2000) Aldicarb poisoning. Hum Exp Toxicol. 19(12): 657-62.

[9] Lifshitz M, Shahak E, Bolotin A, Sofer S (1997) Carbamate Poisoning in Early Childhood and in Adults. Clin Toxicol 35(1): 25-27.

[10] Caracciolo AB, Grenni P, Ciccoli R, Di Landa G, Cremisini C. (2005) Simazine biodegradation in soil: analysis of bacterial community structure by in situ hybridization. Pest Manag Sci. 61(9): 863-9.

[11] Fenyvesi E, Gruiz K, Verstichel S, De Wilde B, Leitgib L, Csabai K, Szaniszlo N. (2005) Biodegradation of cyclodextrins in soil. Chemosphere. 60(8): 1001-8.

[12] Duraes Sette L, Mendonca Alves Da Costa LA, Marsaioli AJ, Manfio GP (2004) Biodegradation of alachlor by soil streptomycetes. Appl Microbiol Biotechnol. 64(5): 712-7.

[13] Tavares A, Selstedt A (2000) A simple, rapid and non-destructive procedure to extract cell wall-associated proteins from Frankia. J Microbiol Methods. 39(2): 171-8.

[14] Burgess JL, Bernstein JN, Hurlbut K (1994) Aldicarb poisoning. A case report with prolonged cholinesterase inhibition and improvement after pralidoxime therapy. Arch Intern Med. 154(2): 221-4.

[15] Leikin JB, Thomas RG, Walter FG, Klein R (2002) Meislin HW. A review of nerve agent exposure for the critical care physician. Crit Care Med. 30(10):2346-54.

[16] Abu-Qare AW, Abou-Donia MB (2002) Sarin: health effects, metabolism, and methods of analysis. Food Chem Toxicol. 40(10): 1327-33.

[17] Parsons JR, Dick TH, Laar A, Hutzinger O (1988) Biodegradation of chlorinated biphenyls and benzoic acids by a Pseudomonas strain. Appl Microbiol Biotechnol. 29, 81-84.

[18] Valo R, Apajalahti J, Salkinoja-Salonen M (1985) Studies on the physiology of microbial degradation of pentachlorophenol. Appl Microbiol Biotechnol. 21: 313-319.

[19] Sun H, Xu J, Yang S, Liu G, Dai S (2004) Plant uptake of aldicarb from contaminated soil and its enhanced degradation in the rhizosphere. Chemosphere. 54(4): 569-74.

[20] Cheng TC, DeFrank JJ, Rastogi VK (1999) Alteromonas prolidase for organophosphorus G-agent decontamination. Chem Biol Interact. 119-120: 455-62.

[21] LeJeune KE, Russell AJ (1999) Biocatalytic nerve agent detoxification in fire fighting foams. Biotechnol Bioeng. 62(6): 659-65.

[22] Lei Y, Mulchandani A, Chen W. (2005) Improved degradation of organophosphorus nerve agents and p-nitrophenol by Pseudomonas putida JS444 with surface-expressed organophosphorus hydrolase. Biotechnol Prog. 21(3): 678-81.

[23] Edgehill RU, Finn RK (1983) Microbial Treatment of Soil to Remove Pentachlorophenol. Appl Environ Microbiol. 45(3): 1122-1125.

[24] Tiryaki O, Yucel U, Sezen G (2004) Biodegradation of trifluralin in Harran soil. J Environ Sci Health B. 39(5-6): 747-56.

[25] Liu G, Dai S, Qian Y, Gan Q (2003) Experimental study on effect of anion surfactant on degradation rate of aldicarb in soil. J Environ Sci Health B. 38(4): 405-16.