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Bioremediation of malathion in aquatic system by different microbial isolates

Ahmed H. Massoud*; Aly S. Derbalah*; El-Sayed B. Belal and Ismail I. El-Fakharany***

*Pesticides Department, Fac. of Agric., Kafr El-Sheikh Univ.

** (Agric. Microbiology), Agric. Botany Dept., Fac of. Agric., Kafr El-Sheikh Univ.

ABSTRACT

Efficiencies of five microbes isolated from cucumber leaves were evaluated for malathion degradation at recommended dose level (50µg/ml) in aquatic system. The identified isolates were belonging to bacterial group (*Pseudomonas* spp EM1, EM5 and EM6) and fungal group (*Asperigillus sp.* (EM8) and *Penicillium* sp. (EMT)). Moreover, the effect of pH and temperature on the growth ability of the different tested isolates was investigated. The results showed that the optimum pH for the growth of the malathion-degrading isolates (bacterial and fungal one) was 7.A temperature of 30°C appears to be the optimum for growth of either the fungal or bacterial isolates. The degradation rate of malathion by different microbial isolates was very high, since 50% of malathion initial concentration (50µg/ml) decomposed within one week. Moreover, almost 100% of the malathion initial concentration degraded within two weeks by all microbial isolates. *Asperigillus* sp. isolate showed the highest ability for degrading malathion followed by *Penicilluim* sp. and *Pseudomonas* spp., respectively. The degradation rate of malathion by fungal isolates was faster than the bacterial one. The tested microorganisms isolated from cucumber leaves are promising for detoxification of malathion in aquatic system.

Keywords: malathion, biodegradation, water, microorganisms, vegetables

INTRODUCTION

The wide spread use of pesticides in the recent decades has resulted in problems caused by their interaction with biological systems in the environment. The problems of pesticides come from their high residual level in agricultural crops especially vegetables that are cultivated under green house conditions. Malathion is an organophosphate insecticide that has been suited for the control of sucking and chewing insects on field crops, fruits, vegetable, livestock, and also extensively used to prevent mosquitoes, flies,

household insects, animal parasites, and head body lice as substitute for DDT (Rettich, 1980, Chambers, 1992 and Barlas, 1996). A major amount of malathion and its residues exposed to the environment are rapidly absorbed by practically all routes including the gastrointestinal tract, skin, mucous membranes, and lungs (Indeerjeet *et al.,* 1997). In particular, it persistently remains in oily products for a long period (Racke, 1992). From animal testing and use experience, the toxic effect of malathion has been shown to affect the central nervous system of invertebrates, immune system of higher vertebrate wildlife, and adrenal glands, liver and blood of fish (Senanayake and Karalliedde, 1987, El-Dib *et al.,* 1996 and Galloway and Handy, 2003).

Malathion also produced detectable mutations in three different types of cultured human cells, including white blood cells and lymph cells (Gallo and Lawryk, 1991 and U.S. Public Health Service, 1995). It is important to note that the carcinogenicity of malathion is typically reported to occur at doses which also produce signs of maternal toxicity, such reduced food intake and body weight, and gross morbidity (Menzie, 1980). In addition, there have been many reports on the carcinogenic effects of malathion in female rats and mice (National Cancer Institute, 1979, Gallo and Lawryk, 1991 and U.S. Public Health Service, 1995) and on the ecological effects of malathion in birds, fish, and honeybees (Menzer, 1987, Kidd and James, 1991). Moreover, organophosphorous insecticides like malathion have been known to potentially cause adverse effects on human health by inhibition of acetylcholine esterase activity in the body (Chambers, 1992 and Racke, 1992). Considering the toxic effect of pesticides such as malathion, remediation technologies for these chemo-pollutants in the environment are in demand. Bioremediation of chemo-pollutants become the method of choice because it is economically feasible and safer than chemical remediation technologies. Microorganisms can use a variety of xenobiotic compounds including pesticides for their growth by mineralizing and detoxifing them. Bioremediation is an accepted technology for accelerating the rate of cleanup of contaminated water. However, the process is often limited by the lack of adapted micro-organism (Grigg *et al.,* 1997).

Hence, it is very important to find a novel biocatalyst that is effective in the degradation of malathion in the environment at the recommended dose level. Therefore, this study attempt to isolate and identify different fungal and bacterial microbes for bioremediation of malathion in aquatic system, to evaluate malathion degradation potential by these microbial isolates and to

investigate the optimum pH and temperature for the growth ability of the tested microbial isolates.

MATERIALS AND METHODS

1. Chemicals

Tested pesticide (malathion) was obtained from the Environmental Protection Agency (EPA), USA. The organic solvent used was acetonitrile (HPLC grade) which was obtained from Merck-Co., Germany.

2. Degradation kinetics

2.1. Media: M9-Minimal Medium as mineral salt medium (MSL) and Luria Bertani (LB) were used through this study as described by Sambrook *et al.*, (1989) as well as potato dextrose agar (PDA) which was used also in the present study.

2.2. Isolation by enrichment culture: Enrichment cultures of microorganisms capable of degrading of malathion were established from leaves of cucumber plants: Samples of cucumber leaves were collected from Kafr Elsheikh Governorate, Egypt that was previously treated with malathion. 10 g from leaves were suspended in 90 ml sterilized mineral salt medium in 500 ml bottle containing (50 µg a.i/ml) from malathion as a sole source of carbon and phosphorus, incubated at 30°C and shacked at 150 rpm for 14 days. After that 10 ml of cultures were transferred into fresh 90ml MSL containing same concentration from malathion. This procedure was repeated four times. Dilution series were prepared after the final time from enrichment culture in glass tube containing 90 ml MSL liquid medium up to $1{:}10^{-6}$ and then 100 µl of it was spreaded on plates MSL+ malathion (50 µg a.i/ml) by using drigalisky triangle. The plates were sealed in polyethylene bags and were incubated at 30°C for 7days monitored for appearance of colonies. Single colonies growing on these dilution plates were isolated by picking the colonies using sterile inoculation needle and were further purified by the standard spatial streaking for bacterial isolates on complex agar media or using acidic complex medium or addition of ampicilline 800 mg/l to complex medium for fungal isolates (LB for bacteria isolates and PDA for fungal one).

The isolated colonies were then tested for their ability to grow in MSL medium containing 50 µg a.i/ml of malathion as a sole source of carbon (Bourquin, 1977; Hasan, 1999) and phosphorus (Rosenberg and Alexander,

1979; Subramanian, *et al.*, 1994). One treatment contained the medium and malathion and the other contained the medium and the isolate (without malathion) as control. The cultures were shaken at 150 rpm and 30°C for 14 days and then checked for an increasing in the intracellular protein for bacterial isolates and mycelia dry weight for fungal isolates. The bacteria cells were digested as described by Belal (2003) and the protein content was determined according to the method described by Lowry *et al.*, (1951) using bovine serum albumin as standard protein. The mycelia dry weight for fungal isolates was determined as described by Belal (2003).

2.3. Identification: The selected malathion degrading bacterial isolates were identified as described by Bergy's manual of Systematic Bacteriology (1984). Also, the selected malathion degrading fungal isolates were identified according to Barnett and Barry (1972).

2.4. Optimization of the cultivation condifions (pH and temperature): Thirty ml of MSL medium supplemented with 2% glucose/L for fungal isolates and 1% for bacterial isolates were used to determine the optimum temperature and pH for the malathion degrading isolates. Glucose was used as a sole source of carbon instead of the malathion in MSL liquid medium to abbreviate the incubation time. MSL medium was inoculated by 1 ml from fungal suspension at 104 cfu/ml or bacterial cell suspension at 107 cfu/ml, respectively. To determine the optimum pH, experiments were carried out at pH 4, 5, 6, 7 and 8 for fungal isolates and at pH 6, 7 and 8 for the bacterial one. Cultures were incubated on a rotary shaker at 30°C and 150 rpm for 7 and 3 days for fungal and bacterial isolates, respectively. To determine the optimum temperature, MSL medium with pH of 7 was incubated at 20, 30 and 40°C, then incubated at 150 rpm for 7 and 3 days for fungal and bacterial isolates, respectively. The growth for fungal isolates was determined as mycelia dry weight of biomass (g) as described by Belal (2003). However, for bacterial isolates the growth was determined as intracellular protein content (µg/ml) as described by Belal (2003).

2.5. Biodegradation of malathion by the tested microbial isolates: Selected isolates were cultured onto MSL + malathion for 7 days and then the growing colonies were washed with 3ml sterilized MSL liquid medium. The cell suspension (107cfu/ml for bacterial isolates and 104cfu/ml for fungal isolates) was then used to inoculate 100ml MSL liquid medium containing 50 µg a.i/ml of malathion. The cultures were incubated at 30°C and 150 rpm for 0, 2, 4, 6, 8, 10, 12 and 14 days. Control flasks of equal

volume of liquid mineral medium and pesticide without any microbial population were run in parallel at all intervals to assess any biotic losses. The growth representing in intracellular protein content for the bacterial isolates and mycelia dry weight for the fungal one were determined in each treatment as mentioned above.

2.6. Analytical procedure: The incubated samples were analyzed directly after passing in Syringe filter (0.2μm) by HPLC which consists of a pump (Lc-10 Ai Shimadzu) a sample injector (Rheolyne Mode 1296, sample size 50μl) and UV detector (SPD-10A, Shimadzu). The column was an Ultron VX-ODS (Suplecosil LC-18, particle size 5μm supelco) 250 mm x 4.6mm i. d. a. guard column (Suplecosil LC-18, 5μm, and 10 mm x 4.6 mm. i. d. was used as a filter in the front of analytical column. A mixture of acetonitrile (HPLC grade) and distilled water (60:40) was used as mobile phase under isocratic mode. The flow rate was set at 0.1 ml/min. The detector wavelength was 254 nm for malathion. Standard solutions of tested pesticide were prepared from technical grade material and were injected three times wherever culture samples were analyzed.

2.7. Calcualtion of degradation rate and half-lives of malathion by different tested isolates: In order to determine the degradation rate, plots of Ln concentration against incubation time were made. The degradation rate constant (slope), k, was calculated from the first order equation: $Ct = C_o$ e-kt where $(C)_t$ represents the concentration of the pesticide at time t, $(C)_o$ represents the initial concentration, and k is the degradation rate constant. When the concentration falls to 50% of its initial amount, the half-life ($t_{1/2}$) can be determined by $t_{1/2} = 0.693/k$, according to the method described by (Derbalah et al., 2003).

RESULTS AND DISCUSSION

1. **Isolation of the malathion–degrading isolates:** Leaves of cucumber plants treated previously with malathion were used to isolate the malathion–degrading microorganisms in the present study. By using enrichment techniques, a total of five morphologically different microorganisms capable of degrading malathion were isolated from the both described microbial sources Fig.(1).

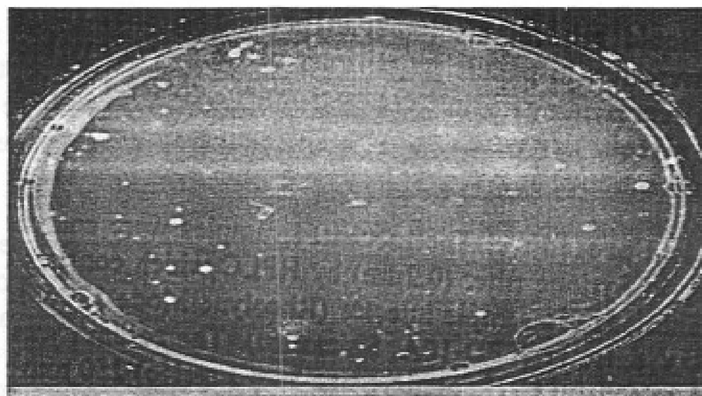

Fig. (1). Initial screening of malathion degrading microorganisms.

A preliminary classification based on the morphology of the isolates revealed that the malathion–degrading organisms belong to the group of bacteria as well as to the group of fungi. Three bacterial isolates of 5 were isolated from soil samples. The three bacterial isolates were gram-negative, motile, rods and were oxidase positive. These bacterial isolates were identified according to the morphological and physiological characteristics as *Pseudomonas* spp. (EM1, EM5 and EM6). Our results are in agreement with previous findings reported by Karpouzas *et al.,* (2005), who found that enrichment culture technique led to the isolation of two bacterial strains, which were able to degrade cadusafos rapidly in liquid cultures. The application of malathion promotes the evolution of microorganisms that are capable of degrading these xenobiotic compounds in the soil (Chaudhry and Ali 1988). On the other hand, 2 fungal isolates of five were isolated from washing leaves of cucumber Fig.(2b). The two fungal isolates were identified according to the morphological and physiological characteristics as *Aspergillus* sp. (EM8) and *Penicillium* sp. (EM8). It was clear that fungi play an outstanding role in degrading of malathion, since the majority of strains belong to this group. It is known that many genera of fungi play an important role in degradation of most agricultural wastes, pesticides and biodegradable plastic (Bumpus and Aust 1987 and Belal, 2003). Due to the paucity of growth which was generally observed on MSL + malathion. The bacterial isolates were also routinely streaked onto plates of LB for bacterial isolates but the fungal isolates were further purified by using acidic complex medium (PDA) or addition of ampicilline 800 mg/l to complex medium (PDA).

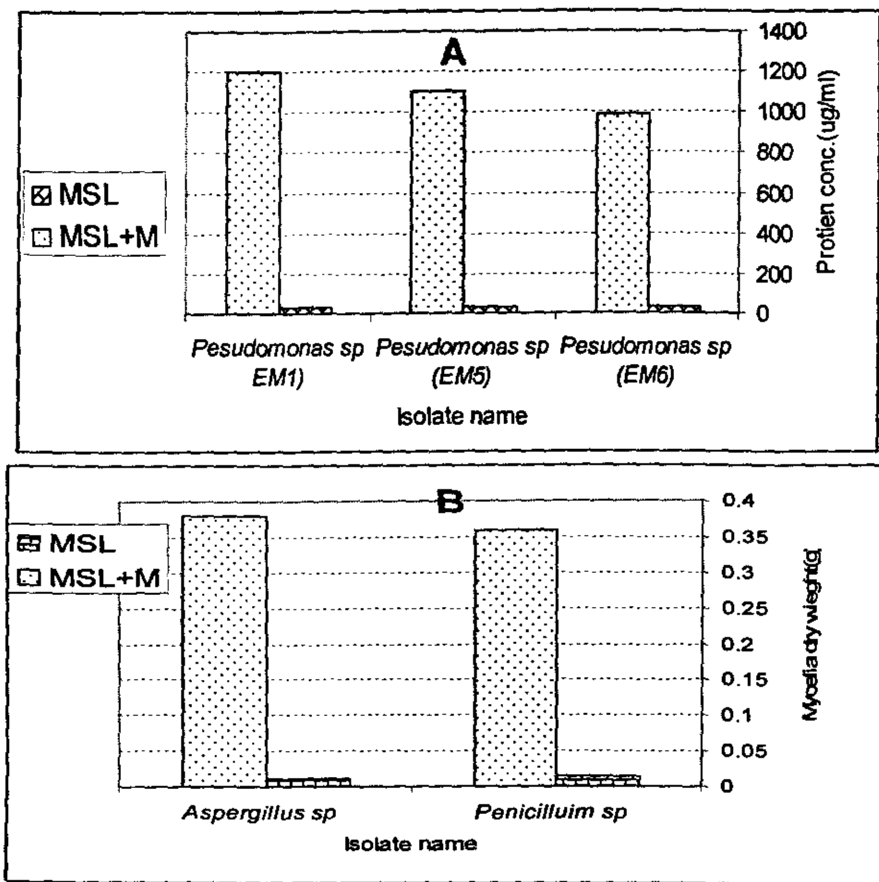

Fig.(2) Growth ability of both bacterial (A) and fungal (B) isolates in MSL supplemented with the malathion.

The growth ability of tested microbial isolates on MSL supplemented with malathion as a sole source of carbon and phosphorus compared with the growth of the isolates in MSL (without malathion) presented in Fig(2ab). The results indicates that these isolates may be promising in malathion degradation since the growth of the tested isolates in supplemented with malathion was much faster than that of MSL medium (no malathion).

2. Optimization the growth conditions of tested isolates: Normally, the pH and temperature influence the growth of microorganisms and hence, these factors will influence also the degradation process of the pesticides. Karpouzas and Walker (2000) reported that the degradation of ethoprophos by *Pseudomonas putida* strains epI and II was affected by pH and

temperature. The question now is, what are the optimal conditions (pH and temperature) for the growth of the malathion–degrading isolates? To determine the optimal growth conditions, glucose was used as a sole source of carbon instead of the malathion in MSL liquid medium to abbreviate the incubation time.

2.1. Optimum pH: The influence of pH on biomass yield of the selected isolates is shown in Fig. 3(ab). Generally, the optimum pH was 7 for all the isolates from either bacteria or fungi. The maximum mycelia dry weight for fungal isolates and intracellular protein content for bacterial isolates were recorded at pH 7 Fig.(3ab). The two fungal isolates grew at quite wide range of pH from 4 to 8 Fig.(3b). This variation is very useful to use these isolates in degradation test in different environments at different pH. Therefore, it can be expected that these isolates can tolerate the pH change during the degradation process thereby increase the degradation potential for these isolates.

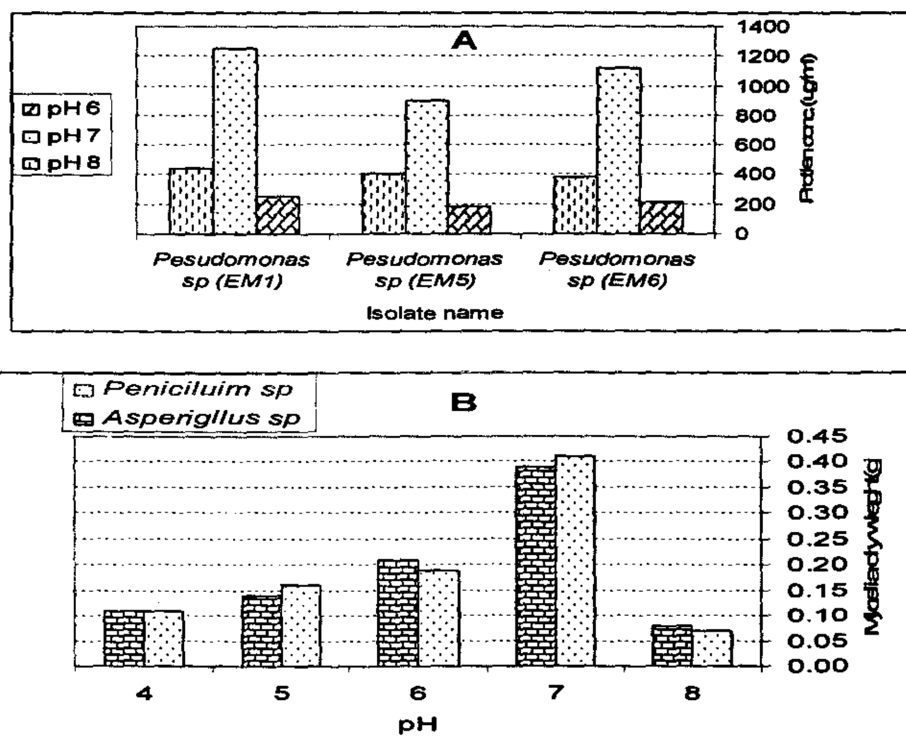

Fig. (3) Effect of pH on growth ability of both bacterial (A) and fungal (B) isolates.

2.2. Optimum temperature: The effect of different temperatures on growth of both fungal and bacterial isolates is shown in Fig.4 (ab), respectively. A temperature 30°C appears to be the optimum for growth of either fungal or bacterial isolates Fig.(4ab). The four isolates EM1, EM5, EM8 and EMT exhibited growth at 40°C but the EM6 did not grow at 40°C. Therefore, the bacterial and fungal isolates were used for further studies under the optimum growth conditions in order to evaluate their degradation potential for malathion at different incubation periods (0,2,4,6,8,10,12 and 14 days).

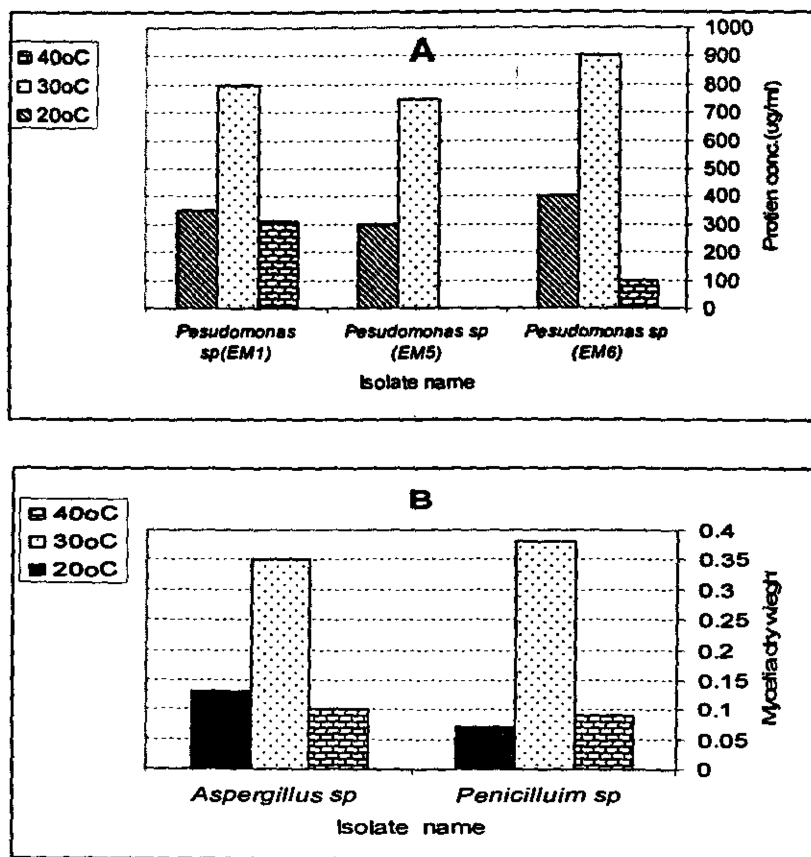

Fig.(4) Effect of temperature on the growth ability of both bacterial(A) and fungal (B) isolates.

3. Biodegradation of malathion by the selected isolates: Determination of degradation rate and half-life of malathion will provide a useful tool for predicting the microbial fate of malathion in the aquatic system and subsequently give a good approximation of what can happen in the

environment. The degradation rate and half-lives of malathion by the tested microbial isolates is presents in Table 1. The results showed that the degradation rate of malathion by different microbial isolates was very high, since 50% of malathion initial concentration (50 μg/ml) decomposed within one weeks and almost 100% of its initial concentration degraded within two weeks. The degradation of malathion by the tested microbial isolates followed the first order equation. Considering the degradation rate of malathion, *Asperigillus* sp. isolate showed the highest ability for degrading malathion followed by *Penicilluim* sp. and *Pseudomonas* spp., respectively (Table1). Half-life values of malathion were 5.6, 6.1, 6.6, 7 and 7.45 day for *Asperigillus* sp(EM8), *Penicilluim* sp. (EMT) and *Pseudomonas* spp. (EM1, EM5 and EM6) respectively (Table 1). On the other hand, malathion degradation percentage reached to 0.06% at the end of the incubation period in control or non-inoculated samples. In the light of Table (1), the degradation rate of malathion by fungal isolates was faster than the bacterial one.

Table (1): Degradation rate constant and half-lives of malathion by the tested microbial isolates.

Isolate name	Degradation rate constant (day^{-1})	Half-life ($t_{1/2}$) (day)	R
Pseudomonas spp.(EM5)	0.09 ± 001	7.45 ± 0.3	0.99
Pseudomonas spp.(EM1)	0.097 ± 0.002	7.0 ± 0.23	0.99
Pseudomonas spp.(EM6)	0.10 ± 0.001	6.6 ± 0.25	0.99
Asperigillus spp. (EM8)	0.12 ± 0.01	5.6 ± 0.2	0.99
Penicilluim spp.(EMT)	0.11 ± 0.02	6.1 ± 0.27	0.99

The main degradation process of malathion by bacterial isolates may be due to the presence of hydrolytic enzymes constitutive in bacterial isolates such as esterases. On the other hand, the mechanism of malathion degradation by fungal isolates could be attributed to the activity of fungal cutinase enzyme (Kim *et al.,* 2005). However, the faster degradation rate of malathion by fungal isolates than the bacterial one may be due to that the fungal cutinase enzyme that acts as malathion degrader much faster and

earlier than the esterase (bacterial enzyme); which agree with the explanation of Kim *et al.*,(2005). Degradation of malathion by *Asperigillus* sp.(EM8) and *Penicillium* sp.(EMT) have been reported by Hassan (1999). The growth response of malathion degrading isolates (representing in protein concentration of intercellular protein for bacterial isolates and mycelia dry weight for fungal isolates) was increased gradually by increasing malathion degradation percentages as shown in fig.(5 and 6a ,b).

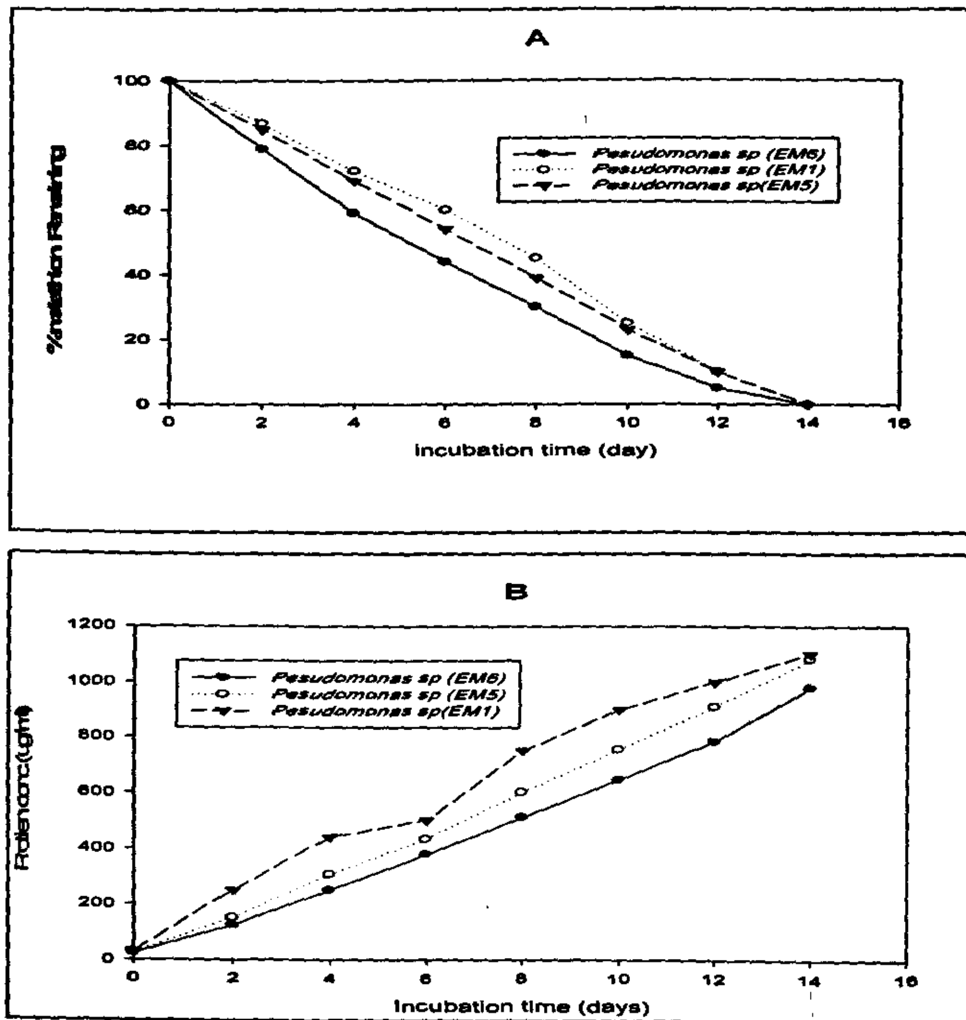

Fig. (5) Biodegradation of malathion by the tested bacterial isolates (A) and their growth response (B) at incubation times.

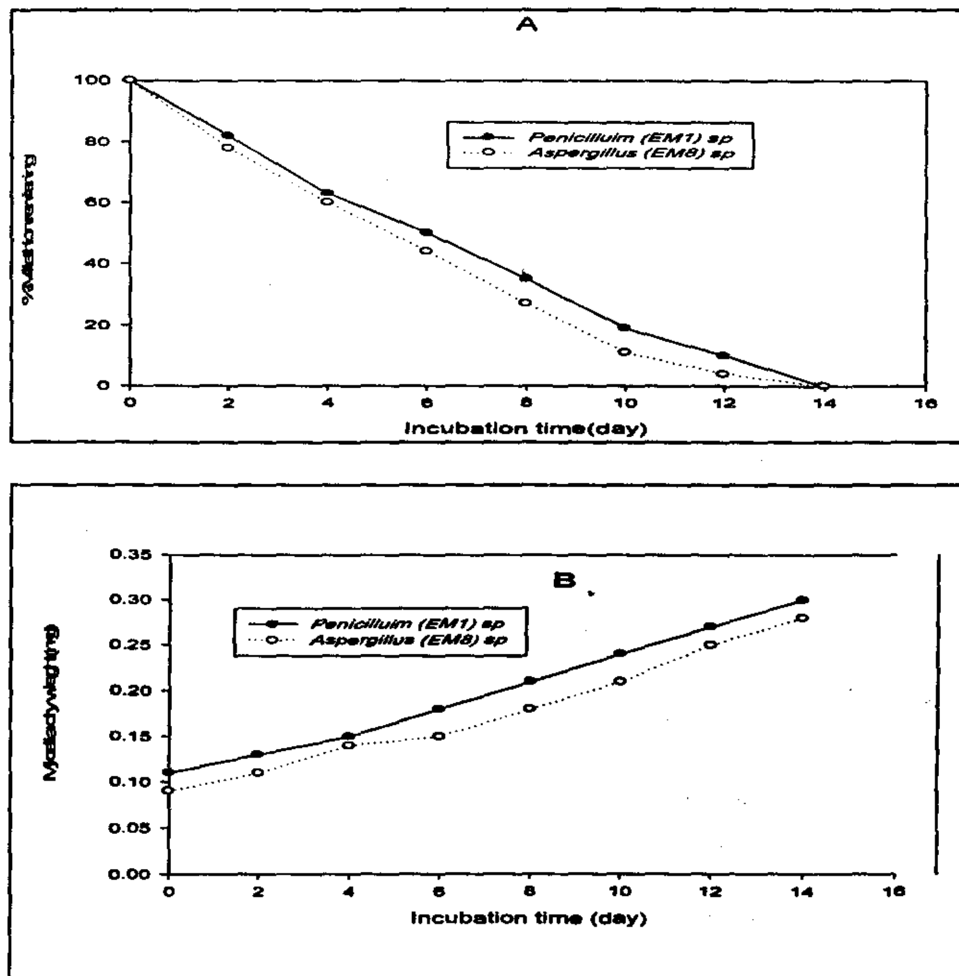

Fig. (6) Biodegradation of malathion by the tested fungal isolates (A) and their growth response(B) at incubation times.

CONCLUSION

The tested microbial isolates showed high ability in malathion degradation. Moreover, the ability of the tested microorganisms which were isolated from cucumber leaves to detoxify malathion at recommended dose level in this study considered to be the key step in the detoxification of malathion residues in vegetable crops especially under green house conditions. The isolation of these microorganisms from cucumber leaves

may lead to use these isolates for significantly reducing malathion residue in vegetable crops especially under green house conditions.

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المعالجة البيولوجية لمبيد الملاثيون فى الوسط المائى بواسطة العزلات الميكروبية

د.أحمد حافظ مسعود* د. على سليمان درباله* د. السيد بلال عبد المطلب بلال** د. اسماعيل ابراهيم الفخرانى*
*قسم المبيدات- كلية الزراعة- جامعة كفرالشيخ
**شعبة الميكروبيولوجي - قسم النبات الزراعي- كلية الزراعة- جامعة كفرالشيخ

تم تقييم كفاءة خمس ميكروبات بالنسبة لتحطيم مبيد الملاثيون عند مستوى تركيز الجرعة الموصى بها و هذه الميكروبات تم عزلها من أوراق الخيار التي سبق معاملتها بمبيد الملاثيون. العزلات التى تم تعريفها منها ثلاثة بكتيرية و هي (*Pseudomonas* spp. EM1, EM5 and EM6)، وعزلتين فطريتين و هما (*Asperigillus* sp. (EM8) and *Penicillium* sp. (EMT)). تم أيضا دراسة تأثير رقم الحموضة ودرجة الحرارة على نمو العزلات المختبرة.أوضحت النتائج إن رقم الحموضة ٧ هو الرقم الأمثل لنمو العزلات. أيضا درجة الحرارة ٣٠ منوية هي درجة الحرارة المثلى لنمو العزلات.معدل تحطم الملاثيون بواسطة العزلات المختلفة كان عاليا حيث أن التركيز الابتدائي للمبيد وصل إلى نصفه بعد التحصين لمدة أسبوع مع العزلات تحت الدراسة. تم تحطيم المبيد كاملا خلال أسبوعين من التحضين مع العزلات المختبرة. أكفا العزلات في تحطيم المبيد كانت فطر *Asperigillus* sp. يليها *Penicilluim* sp. ثم *Pseudomonas* spp. EM1, EM5 and EM6 على الترتيب. تحطم الملاثون بواسطة العزلات الفطرية كان أسرع منه فى حالة العزلات البكتيرية. الميكروبات المعزولة من على أوراق الخيار أظهرت كفاءة عالية في إزالة سمية الملاثيون في الوسط المائي.