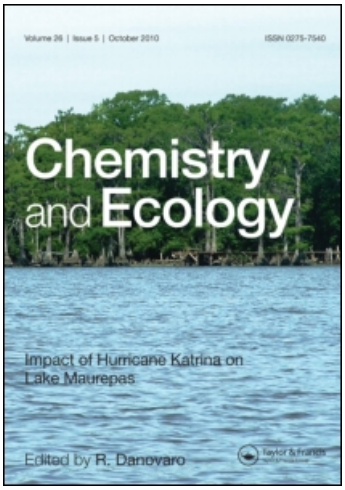

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A. S. Derbalah[a]; E. B. Belal[b]
[a] Pesticides Department, [b] Agricultural Microbiology branch, Agricultural Botany Department, Faculty of Agriculture, Kafr El-Sheikh University, Kafr El-Sheikh, Egypt

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Biodegradation kinetics of cymoxanil in aquatic system

A.S. Derbalah[a]* and E.B. Belal[b]

[a]*Pesticides Department;* [b]*Agricultural Microbiology branch, Agricultural Botany Department, Faculty of Agriculture, Kafr El-Sheikh University, Kafr El-Sheikh, Egypt*

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A potential method to detoxify pesticides in aquatic system is using bioremediation. In this study, four microorganisms (*Pseudomonas* sp (EB11), *Streptomyces* sp. (EB12), *Aspergillus niger* (EB13) and *Trichoderma viride* (EB14) were isolated from cucumber leaves previously treated with cymoxanil using enrichment technique. These strains were evaluated for their potential to detoxify cymoxanil in aquatic system at the concentration level of 5×10^{-4}M. The effect of pH and temperature on the growth ability of the tested strains was also investigated by measuring the intracellular protein and mycelia dry weight for bacterial and fungal strains, respectively. Moreover, the remaining toxicity of cymoxanil after 28 days of incubation with tested strains was evaluated to confirm the complete removal of any toxic materials (cymoxanil and its metabolites). The results showed that the optimum pH for the growth of cymoxanil degrading strains (bacteria and fungi) was 7. A temperature of 30°C appears to be the optimum for the growth of either fungal or bacterial strains. *Pseudomonas* sp. (EB11) was the most effective strain in cymoxanil degradation followed *Streptomyces* sp (EB12), *Trichoderma viride* (EB14) and *Aspergillus niger* (EB13), with half-lives of 4.33, 9.5, 17.3 and 24.7 days, respectively. The degradation of cymoxanil by bacterial strains was much faster than fungal one. There is no remaining toxicity of cymoxanil detected in aqueous media previously treated with *Pseudomonas* sp. (EB11) for 28 days. The results suggest that bioremediation by *Pseudomonas* sp. (EB11) are promising for the detoxification of cymoxanil in aqueous media.

Keywords: cymoxanil; biodegradation; water; microorganisms; toxicity

1. Introduction

Chemical release into the environment is one of the major patterns of anthropogenic pollution. Xenobiotics tend to persist in the natural cycles unless they are degraded. Therefore developing efficient treatment systems enables us to reduce pollution, since they possibly lead to innocuous substances [1]. Considering both natural and laboratory-controlled scales, bacteria are the most significant tool involved in ultimate biodegradation [2]. Bioremediation, as one of the most environmentally-sound and cost-effective methods for the decontamination and detoxification of a pesticide-contaminated environment is discussed, especially considering the factors affecting the biodegradability of pesticides such as biological factors and the characteristics of the chemical compounds. In situ and ex situ bioremediation as possible types of bioremediation activities

*Corresponding author. Email: aliderbalah@yahoo.com

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are weighted up. However, after remediation toxicity assessment are needed, firstly, to provide valuable and complementary information to compound analysis. Secondly, the major advantage of toxicity tests is the direct assessment of the potential hazard to the environmental system by both original pollutants and its metabolites [3]. Cymoxanil [1-(2-cyano-2-methoxyiminoacetyl)-3-ethylurea] is an aliphatic nitrogen fungicide, effective against grape downy mildew. Data concerning the biological detoxification of cymoxanil in aquatic system are not available. Thus, studies on microbial detoxification are in demand which will be the key step in this way. Therefore, this study attempts to isolate and identify different fungal and bacterial microbes for bioremediation of cymoxanil in aquatic system, to evaluate cymoxanil degradation potential of these microbial strains, to investigate the optimal environmental factors such as pH and temperature for the growth of the tested microbial strains and finally to confirm the complete detoxification of cymoxanil by measuring the toxicity of the treated solution against sensitive target.

2. Materials and methods

2.1. *Chemicals*

The tested pesticide cymoxanil (technical grade) 96.4% was supplied from Ciba-Geigy company. The organic solvent used was acetonitrile (HPLC grade) and was obtained from Merck-Co., Germany.

2.2. *Microbial degradation of the tested pesticide*

2.2.1. *Media*

M9-Minimal Medium as mineral salt liquid (MSL) medium and Luria Bertani (LB) medium were used through this study as described by [4] as well as Potato Dextrose Agar (PDA) medium.

2.2.2. *Isolation by enrichment culture*

Enrichment cultures of microorganisms capable of degrading cymoxanil were established from leaves of cucumber plants. Samples of cucumber leaves were collected from Kafr El-Sheikh Governorate, Egypt, which had previously been treated with cymoxanil. 10 g from leaves were added to 90 ml sterilised mineral salt medium in 5×10^{-1} l bottle containing 5×10^{-4}M of cymoxanil as a sole source of carbon, then incubated at 30°C and 150 rpm for 14 days. After that 1×10^{-2} l of cultures were transferred into fresh 9×10^{-2} l MSL medium containing the same concentration of cymoxanil. This procedure was repeated three times. Dilutions series were prepared after the final time from enrichment culture in glass tube containing 9×10^{-3} l MSL medium up to $1{:}10^{-6}$ and then 1×10^{-4} l of it was spread on plates containing MSL medium + cymoxanil (5×10^{-4}M) by using a drigalisky triangle. The plates were sealed in polyethylene bags and were incubated at 30°C for 7 days in order to monitor the appearance of colonies (Figure 1). Single colonies growing on these dilution plates were isolated by picking the colonies using sterile needle inoculation and were further purified by the standard spatial streaking for bacterial strains on complex agar media or using acidic complex medium or addition of ampicilline 800 g l^{-1} to complex medium for fungal isolates (LB for isolated bacteria and PDA for isolated fungi).

The isolated colonies were then tested for their ability to grow in MSL medium containing (5×10^{-4}M) of cymoxanil as a sole source of carbon. One treatment contained the medium and cymoxanil and the other contained the medium and the strain (no cymoxanil). The cultures were shaken at 150 rpm and 30°C for 28 days and then checked for any increase in the intracellular

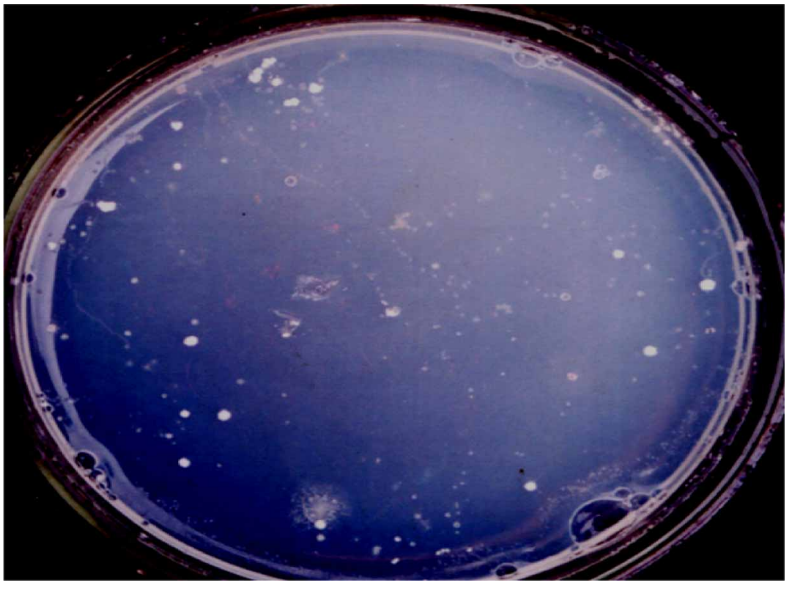

Figure 1. Initial screening of cymoxanil-degrading microorganisms.

protein for bacterial strains and mycelial dry weight for fungal ones. Growth of the bacterial cells was determined as intracellular protein content ($\mu g\,ml^{-1}$) in liquid media after 3 days where the bacterial cells, were first digested and afterwards the protein content was determined in the supernatant as described elsewhere [5]. The mixture was then centrifuged and the supernatant was used for protein determination as described by [6] using bovine serum albumin as standard protein. The growth was determined as mycelial dry weight of biomass (g) for fungal strains after 7 days, the biomass was determined by filtering the content of culture broth through a pre-dried and pre-weighed filter paper and then drying it till a constant weight was achieved, as described by [5].

2.2.3. *Identification*

The selected cymoxanil degrading bacterial strains was identified according to the morphological and physiological characteristics as described by [7]. Also, the selected cymoxanil degrading fungal strains identified according to the method described by [8].

2.2.4. *Effect of environmental factors (pH and temperature) on the growth of tested strains*

A 3×10^{-2} l MSL medium supplemented with 2% glucose/l for fungal strains and 1% for bacterial strains were used to determine the optimum temperature and pH for the cymoxanil degrading strains. Glucose was used as a sole source of carbon instead of the cymoxanil in MSL medium to abbreviate the incubation time. MSL medium was inoculated by 1 ml of fungal suspension at 10^4 cfu ml^{-1} or bacterial cell suspension at 10^7 cfu ml^{-1}, respectively. To determine the optimum pH, experiments were carried out at pH of 4, 5, 6, 7 and 8 for fungal strains and at pH of 6, 7 and 8 for bacterial strains. The cultures were incubated on a rotary shaker at 30°C and 150 rpm for 3 and 7 days for fungal and bacterial strains, respectively. Also, to determine the optimum temperature MSL medium with pH 7 was incubated at 20, 30 and 40°C and 150 rpm for 7 and 3 days for fungal and bacterial strains, respectively. The growth was determined as mycelial dry weight of biomass

(g) after 7 days as described by [5]. Growth of the bacterial cells was determined as intracellular protein content ($\mu g\,ml^{-1}$) in liquid media after 3 days as described by [5,6].

2.3. *Evaluation of degradation potential*

Selected strains were cultured onto MSL medium + cymoxanil for 7 days and then the growing colonies were washed with 3×10^{-3} l sterilised MSL medium. The cell suspension (10^7 cfu ml^{-1}) for bacterial and fungal (10^4cfu ml^{-1}) strains was then used to inoculate 1×10^{-1} l of MSL medium containing (5×10^{-4}M) of cymoxanil. The cultures were incubated at 30°C and 150 rpm for 0, 7, 14, 21 and 28 days. The % of degradation and the half-life of cymoxanil were determined as described afterward. Control flasks of equal volume of liquid mineral medium and pesticide without any microbial population were run in parallel at all intervals to assess abiotic losses. The growth representing in intracellular protein content for bacterial strains and mycelial dry weight for fungal ones was determined in each treatment as mentioned above.

2.4. *HPLC analysis*

The incubated samples were analysed directly after passing through a Syringe filter (0.2 μm) using HPLC. The instrument consists of a pump (Lc-10 Ai Shimadzu) a sample injector (Rheolyne Mode 1296, sample size 50 μl) and UV detector (SPD-10A, Shimadzu). The column was an ultron VX-ODS (suplecosil LC-18, particle size 5 μm supelco) 250 mm × 4.6 mm ID. A gurard column (suplecosil LC-18, 5 μm, 10 mm × 4.6 mm ID) was filtered 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^{-1}. The wavelength was 238 nm for cymoxanil [9]. Standard of tested pesticide were prepared from technical grade material and were used wherever culture samples were analysed.

2.5. *Calculation of degradation rate and half-lives of cymoxanil by different tested strains*

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: $C_t = C_o\ e^{-kt}$ 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 [10].

2.6. *Toxicity evaluation*

First, seven phytopathogenic fungi comprising *Alternaria solani, Helmonthosporium* sp., *Fusarium* sp., *Rhizopus* sp., *Mucor* sp., *Sclerotium rolfsii* and *Pythium debaraynum* were evaluated to select the most sensitive fungus to cymoxanil. The results indicated that *Altenaria solani* was the most sensitive to cymoxanil than the other tested phytopathogenic fungi as shown in Table 1. Therefore, this fungal pathogen was used in the further experiment as sensitive target to determine the remaining toxicity of cymoxanil after incubation with the tested microbial strains.

Alternaria solani was treated with the supernatants of different tested strains after 28 days of incubation with cymoxanil. The test was subject to standardized as follows: Potato Dextrose agar (PDA) medium was poured into petri-dishes (9 cm diameter, 15 ml/dish), after solidification, wells were punched in each plate. The plates were inoculated in the centre with a disk (5 mm diameter) bearing the mycelium growth from *Alternaria solani* culture (5-day-old culture). After

Table 1. Bioassay of cymoxanil at different concentration levels against some phytopathogenic fungi.

	Diameter of inhibition zone (mm) against different phytopathogenic fungi		
Microorganisms	5×10^{-5}M	25×10^{-5}M	5×10^{-4}M
Alternaria solani	3	9	20
Helmonthosporium sp.	2	6	16
Fusarium sp.	1	6	12
Sclerotium rolfsii	3	5	11
Rhizopus sp.	0	3	5
Mucor sp.	0	2	6
Pythium debaraynum	0.5	4	9

that, 50 μl from the supernatant was put into the punched holes (diameter 5 mm) in PDA, where the culture broth was obtained by culturing of the cymoxanil degrading strains with cymoxanil in MSL medium after 28 days as mentioned above. The culture broth was filtrated through a sterile membrane filter (0.2 μm). On the other hand, 50 μl from sterilized liquid medium was put in punched holes which were used as control treatments. Experiments were made in three replicates. Plates were incubated at 30°C until full growth of control treatments. Diameter of inhibition zone (mm) surrounding each hole was recorded.

3. Results and discussion

3.1. *Microbial degradation of cymoxanil*

3.1.1. *Isolation of the cymoxanil-degrading microbes*

Leaves of cucumber plants treated previously with cymoxanil were used to isolate the cymoxanil-degrading microorganisms in the present study. By using enrichment techniques, a total of four morphologically different microorganisms capable of degrading cymoxanil were isolated from the described microbial source (Figure 2A and B). A preliminary classification based on the morphology of the strains revealed that the cymoxanil-degrading strains belong to the group of bacteria as well as to the group of fungi. One of the two bacterial strains was gram-negative, motile, rods and were oxidase positive, identified according to these morphological and physiological characteristics as *Pseudomonas* sp. (EB11). The other strain of this group was filamentous, no-motile, Gram-positive bacteria, and oxidase negative, identified according to these morphological and physiological characteristics as *Streptomyces* sp. (EB12). Our results are in agreement with previous findings reported by [11], who found that enrichment culture technique led to the isolation of two bacterial strains, which were able to degrade cadusafos pesticide rapidly in liquid cultures. The application of pesticides promotes the evolution of microorganisms that are capable of degrading these xenobiotic compounds in the soil [12]. This reference also reported that, actinomycetes have considerable potential for the biotransformation and biodegradation of pesticides. Members of this group were gram-positive bacteria and have been found to degrade pesticides with widely different chemical structures, including organochlorines, *s*-triazines, triazinones, carbamates, organophosphates, organophosphonates, acetanilides, sulfonylureas and herbicide metolachlor [13–15].

On the other hand, the cymoxanil-degrading fungal strains were identified as *Aspergillus niger*. (EB13), and *Trichoderma viride* (EB14). It is known that many genera of fungi play an important role in degradation of most agricultural wastes, pesticides and biodegradable plastic [5,16]. Due to the paucity of growth this was generally observed on MSL medium supplemented with cymoxanil.

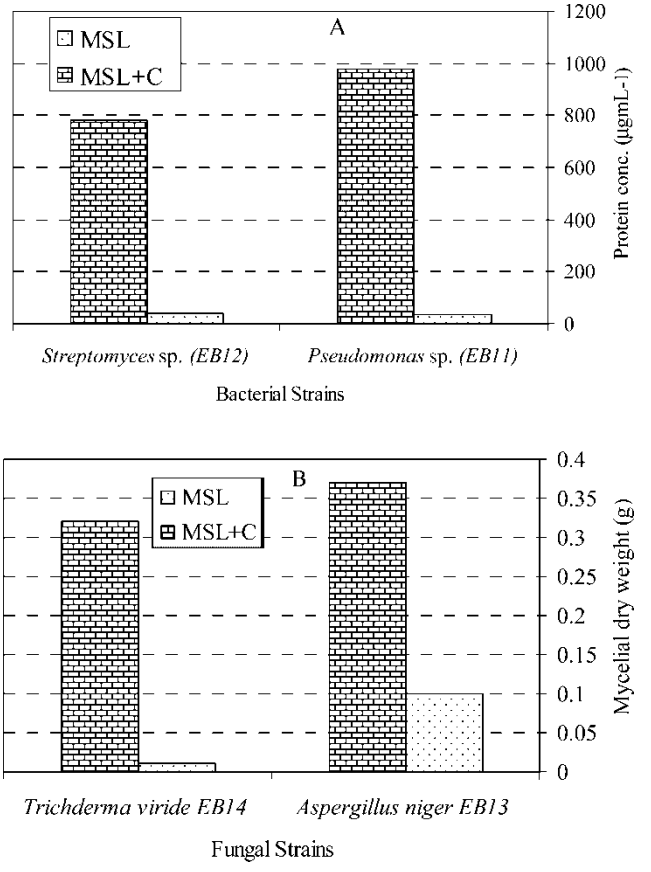

Figure 2. Growth ability of the bacterial (A) and fungal (B) strains in MSL medium supplemented with and without the cymoxanil.

The bacterial strains were also routinely streaked onto plates of LB medium but the fungal strains were further purified by using acidic complex medium (PDA) or addition of ampicilline 800 g l^{-1} to complex medium (PDA).

Results in Figure 2 (A and B) show that the tested strains were grown successfully on MSL medium supplemented with cymoxanil as the sole source of carbon. This indicates that these strains may show a high potential for cymoxanil degradation. The obtained results were compared with the growth of the strains in MSL medium only (no cymoxanil enriched).

3.2. *Effect of environmental factors (pH and temperature) on the growth of the tested strains*

3.2.1. *Effect of pH*

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 [17] reported that the degradation of ethoprophos by *Pseudomonas putida* strains epI and II affected by pH and temperature. Hong *et al.* [18] found that various factors including pH and temperature affected degradation of fenitrothion-contaminated soil using *Burkholderia* sp. FDS-1.

Therefore, the influence of pH on biomass yield of the tested strains was carried out as shown in Figure 3A and B. Generally, the optimum pH was 7 for all the strains either bacteria or fungi. The maximum mycelial dry weight for fungal strains and intracellular protein content for bacterial ones

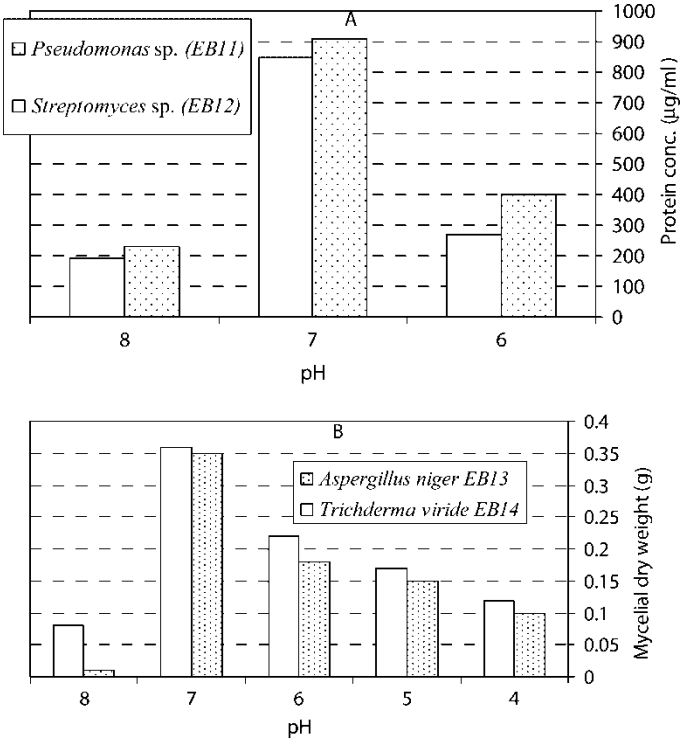

Figure 3. Effect of pH on growth of the bacterial (A) and fungal (B) strains.

were recorded at pH 7. The two fungal strains grew at quite a wide pH from 4 to 8. This variation is very useful to use these strains in degradation test in different environmental conditions of pH. Therefore, it can be expected that these strains can tolerate the pH change during the degradation process thereby increase the degradation potential for these strains. In spite of this, most bacterial strains still prefer the neutral pH but the tested bacterial strains in this study can grow at a range from pH 6–8.

3.2.2. *Effect of temperature*

The effect of different temperatures on the growth of the fungal and bacterial strains, respectively, is shown in Figure 4 (A and B). A temperature of 30°C appears to be the optimum degree for the growth of either fungal or bacterial strains. Moreover, the tested strains (*Pseudomonas* sp. (EB11), *Streptomyces* sp. (EB12), *Aspergillus niger* (EB13), and *Trichoderma viride* (EB14)) exhibited growth at 40°C. Therefore, the bacterial and fungal strains were used for further studies under the optimum growth conditions (pH and temperature) in order to evaluate their degradation potential for cymoxanil at different incubation times (0 , 7, 14, 21 and 28 days).

3.3. ***Biodegradation potential of the tested strains***

The ability of different tested strains to degrad cymoxanil was evaluated as shown in Table 2. The results indicate that *Pseudomonas* sp. (EB11) was the most effective strain in cymoxanil degradation with half-life time of 4.33 days followed *Streptomyces* sp. (EB12), *Trichoderma viride* (EB14) and *Aspergillus niger* (EB13), their half-lives were 9.5, 17.3 and 24.7 days, respectively.

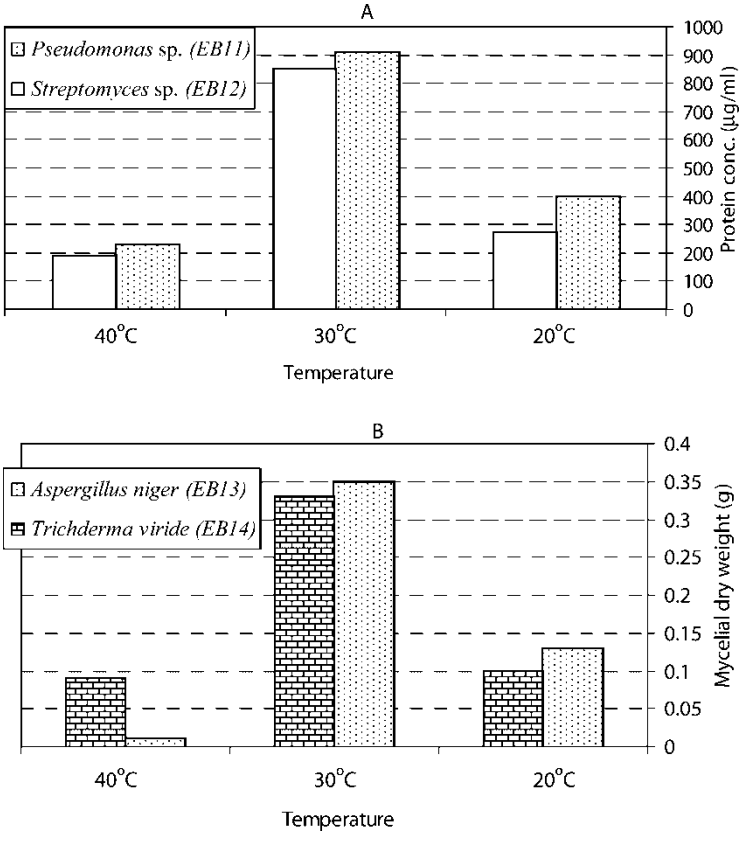

Figure 4. Effect of temperature on growth of the bacterial (A) and fungal (B) strains.

These results of half-lives confirm that the addition of 10 ml of the culture (in materials section in isolation technique) to another new medium containing cymoxanil will not exceed the cymoxanil concentration level. Within 14 days (the incubation time with the mixed culture before transferring to new media) the cymoxanil will have completely been degraded by different tested strains. The degradation of cymoxanil by bacterial strains was much faster than fungal one. More than 98.5% of cymoxanil initial concentration was degraded within 4 weeks by *Pseudomonas* sp. (EB11) while the degradation percentage was 87, 66 and 55.5% for *Streptomyces* sp. (EB12), *Trichoderma viride* (EB14) and *Aspergillus niger* (EB13), respectively. This is suggesting that different microbial types, which may be using different enzymes, have different degradation preferences. On the other hand, no cymoxanil degradation was recorded at the end of incubation time in control or non-inoculated samples. The trend of cymoxanil degradation by *Pseudomonas* sp. (EB11) and

Table 2. Degradation rate constant and half-lives of cymoxanil by tested microbial strains.

Type of microbial isolates	Degradation rate constant (day-1)	Half-life (T1/2) (day)	R2
Pseudomonas sp. (EB11)	0.16 ± 0.01	4.33 ± 0.3	0.99
Streptomyces sp. (EB12)	0.073 ± 0.005	9.5 ± 0.23	0.99
Aspergillus niger (EB13)	0.028 ± 0.002	24.7 ± 2	0.99
Trichoderma viride (EB14)	0.04 ± 0.003	17.3 ± 0.6	0.99

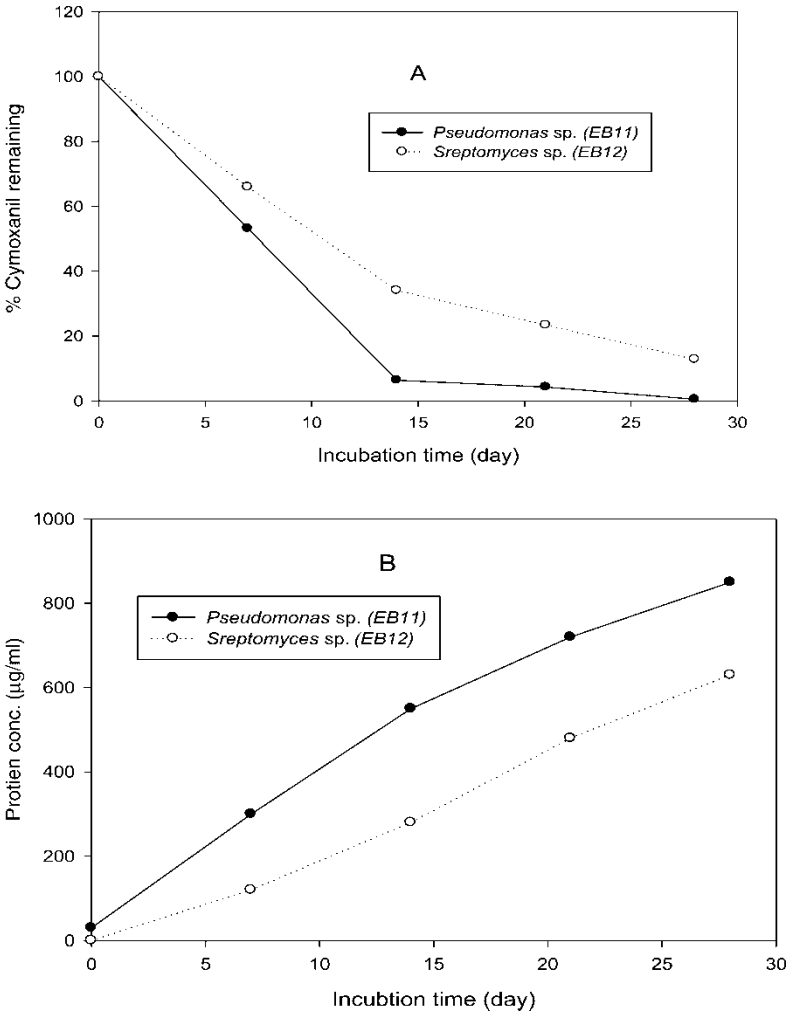

Figure 5. Biodegradation of cymoxanil by the bacterial strains (A) and their growth response (B).

Streptomyces sp. (EB12) was faster than the other tested strains which reflects the significant role of bacteria involved in the ultimate biodegradation [2]. Many authors reported earlier that *Pseudomonas* has considerable potential for the biotransformation and biodegradation of pesticides. Members of this group are gram-negative bacteria and have been found to degrade pesticides with widely different chemical structures [5,19–22]. The degradation of cymoxanil may be attributed to the secretion of enzymes from either tested bacterial or fungal strains which is capable of degrading of pesticides [23]. The growth response of cymoxanil degrading strains (representing as mycelial dry weight for fungal strains and the concentration of intercellular protein for bacterial one) was increased gradually with the increasing cymoxanil degradation rate as shown in Figures 5 and 6 (A and B).

3.4. *Toxicity evaluation*

The remaining toxicity of cymoxanil at concentration level of 5×10^{-4}M in the aqueous solutions after 28 days of incubation with the tested microbial strains was evaluated using *A. solani*.

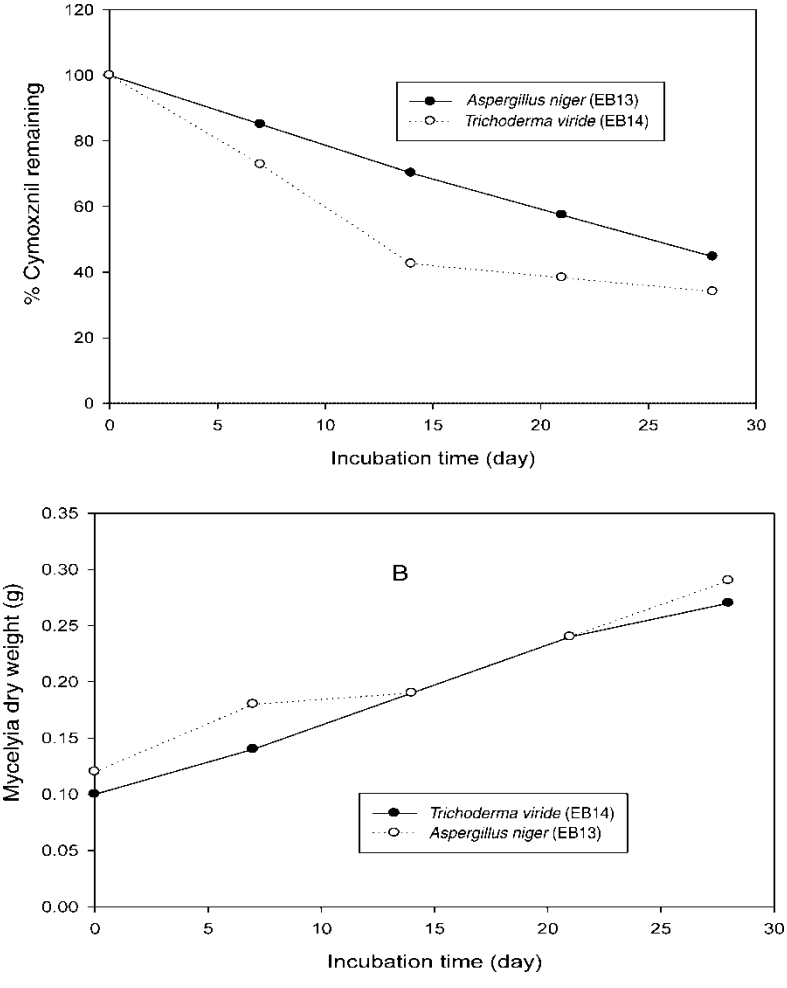

Figure 6. Biodegradation of cymoxanil by the fungal strains (A) and their growth response (B).

Table 3. Toxicity of cymoxanil solutions against *Alternaria solani* before and after 28 days of treatment with different microbial strains.

Supernatant of different cymoxanil degrading strains	% of inhibition against *Alternaria solani*
Pseudomonas sp. (EB11)	0
Streptomyces sp. (EB12)	10
Aspergillus niger (EB13)	50
Trichderma viride (EB14)	40
Control (cymoxanil only)	100

The results in Table 3 showed that the supernatant of cymoxanil after 28 days of incubation with *Pseudomonas* sp. (EB11) had no antifungal activity and could be detected against *A. solani* as a test organism. However, the inhibition percentages of *A. solani* growth by cymoxanil aqueous supernatants treated with *Streptomyces* sp. (EB12), *Trichoderma viride* (EB14) and *Aspergillus niger* (EB13), were 10, 40 and 50%, respectively. The obtained results were compared with control treatment which was cymoxanil only against *A. solani* without any tested microbial strains at

same conditions. This implies that the aqueous solution spiked with cymoxanil was completely detoxified after 28 days of treatment with *Pseudomonas* sp. (EB11) strain. The bioassay test confirms also the aforementioned obtained results with analysis by using HPLC.

4. Conclusion

The tested bacterial strains showed high ability in cymoxanil degradation. No toxicity of cymoxanil was found in the aqueous media after 28 days of treatment with *Pseudomonas* sp. (EB11) strain. The losses in cymoxanil mainly related to the degradation by the tested strains and the possibility for adsorption on the biomass not significant due to the high water solubility and low adsorption coefficient of cymoxanil. This study considered the first report in cymoxanil biodegradation and the key step for further studies in the detoxification of cymoxanil. Moreover, the isolation of the tested microorganisms from cucumber leaves could lead to the significant reduction of cymoxanil residues to the maximum residue limits especially in vegetables crops under green house conditions.

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