

Biodegradation α -endosulfan and α -cypermethrin by *Acinetobacter schindleri* B7 isolated from the microflora of grasshopper (*Poecilimon tauricola*)

Ozlem Gur Ozdal (✉ ozlemgur55@gmail.com)

Ataturk University <https://orcid.org/0000-0003-3460-3638>

Omer Faruk ALGUR

Ataturk University

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Abstract

Extensive applications of pesticides have led to the contamination of ecosystem. Therefore, the isolation of new pesticide degrading bacteria is important. For the biodegradation of α -endosulfan and α -cypermethrin, new bacteria isolates were isolated from grasshopper (*Poecilimon tauricola*). According to different tests, these isolated bacteria were identified as *Pseudomonas aeruginosa* B5, *Acinetobacter johnsonii* B6, *Acinetobacter schindleri* B7, *Bacillus megaterium* B9 and *Brevibacillus parabrevis* B12. The first two of these bacteria have been isolated as those that can use only α -endosulfan and the last two only use α -cypermethrin. Moreover, *A. schindleri* B7 was determined to be able to degrade both pesticides. When glucose was added to non-sulfur medium containing α -endosulfan (100 mg/L) and minimal salt medium containing α -cypermethrin (100 mg/L), both pesticide degradation and bacterial growth was increased. As a result, *A. schindleri*, a new gram negative bacterium, can inevitably be used in the biological treatment of environments exposed to pesticides.

Introduction

Pesticides are used against pests in order to obtain more products and their use is increasing day by day. These chemicals, which are toxic to the environment, are widely used for the protection of stored products outside agricultural areas, against insects and weeds damaging forest trees (Jiang et al. 2019; Gao et al. 2020). According to their chemical structure, the most important pesticide groups are carbamates, organochlorinates, organophosphorus and synthetic pyrethroids. Endosulfan is an insecticide in the cyclodine group of organochlorinated pesticides (Ozdal et al. 2016). The use of endosulfan is prohibited in many countries and in our country. However, studies have shown that endosulfan is produced illegally and continues to be used out of control (Gao et al. 2020). Endosulfan and disintegration products have been identified in atmospheres, soil, food products, groundwater and surface waters. It can remain intact in nature for a long time and threatens the environment and public health. It has immunosuppressive, neurotoxicity, hepatotoxicity, respiratory toxicity, reproductive toxicity, and mutagenic effects in mammals (Sebastian Raghavan 2017; Ahmad 2020; Nazir et al. 2021). Cypermethrin is commercially available synthetic pyrethroid and generally used to control insects and pests in many environments. Synthetic pyrethroids usually have less toxicity for organisms than organophosphate and organochlorines. However, they have negative effects on the environment and human health due to their high rate of use (Gur et al. 2014; Aguilá-Torres et al. 2020).

The breakdown of pesticide in nature can be biotic or abiotic conditions. Pesticide is biodegradable in nature, this is a result of microorganism activities. Endosulfan can be used by microorganisms as a source of carbon and/or sulfur (Mudhoo et al. 2019; Rodriguez-Peña et al. 2020). Also, α -cypermethrin as the sole carbon source for growth and energy metabolism can be used by microorganisms (Gur et al. 2014; Ramya and Vasudevan 2020).

Insects are the richest species in living organisms. Bacteria compose a significant portion of the microbial flora of insects. The symbiotic interactions between microorganisms and insects can be in very

different ways (defend against pathogens, produce enzymes that aid digestion, decomposition of harmful compounds) (Paniagua Voirol et al. 2018). Insect microflora forms a suitable habitat for microorganisms that produce some important enzymes. The effects of insect microflora, especially in the transport of pathogen and antibiotic resistant microorganisms have been investigated (Pai et al. 2005). Insect guts provide a suitable medium for gene transfer between bacteria. Microorganisms can adapt to new environments by acquiring different characteristics with horizontal gene transfer, conjugative plasmids and simple mutations (Itoh et al. 2018; Pietri et al. 2018; Ramakrishnan et al. 2019). According to this information, it is highly possible to isolate pesticide resistant microorganisms from insect intestines.

Generally, bacterial strains such as *Pseudomonas*, *Bacillus*, *Rhodococcus*, *Stenotrophomonas*, *Alcaligenes*, *Serratia* and *Streptomyces* have been used in the breakdown of many pesticides (John et al. 2016; Ozdal et al. 2016; Gaonkar et al. 2019; Jiang et al. 2019). *Acinetobacter schindleri* is a Gram negative, nonmotile, coccobacilli, nonspore forming and aerobic bacterium. *A. schindleri* can be found in different environments and is seldomly used for biodegradation of pesticides. Some studies indicate that *A. schindleri* can biodegrade different chemicals such as polyhydroxyalkanoates (Boyandin et al. 2012), organic pollutants decachlorobiphenyl (Zenteno-Rojas et al. 2019) and acetate (Sigala et al. 2017). The aim of this study is to biodegrade α -endosulfan and α -cypermethrin using *A. schindleri* B7 isolated from insect flora and to determine their degradation products.

Materials And Methods

Chemicals

Pesticides (α -endosulfan and α -cypermethrin) and their degradation products were of analytical purity and they were dissolved in acetone.

Isolation of endosulfan degrading microorganisms

Grasshoppers (*Poecilimon tauricola* Ramme 1951, Orthoptera) were taken from farmland (Uzunyayla Village, Erzurum, Turkey) contaminated with pesticide. The grasshoppers were individually placed in sterile capped vials containing 70% ethanol and gently shaken for 3 minutes and then washed several times with sterile distilled water for surface sterilization (Okay et al. 2013; Ozdal et al. 2016). After alcohol removal with sterile physiological water (SFS), they were crushed in a sterile mortar with SFS and homogenized. Serial dilutions of homogenate were prepared and 0.1 mL of liquid was added into non-sulfur medium (NSM) containing α -endosulfan (100 mg/L) and minimal salt medium (MSM) containing α -cypermethrin (100 mg/L). Cultures incubated for 7 days at 30 °C and 150 rpm were transferred to the media under the same conditions with 0.1 mL and this process was repeated every 7 days and continued for 21 days. Then, 0.1 ml of the enrichment culture was taken and inoculated on the prepared solid NSM and MSM. The petri dishes were incubated at 30 °C and at the end of 72 hours, the colonies of all different characteristics, primarily those that grow densely, were transferred to TSA medium and isolated. To prepare solid media, agar (15 g/L) was added to NSM and MSM. All media were sterilized at 121 °C

for 15 minutes and after cooling to 45 °C, α-endosulfan and α-cypermethrin were added. The contents of NSM and MSM are given in Table 1.

Identification of endosulfan degrading microorganisms

Species growing on NSM agar containing α-endosulfan and MSM agar containing α-cypermethrin were defined by the morphology and biochemical methods. Isolated bacteria were also identified according to fatty acid methyl ester (FAME) profiles using the MIS software package (Ozdal et al. 2016). The isolate that degraded both pesticides was determined by 16S rDNA analysis (Gur et al. 2014).

Preparation of uniform bacterial inoculum

Tryptic Soy Broth was used to reproduce bacteria. Bacteria grown in broth medium were centrifuged for 5 minutes at 5000 rpm, washed several times with 0.9% sterile physiological water and adjusted to optical density (OD_{600}) of 0.5.

Biodegradation of α-endosulfan and α-cypermethrin by *Acinetobacter schindleri* B7

100 mg/L α-endosulfan and α-cypermethrin were added into 100 mL of NSM and MSM in 250 mL conical flasks, and 1 mL of inoculum (OD_{600} 0.5) was inoculated (Ozdal et al. 2017). The flasks were shaken at 150 rpm at 30 °C for 10 days and a pH of 8.0. The effects of glucose on bacterial growth, biodegradation of pesticides and degradation products were also investigated.

Analytical methods

HPLC was used for the determination of pesticides (α-cypermethrin and α-endosulfan) and their biodegradation products. At the end of certain incubation periods, the samples were extracted with ethyl acetate (1:1, v/v). Upper phases were dried with sodium sulfate anhydrous, and concentrated with a rotary evaporator. The biodegradation samples of α-endosulfan were analyzed by HPLC with ODS C18 Hypersil Column (250 × 4.6 mm, 5 µm) equipped with UV–VIS detector at 214 nm. A mixture containing acetonitrile/water (70:30, v/v) was used as the mobile phase (Ozdal et al. 2017). The retention times were as follows: α-endosulfan, 5.323 min; endosulfan sulfate, 7.873 min; endosulfan ether, 8.976 min; endosulfan lactone, 10.035 min; endosulfan diol, 12.086 min, respectively. The biodegradation samples of α-cypermethrin were analyzed by HPLC using a SUPELCOSIL C18 DB column (250 × 4.6 mm, 5 µm) with a acetonitrile:water (85:15) mobile phase. The solutes were detected using a UV-Vis detector at 235 nm (Gur et al. 2014). The retention times were as follows: α-cypermethrin, 4.603 min; 3-phenoxybenzoic acid, 2.119 min; phenol, 8.298 min; 3-phenoxybenzaldehyde, 2.273 min; cis,cis-muconic acid, 3.573 min, respectively. Bacterial growth was determined spectrophotometrically at 600 nm.

Results

Five bacteria capable of breaking down the α-endosulfan and/or α-cypermethrin were successfully isolated from NSM-agar or MSM-agar plates (Table 2). Among these 5 bacterial isolates, 3 were Gram-

negative. All the isolates were catalase positive. Of these isolates, 2 were oxidase positive, 4 were rod and 1 was cocobacilli. According to biochemical test and physical properties, α -endosulfan degrading isolates were identified as *Pseudomonas aeruginosa* B5, *Bacillus megaterium* B9, *Acinetobacter schindleri* B7 and α -cypermethrin degrading isolates were identified as *Acinetobacter johnsonii* B6, *Acinetobacter schindleri* B7 and *Brevibacillus parabrevis* B12. *A. schindleri* B7, which can use both pesticides among the isolated bacteria, was used in subsequent studies.

Genetic analysis of *A. schindleri* B7 strain causing the α -endosulfan and α -cypermethrin biodegradation was performed. A 910-bp 16S rRNA sequence of the strain was screened with BLAST search program of GenBank's. The nucleotide sequence was registered at GenBank with accession number KC453989. A phylogenetic tree was shown in Figure 1. According to these results, this isolate was identified as *A. schindleri* strain B7.

It was determined that *A. schindleri* B7 utilized α -endosulfan as sole sulfur and carbon source, and α -cypermethrin as the sole carbon source. In different NSM (α -endosulfan + glucose, α -endosulfan, and glucose), bacterial growth were significantly altered. Further bacterial growth (OD_{600} 1.74) was determined when only glucose was added to NSM. Bacterial growth in the presence of α -endosulfan and glucose was 1.58 (OD_{600}), and it decreased up to 0.61 (OD_{600}) when glucose was removed (Figure 2a).

In different MSM (α -cypermethrin + glucose, α -cypermethrin, and glucose), bacterial growth were significantly changed. Maximum bacterial growth (OD_{600} 1.75) was achieved with the supplement of glucose to MSM. Bacterial growth in the presence of cypermethrin and glucose was 1.57 (OD_{600}) and dropped to 0.59 (OD_{600}) when glucose was not added (Figure 2b).

The percentage of biodegradation of α -endosulfan and α -cypermethrin was determined with HPLC on the tenth day. It was observed that the α -endosulfan biodegradation rate for *A. schindleri* B7 was different at the same incubation period when inoculated in NSM supplemented with or without glucose. As seen in Figure 3a, the addition of glucose increased biodegradation of α -endosulfan. In the absence of glucose, the α -endosulfan biodegradation efficiency of *A. schindleri* B7 was 59.74% at the end of 10 days. The addition of 1 g/L of glucose increased α -endosulfan biodegradation efficiency to 67.31%, which corresponds to an increase of 12.72%. As shown in Figure 3a, endosulfan diol, endosulfan ether and endosulfan lactone were defined as degradation products in the biodegradation of α -endosulfan by *A. schindleri* B7. Intermediate products ratios increased when glucose was added to NSM. Endosulfan diol was determined as the main metabolite in both media. Also, endosulfan sulfate, a toxic metabolite, was not formed in both media.

As seen in Figure 3b, it was determined that *A. schindleri* B7 biodegraded α -cypermethrin 68.4% in glucose-added medium and 59.53% in glucose-free medium. The addition of glucose (1 g/L) increased the α -cypermethrin biodegradation efficiency to 68.4%, corresponding to an increase of 14.90%. As a result of α -cypermethrin biodegradation, in the glucose containing medium, more muconic acid, 3-

phenoxybenzoic acid and 3-phenoxybenzaldehyde were formed compared to the medium without glucose. But, less phenol was formed in glucose containing medium.

When Figure 2 and Figure 3 are analyzed together, presence of glucose enhanced the growth of the *A. schindleri* B7 and biodegradation of α -endosulfan and α -cypermethrin. The increase in pesticides biodegradation was determined to be related to the increase in bacterial density.

Discussion

Insects are in close interaction with bacteria (Jahnes and Sabree 2020; Ozdal and Algur 2020). Therefore, insects are the source for the isolation of many industrially important microorganisms. *Serratia marcescens* MO-1 isolated from grasshopper has both chitinase activity (Okay et al. 2013) and the ability to produce prodigiosin pigment (Kurbanoglu et al. 2015) which has antimicrobial, antioxidant and anticancer properties. Also, *Pseudomonas aeruginosa* OG1 isolated from cockroaches (*Blatta orientalis*) can produce pyocyanin pigment (Ozdal, 2019) and rhamnolipid biosurfactants (Ozdal et al. 2017b). Similarly, the digestive systems of different animal groups are also rich in microorganisms (Das et al. 2014).

In this study, α -endosulfan and α -cypermethrin degradation was achieved by utilizing bacteria isolated from grasshopper microflora (Table 2). These bacteria were identified as *P. aeruginosa* B5, *A. schindleri* B7, *B. megaterium* B9, *B. parabrevis* B12 and *A. johnsonii* B6. These species have previously been used in the studies of degradation of different toxic compounds (Ozdal et al. 2016; Ozdal and Algur 2020).

In recent years, bacteria isolated from insect flora have been indicated to be a significant source to biodegradation of pesticide (Table 3). Isolation of microorganisms that break down pesticides is made from different environments where pesticides are used (Gaonkar et al. 2019). Many studies have shown that pesticide-resistant insects' microbiota provides a favorable environment for the isolation of potential microorganisms for biodegradation against specific pesticides (Ozdal et al. 2016; Itoh et al. 2018; Pietri et al. 2018; Pietri and Liang 2018). Some symbiotic microorganisms mediate the detoxification of pesticides, providing pesticide resistance to their hosts (Cheng et al. 2017).

It has been known that a carbon source (at low concentrations) other than the target chemical affects the rate of degradation of organic compounds. Of course, the presence of a carbon source in the growth medium significantly affects bacterial metabolism. According to results, the addition of glucose accelerated the growth of the bacteria as well as the biodegradation process (Figure 2). The increase in biodegradation of the pesticide was found to be due to the increase in cell concentration (Gur et al. 2014; John et al. 2016).

Endosulfan is generally biodegraded by oxidation and hydrolysis pathways. In the oxidation pathway, endosulfan sulphate is formed. In the hydrolysis pathway, endosulfan diol, endosulfan ether and endosulfan lactone, which are less toxic than endosulfan, are formed (Mudhoo et al. 2019).

Microorganisms that produce endosulfan sulphate are not suitable for bioremediation applications

because endosulfan sulphate is very toxic to organisms. Some microorganisms degrade endosulfan through oxidation by forming endosulfan sulphate. It is thought to be very valuable that bacterial species carrying the hydrolytic pathway for degradation of endosulfan. Many bacteria (*Pseudomonas*, *Rhodococcus*, *Stenotrophomonas*, *Ochrobacterum* and *Alcaligenes*) have been found to use the hydrolytic pathway for endosulfan degradation (Mudhoo et al. 2019). Ozdal et al. (2017) reported the biodegradation of α-endosulfan by *Stenotrophomonas maltophilia* OG2 (isolated from the cockroaches, *Blatta orientalis*) with the degradation efficiency of 81.5% in 10 days. α-endosulfan was converted to endosulfan diol, endosulfan ether and endosulfan lactone by bacterial isolate OG2. According to these results, *A. schindleri* B7 breaks down the α-endosulfan by hydrolysis (non-oxidative) which is less toxic pathway (Figure 3a).

1. *schindleri* B7 degraded α-cypermethrin to 3-phenoxybenzaldehyde, 3-phenoxybenzoic acid, phenol and muconic acid, respectively (Figure 3b). Similar results were reported for *Micrococcus* sp. strain CPN 1 (Tallur et al., 2008) and *S. maltophilia* starin OG2 (Ozdal et al. 2014). It can be said that the α-cypermethrin biodegradation pathway in *A. schindleri* B7 is similar to that of these researchers. According to α-cypermethrin biodegradation pathway, *A. schindleri* B7 can degrade harmful metabolites such as 3-phenoxybenzoic acid and phenol.

In conclusion, it was determined that pesticide-degrading new bacteria can be isolated from insects and used in biodegradation studies. The newly isolated *A. schindleri* B7 break down the α-endosulfan in a less toxic hydrolytic route. It is also important that this bacterium breaks down the synthetic pyrethroid α-cypermethrin. This isolated bacterium may be important in the microbiological treatment of pesticides contaminated environments. Many insects living in pesticide contaminated environments should be investigated to isolate new pesticide-degrading microorganism.

Declarations

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Authors' contributions

OGO performed the study design, development and methodology, the collection of all data, and the data analysis/interpretation, and wrote all the manuscript. OFA performed the study design and data analysis.

Conflict of interest

The authors declare that they have no conflict of interest.

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Tables

Table 1. Composition of non-sulfur medium (NSM) and minimal salt medium (MSM) (pH = 8)

	NSM	MSM
a (g/L)		
K ₂ HPO ₄	0.225	
KH ₂ PO ₄	0.225	1.5
NH ₄ Cl	0.225	
MgCl ₂ .6H ₂ O	0.845	
CaCO ₃	0.005	
FeCl ₂ .4H ₂ O	0.005	
(NH ₄) ₂ SO ₄		2
MgSO ₄ .7H ₂ O		0.2
CaCl ₂ .2H ₂ O		0.01
FeSO ₄ .7H ₂ O,		0.001
Na ₂ HPO ₄ .12H ₂ O		1.5
b (mg/L)		
MnCl ₂ .4H ₂ O	198	198
ZnCl ₂	136	136
CuCl ₂ .2H ₂ O	171	171
CoCl ₂ .6H ₂ O	24	24
NiCl ₂ .6H ₂ O	24	24

Table 2. The morphological and biochemical characteristics of bacterial isolates of *Poecilimon tauricola*

Code	B5	B6	B7	B9	B12
MIS results	<i>Pseudomonas aeruginosa</i>	<i>Acinetobacter johnsonii</i>	<i>Acinetobacter schindleri</i>	<i>Bacillus megaterium</i>	<i>Brevibacillus parabrevis</i>
Pigment	Blue-green	-	-	-	-
Shape	Rod	Rod	Coccobacilli	Rod	Rod
Gram	-	-	-	+	+
Catalase	+	+	+	+	+
Oxidase	+	-	-	-	+
α ES	+	-	+	+	-
α CP	-	+	+	-	+

Table 3. Bacteria isolated from insect flora for degradation of different pesticides

Pesticide	Insect	Bacteria	References
Fenitrothion	Bean bug, <i>Riptortus pedestris</i>	<i>Burkholderia</i>	Kikuchi et al. 2012
α-endosulfan, α-cypermethrin	<i>Blatta orientalis</i>	<i>Pseudomonas, Stenotrophomonas, Bacillus, Citrobacter, Acinetobacter</i>	Gur et al. 2014
α-endosulfan	<i>Saga ephippigera</i>	<i>Acinetobacter Iwoffii, Pseudomonas aeruginosa, Stenotrophomonas maltophilia</i>	Gur Ozdal et al. 2016
α-endosulfan	<i>Forficula auricularia</i>	<i>S. maltophilia, Brevibacillus choshinensis</i>	Gur Ozdal et al. 2016
α-endosulfan	<i>Sphodromantis viridis</i>	<i>Stenotrophomonas maltophilia, Acinetobacter calcoaceticus, Pseudomonas aeruginosa</i>	Gur Ozdal et al. 2016
α-endosulfan	<i>Gryllus bimaculatus</i>	<i>Pseudomonas aeruginosa, Bacillus megaterium, Flavimonas oryzihabitans,</i>	Gur Ozdal et al. 2016
Chlorpyrifos, deltamethrin, lamda-cyhalothrin, spinosad, and luferunon	Armyworm, <i>Spodoptera frugiperda</i>	<i>Enterococcus casseliflavus, Enterococcus mundtii, Microbacterium paraoxydans, Pseudomonas stutzeri, Staphylococcus sciuri</i>	de Almeida et al. 2017
Trichlorphon	Oriental fruit fly, <i>Bactrocera dorsalis</i>	<i>Citrobacter freundii</i>	Cheng et al. 2017
Acephate, Imidacloprid, thiamethoxam, buprofezin, etofenprox	Brown planthopper <i>Nilaparvata lugens</i>	<i>Morganella, Weissella, Enterococcus</i>	Malathi et al. 2018
Imidacloprid, chlorpyrifos methyl, methomyl, cyantraniliprole and spirotetramat	Pea aphid <i>Acythosiphon pisum</i>	<i>Serratia symbiotica</i>	Skaljac et al. 2018
Deltamethrin	Grasshopper <i>Poecilimon tauricola</i>	<i>Pseudomonas aeruginosa, Enterobacter intermedium, Serratia marcescens, Bacillus atropheaeus</i>	Gur Ozdal and Algur 2020

Figures

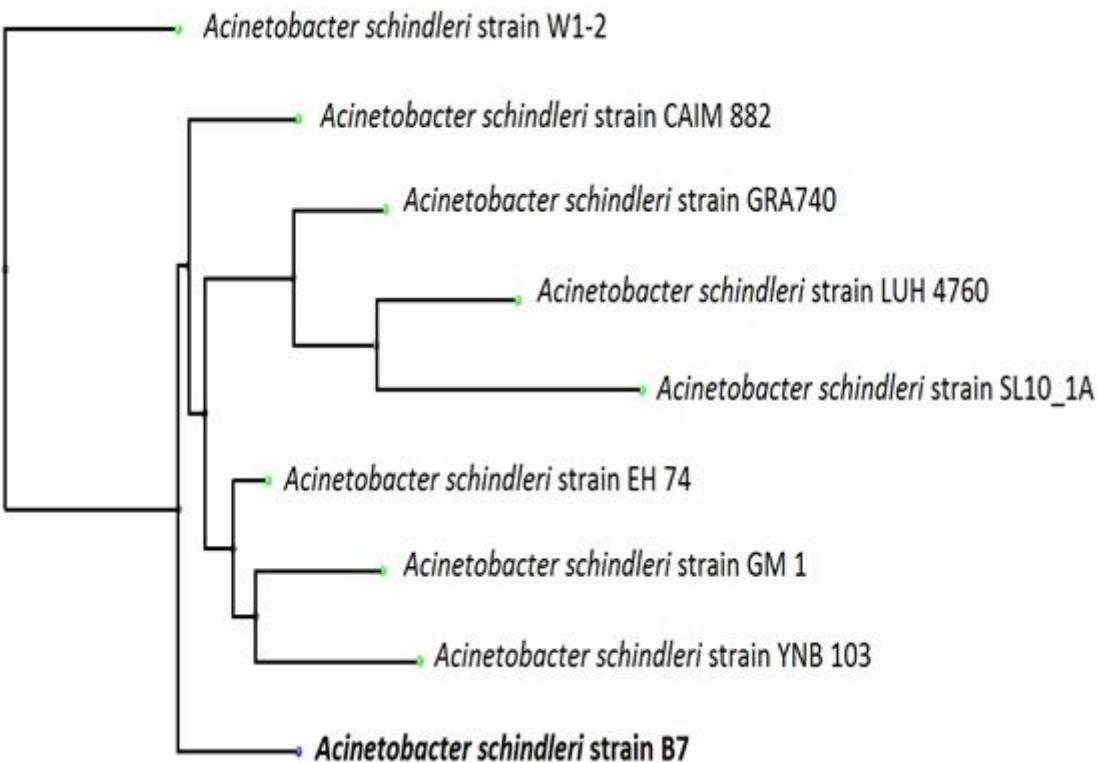


Figure 1

Phylogenetic tree depending on the 16S rRNA sequences of strain B7 and related species

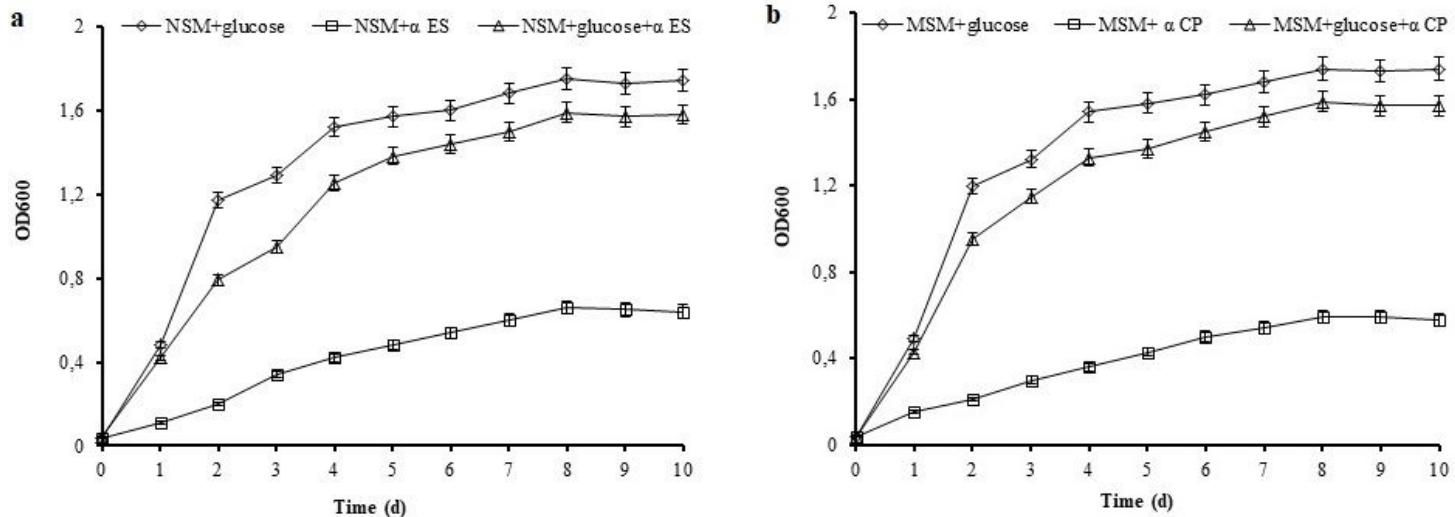


Figure 2

Effects of glucose addition on the growth of *A. schindleri* B7 in the presence and absence of α -endosulfan (a) and α -cypermethrin (b)

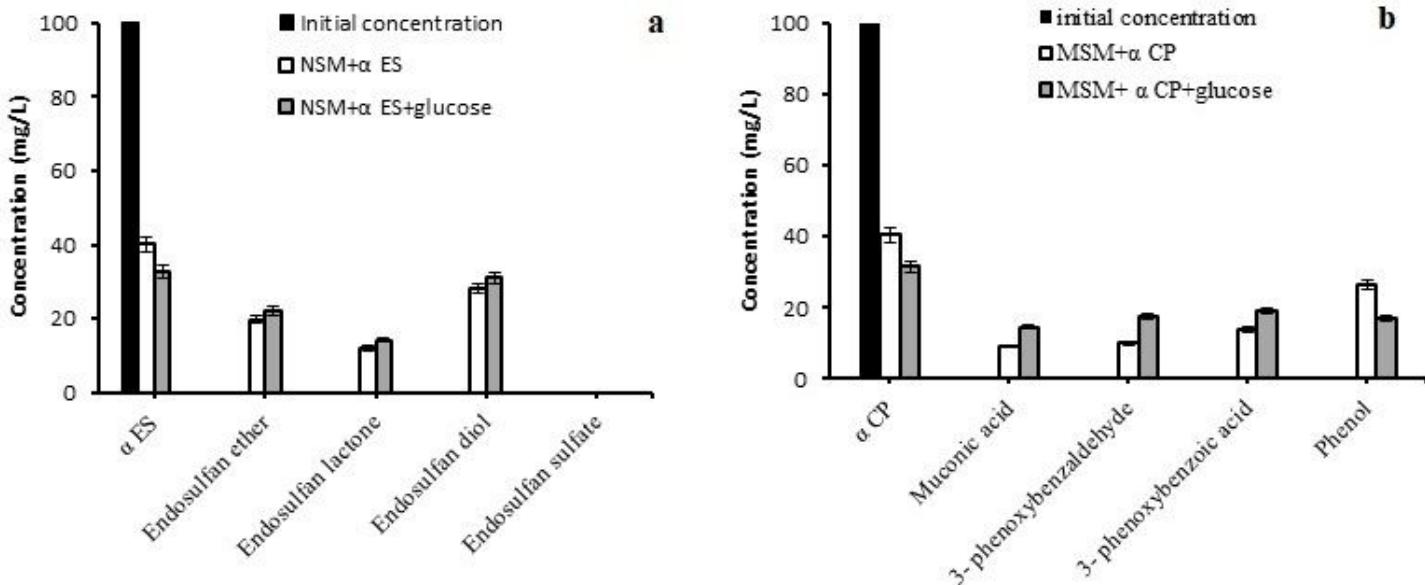


Figure 3

Biodegradation of α -endosulfan (a) and α -cypermethrin (b) in the presence and absence of glucose and its metabolites at 10 days.

Supplementary Files

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- Graphicalabstract.png