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ISOLATION, CHARACTERIZATION AND GROWTH ASSESSMENT OF BIODEGRADING CHLORPYRIFOS-METHYL *BACILLUS* SPECIES ISOLATED FROM ALGERIAN SOIL

Key words: chlorpyrifos-methyl, Algerian agriculture lands, *Bacillus* genus, bacterial growth, bioremediation, soil pollution

Introduction

Pesticides incorporate a broad spectrum of chemicals, including insecticides, fungicides, molluscicides, rodenticides, nematicides, plant growth regulators, weedicides, etc. (Aktar, Sengupta & Chowdhury, 2009). The increasing exponential growth of the world's population is generating a huge use of pesticides in the world to fulfill the augmenting needs for food and fibers (Foong et al., 2020). Agricultural yield has been rising due to the application of pesticides, controlling pests, diseases and helping to keep many dreaded diseases away from society (Naik & Prasad, 2006). However, the overuse of pesticides is proving to be one of the major obstacles to sustainable agricultural production (Agarwal & Pandey, 2017). At present, organophosphates (OPs) are the most widely used pesticides in agriculture due to their lower persistence in the environment and higher efficacy compared to organochlorine and carbamate pesticides (Das & Adhya, 2015; Foong et al., 2020).

Organophosphates are mainly esters, thiols, or amide derivatives of phosphoric or phosphoramidic acids with two organic groups and an additional side chain (Kumar, Kaushik & Villarreal-Chiu, 2016). On the whole, OPs account for more than 36% of the total worldwide sales market (Briceño et al., 2012).

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Chlorpyrifos-methyl (o,o-dimethyl-o--(3,5,6-trichloro-2-pyridyl) phosphorothioate, is a broad-spectrum organophosphate insecticide that is widely used in pest control (Choi & Lee, 2016; Szpyrka, Matyaszek & Słowik-Borowiec, 2017). Although chlorpyrifos-methyl insecticides as well as other OPs are relatively less persistent in the environment and less toxic to non--target animals, their over application and high use frequency can lead to high levels of OP and chlorpyrifos-methyl residues on agricultural products including agricultural crops/fruits, pond/canal water/wells, and many others, which could have negative environmental and public health effects (Matthews, 1990; Abbassy, Salim, Shawir & Nassar, 2017; Bose, Kumar & Vo, 2021). These issues emphasize the necessity of removing chlorpyrifos-methyl residues from contaminated soils, water systems, and crops. Microbial degradation has been found to be the most prominent bioremediation method, as it is the major factor impacting chlorpyrifos--methyl degradation (Kim & Ahn, 2009).

Algeria is currently classified among the countries that use huge quantities of pesticides. There are about 400 registered phytosanitary products in Algeria, of which about 40 varieties are widely used by farmers (Bouziani, 2007). Unfortunately, the determination of the level of contamination of natural compartments is not done systematically. Analyses carried out on water samples taken in the region of Staouli (Algiers Province) and Annaba have shown that in more than 30% of the samples, the concentration of certain organochlorine and organophosphorus molecules exceeds the values recommended by the World Health Organization (WHO). The national use of phytosanitary products in agronomy makes fear a massive pollution of soils, surface waters, water tables and all physical environments in all regions of the country, leading to serious health problems.

Among the remediation technologies proposed for contaminated ecosystems, bioremediation is recognized as one of the most prospective approaches. It is a comparatively inexpensive, simple to use, and environmentally friendly technique (Rayu, Karpousas & Singh, 2012; Huang et al., 2021). The use of microorganisms with the right metabolic pathways is one of the most sustainable alternatives for chlorpyrifos-methyl remediation. To date, a few chlorpyrifos-methyl-degrading bacteria have been isolated from the environment, but studies of these bacteria generally focus only on the concentration of chlorpyrifos-methyl in the biodegradation process, and there is minimal understanding of degradation intermediates and degradation mechanisms (Rayu, Nielsen, Nazaries & Singh, 2017). Excessive use of the pesticide and its accumulation in the soil can changes the physicochemical properties of soil.

The aim of this study is to isolate indigenous bacterial strains from agricultural soils located in northeastern Algeria, and to assess their ability to grow in the presence of chlorpyrifos-methyl as the sole source of energy and carbon. Thus, their growth would make it possible to consider the use of high-performance strains as a means of bioremediation and biodegradation.

Material and methods

Soil sampling

For this work, three experimental agricultural stations, where they were exposed to the application of CM for long

time periods, were selected to isolate CM degrading strains. Samples were collected in the top layer of 0–25 cm from each station. These sites were located at the Technical Institute of Vegetable and Industrial Crops (TIVIC), El Tarf Province located in the far North-East of Algeria. Samples that served for microbiological analysis were collected in sterilized Erlenmeyer of 500 ml capacity, and then conserved at 4°C till use.

Isolation and, identification of soil indigenous bacterial strains

The technique for isolating soil autochthonous bacteria was performed as described by (Aswathi, Pandey & Sukumaran, 2019). Ten grams of each soil sample was added to 90 ml of sterile physiological water and the suspension was gently agitated (150 rpm) at room temperature for approximately 30 min. Decimal dilutions of 10^{-1}–10^{-6} were prepared using sterile physiological water. One-milliliter aliquots were transferred to sterile Petri dishes and held for 10 min before adding Luria–Bertani (LB) agar medium. Plates were incubated at 30°C for 18–24 h, and colonies were purified on LB agar.

Bacterial isolates were identified by using standard methods: morphological identification, Gram staining, oxidase, catalase as well as biochemical tests by using API 20E, API 20NE, API Staph galleries (bioMérieux Inc., France), as well as a molecular method. The PCR amplification and sequencing of genomic DNA for 16s rRNA using universal primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′) was performed in a PCR thermocycler instrument with the following cyclic profile: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 1 min 30 s and final extension at 72°C for 5 min (Batisson et al., 2009). The determined 16S rRNA gene (partial sequence) was aligned with those available in the GenBank database.

Following multiple alignments of the sequence data using ClustalX, phylogenetic analysis was performed using the MEGA 5.0 software packages (Thompson, Gibson, Plewniak, Jeanmougin & Higgins, 1997). Phylogenetic trees were generated using the neighbor-joining method according to the maximum composite likelihood parameter model and evaluated by bootstrap analyses based on 1,000 resembling (Tamura et al., 2011).

A mineral salt medium (MSM) was employed for both strain selection and biodegradation trials of CM-degrading strains. This medium contained per liter of distilled water: 1.6 g K_2HPO_4, 1.6 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 1 g NaCl, 0.02 g $CaCl_2$, 3.5 g $(NH_4)_2SO_4$, 1 ml of $FeSO_4 \cdot 6H_2O$--EDTA. The pH was adjusted at 7.5. A solid MSM obtained by adding 15 $g \cdot l^{-1}$ agar was used to test the capacity of the strains to grow on a solid medium (Rousseaux, Hartmann & Soulas, 2001).

Screening for CM degrading-bacterium

The selection test was based on evaluating bacterial growth on MSM agar plates supplemented with a concentration of CM as the only carbon and energy source. Chlorpyrifos-methyl was sterilized by 0.45 µm membrane filtration and added to the medium to obtain the required concentrations of 25, 50, 100 and 200 $mg \cdot l^{-1}$. Plates were incubated in the dark at 30°C for 24 h to 15 days. Strains were then rated as negative and positive growth. Bacterial strains that

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had shown higher growth on MSM agar were then tested for CM biodegradation in MSM liquid media (Pankaj et al., 2016).

Bacterial growth in MSM liquid media

A suspension of the isolates of interest was made by transferring a few pure bacterial colonies into sterile physiological water. Growth assays with CM as the only carbon and energy source were performed in sterile 500 ml Erlenmeyer flasks containing 100 ml of sterile MSM broth. The CM was introduced as a stock solution to have a dose of 50 $mg \cdot l^{-1}$. The Erlenmeyer flasks were incubated at 30°C ±2°C in an incubator with shaking at 150 rpm under aerobic conditions and protected from light. Samples of the liquid medium were taken in a sterile manner at different time intervals to measure the bacterial concentration which was determined by monitoring the optical density (OD) at 600 nm using a UV spectrophotometer (Secomam Uviline, 9000). Samples without pesticides were used as abiotic controls. All measurements were performed in triplicate (Uniyal, Sharma & Kondakal, 2021).

Results and discussion

Screening and characterization of CM-degrading bacteria

Pesticides are degraded by microorganisms that utilize them as carbon sources and electron donors. However, their degradation depends on various environmental factors and on physiological, ecological, biochemical and molecular aspects. Microorganisms with the potential to degrade pesticides have been isolated and characterized in different laboratories around the world. However, the molecular technology of DNA sequencing has expanded our understanding of the mechanisms, occurrence and identification of effective microorganisms for bioremediation of polluted ecosystems (Ortiz-Hernández, Sánchez-Salinas, Dantán-González & Castrejón-Godínez, 2013).

In our research, several strains between Gram-positive and Gram-negative were isolated from the three agricultural stations. Among them, several strains had the ability to grow on MSM agar supplemented with increasing doses of CM that has been used as the sole source of carbon and energy. As a result, four bacterial strains showed the best growth capacity at 50 $mg \cdot l^{-1}$ of CM, in which the strains has been coded as and MC09 (Tables 1 and 2). Such strains were therefore selected for the growth test on liquid MSM.

TABLE 1. Results of the growth test of strains according to their development capacity on MSM agar in the presence of different doses (25, 50, 100, and 200 $mg \cdot l^{-1}$) of chlorpyrifos-methyl as a sole carbon and energy source

Tested isolate	25 $mg \cdot l^{-1}$	50 $mg \cdot l^{-1}$	100 $mg \cdot l^{-1}$	200 $mg \cdot l^{-1}$
JC415	++	+	+	-
SDP1	+++	++	+	+
SDP7	++	+	-	-
S17	+++	+++	++	+
JM56	+	-	-	-
ANT02	+	+	-	-
ANT14	+++	++	+	+
MA26	+	-	-	-
MC09	+++	++	-	-
JC41	++	+/-	-	-

Microscopic, biochemical characterizations, as well as the 16S rDNA sequencing results of the isolated strains are grouped together in Table 2, which shows the biochemical characteristics of the strains tested. Thus, with the exception of the ANT14 isolate, which is catalase negative, the other strains have the same Gram-positive, catalase-positive, oxidase-positive and positive motility characteristics. Consequently, the identification of 16S rDNA revealed various species as mentioned in Table 2.

The obtained tree was drawn with the length of the branches in the same units as those of the evolutionary distances used to deduce a phylogenetic tree. The evolutionary distances were calculated using the composite maximum likelihood method and are expressed in units of the number of base substitutions per site. All positions containing gaps and missing data were removed from the dataset. Phylogenetic analyzes were carried out with MEGA 5.0 software (Tamura et al., 2011). The identified species belong to two very close clusters (Fig. 1).

TABLE 2. Microscopic characteristics, biochemical and 16S rDNA identification of the interesting strains

Isolate	Gram	Oxydase	Catalase	Mobility	Biochemical identification	Identification 16S rDNA
SDP1	+	+	+	+	*Bacillus* sp.	*Bacillus* sp. H1-80
MC09	+	+	+	+	*B. brevis*	*Brevibacterium frigoritolerans* strain WJB99
ANT14	+	+	-	+	*B. thuringiensis*	*Bacillus* sp. strain GL5 (1st strain)
S17	+	+	+	+	*B. lentus*	*Bacillus* sp. strain GL5 (2nd strain)

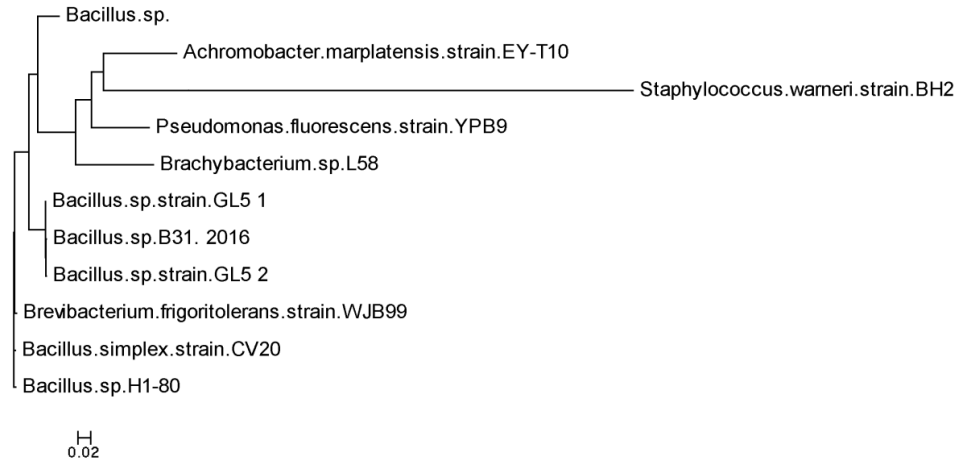

FIGURE 1. Phylogenetic tree and evolutionary relationships between the selected species

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Bacterial growth in MSM liquid media supplemented with CM

Previous studies have shown the ability of microorganisms to degrade various agrochemicals while using them as a source of carbon and nitrogen (Cycoń, Żmijowska, Wójcik & Piotrowska-Seget, 2013). Thus, the demand for eco-friendly solutions and the use of indigenous species to restore pesticide-contaminated soils is growing globally. Biodegradation is considered the best option for *in-situ* restoration operations (Abigail, Samuel & Ramalingam, 2015).

The strains of interest were identified then tested in the presence of increasing concentrations of our insecticide. In the absence of alternative carbon and energy sources, the pesticide added to the solid and then liquid MSM appeared to be degraded and assimilated by our isolates during their development.

The evolution of the strains' growth was monitored by measuring absorbance at 600 nm by spectrophotometer, over relatively long periods. Sterile samples were taken regularly in different time intervals to determine whether there is an increase or decrease in cell biomass under the experimental conditions tested. Using CM as the unique source of energy and carbon, the growth curves obtained with the *Bacillus* strains are shown on Figure 2.

Both strains of *Bacillus* sp. strain GL5 (1st and 2nd strain) reached a maximum OD after 360 and 336 h, respectively (Figs 2 and 3). This phase means high-speed acclimatization of the bacteria with their new conditions of development in the laboratory, that is, there was a real consumption of the active substance available in the medium by our microorganisms.

As we noticed, the obtained curves have a special aspect (absence of stationary phase leading to a bell shape). These isolates show a very particular and characteristic growth. Also, it was demonstrated that the maximum growth of the bacterial cells differs from one

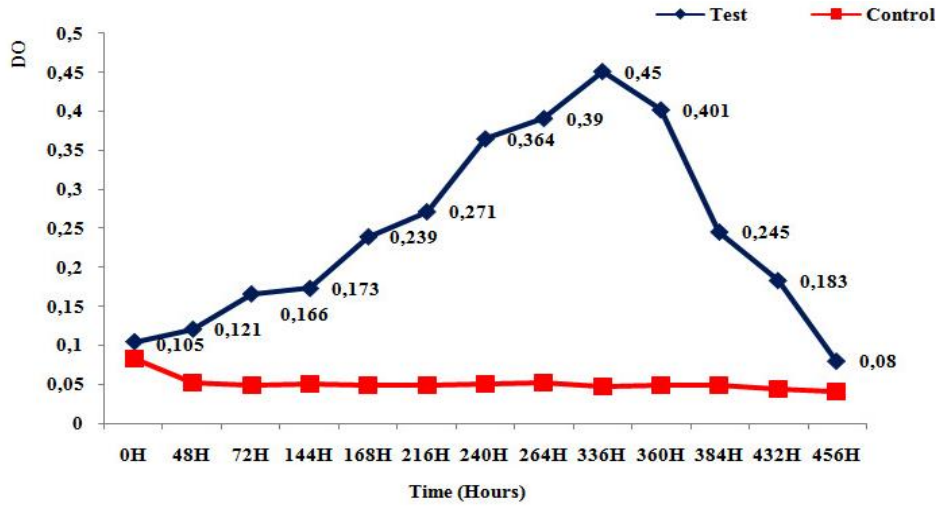

FIGURE 2. Growth of *Bacillus* sp. strain GL5 (1st strain) in the presence of 50 mg·l^{-1} of CM as the unique source of carbon and energy

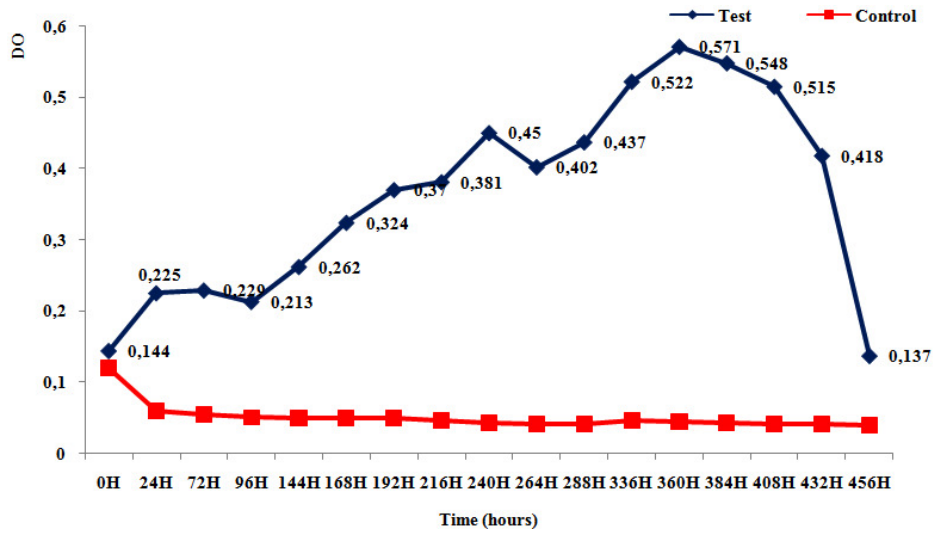

FIGURE 3. Growth of *Bacillus* sp. strain GL5 (2nd strain) in the presence of 50 mg·l^{-1} of CM as the unique source of carbon and energy

strain to another. The absence of a latent phase in the growth curves seems to justify the interesting behavior of *Bacillus* strains with CM.

The appearance of the curves revealed a clear acceleration and duplication of the cell quantities until reaching a maximum absorbance after 48 h of incubation only

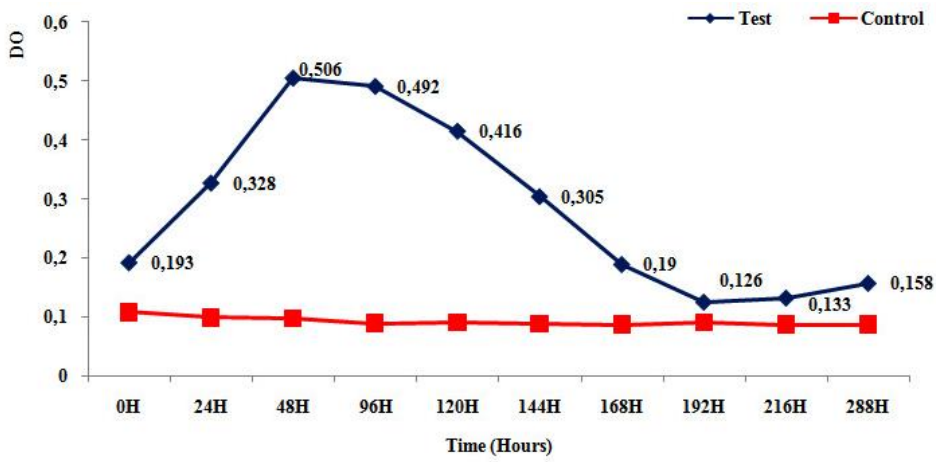

FIGURE 4. Growth of *Bacillus* sp. H1-80 in the presence of 50 mg·l^{-1} of CM as the unique source of carbon and energy

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for *Bacillus* sp. H1-80 (Fig. 4), and 72 h for *Brevibacterium frigoritolerans* strain WJB99 (Fig. 5).

Finally, a common decrease of the optical density in all the species was noted. This stage of growth corresponds to the phase of cellular decline, which is likely linked to possible cell lysis and a lack of substrate in the culture medium.

The slight decline of OD observed with *Brevibacterium frigoritolerans* strain WJB99 (Fig. 5) and *Bacillus* sp. strain GL5 (2nd strain) (Fig. 3) at the end of the 288th and 264th hour of incubation, respectively, could be explained by the production of secondary metabolites more toxic than the starting molecule, thus affecting bacterial growth. Interestingly, these curves showed that no toxic effect on the growth of bacterial cells was observed, and CM has been easily used as an energy source. It should be noted that no previous studies have been conducted on the biodegradation of CM by the bacterium *Brevibacterium frigoritolerans* strain WJB99.

Previous works have already described the resistance of chlorpyrifos to degradation (Mallick, Bharati, Banerji, Shakil & Sethunathan, 1999). Subsequently, studies identified bacteria belonging to the genera *Enterobacter* (Singh, Walker, Morgan & Wright, 2004), *Pseudomonas* (Farhan, Khan, Wahid, Ahmad & Ahmad, 2012), *Bacillus* (Liu, Chen, Shi & Su, 2012; El-Helow, Badawy, Mabrouk, Mohamed & El-Beshlawy, 2013), *Klebsiella* (Ghanem, Orfi & Shamma, 2007) were effectively exhibiting the ability to degrade chlorpyrifos. Other recent investigations state the diversity of chlorpyrifos-degrading bacteria including *Bacillus* (Anwar, Liaquat, Khan, Khalid & Iqbal, 2009). The latter could use as a source of carbon and energy and degrade 3,5,6-trichloropyridinol (TCP),

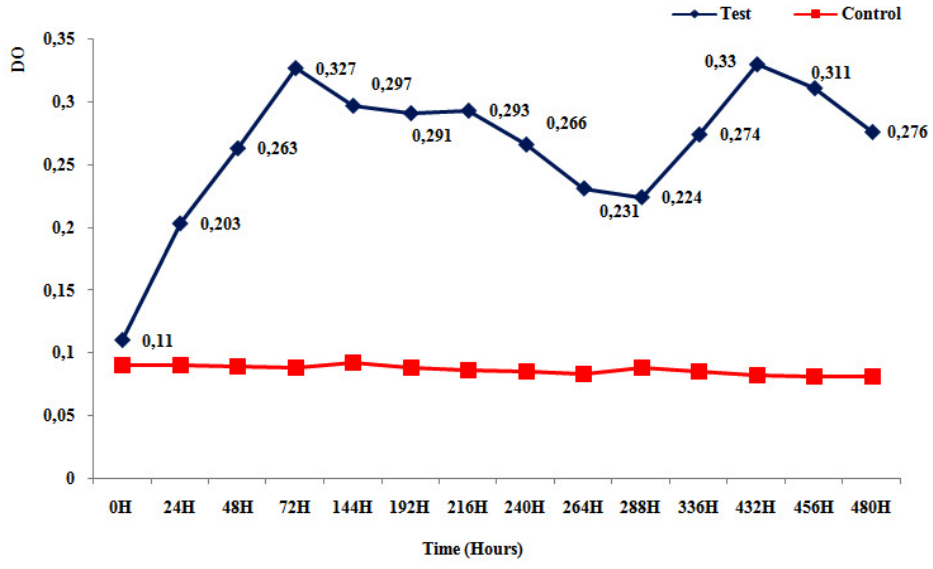

FIGURE 5. Growth of *Brevibacterium frigoritolerans* strain WJB99 in the presence of 50 mg·l^{-1} of CM as the unique source of carbon and energy

a metabolite that is more toxic than CM itself and moderately mobile in the soil.

Many reviews and works have highlighted the importance of *Bacillus* species in the biodegradation and bioremediation of pesticides, especially chlorpyrifos. Maya, Singh, Upadhyay and Dubey (2011) studied the rate of chlorpyrifos biodegradation by three microorganisms of which *Bacillus* sp. were able to remove 52% of the tested doses during ten days. Another study revealed the capability of *Bacillus cereus* to degrade up to 74% of chlorpyrifos with an initial concentration of 100 $mg \cdot l^{-1}$ (Liu et al., 2012). Furthermore, *Bacillus pumilis* C2A1 strain isolated by Anwar et al. (2009) from a cotton crop in Pakistan showed its significant ability to metabolize high doses of CM under different conditions, in addition to TCP at 100, 200, and 300 $mg \cdot l^{-1}$ corresponding respectively to 73, 83 and 87% in liquid medium for ten days.

The diversity of our data observed with the isolated microorganisms is directly linked with the capacity of the soil microflora to adapt to a given pesticide. Generally, the phenomenon of acclimatization and biodegradation occurs when a phytosanitary product is placed in contact with an agricultural plot. This is expressed by a decomposition of the active substance after a very variable duration according to the nature of the product, called the latent period. This first step represents the enzymatic induction that can be described by a physiological and/or genetic adaptation of the microbial populations in question. During a second phase, the degradation of the active substance often results in an exponential decrease of the residual concentration. In case of frequent and successive application of the same product, plus the presence of a very high density of adapted microbial strains, the duration of the latency phase will decrease or even be eliminated. In this case, in adapted soils, 80% of the applied phytosanitary product can be rapidly biodegraded in less than 15 days (Devers-Lamrani, 2008).

Conclusion

In our research, four strains of *Bacillus* degrading chlorpyrifos-methyl were isolated and identified from Algerian agricultural soils exposed to different treatments by different families of phytosanitary products. These are the strains, *Bacillus* sp. strain GL5 (1), *Bacillus* sp. strain GL5 (2), *Bacillus* sp. H1-80 and *Brevibacterium frigoritolerans* strain WJB99 which showed good growth on liquid medium containing 50 $mg \cdot l^{-1}$ of MC as sole carbon source. Depending on the duration of growth, *Bacillus* sp. H1-80 and *Brevibacterium frigoritolerans* strain WJB99 are the most efficient with respectively a maximum of OD after 48 and 72 h of growth. In perspective, optimization studies would be interesting to carry out as well as the sequencing of CM degradation genes, since our strains have not been cited in previous work. Given the high growth capacity of the strains in the presence of CM, they could be very useful for the bioremediation of soils contaminated by this insecticide.

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Summary

Isolation, characterization and growth assessment of biodegrading chlorpyrifos-methyl *Bacillus* species isolated from Algerian soil. Chlorpyrifos-methyl (CM) is a broad-spectrum organophosphate insecti-

cide, which is widely used in pest control. In this research, the isolation, and biochemical and molecular identification of bacterial strains obtained from three soils located in northeastern Algeria were carried out, as well as the evaluation of their ability to grow in the presence of CM. Out of 48 bacterial isolates between Gram-negative and Gram--positive identified, several were able to grow on mineral agar with at least 25 $mg \cdot l^{-1}$ of CM. Four bacteria showed the best growth capacity, were identified as *Bacillus* sp. H1-80, *Brevibacterium frigoritolerans* strain WJB99 and two *Bacillus* sp. strains GL5. The strains were tested for their ability to grow on liquid media with CM as the sole energy and carbon source. In general, these strains showed slow but significant growth visualized by the 600 nm turbidity control, suggesting that they could be used for bioremediation applications of CM polluted soils.