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Biodegradation of commercial Ortiva fungicide by isolated actinomycetes from the activated sludge

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ABSTRACT

Wastewater can be defined as water degraded mainly by human activities. This water must be treated before being reintroduced into the supply circuits. Wastewater from the agricultural sector can be difficult to process because of the presence of many persistent pesticides. The Ortiva fungicide has been used for several years worldwide since 1996. It is very toxic for aquatic organisms and can lead to long-term adverse effects in aquatic environments. Different biological treatment techniques are necessary to overcome the devastating effects of this pollutant. The micro-organisms present in the wastewater sludge are the major players in the suppression of these effects. In this study, we isolated nine actinomycetes strains which are able to degrade the fungicide Ortiva at a concentration of 500 mg/L and at a temperature of 30˚C within 21 d of incubation when supplied as a sole carbon source. The molecular identification by 16S rRNA was possible for only seven actinomycetes under laboratory conditions. These isolates showed a homology to *Nocardia* sp. and *Streptomyces* sp. The biodegradation ability of these strains reveals its potential for further study as a biological agent for the remediation water contaminated with Ortiva.

Keywords: Actinomycetes; Biodegradation; Ortiva; Wastewater sludge; Azoxystrobin

1. Introduction

Pesticides, which are chemicals designed to kill or control pathogens or diseases, have become more widely used in recent years and have contributed to improved crop production and income by substantially reducing the risks of disastrous crop losses [1]. Since most arable crops such as cereals are more widely cultivated and more susceptible to fungal infections, the fungicides are usually used more than any other class of pesticides [2–4].

The commercial Ortiva is a phytosanitary product which belongs to a family of a new generation of active materials developed at the example of nature. It is effective against a large number of diseases, such as *Alternaria*, powdery mildew, disease of spots, and

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rust. This product is used as a fungicide with xylem systematic action. The Ortiva, which belongs to the family of strobilurins, has become one of the most widely used fungicides [5]. The azoxystrobin ($C_{22}H_{17}N_30_5$), as one of the leading worldwide proprietary fungicides, is known to possess broad-spectrum systemic activity against the four major classes of pathogenic fungi [5]. It diffuses from the seed (zone of application) to the surrounding soil, then it is absorbed by the roots during germination to migrate once again to the air parts. The azoxystrobin is marketed under different trade names, such as Bankit, Heritage, Abound, Ortiva, and Dynasty, either as the sole fungicide-active ingredient or in combination with other active ingredients [6].

The azoxystrobin like other strobilurins, such as fluoxastrobin, kresoxim methyl, picoxystrobin, pyraclostrobin, and trifloxystrobin, possess a high antifungal activity but are relatively inert to mammals [7]. The strobilurins function inhibits mitochondrial respiration by binding to the cytochrome b complexes. This binding prevents electron transfer from cytochrome b to c and inhibits energy production via oxidative phosphorylations. The ATP inhibition may result in the death of the micro-organism [5–8].

The azoxystrobin was announced as being very promising molecule because of the extent of their action spectrum and the absence of phytotoxicity. However, the strobilurins cause resistance problems [8].

The azoxystrobin has been reported to have a half-life in soil of less than 14 d [9], but this contradicts with some recent findings which suggest that they persist in soils for up to six months with water solubility from 13 to 121 mg/L [10–12]. Whilst, ecotoxicological testing has established that the azoxystrobin is toxic to freshwater and marine invertebrates and also fish [9], some studies by [13–15] have shown many environmental and health problems because of the continuous and excessive strobilurins use. Other works have studied the azoxystrobin ecological effects on microcosms of brackish water and have found that the azoxystrobin is toxic to copepods of these waters in a much lower concentration of 3 g/L, than previously reported about the monospecific tests made with crustaceans of fresh water [16]. The azoxystrobin and its degradation product R234886 can infiltrate into the loamy soil for a long period of time after the application of pesticide and therefore constitute a potential threat to aquatic vulnerable and drinking water resources [17].

The biodegradation of pesticides is an area of increasing importance because of the enormous potential of micro-organisms to clean up the environment of these dangerous xenobiotics [18,19]. The micro-organisms of wastewater treatment plants are among the main actors of the removal of the harmful effects of these pollutants. The *Actinobacteria* are known by their capacities of biodegradation of most of the polymers. According to the reports of the EFSA [20]; EFSA [15]; and US EPA [9], no study concerning the biodegradation of the azoxystrobin by pure cultures is available. The literature reports some works about the effects and desperation of the azoxystrobin in microcosms of soil and water [21–23]. Consequently, the aim of this study is to isolate and characterize the actinomycetes present in the activated sludge from a wastewater treatment plant. The study also investigates the possibility of these bacteria to grow on a minimal medium supplemented with Ortiva as the sole source of carbon and energy. This work is of a certain importance and can offer micro-organisms capable of being used in systems of cleanup of the environment, such as the bioremediation of the aquatic environments contaminated by this kind of pesticide.

2. Materials and methods

2.1. Sampling

The samples used in this study came from an activated sludge taken from the wastewater treatment station of Ibn Ziad, town of Constantine (Algeria). The samples were taken from the biological basin after an aeration phase. One hundred milliliters were recovered in a sterile flask of 250 mL of volume.

2.2. Isolation of actinomycetes

From the original solution of sludge, a series of decimal dilutions are made, going up to 10^{-5}. For each dilution, two repetitions were established. Two selective culture mediums of actinomycetes were used: starch–casein medium [24] and Olson medium [25]. The nystatin is added aseptically to both mediums as an antifungal agent at a concentration of 100 μg/mL and polymexine at 10 μg/mL as an antibacterial agent active against negative Gram bacteria. These antibiotics are sterilized by filtration through a Millipore filter of 0.22 μm of porosity. One Hundred micro liters of every dilution were inoculated on the surface of Petri dishes containing culture mediums of isolation. The prepared plates were incubated at a temperature of 28°C for 3 weeks. Daily observations were carried out to follow the growth of colonies and avoid contaminations. All colonies approaching their macroscopic appearance to actinomycetes were observed under microscope (S×40)

in the fresh state and after Gram staining. Typical colonies of actinomycetes are transplanted by the streaks method on the same isolation medium for purification.

The isolated and purified actinomycetes colonies are inoculated on Olson medium without antibiotics in inclined agar and incubated at 30˚C for two weeks and then stored at 4˚C. The subculturing is performed every two months. For longer storage, spore suspensions in 20% of glycerol were stored at −20˚C.

2.3. Growth of actinomycetes on Ortiva

This step aims to find *Actinobacteria*, which have a capacity to use Ortiva as the sole source of carbon and energy. The minimal medium used in this study is completely exempt of carbon source. Its chemical composition is: Agar (18 g), KNO_3 (13.76 g), KH_2PO_4 (1.78 g), Na_2HPO_4, $2H_2O$ (4.66 g), Na_2SO_4 (9.68 g), $MgSO_4$, $7H_2O$ (0.8 g), EDTA (10 mg), $FeSO_4$, $7H_2O$ (5 mg), $MnCl_2$, $4H_2O$ (1.22 mg), $ZnSO_4$, $7H_2O$ (0.25 mg), $CuSO_4$, $5H_2O$ (0.2 mg), $CaCl_2$, $2H_2O$ (1 mg), Na_2MoO_4, H_2O (0.2 mg), pH 7. After adjusting the pH to 7.2–7.4 with 8 M NaOH, the solution was autoclaved at 121˚C for 15 min. The Ortiva fungicide was added after being filtered through a membrane of 0.22 µ of porosity at a concentration of 500 mg/L. The bacteria were inoculated by streaking on the surface of the solid medium and incubated for 21 d at a temperature of 30˚C. Non-inoculated minimal plate agar with Ortiva and inoculated minimal plate agar without Ortiva were tested as a control test.

2.4. The actinomycetes identification

2.4.1. The morphological characterization

The morphological characteristics of the efficient isolates were examined according to the identification of actinomycetes [26,27]. The isolates were grown on glucose yeast extract malt agar medium to determine the cultural characteristics, such as shape, margin, elevation, surface appearance of the colonies. The morphological features of the cells and spores were also observed with a microscope according to the technique of slide culture. This consists of inserting delicately sterile lamella into ISP_2, ISP_3, ISP_4, or ISP_5 agar, such that it forms an angle of 45˚ with the surface of plate agar. The bacterial inoculum was then sowed on the lamella in contact with the medium. After 14 d of incubation at 30˚C, the slide was carefully removed from agar, carrying with it fragments of substrate and aerial mycelium; it is then deposited on a blade and examined under an optical microscope (S×100) [27].

2.4.2. The molecular analysis

Seven isolates from the efficient actinomycetes were molecularly identified (D, F, G, L, S, V, and X). The genomic DNA was directly extracted from the strains using PCR colony protocol: for cell lysis, all individual colonies were diluted in 50 µL of MilliQ filtered water and heated at 98˚C in an incubator (Peltier Thermal cycler) from 5 to 10 min. After sufficient mixing, only 1 µL was added to each PCR tube containing the PCR reaction. The 16S rDNA gene fragment was amplified from the genomic DNA bPCR using primers 27F_YM (5´-AGAGTTTGATYMTGGCTCAG-3´) and R1492 (5´-TACGGYTACCTTGTTACGACTT-3´) [28]. Each 50 µL PCR microtube contains 1 µL extracted DNA; 0.5 µL of upstream primer (10 mmol); 0.5 µL of downstream primer (10 mmol); 5 µL of 5× Tampon; 0.5 µL Go Taq DNA Polymerase DNA (5 U/µL); 0.5 µL of dNTP (10 mM); and 17.3 µL of sterile MQ water. Negative control (24 µL mix + 1 µL of water) and positive control (24 µL mix + 1 µL of 16S DNA of *Pseudomonas aeruginosa* $1/1000^{e}$) were included.

The DNA amplification was performed in a thermal cycler with the following conditions: denaturation for 5 min at 95˚C, then 35 cycles of 30 s of denaturation at 95˚C, 30 s of annealing at 52˚C, 1 min and 30 s extension at 72˚C, and finished with a final extension step of 7 min at 72˚C. The purification of the PCR products was performed using the protocol of the kit MinElute PCR Purification Kit. The purified DNA was sequenced according to Sanger method. The nucleotide sequences determined in this study were compared with existing sequences in GenBank by performing a BLASTN to determine their phylogenetic affiliation. The results are expressed as percentage of similarity of the strain to be identified with the most closely related species.

3. Results and discussions

3.1. Isolation of actinomycetes

From the two selective mediums used, colonies of actinomycete isolates were recognized according to their characteristic macroscopic aspect (powdery colonies or hard colonies inlayed in agar plate), and microscopic aspect (filamentous colonies with Gram-positive staining) [29]. After the subculturing, 18 isolates of actinomycetes completely different were purified. These isolates are referred to by code names as follows: A, C, D, E, F, G, H, I, J, K, L, M, O, R, S, U, V, and X.

According to our results, 18 actinomycetes were isolated from activated sludge. The actinomycetes

obtained from both mediums vary in number, 15 actinomycetes are isolated from Olson medium and three from starch–casein agar. According to this, the Olson medium is the medium which allows the isolation of a larger number of this kind of bacteria from water sludge. According to the literature, mesophilic actinomycetes are present in sewage sludge. The genus *Nocardia* often exists; it is the most important genera in water sludge, it forms flocs that facilitate the sedimentation of particles presenting a particular importance [30,31]. The genus *Gordona* was also isolated in activated sludge [32]. The A*ctinobacteria* are parts of accumulating bacteria of the polyphosphates [33].

3.2. Growth of actinomycetes on Ortiva

The growth capacity of actinomycete isolates was tested on a minimal medium with Ortiva added as the sole source of carbon and energy. The obtained results can be seen in Fig. 1. If the bacteria are unable to use this substrate, the growth is weak or absent. This explains the absence of biodegradation's enzymes. On the other hand, the bacteria which exhibit a good growth are able to produce enzymes that degrade this compound.

The use of fungicides in agriculture has two opposite consequences. The first one is to combat pathogens fungi to increase crop yields. The second is the nature of the fungicide, which, under certain conditions, can become pollutants themselves of air, water, food, and soil [34]. The fungal diversity of soil is also affected by some fungicides [23]. The azoxystrobin is known to be photolabile via different pathways, such as photo isomerization, photo hydrolytic, and oxidative cleavages of the aromatic rings and double bonds [35]. The value of the Henry constant of this fungicide is low, and it is unlikely to volatilize, thus it accumulates in aquatic systems and deep groundwater [36]. It is toxic to freshwater and marine invertebrates and also fish [9]. Other studies have shown that the azoxystrobin may persist in water for longer periods of 3–6 months [11] or more than 2 years [10]. The azoxystrobin persistence is influenced by farm management practice with higher breakdown being observed in organically managed soils than conventionally managed soils [12]. It is also affected by the level of humic and fulvic acids in the environment with increasing levels of these compounds generally enhancing photodegradation [37].

Very few information or data exist in the literature on the azoxystrobin degradation in different aquatic systems. Some studies have shown that in water, only 2.5–4.2% of the azoxystrobin was mineralized to CO_2 within 130 d of incubation and the pH of water did not have much effect on the rate of mineralization [38]. In activated sludge, the complete degradation of azoxystrobin affected the microfauna and microflora of sludge and caused an increase of free bacteria and rotifers of Monogononta and Digononta classes. At the microfauna, it is very difficult to determine the influence of this pesticide [39]. However, the emergence and development of new micro-organisms, large holotriches (Colpidium, Chilodonella, Holophrya), was noted because of the appearance of free bacteria on which they feed [39].

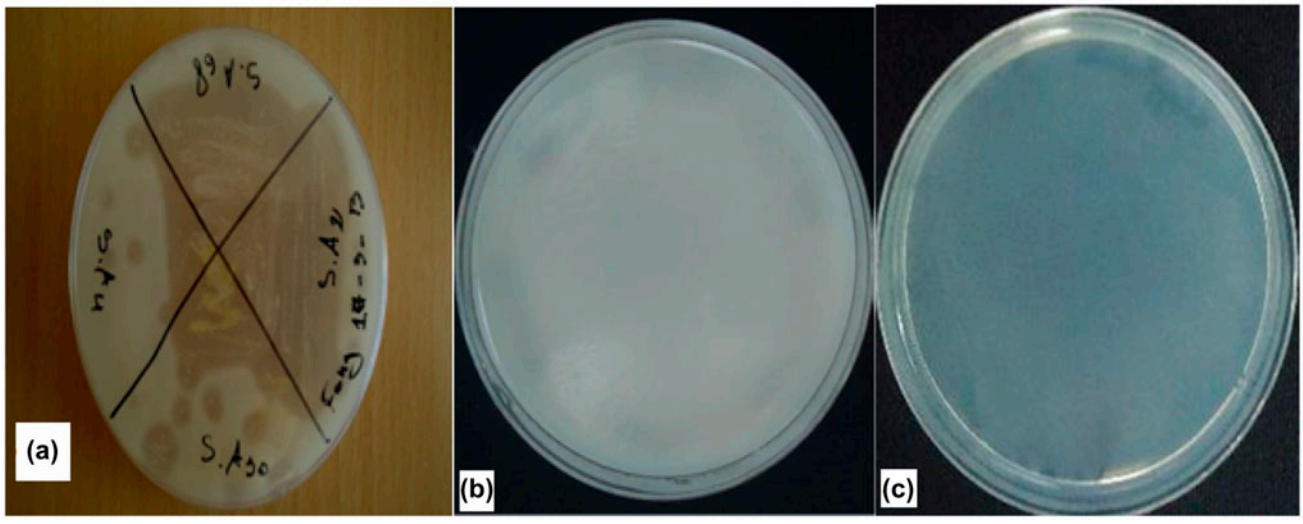

Fig. 1. Growth of the actinomycetes on Ortiva: (a) Good growth of the actinomycetes on Ortiva, (b) Non-inoculated minimal medium with Ortiva, and (c) Inoculated minimal medium without Ortiva.

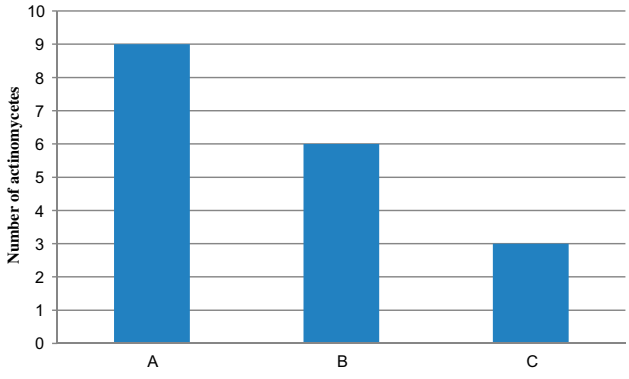

Fig. 2. The number of actinomycetes grown on minimal medium added by 500 mg/L of Ortiva taken as the sole source of carbon and energy: (A) Good growth, (B) Moderate growth, and (C) No growth.

The disappearance of the azoxystrobin applied to tomatoes in greenhouse showed that some residues of the azoxystrobin remained stable [21]. Myung et al. [40] have explored the plant metabolism of three strobilurin fungicides: azoxystrobin, kresoxim-methyl, and trifloxystrobin using wheat cell suspension cultures. The trifloxystrobin and kresoxim–methyl were completely metabolized within 24 h, whereas the metabolism of azoxystrobin was relatively slow with half-lives up to 48 h depending on specific experimental conditions.

The elimination of this molecule is a certain environmental urgency. Wastewater treatment plants are the important structures for treating polluted water before being reintroduced into the supply circuits.

The actinomycetes are among the largest groups with considerable activity in the degradation of natural and synthesis compounds, such as pesticides and other xenobiotics in the environment [41]. Therefore, they play a major role in the remediation of contaminated environments by these components. The actinomycetes belonging to the genera of *Arthrobacter*, *Clavibacter*, *Nocardia*, *Rhodococcus*, *Nocardioides*, and *Streptomyces* are capable of degrading pesticides [42].

In our investigation, 15 preselected actinomycetes were isolated from sludge, which were able to grow well using Ortiva as the sole source of carbon. Among them, nine strains (A, D, F, G, H, L, S, V, X) were the most efficient in degrading Ortiva at the concentration of 500 mg/L at a temperature of 30˚C (Fig. 2).

Growth studies showed that there is a significant degradation of fungicide Ortiva within 21 d period of incubation. This supports the claim that the half-life of the compound was less than 14 d [9].

The works that studied the biodegradation of the azoxystrobin by pure culture of actinomycetes remain largely unknown or poorly reported. A study conducted in Sudan (Khartoum) has reported the ability of the genera *Nocardia*, *Arthrobacter,* and *Mycobacterium* coming from soils to use the azoxystrobin as the sole source of carbon and nitrogen. Among these genres, *Nocardia* had the greatest ability to break the azoxystrobin and identified as *Nocardia brasiliensis* [22].

In a recent work, using sequential soil and liquid culture enrichments, Howell et al. [41] isolated two bacterial strains which were able to degrade the most widely used strobilurin, azoxystrobin when supplied as a sole carbon source. The identification of these bacteria by 16S rRNA showed that the strains showed a homology to *Cupriavidus* sp. and *Rhodanobacter* sp.

3.3. Identification of efficient actinomycetes

The isolates A, D, F, G, H, L, S, V, and X are Gram-positive aerobic bacteria. The color of aerial mycelium and substrate mycelium varies for the different strains. Table 1 represents the color of each isolate spores.

The microscopic observation of the selected isolates by slide culture technique showed filamentous aspects. They form highly branched mycelia that rarely break. At maturity, the aerial mycelium produces chains of variable length spores: straight chains, rectiflexibles chains, or spirals chains of spores. Also, they form partially fast-beaded branching filaments.

The molecular identification of the isolates was possible for only seven actinomycetes in the laboratory conditions. It reveals that the actinomycetes bacteria belong to the genera *Streptomyces* species for F, G, L, V, and X strains and to the *Nocardia* species for the isolates D and S. The taxonomic affiliation of strains is collected in Table 2.

These results are very promising; they show that the actinomycetes isolated from activated sludges can

Table 1
spores color of actinomycete isolates

Isolates	A	D	F	G	H	L	S	V	X
Color	White	Yellow	White	Black	Pink	Gray	Orange	White	Gray

Table 2
The molecular identification of the actinomycetes isolates and their percentage of similarity

Isolate	Affiliation	Percentage of similarity (%)	GenBank source
D	*Nocardia asteroides* strain Z12-8 16S ribosomal RNA gene, partial sequence	99	KJ571110.1
F	*Streptomyces* sp. SAI-13 16S ribosomal RNA gene, partial sequence	99	KM220609.1
G	*Streptomyces microflavus* strain 126,182 16S ribosomal RNA gene, partial sequence	99	JN180196.1
L	*Streptomyces lavendulae* strain FSHJ9 16S ribosomal RNA gene, partial sequence	99	KC626003.1
S	*Nocardia nova* strain GTC 86116S ribosomal RNA gene, partial sequence	99	AB292584.1
V	*Streptomyces flavogriseus* strain P·S.461 16S ribosomal RNA gene, partial sequence	100	KF991651.1
X	*Streptomyces* sp. Sn-23 16S ribosomal RNA gene, partial sequence	100	KJ742904.1

grow on Ortiva as the sole source of carbon. To our knowledge, these strains were the first-reported pure cultures of actinomycetes capable of degrading commercial Ortiva.

4. Conclusion

The wastewater treatment is one of the most complex problems for both developed and developing countries. In conclusion, it has been demonstrated that seven actinomycetes isolated from activated sludges of wastewater treatment plant degrade the Ortiva fungicide at a concentration of 500 mg/L within 21 d of incubation and at a temperature of 30˚C under laboratory conditions. 16S rRNA reveals that the strains showed a homology to *Nocardia asteroides* strain Z12–8, *Streptomyces* sp. SAI-13, *Streptomyces* sp. Sn-23, *Streptomyces lavendulae* strain FSHJ9, *Nocardia nova* strain Z12–8, *Streptomyces flavogriseus* strain P.S.461, and *Streptomyces microflavus* strain 126182. Our strains can be used in systems of cleanup of the environment, such as the bioremediation of the aquatic environments contaminated by this kind of fungicide.

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