ANTIFUNGAL ACTIVITIES OF A NEW STRAIN OF *STREPTOMYCES* ISOLATED FROM AN ALGERIAN SOIL AGAINST PHYTOPATHOGENIC AND MYCOTOXIGENIC *ASPERGILLUS CARBONARIUS* AND *A. NIGER*

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Abstract. *A new strain of Streptomyces was isolated from an Algerian soil and was tested for its antifungal activities against two species of Aspergillus: A. carbonarius and A. niger. Based on its morphological properties this isolate was identified as Streptomyces sp. It showed a good to moderate activity against target strains of two Aspergillus species by using the streak method. The fermentation of this strain in Bennett culture medium exhibited a strong activity against A. carbonarius and least activity against A. niger since the first day of incubation, and then decreased gradually until it becomes zero in the fifth day. No activity was obtained with ISP2 medium despite of the biomass abundance registered. The extraction of the antifungal activities was carried out by using of four organic solvents with different polarities: n-hexan, dichloromethane, ethyl acetate and n-butanol. However, the efficiency of the strain was tested by dish paper diffusion method and was appeared only with ethyl acetate and n-butanol solvents, with a slight advantage to ethyl acetate extract. The diameter of inhibition obtained with ethyl acetate extract against A. carbonarius was 33 mm. The ethyl acetate extract allowed us to detect two superposed active spots with frontal ratio of 0.72 and 0.80 mm. The method used was a combination between Thin Layer Chromatography and Bioautography.*

Keywords: *Streptomyces, Aspergillus carbonarius, A. niger, antifungal activity, phytopathogenic fungi, mycotoxigenic fungi.*

INTRODUCTION

Aspergillus spp. are primarily saprophytic micro-fungi, occurring commonly in soils and on other organic and inorganic substrates. The conidia (asexual spores) are hydrophobic and readily airborne with the capacity to germinate in a wide range of conditions. They are thermotolerant, and capable of growth in temperatures ranging from 12 °C to over 50 °C which has contributed to their success as wide ranging opportunistic pathogens in vertebrates (BHABHRA and ASKEW, 2005). Pathogenic micro-fungi are responsible for many diseases both in plants and animals (PICKFORD and MACINO, 2005). Although they are not considered to be major cause of plant disease, *Aspergillus* species are responsible for several disorders in various plant and plant products. The most common species are *A. carbonarius*, *A. niger* and *A.*

flavus, followed by *A. parasiticus*, *A. ochraceus* and *A. alliaceus*. They can contaminate agricultural products at different stages including pre-harvest, harvest, processing and handling. Changes due to spoilage by *Aspergillus* species can be of sensorial, nutritional and qualitative nature like: pigmentation, discoloration, rotting, development of off-odors and off-flavors. However, the most notable consequence of their presence is mycotoxin contamination of foods and feeds. Because they are opportunistic pathogens, most of them are encountered as storage molds on plant products (KOZAKIEWICZ, 1989). Mycotoxin substances may cause different types of poisoning and, consequently, diverse health problems from acute to chronic problems in both animals and humans (RODRIGUES and NOSUNCHUK, 2020). *Aspergillus carbonarius* is one of the most relevant producers of ochratoxin A in food and feed stuff (KOGKAKI et al., 2015). This mycotoxin is with nephrotoxic, carcinogenic, teratogenic and immunosuppressive properties (BATTAGLIA et al., 1996). In addition, *Aspergillus carbonarius* and *A. niger* is among the predominant *Aspergilli* in vineyards in several countries of the world (SERRA et al., 2003; BOURAS et al., 2005, 2007).

Currently, the antifungal available are inadequate and constricted with limitations such as severe toxicity, narrow antifungal spectrum, emerging drug resistance and high costs (SHARMA et al., 2014). Thus, finding new antifungal drugs with alternative and multi-target mechanisms of action to develop more effective antifungal therapy has become an essential medical priority (LONE and AHMAD, 2020). Unlike antibacterial drugs, the array of available antifungals is somewhat scarcer. Azoles, polyenes, and echinocandins are the three main antifungal classes, being the last considered first-line therapy in many hospitals for the treatment of invasive candidiasis (PAPPAS et al., 2016; COSTA-DE-OLIVEIRA and RODRIGUES, 2020). Reports of resistance to these therapeutic agents have been on the rise, which highlights the need for new antifungals (HAN and HUANG, 2018). It is widely accepted that more than 80% of pharmaceutical substances are either directly derived from natural products or developed from a natural compound (PAN et al., 2013). Approximately 70% of all known antibiotics were isolated from Actinobacteria, especially from the genus *Streptomyces* (XU et al., 2014).

In Algeria, many studies have reported the abundance of Actinobacteria in arid and semi-arid soils and their high potential in producing bioactive secondary metabolites especially new molecules (BOURAS et al., 2006; BOUBETRA et al., 2015; SAKER et al., 2015; KHEBIZI et al., 2018; MERROUCHE et al., 2019; BELGHIT et al., 2021).

The aim of this study was to describe an actinobacterium isolated from soil and its antifungal activities.

MATERIALS AND METHODS

Isolation of the actinobacterial strain

The actinobacterial strain was isolated from a soil sample collected from Djelfa region, Algeria. The dry soil sample was suspended in sterile deionized water and diluted. Aliquots (0.2 mL) of each dilution were spread on chitin-vitamin agar medium (HAYAKAWA et al., 1987), consisting of (per liter of distilled water): 2 g chitin, 0.35 g K_2HPO_4, 0.15 g KH_2PO_4, 0.2 g $MgSO_4$ $7H_2O$, 0.3 g NaCl, 0.02 g $CaCO_3$, 10 mg $FeSO_4$ $7H_2O$, 1 mg $ZnSO_4$ $7H_2O$, 1 mg $MnCl_2$ $4H_2O$ and 18 g agar. The culture medium was supplemented with actidione (50 µg/mL) and penicillin (25 µg/mL) to inhibit the growth of unwanted micro-fungi and bacteria and, respectively. The Petri dishes were incubated at 30 °C for 21 days.

Morphological characteristics

The morphological of actinobacterium was examined by naked-eye after 2 weeks-old cultures grown on ISP2 (International *Streptomyces* Project 2) (SHIRLING et al., 1966). The micromorphology and sporulation was observed by light microscopy.

Physiological analyses

The growth of bacterium at pH 5 and 9 and at 45 and 60 °C, and the growth in the presence of different concentrations of NaCl were studied. Furthermore, seven antibiotics: chloramphenicol (25 mg/mL), erythromycin (10 mg/mL), kanamycin (25 mg/mL), penicillin (25 mg/mL), rifampicin (5 mg/mL), streptomycin (10 mg/mL) and vancomycin (5 mg/mL) were also included in this study.

Antifungal activity

The potential activity of the actinobacterial strain was evaluated against two filamentous micro-fungi: *Aspergillus carbonarius* (M333) and *A. niger* (AN) on solid medium ISP2, by the streak method. Petri dishes were seeded with strain culture by a single streak and incubated at 30 °C for 2 weeks. After that, target microorganisms was inoculated in streaks perpendicular to the strain (a single streak for each at 90° to actinobacterial strain). The antifungal activity was evaluated by measuring the distance of inhibition between target microorganism: *A. carbonarius*, *A. niger* and actinobacterial colony margin, after incubation at 30 °C for 48 h. The used targets *A. carbonarius* and *A. niger* were typically phytopathogenic and mycotoxigenic as noted by BOURAS et al. (2005, 2007).

Kinetics of antifungal production on liquid Bennett

Fermentation was carried out in meat extract-yeast extract-peptone-glucose (Bennett medium) (WAKSMAN, 1966). A seed culture was prepared with the same medium and used to inoculate Erlenmeyer flasks of 500 mL, each one containing 100 mL of medium. The cultures were incubated for 8 days on a rotary shaker (250 rpm) at 30 °C. A volume of 2 mL aliquots were collected regularly to estimate antifungal activity by the agar well method against *Aspergillus carbonarius* (M333) and each 10 mm diameter well was filled with 200 µL of supernatant sample. The growth (dry cell weight of mycelium) and the pH were also measured.

Extraction of antifungal production

The culture broth (from Bennett medium) was centrifuged to remove biomass. The cell-free supernatant was extracted with an equal volume of organic solvent. Four extraction solvents were tested for effectiveness, including *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol. Each organic extract was concentrated to dryness. The resulting dry extract was recuperated in 1 mL of methanol and bioassayed against *A. carbonarius* and *A. niger* by paper disk diffusion method (JORGENSEN and FERRARO 2009).

RESULTS AND DISCUSSIONS

Morphological and physiological characteristics

The strain showed very good growth on ISP2 after 2 weeks of incubation at 30 °C. The aerial mycelium was yellowish white. Substrate mycelium was not fragmented and its color was dark brown. The physiological properties of the strain are shown in Table 1. The optimal growth of the strain was obtained at 30 °C and at pH 7. It was able to grow at 7% of NaCl but not at 10%. It was able to grow at pH 5 and 9, at 45 °C but not at 60°C and in the presence of nitrate reduction. The isolated strain was resistant to all antibiotics tested except kanamycin and rifampicin. Based on its morphological properties, the isolated strain exhibited characteristics of the genus *Streptomyces*. Currently, this genus contained 1116 species including 69 subspecies (http://www.bacterio.net/streptomyces.html) (EUZEBY, 2022).

Table 1

Selected physiological characteristics of the actinobacterial strain

Test	**Result**
Resistance to antibiotics (mg/mL)	
Chloramphenicol (25)	+
Erythromycin (10)	+
Kanamycin (25)	−
Penicillin (25)	+
Rifampicin (5)	−
Streptomycin (10)	+
Vancomycin (5)	+
Growth with (% w/v)	
NaCl (7%)	+
NaCl (10%)	−
pH 5	+
pH 9	+
45 °C	+
60 °C	−
Nitrate reduction	−
Production of melanoid pigments	−

Antifungal activity

The antifungal activity of actinobacterium against *A. carbonarius* and *A. niger* is shown in Table 2. This strain showed a good activity against *A. carbonarius* and moderate activity against *A. niger*. These results were observed on ISP2 medium within 7 days after streaking of the test organisms. According to the report of DAHIYA et al. (2006), many species of Actinobacteria have the capacity to inhibit phytopathogenic and/or mycotoxigenic micro-fungi.

Table 2

Antifungal activity of the *Streptomyces* strain on ISP2 medium.

Test organism	**Distance of inhibition (mm)***
Aspergillus carbonarius (M333)	15.5 ± 0.7
Aspergillus niger (AN)	7.0 ± 0.5

*Each value represents the average of three measurements

Kinetics of antifungal production

The kinetics of growth, antifungal production and pH were monitored in Bennett broth culture, as shown on Figures 1 and 2. The isolate did not show any antifungal activity in ISP2 broth culture (data not shown). The antifungal activities were detected in Bennett medium and reached the maximum inhibition from the first day of fermentation against both *Aspergilli*, then decreased until total disappearance in the fourth and fifth day of incubation. There are many antifungal compounds biosynthesized by strains of *Streptomyces* that appear on the first day of culture as reported by AL-ASKAR et al. (2011) and AYARI et al. (2015). The antifungal activity against *Aspergillus carbonarius* (M333) was stronger than that detected against *A. niger* (AN). The biomass increased from the first day, reached the maximum at day 3, and then decreased slightly after. The pH varied between 6.7 and 7.7 during the incubation period. The production of secondary metabolites in Actinobacteria is greatly affected by various fermentation parameters, such as nutrients availability, pH, aeration, temperature, mineral salts, heavy metals, precursors, inducers, and inhibitors, which often vary from organism to organism (ABDEL-RAZEK et al., 2020).

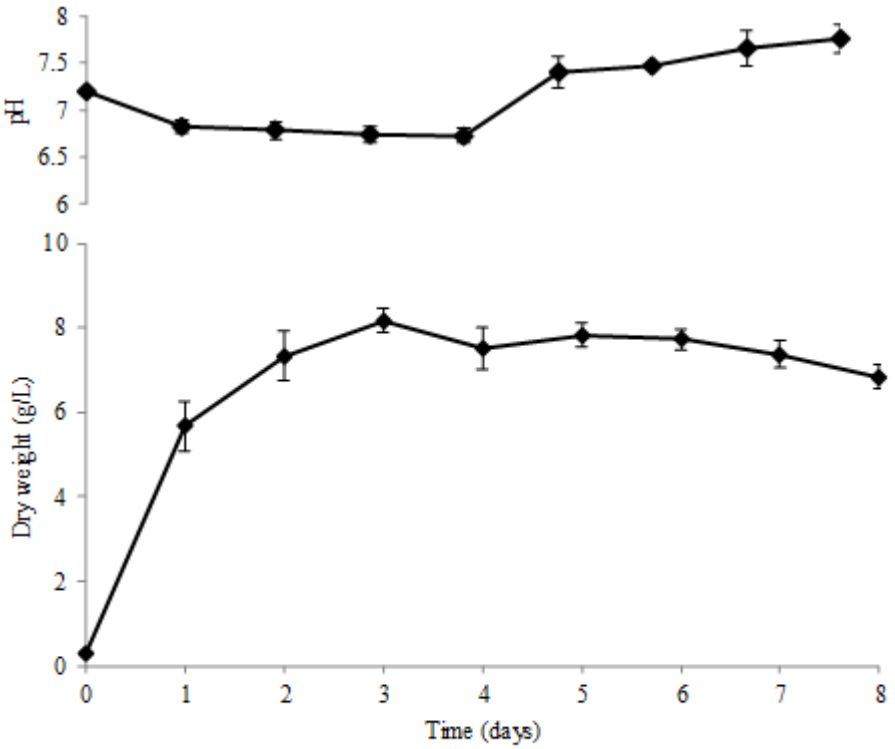

Fig. 1. Time course of growth and pH on Bennett broth medium

Each measure represents average ± standard deviation from three replicates per treatment.

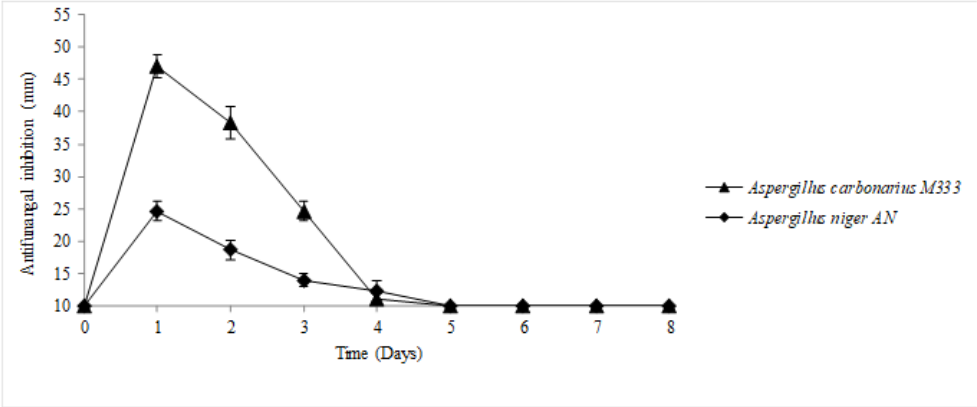

Fig. 2. Time course of antifungal activity on Bennett broth medium against *Aspergillus carbonarius* (M333) and *A. niger* (AN).

Measurements of activity against micro-fungus represent the diameters of inhibition with including the diameter of wells (10 mm). Each measure represents average ± standard deviation from three replicates per treatment.

Extraction of antifungal production

The antifungal compounds were extracted from broth culture (Bennett medium) with ethyl acetate and *n*-butanol, but not with *n*-hexane or dichloromethane. The diameter of inhibition obtained with ethyl acetate extract against *Aspergillus carbonarius* was 33 mm (figure 3). The active extract was concentrated to dryness using the rotavapor, recuperated in methanol and analyzed by TLC developed in ethyl acetate-methanol system (100:15 v/v). Using bioautography the ethyl acetate extract allowed us to detect two active superposed bands, named fraction 1 and 2 with frontal ratio of (R_f = 0.72 and R_f = 0.80 mm).

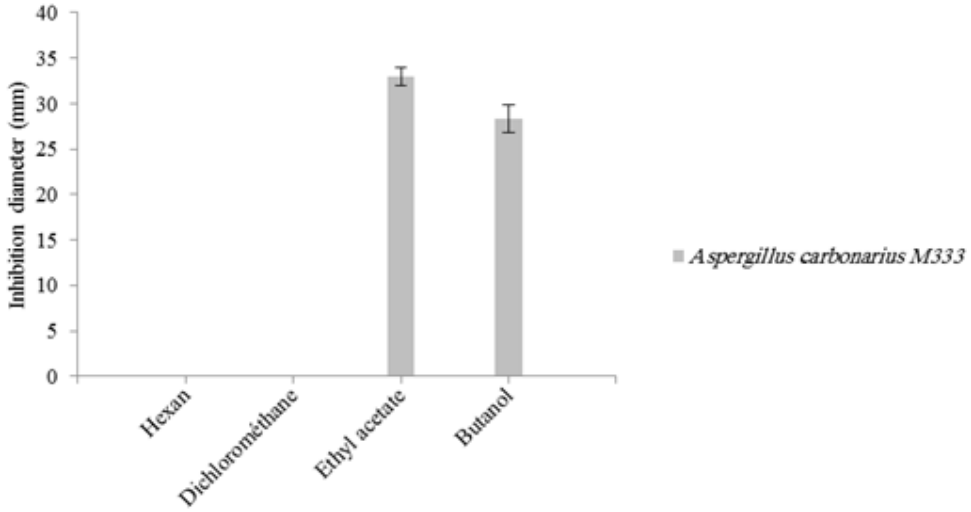

Fig. 3. Anti-fungal activity of organic extracts

CONCLUSIONS

In this study, the actinobacterial strain was isolated from Algerian soil (Djelfa region). Based on morphological characteristics, this strain was related to *Streptomyces* genus. Antifungal assay indicated that it has well to moderate activity against *Aspergillus carbonarius* (M333) and *A. niger* (AN). The kinetics of antifungal production showed a strong activity against *A. carbonarius* since the first day of incubation. The separation of ethyl acetate extract with TLC and the detection of active spots by bioautography indicated the presence of tow active superposed spots.

BIBLIOGRAPHY

ABDEL-RAZEK, A.S., EL-NAGGAR, M.E., ALLAM, A., MORSY, O.M., 2020 – Othman, S.I. Microbial Natural Products in Drug Discovery. Processes, 8: 1-19.

Al-Askar, A.A., Abdul Khair, W. M., Rashad, Y. M., 2011 – In vitro antifungal activity of *Streptomyces spororaveus* RDS28 against some phytopathogenic fungi. African Journal of Agricultural Research, 6 (12): 2835-2842.

AYARI, A., MORAKCHI, H., KIRANE-GACEMI, D., 2015 – Evaluation of antifungal activity of novel marine actinomycete, *Streptomyces* sp. AA13 isolated from sediments of Lake Oubeira (Algeria) against *Candida albicans*. African Journal of Agricultural Research, 10 (6): 156-171.

BELGHIT, S., TOUMATIA, O., BAKLI, M., BADJI, B., ZITOUNI, A., MATHIEU, F., ȘMULEAC, L., BOURAS N., 2021 – Isolation and characterization of a new *Streptomyces* strain LG10 from unexploited Algerian Saharan Atlas. Advanced Research in Life Sciences. 5: 36-42.

BHABHRA, ASKEW., 2005 –Thermotolerance and virulence of *Aspergillus fumigatus*: role of the fungal nucleolus. Medical Mycology, 43 (1): 87-93

BOUBETRA, D., ZITOUNI, A., BOURAS, N., SCHUMANN, P., SPRÖER, C., KLENK, H.P., SABAOU N., 2015 – *Saccharothrix tamanrassetensis* sp. nov., an actinomycete isolated from Saharan soil. International Journal of Systematic and Evolutionary Microbiology, 65: 1316-1320.

BOURAS, N., MATHIEU, F., COPPEL, Y., LEBRIHI, A., 2005 – Aurasperone F- a new member of the naphtho-gamma-pyrone class isolated from a cultured microfungus *Aspergillus niger* C-433. Natural Product Research, 19: 653-659.

BOURAS, N., MATHIEU, F., COPPEL, Y., STRELKOV, S. E., LEBRIHI, A., 2007 – Occurrence of naphtho-gamma-pyrones- and ochratoxin A-producing fungi in French grapes and characterization of new naphtho-gamma-pyrone polyketide (aurasperone G) isolated from *Aspergillus niger* C-433. Journal of Agricultural and Food Chemistry, 55: 8920–8927.

BOURAS, N., MATHIEU, F., SABAOU, N., LEBRIHI, A., 2006 – Nutritional requirements for the production of dithiolopyrrolone antibiotics by *Saccharothrix algeriensis* NRRL B-24137. Enzyme and Microbial Technology, 39: 1423-1429.

COSTA-DE-OLIVEIRA, S., RODRIGUES, A.G., 2020 – *Candida albicans* Antifungal Resistance and Tolerance in Bloodstream Infections: The Triad Yeast-Host-Antifungal. Microorganisms, 8 (2):154.

DAHIYA, N., TEWARI. R., HOONDAL, G.S., 2006 – Biotechnological aspects of chitinolytic enzymes. Applied Microbiology and Biotechnology, 71 (6): 773-782.

EUZEBY, J.P., 2022 – List of bacterial names with standing in nomenclature-genus *Streptomyces*. https://lpsn.dsmz.de/search?word=streptomyces. Accessed 24 Mar 2022.

HAYAKAWA, M., NONOMURA, H., 1987 – Humic acid-vitamin agar, a new medium for the selective isolation of soil actinomycetes. Journal of Fermentation Technology, 65: 501-509.

JORGENSEN, J.H, FERRARO MJ., 2009 – Antimicrobial susceptibility testing: a review of general principles and contemporary practices. Clinical Infectious Diseases, 49:1749-55.

KHEBIZI, N., BOUDJELLA, H., BIJANI, C., BOURAS, N., KLENK, H-P., PONT, F., MATHIEU, F., SABAOU, N., 2018 – Oligomycins A and E, major bioactive secondary metabolites produced by *Streptomyces* sp. strain HG 29 isolated from a Saharan soil. Journal of Medical Mycology, 28: 150-160.

KOGKAKI, E.A., KOGKAKI, P.I., NATSKOULIS, N., MAGAN, E.Z., 2015 – Panagou Effect of interaction between *Aspergillus carbonarius* and non-ochratoxigenic grape-associated fungal isolates on growth and ochratoxin A production at different water activities and temperatures. Food Microbiology, 46: 521-527.

KOZAKIEWICZ, Z., 1989 – *Aspergillus* species on stored products. Mycological Papers, 161: 1-188.

LI HAN, H.S., HUANG, X., 2018 – Potential targets for the development of new antifungal drugs. The journal of antibiotics, 71: 978–991.

LONE, S.A., AHMAD, A., 2020 – Inhibitory effect of novel Eugenol Tosylate Congeners on pathogenicity of *Candida albicans*. BMC Complementary Medicine and Therapies, 20: 1-14.

MERROUCHE, R., YEKKOUR, A., COPPEL, Y., BOURAS, N., ZITOUNI, A., LEBRIHI, A., MATHIEU, F., SABAOU, N., 2019 – A new dithiolopyrrolone antibiotic triggered by a long fermentation of *Saccharothrix algeriensis* NRRL B-24137 in sorbic acid-amended medium. Letters in Applied Microbiology, 69: 294-301.

PAN, S.Y., ZHOU, S.F., GAO, S.H., YU, Z.L., ZHANG, S.F., TANG, M.K., SUN, J.N., MA, D.L., HAN, Y.F., FONG, Y.F., KO, K.M., 2013 – New Perspectives on How to Discover Drugs from Herbal Medicines: CAM's Outstanding Contribution to Modern Therapeutics. Evidence-Based Complementary and Alternative Medicine, 1: 1-25.

PAPPAS, P.G., KAUFFMAN, C.A., ANDES, D.R., CLANCY, C.J., MARR, K.A., OSTROSKY-ZEICHNER, L., REBOLI, A.C., SCHUSTER, M.G., VAZQUEZ, J.A., WALSH, T.J., ZAOUTIS, T.E., SOBEL,

J.D., 2016 – Clinical Practice Guideline for the Management of Candidiasis. Clinical Infectious Diseases, 62: 1-50.

PICKFORD, A., MACINO, G., 2005 – Gene Silencing as a Tool for the Identification of Gene Function in Fungi. Applied Mycology and Biotechnology, 5: 93-116.

RODRIGUES, M.L., NOSUNCHUK, J.D., 2020 – Fungal diseases as neglected pathogens: A wake-up call to public health officials. PLOS Neglected Tropical Diseases, 14: 1-9.

SAKER, R., BOURAS, N., MEKLAT, A., ZITOUNI, A., SCHUMANN, P., SPRÖER, C., SABAOU, N., KLENK, H-P., 2015 – *Prauserella isguenensis* sp. nov., a halophilic actinomycete isolated from desert soil. International Journal of Systematic and Evolutionary Microbiology. 65: 1598-1603.

SERRA, R., ABRUNHOSA, L., KOZAKIEWICZ, Z., VENANCIO, A., 2003 – Black *Aspergillus* species as ochratoxin A producers in Portuguese wine grapes. International Journal of Food Microbiology, 88: 63–68.

SHARMA, S., ALFATAH, M., BARI, V.K., RAWAL, Y., PAUL, S., GANESAN, K., 2014 – Sphingolipid biosynthetic pathway genes FEN1 and SUR4 modulate amphotericin B resistance. Antimicrobial agents and chemotherapy, 58: 2409-2414.

SHIRLING, E.B., GOTTLIEB, D., 1966 – Methods for characterization of *Streptomyces* species. International Journal of Systematic Bacteriology, 13: 313-340.

WAKSMAN, S.A., 1961, – The actinomycetes classification: identification and descriptions of genera and species, vol. 2, Lippincott Williams and Wilkins Company, Baltimore, United States, PP: 363.

XU, S.M., NODWELL, J.R., 2014 –The expression of antibiotic resistance genes in antibiotic-producing bacteria. Molecular Microbiology, 93: 391-402.