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Isolation and phenotypic characterization of actinomycetes from Rabat neighborhood soil and their potential to produce bioactive compounds

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This study aimed to examine the isolation of actinomycetes strains from unexplored Rabat neighbourhood soil followed by phenotypic diversity highlight to access their potential as novel bioactive compounds producers. Twenty nine actinomycetes strains were isolated from southeast of Rabat region. Morphological and cultural characterization, pigment production and antibacterial assay were carried out. All isolates (100%) were able to produce at least one non-diffusible pigment depending on the culture media. Twenty five (86.20%) isolates produce diffusible pigments, 6 (20.69%) produce melanoid diffusible pigments and 5 (17.24%) exhibited antibacterial activities. These results indicate an important potential of the actinomycetes isolated to produce polymorphic secondary metabolites.

Key words: Isolation, actinomycetes, diversity, pigment, bioactive compounds, antibacterial.

INTRODUCTION

The success of natural resources in drug discovery resides in their biodiversity to produce a huge structural diversity of natural compounds. Actinomycetes remain the most prolific producers of microbial-derived natural products, including anticancer, antifungal and antibacterial compounds. Prospection in this field is based on the structural diversity of molecules provided by combinatorial chemistry or natural organic compounds. Natural bioactive substances are screened mostly from plant and microorganisms (Newman and Cragg, 2012; Harvey, 2000).

Among the microorganisms, actinomycetes produce bioactive compounds of high commercial value and continue to be routinely screened for new bioactive substances (Okami and Hotta, 1988; Sanglier et al., 1993; Masayuki et al., 1993; Demain, 1999; Hopwood secondary metabolism biosynthesis pathway. The highest score is assigned to *Streptomyces* genus (Bérdy,

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Table 1. Bacterial count present in soil and number of actinomycetes isolated.

Sample	Count of bacteria × 10^5 UFC/g of soil			Actinomycetes isolated
	Actinomycetes	Other bacteria	Total	
A	12	38	50	14
F	8	26	34	8
C	4	12	16	7
Total	24	76	100	29

(F, Forest soil; A, Agricultural soil and C, Cattle manure deposit).

1995) which is also the most frequently isolated from soil.

Actinomycetes intensive screening over the past several decades provided a wide variety of bioactive compounds. Even with the decrease of new compounds discovery in actinomycetes isolated from the studied accessible soils in the past two decades, huge unexplored regions in the world remain to be studied. In Morocco, only few locations where actinomyctes have been isolated and reported (Barakate et al., 2002;

Hanane et al., 2011; Boussaber et al., 2014).

The isolated strains were mostly attributed to *Streptomyces* genus. Nevertheless, diversity of actinomyctes is still underexplored in our country. In this work, we present phenotypic diversity of strains isolated from Rabat neighbourhood (Morocco) and their potential to provide sources of secondary metabolites.

MATERIALS AND METHODS

Soil sample, actinomycetes screening and storage

Soil samples were collected in winter (February) from three locations in Rabat neighbourhood region. These locations represent three habitats: Forest soil (F), Agricultural soil (A) and Cattle manure deposit soil (C). One sample of each habitat was taken from a depth of 15 cm after removing soil surface, placed in aluminium folium closed tightly and transported aseptically to the laboratory. 1 g of each sediment sample was heated 15 min at 70°C and used for actinomycetes isolation by standard method. 100 µl of diluted (1/2 x 10^{-3} and 10^{-4}) suspensions in NaCl (8.5 g/L) were plated onto (GLM) yeast extract malt extract agar (Kitouni et al., 2005) and starch casein agar (SCA**)** (Kuster and Williams, 1964) supplemented with filter (Millipore 0.45 µm) sterilized cycloheximide (50 µg/ ml). The plates were incubated at 30°C for 1 to 3 weeks. After incubation, total colonies were counted and actinomycetes typically dry, powdery colonies were selected. To get pure cultures, colonies were purified onto ISP2 agar (Shirling and Gottlieb, 1966). Presence of mycelium and spores was examined under light microscope (x1000). KOH test was used to confirm gram positive (Halebian et al., 1981). ISP2 plate and slants of pure cultures were kept at room temperature for short-term storage. For long-term storage, sediment of soil was added and mixed to ISP2 culture slants and stored at room temperature. A second long-term storage was made from a loop-full of the isolate culture dispersed in 20% glycerol and kept at -20°C.

Morphological, cultural and physiological characteristics

All the following determinations were performed by visual observation in the solid media of the International Streptomyces Project (ISP) (Shirling and Gottlieb, 1966):

(1) Colonies morphologies and aerial/substrate mycelium colors of the isolates were determined on ISP2 and ISP4.
(2) Production of diffusible pigments was tested in ISP1, ISP2 and ISP4.
(3) Production of melanin pigment was made in ISP6.

The plates and slant cultures were incubated at 30°C, and observations were recorded on the 7th, 14th and 21th days. Growth rate was estimated on ISP media and GLM visually according to the abundance of mycelium and colonies size.

Antibacterial bioassay

Bioassays of the isolated actinomycetes were performed by disk diffusion assay. Agar disk prepared from lawn culture of each actinomycete isolate on ISP2 medium plate after 7 days of incubation at 30°C. Prepared disks were then aseptically transferred to Mueller Hinton agar (Difco, Germany) plates previously lawn with fresh culture of test bacteria as clinical strains of *Escherichia coli, Klebsiella pneumonie*, *Enterobacter cloacae, Staphylococcus aureus, Escherichia coli* ATCC8739 and *Staphylococcus aureus* ATCC6538. Sterile disk of ISP2 was used as control. After 24 h incubation at 30°C, bioactivity was determined by measuring the diameter of inhibition zone (mm) (Valgas et al., 1981).

RESULTS AND DISCUSSION

Isolation of actinomycetes

Bacterial count in the three samples used showed an important decrease of total bacterial population or actinomycetes from sample A, F to C (Table 1) correlated with increase of their organic matter content (Keen et al., 2011; Moral et al., 2005). These results showed a relationship between organic matter present in these soil samples and bacterial load. High organic matter soil seems to be more favourable to fungi consistent with a reported study (Bailey et al., 2002). Among total flora counted in the three samples, actinomycetes represented 24% indicating a high percent than recently reported (Elbendary et al. 2018). Since we noticed that GLM medium was favourable to non actinomycetes bacteria growth, we used SCA medium agar and we got 5 additional isolates to reach a total of 29 isolates (Table

Table 2. Evaluation of growth and sporulation of all isolates on different media.

Medium	Growth	Sporulation
GLM	Good	Weak
ISP1	Weak	None
ISP2	Good	Good
ISP4	Moderate	Good
ISP6	Weak	None

1). All isolates showed typical characteristics of actinomycetes:

(1) Powdery and dry colony surface appearance on solid media;
(2) Colony inserted in agar medium;
(3) Gram positive in the used KOH test and;
(4) Presence of mycelium and spore as checked by microscopic observation.

Growth and sporulation assessment

On solid media, actinomycetes growth rate presented different degrees of culture density. Slant and Petri dish cultures of all isolates showed best growth on GLM and ISP2. Sporulation of aerial mycelium on solid media was reflected at its surface by the change of the color due to spore pigmentation with powdery aspect. Good sporulation of all isolates was observed on ISP2 agar and ISP4 agar media (Table 2) consistent with reported study (Algafari, 2014).

Non-diffusible and diffusible pigments production

Aerial and substrate mycelium color is an important criterion in identification of actinomycetes (Pridham, 1965; Waksman and Lechevalier, 1953). It reflects the colors of their intracellular pigments. All isolates on ISP2 and ISP4 agar media showed a significant diversity of colors (Table 3). Substrate mycelium on ISP2 showed more isolates with yellow colors shade as previously reported (Ceylan et al., 2008). However, percent of yellow colors shade decreased from 69.23% on ISP2 to 23.07% on ISP4 compared to Ceylan et al. (2008) studies in which 93.3% isolates exhibit yellow shades. In fact, the ratio of non-yellow shade isolates at least in one of the two media became 76.93% and showing more diversity. Consequently, this color diversity reflects the important potential of theses isolates for biosynthesis of different non-diffusible pigments. On the other hand, 25 (86.20%) of the 29 isolates were able to produce diffusible pigments at least in one of the used media: ISP1, ISP2 and ISP4 (Table 4). Production opportunity of these pigments is higher in ISP2 followed by ISP1. In ISP4, only 6 isolates had produced diffusible pigments. Production of melanoid diffusible pigments in ISP6 was detected in 6 isolates (22.22%). Unlike non-diffusible pigments, diffusible pigments with antibacterial activities could exhibit a zone of inhibition in antibacterial assay. This aspect makes it easy to screen for antimicrobial activities (Mamunur et al., 2014) than non-diffusible pigments like the case of prodigiosin (Darah et al., 2014). In the three media used, more isolates produce yellow diffusible pigments as reported in previous studies (Sathi et al., 2002; Franks et al., 2005; Selvameenal et al., 2009; Sasidharan et al., 2013; Indra et al., 2014). However, the profiles of theses isolates for diffusible pigments production in the four media ISP1, ISP2, ISP4 and ISP6 are different. These results suggest the presence of polymorphic biosynthesis processes of these molecules.

Antibacterial activity screening

Screening of the isolated actinomycetes grown on ISP2 medium for antibacterial activity indicated that 5 (17.24%) of the 29 isolates are able to inhibit growth of at least one test bacterium (Table 5). Even there is a large difference in published ratio of isolates exhibiting antibacterial activity (Chaudhary et al., 2013; Ganesan et al., 2017), the study result of 17.24% is closely related to ratio obtained by Parthasarathi et al. (2010) and Chaudhary et al. (2013). Correlation between isolates producing diffusible pigments and exhibiting antibacterial activities has been suggested by Mamunur et al. (2014) and Ceylan et al. (2008). Consistent with these previous reported studies, only MFB-10 was negative in diffusible pigment production test among the 5 isolates exhibiting antibacterial activities. The Values of inhibition zone size showed that Gram positive test bacteria are globally more sensitive to the antibacterial compounds synthesised by these isolates. In Gram negative test bacteria, *E. coli* ATCC8739 was the most resistant to all compounds. MFB-27 and MFB-28 isolates produces the most effective compounds against Gram positive test bacteria. However in this antibacterial assay, isolates were grown on ISP2 medium. Their growth on other media could allow

Table 3. Colors of aerial and substrate mycelia of isolates on ISP2 and ISP4.

Isolate	Mycelium color on ISP2		Mycelium color on ISP4	
	Aerial mycelium	Substrate mycelium	Aerial mycelium	Substrate mycelium
MFB-01	Gray	Beige	White	Gray
MFB-02	Light gray	Yellow orange	Off-white	Brown
MFB-03	Yellow	Yellow	Beige	Yellow
MFB-04	Gray	Yellow brown	Gray	White
MFB-05	White	Light yellow	White	Beige
MFB-06	White	Light yellow	Gray	Gray-Brown
MFB-07	Green bleue	Light green	Light green	Brown
MFB-08	Off-white	Brown chocolate	Gray	Brown
MFB-09	Pearl white	Dark yellow	Pearl white	Yellow
MFB-10	Off-white	Yellow-brown	White	Brown
MFB-11	White-gray	Yellow brown	Gray	Purple-red
MFB-12	White	Light yellow	White	Beige
MFB-13	Green	Light green	Green	Light green
MFB-14	White	Yellow-orange	Pearl white	Light yellow
MFB-15	Gray-green	Black	Gray-brun	Black
MFB-16	Beige	Light yellow	White	Yellow-brown
MFB-17	Off-white	Light yellow	White	Light yellow
MFB-18	White	Yellow-orange	Pearl white	Light yellow
MFB-19	Off-white	Yellow-orange	Pearl white	Brown
MFB-20	White gray	Brown	Gray	Purple-red
MFB-21	White gray	Brown chocolate	Gray	Purple-red
MFB-22	Light yellow	Yellow	Yellow	Dark yellow
MFB-23	Beige	Light yellow	Gray	Orange-red
MFB-24	Gray	Brown	Gray	Purple-red
MFB-25	Black	Black	Black	Black
MFB-26	White gray	Brown orange	Light gray	Gray-brown
MFB-27	White	Light yellow	Black	Gray-brown
MFB-28	White	Light yellow	Light white	Beige
MFB-29	White gray	Brown chocolate	Light gray	Dark brown

Table 4. Diffusible pigments of isolates according to the ISP culture media.

Isolate	Diffusible pigment			Melanin pigment
	ISP1	ISP2	ISP4	ISP6
MFB-01	-	-	-	-
MFB-02	Light yellow	Yellow brown	Dark yellow	-
MFB-03	ND	Light yellow	-	ND
MFB-04	-	-	-	-
MFB-05	-	-	-	-
MFB-06	Light yellow	Light yellow	-	+
MFB-07	Beige	Light green	-	+
MFB-08	-	Dark brown	-	-
MFB-09	Dark brown	Yellow orange	-	+
MFB-10	-	-	-	-
MFB-11	-	Dark yellow	-	-
MFB-12	Light yellow	Dark yellow	-	+
MFB-13	Light yellow	Light yellow	-	-
MFB-14	Dark yellow	Yellow orange	-	+

Table 4. Contd.

MFB-15	Yellow brown	Black	-	-
MFB-16	Yellow brown	Dark brown	-	-
MFB-17	Yellow brown	Dark brown	-	-
MFB-18	Light yellow	Yellow brown	-	-
MFB-19	Light yellow	Yellow brown	-	-
MFB-20	-	Light yellow	-	-
MFB-21	-	Light yellow	-	-
MFB-22	ND	Yellow orange	ND	ND
MFB-23	Beige	Dark yellow	-	-
MFB-24	-	Yellow brown	-	-
MFB-25	Yellow brown	Yellow brown	-	-
MFB-26	Dark brown	Light brown	Light yellow	+
MFB-27	-	Dark yellow	Light yellow	-
MFB-28	-	Yellow green	Light yellow	-
MFB-29	Beige	Dark brown	-	-

Table 5. Inhibition zone diameter of positive isolates on test bacteria.

Isolate	Test bacteria						
	***E. cloacae* (mm)**	***K. pneumonie* (mm)**	***E. coli* (mm)**	***E. coli* ATCC8739 (mm)**	***S. aureus* ATCC29213 (mm)**	***S. aureus* ATCC6538 (mm)**	***M. leutus* ATCC9341 (mm)**
MFB-27	11	10	10	0	25	27	40
MFB-28	10	0	9	0	23	24	35
MFB-10	0	0	0	0	14	10	11
MFB-16	0	12	0	0	0	0	0
MFB-07	12	0	0	0	0	0	0

expression of other bioactive secondary metabolites as exhibited in non-diffusible and diffusible pigments differential expressions which were culture media dependent.

Conclusion

In this assessment work, we showed a high diversity of the actinomycetes isolated and their potential to produce antibacterial compounds. Soil samples used were rich in actinomycetes. All the isolated actinomycetes were able to produce various non-diffusible pigments and most of them secreted various hydrosoluble pigments and/or hydrosoluble compounds with antibacterial activities depending on the media used. This finding suggests the presence of high opportunity if other different culture media could be used to express various bioactive molecules of interest by the isolated actinomycetes strains or further new isolates from this actinomycetes rich soil. More investigations are ongoing in our Laboratory on the pigment antiproliferative activities, molecular identification of isolates showing activities and for structural determination of the active secondary metabolites.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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