INFLUENCE OF CULTURE MEDIA ON ANTIFUNGAL ACTIVITY PRODUCED BY STREPTOMYCES SP. PAL114 ISOLATED FROM GHARDAÏA DATE PALM GROVE SOILS

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Abstract

An actinobacterial strain, named PAL114, isolated from date palm grove soil of Ghardaïa (Algeria) showed an important antifungal activity against various pathogenic and/or mycotoxigenic fungi on different culture media. The highest level of antifungal activity was observed after the 12th day of fermentation. The Bennett medium was found to be the best medium for optimum growth and antifungal activity. The produced metabolites were detected by reverse-phase HPLC using a C18 column and the fractions corresponding to peaks were collected, concentrated then tested against Aspergillus carbonarius *M333 to detect the active fractions. The results showed the presence of novel bioactive metabolites on the 12th day, which exhibited significant antifungal activity.*

Keywords: *Streptomyces, Antagonistic potential, Antifungal production, Palm grove, Ghardaïa.*

Résumé:

Une souche actinobactérienne, PAL114, isolée d'un sol de palmeraie de Ghardaïa (Algérie) a montré une activité antifongique importante contre divers champignons pathogènes et/ou mycotoxinogènes sur différents milieux de culture. Les résultats ont montré que l'activité antifongique la plus élevée est obtenue après le 12ème jour de croissance. Le milieu Bennett s'est avéré être le meilleur milieu pour la croissance optimale et la production de molécules antifongiques. Les métabolites synthétisés ont été détectés par HPLC analytique en phase inverse en utilisant une colonne C18, et les fractions correspondant aux pics ont été récupérées, puis concentrées et enfin testées contre Aspergillus carbonarius *M333 pour détecter les fractions actives.*

Mots clés: *Streptomyces, Potentiel antagoniste, Production d'antifongiques, Palmeraie, Ghardaïa.*

INTRODUCTION

Micro-fungi cause a number of infectious diseases to animals and plants; as well as to humans [1,2]. Moreover, the ability of these filamentous microorganisms to produce mycotoxins can be harmful after ingestion of contaminated food and feed [3]. Current antifungal compounds are limited in their ability to treat infections due to the increase of the resistance in pathogenic and/or mycotoxigenic micro-fungi, and the toxicity of a wide range of antifungal molecules [4,5]. This circumstance motivates the search for new antifungal compounds.

Actinobacteria, a group of Gram positive bacteria, are particularly interesting for their high capacity to produce a large number of bioactive compounds such as antibiotics [6].

Amongst these bacteria, members of the genus *Streptomyces*, which are ubiquitously distributed in nature, are considered to be the most important producers of known antibiotics [7].
Likewise, the ability of *Streptomyces* cultures, to synthesize bioactive products is not a simple procedure, but can be greatly increased or completely lost under different conditions of nutrition and cultivation. Improvement in the bacterial growth and antibiotic production can be carried out by manipulating the nutritional and physical parameters of the culturing conditions [8].
Streptomyces sp. PAL114, a strain isolated from a Saharan soil in Ghardaïa (Mzab, Algeria), has shown to produce in liquid fermentations interesting antimicrobial and anticancer compounds, saquayamycins A and B, as reported by Aouiche et al. [9]. However, the high cost of large scale fermentation technology often limits industrial applications [10]. Among existing technologies in the fermentation industry, solid medium fermentation shows many advantages over fermentation with submerged culture, which include lower cost and high production titers [11]. The solid fermentation media consists of inert carriers with food bases used for biomass and compounds production [12]. In fact, the solid medium fermentation is considered as an alternative to submerged fermentation for production of value added products such as antibiotics, single cell protein, polyunsaturated fatty acids, enzymes, organic acids, biopesticides, biofuel and aroma production [12].
The selection of a suitable fermentation medium plays a vital role in making any industrial process cost effective. Therefore the present work was conducted to find out the most appropriate solid complex medium for the production of *Streptomyces* sp. PAL114-antibiotics.

MATERIALS AND METHODS

Actinobacterial strain

The actinobacterial strain, PAL114, was isolated from a Saharan soil collected in Béni Isguen, Ghardaïa province (Mzab region, Septentrional Sahara), in southern Algeria [9]. The strain was maintained on solid slants of ISP 2 (International Streptomyces Project 2) medium [13] containing (in 1L of distilled water): 4 g Glucose, 10 g malt extract, 4 g yeast extract and 18 g agar. The pH of the medium was adjusted to 7.2 ± 0.3 with a 2 M NaOH solution before autoclaving at 121 °C for 20 min.

Culture media for antibiotic production

The production of antibiotics by the strains PAL114 was studied on four different solid growth media. Each growth medium was incubated at 30 °C in Petri dishes for 7, 10, 12 and 14 days. The composition of each fermentation media is given in Table 1.

Antagonistic properties of the actinobacterial strain

In order to determine its antagonistic activity, strain PAL114 was tested by streak method against 4 micro-fungi, one yeast: *Candida albicans* M3, and three filamentous fungi: *Umbelopsis ramanniana* NRRL 1829, *Aspergillus carbonarius* M333 and *Aspergillus westerdijkiae*. The experiment was done by streaking a straight line of the PAL114 inoculum across the surface of medium on 90-mm diameter Petri dishes and incubated at 30 °C for 7, 10, 12 and 14 days. Then, target-fungi were seeded in streaks perpendicular to the actinobacterial strain (a single streak for each micro-fungus at 90° to actinobacterial strain). The antifungal activity was evaluated by measuring the distance of inhibition between target-fungi and actinobacterial colony margins, after

incubation at 30 °C for 24 h for yeasts, and 48 h for filamentous fungi. All results presented are mean values of three independent experiments.

Table 1- Composition of the different culture media used for the production of antibiotics by the strain *Streptomyces* sp. PAL114[a]

Components (g/L)	ISP2	Bennett	GYEA	NA
Glucose	4	10	10	-
Yeast extract	4	1	10	2
Malt extract	10	-	-	-
Peptone	-	2	-	5
Beef extract	-	1	-	1
NaCl	-	-	-	5

[a] The pH of each medium was adjusted to 7.2 ± 0.3 with a 2 M NaOH solution prior autoclaving (at 121°C for 20 min).
All experiments were performed in the solid medium separately.
NA: Nutrient Agar; GYEA: Glucose Yeast Extract Agar.

Extraction of antifungal compounds from culture medium

At the end of the incubation periods (as described above), the best culture medium was cut into small pieces and extracted with methanol under constant magnetic agitation of 250 rpm for 2 h (under 25 ± 3 °C). The extracts were concentrated to dryness by a rotary evaporator under vacuum at 40 °C. The residues were dissolved in 1 mL of methanol and subjected to biological assay (paper disk of 6 mm in diameter) against *Aspergillus carbonarius* M333. The disks received 50 µL of each extract and were placed on the ISP2 medium inoculated with the target-fungus (*A. carbonarius* M333). Inhibition zones were expressed as diameter (in mm) and measured after incubation at 30°C for 48 h. A paper disk containing the same volume of methanol acted as control.

High performance liquid chromatography (HPLC) analysis

The concentrations of antibiotic components were determined quantitatively by HPLC. A 100 µL volume of each sample was injected into the HPLC system (Agilent 1260) using a reverse phase C18 column (200 × 10 mm, 5 µm) with a continuous linear gradient solvent system from 20 to 100% methanol in water during 55 min, then remained at a steady flow of 100% of methanol for 15 min. A total run time was maintained at a flow rate of 1 mL/min under ambient temperature. The detection of metabolites was carried out at 220 nm. The fractions corresponding to peaks were collected, concentrated and then tested (6 mm paper disk diffusion method) against *A. carbonarius* M333 to detect the active fractions (showing antifungal activity) and distinguish them from the non-active fractions.

RESULTS AND DISCUSSION

Antifungal activity

As shown in Figure 1, among the tested solid growth media, Bennett was found to be the best culture medium as it exhibited the highest antagonistic activity. In fact, appreciable antifungal activity was obtained after 7 and 12 days (maximum) of incubation on Bennett solid medium. As expected, the different available components within the tested culture media directly induced differential production of secondary metabolites (antibiotics in this case).

All the complex culture media used, ISP2, Bennett, NA and GYEA, are able to supply both carbon and nitrogen sources. The selection of appropriate carbon and nitrogen sources can have a great effect on the production levels of secondary metabolites [14]. Nutrients such as amino acids can often act as both carbon and nitrogen sources. Carbon and nitrogen sources, as precursors, stimulated the biosynthesis of secondary metabolites either by increasing the amount of a limiting precursor, and/or by inducing a biosynthetic enzyme (synthase).

Amongst components, the literature referred that the nature and concentration of nitrogen plays an important role in controlling the process of antibiotic biosynthesis in bacteria such as Streptomyces [15,16]. Sanchez and Damian [17] reported that elevated levels of nitrogen affected the biosynthesis of enzymes involved in the production of primary and secondary metabolites as well as the consumption of different media nutrients.

The reduction of antifungal activity in ISP2 and GYEA media (Figures 1B, 1C) was probably due to the lack of complex nitrogen sources in the media (peptone and beef extract). Moreover, the ISP2 and GYEA media contain glucose, which is one of the carbon sources that is metabolized very rapidly and it suppresses secondary metabolite biosynthesis. Consequently, this simple sugar inhibits the formation of most important enzymes in biosynthetic pathways [18].

In contrast to ISP2 and GYEA, the Bennett and NA media (Figures 1A and 1D) both contains peptone and beef extracts as complex nitrogen sources that probably enhanced the production of antifungal compounds.

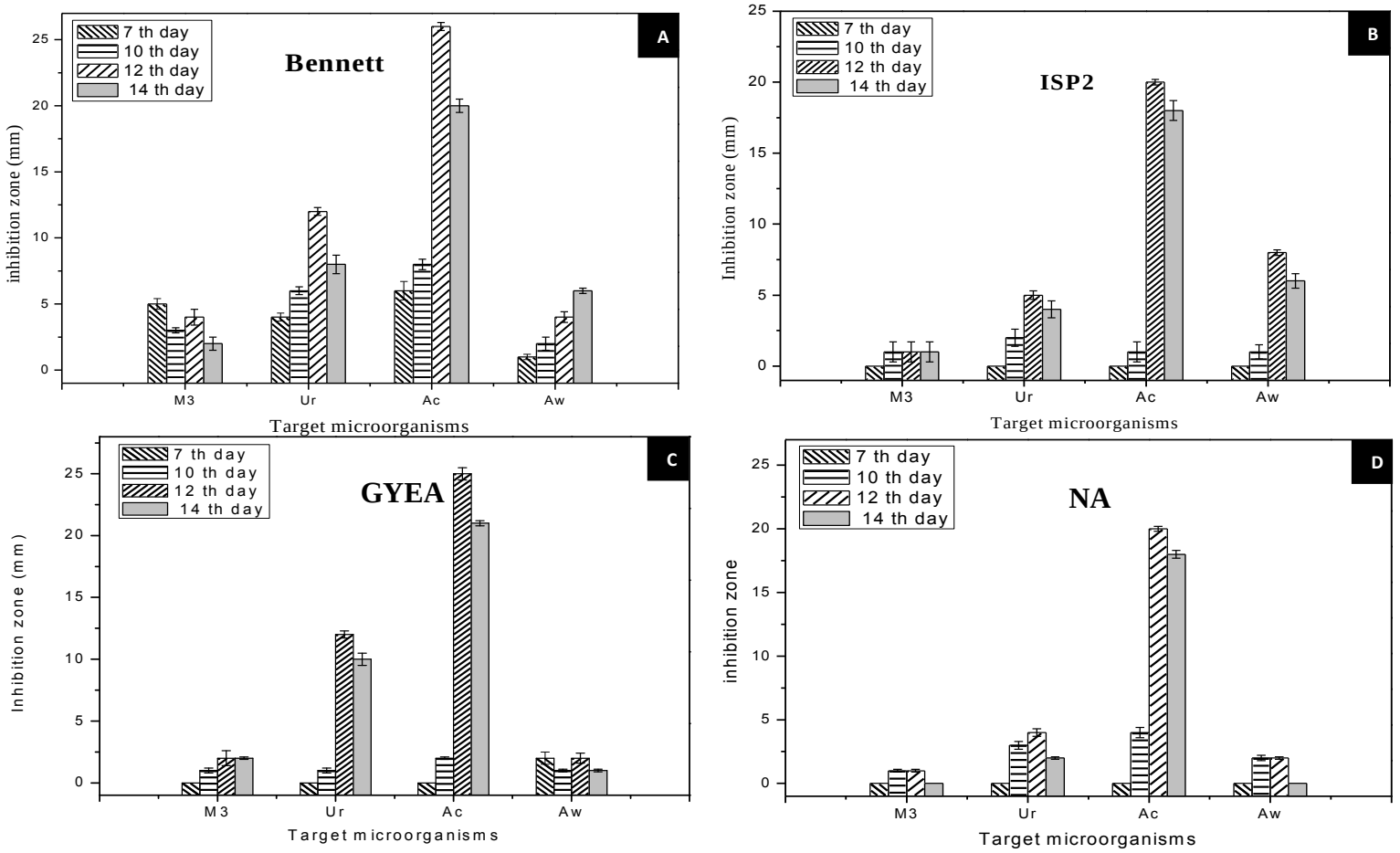

Figure 1. Effect of solid culture media: Bennett (A), ISP2 (B), GYEA (C) and NA (D) media on the production of antifungal activities by *Streptomyces* sp. PAL114.

M3: *Candida albicans* M3, Ur: *Umbelopsis ramanniana* NRRL 1829, Ac: *Aspergillus carbonarius* M333; Aw: *Aspergillus westerdijkiae*.

Antimicrobial compounds production assay

Since solid Bennett was found to be the best medium for production of antifungal activities, it was thus chosen as the production medium for the bioactive compounds.

The antifungal activity exhibited by the strain Streptomyces sp. PAL114 on solid Bennett medium during the fermentation time course was evaluated against three filamentous fungi: *Umbelopsis ramanniana* NRRL 1829, *Aspergillus carbonarius* M333 and *Aspergillus westerdijkiae* as shown in Figure 2. These results revealed that the maximum antifungal activities were obtained 12 days of solid fermentation. Among the targeted fungal strains, *U. ramanniana* NRRL 1829 and *A. carbonarius* M333 were the most sensitive.

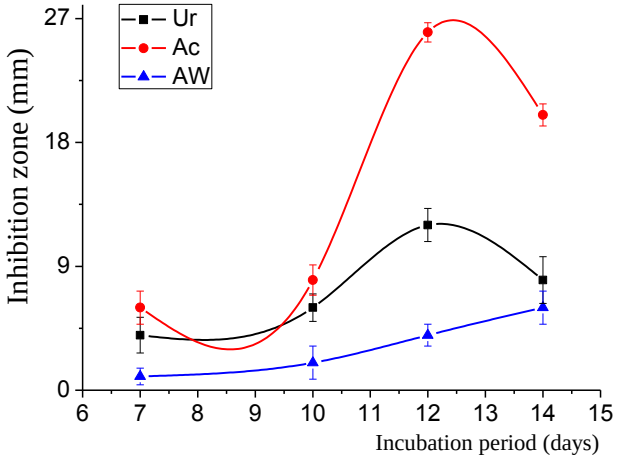

Figure 2. Antifungal activity of Streptomyces sp. PAL114 expressed on solid Bennett medium.

Ur: Umbelopsis ramanniana NRRL 1829, Ac: *Aspergillus carbonarius* M333, Aw: *Aspergillus westerdijkiae*.

HPLC analysis

The antifungal compounds present in the Bennett medium on the 7th and 12th days were analyzed by the HPLC analysis. The HPLC profiles indicated that the Bennett medium supported the biosynthesis of several secondary metabolites at 7th and 12th days of fermentation (data not shown). In order to test their antifungal activity, the HPLC profile obtained on the 12th day of fermentation was divided into four groups designated G1 (retention time (RT) ranging from 0 to 30 min), G2 (RT from 30 to 50 min), G3 (RT from 50 to 60 min) and G4 (RT from 60 to 70 min). Each group of metabolites was tested separately against *Aspergillus carbonarius* M333, and the results obtained are presented in table 2.

Table 2- Antifungal activities of the purified active molecules secreted by the strain *Streptomyces* sp. PAL114 in the Bennett medium at the 7th and 12th day of fermentation.

Fractions	Antifungal activity (mm) against *Aspergillus carbonarius* M333	
	7th day	12th day
G1	-	-
G2	-	-
G3	-	13
G4	-	-

The results indicated that, the best antifungal activity expressed on the 12th day was attributed only to the group G3 (RT ranging from 50 to 60 min), with a diameter of inhibition of 13 mm.

CONCLUSION

Changes in the nature and type of carbon and nitrogen sources present within the complex solid medium tested were shown to affect the formation of cell biomass and also influenced bioactive secondary metabolites biosynthesis in the strain Streptomyces sp. PAL114.

The results of antimicrobial susceptibility tests indicated that antifungal metabolites obtained from the actinobacterium PAL114 were enhanced in the Bennett medium. The preliminary data obtained from this work will be useful for the consecutive full characterization of the Streptomyces strain PAL114 induced antifungal metabolites.

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