



Extraction and preliminary characterization of bioactive molecules produced by a new *Streptomyces* strain

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Abstract

The aim of this study was the description of a new *streptomyces* strain (SP13'), isolated from pottery discharge samples of Boujad region in Morocco. The strain showed interesting antagonistic activity against various test microorganisms. The bioactive metabolites were extracted from both mycelia and culture supernatant of the strain SP13' with n-butanol, ethanol, methanol, hexane, acetone, dichloromethane and chloroforme. The extracts activity was evaluated against two Gram-positive bacteria (*Staphylococcus aureus* ATCC 29213, *Bacillus subtilis*), two gram-negative bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae*), two filamentous fungi (*Rhizomucor* sp. *Penicillium expansum*) and one yeast (*Candida albicans*). The nature of the active extracts was determined by UV spectrophotometry technique. Stability of antimicrobial activity of active extracts was tested by conventional methods according to pH, temperature and light. UV spectra of extracts have shown that the antimicrobial metabolites were a non-polyenic nature. They were not thermostable and there was no significant loss of the antimicrobial activity after treatment with various other factors (pH, light, duration freezing).

Key words: Non-polyene, stability, biological activity, *Streptomyces crystallinus*, pottery discharge.

1 Introduction

Actinomycetes are filamentous bacteria that grow on a wide range of substrates and colonize almost all environments, even those where life is extremely hostile [1]. They are responsible for producing most of bioactive molecules and are the largest supplier of new antibiotics [2]. Antibiotics discovered were mainly isolated from *Streptomyces* species, representing some 70 to 80% of the all isolated compounds. They were primarily active against bacteria and fungi [3]. Though there are number of antibiotics, the need to search for new and efficient antibiotic producing strains keeps rising due to the emergence of drug resistant pathogens [4].

In order to discover original taxa and new bioactive molecules, current researches are often oriented to exploring unusual ecosystems [5]. Morocco presents many unexplored environments, pottery discharge is an environment not yet explored. The *Streptomyces* strain (SP13') was isolated from this particular environment. It was identified as a *Streptomyces* specie very close to the

Streptomyces crystallinus. Based on the 16S rDNA analysis and cultural, morphological and physiological characteristics, this isolate was found to be quite different from *S. crystallinus* and could be new *Streptomyces* specie [6]. It produced interesting bioactive metabolites against various pathogenic fungi and bacteria [7].

The aim of this work was to extract of bioactive molecules by organic solvents, to determine their UV-Visible spectra and to study the stability of their antibacterial activity according to various parameters (temperature, pH and light). Among the used techniques for determination of the polyenic nature of active metabolites produced by actinomycetes [8], spectrophotometry is a direct physical method used by several authors [9, 10, 11] for distinguishing between polyenes and non-polyenes. Indeed, the polyenes have spectra with a peaks series between 260 and 405 nm [12, 13].

Various studies of stability of antimicrobial activity have shown that the secondary metabolites produced by the actinomycetes generally exhibit bioactivity at well defined intervals of temperatures and pH [14, 15, 16].

The present study is aimed to evaluate the stability of antimicrobial activity depending on some physicochemical parameters (temperature, pH, enlightment) of bioactive metabolites produced by the SP13' strain.

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2. Materials and Methods

2.1 UV-visible spectrophotometric study of SP13' extracts

The SP13' strain was inoculated into 10 flasks (250 mL) containing 100 mL of synthetic medium [7]. After incubation at 28°C under stirring for 8 days, the culture medium was centrifuged at 5000 r / min for 20 min. The extraction of active product was performed from the supernatant and pellet by organic solvents with different polarities.

The pellet was washed twice with sterile distilled water, and mixed with five volumes of the each following solvents (n-butanol, ethanol, methanol, hexane, acetone, dichloromethane and chloroform) for twelve hours stirring at room temperature [17]. The obtained mixture was then centrifuged at 4500 r / min for 15 min [18], and the extracts were tested to highlight their antifungal and antibacterial activity by the paper disks technique [14].

In order to determine the solvents able to dissolve the active metabolites contained in supernatant, the following organic solvents were tested: n-butanol, ethanol, methanol, hexane, acetone, dichloromethane and chloroform. After phase separation in separating funnels, the extracts obtained were tested for the detection of antimicrobial activity by the paper disks technique [14, 16, 19].

The following test microorganisms were used during the investigation: two Gram-positive bacteria (*Staphylococcus aureus* ATCC 29213, *Bacillus subtilis*), two gram-negative bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae*), two filamentous fungi (*Rhizomucor* sp. *Penicillium expansum*) and one yeast (*Candida albicans*).

The visible UV spectra were carried out between 200 and 420 nm [20] using a spectrophotometer on the organic extracts exhibiting antimicrobial activity. The blank was constituted by:

- The mixture of sterile culture medium and solvent for the absorbance measurement of extracts from supernatant;
- The only solvent for the absorbance measurement of extracts from pellet.

The measurements were performed in quartz cuvettes with a 1 cm optical path.

2.2 Stability of antimicrobial activity of active extracts

Stability of bioactive molecules contained in the active extracts was highlighted by the action of some

physicochemical factors (temperature, pH, light). The extracts were sterilized by filtration through a Millipore membrane (0.45 microns) [18].

The antimicrobial activity of the treated extracts was tested against three microorganisms: Gram-positive bacteria (*S. aureus* ATCC 29213), a gram negative bacteria (*E. coli* ATCC 25922) and a filamentous fungus (*Rhizomucor* sp.) by the agar diffusion method using paper discs technique, the activity was compared to that obtained with the untreated extracts.

2.2.1 Stability of antimicrobial activity of active extracts according to pH

Volumes of 5 ml of crude active extracts of SP13' strain were adjusted at pH values ranging from 2 to 10 with sterile solutions of NaOH and HCl [21].

2.2.2 Stability of antimicrobial activity of active extracts according to temperature

Fractions of 5 mL of active extracts strain SP13' were subjected to a treatment by different temperatures:

- - 20 °C and room temperature for 24 hours;
- 50 ° C and 70 °C for 30 min;
- 100 °C for 15 min.

2.2.3 Stability of antimicrobial activity of active extracts according to light

To evaluation the effect of light on the extracts antimicrobial activity of SP13' strain, fractions of 5 mL of gross active organic extracts were placed at room temperature and exposed to lamp action (75 Watt) or in the dark with a 24 hour exposure time.

3 Results and Discussion

3.1 UV-visible spectrophotometric study extracts from SP13' strain

3.1.1 Evaluation of the extracts antimicrobial activity

All of the obtained organic extracts from SP13' cultures were tested for their antifungal and antibacterial activities. Some of these extracts showed no antimicrobial activity, while others are active against at least one test microorganism. The inhibitory effect results of these extracts on different tests microorganisms (fungi, bacteria and yeast) are presented in tables 1 and 2.

Table 1: Antimicrobial activity of mycelium organic extracts

Microorganisms test	Ethanol	Methanol	Acetone	Hexane	Butanol	Dichloromethane	Chloroforme
Gram- positive bacteria	<i>S. aureus</i> ATCC 29213	0	1	1	0	0	1
	<i>B. subtilis</i>	1	1	1	0	0	0
Gram- negative bacteria	<i>E. coli</i> ATCC 25922	0	1	0	0	0	0
	<i>K. pneumoniae</i>	0	1	0	0	0	1
filamentous fungi	<i>Rhizomucor</i> sp.	0	1	0	0	0	0
	<i>P. expansum</i>	0	0	0	0	0	1
yeast	<i>C. albicans</i>	0	1	1	0	0	0

0: Absence of activity ; 1: Presence of activity

Table 2: Antimicrobial activity of supernatant organic extracts

test microorganisms	Ethanol	Methanol	Acetone	Hexane	Butanol	Dichloromethane	Chloroforme
Gram- positive Bacteria	<i>S. aureus</i> ATCC 29213	0	0	0	0	1	0
	<i>B. subtilis</i>	0	0	0	0	1	1
Gram- negative Bacteria	<i>E. coli</i> ATCC 25922	0	0	0	0	1	1
	<i>K. pneumoniae</i>	0	0	0	0	0	0
filamentous fungi	<i>Rhizomucor</i> sp.	0	0	0	0	1	0
	<i>P. expansum</i>	0	0	0	0	1	1
yeast	<i>C. albicans</i>	0	0	0	0	1	1

0: Absence of activity ; 1: Presence of activity

The antimicrobial activity of organic extracts from SP13' strain varies according to solvent nature and microorganism tested. Indeed, no antibacterial activity was detected from hexanoic and methanolic extracts of pellet and ethanolic, methanolic, hexanolic and acetonic extracts of supernatant. In contrary, the ethanolic, methanolic, acetonic, chloroformic and dichlorométhanolic extracts of pellet and, butanolic, chloroformic and dichlorométhanolic extracts of supernatant showed antimicrobial activity against at least one studied test microorganism.

These results reflected the production of two active metabolites categories by the investigated strain:

- A class of mycelial origin metabolites soluble in organic solvents having different polarities;
- A class of metabolites released in the medium of culture, essentially soluble in butanol which is a nonpolar solvent, and chloroform and dichloroforme which are solvents of intermediate polarity.

The most intense activity was obtained by the discs impregnated with the methanolic extracts of pellet and with butanolic extracts of supernatant. Thus, the butanol and methanol are considered the best solvents for extracting of bioactive compounds produced by SP13' strain against seven microorganisms studied.

3.1.2 Study of UV-Visible absorption spectrum

UV-Visible spectra of the active extracts of SP13' strain is presented in Figures 1, 2. The spectra analysis showed the absence of the characteristic peaks of polyenes. The absorption maxima are obtained respectively at 330 nm for the methanolic extract of mycelium and at 250 nm for the butanolic extract of supernatant. In contrary, the spectra of active extracts from Rhc2 reference strain [19] which produced polyenic substances, revealed the existence of peaks specific of polyenic structure (Figure 3).

This result indicates that the *Streptomyces* strain SP13' produces two non-polyenic bioactive molecules, a mycelial original molecule and another liberated into the culture medium.

3.2 Study of antimicrobial activity stability of bioactive metabolites produced by SP13' strain

The butanolic extracts from supernatant and the methanolic extracts from pellet are showed strong activity against *S. aureus* ATCC 29213, *E. coli* ATCC 25922 and *Rhizomucor* sp. These microorganisms are selected for stability tests of the antimicrobial activity according to temperature, pH, light and darkness.

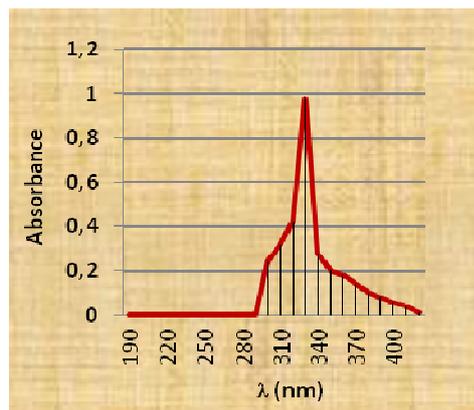


Figure 1: UV-Visible spectrum of methanolic extract from pellet of SP13'

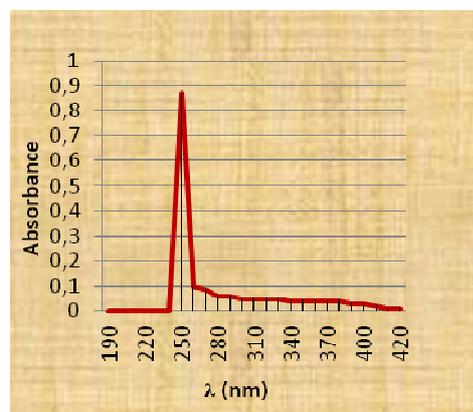


Figure 2: UV-Visible spectrum of butanolic extract from supernatant of SP13'

3.2.1 Stability of active extracts antimicrobial activity according to pH

The variation results of activity of the active extracts from SP13' strain according to pH are summarized in tables 3 and 4.

Table 3 : Antimicrobial activity of methanolic extracts from pellet according to pH

Test microorganisms	Diameters average of inhibition zones in mm								
	pH 2	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10
<i>S. aureus</i> (ATCC 29213)	0	1	4,33	2,33	7,66	6,33	5,33	0	0
<i>E. coli</i> (ATCC 25922)	0	1	0	1	1,33	6,66	8,33	7	4,33
<i>Rhizomucor</i> sp.	0	2	1,66	3,66	3	3,33	1,23	0	0

The values represent the average of three repeats

Table 4: Antimicrobial activity of butanolic extracts from supernatant according to pH

Test microorganisms	Diameters average of inhibition zones in mm								
	pH 2	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10
<i>S. aureus</i> (ATCC 29213)	0	1,66	2,33	3	8	9,33	7,33	2	0
<i>E. coli</i> (ATCC 25922)	0	0	7	6,33	1	3,66	10,33	3,33	0
<i>Rhizomucor</i> sp.	0	0	4	7,66	8	2	1	0	0

The values represent the average of three repeats

Table 5 : Antimicrobial activity of butanolic extracts from supernatant according to temperature and light

Test microorganisms	Diameters average of inhibition zones in mm					
	- 20°C	50°C	70°C	100°C	Light	Obscurity
<i>S. aureus</i> (ATCC 29213)	6	0,33	0	0	8	7,33
<i>E. coli</i> (ATCC 25922)	8	4	1	0	7	3,66
<i>Rhizomucor</i> sp.	2	5	3,33	0	8	6,66

The values represent the average of three repeats

Table 6: Antimicrobial activity of methanolic extracts from pellet according to temperature and light

Test microorganisms	Diameters average of inhibition zones in mm					
	- 20°C	50°C	70°C	100°C	Light	Obscurity
<i>S. aureus</i> (ATCC 29213)	5,33	2,66	1,66	0	6	8
<i>E. coli</i> (ATCC 25922)	7,33	4	0	0	6	4
<i>Rhizomucor</i> sp.	4	7	4	0	10	7

The values represent the average of three repeats

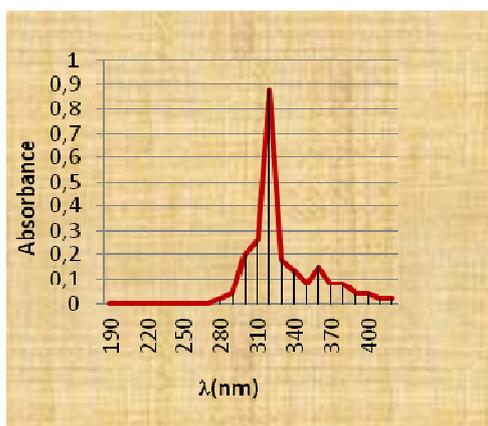


Figure 3: UV-Visible spectrum of chloroformic extract from supernatant of Rhc2

Results showed that the organic extracts activity against the three microorganisms studied was maintained at different pH values.

- The activity against *S. aureus* ATCC 29213 was saved at pH values from 4 to 8 with optimal activity

at pH = 6 for the methanolic extract and at pH = 7 for butanolic extract;

- The activity against *E. coli* ATCC 25922 has an optimum at pH = 8 and two optimum methanolic extract for butanolic extract: the first at pH = 5 and the second at pH = 8;
- The activity against *Rhizomucor* is interesting at acid pH, it is optimal at pH = 6 for methanolic extract and at pH = 5 for the butanolic extract.

The comparison between diameters of inhibition zones of treated extracts with pH and those untreated allow concluding that the pH has a significant effect on the stability of antimicrobial molecules and on their activity persistence. The bioactive molecules produced by SP13' strain maintain their stability in the pH range 3 to 8, while a strong acidity or high alkalinity causing a total loss of antimicrobial activity. These results suggested that the SP13' strain produces several bioactive molecules. Indeed, studies by Florey et al. (1949) [22], have shown that a compound soluble in an organic solvent at any pH, presents itself this pH value (if it is soluble in an acidic solvent it is itself acid). Hence the active extracts of SP13' strain probably contained:

- Acidic antimicrobial molecules having activity against *Rhizomucor* sp.,
- Basic and acidic antimicrobial molecules having activity against *E. coli* ATCC 25922
- Neutral pH substances active against *S. aureus*.

3.2.2 Stability of antimicrobial activity according to temperature and light

The inhibition zone diameters caused by the various organic extracts from SP13' strain and maintained at different temperatures and/or exposed to light or darkness are grouped in tables 5 and 6.

The analysis of these results showed that the antimicrobial activity of active extracts against test microorganisms was reduced from 50 % to almost 100 % after heat treatment. The Heating of extracts for 15 min at 100°C caused a complete loss of antimicrobial activity. However, the exception of contained bioactive molecules in the active extracts against *Rhizomucor* sp. which had lost the half of their activity after freezing, the antimicrobial activity was shown insensitive to freezing at -20°C. The effect of light and darkness exposure of extracts did not present a significant effect on the active extracts against *E. coli*.

These results suggested that the temperature has a significant effect on the stability of bioactive molecules and hence the stability of their antibacterial activity. The disappearance of antibacterial activity after 15 min heating at 100°C indicated the absence of thermostable molecules in the organic extracts of SP13' strain.

4 Conclusion

The present study shows that the extracts from the new *Streptomyces* strain SP13' displayed a strong potential alternative against pathogenic bacteria and fungi. The antimicrobial activity of organic extracts varies according to the solvent nature used. We can conclude that this strain is able to produce many bioactive compounds:

- Acidic molecules presenting antimicrobial activity against *Rhizomucor* sp.;
- Acidic and basic molecules presenting antimicrobial activity against *E. coli* ATCC 25922;
- Neutral molecules presenting antimicrobial activity against *S. aureus*

The stability study of antimicrobial activity according some parameters allow us to conclude that the antimicrobial compounds produced by SP13' were not thermostable and they preserved their stability at pH ranging from 3 to 8.

The characteristic UV spectra of active extracts revealed that this strain produced non-polyenic antimicrobial substances.

Briefly, the new *Streptomyces* SP13', isolated from a pottery discharge, produced interesting bioactive molecules with a non-polyenic structure. This clearly demonstrated the interest that may have actinomycetes isolated from unusual environment and encourages further study both on the actinomycete strain and on the biological activity of secondary metabolites. Thus, it is necessary to:

- Extract, purify and characterize the active principles of bioactive metabolites contained in the active extracts
- Deepen the study of bioactive molecules purified so as to know their antioxidant, anticancer, antimutagenic, toxic and genotoxic effects on animal cells.

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