ANTIMICROBIAL SCREENING OF *Saccharothrix tamanrassetensis* DSM 45947 AND EFFECT OF CARBON AND NITROGEN SOURCES ON ANTIBIOTIC PRODUCTION

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(Received 21 November 2019 - Accepted 24 October 2020)

Abstract.- *The Algerian Saharan actinobacterium Saccharothrix tamanrassetensis DSM 45947, exhibited an antagonist activity against Gram-positive bacteria, yeasts and filamentous pathogenic, toxigenic and phytopathogenic fungi. The antimicrobial potential of the strain was examined by PCR for the presence of type I and II of polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) gene clusters. The latter gene was detected, but the first two genes were absent. Production of antimicrobial (antifungal and antibacterial) activity was investigated by following kinetics in a semi-synthetic broth supplemented with various carbon and nitrogen sources. The highest activity was obtained with corn steep liquor and sucrose. Antimicrobial compounds were extracted from the culture filtrate. A strong activity was detected in the aqueous phase extract; however the activity in the organic extract was weak. The crude extracts were injected in HPLC for purification. Each fraction was tested for antimicrobial activity. The results revealed 7 active peaks in the aqueous extract, suggesting the presence of novel antibiotics.*

Key words: Saccharothrix tamanrassetensis, *antagonist activity, PCR, genes, carbon and nitrogen sources; production kinetics, HPLC.*

CRIBLAGE ANTIMICROBIEN DE *Saccharothrix tamanrassetensis* DSM 45947 ET EFFET DES SOURCES DE CARBONE ET D'AZOTE SUR LA PRODUCTION D'ANTIBIOTIQUES

Résumé.- *L'actinobactérie saharienne algérienne Saccharothrix tamanrassetensis DSM 45947 a montré une activité antagoniste contre les bactéries à Gram positif, les levures et les champignons pathogènes, toxinogènes et phytopathogènes. Le potentiel antimicrobien de la souche est examiné par PCR pour la présence des types I et II de polyketide synthase (PKS) et du groupe de gènes non-ribosomal peptide synthetase (NRPS). Ce dernier gène a été détecté, mais les deux premiers gènes étaient absents. La production de l'activité antimicrobienne (antifongique et antibactérienne) a été étudiée en suivant des cinétiques en milieu semi-synthétique additionné de différentes sources de carbone et d'azote. L'activité la plus élevée est obtenue avec le corn steep liquor et le saccharose. Les composés antimicrobiens sont extraits à partir du filtrat de culture. Une forte activité antibiotique est détectée dans l'extrait de la phase aqueuse, en revanche l'activité de la phase organique est faible. Les extraits bruts sont injectés en HPLC pour purification. Chaque fraction a été testée pour l'activité antimicrobienne. Les résultats ont révélé la présence de 7 pics actifs dans l'extrait aqueux, suggérant la présence de nouveaux antibiotiques.*

Mots clés: *Saccharothrix tamanrassetensis, activité antagoniste, PCR, gènes, sources de carbone et d'azote, cinétiques de production, HPLC.*

Introduction

There is clearly a need for new antimicrobials to combat resistant pathogens in serious and life-threatening diseases [1,2]. Microbial natural products continue to represent an important route to the discovery of new antimicrobial compounds [3]. Actinobacteria have proved to be important sources of industrially useful antibiotics [4,5]. In order to maximize the success rate of drug discovery using natural products, novel antibiotic screening strategies focused on rare actinobacteria strains that are isolated from extreme environments [6]. Many reports have shown that such strains demonstrated novelty and chemical diversity in the produced antibiotics. Microbial groups living under extreme conditions exhibit a variety of physiological and metabolic features allowing them to adapt to these particular environments. They possess a genetic diversity and specific secondary metabolite producing profiles for synthesizing novel antibiotics [6-8]. The Algerian arid soils, exposed to an arid climate, are special ecosystems that have been widely explored in our laboratory. A remarkable diversity of new species has been discovered; most of which showed a strong biosynthetic potential in producing novel antibiotics [9-17].

Since its first description [18], the rare genus *Saccharothrix* has been shown to produce a vast diversity of different bioactive secondary metabolites, including the antiviral agent fluvirucin from *Saccharothrix mutabilis* [19] and the antibacterial agent saccharomicin from *Saccharothrix espanaensis* [20].

The Algerian Saharan soils have been shown to be rich in species and strains of the *Saccharothrix* genus [21] including new species, such as *S. algeriensis* NRRL B-24137^T [22], *S. hoggarensis* DSM 45457^T, *S. saharensis* DSM 45456^T, *S. tamanrassetensis* DSM 45947^T [23-25], *S. isguenensis* DSM 46885^T and *S. ghardaiensis* DSM 46886^T [26,27]. The obtained promising results emphasize the need to continue the research into *Saccharothrix* antimicrobial compounds production. Furthermore, many researches have shown the ability of Saharan *Saccharothrix* strains to produce new or already known antibiotics, such as dithiolopyrrolones [28,29], anthracyclines [30] and chloramphenicol [31].

In this context, the new species *Saccharothrix tamanrassetensis* DSM 45947^T isolated from a Saharan soil sample, was investigated for its antimicrobial activity by the cylinder plating method and by screening the gene fragments of NRPS, PKS-I and PKS-II domains. Production, extraction and preliminary purification of antimicrobial compounds were also carried out.

1.- Material and methods

1.1.- The actinobacterial strain

Saccharothrix tamanrassetensis DSM 45947 was isolated in our laboratory from a soil sample collected from Tamanrasset (Hoggar region), in southern Algeria [6]. The strain was characterized taxonomically as a new species by BOUBETRA *et al.* (2013a) [23]. The strain was maintained as spore suspension in 25% glycerol at -20 °C.

1.2.- Antagonistic properties of *S. tamanrassetensis*

Antimicrobial screening was carried out using the cylinder plating method against a total of 40 different microorganism strains. The target tested strains were mostly human

pathogens or phytopathogens. They included seven Gram-positive bacteria (*Bacillus subtilis* ATCC 6633, *Enterococcus feacalis* Ef B1, *Listeria monocytogenes* ATCC 13932, methicillin resistant *Staphylococcus aureus* ATCC 25923, *S. aureus* ATCC 25922, *S. aureus* ATCC 43300 and *S. aureus* 639c); five Gram-negative bacteria (*Klebsiella pneumoniae* CIP 82.91, *Acinetobacter baumanii* E16, *Escherichia coli* E52, *Pseudomonas aeruginosa* ATCC 27853 and *Agrobacterium tumefaciens* At2410); twenty filamentous fungi (*Fusarium solani* Fsol, *F. sporotrichioides* Fs, *F. culmorum* Fc1, *F. proliferatum* FprX20, *F. oxysporum* f. sp. *lini* Fol, *F. equiseti* Fe, *F. moniliforme* FmX22, *F. oxysporum* f. sp. *albedinis* Foa, *F. oxysporum* f. sp. *radicis lycopersici* Forl, *Penicillium expansum* Pe1, *P. glabrum* Pg1, *Umbelopsis ramanniana* NRRL 1829, *Aspergillus carbonarius* M333, *A. niger* OT304, *A. westerdijkiae* ATCC 3174, *A. parasiticus* CBS 100926, *A. flavus* Af3, *Trichoderma* sp. TdR1, *Alternaria* sp. AltX1 and *Botrytis cinerea* BcvX1); seven yeasts (*Candida albicans* IPA200, *C. albicans* IPA988, *C. tropicalis* CtY20, *C. glabrata* CgY21, *C. pseudotropicalis* CpsY22, *C. zeylanoides* CzY23 and *C. guilliermondii* CgY24) and a mycoplasma strain (*Ureaplasma parvum* ATCC 27813). The strains without an official accession number belong to our laboratory collection.

Cylinders were cut from 10 days old actinobacterium strain culture on ISP2 agar medium which were transferred, each one on a semi-solid ISP2 plate freshly seeded with a test strain. The plates were then incubated at 30°C and the diameters of the inhibition zones were measured after 24 h for the bacteria and 48 h for the yeasts and micro-fungi. All tests were conducted in triplicate ± standard deviation (SD).

1.3.- DNA preparation and detection of PKS-I, PKS-II and NRPS sequences

Genomic potential for producing bioactive metabolites of *Saccharothrix tamanrassetensis* DSM 45947 was evaluated. The strain was grown in 100 ml of ISP2 broth culture. Biomass was harvested by centrifugation for 10 mn (at 8000 rpm) and washed twice with double-distilled water. Chromosomal DNA was prepared using a DNA extraction kit (JetFlex, Germany).

The following specific degenerate primers were used to detect the genes encoding the polyketide synthetase I (PKS-I), polyketide synthetase II (PKS-II) and non-ribosomal peptide synthetase (NRPS) genes: PKS-I: K1F/M6R (5′-TSAAGTCSAACATCGGBCA3′/5′-CGCAGGTTSCSGTACCAGTA-3′; [32]; PKS-II: KSα/KSβ (5′-TSGCSTGCTTGGAYGCSATC3′/5′-TGGAANCCGCCGAABCCTCT-3′ [33] and NRPS: A3F/A7R (5′-GCSTACSYSATSTACA CSTCSGG-3′/5′-SASGTCVCCSGTSCGGTAS-3′ [32].

The 50 µl PCR reaction mixture contained 20-40 ng of DNA template, 2 µm of each primer, 0.4 µm of dNTP mixture, 10 × reaction buffer containing $MgCl_2$, 5% DMSO and 2.5 U of *Taq* DNA polymerase. The PCR thermal cycling program comprised an initial denaturation at 98°C (for 4 mn), followed by 30 cycles comprising a denaturation step at 94°C (1 mn), an annealing step of 1 mn at 57.5°C with K1F/M6R, at 58°C with KSα/KSβ, at 62°C with EdyA/EdyE and at 57°C with A3F/A7R and termnated with an extension step of 1 mn at 72°C. A final extension was performed at 72°C for 10 mn. All of the amplification products were separated by electrophoresis in an 0.8% agarose gel and bands of 1200-1400, 600 and 700-800 bp were classified as products of the PKS-I, PKS-II and NRPS genes, respectively.

1.4.- Selection of the optimal carbon and nitrogen sources

In order to investigate the effect of the nitrogen and carbon sources on the antimicrobial activity produced by *S. tamanrassetensis*, a basal semi-synthetic medium (SSM) was used. This medium, used for both pre-culture and production of antibiotics, consisted of (1 g/l of distilled water): NaCl 2.0, $CaCl_2$ 1.0, K_2HPO_4 1.0, KH_2PO_4 0.5, $MgSO_4.7H_2O$ 0.2, $FeSO_4.7H_2O$ 0.01, pH 7.2) [34].

For inoculum preparation, spore suspension of *S. tamanrassetensis* maintained in glycerol (25%) was transferred to ISP2 solid medium plates. After 10 days of incubation, the seed inoculation culture was prepared by using spore cakes as described by Ahsan et al. [35]. Two spore cakes (4 mm diameter), were transferred to a 250 ml flask containing 50 ml of the culture medium, then incubated under permanent agitation (250 rpm) at 30°C for 48 h. Aliquots 5% (v/v) of this seed culture were used to inoculate 100 ml of the fermentation medium contained in 500-ml Erlenmeyer flasks.

For the selection of the best nitrogen and carbon sources, twelve nitrogen sources and five carbon sources were investigated. The nitrogen sources included corn steep liquor (5% w/v); peptone, tryptone, casein, yeast extract, malt extract (0.25 % w/v), and arginine, valine, leucine, methionine, cystine and lysine at 0.05 % (w/v). These broths were supplemented with glucose (1%) as the carbon source. Carbon sources included glucose, sucrose, fructose and dextrin at 1% and sodium acetate at 0.1% (w/v). In these broths, corn steep liquor (5% v/v) was added as the nitrogen source. Final media pH was adjusted to 7.2. Cultures were incubated on a rotary shaker (250 rpm) at 30°C for 10 days. All results presented are mean values of three independent experiments.

1.5.- Antimicrobial assay

The antimicrobial activity of the collected culture filtrates was determined each 24 h using the agar well diffusion method against methicillin-resistant *Staphylococcus aureus* 639c (MRSA) and *Umbelopsis ramanniana* NRRL1829 (UR). The bacterial suspension was prepared in sterile distilled water and adjusted as inoculum to a final concentration of 1.2×10^8 CFU/ml, while the micro-fungal suspension was adjusted to a final concentration of 0.4×10^6 CFU/ml [36]. Petri dishes were poured with Mueller-Hinton (for MRSA) or Sabouraud (for UR) agar (12 g/l agar) culture media, previously inoculated with each microbial suspension. Wells of 7 mm were cut out from the agar plates and filled with 100 µl of culture filtrate. The plates were incubated at 30°C after a diffusion process of 2 h at 4°C. The antibacterial and antifungal activities were evaluated by measuring the diameter of inhibition zones around the wells after 24 h of incubation for MRSA and 48 h for UR. Results are expressed as mean of triplicates and standard error of the mean.

1.6.- Production and extraction of antibiotics in the selected culture medium

Before culture productions, time course of pH, growth and antibiotic production was monitored in agitated 500 ml Erlenmeyer flasks containing 100 ml of the culture medium supplemented with the best carbon and nitrogen sources (250 rpm, 30°C, 10 days).

The antimicrobial activity was daily assayed against MRSA and UR, by the agar well diffusion method previously described. The pH values were measured with a pH

meter (HANNA instruments). Growth was measured as the dry cell weights (DCWs), with the method of PFEFFERLE *et al*. (2000) [37].

Four ml of homogenized culture broth were collected in sterile pre-weighed Eppendorf tubes and were centrifuged at 13000 rpm for 10 mn. The supernatant was recuperated for antimicrobial activity and pH measurements. The Eppendorf tube containing the pellet was used for measuring growth (by measuring the dry weight of the biomass). The pellet was washed three times with sterile distilled water. The Eppendorf tubes containing the pellets were dried at 105°C for 24 h, cooled in a desiccator, and weighed (KERN analytical balance). The results were expressed as g/l.

For the production of antibiotics, fermentations were carried out in 500 ml Erlenmeyer flasks containing 100 ml of the selected medium (250 rpm, 30 °C, 8 days). The extraction of active compounds was realized on the day of optimal production rate. The culture broths were centrifuged to remove the biomass and then filtered. The whole cell-free supernatant was extracted with an equal volume of dichloromethane. The organic and aqueous phases were recovered separately. The organic phase was dehydrated by passing through anhydrous sodium sulfate to remove residual traces of water and hydrophilic contaminants. This organic phase and the aqueous phase were concentrated to dryness and bioassayed against the indicator organisms (MRSA and UR) by the agar well diffusion method. The dry crude extracts were kept at -20°C for HPLC analysis.

1.7.- High performance liquid chromatography HPLC analysis

The active organic extract was dissolved in 1 ml of MeOH and subjected to semi-preparative HPLC purification on a Waters system using a reverse phase C18 column (200 mm × 10 mm, 5 µm) with a continuous linear gradient solvent system from 30 to 100% methanol in water during 40 mn (tab. I), a flow rate of 1 ml/mn, and UV detection at 220 and 260 nm. The fractions corresponding to peaks were collected, concentrated and then tested against MRSA and UR to detect the active fractions.

Table I.- HPLC program for organic extract purification

	Time (mn)	**Methanol (solvent A %)**	**Water (solvent B %)**
Pre-run	10	30	70
Run	0.00	30	70
	40	100	0
Post-run	50	100	0

The aqueous dry extract was recovered in minimum of ultrapure water and analyzed by HPLC using an amphiphilic Atlantis Prep T3 semi-preparative column (250 mm × 10 mm, 5 µm, Waters, Milford, MA) with a discontinuous gradient of methanol–water during 40 mn conforming to program in Table II, a flow rate of 1.5 ml/mn and UV detection at 220 and 254 nm. All the fractions were collected and tested against MRSA and UR.

Table II.- HPLC program for hydrophilic extract purification

	Time (mn)	Water (solvent A %)	Methanol (solvent B %)
Pre-run	10	100	0
Run	0.00	100	0
	22	25	75
	38	0	100
	40	100	0
Post-run	50	100	0

2.- Results

2.1.- Antimicrobial screening by the cylinder plating method

The antimicrobial activity of *S. tamanrassetensis* DSM 45947 by the cylinder plating method is shown in Table III. This strain was active against almost all target microorganisms, and the values of the inhibition diameters ranged from 5 to 25 mm. However, no activity was observed for four bacteria tested: methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 25922, *Enterococcus faecalis* Ef B1, *Klebsiella pneumoniae* CIP 82.91 and *Escherichia coli* E52; and four yeasts tested: *Candida glabrata* CgY21, *C. albicans* IPA988, *C. albicans* IPA200 and *C. guilliermondii* CgY24; and four micro-fungi tested: *Fusarium solani* (Fsol), *F. sporotrichioides* (Fs), *F. proliferatum* FprX20 and *Aspergillus parasiticus* CBS 100926.

Table III.- Antimicrobial activity of *S. tamanrassetensis* DSM 45947 screened by using the cylinder plating method on ISP2 medium

Test microorganisms	Inhibition diameter (mm) ± SD
Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 25923	18 ± 1.15
Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300	21 ± 2.40
Methicillin-resistant *Staphylococcus aureus* (MRSA) 639c	36 ± 1.15
Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 25922	0
Bacillus subtilis ATCC 6633	23 ± 0.67
Listeria monocytogenes ATCC 13932	15 ± 1.33
Enterococcus faecalis Ef B1	0
Acinetobacter baumanii E16	14 ± 0
Klebsiella pneumoniae CIP 82.91	0
Escherichia coli E52	0
Agrobacterium tumefaciens At2410	15 ± 1.33
Pseudomonas aeruginosa ATCC 27853	13 ± 0.67
Ureaplasma parvum ATCC 27813	19 ± 0.67
Candida tropicalis CtY20	8 ± 4.00
Candida glabrata CgY21	0
Candida albicans IPA 988	0
Candida albicans IPA 200	0
Candida pseudotropicalis CpsY22	13 ± 0.67

Candida zeylanoides CzY23	13 ± 0.67
Candida guilliermondii CgY24	0
Fusarium solani Fsol	0
Fusarium sporotrichioides Fs	0
Fusarium oxysporum f. sp *albedinis* Foa	12 ± 0.67
Fusarium culmorum Fc1	8 ± 4.0
Fusarium proliferatum FprX20	0
Fusarium oxysporum f. sp. *lini* Fol	14 ± 1.15
Fusarium oxysporum f. sp. *radicis lycopersici* Forl	8 ± 4.00
Fusarium equiseti Fe	13 ± 0.67
Fusarium moniliforme FmX22	15 ± 0.67
Umbelopsis ramanniana NRRL 1829	29 ± 0.67
Penicillium glabrum Pg1	8 ± 4.00
Penicillium expansum Pe1	9 ± 4.37
Aspergillus carbonarius (M333)	17 ± 0.67
Aspergillus westerdijkiae ATCC 3174	13 ± 0.67
Aspergillus flavus Af3	13 ± 0.67
Aspergillus niger OT304	8 ± 4.00
Aspergillus parasiticus CBS 100926	0
Trichoderma sp. TdR1	13 ± 0.67
Alternaria sp. (AltX1)	13 ± 0.67
Botrytis cinerea (BcvX1)	15 ± 0.67

The human pathogenic methicillin resistant *Staphylococcus aureus* 639c (MRSA) and the fungus *Umbelopsis ramanniana* NRRL 1829 (UR) are among the most sensitive target germs. They were selected as the indicator microorganisms for determining the antimicrobial activity in the subsequent experiments.

2.2.- Screening of genomic potential for antibiotic production

The antimicrobial biosynthetic gene diversity in *Saccharothrix tamanrassetensis* was examined by screening the presence of PKS-I, PKS-II and NRPS sequences using PCR. The results are shown in figure 1. NRPS gene was detected in *S. tamanrassetensis*; however, PKS-I and PKS-II were not detected. The bands observed on agarose gel at 750 bp indicated the presence of NRPS genes. This result reveals the antimicrobial potential of *S. tamanrassetensis*.

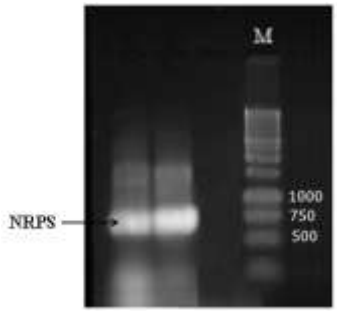

Figure 1.- Polymerase chain reaction analysis of *S. tamanrassetensis* DSM 45947 for PKS-I, PKS-II and NRPS
Test was conducted in duplicate. M: molecular weight marker (1 kb).

2.3.- Selection of the optimal carbon and nitrogen sources

The influence of nitrogen and carbon sources was studied in Erlenmeyer flasks. The results of antimicrobial activity production are illustrated in figure 2. Corn steep liquor (CSL) proved to be the best source for antimicrobial activity (24.0 mm and 22.0 mm) against UR and MRSA, respectively. This strong activity with CSL was followed by those obtained with peptone (24.0 mm and 21.0 mm) and yeast extract (22.0 mm). No antimicrobial activity was observed when the medium was supplemented with methionine, cystine or lysine. Corn steep liquor was retained as nitrogen source for the next kinetics.

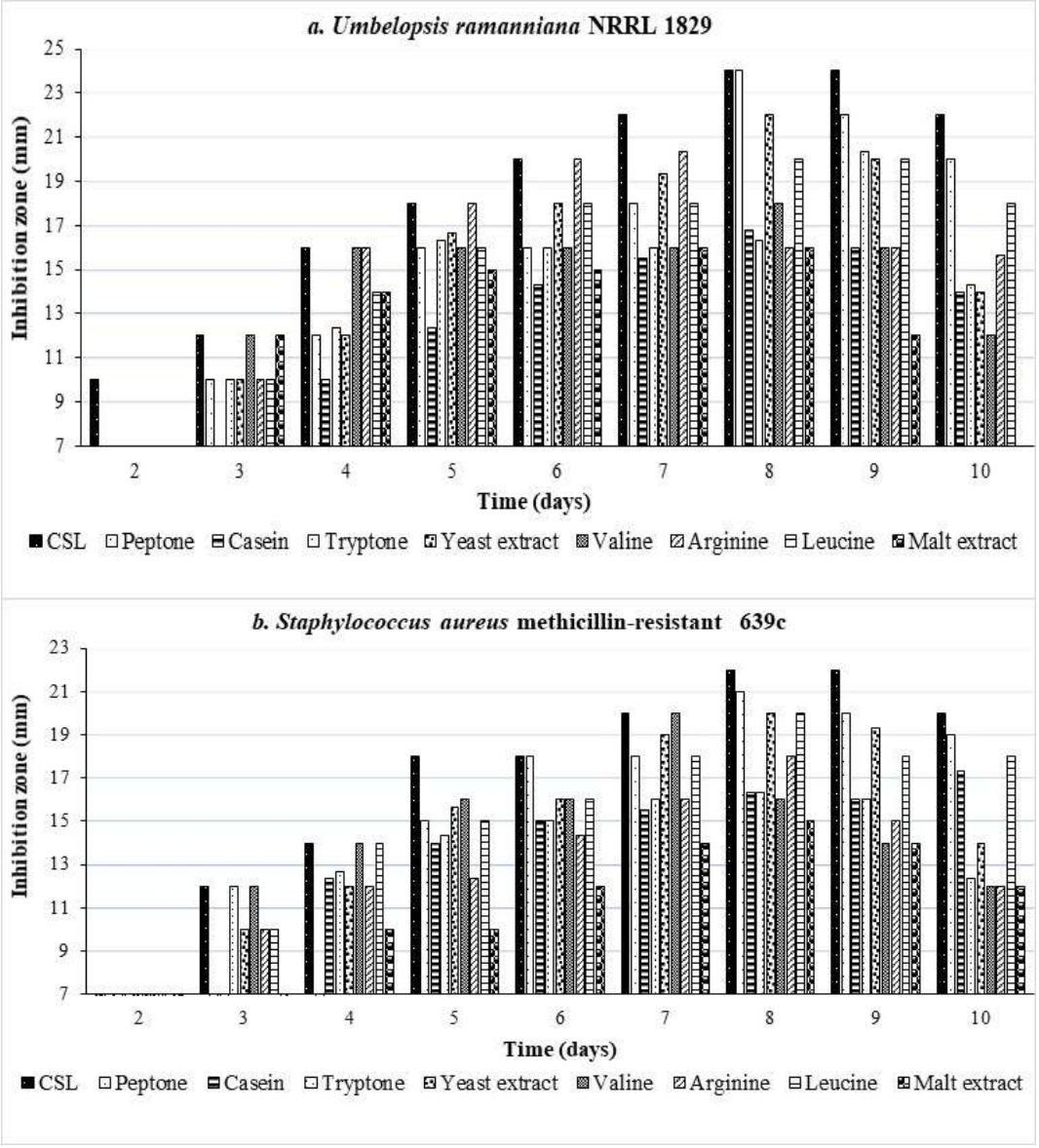

Figure 2 (a, b).- Influence of nitrogen sources on antibiotic production by *S. tamanrassetensis*.
Medium supplemented with glucose at 1%.

The results of effect of carbon sources on antimicrobial activity are illustrated in figure 3. The highest antimicrobial activity was obtained in the presence of sucrose (32.0 mm and 28.0 mm), followed by fructose (28.0 mm and 24.0 mm) and glucose (22.0 mm

and 22.00 mm) against UR and MRSA, respectively. No or very weak activity production was noticed during cultivation in the presence of both dextrin and sodium acetate (≤16.0 mm). Sucrose is better than glucose as it is cheaper and do not cause catabolite repression. Thus, for the antibiotic production, sucrose and corn steep liquor were selected as carbon and nitrogen sources, respectively.

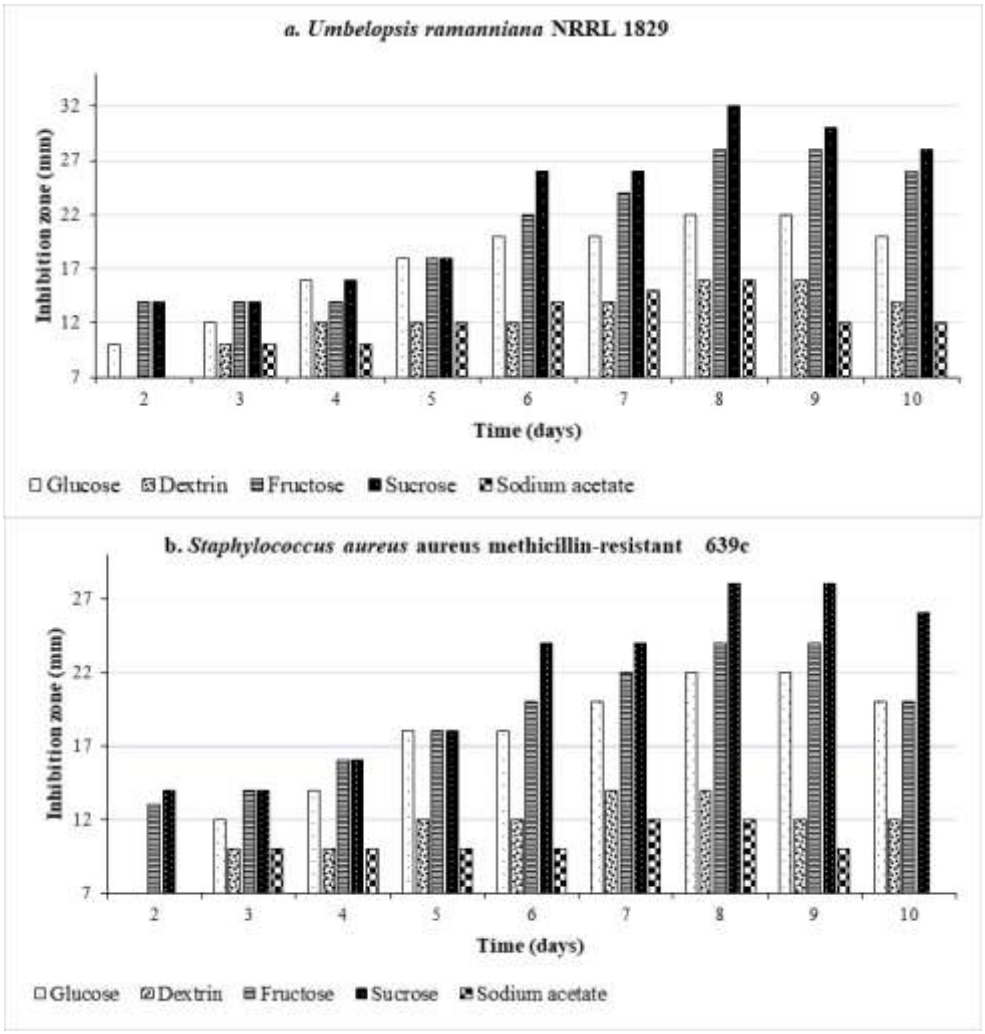

Figure 3 (a, b). - Influence of carbon sources on antibiotic production by *S. tamanrassetensis*
Medium supplemented with corn steep liquor at 5%.

2.4.- Production of antibiotics in the selected culture medium

Time course of antibiotic production, pH and growth of *S. tamanrassetensis* was followed on the basal semi-synthetic medium supplemented with CSL (5%) and sucrose (1%). The antibacterial and antifungal activities against methicillin-resistant *Staphylococcus aureus* 639c and *Umbelopsis ramanniana* NRRL 1829 were detected after few hours of fermentation, and the maximum observed after 8 days of fermentation (fig. 4). The pH varied between 7.0 and 8.2 during 10 days of fermentation. The growth of *S. tamanrassetensis* was perfectly correlated to antimicrobial activity.

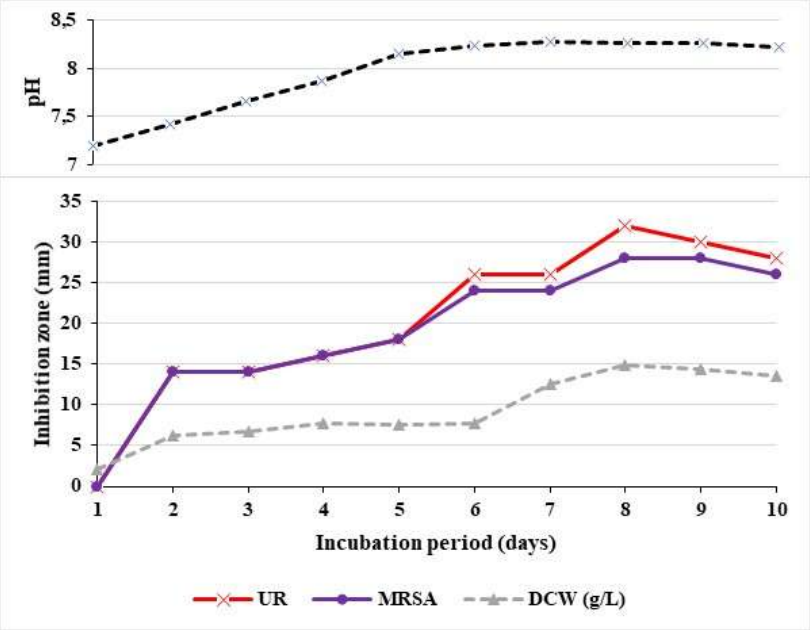

Figure 4.- Time course of growth, pH and antimicrobial activity (against MRSA and UR) on SSM supplemented with CSL and sucrose

For the production of antibiotics, *S. tamanrassetensis* was cultured during 8 days in 500 ml Erlenmeyer flasks containing 100 ml of the selected medium. Active compounds were extracted with dichloromethane from 6l of cell-free culture filtrate. The organic and aqueous extracts were concentrated and assayed for antimicrobial activity against MRSA and UR.

The dichloromethane (organic) extract was yellow. Diameters of inhibition zones were 15 mm against UR and 17 mm against MRSA (wells of 7 mm in diameter are included).

The aqueous extract was brown. It was dissolved in ultrapure water and tested against target germs. The antimicrobial activities of the aqueous extract were more potent than that of the organic phase. Inhibition zones diameters were 20 mm for UR and 24 mm for MRSA (wells of 7 mm in diameter are included).

2.5.- Purification by high performance liquid chromatography (HPLC) and detection of biological activity

The yellow organic extract was dissolved in methanol and purified by high performance liquid chromatography (HPLC) in reverse phase column. The chromatogram of the first round of HPLC injection showed the presence of 16 main peaks numbered from 1 to 16 (fig. 5). Each main peak was collected separately and tested against UR and MRSA. The results of fraction color, retention time and antimicrobial activity were illustrated in table IV.

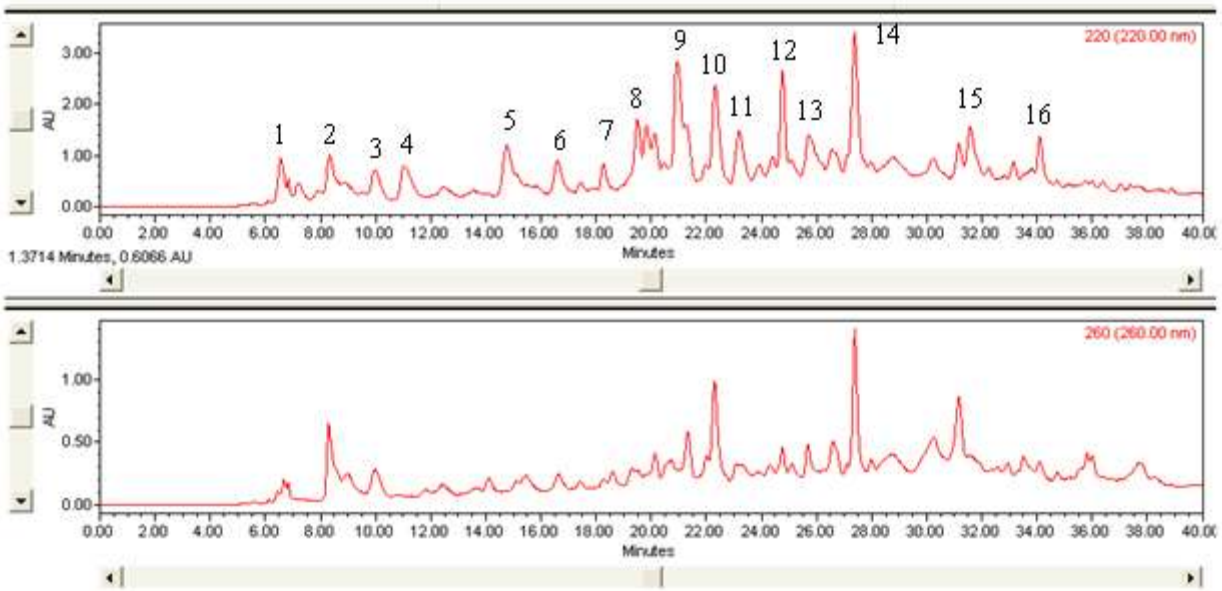

Figure 5.- HPLC profile of the first round of HPLC injection of the crude organic extract (C18 column, continuous linear gradient solvent system, detection at 220 and 260 nm)

Table IV.- Antimicrobial activities, retention times and colors of the main peaks obtained in the first HPLC injection of the organic extract

Peaks	Retention Time (mn)	Color	Antimicrobial activity (Inhibition diameter in mm)	
			MRSA*	UR**
1	6.60	Not colored	0	0
2	8.55	Not colored	0	0
3	10.02	Not colored	0	0
4	11.22	Not colored	0	0
5	14.75	Not colored	0	0
6	16.53	Not colored	0	0
7	18.14	Not colored	0	0
8	19.51	Not colored	0	0
9	20.90	Not colored	9	8.5
10	22.40	Not colored	10	9
11	23.19	Not colored	8.5	9
12	24.92	Not colored	11	11
13	25.75	Not colored	9	9
14	27.52	Pale yellow	12	11.5
15	31.50	Pale yellow	9	10
16	34.40	Pale yellow	8.5	9

Values of inhibition diameters include the diameter of the well (7 mm).
* methicillin-resistant *Staphylococcus aureus* 639c; ** *Umbelopsis ramanniana* NRRL 1829.

The antimicrobial activity of the organic extract was detected in the fractions eluted between 20 to 34.40 mn. A moderate to weak activity was detected in fractions corresponding to peaks 12 (RT 24.92 mn) and 14 (RT 27.52 mn). A weak antibacterial and antifungal activity was detected in the peaks 9, 10, 13, 15 and 16, and no activity was

observed against MRSA and UR in the peaks 1 to 8.

The brown aqueous extract was dissolved in ultrapure water and purified by HPLC) in amphiphilic column. The chromatogram of the first round of HPLC injection showed the presence of eight main peaks numbered from A1 to A7 (A for aqueous) (fig. 6). Each main peak was collected separately and tested against UR and MRSA. The results of fraction color, retention time and antimicrobial activity were illustrated in table VI.

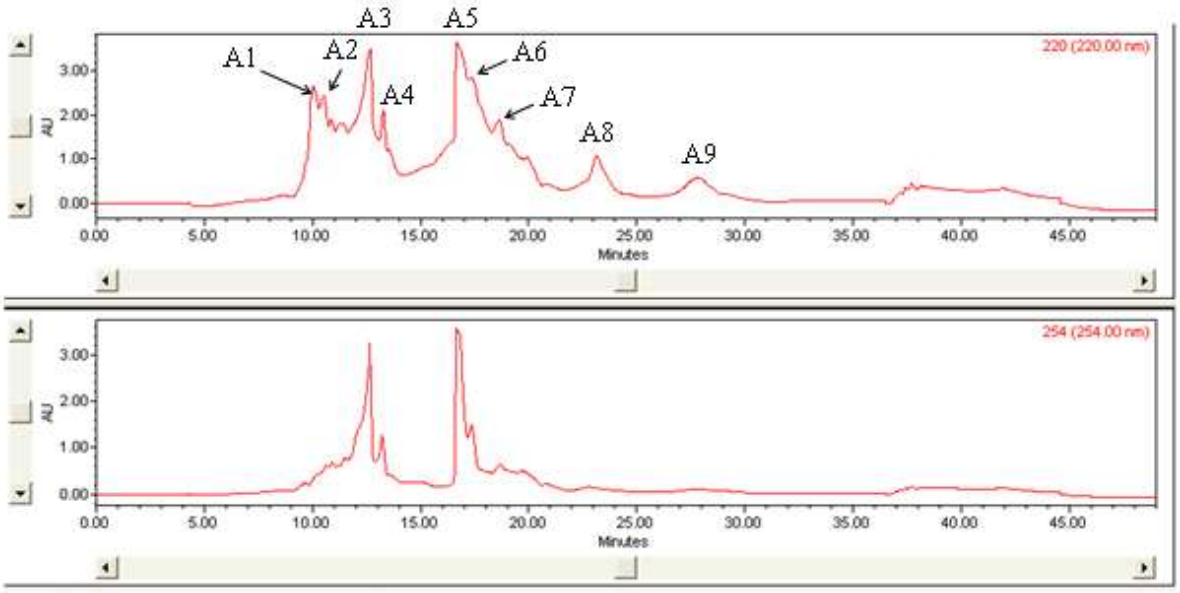

Figure 6.- HPLC profile of the first round of HPLC injection of the crude aqueous extract (amphiphilic column, discontinuous gradient solvent system, detection at 220 and 254 nm)

Table V.- Antimicrobial activities, retention times and colors of the main peaks obtained in the first HPLC injection of the aqueous extract

Peak	Retentions Time (mn)	Color	Antimicrobial activity (Inhibition distance in mm)	
			MRSA*	UR**
A1	9.89	Pale brown	12	42
A2	10.59	Pale brown	11	40
A3	12.63	Pale Yellow	14	0
A4	13.67	Yellow	0	0
A5	16.96	Pale Yellow	30	28
A6	17.31	Not colored	14	22
A7	18.55	Not colored	22	14
A8	23.13	Not colored	22	14
A9	27.57	Not colored	0	0

Values of inhibition diameters include the diameter of the well (7 mm).
* methicillin-resistant *Staphylococcus aureus* 639c; ** *Umbelopsis ramanniana* NRRL 1829.

Strong biological activities are recorded against MRSA in the fractions A5 (RT 16.96 mn), A7 (RT 18.55 mn) and A8 (RT 23.13 mn). The A1 (42 mm), A2 (40 mm) and

A5 (28 mm) fractions exhibited very strong to strong antifungal activity. Each active fraction was active against both MRSA and UR, except for A3 (RT 12.63 mn) which was active only against MRSA. No activities were observed in fractions A4 (RT 13.67) and A9 (RT 27.57).

3.- Discussion

Several studies reported the production of various antimicrobial compounds by *Saccharothrix* strains isolated from Saharan soil samples, such as dithiolopyrrolones [28,29], anthracyclines [30] and chloramphenicol [31].

In the primary antimicrobial screening, *Saccharothrix tamanrassetensis* showed an interesting antimicrobial activity. This included the human pathogenic MRSA 639c, mycotoxigenic and plant pathogens. The increasing of antimicrobial resistance is of particular concern in medicine and public health, and has created a serious need to develop new antibiotics with novel mechanisms of action [1]. Many nosocomial pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Enterococcus* spp. exhibit resistance to multiple drug classes, making treatment a challenge [38,39]. The studied strain exhibited an antifungal activity against *Aspergillus carbonarius* and *A. westerdijkiae*, which are mycotoxigenic micro-fungi. They produce ochratoxin A, a dangerous toxin for humans and animals, that can cause poisoning, immune deficiency and cancer [40]. *Fusarium oxysporum* f. sp. *albedinis* and *Fusarium oxysporum* f. sp. *radicis-lycopersici* are plant pathogens. The former is the agent of vascular wilt (fusariosis) of date palm and causes important deteriorations to the plant [41,42]. The latter is one of the most destructive diseases of tomatoes. These results reveal that *S. tamanrassetensis* could be considered as a potential promising candidate for production of novel antibiotics.

Further investigations on the antimicrobial activity screening of *S. tamanrassetensis* were devoted to the evaluation by PCR of the presence of genes encoding polyketide synthases (type I and II) and NRPS using primers for highly conserved regions encoding enzymes associated with biosynthesis of polyketides and peptides. A positive PCR amplification was obtained for NRPS. In contrast, PKS-I and PKS-II sequences were negative. Many antibiotics produced by actinobacteria have been biosynthesized through a pathway involving non-ribosomal peptide synthetases (NRPS) and/or polyketide synthases (PKS) [32,43-44]. The high frequency of NRPS genes in this actinobacterial strain may be evidence of the high potential of extremophilic actinobacteria to produce a large number of bioactive secondary metabolites [45]. Furthermore, the low occurrence of PKS-I genes in actinobacterial strains has been reported by several authors [45,46].

A lot of the genome mining methods are based on the detection of domains and protein families, well known to be involved in secondary metabolism such as NRPS and PKS [6,47]. The information obtained on the biosynthetic pathway involved in the production of a secondary metabolite can be used to predict the chemical structure of the resultant compound. Intensive research on these enzymes led to the identification and characterization of more than 23000 natural products of PKS and NRPS, which have been widely used in medicine as antibiotics and antitumor agents [3,48-52]. Soil actinobacteria are a potential source for novel PKSs and NRPs [53,54]. However, the PKS and NRPS biosynthetic genes of the Algerian Saharan soil strains are rarely reported. The NRPS gene detected in *S. tamanrassetensis* could be involved in the antibacterial and antifungal activities observed in this species against the target microorganisms, and suggested a

possibility for biosynthesis of peptide or polypeptide antibiotics.

Natural products biosynthesized by NRPSs are some of the most important drugs currently used clinically for the treatment of a variety of diseases. They included the most important antibacterial, antifungal, antiviral, immunosuppressant, and anticancer drugs [55]. Examples of NRPS antibiotics included capreomycin produced by *Saccharothrix mutabilis* subsp. *capreolus* strain ATCC 23892. It is used in human medicine as an antitubercular antibiotic, and is an essential component of the drug arsenal for the treatment of multidrug-resistant tuberculosis [56]. Daptomycin is produced by *Streptomyces roseosporus* NRRL11379 and used for the treatment of skin infections caused by Gram-positive pathogens, including methicillin- and vancomycin-resistant *Staphylococcus aureus* [57,58], as well as the treatment of bacteremia and endocarditis [59]. The cyclosporins are another example of NRPS produced by the fungal species *Tolypocladium inflatum*, with cyclosporin A having the most interesting biological activities [60]. Initially investigated as a potential fungicide, cyclosporin A was later identified as a potent anti-inflammatory and immunosuppressant agent by suppressing T-lymphocyte activation [61]. This activity prompted its clinical use in transplant surgeries to prevent graft rejection and the treatment of autoimmune diseases [61,62]. Vancomycin and teichoplanin are glycopeptides and two clinically relevant drugs. Vancomycin, produced by the actinobacterium *Amycolatopsis orientalis*, is used against some methicillin-resistant *Staphylococcus aureus* infections, and is also used to treat serious infections and endocarditis caused by *Staphylococcus*, *Streptococcus*, and *Corynebacterium* [63-65]. Teichoplanin, produced by the actinobacterium *Actinoplanes teichomyceticus*, is used for vancomycin-resistant enterococci [66]. The bleomycins are a family of glycosylated peptides that were found to have anticancer activities [67,68]. Future research in our laboratory is aimed at exploiting NRPS products of the new Saharan strain *S. tamanrassetensis* to the development and discovery of potential novel antibiotics.

Kinetics of antibiotic production by *S. tamanrassetensis* was initially studied by BOUBETRA *et al.* (2013a) [23]. These authors reported an antimicrobial activity against Gram-positive and Gram-negative bacteria and filamentous fungi, but not against yeasts. Time course of antibiotic production, growth and pH were monitored in ISP2 broth cultures. Purification and characterization of the antimicrobial products led to the identification of two new antibiotics purified from the organic extract. However, it is well known that antibiotic production depends considerably on culture conditions. The culture medium composition plays a major role in the biosynthesis of bioactive metabolites [69]. Minor changes in the type and concentration of carbon and nitrogen sources can affect antibiotic biosynthesis [70].

In this work, the authors proposed that selecting carbon and nitrogen sources in a basal semi-synthetic medium (SSM) can provide novel drug candidates. Corn steep liquor and sucrose were found to support maximum antimicrobial production. These nitrogen and carbon sources could be important sources for antibiotic biosynthesis in *S. tamanrassetensis*. CSL is a source of proteins, amino-acids, minerals, vitamins and microelements [71,72]. Our results were in agreement with many works in which, CSL is employed as a nitrogen source for secondary metabolite production. SARADA and SRIDHAR [73] found that CSL was the best source to enhance the production of cephamycin C by *Streptomyces clavuligerus*. The same was observed by SHARMA *et al.* (2013) when using CSL among several agro-industrial wastes [74]. The pullulan produced by *Aureobasidium pullulans* RBF 4A3 was enhanced. The result obtained by addition of

sucrose agrees with those obtained by ELIBOL *et al.* (2004), THAKUR *et al*. (2009) and ISLAM *et al*. (2009) concerning antibiotic production by *Streptomyces coelicolor* A3(2); *Streptomyces* sp. 201 and *Streptomyces albidoflavus*, respectively [75-77]. Sucrose was proved to be the optimal carbon source for enhancing of antifungal activity by *Xenorhabdus stockiae* PB09 [78].

The highest antibacterial activity against methicillin-resistant *Staphylococcus aureus* 639c and *Umbelopsis ramanniana* NRRL 1829 were observed both after 8 days of fermentation. This means that the strain first grows to form a considerable amount of biomass followed by the production of secondary metabolites (antibiotics in this case). Fast growth actinobacterial strains produce antibiotics in the first days of fermentation. However, slower growth strains with a slow growth rate produce antibiotics during the last days of fermentations [79]. Detection of a strong activity in the aqueous extract suggests an hydrophilic nature of the produced antibiotics. Solvent extraction (with dichloromethane) appeared necessary, since it allowed the elimination of impurities, and therefore facilitated the purification by HPLC. The first injections of the crude aqueous extract in HPLC system allowed the detection of 7 compounds with strong to very strong biological activity.

In the previous studies on *Saccharothrix tamanrassetensis* DSM 45947, BOUBETRA *et al.* (2013a) used the ISP2 medium to characterize three new antibiotics from the organic extract [23]. The aqueous phase was also inactive. In our case, the semi-synthetic medium supplemented with CSL and sucrose was used for antibiotic production. HPLC results of the aqueous extract reveal the presence of seven active fractions. These results highlight the antimicrobial diversity of *Saccharothrix tamanrassetensis* DSM 45947, and encourage further investigations on the produced antibiotics.

Conclusion

Saccharothrix tamanrassetensis DSM 45947 exhibited strong antimicrobial potential. Analysis of biosynthetic genes for the production of antimicrobial compounds revealed the presence of NRPS, and therefore the possibility of producing peptides or polypeptides antibiotics. Testing of carbon and nitrogen sources in the semi-synthetic medium led to the selection of corn steep liquor and sucrose for the formulation of a medium favorable for an optimal antimicrobial activity production. Strong antibacterial and antifungal activities were produced in the selected culture medium. After solvent extraction of the active compounds, the strong activity remained in the aqueous phase. Preliminary HPLC purification revealed seven active products with strong to very strong antibacterial and antifungal activity. These results compared with that obtained by BOUBETRA *et al.* [23] suggest the presence of novel antibiotics. Final purifications and further chemical analyses of the bioactive compounds are recommended.

In memory of Professor Nasserdine SABAOU (1956-2019)

Nasserdine SABAOU, a great scientist and specialist in Microbiology, has served as a professor at the Department of Natural Sciences of the "Ecole Normale Superieure de Kouba, Algiers (Algeria)", and was the former head of the "Laboratoire de Biologie des Systèmes Microbiens (LBSM)". As researcher, his work mainly focused on systematic and antimicrobials of actinobacteria especially those of Saharan soils. Other interests included toxigenic fungi and mycotoxins. During his scientific career, he required clarity, accuracy, and honesty in scientific reporting. He was a role model for many of us. We were influenced for many years by his exceptional professional competence and his love for science.

As the instigator of this work, Professor SABAOU continuously provided advice and comments. His tireless fellow-up and proofreading assisted us in producing this article. We are honored to dedicate this paper to his memory. *God Bless Him.*

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