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Research Article

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Antibacterial and antioxidant activities of *Streptomyces* sp. strain FR7 isolated from forest soil

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Abstract

Actinomycetes produce secondary metabolites with many bioactivities such as antimicrobial, which can be useful as alternatives against resistant bacterial strains. Therefore, the screening of new habitats is likely to provide new strains with high potential. In this work, the antimicrobial capacity was used to select *Streptomyces* sp. strains isolated from Raf Raf forest (Tunisia). From the strain displaying higher activity, FR7, an ethyl acetate extract was prepared under optimized culturing conditions (10 days at 30°C in ISP2 medium with initial pH 8), showing significant antimicrobial activity against *Micrococcus luteus* and *Staphylococcus aureus* (MIC = 5 μg ml^{-1}), and *Listeria monocytogenes* and *Pseudomonas aeruginosa* (MIC = 20 μg ml^{-1}). The extract displayed strong DPPH radical scavenging activity (IC_{50} = 1.3 μg ml^{-1}) and protection of yeast cells from H_2O_2-induced oxidative stress determined by flow cytometry with dichlorofluorescein diacetate. The crude extract showed the presence of polyketides, with methylsalicylic acid as moiety, a large and diverse group of secondary metabolites with a wide range of bioactivities, including antioxidant and antibacterial. Based on 16S RNA gene sequences, strain FR7 was identified as belonging to genus *Streptomyces* with high resemblance to *S. iakyrus*. *Streptomyces* sp. FR7 has great potential as a source of antibacterial and antioxidant metabolites.

Significance and impact of the study

The widespread use of antibiotics over decades has triggered the selection of resistant strains that menace the efficacy of therapeutic interventions. The finding of polyketides in the extract of *Streptomyces* sp. strain FR7 with antibacterial activity might be important to overcome this problem. Polyketides are an extremely diverse family of compounds with many biological activities. Therefore, it is highly likely that new compounds are present in this extract that can be used in medicine, veterinary, or agriculture. In addition, the antioxidant activity of the extract, presumably also from polyketides, has the potential of human preventive application against degenerative diseases.

Keywords: actinomycetes, *Streptomycetes*, antimicrobials, antioxidants, soil, polyketides

Introduction

Antimicrobial resistance has developed as one of the major urgent threats to public health mainly by misusing and overusing different antibacterial agents in the health care setting as well as in the agricultural industry (Hofer 2019). According to OECD (The Organisation for Economic Cooperation and Development), in the next 30 years, 2.4 million people in Australia, Europe, and North America will die from infections with resistant microorganisms (Co-operation and Staff 2019). New antimicrobial compounds from different sources are one of the solutions to overcome resistance by such strains. In addition, with the increase of life expectancy in developing and developed countries, degenerative diseases like cancer, diabetes, and several neurological disorders have been increasing. The fact that these diseases have been associated to oxidative stress (Barnham et al. 2004, Giacco and Brownlee 2010, Singh et al. 2019, Hayes et al. 2020, Zhang et al. 2020) makes the search for new antioxidant compounds from natural sources a promising approach to promote health care. Furthermore, to delay oxidative processes, several synthetic antioxidants, such as butylated hydroxyanisole and butyl hydroxytoluene, have been employed as food preservatives. However, cytotoxicity concerns have been raised for such compounds (Vandghanooni et al. 2013, Xu et al. 2021), so attempts have been made to select antioxidant compounds from natural sources (Lee et al. 2014).

Actinomycetes have attracted attention by producing bioactive secondary metabolites, which are used in medical and agricultural applications, due to their antibiotic (Alqahtani et al. 2022), antifungal (Qi et al. 2022, Zhou et al. 2022), anticancer (Santos et al. 2019, Osama et al. 2022), antioxidant (Pinto-Almeida et al. 2021, Rani et al. 2021), bioherbicide (Shi et al. 2019, Kim et al. 2020), and bioinsecticide (Amelia-Yap et al. 2022) properties. Actinobacteria are Gram-positive bacteria characterized by the formation of substrate and aerial mycelium, presence of spores and high content of G + C (Kekuda et al. 2015). Among all known actinomycetes, species of the genus *Streptomyces* present the richest source of bioac-

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tive molecules, providing about 80% of the naturally occurring antibiotics (Harir et al. 2018). Although many antibiotics have already been isolated from bacteria of the genus *Streptomyces*, they represent only a small fraction of the bioactive compounds produced by these bacteria (Watve et al. 2001) and it becomes important to find optimal conditions for the growth and production of compounds of biotechnological interest (Kadiri and Yarla 2016, Al-Ansari et al. 2020, Zhang et al. 2022).

As *Streptomyces* strains isolated from soil samples have been found to be rich in bioactive secondary metabolites (Anderson and Wellington 2001, Biswas et al. 2012), in this work, this habitat was considered to screen strains of this genus in order to find new promising sources of bioactive metabolites. The focus of the work was on antibacterial and antioxidant activities to meet the need for new antibacterial and antioxidant agents. In addition, optimal growth conditions were assessed in order to provide a perspective of future production of bioactive metabolites.

Materials and methods

Chemical compounds

The radical DPPH, fluorochrome H_2DCFDA, hydrochloric acid, acetic acid, and ethylenediaminetetraacetic acid (EDTA) were purchased from Thermo fisher scientific; hydrogen peroxide, ethanol, and ethyl acetate from Merck; BHA, ethidium bromide, and nalidixic acid from Sigma–Aldrich. Furthermore, nystatin and streptomycin were obtained from Gibco and Tris from Fisher Bioreagents.

Bacterial and yeast strains and media

Bacterial strains used in this work in the antimicrobial activity were *Micrococcus luteus* YSP7, *Bacillus cereus* ATCC 7064, *Escherichia coli* DH5-alpha, *Listeria monocytogenes* CECT 4031T, *Pseudomonas aeruginosa* ATCC 10145, *Staphylococcus aureus* ATCC 6538, and *Staphylococcus epidermidis* CECT 4183. *Saccharomyces cerevisiae* strains were BY4741 (*MATa his3D1 leu2D0 met15D0 ura3D0)* and the derived mutant strain *yap1*, defective in the gene *YAP1* (*MATa his3D1 leu2D0 met15D0 ura3D0 YML007w:: kanMX4*; Euroscarf, Germany). The composition of media for cultures of bacteria: ISP2 (International Streptomyces Project), ISP3 (oatmeal agar medium), ISP5 (glycerol-asparagine agar), GLM (glucose-yeast extract malt), SCA (starch-casein agar), AIA (actinomyces isolation agar), LB (Luria–Bertani), and yeasts: YPD (yeast extract peptone dextrose), is presented in Table S1. All isolation media were pH adjusted from 7.1 to 7.2. The growth of yeast strains cultures was monitored spectrophotometrically at 600 nm (OD_{600}), while the assessment of growth of cultures of actinomycetes was restricted to the determination of dry weight. For dry weight measurements, 100 ml of liquid culture was withdrawn from the flask at the assigned time, the whole content was centrifuged at 10 000 × *g* for 30 min and the cell pellet was dried in a hot air oven at 90°C for 10 h and the dry cell weight was measured (Ye et al. 2009).

Collection of actinomycetes samples

Soil samples were collected from the Aleppo pine forest of Raf Raf city (latitude 37°11′27″ N, longitude 10°11′00″ E), marine sediment, and salty water were obtained from Sidi Ali El Mekki lagoon (latitude 37°10′47″ N, longitude 10°16′44″ E) in Ghar El-Melh coast located in Bizerte, north-eastern Tunisia. Approximately 100 g of soil and marine sediment at 5–25 cm depth were collected and placed in sterile bags. Seawater samples were collected directly into 50 ml sterile falcon tubes. The samples were transported aseptically in a 4°C refrigerator to the laboratory until use to isolate actinomycetes.

Isolation of actinomycetes strains

Actinomycetes were isolated according to Das et al. (2008) with some modifications. Briefly, from each sample (1 g of soil was suspended in 1 ml of sterile deionized water and serially diluted to 10^{-5}). One millilitre of diluted sample was spread over the surface of a solid ISP2 medium supplemented with nystatin (50 μg ml^{-1}) and nalidixic acid (10 μg ml^{-1}), to suppress fungal growth and undesirable bacterial growth, respectively. Then, the plates were incubated at 30°C, for 7–21 days. Bacterial colonies were subscultured on different solid media like SCA, GLM, or ISP2, incubated under the same conditions until the development of colonies, and then maintained at 4°C for further study.

Morphological characterization of actinomycetes strains

FR1 and FR7 strains (obtained from the soil of the forest), LSG and LS3 strains (obtained from the marine sediment of the lagoon), and LEG1 (obtained from the salty water of the lagoon) were selected for morphological characterization. The morphological characterization of strains was based on macroscopic and microscopic observation, the mycelium structure, colour, and spore chain arrangement were examined under oil immersion (magnification: 1000×) and the observed morphology of the isolates was compared with Bergey's Manual of Determinative Bacteriology (Bergey and John 2000). The microscopic characterization was examined using the Gram staining method.

Actinomycetes antibacterial activity screening

Antimicrobial activity of the isolated actinomycetes strains (FR1, FR7, LSG, LS3, and LEG1) was assessed through the agar-well diffusion method (Nandhini et al. 2018) against different pathogenic bacterial strains namely, *B. cereus*, *E. coli, L. monocytogenes*, *M. luteus, P. aeruginosa, Staph. aureus*, and *Staph. epidermidis*. Cultures of bacterial test organisms were grown in LB medium at 37°C, 200 rpm, overnight. Twenty millilitres of melted LB agar were cooled to 55°C, mixed with 1 ml of the test organism suspension (adjusted to $OD_{600} = 0.1$), and poured onto a Petri dish. In each Petri dish, 6 mm wells were made to load with 70 μl of the supernatant of actinomycetes cultures. Supernatants were prepared from actinomycetes cultures in ISP2 media incubated for 10 days at 30°C, 200 rpm, after centrifugation at 10 000 × *g* for 15 min. The plates were kept in the refrigerator to diffuse the supernatant for 2 h, and then they were incubated at 37°C for 24 h. The inhibition zones around the wells were measured in millimetres.

Molecular characterization of the selected strain

From a culture grown on ISP2 medium, incubated at 30°C, 200 rpm, for 72 h, cells of isolated strain FR7 were harvested by centrifugation at 14 000 × *g* for 5 min at 4°C. The genomic DNA was purified according to the protocol described by Nikodinovic et al. (2003). The DNA was dried at room temperature and dissolved in TE buffer (10 mmol l^{-1} Tris.HCl,

pH 8.2, and 10 mmol l^{-1} EDTA). Amplification of 16S ribosomal sequence from genomic DNA was carried out by using the universal primers fD1 (5′-CCGAATTCGTCGACAACAGAG TTTGATCCTGGCTCAG-3′) and rD1 (5′-CCCGGGATC CAAGCTTAAGGAGGTGATCCAGCC-3′ (Weisburg et al. 1991). The PCR reaction was performed according to Keikha et al. (2015): 35 cycles of 1 min denaturation at 94°C, primer annealing at 63°C for 1 min, primer extension at 72°C for 2 min and a final extension step at 72°C for 10 min. The PCR reaction mix was separated by electrophoresis in a 1% agarose gel in TAE buffer (40 mmol l^{-1} Tris.HCl, 20 mmol l^{-1} acetic acid, 1 mmol l^{-1} EDTA, and pH 8.3) and then visualized by ultraviolet fluorescence after ethidium bromide staining. The sequencing of the purified PCR products was performed using an automatic sequencer (Applied Biosystems). The nucleotide sequence of the 16S rRNA gene was deposited at the GenBank under the accession number MG008495.1 and compared to all sequences available in GenBank using BLAST software from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). Closest sequences were selected for construction of phylogenetic relationships using MEGA version 11 (Tamura et al. 2021).

Optimization of actinomycete FR7 strain cultures growth conditions and the effect on antibacterial activity

To determine the favourable conditions for optimum growth and to improve the production of antibacterial metabolites by *Streptomyces* sp. strain FR7, the effect of the culture conditions namely, medium composition, temperature, incubation period, pH, and carbon source were determined. All experiments were set up according to the conditions to test and samples of the cultures were taken to assess growth by determination of dry mass (see above) and to obtain the supernatant to assess the antibacterial activity by the agar-well diffusion assay (see above). To study the influence of media on the antibacterial activity and growth of *Streptomyces* sp. strain FR7, six different liquid media (ISP2, ISP3, ISP5, AIA, SCA, and GLM; described in Table S1) were used in 250 ml Erlenmeyer flasks. Except for testing time of incubation and temperature, the cultures were incubated at 200 rpm for 10 days at 30°C. For the influence of temperature, cultures were incubated in ISP2 medium for 10 days at 10°C, 15°C, 20°C, 25°C, 30°C, 35°C, or 40°C. For the influence of the incubation period, cultures were incubated in ISP2 medium, at 30°C and samples were harvested on days 2, 4, 6, 8, 10, 12, and 14. For the influence of initial pH, the ISP2 medium was adjusted at different pH values (3–10), inoculated with the strain under study and incubated at 30°C for 10 days at 200 rpm. Additionally, the influence of the carbon source on growth and antibacterial activity was assessed by cultivating FR7 strain in ISP2 media with 1% (w/v) glucose, 1% (w/v) fructose, 1% (w/v) starch, or 1% (w/v) sucrose at 30°C, 200 rpm for 10 days.

Extraction of antimicrobial metabolites from *Streptomyces* sp. FR7 strain

Streptomyces sp. strain FR7 culture was grown in ISP2 broth or GLM broth at 30°C for 10 days at 200 rpm. The culture was centrifuged at 14 000 × *g* for 15 min, the supernatant was filtered with filter paper (Whatman N° 1), and an equal volume of ethyl acetate was added to the filtrate and shaken for 20 min. The organic phase was separated from the aqueous phase and concentrated in a rotavapor at 30°C–45°C to obtain the crude extract.

Bioactivities of *Streptomyces* sp. strain FR7 extract

Antibacterial activity

For assessment of antibacterial activity, MIC was determined for the *Streptomyces* sp. FR7 strain crude extract against bacteria, using 96-well microtiter plates (Al-Dhabi et al. 2020). Overnight cultures of test bacteria were adjusted to OD_{600} of 0.1 and distributed to wells. The treatment mixture in each well was prepared with bacterial suspension, deionized water, and crude extract diluted in ethanol to make different final concentrations (5, 10, 25, 50, and 100 μg ml^{-1}). The negative control was prepared by replacing the extract with ethanol (solvent of the extract). The positive control was prepared in a similar way with streptomycin to make final concentrations ranging from 5 to 100 μg ml^{-1}. After 17 h incubation at 37°C, the MIC was estimated as the lowest concentration of the extract inhibiting the growth of the test cultures by measuring the OD_{600} with a microplate reader (Molecular Devices, SpectraMax Plus).

Antioxidant activity

Antioxidant activity of the crude extract was assessed by the DPPH method in 96-well microplates, according to (Lee et al. 2014), with some modifications. The crude extract of *Streptomyces* sp. strain FR7 was diluted in absolute ethanol to make concentrations ranging from 10 to 300 μg ml^{-1}. In 96-well microplates, 190 μl of DPPH (0.008%) was mixed with 10 μl of the extract at different concentrations. The reaction was allowed to proceed for 30 min at room temperature in the darkness. Solutions of the standard compound BHA were treated the same way. The absorbance of the mixtures was measured at 517 nm, using a microplate reader (Molecular Devices, SpectraMax Plus) and ethanol was used as a blank. The negative control was prepared with DPPH and ethanol to replace the sample extract. The scavenging activity of the crude extracts was calculated and compared to the antioxidant standard BHA. Results were expressed as a percentage decrease with respect to control values, according to the following equation:

$$\text{DPPH Radical scavenging} = \frac{Ac - At}{Ac} \times 100,$$

where *Ac* is the absorbance of the control and *At* is the absorbance of the test sample.

Antioxidant activity was also assessed in cellular context by flow cytometry. Hydrogen peroxide (5 mmol l^{-1}) was used to induce oxidative stress in yeast cells. *Saccharomyces cerevisiae* parental strain BY47141 and the derived mutant *ypa1* were used as models of study. Yeast cells were cultivated in YPD medium, incubated at 30°C and 200 rpm. Exponentially growing cultures (OD_{600} of 0.4–0.8) were centrifuged (2 min, 14 000 × *g*, 4°C), the supernatants were discarded, and cells were washed twice under the same conditions with the same volume of ice-cold PBS (137 mmol l^{-1}, NaCl, 2.7 mmol l^{-1}, KCl, 10 mmol l^{-1}, Na_2HPO_4, 1.8 mmol l^{-1}, KH_2PO_4, and pH 7.4). The suspension was diluted to $OD_{600} = 0.02$ and 500 μl were collected and kept on ice until cytometry analysis for the measurement of autofluorescence. Cells were loaded with 50 μmol l^{-1} H_2DCFDA as a redox-sensitive fluorescent probe,

by incubation at 30°C, 200 rpm, for 1 h in the dark. Then, cells were washed twice by centrifugation as described above with the same volume of ice-cold PBS. Treatments were made with aliquots of 500 μl, mixed with crude extract at different concentrations and 5 mmol l^{-1} H_2O_2. For the positive control, an aliquot of cell suspension was mixed with H_2O_2 only (same concentration as above) and for the negative control the cell suspension was mixed with ethanol (extract solvent; the same amount as the maximum concentration of extract tested). Mixtures were incubated for 90 min at 30°C, 200 rpm, in the dark. About 20 000 cells of each sample were analysed by flow cytometry in an XLTM Epics® cytometer (Beckman Coulter) equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. The green fluorescence was collected on a 488 nm blocking filter, 550 nm long-pass dichroic and 225 nm band-pass filter. The data was analysed, and histograms were done using Flowing Software.

Electrospray ionisation mass spectrometry (ESI-MS)

Streptomyces sp. strain FR7 extract was analysed by electrospray ionization mass spectrometry (ESI-MS and ESI-MS-MS) and performed on a linear ion-trap mass spectrometer (Thermo LxQ) equipped with an electrospray ionisation source. The lyophilized sample was dissolved in water/methanol (30/70 v/v) mixture and analysed by ESI-MS and ESI-MS-MS at a flow rate of 8 μl min^{-1} under ion spray voltage 5 kV, capillary temperature 250°C, tube lens offset 120 V, and a capillary voltage of 20 V. MzCloud Mass Spectral database was used for compound identification.

Statistical analysis

All experiments were performed at least three times in independent assays. Results are presented as mean ± standard deviation (SD) and P value < 0.05 was considered statistically significant. Details on specific statistical analysis are referred to in the legend of the figures. All data were plotted by *GraphPad Prism* software (GraphPad Inc v8., USA).

Results and discussion

Actinomycetes isolation and antibacterial activity screening

From the Raf Raf forest and the Ghar El-Melh lagoon, five isolated strains (FR1, FR7, LS3, LEG1, and LSG) were preliminarily identified as actinomycetes based on macroscopic and microscopic characteristics (colour of aerial and substrate mycelium, size, texture, diffusible pigment production; not shown) and microscopic (spore arrangement in cover-slip method; not shown) features of Bergey's Manual of Determinative Bacteriology (Bergey and John 2000) and were chosen for further antibacterial screening.

To select the best performing strain in terms of antibacterial activity for further characterization, the supernatant of cultures in ISP2 of each strain was tested using the agar-well diffusion test. Five isolates (FR1, FR7, LS3, LEG1, and LSG) displayed a large spectrum of activity against the tested Gram-positive microorganisms (Table 1), while only the isolate obtained from the soil of Raf-Raf forest (FR7) showed activity against Gram-negative bacteria, namely *E. coli*. From all active strains, FR7 showed a wider spectrum of antimicrobial activity and higher growth inhibition zones, with 21.0 ± 0.1, 19.8 ± 0.1, and 18.9 ± 0.0 mm against *M. luteus*, *Staph. aureus*, and *L. monocytogenes*, respectively, and 12.4 ± 0.1 mm against *E. coli* (Table 1).

The strain FR7 was selected for further studies due to the remarkable performance in antibacterial activity, which is a common trait in extracts from species of *Streptomyces* genus isolated from soil samples (Radhakrishnan et al. 2016, Rathod et al. 2018, Gacem et al. 2020, Elnady et al. 2022). The nucleotide sequences of the 16S rRNA gene (Table S2) were obtained and deposited in GenBank as *Streptomyces* sp. strain FR7 under the accession number MG008495.1. To study the similarity of strain FR7 with other species of *Streptomyces*, the evolutionary history was inferred using the neighbour-joining method from partial sequences of the 16S ribosomal RNA gene (Fig. 1). Strain FR7 clustered closely to *Streptomyces iakyrus* NBRC 13401, which correlated with the BLAST 100% similarity between both strains (not shown). However, considering the short sequence of FR7 16S ribosomal RNA gene used (970 bp), assumption of species identity should only be assumed upon phylogeny analysis from longer sequences. Therefore, in accordance with the morphology of the strain, phylogenetic analysis indicates that strain FR7 belongs to the genus *Streptomyces* and is close to *S. iakyrus* NBRC 13401.

The genus *Streptomyces* has been found in different environments such as soil, sea, freshwater, and lakes. Typically, these environments provide complex and adverse conditions where the production of secondary metabolites might help in their survival and resistance in changes of environmental conditions (Xiong et al. 2004). Furthermore, *Streptomyces* strains are still considered the most promising candidates to explore potentially antibiotic compounds (Xia et al. 2020).

Optimization of culture conditions for improvement of bioactivity of *Streptomyces* sp. strain FR7 and assessment of antibacterial properties

In order to determine the favourable conditions for an optimum growth and to improve the antibacterial activity of the selected strain, the effect of the culture conditions (such as medium, incubation period, pH, temperature, and carbon source) on growth (dry mass) and antibacterial bioactivity (agar-well diffusion) was performed. In most cases, final biomass and antibacterial activity correlated (Fig. 2), suggesting that improvement of cell proliferation promotes higher yield in antibacterial metabolites. Our results showed that ISP2 was the most suitable medium for cell proliferation and antibiotic production (Fig. 2A) and are consistent with a study, which recorded maximum production of antibiotics by *Streptomyces* sp. KOD10 in ISP2 medium compared to other media tested (Sharon et al. 2014). This emphasizes the importance of the ingredients of ISP2 medium in antibiotic production in actinomycetes as demonstrated by Iwai and Omura (1982). The combined utilization of a variety of nitrogen sources and carbon sources promoted the production of antimicrobial compounds, compared to the use of single nitrogen or carbon sources (Yu et al. 2022). Possibly due to this, in media without the presence of these two components, such as ISP3 and AIA, the final biomass and the activity against the bacteria tested were lower (Fig. 2A). Another interesting fact to observe was the difference in antimicrobial activity between the ISP2 and GLM media, both having glucose as a carbon source and the presence of nitrogen sources. The high concentrations of glucose found in the GLM medium could lead to catabolic

Table 1. Antibacterial activity screening of actinomycetes isolated strains (FR7, FR1, LEG1, LSG, and LS3), in agar-well diffusion method, against different bacterial strains.

Test microorganisms	Antibacterial activity of actinomycetes strains (inhibition zone in mm)				
	FR7	FR1	LEG1	LSG	LS3
M. luteus YSP7	21 ± 0.1[a]	17.2 ± 0.2[c]	18.9 ± 0.0[b]	16.8 ± 0.2[d]	–
Staph. aureus ATCC 6538	19.8 ± 0.1[a]	16.6 ± 0.0[b]	15.1 ± 0.1[c]	14 ± 0.0[d]	–
L. monocytogenes CECT 4031T	18.9 ± 0.0[a]	12.7 ± 0.1[c]	13 ± 0.1[b]	3.2 ± 0.1[d]	–
Staph. epidermidis CECT 4183	13.5 ± 0.3[a]	–	2.1 ± 0.1[c]	12.9 ± 0.1[b]	–
P. aeruginosa ATCC 10 145	16.8 ± 0.1	–	–	–	–
B. cereus ATCC 7064	16.8 ± 0.1[a]	15.3 ± 0.1[b]	–	2.7 ± 0.0[c]	–
E. coli DH5-alpha	12.4 ± 0.1	–	–	–	–

Results are expressed in inhibition zone (mm) ± SD and are representative of at least three independent assays (–) means no antibacterial activity. Within a row, different letters indicate significant differences between the actinomycetes strains tested, determined by one-way ANOVA followed by Tuckey post hoc test ($P < 0.05$).

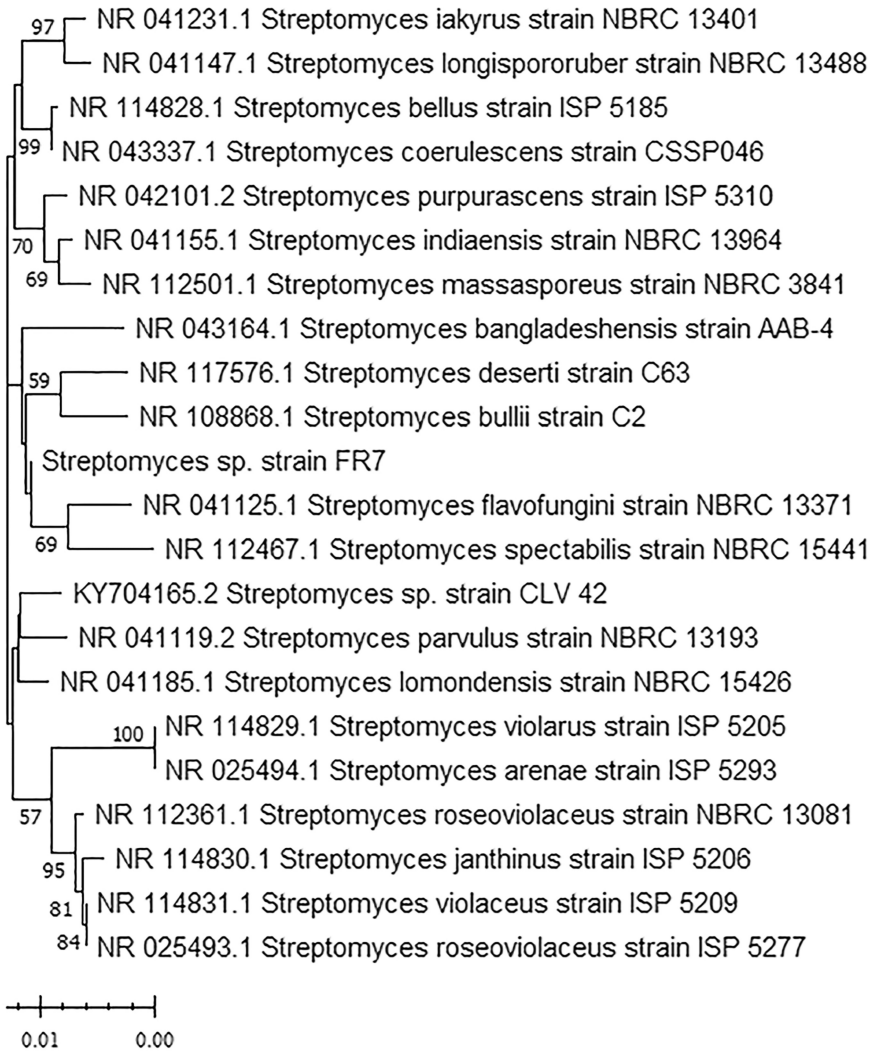

Figure 1. Neighbour-Joining phylogenetic tree based on 16S rRNA gene partial sequences showing the relationship between strain FR7 and closely related *Streptomyces* type strains. Numbers on the branches represent the confidence limits estimated from bootstrap analysis with 1000 replicates. Accession sequences of selected sequences are depicted in the tree.

repression and inhibit the synthesis of secondary metabolites (Romero-Rodríguez et al. 2016).

Besides the medium, our results indicate that 10 days incubation (Fig. 2B), initial pH 8 (Fig. 2C), 30°C (Fig. 2D) and the use of glucose as sole carbon and energy source (Fig. 2E) were the optimal conditions for highest biomass and antibacterial activity. Globally, our results on time of incubation (Fig. 2B) are in agreement with the usual profile of growth and antibiotic metabolites production of *Streptomyces* over time (Sejiny 1991), with maximum antibiotic activity recorded on the ninth or tenth day, when cultures reach the stationary phase (Ripa et al. 2009). Regarding pH, usually, actinomycetes pre-

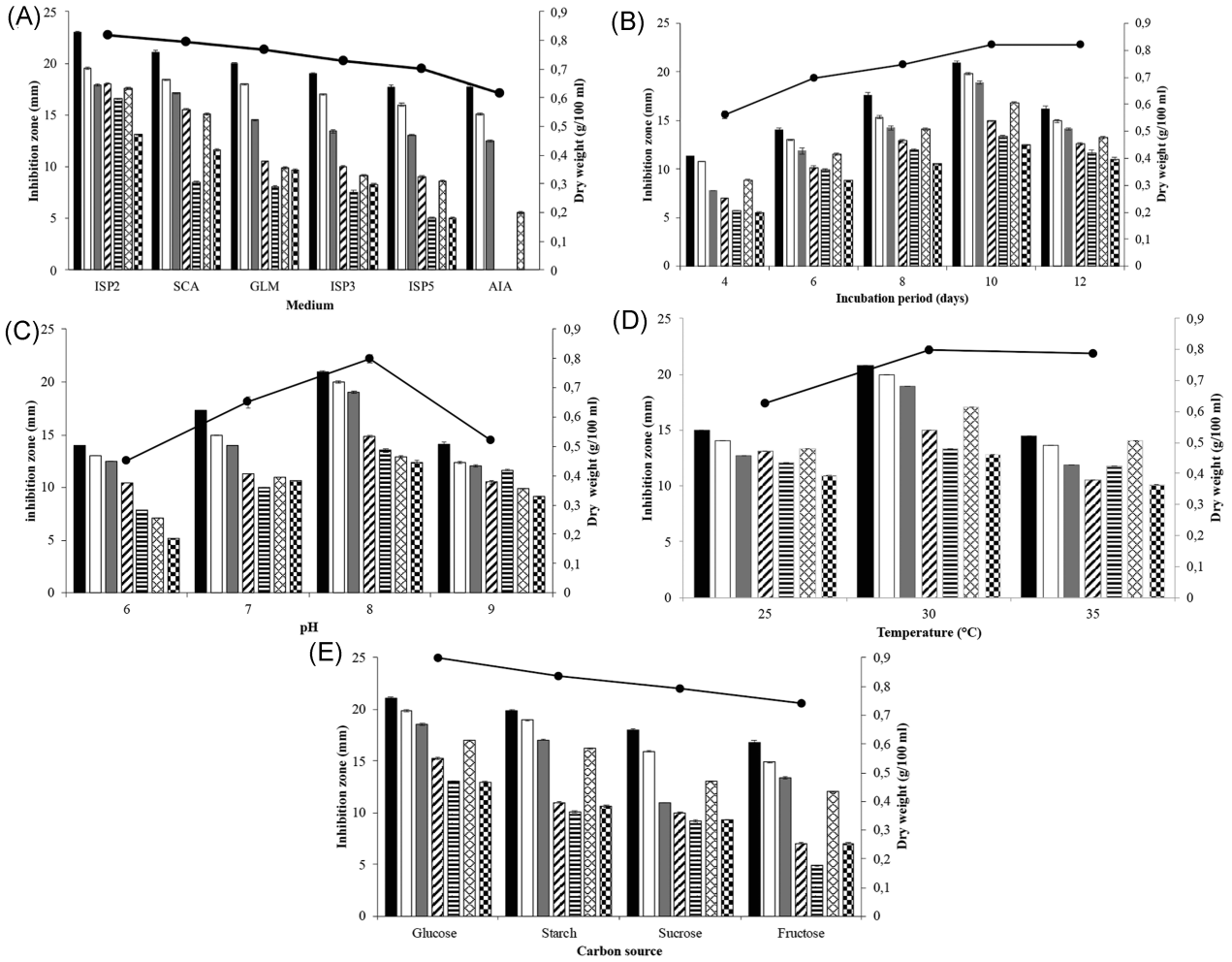

Figure 2. Optimization of culture conditions of *Streptomyces* sp. strain FR7 for maximal growth and antibacterial activity. (A) influence of different media composition (30°C/10 days); (B) effect of incubation period (30 °C, ISP2 medium); (C) impact of initial pH (30°C/10 days, ISP2 medium); (D) effect of temperature (10 days, ISP2 medium); and (E) Impact of carbon source (30°C/10 days, ISP2 medium). Dry weight (line graph) is represented by circles and the antibacterial activity (bar graph) is presented against *M. luteus* YSP7 (black), *Staph. aureus* ATCC 6538 (white), *Staph. epidermidis* CECT 4183 (grey), *L. monocytogenes* CECT 4031T (oblique lines), *B. cereus* ATCC 7064 (horizontal lines), *P. aeruginosa* ATCC 10145 (grid) and *E. coli* DH5-alpha (black and white squares). Results are presented as the mean ± standard deviation from three independent assays.

fer neutral or alkaline soils with pH between 5 and 9 (Goodfellow and Williams 1983). Biomass and antibacterial activity variation with initial pH (Fig. 2C) are quite comparable to those reported in the literature and suggest that maximal performance is achieved with an initial pH 8 in the medium (Singh et al. 2009, Managamuri et al. 2017). Our results on the influence of temperature (Fig. 2D) are in agreement with the report of Oskay (2011) and Vijayakumar et al. (2012), in which antimicrobial production and highest growth of *Streptomyces* sp. KGG32, *Streptomyces werraensis*, and *Streptomyces* sp. VPTS3-1 were observed at 30°C. The preference for glucose as carbon and energy source observed for *Streptomyces* sp. FR7 (Fig. 2E) has been reported also for *Streptomyces tanashiensis* and *Streptomyces* sp. (Singh et al. 2009, Haque et al. 2017).

After determining the most suitable culturing conditions of *Streptomyces* sp. FR7 for growth and antibacterial activity, we assessed the property of the ethyl acetate extract from *Streptomyces* sp. strain FR7 cultured under optimize conditions with a panel of several test microorganisms. After 10 days of incubation at 30°C in ISP2 medium with glucose as carbon source with initial pH adjusted to 8, the culture was used to prepare the extract, which was used in the minimum inhibitory concentration (MIC) determination experiments. As shown in Table 2, MIC values ranged from 5 to 100 $\mu g\,ml^{-1}$ over a wide range of Gram-positive bacteria and two Gram-negative bacteria. Among the tested bacteria, *M. luteus*, *Staph. aureus*, and *P. aeruginosa* were the most sensitive to *Streptomyces* sp. FR7 ethyl acetate extract, MICs attaining 5, 5, and 20 $\mu g\,ml^{-1}$, respectively. Remarkably, these values were lower than the standard broad-spectrum antibiotic, streptomycin. The MIC found in our work displayed higher antimicrobial activity than the ethyl acetate extract of *Streptomyces lavendulae* strain SCA5, which presented MIC values of 125 and 500 $\mu g\,ml^{-1}$ against *M. luteus* and *Staph. aureus*, respectively (Saravana Kumar et al. 2014). The same was observed in the work of Al-Dhabi et al. (2020), in which the extract from *Streptomyces* sp. presented MIC values of 500, 500, and 62.5 $\mu g\,ml^{-1}$ against *B. subtilis*, *Staph. aureus*, and *P. aeruginosa*, respectively.

Our results suggest effective antimicrobial activity of the ethyl acetate extract at very low concentrations against Gram-

Table 2. MIC of the *Streptomyces* sp. strain FR7 ethyl acetate extract against bacteria.

Test strain	MIC (μg ml^{-1})	
	Ethyl acetate extract	Streptomycin
M. luteus YSP7	5	6.25
Staph. aureus ATCC6538	5	6.25
L. monocytogenes CECT4031T	20	8
B. cereus ATCC 7064	100	4
Staph. epidermidis CECT 4183	100	50
P. aeruginosa ATCC 10 145	20	25
E. coli DH5-α	100	25

positive and Gram-negative bacteria. Furthermore, as far as we could find in the literature, antibacterial activity of ethyl acetate extracts is less pronounced than streptomycin against the bacterial strains tested. The extract of *Streptomyces* sp. strain FR7 reported in this study, displayed comparable or higher activity than streptomycin against *M. luteus*, *Staph. aureus*, and *P. aeruginosa*, indicating that this strain is a promising source of highly active antibacterial metabolites.

Streptomyces sp. FR7 strain extract has remarkable antioxidant activity

Due to the presumable richness in secondary metabolites of *Streptomyces* isolated from very diverse habitats, other bioactivities than antibacterial are likely to be present in the extract. Hence, we hypothesized that *Streptomyces* sp. FR7 extract could also have antioxidant secondary metabolites. Remarkably, the ethyl acetate extract exhibited the ability of scavenging free radicals by reducing the purple-coloured 2,2-diphenyl-1-pircrylhydrazyl (DPPH) radical, showing an IC_{50} value of 1.3 μg ml^{-1}, which is lower than that of the pure standard antioxidant compound butylhydroxytoluene (IC_{50} = 50 μg ml^{-1}; BHA). Therefore, the ethyl acetate extract of *Streptomyces* sp. strain FR7 showed remarkably radical scavenging activity in the DPPH method, this activity was higher than *Streptomyces chrysomallus* and *Streptomyces* sp. with IC_{50} values of 15 mg ml^{-1} and 10.6 $\pm$ mg ml^{-1}, respectively (Andriambeloson et al. 2016, Tangjitjaroenkun 2018).

The radical scavenging capacity displayed by the *Streptomyces* sp. FR7 extract was further investigated in order to assess if it could decrease intracellular oxidation of cells upon an oxidative challenge. Hence, we selected *Saccharomyces cerevisiae* as a model of eukaryotic cells to assess intracellular oxidation by flow cytometry with the redox-sensitive probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA). In addition, we used a mutant strain affected in the gene encoding for the transcription factor Yap1, which has been implicated in a cellular pathway that controls the oxidative stress response (Lee et al. 1999). Strains inactivated for this regulator are hypersensitive to H_2O_2 since they are defective in transcription of genes encoding antioxidant enzymes (Morgan et al. 1997).

After incubation of wild type yeast cells with H_2O_2, an increase of the fluorescence was noticed when compared to negative control (Fig. 3A), suggesting increase of intracellular oxidation. However, when cells were incubated with H_2O_2 and increasing concentrations of the extract, a shift of the cell population was observed to lower levels of fluorescence, indicating that the extract is counterbalancing the oxidative

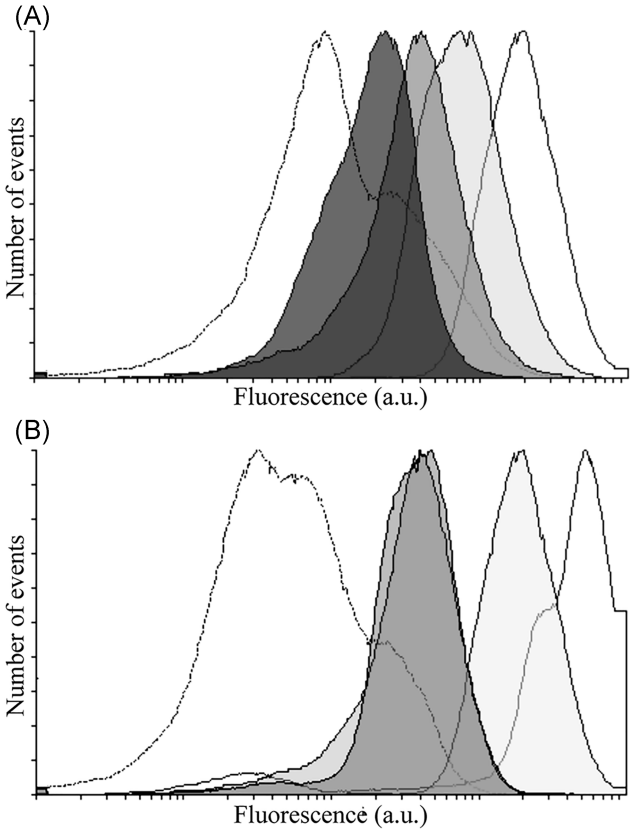

Figure 3. Intracellular oxidation of *Saccharomyces cerevisiae* cells upon treatments with H_2O_2 and extract from *Streptomyces* sp. strain FR7. Yeast wild type cells (A) and *yap1* mutant cells (B) were incubated for 1 h with 5 mmol l^{-1} H_2O_2 and 100 μg ml^{-1} (light grey), 200 μg ml^{-1} (medium grey) or 500 μg ml^{-1} (dark grey) extract from *Streptomyces* sp. strain FR7 and analysed for fluorescence by flow cytometry with the redox-sensitive probe H_2DCFDA. The negative control (dashed line) represents cells incubated only with ethanol, the extract solvent, and the positive control (solid line) represents cells incubated with 5 mmol l^{-1} H_2O_2 only. Data are from a representative experiment from three independent replicas. a.u. means arbitrary units.

stress imposed by H_2O_2 in a dose-dependent manner. Interestingly, in similar experiments with the mutant strain *yap1*, the results obtained were comparable to those of the wild type strain (Fig. 3B). This suggests that the extract exerts its antioxidant activity by reactive oxygen species scavenging since it is still able to decrease intracellular oxidation in stressed cells with defective antioxidant defences. Similarly, to the wild-type strain, the antioxidant effect was dose-dependent and it is interesting to note that, in accordance to the fact that

the *yap1* strain is highly sensitive to oxidative stress, the difference between the positive control and the negative control is considerably higher than in the case of the wild type strain.

The presented results of antioxidant activity clearly point to a strong reactive oxygen species scavenging activity of the extract rather than induction of cellular antioxidant defences. Extracts from other *Streptomyces* strains have been reported as having antioxidant activity like the protective effect of the crude extract against H_2O_2 of *Streptomyces* sp. MUM212, isolated from mangrove soil in Kuala Selangor, Malaysia, on Vero cells (Tan et al. 2017) and the extract of *Streptomyces variabilis,* isolated from sea sediment (Dholakiya et al. 2017). Accordingly, it is expected that strains with antioxidant secondary metabolites would be resistant to oxidative stress as is the case of *Streptomyces* sp. H-KF8 (Undabarrena et al. 2017).

Electrospray ionisation mass spectrometry (ESI-MS) of *Streptomyces* sp. strain FR7 crude extract

In order to identify the natural compounds produced by *Streptomyces* sp. strain FR7, the crude extract was analysed by ESI-MS in both positive and negative ionisation modes, using full scan mass measurements (Fig. S1). ESI-MS/MS spectrum of the ion with *m/z* 913 (Fig. S1A), which corresponds to the one with higher abundance shows fragmentations with several losses of 58 amu. Successive eliminations of CH_3-$CO^-CH_2{}^+H$ groups from polyketide compounds, after a MacLafferty rearrangement involving the transfer of hydrogen from an alkyl group at the beta position, can be foreseen in this case. This assumption is in agreement with the fact that the biosynthesis of polyketides can be carried out by *Streptomyces* since it involves a group of enzyme activities called polyketide synthases present in this genus (Risdian et al. 2019). These losses were accompanied by losses of water molecules (18 amu), indicating also the presence of OH groups. The ions obtained with negative ionization were identified by *m/z* cloud database using their MS and MS/MS spectra (Fig. S1B). The mass spectrum data [ESI(-)-MS] and the attempt to identify the compounds in the *Streptomyces* sp. strain FR7 ethyl acetate extract are shown in Table S3, the main ions correspond to methylsalicylic acid, 2-[(3-eth-1-ynylphenyl)imino]methyl-4-nitrophenol, and 1-[2-(2-thienyl)vinyl] benzoic acid with, respectively, 93%, 74%, and 60% matches.

The polyketides compounds are related to piericidins and iakyricidins (reviewed by Azad et al. 2022), the latter being recently found in *S. iakyrus* SCSIO NS104 (Li et al. 2019). In accordance with the phylogenetic analysis of *Streptomyces* sp. strain FR7, piericidins are mainly produced by *Streptomyces* (Zhou and Fenical 2016; Azad et al. 2022) while iakyricidins are from *S. iakyrus* (Li et al. 2019), the species that clustered closely with strain FR7 in the phylogenetic analysis (Fig. 1). In addition, *Streptomyces* strains identified as sources of piericidins have been isolated mainly from marine habitats (Chen et al. 2014), insects (Ortega et al. 2019) and soil (Zhou and Fenical 2016). The habitat from which *Streptomyces* sp. strain FR7 was isolated correlates with the presence of polyketides in this strain. Therefore, the presence of polyketides may underlie the antibacterial activity of the *Streptomyces* sp. FR7 extract since it has been shown that polyketides from *Streptomyces* spp. may present antibacterial properties (Almalki 2020, Peng et al. 2020). Several examples of antioxidant polyketides can be found reported in the literature synthesized by the edible mushroom *Cortinarius purpurascens* (Bai et al. 2013), the lichen endophytic fungus *Preussia* sp. (Paudel et al. 2018), and *Penicillium citrinum* (Samanthi et al. 2015). Piericidins have also been associated with antioxidant properties, such as piericidin A, which was reported to induce expression of the antioxidant protein peroxiredoxin 1 in cellosaurus cell line ACHN (Zhou et al. 2019). So, taking these examples and the wide array of biological activities of polyketides, it is tempting to consider that polyketides may be also responsible for the remarkable antioxidant properties of the *Streptomyces* strain FR7 extract.

Regarding the negative ionization, the compound methylsalicylic acid found in the *Streptomyces* sp. FR7 extract is also found for *Streptomyces antibioticus* DSM 40725 (Shao et al. 2006, Bai et al. 2013). Interestingly, synthesis of methylsalicylic acid is catalysed by an iterative type I polyketide synthase 6-methylsalicylic acid synthase (Shao et al. 2006), which further supports the presence of polyketide synthesis catalysing enzymes. The presence of methylsalicylic acid moieties in bioactive metabolites from *Streptomyces* strains has been described frequently. Examples of such compounds are the antibacterial and antitumor metabolite pactamycin isolated from *Streptomyces pactum* ATCC 27456 (Ito et al. 2009) and *Streptomyces* sp. a- WM-JG-16.2 (Iwatsuki et al. 2012), the cytotoxic nataxazole from *Streptomyces* sp. Tu¨ 6176 (Cano-Prieto et al. 2015) and the antibacterial polyketomycin from *Streptomyces diastatochromogenes* (Daum et al. 2009).

Based on these observations, in this work, we provide evidence suggesting the production of bioactive metabolites with methylsalicylic acid moieties in *Streptomyces* sp. strain FR7. To our knowledge, for the compounds 2-[(3-eth-1- ynylphenyl)imino]methyl-4-nitrophenol and 1-[2-(2-thienyl)vinyl]benzoic acid, detected in the negative ionization analysis, there are no reports in the literature associating them to *Streptomyces*. Nonetheless, isolation of benzoic acid derivatives has been reported from *Streptomyces* sp. CP27-53 with sirtuin inhibition activity (Chen et al. 2014).

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Supplementary data

Supplementary data is available at *LAMBIO Journal* online.

Conflict of interests

No conflict of interest declared.

Author contributions

Imen Weslati (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft), Luara Simões (Data curation, Formal analysis, Validation, Visualization, Writing – review & editing), Ana Teixeira (Data curation, Formal analysis, Validation, Visualization, Writing – review & editing), Pier Parpot (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing), Aly Raies (Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision), and Rui Oliveira (Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing)

Data availability

The data that supports the findings of this study are available in the supplementary material of this article.

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