

Full Length Research Paper

Isolation, characterization and antimicrobial activity of a *Streptomyces* strain isolated from deteriorated wood

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Emergence of drug resistance among pathogenic bacteria to currently used antibiotics has made the search for novel bioactive compounds from natural and unexplored habitats a necessity. In this study, we reported the isolation, characterization and antimicrobial activity of an actinomycete strain isolated from deteriorated wood of an old house located in the Medina of Fez. The isolate, named H2, was identified by 16S rDNA sequencing and was shown to belong to the genus *Streptomyces*. The isolate was screened for antimicrobial activity against Gram positive bacteria, Gram negative bacteria, Mycobacteria, yeasts and fungi. Partial characterization of the active substance (resistance to proteinase K and heat) showed that it would be of non-protein nature. The kinetics of production of the active substance showed that the maximum production occurs between the 7th and 10th day of fermentation. In addition, organic extract of the isolate was able to release genomic DNA of *Staphylococcus aureus* suggesting that it acts probably on the bacterial cell wall. Thin layer chromatography (TLC) of the ethyl acetate extract followed by bioautography has allowed localizing the active substances. This will open the way to further investigations to demonstrate their potential importance in combating pathogenic bacteria.

Key words: Actinomycetes, *Streptomyces*, antimicrobial activity, molecular identification.

INTRODUCTION

Since the discovery of antibiotics, bacterial resistance to these chemicals has continued to evolve. Thus, we are witnessing more and more multiresistant bacteria that pose a serious public health problem. Therefore, novel antibiotics against drug resistant bacteria are urgently needed. Microbial natural products have been one of the major sources of novel drugs.

Among microorganisms, actinomycetes are the most economically and biotechnologically useful prokaryotes (Lam, 2006; Valli et al., 2012). They produce antibiotics

and other industrially important secondary metabolites (Okami and Hotta, 1988; Koehn and Carter, 2005; Nermeen and Gehan, 2006; Kekuda et al., 2010; Naine et al., 2011).

From all the genera of actinomycetes, the genus *Streptomyces* is represented in nature by the largest number of species and varieties. These species can produce a large number of antibiotic and active secondary metabolites (Devi et al., 2006). In fact, 80% of the recognized antibiotics are sourced from this genus

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(Procópio et al., 2012), and many representatives of this group produce substances of high commercial value and are being extensively screened for novel bioactive compounds (Anderson and Wellington, 2001; Vijayakumar et al., 2007).

Exploring new habitats is one of the most promising ways to isolate new strains of actinomycetes endowed with antimicrobial activity (Zitouni et al., 2005; Khanna et al., 2011; Wadetwar and Patil, 2013).

Thus, we report here the isolation and molecular identification of a *Streptomyces* strain endowed with antibacterial activity. Partial characterization of the active substance as well as its mode of action is also reported.

MATERIALS AND METHODS

Sample collection

Pieces of deteriorated wood were taken from an old house, built 450 years ago, located in the former Derb Lamté in the Medina of Fez and were transported aseptically in sterile containers to the laboratory.

Isolation and culture conditions

Ten grams of wood samples were crushed and suspended in physiological water, then vigorously mixed for 2 h. The suspension was serially diluted up to 10^{-7} . An amount of 0.1 ml of each dilution was spread on the surface of the Actinomycetes Isolation Agar medium (AIA) (Thakur et al., 2007) and incubated at 30°C. After 2-4 weeks of incubation, the plates were examined for the presence of actinomycetes colony. The colonies of actinomycetes were recognized according to their macroscopic and microscopic characteristics (optical microscopy and Gram stain) then purified and conserved at 4°C for short periods and at -20°C in glycerol stock (20%, v/v) for a longer period. Twelve isolates showing different morphological characteristics were obtained. In this work, an isolate named H2 was chosen for the study of its antimicrobial activity.

Characterization of the isolate H2

The isolate H2 was tested for its ability to grow at pH 5 to 10 and at a temperature range of 25 to 42°C. Thus, actinomycetes cultures were spot inoculated onto plates of ISP2 (Shirling and Gottlieb, 1966) media to pH 5, 6, 7, 8, 9 and 10. The plates were checked for growth after seven days of incubation at 30°C. The same procedure was followed for the temperatures test except the use of ISP2 medium at pH 7.2. The temperatures tested were 25, 30, 37 and 42°C.

Actinomycetes synthesize and excrete dark pigments, melanin or melanoid, which are considered to be a useful criterion for taxonomical studies (Zonova, 1965; Arai and Mikami, 1972). Therefore the production of melanoid pigments was carried out on ISP6 and ISP7 agar (Shirling and Gottlieb, 1966).

DNA extraction, PCR amplification and sequencing of 16S rDNA

The isolate H2 was grown for 4 days at 28°C with agitation in 10 ml of ISP2 medium. Biomass was harvested by centrifugation at 8000

rpm for 10 min and washed twice with sterile distilled water. The pellet was dispersed in 800 µl of the aqueous lysis solution (100 mM Tris-HCl, pH 7; 20 mM EDTA; 250 mM NaCl; 2% SDS; 1 mg/ml lysozyme). The suspension was incubated at 37°C for 60 min. About 10 µl of a proteinase K solution (20 mg/ml) was added, and the lysis solution was reincubated at 65°C for 60 min. The lysate was extracted with an equal volume of phenol and centrifuged at 7000 rpm for 10 min. The aqueous layer was reextracted with phenol (50-50%, v/v) and then by chloroform (50-50%, v/v). DNA was recovered from the aqueous phase by the addition of NaCl (150 mM final concentration) and two volumes of cool 95% (v/v) ethanol prior to centrifugation. The precipitated DNA was cleaned with 500 µl of 70% (v/v) ethanol, centrifuged at 7000 rpm for 10 min, resuspended in 20 µl of TE buffer (10 mM Tris-HCl, pH 7.4; 1 mM EDTA, pH 8), and stored at -20°C (Duraipandian et al., 2010).

The 16S rDNA gene of the isolate H2 was amplified by polymerase chain reaction (PCR) using primers fD1 (5' AGAGTTTGATCCTGGCTCAG 3') and Rs16 (5'TACGGCTACCTGTTACGACTT 3') (Weisberg et al., 1991). The same primers were then used separately in two sequencing reactions from the two ends of the amplified fragment (about 1.5 kbp). The two sequences obtained were compared for similarity with those contained in genomic database banks, using the NCBI BLAST (Altschul et al., 1997).

Antimicrobial activity according to the culture medium

The actinomycete isolate was sown in scratches tightened in different agar media GLM (Kitouni et al., 2005), GYEA (Athalye et al., 1985), ISP2 (Shirling and Gottlieb, 1966), Bennett (Badji et al., 2005) and ISP1 (Shirling and Gottlieb, 1966) to show which medium stimulates maximum antimicrobial activity. After incubation for 7 days at 30°C, agar cylinders were then taken with hollow punch (Shomura et al., 1979; Saadoun and Al moumani, 1997; Petrosyan et al., 2003) and placed on Mueller Hinton agar plates, previously seeded with the test microorganism (10^5 - 10^6 CFU/ml). Plates were kept at 4°C for 2 h, and then incubated at 30°C for yeasts and at 37°C for bacteria and observed for antibiosis after 24 to 48 h. The bacteria and yeasts used as target were *Escherichia coli* DH5α, *E. coli* CIP 7624, *Staphylococcus aureus* CIP 53154, *Mycobacterium smegmatis* MC² 155, *Mycobacterium aurum* A⁺, *Bacillus subtilis* CIP 5262, *Bacillus cereus* CIP 14579, *Pseudomonas aeruginosa* 27853, *P. aeruginosa* A22, *Candida albicans* and *Candida tropicalis*.

Screening of antifungal activity

Antifungal activity was evaluated on ISP2 medium by the double layer method against a strain of *Aspergillus niger*. The isolate H2 was sown by touch in the center of Petri dish and incubated at 30°C during 7 days. The culture was covered by 8 ml of ISP2 medium containing 10 g of agar already sowed by *A. niger*. The diameter of inhibition was determined after 48 h of incubation at 30°C (Boughachiche et al., 2005).

Extraction of antimicrobial metabolites

The isolate of actinomycete was cultivated in ISP2 broth medium in 500 ml Erlenmeyer flask containing 100 ml of medium. The culture was incubated at 30°C for 7 days under constant agitation of 250 rpm. The production medium was centrifuged for 20 min at 8000 rpm to remove the mycelium. Ethyl acetate was added to the supernatant in the ratio 1:1 (v/v) and shaken vigorously for 2 h at room temperature. The organic extract was evaporated to dryness using a Rotavapor (Zitouni et al., 2005). The resulting dry extract

was recuperated in 1 ml of methanol and subjected to biological assay (disk of 6 mm in diameter) against *S. aureus*. The control corresponded to a disc containing an identical volume of methanol. The experiment was repeated twice. The dry extract was recovered in 1 ml of physiological water (NaCl 0.9%) instead of methanol to evaluate its effect on *S. aureus* cell wall (see below "extraction of staphylococcal DNA by the organic extract of the isolate H2").

Kinetics of antimicrobial activity of the isolate

Kinetics of antimicrobial activity was assessed on ISP2 broth. Pre-culture of the isolate H2 was carried out in 250 ml Erlenmeyer flask containing 50 ml of the ISP2 medium. After incubation for 48 h at 30°C under constant agitation at 250 rpm, the flask was homogenized and 5 ml of pre-culture was used to inoculate a 500 ml flask containing 100 ml of ISP2 medium. The flask was then incubated under the same conditions as above (Boudjella et al., 2006). The activity against *S. aureus* was regularly recorded each day by the agar diffusion method (well technique). The experiment was repeated twice.

Partial characterization of the antimicrobial products

The sensitivity to heat was examined by boiling the supernatant of the isolate H2 to 60, 80 and 100°C for 15 min then the treated supernatant was tested against *S. aureus* to determine its antibacterial activity by the well method. After incubation for 24 h at 37°C, the inhibition zone was measured. In addition, the sensitivity of organic extract to proteinase K was tested as follow: 40 µl of a Proteinase K solution (1 mg/ml) was mixed with 100 µl of the organic extract (using the protocol described previously) and incubated for 3 h at 37°C (Wu et al., 2005). Then, the effect of the Proteinase K treated extract was tested against *S. aureus* using the well method. The control was a solution of Proteinase K at the same concentration (the experiment was repeated twice).

Extraction of staphylococcal DNA by the organic extract of the isolate H2

A volume of 1 ml of an overnight culture of *S. aureus* was centrifuged at 5000 rpm during 5 min. The bacterial pellet was re-suspended in 360 µl of the organic extract recuperated in 1 ml of physiological water. This bacterial suspension was incubated at 37°C during 5 min and then centrifuged at 5000 rpm for 5 min. DNA was precipitated with two volumes of cool 95% (v/v) ethanol and 40 µl of NaCl 5 M. After incubation at -20°C during 30 min and centrifugation at 10000 rpm for 20 min, the pellet was dried and re-suspended in 20 µl of sterile distilled water. The control used corresponded to the same protocol as above, except for the use of 360 µl of physiological water instead of the organic extract (Hassi et al., 2007).

The genomic DNA obtained was detected by electrophoresis on 1% of agarose gel in a TAE buffer (Tris base 242 g; glacial acetic acid 57.1 ml; 0.5 M EDTA pH 8.0; H₂O 1000 ml) and was visualized by ultraviolet (UV) fluorescence after ethidium bromide staining. The experiment was repeated three times.

Thin layer chromatography

Ethyl acetate extract was used for primary analysis of the antibacterial substances. It was performed by thin layered chromatography (TLC) on silica gel slides by using chloroform-methanol (9:1, v/v) as solvent system. The chromatogram was observed under UV light. The experiment was repeated three times.

Bioautography

TLC slide was dried and was put in empty sterile Petri plate, in which 15 ml of sterile, LB agar seeded with *S. aureus* was poured. LB agar plate was incubated at 37°C for 24 h. After incubation, zone of inhibition around the spot was observed. The sterile zone on the media proved the presence of active antibacterial components in the studied samples (Holt, 1994). The R_f values of antibacterial compounds were determined.

In order to confirm the results from this experiment, the silica gel around the inhibition zone was eluted using ethyl acetate. After evaporation, the product was recuperated in sterile distilled water and 20 µl was spotted onto a sterile 6 mm diameter paper disc placed at the center of LB agar plate previously inoculated with a liquid culture of *S. aureus*. The experiment was repeated twice.

RESULTS AND DISCUSSION

Isolation of actinomycetes

In this study, an actinomycete isolate named H2 was isolated from deteriorated wood in an old house built 450 years ago located in the former Derb Lamté in the Medina of Fez. The isolate was Gram positive and formed colored tough and filamentous colonies that were hard to pick from the culture media as a characteristic of actinomycetes. It also produced colored pigments (purple), which were secreted into the culture media.

Characterization of the isolate

The growth of the isolate H2 was tested on ISP2 media at different pH and temperatures. We found that the isolate grew at pH and temperatures ranged from 5 to 10 and from 25 to 37°C, respectively.

For most actinomycetes, the optimum growth temperature is 23-37°C (Breidt et al., 1995; Stal and Moezelaar, 1997; Spyropoulou et al., 2001; Chen et al., 2003) whereas Goodfellow and Williams (1983) reported that most of the actinomycetes behave as mesophiles with an optimum growth at 30°C. There are also thermotolerant and thermophilic actinomycetes (Xu et al., 1998).

Streptomycetes are known to prefer neutral to alkaline environmental pH, the optimal growth pH range being 6.5 to 8.0 (Kutzner, 1986; Locci, 1989). However, acidophilic and alkalophilic streptomycetes have also been found (Kontro et al., 2005).

Melanin production was observed on the ISP6 medium but not on the ISP7 medium. This result is comparable with Antonova-Nikolova et al., (2006-2007) who found that *Streptomyces* sp. strain 34-1 synthesizes melanin on the ISP6 medium only and does not possess tyrosinase activity.

Some actinomycetes are capable of producing dark-brown substances in the culture media, generally referred to as melanin or melanoid pigments. Melanins are negatively charged composed of multi-functional polymers and polyphenolic compounds that are produced by various

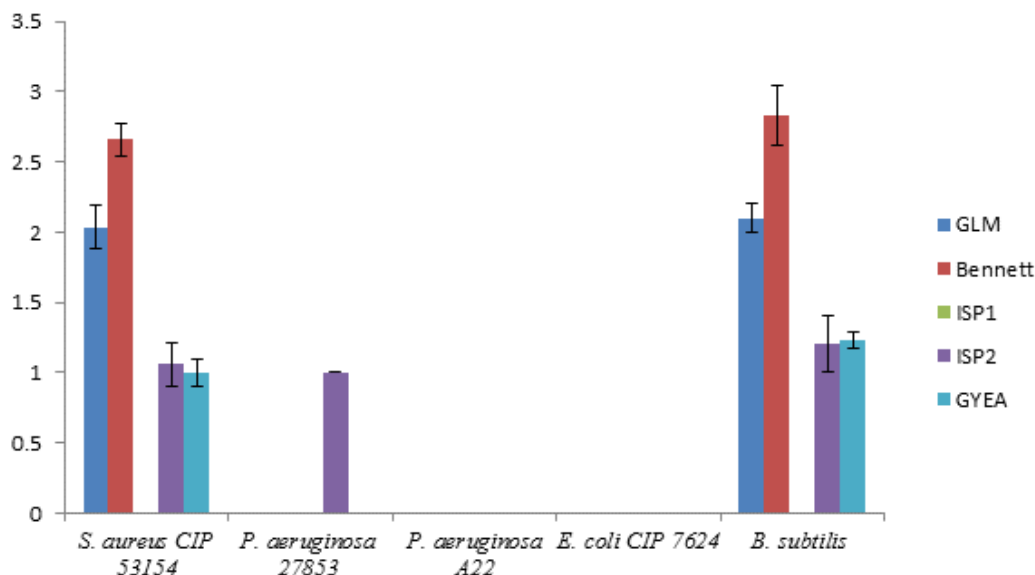


Figure 1. Antimicrobial activity of the isolate H2 on different agar media.

microorganisms by fermentative oxidation. They have also the radioprotective and antioxidant properties that can effectively protect the living organisms from ultra-violet radiation (Dastager et al., 2006). In addition they are used in medicine, pharmacology and cosmetics preparations (Dastager et al., 2006). Vasanthabharathi et al. (2011) found that the melanin pigment obtained from marine *Streptomyces* had antibacterial activity whereas Ali et al. (2011) found that pigment (melanin) produced by *Streptomyces virginiae* had antimicrobial activity against *A. niger*.

Molecular identification of the isolate H2

In this study, universal primers were used for amplification and sequencing the gene 16S rDNA of the isolate H2. The lengths of the 16S rDNA sequences analyzed were 540 and 550 bp for the primers fD1 and Rs16, respectively. Their analysis in comparison with the sequences available in Gen Bank, EMBL, DDBJ and PDB databases showed that the isolate H2 was closely related to *Streptomyces* sp. with 98 and 100% similarity for sequences obtained by fD1 and Rs16, respectively. This result is consistent with literature; in fact, the universal primers seem to be sufficient for identifying the genus but not the species (Boudemagh et al., 2005; Jeffrey, 2008; Arasu et al., 2008). Therefore, it is necessary to consider both the genetic and phenotypic aspects to identify the actinomycetes (Goodfellow et al., 2004). Other techniques were used such as PCR-RFLP gene encoding 65-kilodalton heat shock protein (Steingrube et al., 1997), repetitive intergenic DNA sequences (Sadowsky et al., 1996) and sequencing of the rpoB gene encoding the β subunit of RNA polymerase (Kim et al., 2004).

Antimicrobial activity according to the culture medium

Antibiotic production by actinomycetes is dependent on the composition of the medium (Singh et al., 2009) and especially on carbon and nitrogen sources (Iwai and Omura, 1982).

To determine the best production medium of antimicrobial substances by the isolate H2, the following culture medium were used: GLM, GYEA, ISP2, ISP1 and Bennett (Figure 1). First, we noticed that the spectrum of activity was different depending on the culture medium used. ISP1 seems to be a poor production medium since no activity was observed on it. Badji et al. (2005) found that GYEA and ISP1 media enabled satisfactory production in comparison with Bennett, ISP2 and nutrient agar whereas Cheraiti and Gacemi kirane (2012) showed that the activity of strain Act sp5 was very important on GYEA medium as compared to ISP1, Bennett and ISP2.

No activity was observed on all media for strains *E. coli* CIP 7624 and *P. aeruginosa* A22 indicating that these strains might be resistant to the substances produced by the isolate H2. These substances were active on *S. aureus* CIP 53154 and *B. subtilis* CIP 5262 on GLM, Bennett, ISP2 and GYEA. It is interesting to note that *P. aeruginosa* 27853 was sensitive only on ISP2 medium. This medium is recognized in the literature as a good production medium (Badji et al., 2006, 2007). So, in the following, ISP2 medium were used as the production medium.

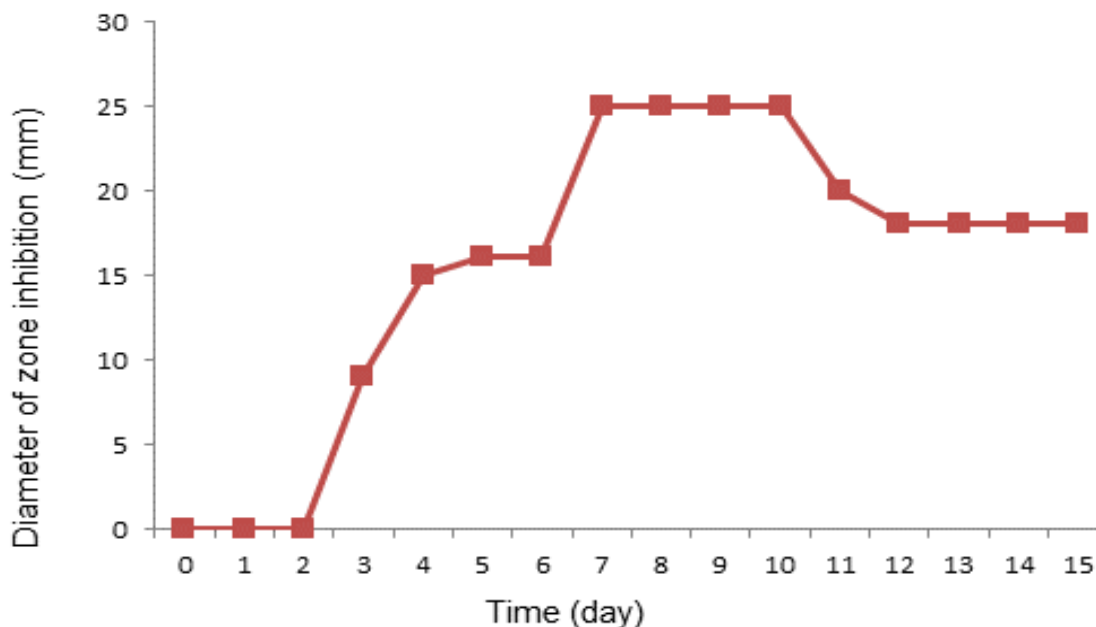
Screening of antimicrobial activity of the isolate H2 on ISP2 medium

The isolate H2 was active against Gram positive bacteria

Table 1. Antibacterial activity of the isolate H2.

Test isolate*								
EcDH5α	Ec CIP	Bs	Bc	Pa A22	Pa	Sa	Ms	Ma
-	-	+	+	-	+	+	+	+

+ Inhibition; - no inhibition. *EcDH5α, *E. coli* DH5α; Ec CIP, *E. coli* CIP 7624; Bs, *B. subtilis* CIP 5262; Bc, *B. cereus* CIP 14579; Pa A22, *P. aeruginosa* A22; Pa, *P. aeruginosa* 27853; Sa, *S. aureus* CIP 53154; Ms, *M. smegmatis* MC² 155 and Ma, *M. aurum* A⁺

**Figure 2.** Kinetics of antimicrobial activity of the isolate H2 against *S. aureus*.

(*B. subtilis* CIP 5262, *B. cereus* CIP 14579 and *S. aureus* CIP 53154), Gram negative bacteria (*P. aeruginosa* 27853), Mycobacteria (*M. smegmatis* MC² 155 and *M. aurum* A⁺) whereas Gram negative bacterium *E. coli* DH5α, *E. coli* CIP 7624 and *P. aeruginosa* A22 were resistant (Table 1).

Furthermore, no activity was observed on yeasts and fungi tested in this study suggesting that the isolate H2 did not possess an antifungal activity.

Kinetics of antimicrobial activity of the isolate H2

The kinetics of production was carried out on ISP2 broth and the zones of inhibition against *S. aureus* were measured by the agar diffusion method (Figure 2). The antimicrobial activity was detected after 2 days of incubation and the maximum production was detected between the 7th and 10th day of fermentation. This result is consistent with the literature. In fact, it is now established that the optimum production of antimicrobial substances by actinomycetes coincides with the phase of differentiation.

Kojiri et al. (1992) reported that the optimum production by *Streptomyces* sp. was reached at 6th day of incubation whereas Bouras et al. (2013) reported that the time of maximal antimicrobial activity by *Streptomyces* sp. PP14 was obtained always after 7, 8 or 9 day of fermentation.

Partial characterization of the antimicrobial product

The active substance of the isolate H2 inhibited *S. aureus* growth creating an inhibition zone around the wells. This activity was not eliminated upon treatment by Proteinase K (Table 2) or temperature (60, 80 and 100°C) (Table 3). It is concluded that the active substance might be of non protein nature.

Extraction of staphylococcal DNA by organic extract of the isolate H2

The active substance from the isolate H2 was extracted

Table 2. Sensitivity of the active substance from the isolate H2 to Proteinase K.

	Inhibition diameter against <i>S. aureus</i> (cm)		
	Without treatment with Proteinase K	After treatment with Proteinase K	Control
Organic extract of the isolate H2	3 ± 0.28	2.85 ± 0.07	0

The control used was a solution of Proteinase K. Values are means of two replicates ± standard deviation.

Table 3. Sensitivity of the active substance from the isolate H2 to temperature.

	Inhibition diameter against <i>S. aureus</i> (cm)			
	Not subjected to heat treatment	After heat treatment		
		60°C	80°C	100°C
Supernatant of the isolate H2	3 ± 0.14	3 ± 0.00	3 ± 0.14	2,6 ± 0.00

Values are means of two replicates ± standard deviation.

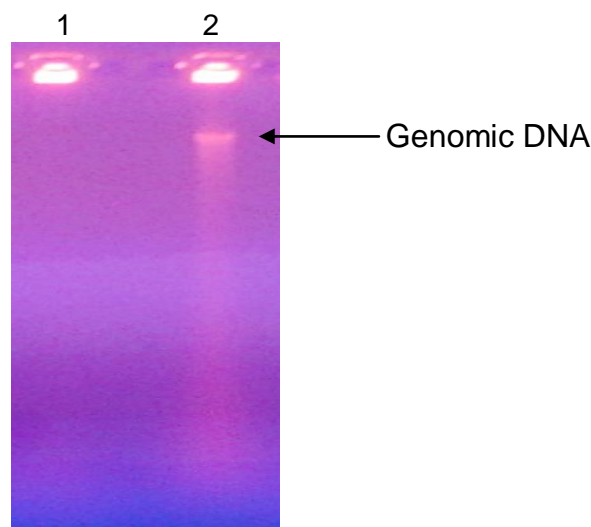


Figure 3. 1% Agarose gel electrophoresis analysis of *S. aureus* genomic DNA extracted with the organic extract of the isolate H2. 1: Control corresponding to DNA extraction of *S. aureus* with physiological water. 2: DNA extraction of *S. aureus* with the organic extract of the isolate H2.

by ethyl acetate, recuperated in physiological water and then tested for its ability to extract genomic DNA from *S. aureus* (previously explained in methods). Figure 3 shows that genomic DNA was obtained when *S. aureus* was treated by the organic extract of the isolate H2 in comparison with the control where organic extract was replaced by physiological water. This extraction was carried out in the absence of conventional agents of lysis, such as lysozyme, SDS or proteinase K, suggesting that the active ingredient acts at the *S. aureus* wall level.

Other active substances produced by actinomycetes

act on cell wall biosynthesis (Schneider et al., 2009; Hendlin et al., 1969; Krogstad et al., 1980; Singh et al., 2003).

More experiments are needed to characterize the structure of the active substance produced by the isolate H2 and to illustrate its effect on the bacterial cell wall of *S. aureus*. The effect might be also studied on other bacteria, mainly Gram negative ones. Thus, a protocol based on this substance could be developed and used in extraction of bacterial genomic DNA.

Thin layer chromatography and bioautography

TLC and bioautography are often used for preliminary characterization of antimicrobial substances from producing microorganisms' organic extract, especially determination of the position of the hot spot on TLC (Considine and Mallette, 1965; Awais et al., 2007).

In this study, chloroform-methanol (9:1, v/v) was used as solvent system to study ethyl acetate extract of the isolate H2. TLC silica gel analysis showed three bands having respectively the following R_f values: B1=0.37; B2=0.6 and B3=0.7 (Figure 4a). Bioautography of the TLC slide against *S. aureus* resulted in the formation of inhibition zones around the components corresponding to B2 and B3 bands (Figure 4b). No inhibition zone was observed around the B1 band.

Silica gel around the three bands was separately eluted and evaporated. The resulting products were separately recuperated in 20 µl of sterile distilled water and tested using the disc method. The results obtained confirms that the active bands were B2 and B3 (Figure 5). Further work is needed to determine the chemical structure of the products contained in the bands 2 and 3. The spectrum of activity of each of the two products should be determined on different bacteria and fungi. It would also

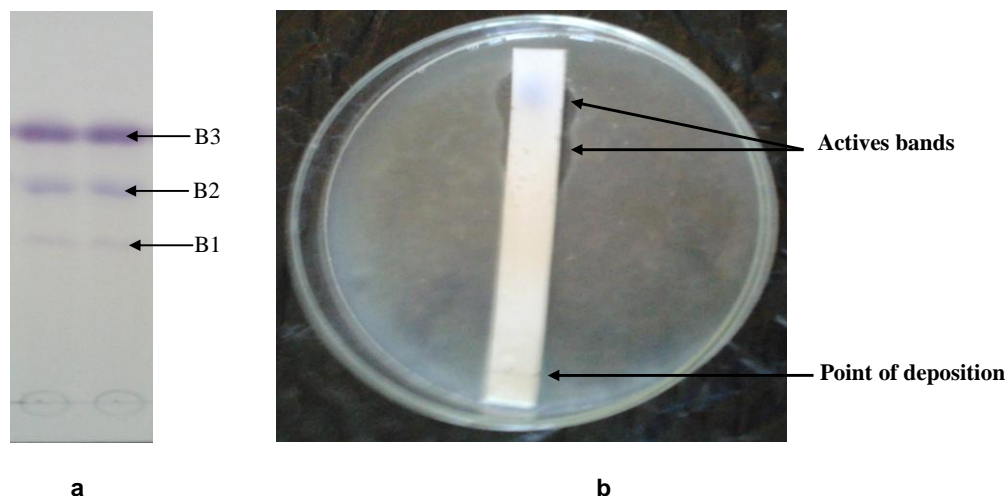


Figure 4. a) TLC analysis of the crude extract of H2 strain after migration; b) Bioautography of the H2 extract against *S. aureus*.

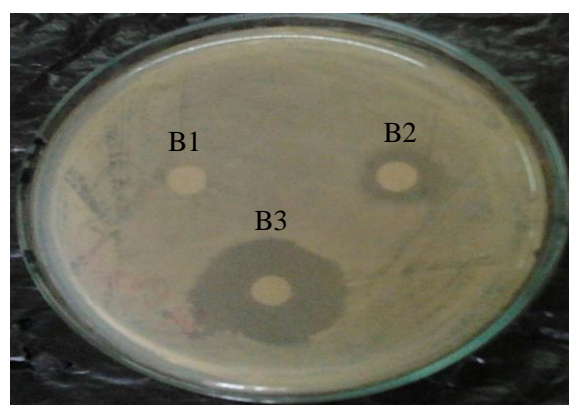


Figure 5. Effect of the three products (B1, B2 and B3) on *S. aureus* growth.

be interesting to see if the two products have a synergic effect or not.

In summary, a *Streptomyces* strain producing at least two antimicrobial substances was isolated from deteriorated wood. The activity of the isolate H2 against *S. aureus*, *Mycobacteria* and *P. aeruginosa* makes it more interesting to study because of the problems of resistance and multi-resistance encountered by these bacteria.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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