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Isolation and Screening of Antagonistic Actinomycetes For Potential Application in The Control of Pathogenic Bacteria in Contaminated Waste

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Abstract

Organic wastes for agricultural use represent a real agronomic interest but also a risk for public and animal health. Actinomycetes present a potential source of bioactive compounds with multiple applications. Among the 88 isolates of actinomycetes obtained from different samples, we selected a strain identified, thanks to macroand microscopic characters, as Streptomyces sp. SCM21. This strain showed these potentialities to produce antibacterial metabolites against the pathogenic bacteria tested (S. aureus, B. cereus and S. enteritidis) when cultivated in liquid and solid fermentation. Solid-state fermentation was conducted using sewage sludge as substrate. This is the first time that sewage sludge has been used to produce antibacterial metabolites by a strain of actinomycetes in solid-state fermentation. In addition, solid-state fermentation moistened with distilled water gave better antibacterial activity with good sporulation of Streptomyces sp. CSM²₁. Thus, the product of solid-state fermentation can be used in the management of contaminated waste to control pathogenic bacteria

Keywords: Actinomycetes, Sewage Sludge, Solid-State Fermentation, Pathogenic Bacteria, Contaminated Wastes

Introduction

Sewage sludge (SS) and animal manure represent a real agronomic interest given their richness in organic matter and nutrients. Without management program, these wastes may pose potential risks to human and environmental health [1]. Among the factors limiting the use of SS and animal manure in agriculture, we note the presence of pathogenic bacteria such as Salmonella spp. Escherichia coli and Listeria monocytogenes [2,1]. However, the application of these wastes in agriculture can increase the risk of contamination [3]. Animal manure is known to harbor a wide variety of microorganisms that can be pathogens. The spread of these pathogens could occur through unplanned and uncontrolled release through runoff from livestock facilities and through infiltration into soils and groundwater [4]. Groundwater contamination by these agents is the cause of the outbreak of waterborne diseases. Microbial contamination of surface water can come from intensive livestock systems. In this production system, pathogens most often enter surface water during rainfall events that promote land flow of pathogens from manure [5].

Wastes recycling and its application in agriculture requires the development of effective treatment techniques [6]. To reduce or eliminate pathogens, physical and/or biological methods are applied [7]. However, physical methods are energy-intensive and expensive processes. Recently, researchers' attention has turned to composting of SS for agricultural and horticultural purposes [8]. Composting is one of the natural processes capable of stabilizing organic waste. This process destroys most pathogens contained in this waste [9]. Nevertheless, several studies have shown that some pathogenic bacteria have the potential to persist in finished compost and in soil amended with compost [10]. Persistence of Listeria spp., Salmonella spp. and non-pathogenic E. coli during composting and survival of Salmonella spp. and non-pathogenic E. coli in mature composts have been reported [2]. Another major preoccupation of the composting application is the possibility of pathogen regrowth, indicating that a small pathogen population that survives the composting process or transferred from the outside environment can multiply to high concentrations under favorable conditions [10,9]. This requires the selection of appropriate, effective and environmentally sound new disposal methods. Figure 1 is a diagram showing how contaminated waste affects public and animal health.

Actinomycetes are responsible for the production of a huge variety of natural products with various biological activities, namely antibacterial, antifungal, anti-protozoan, antiviral, insecticide and herbicide activity [11]. In addition, they play an important role in recycling of organic matter in nature [12]. Actinomycetes produce extracellular enzymes such as amylases, cellulases, chitinases, lipases, proteases, ureases and keratinases which find applications in industry, agriculture and in wastewater treatment [13]. The recovery of organic waste offers an economic and environmental opportunity, which can reduce the problems of its conventional disposal. Solid-state fermentation (SSF) currently has several applications, such as the production of enzymes, antibiotics, organic acids, bioethanol, biodiesel and biosurfactants. Several authors have reported the production of some antibiotics by SSF using organic waste as a support, reflecting the importance of SSF for the production of this type of metabolites [14]. Antibiotic production by SSF has attracted much attention in biotechnology studies for the production of cephamycin, oxytetracycline, iturin, neomycin and rifamycins [15].

This study is intended to isolate and purify actinomycetes from different sites. Then, the bacterial collection is evaluated for these antibacterial potentialities against pathogenic bacteria. The highly antagonistic isolates are then cultured in liquid and solid fermentation to study their ability to produce antibacterial metabolites. The main objective of this study is to have a product of SSF rich in antagonistic agents with their secondary metabolites (antibacterial and enzymes) intended for the control of pathogenic bacteria in contaminated waste.



Figure 1: PM flow path of contaminated waste from livestock and wastewater treatment plants to surface and groundwater, agricultural products and pasture fields. These flows are favored mainly by heavy rains, spreading on agricultural and pasture lands and the presence of surface and groundwater near these stations and contaminated waste storage areas.

Materials and Methods Sample Collection and Isolation of Actinomycetes

Several samples were collected from different localities in Algeria, namely: Seawater, sea sand, soil and SS using sterile sampling bags and vials. Samples were pre-treated in a water bath at 50 °C for 60 min [16,17]. Then, dilutions were prepared of each sample and then inoculated into Petri dishes containing one of the following isolation media: Starch casein agar (SCA) [18], N-Free agar and Carboxymethylcellulose agar (CMC agar). In the case of seawater, 1 ml of sample was spread directly without dilution. The inoculated dishes were incubated at 30 °C until typical actinomycete colonies appeared. These were purified on the isolation medium by successive subculturing and then stored at 4 °C for later use.

Screening of Antagonistic Actinomycetes Strains

Isolated and purified actinomycetes were screened for their antagonism against four pathogenic bacteria (Staphylococcus aureus ATCC 25923, Bacillus cereus ATCC 10876, Escherichia coli ATCC 25922 and Salmonella enteritidis ATCC 13076) using cross streak method [16]. Antagonism of actinomycete isolates is classified according to the mean diameter of inhibition zone: excellent activity (\geq 18 mm), good activity (12-15 mm), moderate activity (10-12 mm) and low activity (\leq 9 mm) [19]. Highly antagonistic isolates were retained for further work.

Production of Antibacterial Metabolites in Liquid Fermentation 500ml Erlenmeyer flasks, each containing 100 ml of Trypticase soy broth (TSB) or Starch casein broth (SCB) liquid culture medium was inoculated with 3 agar discs (5 mm in diameter) of antagonist actinomycetes, then incubated at 30 °C for 10 days with permanent agitation (150 rpm) [20]. After incubation, approximately 4 ml of each culture was centrifuged at $12,000 \times g$ for 10 min at 4 °C to assess the antibacterial activity of crude supernatant by agar well diffusion method. Part of the culture was extracted with an organic solvent. After centrifugation at 4000 rpm for 15 min, the supernatant was mixed with ethyl acetate (1:1, v/v) and kept under permanent agitation (150 rpm) for 60 min [21,20]. The organic phase was separated from the aqueous phase using a separating funnel and ethyl acetate was removed using a RotaVap (50 °C/100 rpm). Then 10% dimethylsulfoxide (DMSO) was added to the crude extract to have a final extract concentration of 10 mg ml-1 [22,20]. The various prepared solutions were sterilized by filtration and evaluated for their antibacterial activity by agar well diffusion method.

Preparation of Actinomycete Inoculums

Three agar discs (5 mm in diameter) of each actinomycete culture cultivated on Trypticase soy agar (TSA) medium for 7 days at 30 °C were introduced into 200 ml flasks containing 30 ml of TSB medium and then incubated at 30 °C for 7 days with permanent agitation (150 rpm). After incubation, each culture was centrifuged (8000 rpm/15 min) and the pellet was resuspended in 20 ml of physiological water. The prepared suspensions were used to inoculate the liquid and solid fermentation.

Effect of Incubation Time on Antibacterial Metabolite Production

The production of antibacterial metabolites by antagonist actino-

mycete isolates was studied as a function of incubation time. 500 ml Erlenmeyer flasks containing 100 ml of TSB were inoculated with 2 ml of each prepared inoculum and then incubated at 30 °C for 14 days with permanent agitation (150 rpm). Samples were taken every two days and centrifuged at $12,000 \times g$ for 10 min at 4 °C. The antibacterial activity of the crude supernatants was evaluated against pathogenic bacteria by agar well diffusion method.

Production of Antibacterial Metabolites by Solid-State Fermentation (SSF)

Antagonist actinomycete isolates were evaluated for the production of antibacterial metabolites by SSF using SS as a substrate (20 g + 15 ml TSB). The medium is spread uniformly in a 250 ml Erlenmeyer flask and then autoclaved. Then, each Erlenmeyer flask was inoculated with 2 ml of each prepared inoculum and then incubated at 30 °C for 12 days. A second SSF was conducted using SS combined with wheat bran and coffee grounds with ratios: 65% (13 g): 10% (2 g): 25% (5 g), respectively. Each medium was prepared either with 20 ml of TSB or distilled water, homogenized and then autoclaved. Each Erlenmeyer was then inoculated with 2 ml of each prepared inoculum and incubated at 30 °C for 12 days. In order to check the production of antibacterial metabolites in the fermented product we carried out aqueous extraction. After incubation, the fermentation media were added with 50 ml of sterile distilled water and then kept under strong agitation (400 rpm for 30 min) [23]. The aqueous phase was centrifuged at $12,000 \times g$ for 15 min and the aqueous extract is evaluated for its antibacterial activity by agar well diffusion method.

Determination of Antibacterial Activity by Agar Well Diffusion Method

A rod of a sterile swab was immersed in a suspension of each pathogenic bacteria (OD600nm = 0.1). Then the rod evenly swabs the entire surface of the Mueller Hinton Agar (MHA). The inoculated dishes were kept at room temperature for 3 to 5 min before the application of the extracts. Wells of 6-mm diameter were created on the MHA medium using sterile Pasteur pipettes. Then, approximately 100 μ L of supernatant, crude extract (10 mg ml-1) or aqueous extract were carefully distributed into each well and allowed to diffuse for 2 h at room temperature (20 °C), then incubated at 37 °C for 24 h. Each treatment was applied in triplicate. After incubation, the inhibition zone around each well was measured. Wells loaded with 100 μ l of 10% DMSO or 100 μ l of aqueous extract of uninoculated medium were used as negative control [22].

Characterization of Selected Antagonist Isolates

The cultural characteristics were studied on different culture media: yeast extract-malt extract agar (ISP-2), oatmeal agar (ISP-3), Inorganic Salt Starch Agar (ISP-4), Glycerol Asparagine Agar Base (ISP-5), SCA and TSA. Arrangement and structure of spores were examined under a light microscope (G×1000) after cultivation by the coverslip culture method [22]. The selected isolates were studied for their growth as a function of temperature (30; 35; 40 and 45 °C), their tolerance to salinity (5; 7 and 15%, w/v) and their enzymatic potential, namely protein hydrolysis [24], starch [25], cellulose [26], lipid [27] and urea [28]; ammonia production [29] and melanin [30]; and gelatin liquefaction [31].

Statistical Analysis

The data collected from experiments are subjected to the analysis of variances (ANOVA) according to the Fisher multiple comparison test (LSD), p < 0.05 is considered significant, using the XLSTAT 2013 software.

Results

Isolation and Purification

Actinomycete colonies appear after 7 to 10 days of incubation on the various isolation media. They are recognized by their morphological appearance (powdery or hard colonies embedded in the agar) Figure 2.



Figure 2: Colonies of actinomycetes isolated on different isolation media. A and C: SCA; B and H: N-Free agar; D, E, F and G: CMC-agar.

Table 1 illustrates the results of isolation of actinomycetes on different isolation media. A total of 88 actinomycete isolates were isolated and purified. Based on origin of isolation, there are 60 isolates from soil samples, 20 isolates from SS, 5 isolates from seawater and 3 isolates from sea sand. SCA culture medium covered more isolates than other media with 43 isolates followed by CMC-agar (31 isolates) and N-Free agar (14 isolates).

 Table 1: Distribution of actinomycete isolates by sample and isolation media.

Sample (code)	N-Free agar	CMC- agar	SCA	Total
Forest soil 1 (SFA1)	2	5	NU	7
Forest soil 2 (SFA2)	0	3	NU	3
Seawater 2 (EMPT)	5	0	NU	5
Sea sand 2 (SMPT2)	1	2	NU	3
Semi-arid soil 1 (SSA1)	2	6	12	20
Semi-arid soil 2 (SSA2)	4	3	23	30
Sewage sludge (CBE)	0	12	8	20
Total	14	31	43	88
NU : Not used in isolation				

Antagonism of Actinomycetes Against Pathogenic Bacteria

Of the 88 actinomycete isolates tested, 15 isolates (17.04%) showed antagonism to at least one pathogenic bacterium. However, only 8 isolates of them showed moderate to strong antibacterial activity. Actinomycetes isolates with significant antibacterial activity were isolated from semi-arid soil and sea sand. These isolates exhibited antagonism against *S. aureus, B. cereus* and *S. enteritidis*. However, all isolates showed no antagonism to *E. coli* Table 2. These results enabled us to retain the CSM_{11}^2 NSA₁ and SSA₁₂ isolates for further work, due to their strong antibacterial activity with a broad spectrum of action.

 Table 2: Antagonism of actinomycetes isolates against pathogenic bacteria (cross-streak method).

Isolate	Origin of isolation	isolation medium	Antagonism against pathogenic bacteria			
			Gram positive		Gram negative	
			B. Cereus	S. aureus	S. enteritidis	E. coli
SSA213	Semi-arid soil 2	SCA	+	+	+	-
SSA23	Semi-arid soil 2	SCA	++	+	+	-
SSA112	Semi-arid soil 1	SCA	++++	++++	++++	-
NSA21	Semi-arid soil 2	N-Free agar	++++	++++	++++	-
CSM21	Sea sand 2	CMC-agar	++++	++++	++++	-
SSA117	Semi-arid soil 1	SCA	++	+	+++	-
NEM22	Seawater 2	N-Free agar	+++	++	+	-
SBE5	SS	SCA	++++	-	++++	-
(+): low activity ($\leq 9 \text{ mm}$); (++): moderate activity (10-12 mm); (+++): good activity (12-15 mm); (++++): excellent activity						

 $(\geq 18 \text{ mm})$; (-): absence of activity.

Production of Antibacterial Metabolites in Liquid Fermentation

The production of antibacterial metabolites in liquid medium by the selected isolates was evaluated using two culture media: TSB and SCB. This production was verified by the well method. Results of antibacterial effect of crude supernatants and organic extracts are presented below Figure 3. The antibacterial activity differs according to the culture medium used. TSB medium is more favorable for the production of antibacterial metabolites against the pathogenic bacteria tested than SCB medium, with the exception of the isolate SSA¹₁₂ against *S. aureus*. In addition, we note that isolate SSA¹₁₂ produces antibacterial substances against B. cereus when cultured in TSB, while no production is observed when cultured in SCB. This activity also differs, quantitatively and qualitatively, according to the actinomycete isolate. The isolate CSM_1^2 shows interesting activities against pathogens (*S. aureus* and *S. enteritidis*) compared to other isolates. In addition, a remarkable difference was observed in the antibacterial activity between the crude supernatant and its organic extract in the three isolates of actinomycetes Figure 3. For example, the crude supernatant of the isolate CSM_1^2 grown in TSB medium has a diameter of inhibition against S. aureus and S. enteritidis of 21.24 and 18.03 mm, respectively, while its organic extract has a diameter of 6.62 and 0 mm, respectively. Additionally, no activity has been reported against *E. coli* in all actinomycete isolates.



Figure 3: Antibacterial activity of crude supernatants and organic extracts of selected actinomycete isolates. (A) Antibacterial activity of isolate SSA_{12}^1 (B) antibacterial activity of isolate CSM_1^2 and (C) antibacterial activity of isolate NSA_1^2 . (SE: SCB Organic extract, SS: SCB Crude supernatant, TE: TSB organic extract and TS: TSB Crude supernatant). a-c, a'-c' and a''-c'' represent the different groups.

Effect of Incubation Time on Antibacterial Metabolites Productions

The kinetics of the production of antibacterial metabolites by the isolates CSM_{1}^2 , NSA_{1}^2 and SSA_{12}^1 were followed in liquid culture Figure 4. Antibacterial activity of the isolate CSM_{1}^2 against *S. aureus*, *B. cereus* and *S. enteritidis* is observed after 6 days of incubation with zones of inhibition of 20.68; 18.35 and 17.80 mm, respectively. Maximum activity was recorded on day 10 against *B. cereus* and *S. enteritidis* with zones of inhibition of 24.15 and 22.83 mm, respectively, while maximum activity against *S. aureus* was detected on day 8. For isolate NSA_{1}^2 its antibacterial activity against S. aureus and B. cereus occurs on day 4 with zones of inhibition of 6.21 and 11 mm, respectively. While its activity against S. enteritidis is recorded on day 8 with an inhibition zone of 6.88 mm. The maximum activity is recorded on day 10 against *S. aureus* and *S. enteritidis* with inhibition zones of 17.59 and 16.10 mm, respectively, while its maximum activity against B. cereus is detected on day 8 with 18.82 mm. Antibacterial activity of isolate SSA112 against *S. aureus* and *S. enteritidis* occurs on day 8 with an inhibition zone of 14.87 and 15.45 mm, respectively. While its activity against B. cereus is recorded on the 6th day with an inhibition zone of 4.70 mm. This isolate shows maximum activity against all pathogenic bacteria on the 10th day of incubation. The isolate CSM_{1}^{2} is marked by its high activity compared to other isolates. Beyond 10 day, the antibacterial activity decreases in all three isolates.



Figure 4: Effect of incubation time on antibacterial activity: (A) antibacterial activity against S. aureus, (B) antibacterial activity against B. cereus, (C) antibacterial activity against S. enteritidis. (D) maximum antibacterial activity of the different isolates on pathogenic germs (well method).

Production Of Antibacterial Metabolites By SSF

The production of antibacterial metabolites by SSF was studied using SS as a fermentation substrate Figure 5-A. This fermentation clearly shows that isolate CSM_1^2 was able to produce antibacterial metabolites that inhibit the three pathogenic bacteria tested with inhibition zones between 14 and 18 mm. In contrast, no inhibition was recorded for isolate NSA_1^2 . While isolate SSA_{12}^1 was able to produce antibacterial metabolites inhibiting only S. enteritidis growth with an inhibition zone of approximately 10 mm. Based on these results, isolate CSM_1^2 was selected for the production of antibacterial metabolites using SS combined with other substrates (spent coffee grounds and wheat bran) Figure 5-B. This second fermentation allowed us to improve the antibacterial activity of the aqueous extract against the three pathogenic bacteria tested with inhibition zones between 17 and 23 mm. In addition, we have found that the use of distilled water instead of TSB in the preparation of the fermenting medium gives significant antibacterial activity against *S. aureus* and *S. enteritidis* with remarkable sporulation Figure 5-C.



Figure 5: Production of antibacterial metabolites by isolates CSM_1^2 , NSA_1^2 and SSA_{12}^1 in SSF after 12 days of incubation. (A) SS as substrate, (B) SS, coffee grounds and wheat bran as substrate and (C) growth of the isolate CSM_1^2 in the SS/coffee grounds/wheat bran mixture moistened with TSB or distilled water.

Cultural, Morphological and Physiological Characteristics of The Isolate Csm²₁

Macroscopic characteristics of the isolate CSM21 cultured on the different culture media are shown in Table 3. These characters differ according to the culture medium used. It is also noted that the media used in the cultural characterization do not give a good sporulation. Growth of isolate CSM21 is abundant on ISP4 medium, moderate on ISP5 and SCA media with low growth on TSA and ISP3 media. It produces a diffusible pigmentation of a light-yellow color in all media except ISP4 medium where it produces a dark yellow pigmentation. This isolate develops a substrate mycelium (MS), usually yellow. The production of aerial mycelium (AM) is observed on all media except TSA, with a white to gray color. This isolate forms colonies with a diameter of 1-8 mm.

Culture medium	Growth	SM color	Diffusible pigmentation	Presence and color of AM
SCA	++	Pale yellow	Light yellow	Grey-white
ISP3	+	Yellow	Light yellow	Grey-white
ISP5	++	Yellow	Light yellow	Yellow-white
ISP4	+++	Yellow-brown	Dark yellow	Grey-white
TSA	+	Yellow	Light yellow	-
(+++) important; (++) average; (+) Low; (-) absent; SM: Substrate mycelium; AM: aerial mycelium				

Isolate CSM_1^2 shows, after 7 days of incubation, an SM with branched filaments gram-positive, fine, uncultivated, non-spore-forming. After 14 days of incubation, we observed hyphae with a light purple color, these are degrading SM. During develop-

ment, segmentation of AM gives rise to spore chains in the form of closed loops, sometimes taking the form of hook-shaped rounded spores Figure 6.



CSM21 after 7 days of incubation CSM21 after 14 days of incubation

Figure 6: Microscopic observation of isolate CSM21 after Gram stain (G×1000). SM: Substrate mycelium; AM: Aerial mycelium; S: Septum; CS: Spore chain.

Isolate CSM21 tolerates temperatures up to 45 °C. For salinity resistance, this isolate tolerates up to 7% NaCl. The results of the enzymatic activities clearly show the active metabolism of

isolate CSM21 via the production of a variety of enzymes. The results of enzymatic activities are reported in Table 4.

Enzymatic activities	results	
Cellulase	+++	
Protease	+++	
Amylase	+	
Lipase	+	
Urease	+	
Gelatinase	+	
Ammonia production	+	
Production of melanoid pigments	-	
(-) negative; (+) low activity; (++) moderate activity; (+++) good activity		

Table 4: Enzymatic activities of isolate CSM21

Discussion

The current study focused on the isolation of actinomycetes from a few sites in Algeria in order to select strains with high antagonistic power. Eighty-eight isolates of actinomycetes were isolated from different samples. Actinomycetes are widely distributed in natural ecosystems including soil, aquatic ecosystems, deserts and even in Antarctica [32,33]. In order to reduce the microbial load in the samples and promote the proliferation of actinomycetes, we performed a preheating. Baskaran et al. [16] reported that the predominance of other bacteria and fungi inhibits the colonization of actinomycetes. Heat treatment is often used to reduce the number of Gram-negative bacteria often invading isolation dishes [17]. Using three isolation media allowed us to cover more species. SCA medium covers more isolates than CMC-agar and N-Free agar media. However, the latter two media allowed us to isolate high antagonistic actinomycetes that SCA medium does not cover. For this, it is interesting to use a range of media for the isolation of antagonistic actinomycetes. Actinomycetes present a potential source of bioactive compounds. They are remarkable antibiotic producers, making quarters out of all known pharmaceuticals [16]. In order to screen our actinomycetes isolates for their antagonism against pathogenic bacteria, we opted for the cross-streak method. The results of this screening showed that the isolates CSM21, NSA21 and SSA112 possessed excellent antibacterial activity against all pathogens tested except E. coli. Baskaran et al. [16] screened 42 actinomycetes isolated from mangrove sediments against pathogenic bacteria and found that 22 species exhibited antagonism, of which isolate A107, identified as Streptomyces spp., had the maximum activity against all pathogens targeted. Ganesan et al. [21] screened 106 strains of actinomycetes against several pathogens using the cross-streak method. Only 41.50% showed good antagonism against the pathogens tested. The cross-streak method has been reported to be used for rapid screening of antagonistic microorganisms [34].

By analyzing the results of the antibacterial activity of the crude supernatants and of the organic extracts (ethyl acetate) of the fermentations in liquid medium we found that the crude supernatants of all the isolates have a greater antibacterial activity than that of the organic extracts which is sometimes absent. The significant antibacterial activity recorded in actinomycete isolates cultured in TSB medium can be explained by the composition of this medium that promotes the production of antibacterial substances. Thus, it has been reported that there are both quantitative and qualitative variations in the antibiotics produced. Substrates and habitats greatly influenced the production of antibiotics by actinomycetes [16]. Contrary to our results, several authors have reported the effectiveness of ethyl acetate in extracting large amounts of antibiotics from crude supernatants of actinomycete cultures [21,19]. This result can be explained by the difference in polarity of the antibacterial metabolites produced by our isolates with that of ethyl acetate. The study of the production of antibacterial metabolites as a function of incubation time showed early and important production in isolate CSM21 against the three pathogenic bacteria. From the economic aspect, it is interesting to select actinomycetes which produce large quantities of antibacterial metabolites in a short time, which reduces production costs. Likewise, many actinomycetes have been reported to begin production of antibiotics after 4-6 days of incubation [18].

With the population explosion, large amounts of waste contaminated with pathogens are generated. It is therefore obvious that this waste must be properly contained and managed because it can cause infections in humans [4], hence the need to develop new effective biological methods to combat these agents. Screening, isolation and characterization of promising strains of actinomycetes producing bioactive compounds is an important area of research worldwide. Actinomycetes, especially Streptomyces species, are widely recognized as important microorganisms with potential applications [32]. It has been reported that members of the genus Streptomyces isolated from terrestrial or aquatic environments are responsible for the largest share of production of antimicrobial substances [35]. View their metabolisms, actinomycetes can be proposed as biological control agents against the presence and persistence of pathogenic bacteria in contaminated waste. To produce antibacterial metabolites

and take advantage of organic waste, SSF was adopted in this study where we used SS as a substrate. We have recorded a considerable production which results in the high diameter of the inhibition zones of the pathogenic bacteria tested, especially when the substrates are moistened with distilled water which makes the production costs less expensive. In addition, SS is a highly available and inexpensive waste. Several authors have reported the production of antibacterial metabolites by actinomycetes in SSF, particularly, by Streptomyces species. Gebreyohannes et al. [22] showed the antibacterial activity of crude extracts from an SSF carried out by strains of actinomycetes using rice grains as a substrate. Khaliq et al. [23] studied the production of tylosin in SSF by Streptomyces fradiae NRRL-2702 and its mutant y-1 using different agro-industrial wastes. Wheat bran, as a solid substrate, gave the best production by the mutant γ -1. Deen et al. [15] showed the efficiency of erythromycin production in SSF by Saccharopolyspora erythraea NCIMB 12462 using several agro-industrial wastes where sugarcane bagasse was found to be the best. Other antibiotics have also been produced by SSF, namely tetracycline by Streptomyces viridifaciens ATCC 11989, neomycin by Streptomyces marinensis, meroparamycin by Streptomyces sp. TUE01 [14]. SSF offers a number of advantages over liquid fermentation. The production medium is often simple, using agro-industrial by-products such as wheat bran, rice bran and wheat straw [15]. In addition, it can promote the production of new secondary metabolites compared to liquid fermentation [35]. The application of antagonistic actinomycetes with significant and diverse enzymatic activity can increase the rate of elimination of pathogenic bacteria in contaminated waste. Parmar et al. [7] have proven the efficacy of proteases in the elimination of pathogenic bacteria in SS.

The selected isolate CSM21 was identified using macro and microscopic characters. Macroscopic examination on different media allows us to follow the growth of colonies, the arrangement of SM and AM as well as the morphology of the spores which can be taken into account when characterizing actinomycetes strains [36]. In addition, the straight to flexible, hook, loop or spiral spore chains are characteristics of the genus Streptomyces which is the most abundant genus in nature. AM consists of three to more mature spores. Colonies can produce a wide variety of pigments responsible for staining SM and AM [36,37]. Actinomycetes are ubiquitous microorganisms that may have existed in normal to extreme ecosystems with acidic or alkaline pH, low or high temperature, high salinity, etc. [38]. A large number of Streptomyces, Nocardia and Micromonospora are grown at temperatures between 25 and 30 °C. Streptomyces sp. JUBM-35-NS-1 and Nocardia sp. JUBM-35-NS-2 exhibit growth at temperatures between 25 and 45 °C and tolerance of up to 8 and 2% NaCl, respectively [12]. Loqman et al. [39] described a new species of Streptomyces named S. thinghirensis which tolerates 7% NaCl with growth occurring between 28 and 42 °C. Marine ecosystems are distinguished from other ecosystems by several characteristics, thus promoting the development of particular microorganisms with particular physiological and cultural characteristics allowing them to produce bioactive secondary metabolites [11]. The results of the cultural and morphological properties obtained allow us to suggest that the CSM21 isolate is affiliated with the genus Streptomyces. However, confirmation

of this identification requires genetic analysis of 16S rDNA.

Screening a collection of actinomycetes for its antagonism to pathogenic bacteria allowed us to select a potential strain affiliated with the genus Streptomyces named CSM21 isolated from sea sand. This strain showed these potentials to produce antibacterial metabolites in liquid and solid fermentation. The recovery of organic waste offers an economic and environmental opportunity, which can reduce the problems of its conventional disposal. For the first time, SS are used as a solid substrate to produce antibacterial metabolites in SSF by actinomycetes. This well-known and available waste can promote the production of new antibacterial molecules. Thus, the use of SSF product rich in antagonist isolate (CSM21), antibacterial metabolites and hydrolytic enzymes in contaminated waste storage areas and in the various waste treatment processes is proposed as one of the biological methods for use in the control of pathogenic bacteria.

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