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# Selective isolation, antimicrobial screening, and phylogenetic diversity of marine actinomycetes derived from the Coast of Bejaia City, Algeria, a polluted and microbiologically unexplored environment

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1        **Selective isolation, antimicrobial screening, and phylogenetic diversity of**  
2        **marine actinomycetes derived from the Coast of Bejaia City, Algeria, a**  
3        **polluted and microbiologically unexplored environment**

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14       **Running title:** Antimicrobial potential of marine Actinobacteria

15  
16  
17

18 **Abstract**

19 **Aims:** The aim of the current study was to evaluate the occurrence of actinomycetes in the Coast of  
20 Bejaia City using different isolation methods, as well as to evaluate their bioactivity.

21 **Methods and Results:** Different selective media and methods were used, leading to the isolation  
22 of 103 actinomycete strains. The number of strains was influenced by isolation procedures and their  
23 interactions based on a three-way ANOVA and a post-hoc Tukey test, which revealed that using  
24 M2 medium, dilution of samples followed by moderate heat treatment, and sampling sediment at  
25 10-20 m yielded the highest numbers of actinomycetes. The isolates were screened for their  
26 antimicrobial activity against human pathogenic microorganisms using agar and well diffusion  
27 methods. Of all the isolates, ten displayed activity against at least one Gram-positive bacterium, of  
28 which P21 showed the highest activity against *Staphylococcus aureus*, Methicillin-resistant *S.*  
29 *aureus*, and *Bacillus subtilis*, with a diameter of 32, 28 and 25 mm, respectively. Subsequently,  
30 active isolates were assigned to *Streptomyces* spp. and *Nocardiopsis* spp. based on 16S rRNA gene  
31 sequencing, including a putative new *Streptomyces* species (S3). The phenotypic characteristics of  
32 the P21 strain were determined, and interesting enzymatic capacities were shown.

33 **Conclusion:** The recovery of actinomycetes along the coast of Bejaia City was influenced by the  
34 isolation procedure. Ten strains displayed interesting antibacterial activity against Gram-positive  
35 bacteria, of which the P21 strain was selected as the most active strain.

36 **Significance and Impact of the Study:** This work provides a new insight into the occurrence of  
37 actinobacteria in the Coast of Bejaia using selective isolation. It suggests also that polluted  
38 environments such as Bejaia Bay could provide access to interesting actinomycetes as sources of  
39 antibiotic leads.

40 **Keywords:** Marine actinomycetes, Polluted marine environment, Selective isolation,  
41 Antimicrobial potential, Phylogenetic diversity.

## 42 **Introduction**

43 For decades, severe infections caused by resistant bacteria have been responsible for a major  
44 global healthcare problem, requiring longer and more complex treatments that are significantly  
45 more expensive to diagnose and treat (Alanis 2005). Moreover, infections generated by Methicillin-  
46 resistant *Staphylococcus aureus* (MRSA) have emerged as a widespread cause of community  
47 infections, which is due to their ability to develop resistance (Chambers and DeLeo 2009; Pasberg-  
48 Gauhl 2014; Barbosa *et al.* 2020). This emerging problem has been observed in many countries,  
49 including Algeria (Mairi *et al.* 2020). Thus, the search for new antibiotics remains a worldwide  
50 research priority.

51 Marine environments present a vast source of new bioactive natural products, but they are  
52 still either unexplored or underexplored (Bhatnagar and Kim 2010, Gogineni and Hamann 2018).  
53 Those environments are characterized by particular conditions such as high salinity, pressure, low  
54 temperatures, and variable oxygen concentrations (Bullet *al.* 2000). Hence, marine organisms  
55 require distinctive metabolic capabilities to adapt and thrive in these environments (Bose *et al.*  
56 2015). An increasing number of studies have been conducted on marine actinobacteria as important  
57 producers of bioactive compounds in recent years (Maldonado *et al.* 2005; Bull and Stach 2007;  
58 Manivasagan *et al.* 2014; Kamjam *et al.* 2017; Barbosa *et al.* 2020; Ibrahimi *et al.* 2020; Goel *et*  
59 *al.* 2021). Interestingly, microorganisms living in unusual growth conditions, such as polluted  
60 environments, could be a significant source of interesting bacteria. Even though bacteria isolated  
61 from polluted environments have been instead investigated for their enzymatic activity their  
62 secondary metabolite production remains poorly understood.

63 Actinomycetes have long been recognized as a major source of natural products,  
64 particularly antibiotics (Bérdy 2005; Kurtböke 2012; Jose and Jha 2016; Jose *et al.* 2021). Recently,  
65 rare actinobacteria have attracted more attention and are defined as strains other than the commonly  
66 known *Streptomyces* or strains with less frequency of isolation under conventional methods (Baltz

2006). They have taken an increasingly important position in the production of compounds of biotechnological interest. They have yielded a wide range of molecules with medicinal, industrial, or agricultural uses (Dhakal *et al.* 2017; Ding *et al.* 2019; Mast and Stegmann 2019), notably those isolated from rare actinobacteria derived from extreme environments (Hui *et al.* 2021).

Although the isolation of rare actinomycetes in the marine environment is relatively low due to the need for appropriate isolation procedures and the application of different selection conditions, they are difficult to cultivate and maintain under conventional conditions (Subramani and Aalbersberg 2013; Azman *et al.* 2015). Therefore, a high number of them are "viable but non-culturable" (VBNC) strains (Bernard *et al.* 2000). Recently, several strategies have been developed that have promoted the growth of some previously VBNC species, such as mimicking the natural environment in terms of pH, oxygen gradient, nutrient composition, etc. (Dhakal *et al.* 2017).

Microorganisms derived from extreme biomes have recently attracted scientists' interest since they could house structurally unique natural products that are still unexploited. For that, the current study focuses on the selective isolation of actinomycetes from an unexplored marine environment along the coastline of Bejaia City (Algeria) and screening of their antimicrobial potential against a set of human pathogenic microorganisms.

## **Materials and methods**

### **Sampling sites**

The sampling was performed in the summer of 2017 from different sites (S1, S2 and S3) in the coastal area of Bejaia City, Algeria (Figure 1), and at different depths that ranged from 0.5 to 20 m with the help of a diver. S1 samples were collected at 10-20 m of depth, S2 at 1-2 m, and S3 samples were collected at 0-0.5 m of depth. The sampling area is polluted by different sources, mainly by the petroleum port (S1), industrial wastes (S2), and domestic discharges (S3). Sediment samples (S1) and water samples (S2 and S3) were taken from different points and were collected in sterile glass bottles, then transferred in a cool box to the laboratory for bacteria isolation.

## 92 **Selective isolation of actinomycetes isolates**

93 To isolate rare genera of actinomycetes, one should avoid the growth of non-actinomycetes  
94 bacteria that affect the isolation of actinomycetes known for their slow-growing. Therefore,  
95 different selective isolation media were used as previously recommended in several studies, such  
96 as: ISP2 (Claverías *et al.* 2015), Starch-Nitrate (Ghanem *et al.* 2000), A4 (Gontang *et al.* 2007),  
97 M2 (Jensen *et al.* 1991), and Chitin-agar (Pisano *et al.* 1986). In addition, the isolation media were  
98 combined with different isolation methods that are detailed in Table 1. The isolation media were  
99 inoculated with 100 µL aliquots of each sample previously pre-treated with methods 1, 2, and 3, as  
100 well as method 4, which consisted of the inoculation of untreated samples onto media with added  
101 antimicrobial compounds. All inoculated plates were incubated at 28 °C until up to 5 months (with  
102 wetted paper towels under the plates to avoid desiccation after 2 months) and examined daily, and  
103 all actinomycetes colonies were streaked into new plates containing the same media of isolation  
104 until a pure culture was obtained. It should be noted that all isolation media were adjusted to pH  
105  $7.2 \pm 0.2$  and prepared with 100% of natural seawater.

## 106 **Preliminary screening of antimicrobial activity**

107 Preliminary screening for antimicrobial production was estimated by the conventional agar  
108 diffusion assay described by Shomura *et al.* (1979) and Engelhardt *et al.* (2010). Briefly, agar discs  
109 (8 mm in diameter) recovered from the actinomycetes culture (M2 medium, 14 days of incubation  
110 at 28 °C) were placed on the surface of Muller Hinton agar pre-seeded with X µl of the target  
111 microorganism (Table 2) at  $10^7$  UFC/mL. Plates were kept for 2 h at 4 °C for good diffusion of  
112 the active compounds. Activity was evaluated by measuring the diameters of the inhibition zones  
113 around the agar disc after 24 h of incubation at 37 °C. The M2 medium was composed of 10 g/L  
114 of starch, 1 g/L of casein bovine milk, 18 g/L of microbiological agar (Jensen *et al.* 1991), and 1

115 mL (per liter of final medium) of trace salt solution prepared according to (Shirling and Gottlieb  
116 1966). All experiments were performed three times independently.

### 117 **Secondary screening of antimicrobial activity**

118 The previously selected isolates were screened a second time by the well diffusion method  
119 to improve the production of antibacterial substances in the broth medium using the supernatant of  
120 the culture prepared on M2 medium (Patil *et al.* 2010). Positive activity was estimated by the  
121 inhibition zone around the culture supernatant well (8 mm in diameter) of actinomycetes isolates.  
122 All experiments were performed three times independently and the results were shown as means  
123 with the standard error as error bars.

### 124 **Molecular identification and phylogenetic analyses of the active isolates**

125 All active isolates were cultured on M2 medium for 14 days at 28 °C. Genomic DNA was  
126 extracted from the isolates using the Wizard® Genomic DNA Purification Kit (Promega, USA)  
127 according to the manufacturer's instructions. PCR and sequencing were performed as previously  
128 described by (Fagervold *et al.* 2013), using universal primers recommended for bacteria 27F mod:  
129 5'AGRGTTCGATCMTGGCTCAG 3' and 1492R mod: 5'TACGGYTACCTTGTTAYGACTT  
130 3'. PCR products were purified using a purification kit (Promega, USA) and sequenced using the  
131 Sanger-dideoxy-termination method on the Bio2Mar platform with an automated sequencer  
132 (3730XL). The 16S rRNA sequences have been deposited in the GenBank database under accession  
133 numbers that are given in parentheses in Figure 5.

134 The 16S rRNA gene sequences were submitted to the EZBiocloud database for  
135 identification (Yoon *et al.* 2017), available at: <https://www.ezbiocloud.net>. The sequences were  
136 imported into the SILVA\_138.1\_SSUref\_NR99\_12\_06\_20\_opt.arb database and aligned using the  
137 arb fast aligner tool. Sequence positions (662 total), excluding ambiguous and missing, and present  
138 in a majority of sequences, were exported and used in phylogenetic reconstruction. Subsequently,

139 a phylogenetic tree was constructed using MEGA X software (Kumar *et al.* 2018) by the Neighbor-  
140 Joining (NJ) method using the p-distance model (Felsenstein 1985), and the robustness of the tree  
141 was estimated by 1000 bootstrap random resampling of the original dataset.

#### 142 **Phenotypic characterization of the selected strain (P21)**

143 The micro-morphology of the selected strain (P21) was established by light microscopy to  
144 visualize mycelia organization and sporulation. The strain was grown on M2 medium at 28 °C for  
145 two weeks before being observed under an optical microscope (Euromex). Furthermore,  
146 morphological and physiological characteristics were carried out as described in the *International*  
147 *Streptomyces Project* (ISP) recommendations by Shirling and Gottlieb in 1966, as well as on three  
148 "no ISP" media, including nutrient agar, Bennet medium (Yokota *et al.* 1993) and GYEA medium  
149 (Athalye *et al.* 1981). Melanoid pigment production was determined on tyrosine agar medium  
150 (ISP7) recommended by (Shirling and Gottlieb 1966). In addition, the utilization of carbohydrates  
151 was tested on a minimal medium (ISP9) containing different carbon sources (D-glucose, sucrose,  
152 xylose, inositol, mannitol, D-fructose, rhamnose, and cellulose) at 1% concentration. Basal medium  
153 without a carbon source served as a control. Moreover, the conditions of growth, such as the effect  
154 of pH (4-14), temperature of growth (4-45 °C), and NaCl tolerance were evaluated (Aouiche *et al.*  
155 2012). Resistance to different antibiotics was carried out using the disc diffusion test as  
156 recommended by the Clinical and Laboratory Standards Institute (CLSI, 2016). The tested  
157 antibiotics were: Penicillin G (6 µg), Ciprofloxacin (5 µg), Clindamycin (2 µg), Tetracycline (30  
158 µg), Gentamicin (10 µg), and Chloramphenicol (30 µg). Other tests on the selected strain were  
159 carried out according to (Gordon and Mihm 1956), such as the ability of the strain to decompose  
160 substrates including casein, starch, and gelatin, and resistance to lysozyme at 0.005% (W/V), while  
161 the tyrosine assimilation was performed according (Kurup and Fink 1975).

#### 162 **Statistical analyses**



163 All experiments were carried out in triplicate. Firstly, the selective isolation data were  
164 analyzed using different statistical tests. The normality of the distribution in the studied groups was  
165 confirmed by the Shapiro-Wilk test. To find out the effect of the isolation methods, the used media,  
166 and the sampling depths on the number of actinomycetes isolated (note, we did not correct for the  
167 dilution in Method 1), isolation data were compared by three-way ANOVA (Analysis Of Variance)  
168 (3WA), followed by the post hoc Tukey test without correction for multiple comparisons. The test  
169 was considered statistically significant if ( $p < 0.05$ ) and was conducted using XLSTAT software  
170 (version 2018.1).

171 Secondly, the preliminary antimicrobial activity screening data were analyzed using  
172 principal component analysis (PCA) and hierarchical cluster analysis (HCA). The statistical  
173 analyses were performed using R software version 4.0.2 ([https://www.r-project.org/https://www.r-](https://www.r-project.org/https://www.r-project.org/)  
174 [project.org/](https://www.r-project.org/)), the RProject for Statistical Computing, with the plugin FactoMineR included in R.  
175 The PCA plot is used to reduce the dimensionality of such datasets, increasing interpretability while  
176 minimizing information loss.

## 177 **Results**

### 178 **Samples collection and isolation of actinomycetes**

179 Three polluted marine sites were sampled at different depths from the coastal areas of Bejaia  
180 City (Algeria), using different methods and media. A total of 103 colonies of presumptive  
181 actinomycetes were observed and isolated. The number of isolates varied with the used methods,  
182 media, and sampling depths. As a result of the selective methods that were used, Method 1 showed  
183 the highest number of isolates with 46, while 12, 20, and 25 isolates were obtained when Methods  
184 2, 3, and 4 were applied (Figure 2A), respectively. Based on the isolation media used, we found  
185 that M2 medium, ISP2 and Chitin-agar medium gave the highest number of isolates with 32, 27  
186 and 22 isolates, respectively. On the other hand, only eleven isolates were obtained with Starch-

187 Nitrate and A4 (Figure 2B). According to the results of sampling depths (Figure 2C), 56 isolates  
188 were obtained at D1 (10-20 m), 33 isolates at D2 (1-2 m), and only 14 isolates were obtained at D3  
189 (0-0.5 m).

190 Furthermore, the isolation variables (methods, media, and depths) showed a significant  
191 effect on the number of actinomycetes isolated ( $p<0.05$ ) based on the three-way ANOVA test. The  
192 M2 medium, Method 1 and D1 affected were the best combination for the recovery of  
193 Actinomycetes ( $p<0.05$ ). Moreover, the post hoc Tukey test revealed a significant interaction  
194 between the different depths/isolation methods ( $p<0.05$ ), but no significant interaction was found  
195 between the different depths/isolation media, or the isolation methods/isolation media.

#### 196 **Preliminary screening assay**

197 The isolates were screened for their antimicrobial activity by the agar diffusion method  
198 against various pathogenic microorganisms (Gram-positive and Gram-negative bacteria, and a  
199 yeast). The results showed that 10 out of 103 isolates exhibited antibacterial activity against at least  
200 one of the pathogenic tested strains, particularly against Gram-positive bacteria including *S. aureus*,  
201 MRSA, and *B. subtilis*, while no activity was detected against the yeast and Gram-negative bacteria.

202 The results of preliminary antimicrobial screening were subjected to a PCA analysis, which  
203 was performed on the active isolates (10 isolates) using nine variables. Results showed that 100%  
204 of the total variation was distributed in three dimensions; the horizontal axis explained 65.64% of  
205 the total variance, and the vertical explained 32.65% (Figure 3A). The results demonstrated  
206 substantial differences between active strains. Moreover, it was found that only three variables  
207 contributed to the construction of this PCA, which are MRSA, *S. aureus*, and *B. subtilis*. Figure 3B  
208 presents the projection of individuals (actinomycetes isolates). Following that, the PCA data were  
209 used for Hierarchical Cluster Analysis (HCA). Therefore, the isolates were grouped into four  
210 clusters of active actinomycetes (Figure 3C) identified by their specific antimicrobial activity

211 against targeted bacteria, according to the results of HCA analysis. The first cluster was presented  
212 by two isolates (S3 and S5) that showed activity only against *B. subtilis* with a 15 mm inhibition  
213 zone, while no activity was shown against MRSA and *S. aureus*. The isolates P19, P24, E, and C15  
214 formed the second cluster, which showed significant activity against MRSA and *S. aureus* that  
215 varied between 11-22 mm, but no activity was observed against *B. subtilis*. The third cluster was  
216 presented by three isolates, S1, MP1m, and S4 that showed relatively similar activities against  
217 MRSA, *S. aureus*, and *B. subtilis*, with an inhibition zone that varied from 11-22 mm. The fourth  
218 cluster was presented by the strongest active isolate, P21, which showed 28, 32, and 25 mm of the  
219 inhibition zones against MRSA, *S. aureus*, and *B. subtilis*, respectively. Thus, cluster 4 is the most  
220 active cluster ( $p<0.05$ ) based on inhibition diameter size with the highest antibacterial mean against  
221 MRSA and *S. aureus*.

## 222 **Secondary screening**

223 A secondary screening of the previously selected active actinomycetes was performed on  
224 M2 medium by the well diffusion method. Except for two isolates (S1 and S3), all active isolates  
225 showed positive activity (Figure 4). The P21 strain was found to be the most active isolate, with  
226 inhibition zones of 25, 33, and 27 mm against MRSA, *S. aureus*, and *B. subtilis*, respectively. The  
227 isolates C15, E, S4 and S5 showed activity against MRSA and *S. aureus* with inhibition zones that  
228 varied between 17-26 mm. The P24 isolate showed an inhibition zone of 9 mm against MRSA. The  
229 MP1m isolate showed 14, 22, and 8 mm of inhibition zones against MRSA, *S. aureus*, and *B.*  
230 *aureus*, respectively, and the P19 strain showed 25 and 17 mm against MRSA and *B. subtilis*.  
231 However, no activity was recorded against Gram-negative bacteria or yeast.

## 232 **Molecular identification and phylogenetic analyses of the active isolates**

233 The 16S rRNA sequences of active isolates were identified using the EZBiocloud database.  
234 Isolates were found to belong to the Actinobacteria phylum and were grouped into two genera:

235 *Streptomyces* (8 out of 10), and *Nocardiopsis* (2 out of 10), with similarity percentages to known  
236 stains ranging from 98 to 100% (Table S1). Isolates P21 and S5 belonging to the *Nocardiopsis*  
237 genus showed 100% similarity to *Nocardiopsis halotolerans* DSM 44410(T) and *Nocardiopsis*  
238 *aegyptia* DSM 44442(T), respectively. Two isolates, C15 and P19, were closely similar to  
239 *Streptomyces xinghaiensis* S187(T) with 99.78% and 99.71% of similarity, respectively. The P24  
240 isolate had a 99.79% similarity with *Streptomyces albidoflavus* DSM 40455(T). The S1 isolate  
241 showed the highest similarity (99.76%) to *Streptomyces hydrogenans* NBRC 13475(T). The S3  
242 isolate was the most closely (98.44%) related strain to *Streptomyces rimosus subsp. rimosus* ATCC  
243 10970(T), which could possibly indicate the presence of a new species within the *Streptomyces*  
244 genus. Moreover, the S4 isolate showed 99.79% similarity to *Streptomyces albogriseolus* NRRL  
245 B-1305(T). Both E and Mp1m isolates were similar to *Streptomyces griseoflavus* LMG 19344(T)  
246 with 99.62% and 99.61% of identity, respectively.

247 A phylogenetic tree based on the 16 rRNA gene sequences (Figure 5) was constructed using  
248 the neighbor-joining method (Saitou and Nei 1987) using *Micrococcus luteus* NCTC 26650 as an  
249 outgroup. The P21 strain formed a monophyletic line with the S5 strain supported by 100% of the  
250 bootstrap replicates. The following strains: C15, E, Mp1m, S4, P19, P24, S3, and S1 appeared in  
251 one cluster as closest neighbors.

### 252 **Phenotypic characterization of a selected strain (P21)**

253 The observation of the P21 strain under an optical microscope showed that it has a  
254 branching substrate mycelium and produces short chains of spores (Figure 6A). On the other hand,  
255 Figure 6B shows the aerial mycelium, which has the typical zig-zag form of *Nocardiopsis* strains  
256 that are fragmented in older cultures. The cultural characteristics of the selected strain are presented  
257 in Table S2. The P21 strain was growing well on all media except nutrient agar, where poor growth  
258 was observed. Differences in the growth rates were observed, and the color of the substrate  
259 mycelium varied from brown-red to white. The production of soluble pigments was observed in

260 some media (ISP2, ISP5, M2, and Bennet) but not in others (ISP3, ISP4, and Nutrient Agar).  
261 Generally, the colors of the aerial mycelium were not the same as those of the substrate mycelium.  
262 Table S3 lists the physiological and biochemical characteristics of the P21 isolate. The selected  
263 strain was not able to assimilate the carbon sources tested. However, it showed good growth when  
264 the medium was supplemented with 10% of sodium chloride, but poor growth was observed at 3-  
265 7% and no growth was found at 0% and 15-30% of NaCl. The optimum range of pH growth was  
266 found between 7 and 10. Moderate growth was observed at pH 3-5, while no growth was observed  
267 at pH 12-14. Moreover, the temperature ranges for growth were between 28 and 40 °C, with an  
268 optimum at 28 °C. The P21 strain was found to be able to degrade a variety of substrates, including  
269 starch, tyrosine, gelatin, and casein from bovine milk. Furthermore, based on the susceptibility test  
270 results, it was found that the P21 strain was resistant to Penicillin G and Clindamycin, but sensitive  
271 to Ciprofloxacin, Tetracycline, Gentamicin, and Chloramphenicol.

## 272 **Discussion**

273 Marine environments are unexplored in Algeria in terms of their microbiota; therefore, the  
274 aim of our study was to isolate actinomycetes from those environments. A total of 103  
275 actinomycetes isolates were obtained from the Coast of Bejaia City using different methods and  
276 media. These isolates were screened for their antimicrobial activity, and ten isolates showed  
277 activity against at least one Gram-positive bacterium (*S. aureus*, MRSA, and *B. subtilis*), while no  
278 activity was found against Gram-negative bacteria or the yeast tested.

279 Different selective methods and media were used in this study to promote the growth of rare  
280 genera of actinobacteria and avoid the growth of terrestrial actinomycetes while reducing  
281 undesirable flora occurring on the isolation plates, as have been reported by others (Pisano *et al.*  
282 1986; Undabarrena *et al.* 2016; Kamjam *et al.* 2017). The application of pretreatments or the  
283 presence of antimicrobial substances influences the number of actinomycetes isolates (Bull and  
284 Stach 2007). In the present study, Method 1 proved to be among the most effective methods for

285 isolating actinomycetes, which is consistent with the observation of Pisano *et al.* (1987) that  
286 recorded that heat pretreatment of either 55 °C or 100 °C is a suitable alternative to the higher  
287 temperature for actinomycetes isolation, the fact that we did not correct the numbers for the initial  
288 dilution makes this result more notable and could also suggest that dilution of inhibiting substances  
289 in this highly polluted samples could have also be in play. The low (103) number of total strains in  
290 540 petri dishes supports the possibility of negative effects of the pollution on the actinomycete  
291 community.

292         Using a filter membrane (Method 3) or adding antimicrobial substances to the isolation  
293 media (Method 4) have been previously reported to be the best methods for actinomycetes isolation  
294 from water and sediments according to (Hirsch and Christensen 1983) and (Bredholdt *et al.* 2007),  
295 but in our case, these methods (3 and 4) gave fewer isolates compared to Method 1. Even though  
296 Bredholt *et al.* (2008) found that heating and exposure of samples to phenol increased the number  
297 of mycelium-forming actinomycetes, this method (2) yielded the lowest number of isolates. Based  
298 on the isolation media used, the M2, ISP2 and Chitin-agar seem to be the best media for isolating  
299 actinomycetes from marine environments. Nevertheless, other studies revealed that low-nutrient  
300 media were more efficient than rich-nutrient media for the isolation of rare marine actinobacteria  
301 (Jensen *et al.* 2005; Maldonado *et al.* 2005). Furthermore, the sampling depth influences the  
302 number of actinomycetes isolated. It was therefore found that the number of isolates increased  
303 proportionally with the depth, and the highest number of isolates was obtained at D1 (10-20 m). A  
304 similar observation was reported previously by (Jensen *et al.* 1991). Nonetheless, based on the  
305 statistical significance of 3WA and Tukey's post hoc test, the best procedure that gave the highest  
306 number of actinomycetes isolates involves using the M2 medium, Method 1, and sampling at 10-  
307 20 m (D1) of depth. According to these findings, we suggest that the differences in the responses  
308 to the isolation conditions between our study and other studies are very likely due to the level of  
309 pollution in the isolation area that affects the abundance of actinobacteria strains. Moreover, the

310 designated methods to isolate actinomycetes from polluted marine environments including  
311 sediment and water samples are not optimal for such biotopes, and more effects are required to  
312 establish specific isolation methods for such environments.

313         The obtained isolates were screened for their antimicrobial activity against nine human  
314 pathogenic bacteria. Targeting more than one pathogenic microorganism to obtain diverse  
315 bioactive bacteria has previously been supported by (Genilloud *et al.* 2011). Ten isolates were  
316 selected among 103 that showed activity against at least one targeted Gram-positive bacteria.  
317 However, this percentage is relatively low in comparison with the results of Peela *et al.* (2005),  
318 who reported that the majority of marine actinomycetes isolated showed antagonistic potential,  
319 including antibacterial and antifungal activities. The low rate of active strains could have several  
320 possible reasons: 1). Is probably due to the fact that the screening of isolates was based on a specific  
321 activity that could be inappropriate for the isolates' potential, and thus we missed other biological  
322 activities. 2). The isolates might possess biosynthesis gene clusters that encode for bioactive  
323 compounds but are, "silent" or "cryptic", which means they are not expressed under laboratory  
324 conditions. 3). The method or medium used is inappropriate for the production of metabolites. For  
325 these reasons, further investigations are being envisaged. Interestingly, the active isolates only  
326 showed activity against Gram-positive bacteria, suggesting that the isolates have developed  
327 specific marine adaptation mechanisms that likely affect secondary metabolite biosynthetic  
328 pathways.

329         The P21 isolate was shown to be the most active strain against Gram-positive bacteria and  
330 belongs to the *Nocardiopsis* genus, which has been described as a producer of a wide range of  
331 bioactive compounds (Fu *et al.* 2013; Hatem Ibrahim *et al.* 2018; Tatar 2020). Although this genus  
332 has received less attention than *Streptomyces*, which has been extensively investigated (Lazzarini  
333 *et al.* 2000; Takahashi *et al.* 2018). Previous studies have shown the anti-MRSA activity of the  
334 *Nocardiopsis* genus, such as the activity of the angucyclines (nocardiopsistins A-C) isolated from

335 a marine sponge-derived *Nocardiopsis* sp. (Xu *et al.* 2018). The P21 strain does not show any  
336 activity against Gram-negative bacteria even though previous studies have provided the  
337 antagonistic potential of *Nocardiopsis* strains against Gram-negative bacteria, such as  
338 *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* (Rajivgandhi *et al.* 2019), *Enterococcus faecium*  
339 and *Candida albicans* (Engelhardt *et al.* 2010). Hence, this difference is likely due to the specific  
340 activity of the secreted molecules by the P21 strain against Gram-positive bacteria, especially  
341 MRSA (Figure 7).

342 Marine sediments harbor a large diversity of culturable actinobacteria (Bredholdt *et al.*  
343 2007; Gontang *et al.* 2007; Claverías *et al.* 2015). In the current work, the phylogenetic diversity  
344 analysis of the active strains showed that the isolates belonged to two genera (*Streptomyces* and  
345 *Nocardiopsis*), which are widely distributed in marine environments worldwide (Claverías *et al.*  
346 2015; Jose and Jha 2016). The low recovery observed both in number and diversity could be due  
347 to the influence of the contaminants that are present in the isolation region, which can reduce the  
348 biological diversity of actinobacteria. In addition, the results of the phylogenetic analysis showed  
349 that the homology of the sequences with the closely related species ranged from 98 to 100%. In  
350 particular, the S3 isolate showed 98.44% similarity to the closest strain, which is below the 98.65%  
351 threshold for this genus (Kim *et al.* 2014). This isolate can thus be considered a presumptive new  
352 *Streptomyces* species. Moreover, several studies in Algeria have recorded the *Nocardiopsis* genera  
353 from soil samples, such as the desert soil (Zitouni *et al.* 2005; Meklat *et al.* 2011), the salt pan of  
354 the Kenadsa region (located in southwest Algeria) (Messaoudi *et al.* 2019), a salt lake in the  
355 Algerian Sahara (Hadj Rabia-Boukhalfa *et al.* 2017), and from the rhizosphere-associated with  
356 semi-arid soil in the North-East of Algeria (Aouar *et al.* 2012). However, *Nocardiopsis* strains had  
357 never been isolated from marine environments in Algeria.

358 Morphological characteristics, mainly based on the substrate mycelium, the aerial hyphae,  
359 pigment production, spore appearance, and chaining, have been proven to be an effective and



360 widely used reference for the preliminary identification of actinobacteria (Taddei *et al.* 2006). The  
361 P21 isolate showed the typical microscopic appearance of most species of the *Nocardiopsis* genus.  
362 It grew well on several media and showed various cultural characteristics that are related to the  
363 available substrate and growth conditions. The P21 strain was susceptible to four antibiotics and  
364 displayed resistance to two antibiotics tested (Penicillin G and Clindamycin). According to  
365 previous reports, antibiotic resistance may reveal an isolate's ability to produce the antibiotic(s) to  
366 which it is resistant (Basu and Paul 1992; Jain *et al.* 2012). Furthermore, biochemical  
367 characteristics showed the potential of the P21 strain to degrade several substrates, which is in line  
368 with previous reports that described the *Nocardiopsis* genus as a potential source of extracellular  
369 enzymes (Bennur *et al.* 2014). Further experiments should be carried out to confirm the enzymatic  
370 abilities of this strain.

371 In conclusion, the Coast of Bejaia City was an interesting source of bioactive actinobacteria  
372 strains with the possibility of the presence of new *Streptomyces* species. *Nocardiopsis* sp. P21 was  
373 found to be the most active strain, with potential for new antimicrobial therapies to fight MRSA  
374 infections. Further studies are required to understand the biosynthetic pathways involved in the  
375 antibacterial activity of the active strains, including genome mining and metabolomics analyses.

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## 584 **Figure legends**

585 **Figure 1** Map showing the sampling sites of actinomycetes isolates. Sampling spots (**S1**:  
586 36°45'29.6"N5°05'50.3"E, **S2**: 36°44'12.1"N5°04'50.7"E, and **S3**: 36°43'55.2"N5°04'37.9"E) were  
587 indicated. The map was generated with the R package ggmap (Kahle and Wickham 2013) under  
588 the development environment Rstudio 1.4.1 (<https://www.R-project.org/>) coupled with R 4.0.2,  
589 then it was polished with the image-editing tool Paint.net 4.2.14.

590 **Figure 2** Actinomycetes counts in sampling sites collected from the Coast of Bejaia (Alegria)  
591 depending; **(A)** isolation method (1, 2, 3 and 4) , **(B)** isolation media (M2, ISP2, Starch-Nitrate,  
592 and Chitin-agar), **(C)** sampling depth (D1: 10- 20 m, D2: 1-2 m and D3: 0- 0.5 m).

593 **Figure 3** Principal component analysis (PCA) and hierarchical clustering analysis of active  
594 actinomycetes based on antimicrobial activity against 9 human pathogenic bacteria. **(A)** Projection  
595 of the evaluated variables into the two-dimensional space, Dim1 and Dim2. **(B)** Projection of the  
596 active strains into the two-dimensional space, Dim1 and Dim2. **(C)** Hierarchical clustering of active  
597 isolates based on antimicrobial activity. **(D)** Actinomycetes isolates hierarchical grouping in a  
598 three-dimensional space.

599 **Figure 4** Average inhibition zones (mm) of antimicrobial activity of active actinomycetes isolates  
600 against targeted microorganisms using the well diffusion method. Error bars indicate  $\pm$  s.e.  
601 averaged over three measurements.

602 **Figure 5** Neighbor Joining-phylogenetic tree showing the evolutionary relationship of bioactive  
603 strains (marked with a triangle) and closely related sequences based on partial 16S rRNA gene

604 sequences. The percentage of replicate trees in which the associated taxa clustered together in the  
605 bootstrap test (1000 replicates) is shown next to the branches (**Felsenstein 1985**). The evolutionary  
606 distances were computed using the *p*-distance method and are in the units of the number of base  
607 differences per site. The accession number is shown in parentheses following the strain name. The  
608 scale bar corresponds to 0.01 substitutions per nucleotide position.

609 **Figure 6** Morphological appearance of P21 strain grown on M2 medium after 2 weeks at 28 °C.  
610 **(A)**: Micro-morphology of Filamentous vegetative mycelia under light microscopy (x40), **(B)**:  
611 macroscopic observation.

612 **Figure 7** Antibacterial activity of the P21 strain against MRSA. Using the agar diffusion method  
613 (A) and well diffusion method (B). The delimited zones on red present the inhibition zones.

#### 614 **Legends for the supporting information**

615 **Table S1** Molecular identification of actives isolates by EZBiocloud database

616 **Table S2** Cultural characteristics of the P21 isolate

617 **Table S3** Physiological and biochemical characteristics of the P21 isolate

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**Table 1** Descriptions of the applied isolation methods

<b>Method</b>	<b>Description</b>
<b>Method 1</b>	Samples were diluted at 1:4 (v/v) with sterile sea water, then vigorously shaken. They were then heated for 6 min at 55 °C (Gontang <i>et al.</i> 2007). A 100 µL volume of the treated sample was inoculated into each media.
<b>Method 2</b>	Samples were heated at 120 °C for 1 h, then exposed to 1.5% phenol for 30 min (Bredholt <i>et al.</i> 2008). A 100 µL volume of the treated sample was inoculated into each media.
<b>Method 3</b>	The agar plates of all media were overlaid with a cellulose ester membrane filter (0.45 µm pore size) and then inoculated with a 100 µL volume of the untreated sample. After 2 weeks, the filters were removed and the plates were reincubated (Hirsch and Christensen 1983).
<b>Method 4</b>	The isolation media were supplemented with filtered (0.2-µm pore size) Nalidixic acid (10 µg/mL), Nystatin (50 µg/mL) (Bredholdt <i>et al.</i> 2007), and Cycloheximide at (50 µg/mL) as recommended by (Bredholt <i>et al.</i> 2008). A 100 µL volume of the untreated sample was inoculated into each media.

**Table 2** Targeted organisms tested to select bioactive Actinomycetes

	<b>Microorganisms tested</b>	<b>References</b>
<b>Gram + Bacteria</b>	Methicillin-resistant <i>Staphylococcus aureus</i> (MRSA)	ATCC 43300
	<i>Staphylococcus aureus</i>	ATCC 25923
	<i>Bacillus subtilis</i>	ATCC 6633
	<i>Enterococcus faecalis</i>	ATCC 10541
<b>Gram – Bacteria</b>	<i>Escherichia coli</i>	ATCC 25922
	<i>Pseudomonas aeruginosa</i>	ATCC 27853
	<i>Salmonella typhi</i>	ATCC 6539
	<i>Klebsiella oxytoca</i> (Flugge)	ATCC 13182
<b>Yeast</b>	<i>Candida albicans</i>	ATCC 10231

ATCC: American Type Culture Collection.

Figure 1

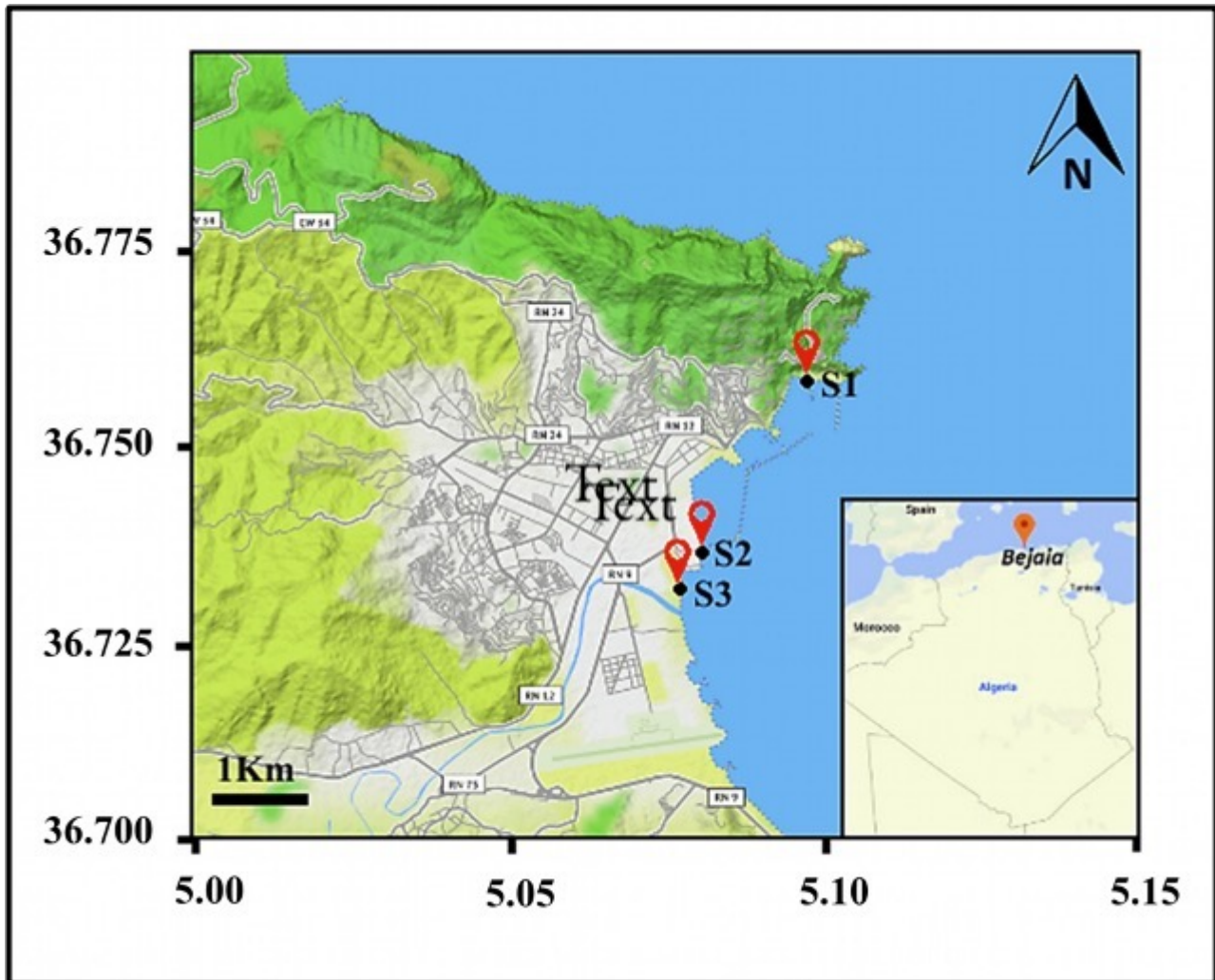


Figure 2

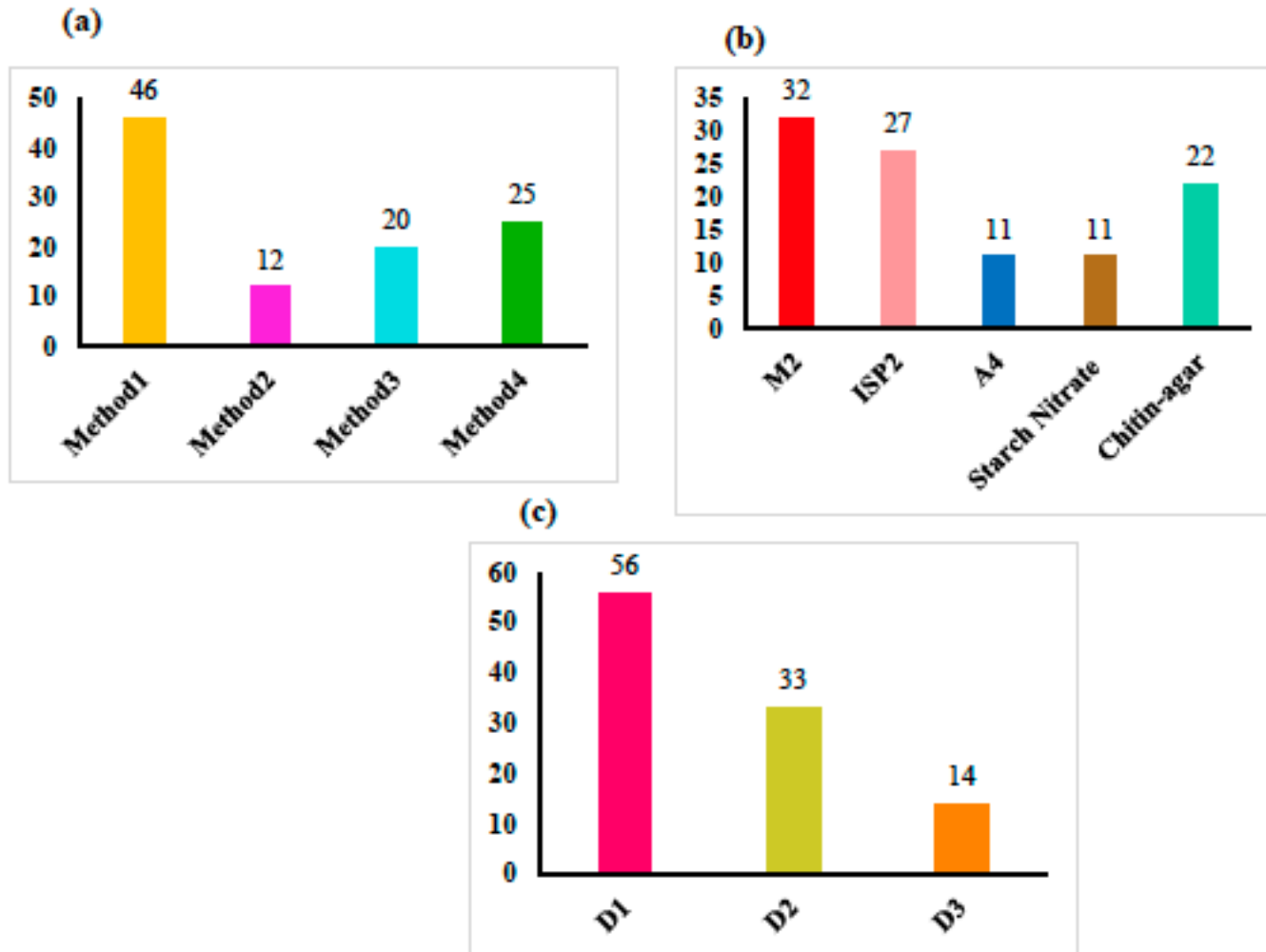


Figure 3

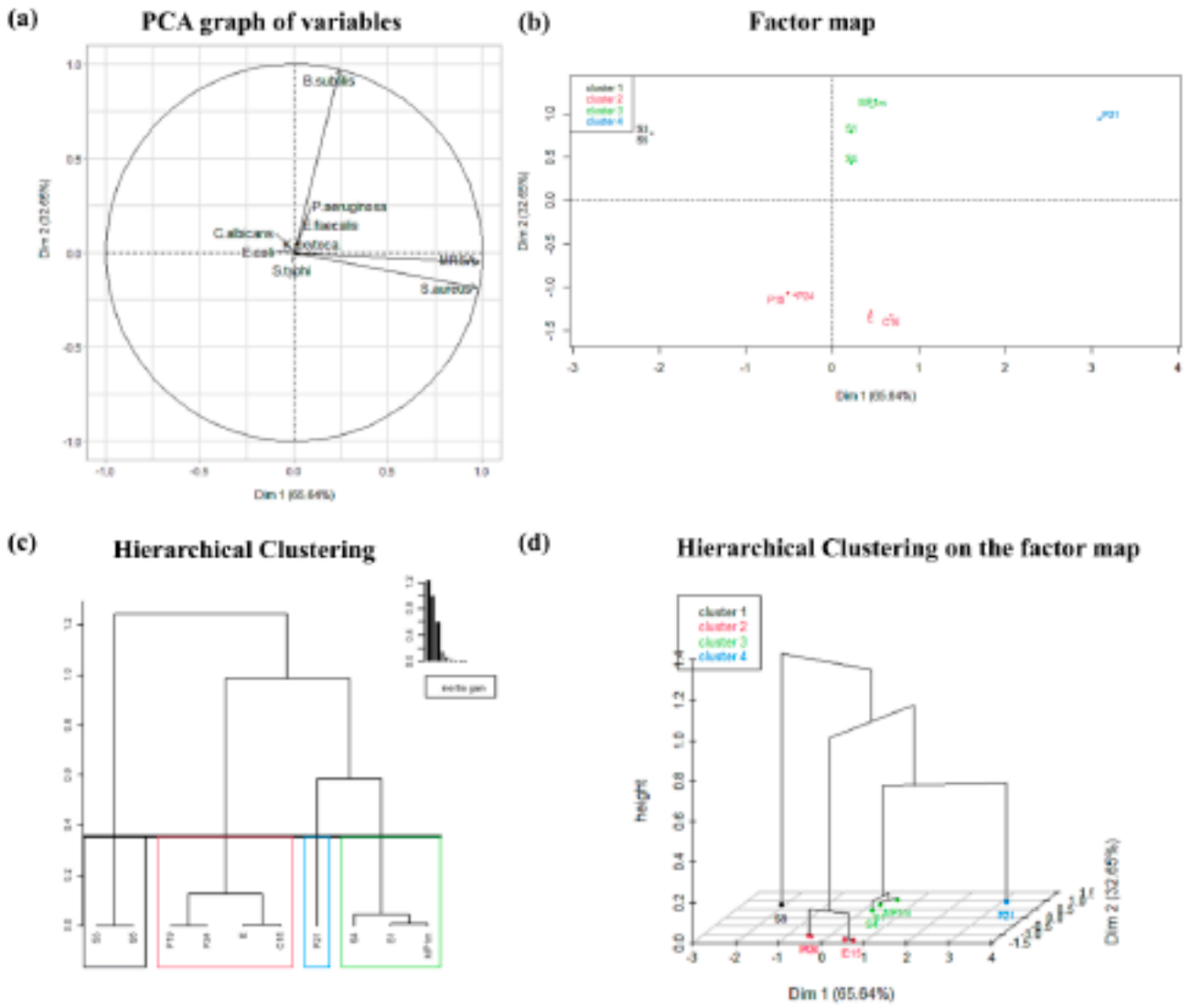




Figure 4

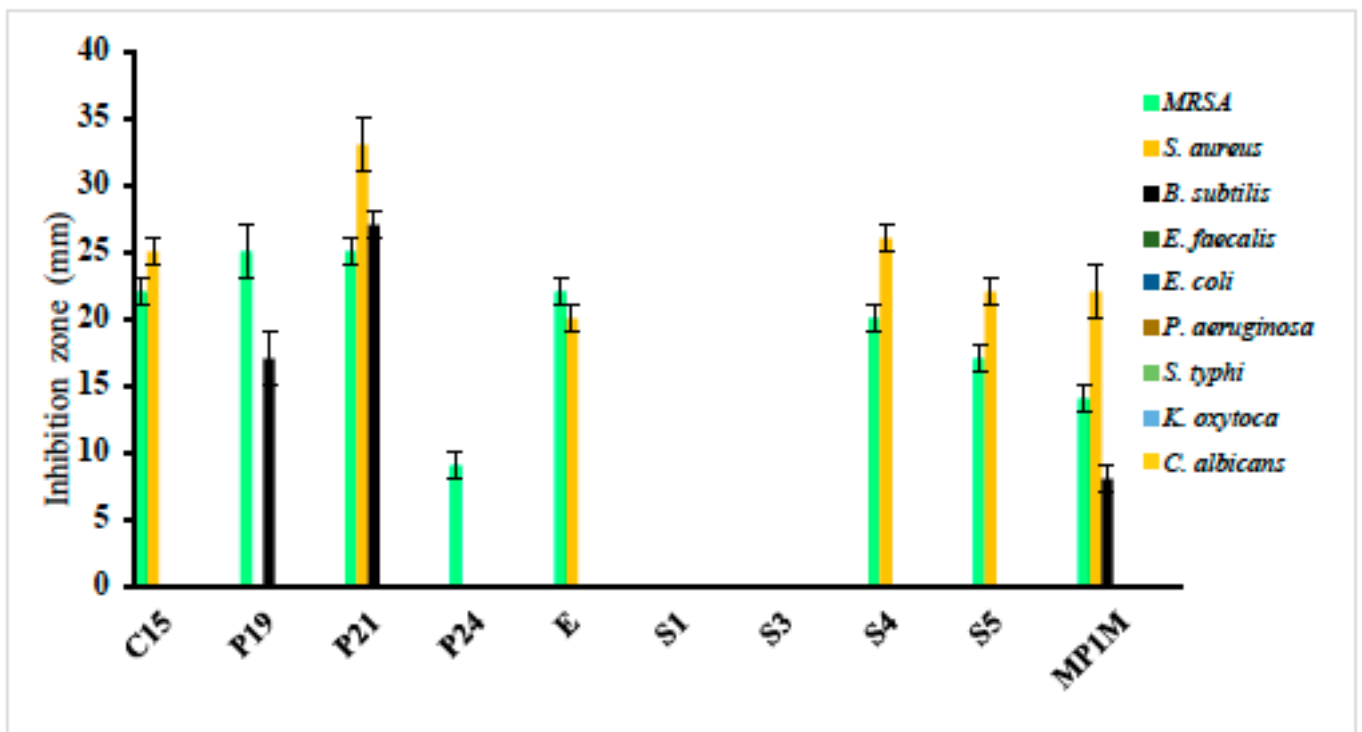


Figure 5

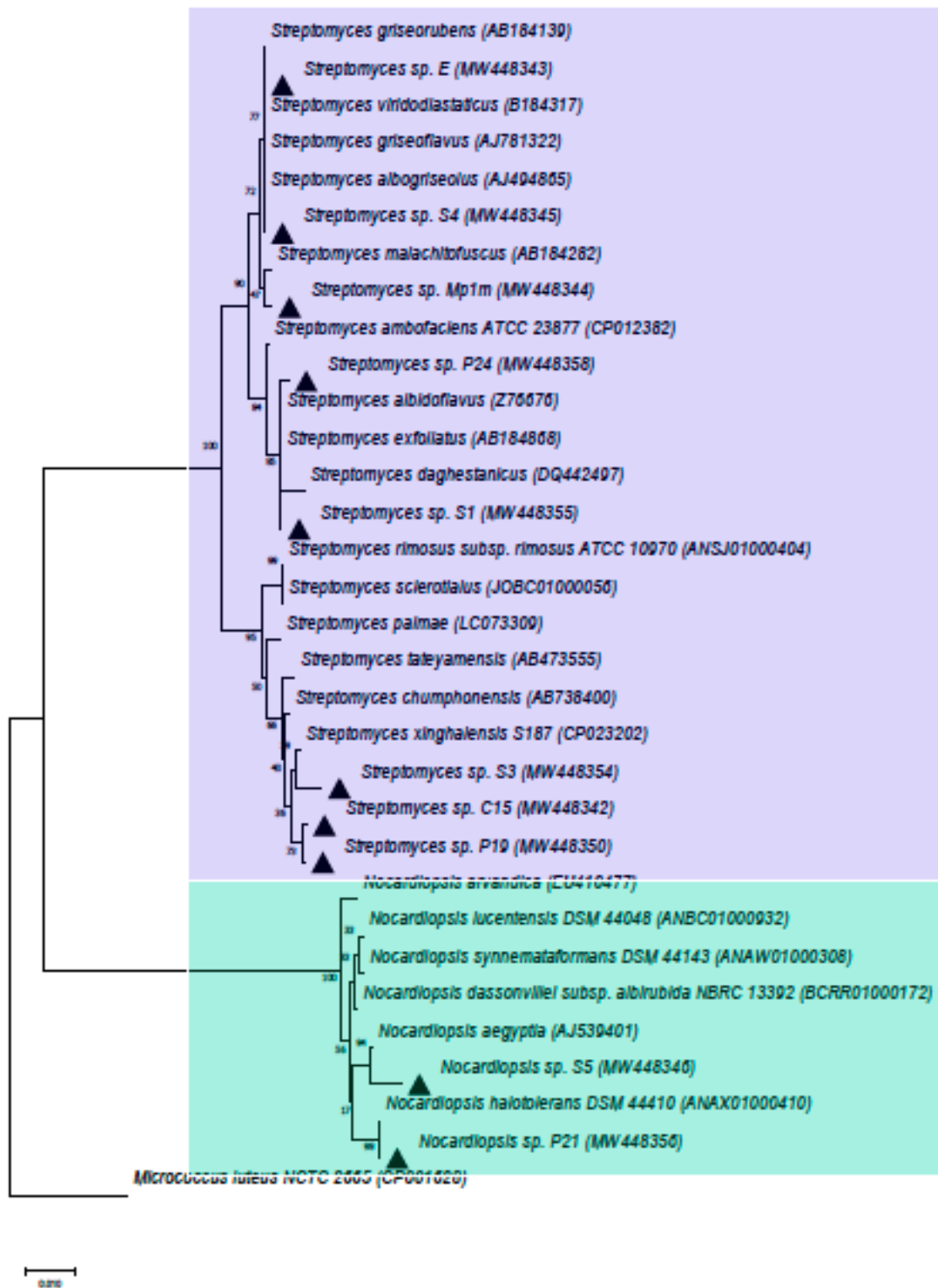


Figure 6

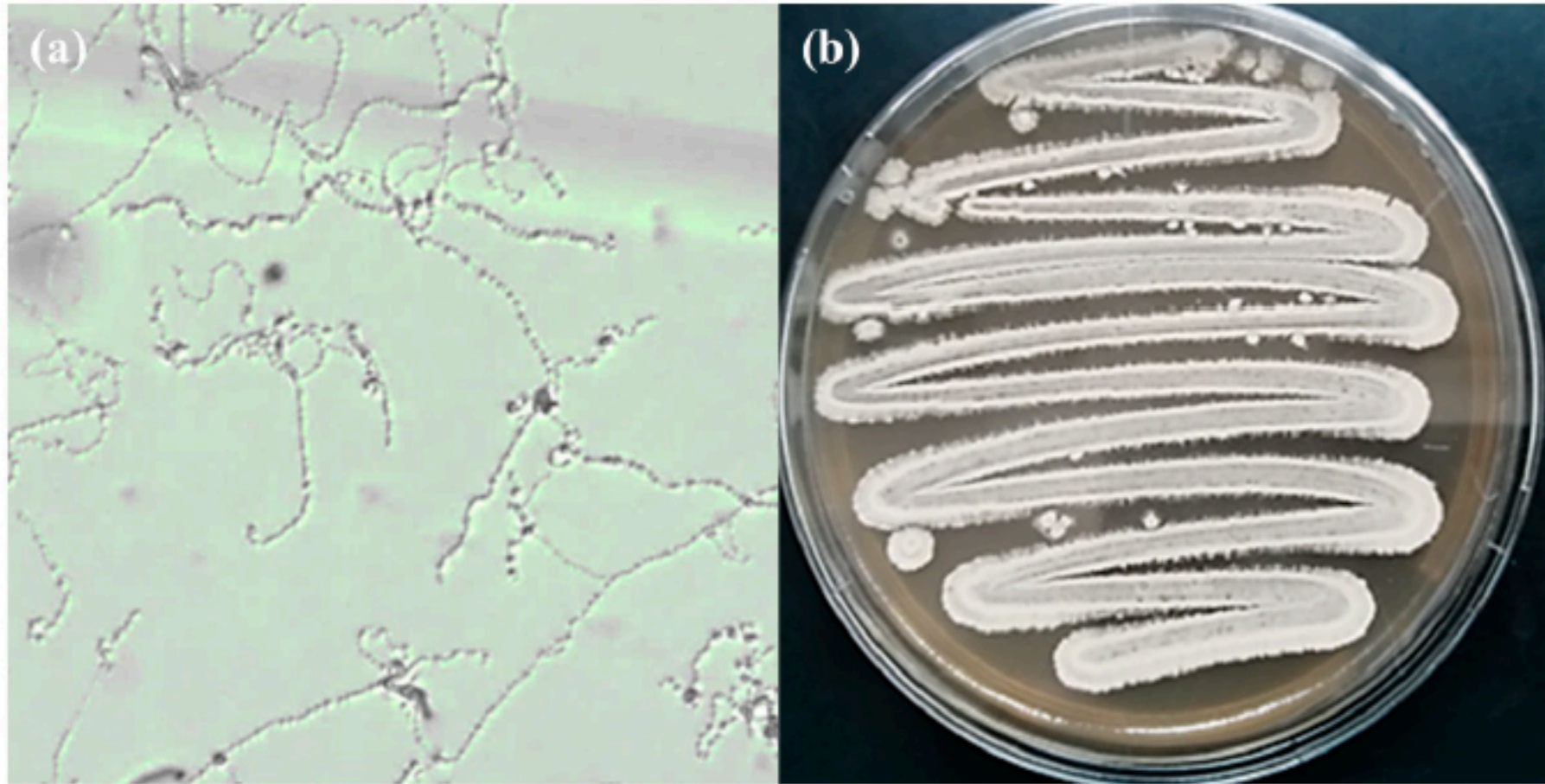


Figure 7

