

Harnessing Biological Activities in Soil-Bacillus Strains to Promote the Discovery of New Bioactive Compounds

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Abstract The biological activities of bacteria of the genus *Bacillus* (MN6, MN7, MN12, MN14, MN17, and MN26) isolated from the soils at Brazzaville were explored. The capacity of six strains to produce hydrolytic enzymes, in particular: proteases, lipases, amylases, cellulases was evaluated. The antibacterial activity of each strain of *Bacillus* for three pathogenic strains (*Escherichia coli* MN40, *Salmonella typhimurium* MN42, *Pseudomonas aeruginosa* MN41) was also tested. The methods of petri dishes containing the substrate relating to the search for each hydrolytic activity were used. All *Bacillus* strains grow under these culture conditions. The growth of each strain of *Bacillus* correlated with the production of protease gave values for the optical density respectively between 0.75 ± 0.2 and 0.99 ± 0.1 , while the production of proteolytic enzyme values between 11.1 ± 0.04 and 20 ± 0.11 . Two strains of *Bacillus* MN7 and MN17 produce amylases. The MN17 strain was tested producing lipase. The MN12, MN17, MN26 strains were tested positive for the production of cellulases. The other three strains of *Bacillus* MN6, MN7, MN14 were tested negative for cellulase production. A principal component analysis revealed that all of the strains have antibacterial activity against at least one of the three pathogenic strains indicated. A principal component analysis indicates that the antibacterials produced by the MN6, MN14 and MN17 strains are very effective at the three pathogenic strains with greater efficacy of MN17 on *E. coli* MN40. MN7, MN12 and MN14 produce antibacterials which neutralize the bacteria *Salmonella typhimurium* MN42, *Pseudomonas aeruginosa* MN41. These bacteria isolated from the soil in Brazzaville, produce bioactive compounds of biotechnological interest.

Keywords: exploitation, biological activities, *Bacillus*, soil, bioactives compounds

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1. Introduction

Soil contains a wide variety of organisms, many of which are microorganisms [1]. These organisms are very diverse with numerous trophic, physico-chemical and physical interactions with each other and with the environment [1]. The microbial diversity in soil ecosystems far exceeds that of eukaryotic organisms, these authors point out that a gram of soil can host up to 10 billion microorganisms, probably thousands of different species. However, less than 1% of all microbial species are easily isolated by standard culture methods [2]. Bacteria are an important part of soil microorganisms [3]. Soil bacteria are involved in biogeochemical cycles and this contributes to improving the quality of the soil, so many elements are recycled. Bacteria in their metabolisms

have several activities including the production of hydrolytic enzymes. Many bacteria produce exocellular enzymes, but the genus *Bacillus* is one of the major producers of these enzymes used in industry.

Among these exocellular enzymes, we cite the proteolytic enzymes or proteases with numerous applications in the industry, some authors have illustrated this in their work in particular [4].

Cellulases or cellulolytic enzymes hydrolyze cellulose, Cellulose is the major constituent of the wall of plant cells, is the most abundant biological polymer on earth. *Bacillus* bacteria form cellulases, are used in the papermaking industry, the detergent industry, in animal feed and in the textile industry [5,6,7,8,9].

Amylases, produced by bacteria of the genus *Bacillus* used in the starch industry and in the food industry (bread-making, brewing, etc.), and the textile industry [10,11,12].

Bacillus bacteria are capable of developing antibacterial substances and are one of the most studied prokaryotic taxa due to their importance in biotechnology, medical science, ecology and other branches of biology. These bacteria are ubiquitous in terrestrial ecosystems, soils, freshwater and marine waters [5].

After proteases and amylases, lipases of microbial origin are considered to have the third highest sales volume, up to billions of dollars, which shows the versatility of their applications, which makes them particularly attractive for industrial applications.

Six strains of bacteria of the genus *Bacillus* were isolated from soil samples in Brazzaville, in the Republic of the Congo were identified by their 16S rDNA and their phylogenetic classification was established [13].

In this work, six strains of bacteria of the genus *Bacillus*: *Bacillus cereus* MN6, *Bacillus pumilus* MN7, *Bacillus thuringiensis* MN12, *Bacillus cereus* MN14, *Bacillus* sp. MN17 and *Bacillus subtilis* MN26 isolated from the soil and already identified by their DNA16S, have been exploited by their biological activities. For each of the strains we carried out a demonstration of the production of the following hydrolytic enzymes: proteases, cellulases, amylases and lipases and an evaluation of the production of each enzymatic type. For each of these strains, we also evaluated the production of antibacterial substances.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

2.1.1. *Bacillus* Strains Whose Biological Activity is to Be Tested

Six *Bacillus* strains isolated from the soil in Brazzaville. These strains available in our laboratory are already identified on the molecular level by their 16S DNA [13]. These strains were grown on liquid LB. We carried out the overnight cultures of the suspensions of the *Bacillus* strains during 18 h of incubation at 37°C. The bacterial cultures were centrifuged at 6000 rpm for 15 minutes at 4°C. The supernatants were collected and used to test the biological activities of each strain; their pH varies from 6 to 9.

2.1.2. The Bacterial Strains Used as a Substrate to Test the Antibacterial Activity

2.1.2.1. Characteristics of pathogenic strains

Three strains of pathogenic bacteria, notably *Escherichia coli* MN40, *Pseudomonas aeruginosa* MN41 and *Salmonella typhimurium* MN42 were used.

Table 1. Characteristics of pathogenic strains used in this study.

Pathogenic strains	Origin	Characteristics
<i>Escherichia coli</i> MN40	Urine	Résistant to penicillin and kanamycin
<i>Salmonella typhimurium</i> MN42	Stool	Résistant to meticcillin and, tetracyclin
<i>Pseudomonas aeruginosa</i> MN41	Waste water	Résistant to imipenem and tetracyclin

2.1.2.2. Demonstration of the antibacterial activity of *Bacillus* strains

The method of diffusion by well described by [14,15] was used. Briefly, the culture was revived on LB for the three strains; a colony of each strain is placed on the tanks with distilled water taken using a 10 ml syringe. Subsequently, the prepared bacterial suspensions are placed in the spectrophotometer to measure the optical density (OD) of each culture. The optical density is between 0.08 and 0.1 which corresponds to 1-2 x 10⁸ CFU / ml for bacteria at a wavelength $\lambda = 625$ nm (turbidity equivalent to 0.5 Mc Farland). The pathogenic strains are inoculated on the Mueller Hinton agar dishes, then allowed to cool. After solidification of the dishes, we made the wells with sterile cones. 100 μ l of the supernatant of each strain of *Bacillus* are placed in the wells, then leave in the open air for about 1 hour before being placed in the oven at 37°C for 24 hours [16]. The revelation is made by observing an inhibition zone around the well.

2.2. Evaluation of Antibacterial Activity

To assess antibacterial activity, we measured the diameters of the Mueller Hinton (MH) nutrient agar inhibition zones around the wells for each petri dish and each strain of *Bacillus*. Each pathogenic strain is placed on a petri dish. And each petri dish includes wells and in each well was placed a culture supernatant of each of the *Bacillus* strains [17]. The appearance of an inhibition zone of diameter greater than 2 mm is indicative of an inhibition of the growth of the pathogen by the strains tested [18]

2.3. Activity of Hydrolytic Enzymes

2.3.1. Lipolytic Activity

In this work, lipolytic activity was based on the esterase research approach to the hydrolysis of Tween 20.

2.3.1.1. Preparation of the tween 20 box

Hydrolysis of Tween 20, Principle: Tween-20 is a polysorbic surfactant presenting a fatty acid ester and a long polyoxyethylene chain, in the presence of the esterase there is hydrolysis of the fatty acid ester which manifests itself by the formation of an opaque halo around the colony on a petri dish [19].

Preparation of the culture medium: This test was carried out in LB agar medium supplemented with tween 20. 2 g of LB are weighed and mixed in 100 ml of distilled water. After homogenization, the broth and the tween 20 are autoclave at 121°C for 15 min. The media are allowed to cool to about 45°C and then 10 drops of tween 20 have been added to the media using a sterile pasteur pipette. Finally, the medium is poured into the petri dishes.

2.3.1.2. Demonstration of lipolytic activity (lipolytic test)

A colony of each strain of *Bacillus* is taken from the petri dish and streaked on the tween 20 dish, spread out by forming a streak. The dishes are incubated at 37°C for 24

hours. After incubation in the incubator, the test is positive with the presence of a clear area at the edge of the streak. This zone characterizes the hydrolysis of the ester for the strain studied [20].

2.3.2. Amylolytic activity

Preparation of the starch box and Demonstration of the Amylolytic activity (amylolytic test)

This test was carried out by cultivating the strain on a nutritive agar containing 1% starch. The dishes are incubated in an oven at 50°C for 48 h. After incubation we soaked the box of lugol, and incubated again in the oven for 30 minutes [21,22,23]. The hydrolysis of starch is thus highlighted by the appearance of a clear zone around the colony, on the other hand a negative result results in a brown color around the culture [24].

2.3.3. Cellulolytic Activity

2.3.3.1. Preparation of the cellulose box

We used the methyl cellulose carboxyl (CMC) whose composition per 100 ml of distilled water is as follows: 1g of CMC, 0.2 g of NaCl, 0.1g of NaNO₃, 0.1K₂HPO₄, 0.1KCl, 0,05 g MgSO₄, yeast extract 0.005 g and 1.7 g of Agar. The mixture was then autoclaved at 121°C for 15 min. While the mixture is cooling. After homogeneous mixing, we poured the petri dishes and left until the medium solidified, five wells were made on the dish containing the CMC. 100 µl of samples were added to each well, and the dishes were incubated at 50°C for 24 h [25].

2.3.3.2. Demonstration of Cellulolytic Activity

After incubation, the CMC dishes were flooded with lugol, incubated for 30 min and then discolored with 1 M NaCl. Samples of bacteria with large clearance zones were observed, which explains why our strains are producers of cellulase [24].

2.3.3.3. Evaluation of cellulolytic activity

We have for each strain tested measured the diameter of the clear area around the well. The different values made it possible to compare the different strains for their production of cellulase.

2.3.4. Proteolytic Activity

Skim milk contains casein, in the presence of bacteria which contain enzymes with proteolytic activity, there is hydrolysis of casein, in this medium the hydrolysis is manifested by the presence of a clear zone around the well in which the supernatant has been deposited [26].

2.3.4.1. Preparation of the casein box

We used the PBS at 0.001N and prepare the casein box. Briefly, we weighed 1g of agarose and mix with 100ml of PBS, the mixture was heated until the agarose was completely dissolved, during cooling, to around 50°C, we added casein (at 2% or simply 10 ml of skimmed milk.), then left in the open air. The gel contained in the box solidified, so we obtained the casein box [26].

2.3.4.2. Detection and evaluation of proteolytic activity

We then made wells on the gel in each petri dish. And finally, we deposited in each well, the supernatants

resulting from the centrifugation of each culture of the Bacillus strains to be tested. The boxes were then placed at 37°C in the oven for 18h. Observation of the clear halo around the well reflects the production of the proteolytic enzyme.

3. Results and Discussion

3.1. Proteolytic Activity

Table 2 shows the variability of the optical density (growth) and that of the proteolytic enzyme production of the used Bacillus strains. Here the casein plate was used. This table shows that the optical density varies between 0.75 and 0.99, this indicates that all the strains grow well under the culture conditions used. The enzymatic production (PE) varies from 10 to 20 mm. All the Bacillus strains used are proteolytic.

Table 2. Variation in OD and enzyme production in the different strains of Bacillus identified

Strains	Optical Density (OD)	Enzyme Production (EP)
MN6	0,76 ± 0,1	19,1 ± 0,02
MN7	0,75 ± 0,2	11,1 ± 0,04
MN12	0,77 ± 0,2	11,3 ± 0,1
MN14	0,78 ± 0,2	15 ± 0,02
MN17	0,77 ± 0,1	20 ± 0,1
MN26	0,99 ± 0,1	10 ± 0,02

3.2. Amylolytic Activity

Figure 1 shows the starch hydrolysis activity of two strains MN7 and MN17. Around the culture for each strain we can observe a clear zone reflecting the hydrolysis of starch. In contrast, in remote areas of the crop, the starch remained without being hydrolyzed.

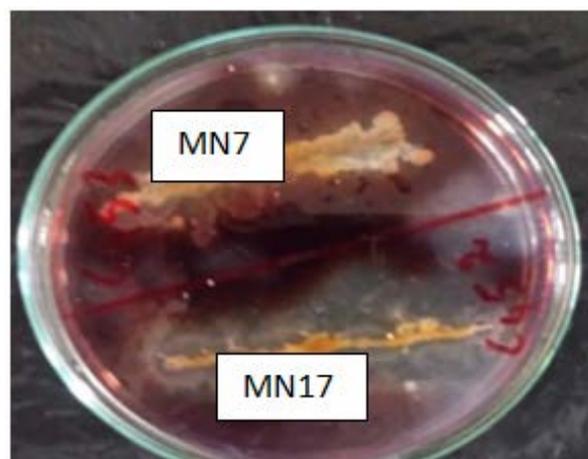


Figure 1. Presence of clear areas around cultures of bacteria MN17 and MN7, reflecting hydrolysis of starch (amylolytic activity).

3.3. Lipolytic Activity

Figure 2 shows the hydrolysis of tween 20 (ester) by the strain MN17 we can observe around the culture a clear zone, implying that the strain MN17 has lipolytic activity.



Figure 2. Hydrolysis of tween 20 by strain MN17, clear area around the culture.

3.4. Cellulolytic Activity

Figure 3 shows the hydrolysis of cellulose by the strains. We observe clear zones (clear halo) around the wells where they were placed on them after culture of the strains MN26, MN17 and MN12, these three strains degraded the translating cellulose. No clear zone was observed around the well of which the supernatant of the strain MN7 was placed, no cellulolytic activity.

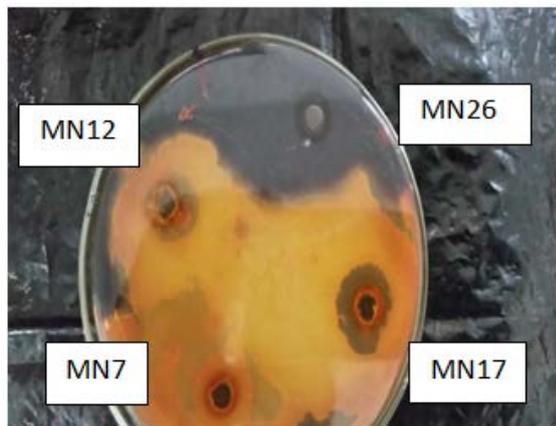
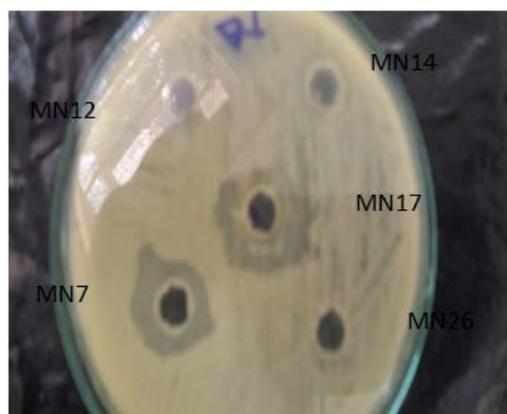
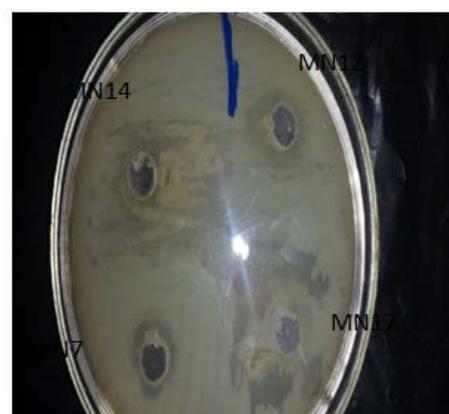


Figure 3. Degradation of cellulose around the wells for strains MN26, MN17 and MN12



(D)



(E)

Figure 5. Antimicrobial activity of Bacillus strains against *E. coli* MN40 (D) and against *Pseudomonas aeruginosa* MN41

The diameters obtained made it possible to evaluate the production of cellulolytic enzyme for different strains. Figure 4 shows the different production profiles of the strains.

In Figure 4 it is clearly shown that three strains MN12, MN17 and MN26 exhibit cellulolytic activity. The MN17 strain with the highest enzyme production, while the MN7 and MN14 strains do not produce a cellulose hydrolyzing enzyme.

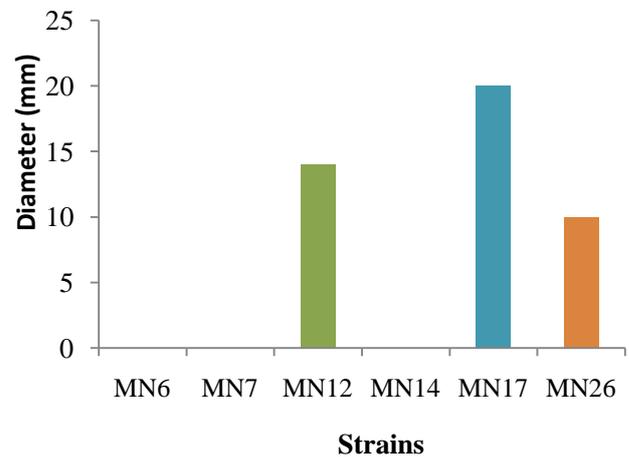


Figure 4. Cellulolytic enzyme production profiles of the bacillus strains used in this study

3.5. Antibacterial Activity

Figure 5 shows the antibacterial activity of the Bacillus strains used; this activity is illustrated by the clear zones around the different wells which have received the supernatants after culture of the different strains. The results of this test are shown in Table 2. The results show a variation in the inhibitory effect depending on the strain of Bacillus and the pathogenic bacteria.

Table 3 presents the different diameters of the six Bacillus strains used, MN6, MN7, MN12, MN14, MN17 and MN26 which are tested on the petri dishes which contain the pathogenic strains. The MN6 and MN26 strains only inhibit the growth of *Salmonella* *typ.* MN42, Strains MN7, MN14 and MN17 inhibits the growth of the three pathogenic bacteria. All strains inhibit the growth of *Salmonella* *typ.* MN42.

Table 3. The evaluation of the activity of Bacillus strains to be tested against the three pathogenic strains

Pathogenic strains	Code	MN6	MN7	MN12	MN14	MN17	MN26
<i>Escherichia coli</i> MN40	<i>Eco</i>	0	15	0	7	19	0
<i>Pseudomonas aeruginosa</i> MN41	<i>Pae</i>	0	13	11	12	8	0
<i>Salmonella typhimurium</i> MN42	<i>Sty</i>	17	10	13	13	8	6

Figure 6 presents correlations of the diameters of the different strains tested as a function of each pathogen. A principal component analysis explains these correlations more clearly. To carry out the principal component analysis (PCA), we have normalized the results of Table 3. We find that the MN7 and MN17 strains have the largest inhibition diameters for the pathogenic strain of *E. coli*. The MN6 and MN26 strains respectively have the minimum and maximum diameters for inhibition of the pathogenic strain *Salmonella typ.*

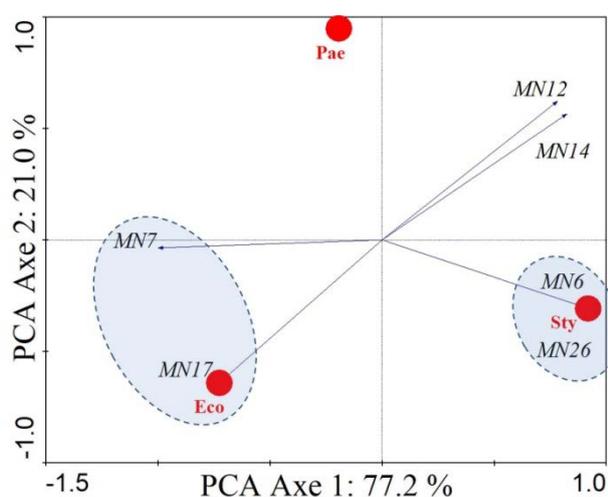


Figure 6. Principal components Analysis (PCA) based on the antibacterial activity of Bacillus strains. Ordination of the strains in the plane defined by axes 1 and 2 of the PCA highlighting the antibacterial activity of the Bacillus strains against the pathogenic strains (*Escherichia coli* MN40, *Pseudomonas aeruginosa* MN41, and *Salmonella typhimurium* MN42)

3.2. Discussion

This work concerns the biological activities of six strains of the Bacillus genus (*MN6*, *MN7*, *MN12*, *MN14*, *MN17* and *MN26*), namely: proteolytic activity, lipolytic activity, amylolytic activity, cellulolytic activity and antibacterial activity against three pathogenic strains. With the increasing presence of multi-resistant bacteria, the search for antibacterial substances is an important challenge. In this work, the exploitation of the biological activities of soil bacteria is a first step towards the discovery of new bioactive molecules of biotechnological interest.

For the proteolytic activity the six strains each have an enzymatic production (varying from 10 to 20 mm while the optical density varies between 0.75 and 0.99) this translates that all the strains grow well under the culture conditions used. results are close to those of [27] who isolated strains that all exhibited proteolytic activity and all growing under working conditions. Our results suggest that each strain has growth and production of different enzymes, as shown in previous work by [28,29].

Figure 1 shows the hydrolysis of starch by two strains *MN7* and *MN17*. For these strains our results are in agreement with those presented by [30] for the study of the enzymatic activities of 23 strains of actinomycetes isolated from the roots of a tomato plant (*Lycopersicon esculentum*) where they have shown that all of their isolates have amylolytic activity. These results are also close to [23], who also highlighted bacterial strains producing amylases.

The strain *MN17* has lipolytic activity shown in Figure 2, the strains *MN12*, *MN26* and *MN7* also show the hydrolysis of the ester, on the other hand, two strains *MN6* and *MN14* do not exhibit the hydrolysis of the ester. These results close to [31] who revealed that the production of lipase by microorganisms could be induced by the addition of tweens. Smibert et al in 2010 also showed that *B.safensis* has the capacity to produce lipase [32].

For the production of cellulolytic enzyme, the different strains of Bacillus, *MN12*, *MN26*, *MN17* exhibited significant cellulolytic activity. These results are similar to those of [33,34] who conducted this work on fungal strains isolated from extreme environments show the capacity of these molds in particular the genus *Trichoderma* to produce the cellulase.

The antibacterial activity of Bacillus strains led us to observe inhibitions of the *MN6*, *MN7*, *MN12*, *MN14*, *MN17* and *MN26* strains. These results are in agreement with those found by [35,36]. And also to those of [37] have isolated actinomycetes from different soil samples and confirm that actinomycetes isolated from these extreme media have a remarkable antimicrobial power compared to their isolated counterparts from normal natural environments (rhizosphere, fresh water, floristic soil, ... etc.).

These results suggest that different ecosystems, and particularly soils, are capable of harboring microorganisms possessing antibacterial activity. The principal component analysis shown in Figure 6 allows, depending on the two axes, to make a more refined interpretation of the results, while *MN7* and *MN17* register as having the largest inhibition diameters for *E. coli*. This indicates that the antibacterials produced by these two strains must be isolated, purified and identified, and would represent interesting antibacterial bioactive molecules. *MN6* and *MN26* have the largest diameters of inhibition and the lowest for the *Salmonella typ* strain, respectively. indicates that for this pathogenic strain, the antibacterial developed by *MN26*, is very interesting and deserves to be isolated. These results are in harmony with those found by [38,39] who showed the antibacterial activity of traditional herbs against strains resistant to antibiotics, particularly methicillin. It also demonstrates the importance of research into new antimicrobials in the face of multidrug-resistant strains.

4. Conclusion

Microorganisms are in great demand because of their roles in many applications in several fields including industry, agriculture, detergents, petrochemicals, soil bioremediation and many others. The biological activities of microorganisms, including the production of hydrolytic enzymes and the production of antibacterials, must be explored and highlighted to open the way to the purification, isolation and identification of these bioactive substances. This work has paved a path that researchers can use to move towards the discovery of bioactive compounds of biotechnological interest. Six strains of *Bacillus* isolated from soil can produce bioactive compounds

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