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Molecular Identification, Phylogenetic Classification and Proteolytic Capacity of Cultivable Bacteria Isolated from Soils in Brazzaville, Republic of Congo

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HISTORY

Received: 24thSeptember 2019
Received in revised form: 25th of October 2019
Accepted: 18th of November2019

KEYWORDS

molecular identification
phylogenetic classification
proteolytic activity
bacteria
soils

ABSTRACT

Soils present a biodiversity of bacteria, part of this microflora is cultivable, it can be estimated, characterized and identified. Four zones each comprising three sites were sampled in Brazzaville in the Republic of Congo. Phenotypic characterization, molecular identification and proteolytic capacity of soil bacteria were conducted. One of the factors, the pH was measured, the pH values were between 6 and 7. From the classical microbiology techniques, we counted the total flora of the microorganisms in (CFU / g.103), it is between 47 ± 11.2 and 214 ± 58.2 and that of bacteria of the genus *Bacillus* from 51.3 ± 5.0 to 74 ± 30.7. Cocci and bacilli were obtained, Gram- and Gram + bacteria distinguished, all bacteria were catalase +, some had sporulation, others not. Thirty-one (31) isolates were phenotypically characterized and 16S rDNA PCR was performed. Ten (10) strains were sequenced and the phylogenetic classification of the identified strains presented. Blastn's research on the 16S rDNA sequences of the different strains shows similarity rates (96.77% -100%) and E. value (9.00E-93 -0.0), these two indices allowed the identification of the strains studied. The percentages of the identified strains are as follows: *Bacillus cereus (20%) = MN6 and MN14, Bacillus pumilus (10%) = MN7, Bacillus thuringiensis (10%) = MN12, B sp. (10%) = MN17, Bacillus subtilis (10%) = MN26, Staphylococcus haemolyticus (10%) = MN8, Staphylococcus saprophyticus (10%) = MN21, Staphylococcus sp. (10%) = MN22, Staphylococcus gallinarum (10%) = MN24.* The phylogenetic tree shows that the bacteria belong to two main monophyletic genera, the genus *Bacillus* and the genus Staphylococcus. The proteolytic capacity of *Bacillus* bacteria was assessed in parallel with growth. The optical density was between (0.8- 0.93) and proteolytic enzyme production between (9-20mm). The parallelism between growth and enzymatic production shows that the two phenomena are distinct.

INTRODUCTION

Soil is a complex heterogeneous environment that contains a wide variety of communities and bacterial species [1]. As a result, access to bacterial diversity in an exhaustive manner is one of the major challenges of the last decades in soil microbiology and the techniques dedicated to it have been constantly evolving [2]. Before the development of molecular techniques, the identification and taxonomic classification of bacteria required their isolation, cultivation and characterization on the basis of morphological, physiological, metabolic, biochemical and ecological criteria. These criteria of classification quickly found their limits because of the phenotypic and physiological diversity of the bacteria but also by the simplicity of the classification engendered [3].

Pliant this problem, the development of approaches Molecular revolution has revolutionized the world of soil microbiology by enabling the refinement of bacterial classification and by significantly increasing the number of species considered, compared to methods based on only culturable bacteria [4]. Among these bacteria are bacteria of the genus *Bacillus* which, like other bacteria, can be characterized on a molecular level by 16S rDNA. Comparison of genomic sequences of bacterial species shows that the 16S rRNA gene is highly conserved within the species and between species of the same genus and can be used as a tool for the specification of bacteria [4,5]. This method has been used successfully on a large number of bacterial species [4,6].

Bacteria of the genus *Bacillus* have several subspecies, the species have several properties, including the production of proteolytic enzymes) [7,8]. *Bacillus* bacteria produce proteolytic enzymes depending on culture conditions, and each strain has optimal growth and enzyme production conditions [9-12]. In this work, four soil samples taken from different locations in Brazzaville in republic of Congo were used to characterize bacterial biodiversity. After enumeration, isolates were identified by PCR of their 16S rDNA. Dix (10) isolates of which the PCR products were sequenced correspond to six bacteria of the genus *Bacillus* and four of the genus Staphylococcus. The phylogenetic classification based on the gene encoding 16S rRNA was developed, and the property to produce proteolytic enzymes evaluated for bacteria of the genus *Bacillus*.

MATERIALS AND METHODS

Soil sampling and pH measurement.
From November 2016 to September 2017, 12 soil samples were collected in clean, dry, sterile glass tubes with a sterile spatula. Collection took place in four areas of Brazzaville and for each zone three samples were taken. All samples were transferred to the laboratory; under sterile conditions for analysis. The map in **Table 1** shows the different sampling sites in the city of Brazzaville (Republic of Congo).

Table I. Distribution of sampling areas of soil samples in Brazzaville.

Areas	Stations (GPS)	Sites
Town center	194	Beach Market
	195	Bassin of Congo Terminal (TBC)
	196	Mamiwata
Boulevard	197	Puma Service Station
	198	Foreign Affairs Strangers
	199	Tennis Club
Marien Ngouabi	200	School years
University	201	Campus I
	202	University library
Sciences Faculty	203	gardin
and Technologies	204	High School Pierre Savorgnan de Brazza
	205	Saint Exupery School

A pH meter was used to measure the pH values of the different soil samples .

Culture conditions and enumeration
One gram of each of the twelve samples of the sols was diluted in a tube containing 10 ml of distilled water and seeded by plating on nutrient medium (PCA), to count the total flora.To obtain the representative isolates of bacteria of the genus *Bacillus*, the 3 samples from each zone were mixed forming composites, then diluted and seeded by plating on Mossel nutrient medium, the dishes were incubated at 37 ° C for about 12 hours. The colonies obtained were counted by counting. The total viable count was performed using the casting method described by [13]. The counting unit is then expressed as colony forming (CFU) per gram of soil.

$$CFU / g = N / v * d$$

N: colony number, V: dilution volume, d: dilution

Isolation and phenotypic characterization of isolates
The colonies obtained on PCA medium were purified on LB and those obtained on Mossel purified on Mossel. The isolates obtained were prepared for the tests [14]. The purification was done by three successive episodes. From the bacterial culture, isolate colonies were identified according to Bergey's manual on identification of *Bacillus* species modified by [15]. a classification manual of the genus *Bacillus* [15], and also according to [16]. The dishes were incubated at 37 ° C for about 12 hours.

The isolates were stored in liquid LB broth, a 1% glycerol supplement and placed at + 4°C after a 24-hour incubation at 37°C.The phenotypic characteristics of the colonies were determined with a culture of 12 h incubated at 37°C in nutrient agar. The morphology of the colonies (shape, size, color, appearance, contour) was supplemented by a microscopic examination carried out between slide and coverslip from the cultures used to study the cultivation characteristics. The microscopic examination (the shape, clustering mode and cell mobility), Gram stain, catalase assay [17], and Sporulation [18] were performed. have been identified morphologically and physiologically up to the genus level, according to the (Bergey's) manual method of bacteriology and [14].

Molecular identification

Extraction of genomic DNA
Extraction of the bacterial genomic DNA was performed using a commercial DNA microorganism (Nucleospin Microbial DNA) genomic extraction kit. Proteinase K and RNase have been added to eliminate protein and RNA contaminations on DNA, which allows the production of good quality DNA according to the manufacturer's instructions described in the manual. Using 31 isolates, the DNA of each bacterium was extracted by adding in a microtube some colonies of bacteria and 1000 ml of liquid LB. The mixture was vortexed for 1 minute. We centrifuged the tubes at 1000 x g for 3 minutes, then discarded the supernatant, and then took 100 µl of BE that was deposited in the tubes that contained the pellet.

The mixture was pipetted into Nucleospin Bead Tube Type B, and 40 µl MG Buffer, 10 µl Proteinase K was added, then stirred for 6 min, centrifuged at 11000 x g for 30 sec. Then 600 µl of Buffer MG was added, vortexed for 3 s and centrifuged at 11000 x g for 30s. The Nucleospin Microbial DNA Colum Load tubes 500-600 µl sample was changed and centrifuged at 11000 x g for 30 sec. The first wash consists of adding 500 µl of BW and centrifuging at 11,000 × g for 30 sec. For the second wash the columns were changed and added with 500 µl of BW and centrifuged at 11000 x g for 30 s. 100 µl of BE was added and let stand for 1 min, centrifuged again at 11000 x g for 30 s. DNA in the volume of 100 µl was eluted and were kept at - 20° C for later use.

Primers design and PCR reaction
Universal primers to amplify 16S rDNA were (fD1; 5'-AGAGTTTGATCCTGGCTCAG -3 'and rP2; 5' ACGGCTACCTTGTTACGACTT -3 ') [19]. In a total volume

of 50 microliters, the PCR reaction mixture was prepared as follows: 1 µl deoxynucleotide triphosphate (dNTP), 10 µl buffer, 32.75 µl of distilled water and 0.25 µl of enzyme 2 µl primer fD1, 2 µl rP2 primer and 2 µl of DNA. The amplification is carried out in a thermocycler (BIO-RADT100Thermal Cycler) The PCR amplification conditions were as follows: an initial denaturation step at 95 ° C. for 5 min, followed by 30 cycles each comprising a denaturation step at 95 ° C. for 30 s, hybridization at 55 ° C for 30 s, initial elongation 72 ° C for 1 minute 30 s and finally a final elongation step 72 ° C for 10 minutes

Agarose gel electrophoresis of PCR products
The PCR products were analyzed by electrophoresis on 0.8% agarose gel at 100 volts for 45 minutes with TBE buffer. The coloration was made with a solution of 1 µg/ml of ethidium bromide. The gel was visualized by Transilluminator UV type BIOBASE and recorded by the digital camera.

Sequencing of amplicons and assembly
The PCR products resulting from the amplification of the gene coding for the 16S rRNA were purified using the NucleoFast 96 PCR plate (Macherey-Nagel EURL, France) and sequenced by the company Masrogene using the BigDye terminator chemistry on an ABI sequencer. 3730 (Applied Biosystems, Foster City, California, USA). DNA Baser sequence Assembler was used for sequence assembly. The resulting sequences were processed with Bioedith Aligner software for alignments.

Proteolytic enzyme assay
To test the production of the proteolytic enzyme we used the modified techniques [20]. The bacteria were cultured in LB medium with stirring at 37 ° C, for 24 hours after culture the medium was centrifuged for 5 minutes at 5000rpm and the supernatant recovered. In a 250 ml Erlenmeyer flask containing 100 ml of 0.1 N PBS, we dissolved 1 g of agarose, heated to complete dissolution, after cooling to 55-60 ° C, we added 10 ml of skimmed milk, homogenized the mixture. We then sank in the Petri dishes; after solidification, we prepared wells in the gel and place in each well 50 ml of supernatant resulting from the centrifugation of the culture. The boxes are placed in an oven at 37 ° C for about 12 hours. Observation of a clear translucent zone indicates that the strain produces a caseinolytic proteolytic enzyme (caseinolytic protease) [24, 22, 23 ,12].

Analysis and treatment of results
The texts were entered with world, the tables and graphs of different **Fig. 6** were made by Excel. The optical density was measured by a Zuzi spectrophotometer, model 4211/50. Sequence analysis was performed by Blastn (Basic Local Alignment Search Tool) available on the National Biotechnology Information Center database (http://www.ncbi.nlm.nih.gov)Blastn has, with its parameters, in particular the similarity rate, identified the different isolates according to equivalent strains on the basis of data used. The phylogenetic tree was constructed by MEGA 7 using the neighbor joining method with 1000 bootstrap replicates. The scale bar indicates the number of differences in nucleotide substitutions per sequence.

The study of evolutionary divergence was performed as a measure of the notation similarity matrix; the lower score meant a close neighbor with a similar evolutionary origin. The total variability of the sequence was calculated as the mean distance [36 -38].

RESULTS

pH values of the samples

Table 2. pH values of different soils for each GPS station.

GPS Station	pH
194	6.5
195	6.5
196	6.6
197	6.6
198	6.7
199	7.0
200	6.8
201	6.5
202	6.4
203	6.7
204	6.5
205	7.0

Note pH value is between 6.5 and 7

Enumeration

Enumeration of total flora
Table 3 shows the results of enumeration of the total flora of the different soil samples taken from different areas in Brazzaville.

Table 3. Enumeration of the total flora of bacteria on PCA.

Tubes	T1	T2	T3	T4	T5	T6
UFC/g.10^3	60±4	55.6±4.5	30.8±4.7	47±11.2	214±58.2	130±9
Tubes	T7	T8	T9	T10	T11	T12
UFC/g.10^3	186±17.0	210±26.4	87.3±11.05	76±31.7	113.3±30.5	153.33±3

This table shows that the total flora is variable from one sample to another, it is, the low bacterial biomass is tube 3 while the strongest is in tube 5.

Enumeration of bacteria of the genus *Bacillus* on Mossel
Table 4 shows the count of bacteria of the genus *Bacillus* on mossel medium, C1, C2, C3 and C4 represent the 4 sampling zones, each of which comprises 3 sites:

Table 4. Enumeration of bacteria of the genus *Bacillus* on Mossel.

Composite	C1	C2	C3	C4
UFC/g.10^3	51.3±5.0	54.6±11.2	62.2±36.2	74±30.7

This table shows a variation of the number of bacteria of the genus *Bacillus* according to the zone. In zone C4 these bacteria are more represented and in C1 they are less represented

Isolation and Phenotypic Characterization of Isolates

The different characteristics of bacteria is shown in **Table 5**.

Table 5. shows the phenotypic characteristics of the 31 isolates, the characterization procedures are described in material and methods.

Isolates	Colony form	Colour colony	Aspect colony	Form of cell	Arrang-ement	Gram	Catal-ase	Sporul-ation	Mobi-lity
MN1	Rod	Yellow	Round	Mobile Bacillus	Isolated	+	+	+	+
MN2	Rod	Red	Bomb	Mobile Bacillus	Isolated	+	+	+	+
MN4	Rod	Yellow	Plat	Mobile Bacillus	Isolated	+	+	+	+
MN5	Rod	Yellow	Round	Mobile Bacillus	Isolated	+	+	+	+
MN6	Rod	Yellow	Round	Mobile Bacillus	Isolated	+	+	+	+
MN7	Rod	yellow	Round	Chain Bacillus	Isolated	+	+	+	+
MN8	Shell	yellow	Round	Cocci mobile	heap	+	+	+	-
MN9	Rod	Yellow	Bomb	Chain Bacillus	Isolated	+	+	+	+
MN10	Rod	Yellow	Round	Mobile	Isolated	+	+	+	+
MN12	Rod	Yellow	Plat	Cocci chain	Isolated	+	+	+	+
MN13	Rod	Red	Round	Mobile Bacillus	Isolated	+	+	+	+
MN14	Rod	Yellow	Round	Mobile Bacillus	Isolated	+	+	+	+
MN15	Rod	Yellow	Round	Mobile Bacillus	Isolated	+	+	+	+
MN16	Rod	Yellow	Bomb	Bacillus chain	Isolated	+	+	+	+
MN17	Rod	Red	Plat	Mobile Bacillus	Isolated	+	+	+	+
MN18	Rod	Red	Plat	Mobile Bacillus	Isolated	+	+	+	+
MN19	Rod	Yellow	Plat	Mobile Bacilllus	Isolated	+	+	+	+
MN20	Rod	Red	Round	Bacille chain	Isolated	+	+	+	+
MN21	Shell	white	Plat	Cocci mobile	heap	+	+	+	-
MN22	Shell	Yellow	Bomb	Cocci mobile	heap	+	+	+	-
MN23	Rod	Yellow	Plat	Cocci mobile	Isolated	+	+	++	+
MN24	Shell	Yellow	Round	Cocci mobile	Isolated	+	+	+	-
MN25	Rod	Red	Round	Mobile Bacillus	Isolated	+	+	+	+
MN26	Rod	Yellow	Round	Mobile Baclillus	Isolated	+	+	+	+
MN27	Rod	Yellow	Plat	Mobile Bacillus	Isolated	+	+	+	+
MN28	Rod	Yellow	Bombe	Mobile Bacillus	Isolated	+	+	+	+
MN29	Rod	Red	Round	Mobile Bacillus	Isolated	+	+	+	+
MN30	Rod	Yellow	Round	Mobile Bacillus	Isolated	+	+	+	+
MN31	Rod	Red	Round	Mobile Bacillus	Isolated	+	+	+	+
MN32	Rod	Red	Round	Mobile Bacillus	Isolated	+	+	+	+
MN33	Rod	Yellow	Round	Mobile Bacillus	Isolated	+	+	+	+

Molecular identification

Agarose gel electrophoresis of the PCR amplification products of the gene encoding 16S rRNA from the universal primers is shown in **Fig.** 1.

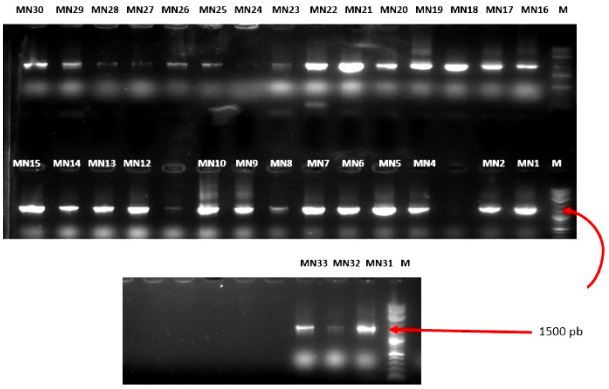

Fig. 1. 0.8% agarose gel electrophoresis of PCR products of the 16S rRNA gene from 31 isolates.M = molecular weight marker, MN1, MN2, MN4, MN5, MN6, MN7, MN8, MN9, MN10, MN12, MN13, MN14, MN15, MN16, MN17, MN18, MN19, MN20, MN21, MN22, MN23, MN24, MN25, MN26, MN27, MN28, MN29, MN30, MN31, MN32, MN33 different isolates.

Sequence analysis

The ten sequenced PCR products were analyzed by Blastn which is a local similarity search program that uses a heuristic method [36,37]. The significance of the alignments is evaluated statistically according to the length and composition of the sequence, the size of the bank and the scoring matrix used. Blast presents its results with a list of sequences with significant alignment, each associated with a score and an E. Value. The lower the E. Value, the higher the alignment score is significant. The search for the homologues for each sequence entry makes it possible, by the similarity percentage, to specify the equivalent strain on the basis of data consulted, Table VI presents the results obtained for the identification of each isolate.

Table VI. Blastn Results for Strain Identification by Similarity of 16S rDNA.

code	Max Score	E. Value	% de similarity	Accession Number	Equivalent Strain
MN7	353	9,00E-93	100.00%	MK601669.1	*Bacillus pumilus*
MN6	1814	0.0	97.90%	CP018931.1	*Bacillus cereus*
MN8	398	4,00E-106	99.10%	KX009746.1	*Staphylococcus haemolyticus*
MN12	296	1,00E-75	97.14%	HQ917121.1	*Bacillus thuringiensis*
MN14	418	3,00E-112	99.56%	HQ694071.1	*Bacillus cereus*
MN17	712	0.0	98.04%	JN874746.1	*Bacillus sp*
MN21	737	0.0	97.03%	KT003250.1	*Staphylococcus saprophyticus*
MN22	102	3,00E-17	96.77%	AB740361.1	*Staphylococcus sp*
MN24	1162	0.0	96.84%	MK408479.1	*Staphylococcus gallinarum*
MN26	411	5,00E-110	99.12%	JX683721.1	*Bacillus subtilis*

This table shows that: the NCBI BLAST homology had a similarity of 100% with MN7 *Bacillus* pumilus (MK601669.1), MN26 99.12% *Bacillus* subtilis (JX683721.1), MN14 99.56% *Bacillus* cereus (HQ694071.1), MN17 98.4% *Bacillus* sp (JN874746.1) MN12 97.14 *Bacillusthuringiensis* (HQ917121.1), MN6 97.90% *Bacillus* cereus (CP018931.1) and MN8 99.10% *Staphylococcus haemolyticus* (KX009746.1), MN2 97.3% *Staphylococcus saprophyticus* (KT003250.1).

Representativity of strains identified by 16S rDNA

Fig. 2 shows the different percentages of the strains identified, we can note the presence of two genera bacteria of the genus *Bacillus* and *Staphylococci*.

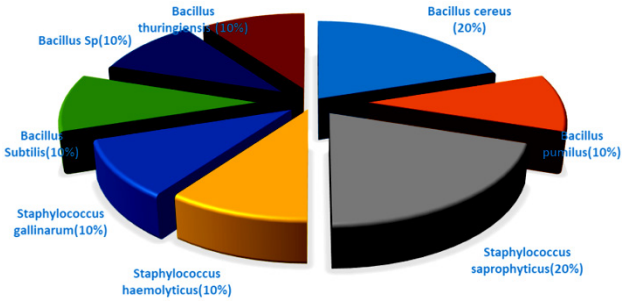

Fig. 2. Representability of strains identified by 16S rDNA.

Phylogenetic classification of strains identified by the gene encoding 16S rRNA

The multiple alignment of the sequences of the bacterial isolates is shown in **Fig. 3**. This alignment makes it possible to observe that the gene encoding the 16S rRNA exhibits both variable regions and non-variable regions, but the conserved areas are larger than the non-conserved regions. There is the presence of indels (insertions deletions.

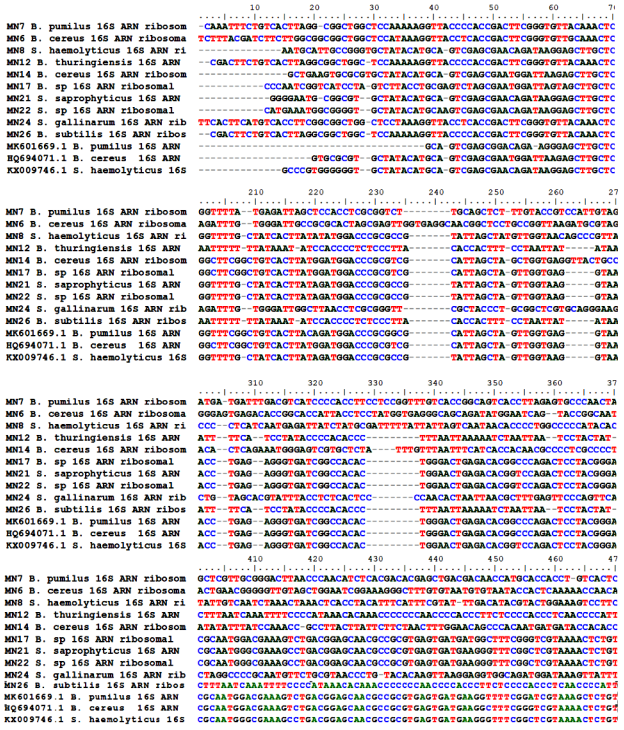

Fig. 3. Part of the multiple alignment of the 16 S rRNA gene sequences of (13) strains of which there are (10) identified strains (MN7, MN6, MN8, MN12, MN14, MN21, MN22, MN26, MN24, MN17) and (3) counterparts (MK601669.1), taken from the database.

The phylogenetic tree

The evolutionary history has been deduced using the neighboring junction method [31]. The optimal tree (**Fig. 4)** with the sum of the branch length = 3.14669626 is shown. The tree is drawn to scale, with branch lengths in the same units as the evolutionary distances used to derive the phylogenetic tree. Evolutionary distances were calculated using the Poisson correction method [32] and are expressed in units of the number of amino acid substitutions per site. The analysis involved 13 nucleotide sequences. All positions with gaps and missing data have been eliminated. Evolutionary analyzes were conducted in MEGA7 [33].

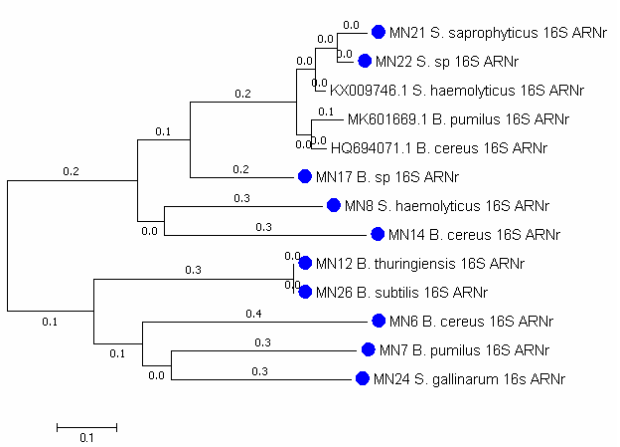

Fig. 4. The phylogenetic tree of identified strains and their homologous.

Proteolytic activity of bacteria of the *Bacillus* genus isolated and identified by their 16S rDNA.

Proteolytic enzyme production

Fig. 5 shows the demonstration of proteolytic enzyme production by bacteria of the genus *Bacillus* identified by their 16S rDNA. The translucent stain testifies to the digestion of the casein, it is the results of the supernatants resulting from the centrifugation of the cultures of which 50 microliters were deposited in the wells of the Gel, and previously placed in the oven during about 12 h. **Fig. 5** shows that the diameter of the translucent zone is variable from one strain to another, MN6 (19 mm), MN17 (20 mm), MN14 (15 mm) has a larger enzyme production whereas, MN26 (10mm), MN7 (11mm) and MN12 (11.3mm) show low enzyme production.

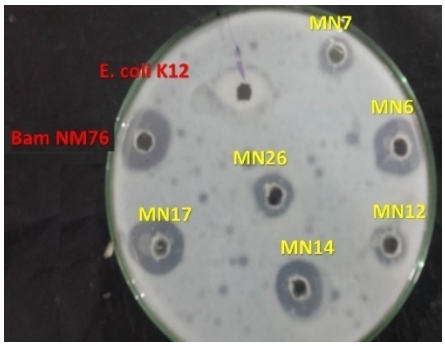

Fig. 5. Digestion of casein for different strains, clear areas indicate proteolysis.MN6, MN7, MN12, MN14, MN17 and MN26 are the different strains of bacteria of the genus *Bacillus* isolated and identified in this study. E. coli K12 which is the negative control and Bam NM76 positive control.

Simultaneous evaluation of proteolytic enzyme production and growth (OD)

Clear area diameters representing proteolytic enzyme (EP) production were measured in correlation with optical density (expression of OD growth), **Fig. 6** shows the results obtained.

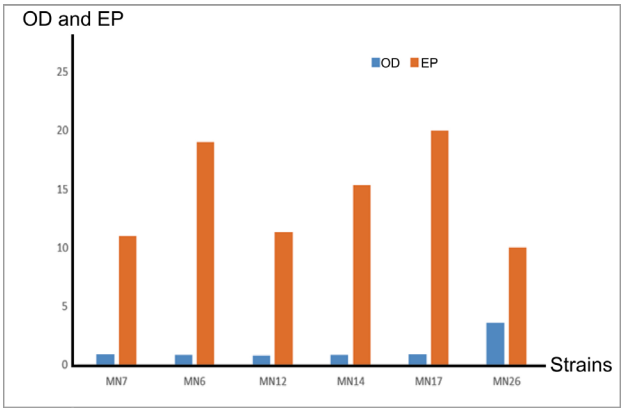

Fig. 6. Variation in OD and proteolytic enzyme production for different strains of *Bacillus*isolated and identified.
Note: OD = optical density EP = enzyme production

The parallelism between OD and EP shows that all the strains used showvariable growth from one strain to another. All the identified *Bacillus* strains produce the enzyme. Enzyme growth and production are two related but distinct phenomena. Strains MN6, MN14, and MN17, which show significant enzyme production, do not have the highest OD, however strains MN26, MN7 and MN12 show less significant enzyme production, strain MN26 has higher OD.

DISCUSSION

In this study, bacterial strains from soil samples collected at 12 sites in Brazzaville, Republic of Congo, were isolated and characterized phenotypically and molecularly. The gene encoding 16S rRNA has been used to characterize Bacterial Biodiversity and the proteolytic capacity of bacteria of the genus *Bacillus* evaluated. The pH values of the samples are between 6 and 7. Determining the pH of the soil is equivalent to measuring its acidity or its basicity, which depends on the proton concentration, that is to say on H3O + ions. the main factors that influence bacterial growth.

Indeed, the survival of microorganisms differs from one group to another depending on the degree of acidity of the medium. [27] and [28] pH is an environmental parameter that determines the development of soil microflora since its fluctuations affect both biotic and abiotic factors of this ecosystem. It particularly influences the heterogeneity of the bacterial communities that remain there [29]. Most bacteria have been shown to grow at near-neutral pH levels, with the optimum growth being between pH 6 and pH 8 [38]. Indeed, the bacterial growth is exponential when the pH values are close to 7 whereas it is slower at lower pH [27]. These results, similar to ours, explain the growth of bacteria, because the pH of the soils of our study are between 6 and 7. Indeed, the soils of Brazzaville are rather acidic in general [31].

The enumeration of the total flora showed a variation of the CFU / g according to each sample these numbers are between 30.8 ± 4.7 CFU / g.103 (for the sample of the tube 3) to 214 ± 58.2 CFU / g 10^3 (for the sample of tube 5). All soils contain bacteria. A [26] also showed that a soil contaminated by hydrocarbons contains bacteria, these results are similar to ours because of the presence of bacteria in all the soil. For bacteria of the genus *Bacillus*, the highest bacterial biomass was in the composite (C4) and the lowest in the composite (C1). Here again the variation of CFU / g as a function of the composite. All isolates are catalase +, gram + and all show sporulation. On the other hand, the shape, the color and the appearance of the colonies, the arrangement and the mobility of the cells vary according to the isolates, 27 isolates have the rod shape while 4 present the cocci form. 21 isolates are yellow in color and 12 in red, 18 isolates are of rounded form, 5 isolates are bomb and 8 isolates are flat. These results are similar with de [25] who worked on different food environments. Moutou worked on foods that are also environments, and also found isolates with different morphological characteristics.

Agarose gel electrophoresis at 0.8% PCR products of the 16S rRNA genes of the isolates in this study showed a length of approximately 1500 bp. The study [25] however showed a length of about 1380 bp that are different from our study. In reality, the 16S rRNA is a marker of molecular identification. According to **Fig. 3**, which showed some differences among the nucleotide sequences that were aligned, these results are supported by 16S rRNA, sequencing as a more potent bacterial identification technique, and these results are in agreement with previous studies [34] have successfully used 16S rRNA gene sequencing to identify pathogenic bacteria in the clinical laboratory. [4] Used sequencing of the 16S rRNA gene for bacterial identification and found new bacteria in clinical microbiology laboratories.

Fattahi et al. [35] developed 16S rRNA as a PCR target for the detection of *E. coli* in rainbow trout. Most sequences showed 99% of identity with the closest sequences already presented in the NCBI databases. MN7 has 100% similarity, MN26 99%, and (MN14, MN17) 98%, (MN6, MN12) 97% for *Bacillus* and 99% MN8, MN21% and (MN22, MN24) 96% for Staphylococci. **Table 4** of Blast shows the correlation between E, score and similarity for strains. Strains MN6, MN24, MMN17 and MN21 show low E. values, and the alignment score is significant. The results are expressed as percent similarity of strains found to identify for our study with closest species (homologs). With regard to the intervals (10-100,110-200, 210-300,310-400,410-500) one observes the **Fig. 3** which presents the indel (insertion or deletion) on the first alignment, the second and the third while the fourth does not has almost no indel from which we can talk about the conservation of nucleotides.

The phylogenetic tree represents the phylogenetic relationships between the newly obtained sequences and reference sequences in the databases. It was built by the distance method by Mega 7 software. **Fig. 6** shows the parallelism between proteolytic enzyme production and growth, our results show that the growth and production of enzyme vary from one strain to another, isolates MN17 has the largest enzyme production (20mm), while MN26 has the lowest production (10mm), these results are similar to those of [12] which worked on the ntoba mbodi fermented food and also found variation in growth and proteolytic enzyme production as a function of the isolates. Our results suggest that each strain has different growth and enzyme production conditions, as shown by previous work by [39, 40].

CONCLUSION

At the end of our study, we were able to enumerate, isolate, identify and characterize on a phenotypic and molecular level the bacteria present in the soils. The ten of the 31 isolates identified and characterized on the molecular level have allowed after sequencing to distinguish by phylogeny two coherent monophyletic groups bacteria of the genus *Bacillus* and the genus Staphylococcus. The six bacteria of the genus *Bacillus* showed different proteolytic activity and growth showing that growth and proteolytic activity are two distinct phenomena. The 16SrRNA remains a useful tool for the biodiversity of culturable bacteria.

ACKNOWLEDGEMENTS

We thank the members of the Department of Molecular and Cellular Biology of the Faculty of Sciences and Technics of the University Marien Ngouabi, the members of the Molecular and Biology Laboratory of the National Institute of Research in Natural and Exact Sciences (IRSEN)as well as the members of the National Laboratory of Public Health (LNSP) of the Republic of Congo for facilitating our access to their technical platform for our analysis. We express our gratitude to Mrs. Annie Justine Pahou Bilimba for supplying us with reagents.

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