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Diversity of Cellulolytic Bacteria in Soils Revealed by 16S rRNA Gene Sequençing Analysis in Brazzaville, Republic of Congo

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A B S T R A C T

The study was aimed to isolate bacteria from different soil samples and phenotypically characterize the bacterial isolates. Subsequently, selected isolates were tested for their ability to produce cellulolytic enzymes and molecularly identified through 16S rRNA gene sequencing and phylogenetic analysis. Four sites each constituting a sampling point were sampled in Brazzaville in the Republic of Congo. pH measurement, Enumeration, phenotypic and16S rRNA gene molecular characterization as well as cellulolytic potential of isolates were performed. The pH whose values vary between 6.5 and 7 was measured. From the basic classical techniques of microbiology, the total aerobic mesophilic flora on Plate Count Agar (PCA) medium in CFU / g at 10^2 gave values between 12 ± 4.3 to 22.65 ± 7 and the bacteria of the genus Bacillus on Mossel, values between 16.5 ± 0.7 to 20.5 ± 2.1. Bacteria in the form Cocci and bacillus, gram negative and gram positive, all catalase positive were obtained. Out of a total of 84 isolates phenotypically characterized in this study: 10 isolates were molecularly identified, but only 4 were submitted to the GenBanck database whose accession numbers for each strain are as follows: *Pantoea dispersa* MLTBY6 (MT646430.1); *Pseudomonas aeruginosa* MLTBM2 (MT646431.1); *Bacillus subtilis* MLTBC5 (MT674681.1) and *Pseudomonas monteilii* MLTBC10 (MT674682.1). The phylogenetic classification inferred with MEGA.7 clearly show the bacterial diversity of the studied soils. The search for bacteria with cellulolytic potential in the soil opens a door for the production of cellulolytic enzymes for industrial production. This study, which provides an overview of the microbiological diversity of soil samples in Brazzaville, was to be extended to the whole city of Brazzaville, as well as to other cities in the country. This will open up prospects for new approaches to identification using targeted meta-genomics.

Introduction

Soil is the shallow, loose layer of the earth's crust that extends vertically from weathered rock to the surface and generally varies in thickness from a few centimeters to a few meters. Horizontally, it stretches almost continuously over the surface of the continents, only interrupted by rock outcrops, rivers and human constructions (Bruand *et al.,* 2009).

During the twentieth century, human activities have intensified and this is, in part, due to the increase in the world population and the growing need for associated food, but also because of high demand from part of this population. To develop their well-being. This intensification of human activities has caused an alteration of our environment and is the cause of a significant erosion of biodiversity on a planetary scale. Ecosystem assessment has shown that the rate of species extinction has increased by a factor of 10, 100 or even 1000 in some cases since the start of the industrial era (McMahon *et al.,* 2005). Such loss of biodiversity leads to changes in biological processes and the stability of ecosystems (Balvanera *et al.,* 2006). From an ecological point of view, the soil represents one of the most important reservoirs of biological diversity on our planet and above all one of the last ramparts for this biodiversity (Swift *et al.,* 1998). It is also the site of many biological and ecological processes and therefore provides a large number of ecosystem services resulting from the complexity of taxonomic and functional assemblages of native communities and interactions between organisms (Coleman *et al.,* 2005). However, the number of studies on the quantification of soil biodiversity and its translation into biological functioning is much lower compared to work on the diversity of living organisms on its surface (especially plants) and consequently, our level of knowledge remains limited.

This is all the more true for the indigenous microbial communities which are still considered, on the basis of a postulate proposed by Beijerinck *et al.,* (2013): “tout est tout” (“everything is everywhere”), as ubiquitous organisms with a strong functional redundancy. As such, the telluric microbial communities still appear recurrently as a functional "black box" generating flux, the intensity of which depends only on abiotic factors such as temperature, humidity, pH, etc., excluding the hypothesis that the diversity and composition of microbial communities as well as trophic interactions (competition, commensalism, etc.) between populations can play a functional role (Gignoux *et al.,* 2001). In biogeochemical cycles such as carbon or nitrogen, bacteria are of considerable importance because they play a fundamental role in fixing atmospheric nitrogen, a function that has been studied extensively for several decades (Vitousek *et al.,* 1991). Most bacteria are harmless to humans, and some are even essential for the body to function properly. However, there are many pathogenic species that cause infectious diseases such as cholera, syphilis, plague, anthrax, tuberculosis (Kumar *et al.,* 2009; Xu *et al.,* 2010).

However, the majority of these bacteria have not yet been identified or characterized because they remain recalcitrant to in vitro culture on culture media that may have been offered to them (Rappe *et al.,* 2003). These limits imposed by in vitro culture have allowed the development of independent approaches. The advent of molecular biology (PCR) and meta genomics techniques in the 1990s restricted the study of bacteria to their DNA directly extracted from the environment (McHardy *et al.,* 2007). Recently new DNA sequencing techniques have made it possible to obtain huge databases of sequences from uncultured bacteria (Roesch *et al*., 2007; Srinivasan *et al.,* 2015), allowing a variety of genetic applications. Bacteria in their metabolisms have several activities including the production of hydrolytic enzymes. Many bacteria produce exocellular enzymes, among these enzymes are cellulolytic enzymes. Cellulose is the most abundant molecule on earth. It is the basic polysaccharide structure of plant cell walls and accounts for around 50% of the total mass of organic matter (Deguchi *et al.,* 2006). Cellulose is a polyholoside with a long chain of cellobiose units, made up of an hydroglucoses linked by β-1,4 bridges. It is present in amorphous or crystalline form, generated by the establishment of hydrogen bonds between the different chains (Oh *et al.,* 2005). In plants, cellulose is present in the form of microfibrils with a diameter of 20 - 30 nanometers, and a length of 100 - 40,000 nanometers. Each cellulose molecule contains 2,000 to over 15,000 cellobiose subunits. In nature, cellulose is broken down by a group of enzymes of fungal or bacterial origin known as cellulases.

Cellulases are a group of hydrolytic enzymes capable of degrading lignocellulosic materials. According to Acharya (2008), they are synergistic enzymes used to degrade cellulose into glucose or other oligosaccharide compounds by hydrolysis of its β (1-4) glycosidic linkage. They are produced mainly by bacteria (Xu,

2002). There are three main types of cellulolytic enzymes that can degrade cellulose: endo-1,4-β-D-glucanases (EC 3.2.1.4), exoglucanase or exo - cellulase (EC 3.2.1.91) and β- glucosidase (EC 3.2.1.21) (Xu, 2002).

Cellulases are produced by numerous microorganisms: by bacteria, actinomycetes and fungi (Kuhad *et al.,* 2011). Cellulase-producing bacteria include:

Acinetobacter junii; Bacillus subtilis; Bacillus pumilus; Bacillus amyloliquefaiens; Bacillus licheniformis; Pseudomonas aeruginosa...

In this work, a soil exploration was carried out in Brazzaville. pH measurement, Enumeration, phenotypic and16S rRNA genemolecular characterization of. Cellulolytic bacteria from soil have been performed, and cellulolytic enzyme production have been evaluated. The phylogenetic classification has also been tried. The choice of this study is based on the low number of studies on soil microbiology in our country and the lack of much information and knowledge on soil biodiversity in Congo.

Materials and Methods

Samples Collection

Between July 2019 and October 2020, four soil samples were collected using a sterile spatula, introduced into a sterile Pyrex tube and then passed through a cooler at the cell and molecular biology laboratory of the Faculty of Science and techniques of the Marien NGOUABI University for microbiological analyzes as shown in Table 1. The four samples were collected in different sites or stations in Brazzaville, all sites were distant from each other in less than two kilometers. As it is known that soil composition will influence the bacterial composition, we just deal with the distance factor.

Enumeration

The count of soil bacteria was determined from a soil solution according to the method described by (Afnor *et al.,* 1999).

One g of each sample (soil) were weighed using an OHAUS PIONEER type precision balance and poured into a pyrex tube containing 9ml of sterile physiological water (0.9% NaCl, B. Braun Melsungen AG 34209 Melsungen, Germany). After 1 minute of homogenization, a series of cascade dilutions or decimal dilutions (10^{-1} to 10^{-7}) were prepared from this stock solution.

The inoculation was carried out by spreading 0.1 ml of the inoculum on the petri dishes containing the agars (PCA) for the determination of the total aerobic mesophilic flora (FMAT) and the agar (Mossel) for the isolation of bacteria. Of the genus Bacillus, because of Bacillus have been shown to be one of the most important genus in cellulolytic bacteria (Amika *et al.,* 2018). Two petri dishes were inoculated by dilution. The dishes were incubated in an oven at 37 degree Celsius for 24 hours. The calculation of the number of microorganisms was made from the number of colonies obtained in the dishes corresponding to the dilutions giving a significant result.

Each colony is considered to have been generated by one or more microorganisms, thus forming a CFU. The number of microorganisms or CFU per gram of product (soil) was determined using the following equation: CFU / g = N / (Vd) (Hamdi *et al.,* 2012).

N: number of colonies, V: dilution volume, d: dilution factor

Isolation and phenotypic characterization of isolates

Isolation and phenotypic characterization of isolates from colonies obtained on PCA medium were purified on LB and those obtained on Mossel were purified on Mossel. The pure isolates thus obtained were prepared for the tests (Austin *et al.,* 1998). The purification was carried out in three successive stages. From bacterial culture, isolated colonies were identified as described according to Bergey's manual on the identification of Bacillus species modified by Wulff *et al.,* (2002). The dishes were incubated at 37 °C for approximately 24 hours.

The isolates thus purified were stored in LB liquid broth, 1% glycerol supplement and placed at + 4 ° C after a 24-hour incubation at 37 °C. The phenotypic characteristics of the pure colonies were determined with a 24 h culture incubated at 37 °C. Morphological observation of pure colonies (shape, size, color, appearance and outline) was supplemented by microscopic examination performed between slip and slip cultures used to study culture characteristics. Microscopic examination (shape, mode of clustering and cell mobility), Gram stain, catalase test,

and sporulation were performed (Gusils *et al.,* 2010). Isolates were identified morphologically and physiologically down to the genus level, according to the method described in the manual (Gibson *et al.,* 1974; Hans-Jiirgen *et al.,* 1996).

Cellulolytic activity

Production of cellulolytic enzymes Assay

The production of cellulolytic enzymes has been studied using modified techniques (Puri *et al.,* 2002). Each selected pure cellulose-producing isolate was inoculated into an Erlenmeyer flask containing 20 ml of LB nutrient broth and placed in a rotary oven at the speed of 20 rpm at 37 ° C. for 24 hours. 10ml of the solution was centrifuged at 6000trs / minute for 10 minutes. The medium (CMCA) whose chemical composition is: 0.5g of $NH_4H_2PO_4$, 0.1g of KCl, 0.5g of $MgSO_4$. $7H_20$, 0.5g of yeast extract, 13g of cellulose and 1.5g of Agar. sterilized in the autoclave was poured into the petri dishes. After solidification of the agar, the then were made. 50 µl of the supernatant were placed in the wells, the petri dishes were incubated at 37 ° C. for 48 hours.

Assessment of Cellulolytic Enzyme Production

The revelation was made with the lugol. The observation of a clear area on the petri dish containing the cellulose (Sauvageon *et al.,* 2012).

Molecular identification of isolates

Extraction of genomic DNA

DNA extraction was done from pure isolates using the qiAmp DNA kit (qiagen, Hilden, Germany) following the manufacturer's recommendations. DNA extraction involves cell lysis in the bacterial sample, followed by attachment of genomic DNA from the cell lysate to the membrane of a spin column, washing of the membrane, and elution of genomic DNA.

For this purpose, the Eppendorf tubes have been identified. After that, the bacterial cells were suspended in 200µl of PBS, 20µl of proteinase k was added, 200µl of buffer AL was added, then the tubes were vortexed for 3 seconds, incubate in a water bath at 58 ° C for 10 minutes. After incubation, 200 µl of ethanol (96-100%) was added to the tubes, the tubes were vortexed for 3 seconds. The supernatant was transferred to the DNeasy mini spin placed in a 2ml tube collector, the tubes were centrifuged at 8000 rpm for 1 minute. The collection tubes containing the precipitate were discarded, then the DNeasy mini spin column was placed in new collection tubes, 500µl of buffer AW1 (washing solution) was added to the column, the collection tubes and the columns were added was placed in the centrifuge, centrifugation at 8000 rpm was carried out for 1 minute. Subsequently the columns were removed from the collection tubes, the supernatant was discarded, the columns were placed in the new collection tubes previously placed on the rack. 500 µl of buffer AW2 (washing solution) were added to the columns, the collection tubes and the columns were placed in the centrifuge, centrifugation at 8000 rpm was carried out for 3 minutes, the columns were removed from the tubes collectors, the supernatant was discarded. The columns were placed in the new Eppendorf tubes placed on the rack, 200 µl of buffer AE were added to the columns, the Eppendorf tubes containing the columns were incubated at room temperature for 1 minute, placed in the centrifuge, then a Centrifugation at 6000 Xg revolutions was performed for 1 minute to separate the DNA from the matrix (the filtrate contains DNA). The resulting DNA was stored in the tubes.

PCR amplification of the 16S rRNA gene

Designation and synthesis of primers

To perform the PCR, in order to amplify the 16S rRNA, the following universal primers were chosen with reference to Weisburg *et al.,* (1991). These same primers were also used by Ngo-Itsouhou *et al.,* (2020); Soloka *et al.,* (2020). These primers were synthesized by the Macrogen Company France The sequences and orientation of these primers are: fD1 5′AGAGTTTGATCCTGGCTCAG-3 'and Rp15'ACGGCTACCTTGTTACGACTT-3'.

Composition of the reaction mix and PCR conditions

In a final volume of 50 microliters, the PCR reaction mixture was prepared as follows: 37.75 µl of distilled water, 2 µl of Fet R primer with a concentration of 20 µM per primer, 2 µl of dNTPs (10 µM), 0, 25µl of the enzyme (One Tap DNA polymerase 5000U / ml), 10µl of Buffer (One Taq standard Buffer 5X). Amplification was

performed in a thermal cycler (BIO-RADT100Thermal Cycler) with the following conditions:

A first denaturation at 95 ° C for 5 minutes, followed by 30 cycles each comprising a denaturation step at 95 ° C for 1 minute, a hybridization at 55 ° C for 1 minute, and an initial elongation at 72 ° C for 1 minute. Finally, a final elongation at 72 ° C for 5 minutes.

DNA Agarose Gel Electrophoresis

Agarose Gel Preparation

Two agarose gels were prepared in this study: the 0.8% gel for the genomic DNA and the 1% gel for the PCR fragments.

The concentration of the gel was prepared according to the size of the gene to be visualized. In two 250 ml Erlenmeyer flasks, we dissolved 0.8g and 1g of agarose respectively in 100ml of TBE buffer (Tris- Borate-EDTA: 89mM; 89mM; 2.5mM), the mixture was heated on a hot plate until 'to boiling or complete disappearance of the agarose. We had allowed the gel to cool to a temperature of 50 to 60 ° C, we had placed the comb in the mold, the gel was poured into the mold (tank), we had waited at least 30 to 45 minutes for the polymerization of the gel, we had removed the comb and it forms the wells in the gel.

Sample processing, migration and visualization

The DNA samples were processed before they were placed in the wells. For this, we used Midori and Buffer Dye, the quantities are as follows: 5µl of DNA, 0.5µl of midori, 1µl of Buffer Dye. Midori is an intercalating agent, it fits between nucleotides and allows nucleotide fluorescence and Buffer Dye, allows DNA to be deposited and maintained at the bottom of wells. The gel was subjected to an electrical voltage. In our study, the gel was subjected to a voltage of 100V for 45, we had allowed the bands to migrate so that the blue line arrived in the middle or until the end of the gel, we stopped the feed when the dye has traveled the required distance or allow the migration to arrive up to 1cm from the edge of the tank (this line is called the migration font). After migration, the gel was incubated in TBE buffer containing 1 µg / ml of Etidium Bromide (BET) for 30 minutes. Visualization was done under a UV fluorescence lamp (6W 254NM).

Sequencing

The sequencing of the amplicons and the assembly of the PCR products resulting from the amplification of the genetic coding of 16S RNA were purified using the NucleoFast 96 PCR plate (Macherey-Nagel EURL, France) and sequenced by the company Macrogen using BigDye terminator chemistry on ABI sequencer. 3730 (Applied Biosystems, Foster City, California, USA).

The baser DNA sequence assembler was used for the sequence assembly. The resulting sequences were processed with Bioedith Aligner software for alignments (Soloka *et al.,* 2020).

Statistical Analysis

Statistical analyzes of data related to graphs and other charts were performed using Microsoft Excel. The in-silico analysis of the sequencing products was performed as follows: Sequence assembly with Codon Code Aligner and DNA Baser assemble software. In-silico analysis was performed from NCBI (National Center for Biotechnology Information, Los Alamos, USA) using BLAST (Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov), which is an algorithm used by a family of five programs which allow the alignment of a new sequence with respect to a database. The BLAST program uses the algorithm developed by Altschu *et al.,* (1990) to find similar segments between a query sequence (or "query" sequence) and all the sequences present in the nucleic acid or protein bank.

The sequences are classified according to a "score" which depends on the homology with the request sequence, on the size of the bank and the value of the "E-Value." The smaller this is, the greater the homology between the request sequence and that of the library (Rouhou *et al.,* 2006). Ranked reference species reported percentages of similarity ≥99%, the isolate unknown will be assigned to this species; If the percentages are between 97% and 99% the unknown isolate will be assigned to the corresponding genus; If the percentages are ≤97%, the unknown isolate will be assigned to a family.

Alignment of nucleïc sequences was done on Bioedith. Phylogenetic and molecular evolution analyzes were performed using MEGA 7 (Sneath *et al.,* 1973; Tamura *et al.,* 2004; Kumar *et al.,* 2016).

Results and Discussion

Sampling

The table 1presents the Stations, GPS coordinates, pH, and bacterial count in UFC/g of the different samples collected in the city of Brazzaville. The pH is about 6,5 – 7 for the stations of all station. Samples of station 2 and 3 are showing more microorganisms then station 1 and 4

Enumeration

The table 1 shows the variation of the total aerobic mesophilic flora on the PCA nutrient agar and bacteria of the genus Bacillus on mossel medium isolated from the soils sampled at different stations in Brazzaville.

Isolation and phenotypic characterization of isolates

Isolation and phenotypic characterization was carried out according to the macroscopic (appearance, shape, color, outline of the colonies), microscopic (mobility, shape of the colonies) and biochemical characteristics (catalase test, potash test, Grams test and the sporulation test). Table below2 the isolation and phenotypic characterization of the isolates.

Macroscopic characterization of isolates

Macroscopic characterization of isolates was done according to colony color, shape and appearance. Table: 2 below presents the phenotypic characterization of the isolates according to the macroscopic characters. This table shows that, out of a total of 84 isolates, 8 are whitish in color, polylobed and slimy in appearance, 11 are whitish in color, circular in shape and slimy in appearance and 65 are yellow in color, are circular in shape and have a slimy appearance

Microscopic characterization of isolates

The microscopic characterization of the isolates was made according to the shape and the mobility of the bacteria. Table: 2 below presents the characterization of the isolates according to the microscopic characteristics. This table shows that out of a total of 84 isolates, 36 are rod-shaped, are movable, 34 are rod-shaped are immobile, 5 are rounded and are movable, and 9 are rounded and are immobile.

Biochemical characterization of isolates

The biochemical characterization of the acete isolates made according to the catalase test, the potash test, the gram test and the sporulation test. Table: 2 below shows the biochemical characterization of the isolates. This table shows that all 84 isolates are catalase positive, 47 are Gram positive and sporulent, 17 are Gram negative and sporulent, 8 are Gram negative and non-sporulent, and 12 are Gram positive and non-sporulent.

Cellulolytic Enzyme Production

Cellulolytic enzyme Assay

Figure 1: (A, B, C, D, E, F, G, H) shows the demonstration of cellulolytic enzyme production by the presence of a halo around the well.

This figure shows that, the diameters of the halos differ from one isolate to another, but also from one sample to another. The spot around the well is used as an indicator of cellulase, which is an enzyme capable of hydrolyzing cellulose.

Evaluation of cellulolytic enzymes production

Figures 2: below show the histograms showing the diameters in mm of the evaluation of cellulolytic enzyme production of isolates obtained from soils by the sample.

Figure 2 below shows the histogram showing the evaluation of the production of cellulolytic enzymes in Sample 1 (Faculty of Science and Technology). These results show that the hydrolysis diameters vary between 18 mm to 28 mm. The F4 isolate has a larger diameter than the other isolates in this sample. Out of a total of 17 isolates obtained for this sample, only 10 isolates synthesized cellulase, an enzyme capable of hydrolyzing cellulose, ie a percentage of 58.82%. The hydrolysis of cellulose by cellulases is observed by a halo around the wells as shown in Figure 1.

Figure 2 below shows the histogram of the evaluation of cellulolytic enzyme production of isolates obtained from the soil of sample 2 (Boulevard Alfred Raoul). These results show that the hydrolysis diameters vary between 12 mm to 43 mm. Isolate B11 has a larger diameter than the other isolates in this sample. Out of a total of 36 isolates obtained for this sample, only 18 isolates

hydrolyzed cellulose, that is, have hydrolytic activity, ie a percentage of 50%.

Figure 2 below presents the histogram showing the evaluation of cellulolytic enzyme production of isolates obtained from the soil of sample 3 (Campus I). These results show that the hydrolysis diameters vary between 14mm to 25mm. The C9 isolate has a larger diameter than the other isolates in this sample. Out of a total of 20 isolates obtained for this sample, only 15 isolates hydrolyzed cellulose, that is to say have hydrolytic activity, ie a percentage of 75%.

Figure 2 below shows the diameters in mm of the evaluation of the production of cellulolytic enzymes of the isolates obtained from the soil of sample 4 (Port of Yoro). These results show that the hydrolysis diameters vary from 15mm to 20mm. The Y7, Y2, YP2, Y3 and Y5 isolates have larger diameters than the other isolates in this sample. Out of a total of 12 isolates obtained for this sample, only 9 isolates hydrolyzed cellulose, that is to say have hydrolytic activity, ie a percentage of 75%.

Molecular identification of isolates

DNA agarose gel electrophoresis

0.8% agarose gel electrophoresis of the genomic DNA of isolates obtained from soils

The results of the electrophoresis of the genomic DNA of the isolates obtained from the soils are presented in the figure below 3.

1% agarose gel electrophoresis of PCR products of the gene encoding 16S rRNA from isolates obtained from soils

Molecular identification by amplification of the gene encoding 16S rRNA of pure isolates obtained from soils revealed bands of around 1500bp as shown in Figure 4

Bioinformatics analyses

Blastn Analysis

The sequenced PCR products were analyzed by Blastn. 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. The search for homologs for each sequence entry makes it possible, by the percentage of similarity, to specify the equivalent strain on the basis of the data consulted, Table 3shows the results obtained for the identification of each isolate.

This table shows that: NCBI BLAST homology had 100.00% similarity to M2 *Pseudomonas aeruginosa* (MN646431.1), 100.00% to Y6 *Pantoae dispersa* (MT646430.1), 100.00% to C5 *Bacillus subtilis* (MT674681.1), 100.00% with C10 *Pseudomonas montelii* (MT674682.1), 97.50% with B7 *Lysinibacillus fusiformis* (MN904872.1), 89.55% with B3 *Enterococcus faecium* (MH476286.1), 97.14% with B5 *Bacillus subtilis* (KY905360.1), 91.38% with C13 *Botryllus schlosseri clone* (KR911865.1), 96.95% with YP1 *Staphylococcus saprophyticus* (MN173448.1) and 98.39% with *Staphylococcus edaphicus* (MW033681.1)

Strains identified with their accession numbers in GenBanck

The table below presents four (4) of the ten (10) strains identified. This table shows four strains submitted to GenBanck with their accession numbers.

Phylogenetic classification of strains identified by the 16S rRNA gene

Multiple sequence alignment of isolates obtained from soils

Figure 6 below shows the analysis of the multiple alignment of the different sequences (10) of the 16S rRNA gene of strains of bacteria of the genus *Bacillus*, *Pseudomonas*, *Pantoea*, *Staphylococcus*, *Botryllus*, *Lysinibacillus* and *Enterococcus* isolated from soils. although there are two areas. An area where nucleotides have been highly conserved (110-170) and an area where nucleotides have not been conserved (170-177). However, some areas are less similar in that some indels have been removed with the BioEdit program to allow proper alignment of the sequences for better construction of the phylogenic tree. The presence of hypervariable regions of the 16S rRNA gene provides specific information for the species, and, therefore, is very important for the identification of the latter. This figure allows us to see that this gene is less conserved at the beginning and very conserved in the middle and allowed the identification of the species and the genus.

Phylogenetic analysis

Figure 7 below shows the phylogenetic tree constructed from the nucleotide sequences of the 16S rRNA gene from strains isolated from soils. The homologous sequences were uploaded in FASTA format via GenBank. The Bio Edit program did alignment of all sequences (query sequences and peers). The phylogenetic tree was performed using MEGA version 7. In this work, we have used ten query sequences of different kinds. For each genus, a closest homolog was chosen and the phylogenetic tree was constructed based on the query sequences and their closest counterparts. We note that, the evolutionary history was deduced using the UPGMA method (Sneath *et al.,* 1973). The optimal tree with the sum of branch length = 5.18107810 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004) and are in the units of the number of base substitutions per site. The analysis involved 17 nucléotide séquences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 115 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.,* 2016).

The count of total aerobic mesophilic flora on PCA varies greatly from sample to sample. It shows that, the values are between (12 to 22.65) 10^2 CFU / g. the total aerobic mesophilic flora is higher in the samples from the Port of Yoro and Boulevard Alfred Raoul than those from the Campus la forêt and the Faculty of Sciences and Techniques. These results are similar to those found by Soloka (2020); Ngo-Itsouhou (2020), who, working respectively on soils and seeds of NTETE squash in Brazzaville, demonstrated that the total aerobic mesophilic flora of soils and seeds of NTETE squash varies from one sample to another. As soil is a reservoir of biodiversity, this is confirmed by the fact that the soil is home to many species, among so many other microorganisms. The quantitative microbial diversity from one soil sample to another can be explained simply by the fact that environmental conditions depend from one point to another. We also did the enumeration on mossel medium which is a specific medium for the isolation of bacteria of the genus Bacillus. The count on this medium shows that the values of bacteria of the genus Bacillus vary from (20.5 to 12) 10^4 CFU / g. The quantity of bacteria of the genus Bacillus are more numerous in the samples from the Port of Yoro and Campus I than those from the samples from Boulevard Alfred Raoul and the Faculty of Science and Technology.

Numerous studies on the ground (Anderson *et al.,* 1994; Ngo-Itsouhou *et al.,* 2020) have also shown that the soil contains bacteria of the genus Bacillus. Our results on bacteria of the genus Bacillus shows that, all bacteria are catalase positive, Gram +, rod-shaped, isolated, grouped in two or in clusters, all yellow in colour. This phenotypic identification collaborates with that found by (Etienne *et al.,* 2020).

For the cellulolytic activities, Figure: 1 (A, B, C, D, E, F, G, H) presents the demonstration of the production of cellulolytic enzymes of the isolates obtained from the soils by the appearance of a zone clear whose hydrolysis diameter synonymous with the hydrolysis of cellulose. This figure shows that our isolates have a halo or clear zone (12 to 32 mm in diameter) on the petri dish containing cellulose as a source of carbon plus agar around the well. These results allow us to state that our isolates synthesized cellulase which is a synergistic enzyme used to break down cellulose into glucose or other oligosaccharide compounds by hydrolysis of its β (1-4) glycosidic bond. They are produced by various fungi, bacteria, protozoa, plants and animals (Xu *et al.,* 2002). Our results are close to those found by (Sauvageon *et al.,* 2012) who, working on the hydrolysis of maize fertilizers by cellulases produced by *Aspergillus niger* and Penicillium sp which obtained a hydrolysis zone of 30 mm by *Aspergillus niger* and of 16 mm by *Penicillium decumbens*. Our results are similar to those found by (Moïse *et al.,* 2020) who conducted this study on bacteria of the genus *Lysinibacillus louembei* isolated from Ntoba-Mbodi have the capacity to secrete cellulolytic enzymes.

Our results on cellulolytic enzyme production are similar to those found by Ngo-Itsouhou *et al.,* (2019) who working on bacteria of the genus *Bacillus* isolated from soils, demonstrated that bacteria of the genus *Bacillus* have the potential to produce cellulases. Our results are also similar to those found by Hind *et al.,* (2013); Fergani *et al.,* (2015), who, working on isolated fungal strains Strains isolated from extreme environments show the ability of these mussels in particular the genus Trichoderma to produce cellulase.

Table.1 Stations, GPS coordinates, pH, and bacterial count in UFC/g of the different samples collected in the city of Brazzaville.

Samples	Sampling Stations	pH	Longitude latitude	UFC/g	
				PCA	Mossel
1	Faculty of Sciences and Techniques	6,5	179.56568526223E 85.766725306283N	12,4 ± 2,25(10^3)	16,5 ± 1,5(10^5)
2	Alfred Raoul Boulevard	6,5	179.47831417974E 85786865397005N	22 ± 2,2(10^3)	18 ± 1,8(10^5)
3	Yoro Port	7	178.89485778543E 85.808834987185N	22,65 ± 2,26(10^3)	20,55 ± 2,05(10^5)
4	Campus I	6,5	179.66937801832E 85.775522073034N	16,65 ±1,65(10^3)	20,5 ± 2,05(10^5)

Table.2 Phenotypic Characteristics of isolates of this study

Isolate	Morphologic Characteristics					Biochemical characteristics			
	colonies			Isolate cells					
	Colony coulour	Colony form	Colony aspect	Mobility	Form	Catalase	Gram	KOH	Sporulation
Faculty of Sciences and Technique									
F1	Whitish	Polylobed	Stichy	Motionless	Stick	+	+	-	+
F2	Whitish	Circular	Sticky	Mobile	Round	+	+	-	-
F3	Whitish	Circular	Sticky	Mobile	Round	+	+	-	+
F4	Whitish	Polylobed	Sticky	Mobile	Stick	+	-	+	-
F5	Whitish	Circular	Sticky	Motionless	Round	+	-	+	+
F6	Whitish	Circular	Sticky	Motionless	Round	+	+	-	-
F7	Whitish	Polylobed	Sticky	Motionless	Round	+	+	-	+
F9	Whitish	Polylobed	Sticky	Motionless	Stick	+	+	-	-
F10	Whitish	Circular	Sticky	Mobile	Stick	+	+	-	-
M1	Yellow	Circular	Sticky	Mobile	Stick	+	+	-	+
M2	Yellow	Circular	Sticky	Mobile	Stick	+	-	+	+
M3	Yellow	Circular	Sticky	Mobile	Stick	+	+	-	-
M4	Yellow	Circular	Sticky	Mobile	Stick	+	+	-	+
M5	Yellow	Circular	Sticky	Mobile	Stick	+	+	-	-
M6	Yellow	Circular	Sticky	Mobile	Stick	+	+	-	+
M7	Yellow	Circular	Sticky	Mobile	Stick	+	+	-	+
Alfred Raoul Boulevard									
B1	Yellow	Circular	Sticky	Motionless	Round	+	-	+	+
B2	Yellow	Circular	Sticky	Mobile	Round	+	-	+	+
B3	Yellow	Circular	Sticky	Mobile	Round	-	+	-	+
B4	Yellow	Circular	Sticky	Motionless	Stick	+	+	-	+
B5	Yellow	Circular	Sticky	Motionless	Stick	+	+	-	+
B6	Yellow	Circular	Sticky	Motionless	Round	+	-	+	+
B7	Yellow	Circular	Sticky	Mobile	Stick	+	+	-	+
B8	Yellow	Circular	Sticky	Motionless	Stick	+	-	+	+
B9	Yellow	Circular	Sticky	Motionless	Stick	+	+	-	+

B10	Yellow	Circular	Sticky	Motionless	Stick	+	+	-	+
B11	Yellow	Circular	Sticky	Motionless	Stick	+	-	+	+
B12	Yellow	Circular	Sticky	Motionless	Stick	+	+	-	+
B13	Yellow	Circular	Sticky	Motionless	Stick	+	+	-	+
B14	Yellow	Circular	Sticky	Motionless	Stick	+	-	+	+
B15	Yellow	Circular	Sticky	Motionless	Stick	+	+	-	+
B16	Yellow	Circular	Sticky	Motionless	Stick	+	+	-	+
B17	Yellow	Circular	Sticky	Motionless	Stick	+	+	-	+
B18	Yellow	Circular	Sticky	Motionless	Stick	+	+	-	+
B19	Yellow	Circular	Sticky	Motionless	Stick	+	+	-	+
B20	Yellow	Circular	Sticky	Motionless	Stick	+	-	+	+
B21	Yellow	Circular	Sticky	Motionless	Stick	+	+	-	+
B22	Yellow	Circular	Sticky	Motionless	Round	+	-	+	+
B23	Yellow	Circular	Sticky	Mobile	Stick	+	-	+	-
B24	Yellow	Circular	Sticky	Mobile	Stick	+	-	+	+
B25	Yellow	Circular	Sticky	Motionless	Stick	+	-	+	-
B26	Yellow	Circular	Sticky	Motionless	Round	+	+	-	+
B27	Yellow	Circular	Sticky	Motionless	Stick	+	+	-	+
B28	Yellow	Circular	Sticky	Motionless	Round	+	+	-	-
B29	Yellow	Circular	Sticky	Motionless	Stick	+	+	-	+
B30	Yellow	Circular	Sticky	Motionless	Stick	+	+	-	+
B31	Yellow	Circular	Sticky	Motionless	Stick	+	-	+	+
B32	Yellow	Circular	Sticky	Motionless	Stick	+	-	+	+
B33	Yellow	Circular	Sticky	Motionless	Stick	+	+	-	-
B34	Yellow	Circular	Sticky	Motionless	Stick	+	+	-	-
B35	Yellow	Circular	Sticky	Motionless	Stick	+	+	-	+
B36	Yellow	Circular	Sticky	Motionless	Stick	+	+	-	+
Yoro Port									
Y1	Yellow	Circular	Sticky	Mobile	Stick	+	+	-	+
Y2	Yellow	Circular	Sticky	Mobile	Stick	+	-	+	-
Y3	Yellow	Circular	Sticky	Mobile	Stick	+	-	+	-
Y4	Yellow	Circular	Sticky	Motionless	Stick	+	+	-	+
Y5	Yellow	Polylobée	Sticky	Motionless	stick	+	-	+	+
Y6	Yellow	Circulaire	Sticky	Motionless	Stick	+	-	+	+
Y7	Yellow	Circulaire	Sticky	Motionless	Stick	+	-	+	+
Y8	yellow	Circulaire	Sticky	motionless	Stick	+	-	+	-
YP1	Whitish	Polylobed	Sticky	Mobile	Round	+	+	-	+
YP2	Whitish	Polylobed	Sticky	Mobile	Stick	+	-	+	-
YP3	Whitish	Polylobed	Sticky	Motionless	Stick	+	-	+	+
YP4	Whitish	Polylobed	Sticky	Motionless	Round	+	+	-	-
Campus I									
C1	Whitish	Circular	Sticky	Mobile	Stick	+	+	-	+
C2	Whitish	Circular	Sticky	Mobile	Stick	+	+	-	+
C3	Whitish	Circulr	Sticky	Mobile	Stick	+	+	-	+
C4	Whitish	Circular	Sticky	Mobile	Stick	+	+	-	+
C5	Whitish	Circular	Sticky	Mobile	Stick	+	+	-	+
C6	Whitish	Circular	Sticky	Mobile	Stick	+	+	-	+

C7	Yellow	Circular	Sticky	Mobile	Stick	+	+	-	+
C8	Yellow	Circular	Sticky	Mobile	Stick	+	+	-	+
C9	Yellow	Circular	Sticky	Mobile	Stick	+	+	-	+
C10	Yellow	Circular	Sticky	Mobile	Stick	+	-	-	+
C11	Yellow	Circular	Sticky	Mobile	Stick	+	+	-	+
C12	Yellow	Circular	Sticky	Mobile	Stick	+	+	-	+
C13	Yellow	Circular	Sticky	Mobile	Stick	+	+	-	+
C14	Yellow	Circular	Sticky	Mobile	Stick	+	+	-	+
C15	Yellow	Circular	Sticky	Mobile	Stick	+	+	-	+
C16	Yellow	Circular	Sticky	Mobile	Stick	+	+	-	+
C17	Yellow	Circular	Sticky	Mobile	Stick	+	+	-	+
C18	Yellow	Circular	Sticky	Mobile	Stick	+	+	-	+
C19	Yellow	Circular	Sticky	Mobile	Stick	+	+	-	-
C20	Yellow	Circular	Sticky	Mobile	Stick	+	+	-	+

Figure.1 Assay of cellulolytic enzyme production by halo around the well for different bacterial isolates of this study.

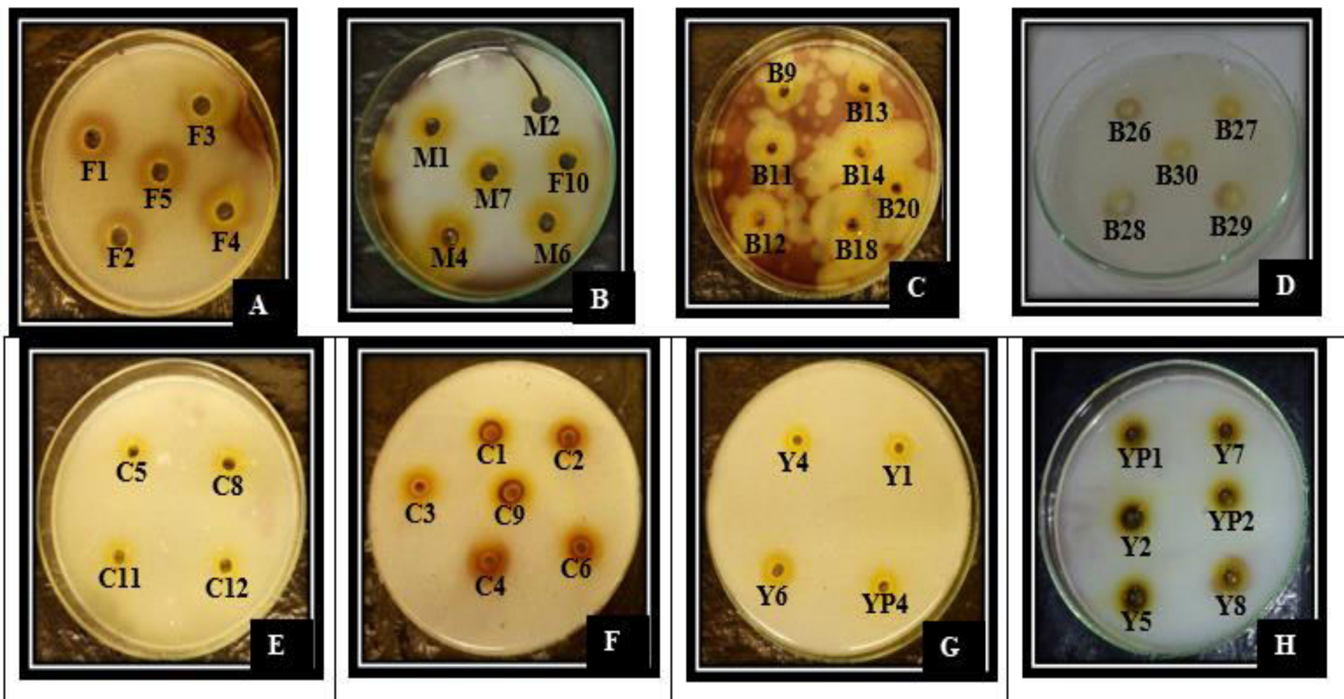


Table.3 Molecular Identification of strains by 16S rDNA similarity using BLASTn

Sequencing Code of isolates	Max score	E. value	% of similarity	Equivalent strain in GenBank	Accession numbersof equivalent strains GenBank
C5	492	7e-135	100.00%	*Bacillus subtilis*	KJ655538.1
C10	466	3e-155	100.00%	*Pseudomonas monteilii*	KPO56325.1
M2	2509	0.0	100.00%	*Pseudomonas aeruginosa*	MN911373.1
Y6	2480	0.0	100.00%	*Pantoea dispersa*	MT367860.1
B7	1502	0.0	97,50%	*Lysinibacillus fusiformis*	MN904872.1
B3	86.1	1e-12	89.55%	*Enterococcus faecium*	MH476286.1
B5	117	4e-22	97.14%	*Bacillus subtilis*	KY905360.1
C13	472	1e-128	91.38%	*Botryllus schlosseri clone*	KR911865.1
YP1	1687	0.0	96.75%	*Staphylococcus saprophyticus*	MN173448.1
YP4	1967	0.0	98.39%	*Staphylococcus edaphicus*	MW033681.1

Table.4 Accession numbers in GenBank of new bacterial strains identified in this study

Identified bacterial strains	New Accession numbers
***Pantoea dispersa* strain MLTBY6**	MT646430.1
***Pseudomonas aeruginosa* strain MLTBM2**	MT646431.1
***Bacillus subtilis* strain MLTBC5**	MT674681.1
***Pseudomonas monteilii* strain MLTBC10**	MT674682.1

Figure.2 Profile of cellulolytic enzyme production of bacterial isolates from sample1-4

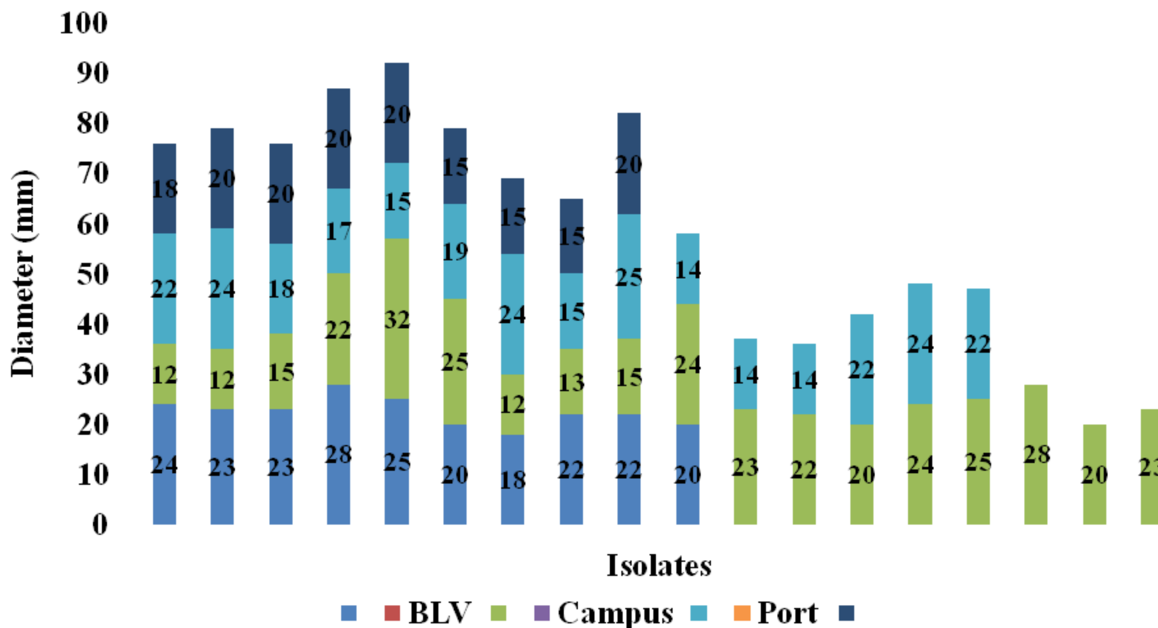
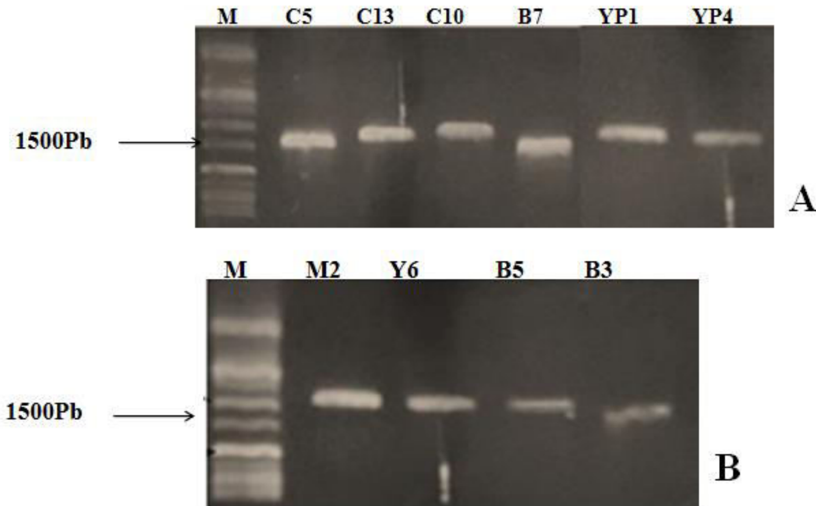
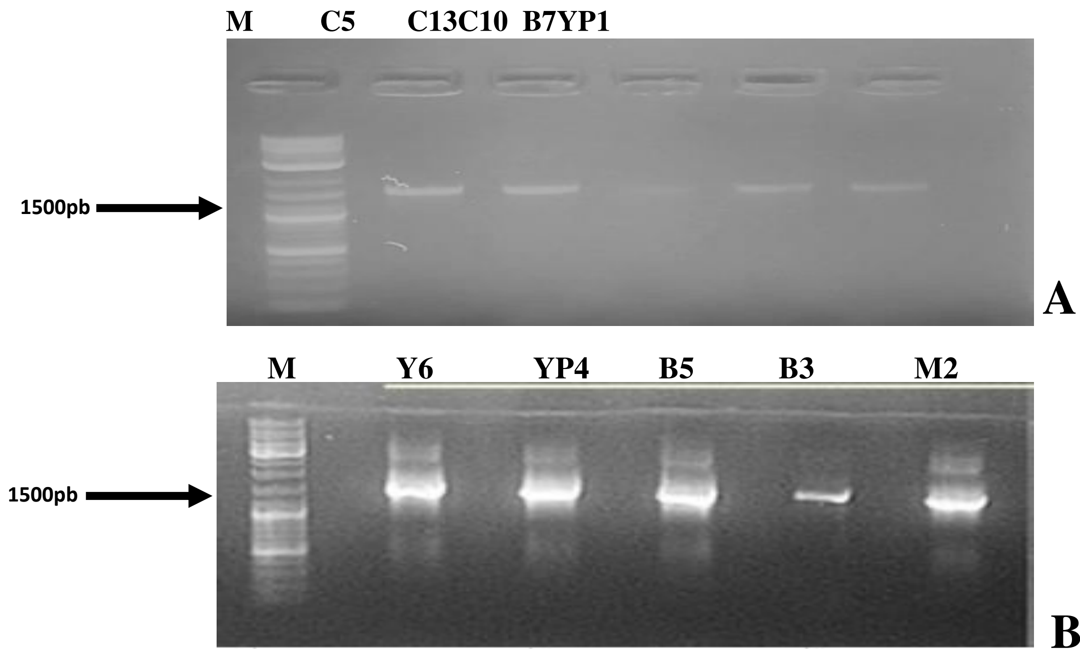


Figure.3 0,8% of Agarose Gel Electrophoresis of Genomic DNA of isolates in this study.



In A : M= DNA molecular weight marker ; fromC5 à YP4 DNA fragments.
In B : M=DNA molecular weight marker; M2 à B3 DNA fragments.

Figure.4 1% of Agarose Gel Electrophoresis of PCR amplied 16rDNA fragments of isolates in this study
In A : M= DNA molecular weight marker; *C5= Bacillus subtilis, C13= Botryllus schlosseri clone, C10= Pseudomonas monteilii, B7= Lysinibacillus fusiformiset YP1=Staphylococcus saprophyticus*are DNA fragments.



In B : M= DNA molecular weighi marker, Y6= *Pantoea dispersa*, YP4= *Staphylococcus edaphicus,B5= Bacillus subtilis, B3= Enterococcus faecium,* M2= *Pseudomonas aeruginosa* are DNA fragments.

Figure.5 Part of the multiple alignment of the ten bacterial strains of this study : Y6 (*Pantoea dispersa*), C10 (*Pseudomonas monteilii*), C5 (*Bacillus subtilis*), M2 (*Pseudomonas aeruginosa*), B7 (*Lysinibacillus fusiformis*), B3 (*Entérococcus faecium*), B5 (*Bacillus subtilis*), C13 (*Botryllus schlosseri clone*), YP1 (*Staphylococcus saprophyticus*) et YP4 (*Staphylococcus edaphycus*) identified by the gene encoding 16S rRNA.

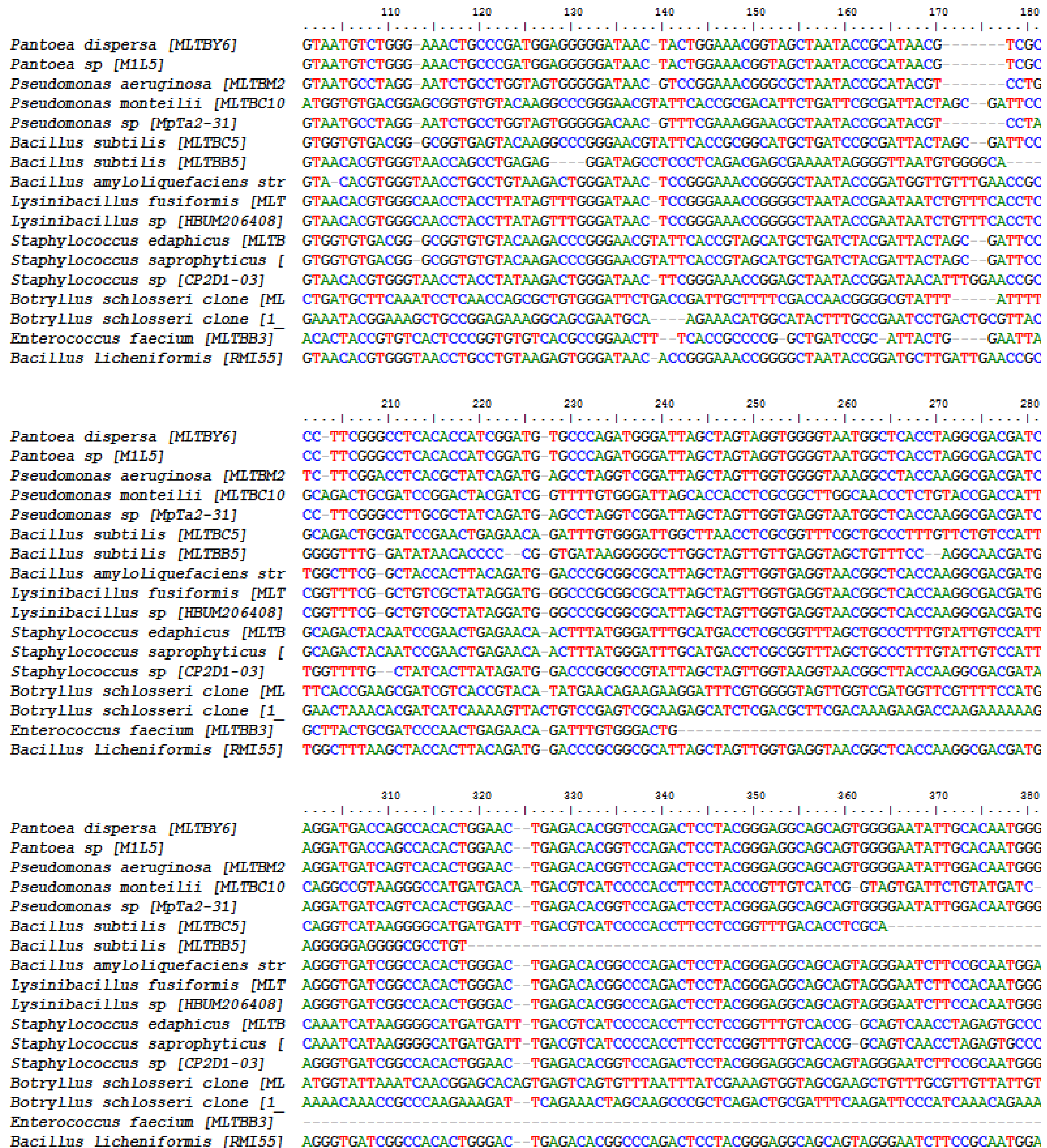
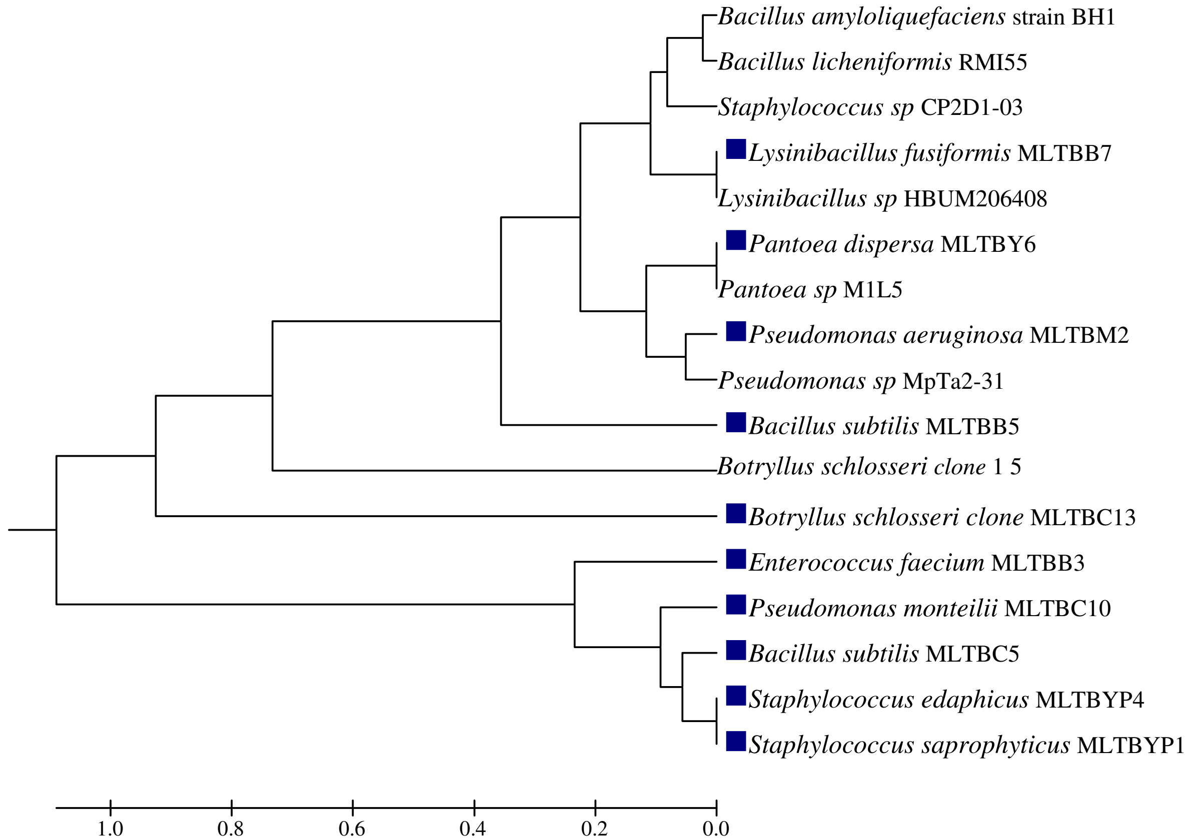


Figure.6 Phylogenetic tree constructed from the alignment of the nucleotide sequences of genes encoding 16S rRNA of strains obtained from soils and their homologues



In terms of molecular identification, electrophoresis on agarose gel at 0.8% of the genomic DNA and at 1% of the PCR products of the 16S rRNA genes of our isolates obtained from the soils represented in the figure. 3 (A, B, C) and Figure 4 (A, B, C) show bands up to 1500bp. This claim has long been endorsed by numerous studies which demonstrate not only the importance of 16S rRNA in the molecular characterization of prokaryotes, but also in the molecular identification of both genus and species (Dauga *et al.,* 1997); (Kim *et al.,* 2014); (Stackebrandt *et al.,* 1994). Molecular identification by the gene encoding 16S rRNA allowed us in this study to assess the microbial diversity of soils. This diversity has allowed us to obtain different genera among which: *Pseudomonas*, *Bacillus*, *Pantoea*, *Lysinibacillus*, *Enterococcus*, *Botryllus* and *Staphylococcus*. Our results with the 16S rRNA gene give a diversity of seven genera in soils, therefore more extended than in 30, also working on soils in Brazzaville and using the same techniques could only count two genera: Bacillus and staphylococci. Indeed, this difference is attributable to the different environments; each soil-sampling medium has different factors (pH, temperature, salinity, etc.). The 16S rDNA gene which encodes 16S rRNA (16S ribosomal RNA) is one of the best-conserved genes among prokaryotic organisms (*Eubacteria* and *Archaea*) (Soloka *et al.,* 2020). It was chosen as a phylogenetic marker by constituting an efficient and reliable basis of comparison to be able to both compare and differentiate between bacteria. Indeed, 16S rDNA comprises very conserved internal sequences which make it possible to select universal primers for the amplification of 16S rDNA from the majority of existing bacteria, such is the case in this study, these universal primers initially designated by Weisburg *et al.,* (1991), used here have also been used by Ngo-Itsouhou *et al.,* (2020); Soloka *et al.,* (2020).

Contains variable internal sequences, which, once analyzed, make it possible to distinguish between bacterial species and to classify them according to their phylogeny. It is in this perspective that we also use this technique to assess diversity and identify our isolates obtained from soils

The approximate size is approximately (~ 1500 bp) (Soloka *et al.,* 2020). The correlation of the identity percentages between 16S rDNA sequences (Laurent *et al.,* 1999) allows us to appreciate the sequences which share less than 97% similarity correspond to different species. This technique has greatly contributed in the classification and identification of actinomycetal bacteria (Kim *et al.,* 2003). Through 16S RNA sequencing, some non-mycelial bacterial genera have been included in the order Actinomycetales, while others have been excluded (Labeda *et al.,* 2012); (Soloka *et al.,* 2020).

The present study results agree with those found by Ahombo *et al.,* (2020); Ngo-Itsouhou *et al.,* (2020); Fossou *et al.,* (2012); Moutou *et al.,* (2018) who, working with pumpkin seeds, soils and sewage have also shown that identification by 16S rRNA can assess bacterial diversity. But also who have proven that the size of the bands expected during the amplification of rRNA16 is 1500bp. On the other hand, our results do not agree with those found by Sauvageon *et al.,* (2012) who found bands by amplifying the 16S rRNA of 1380bp. The 16S rRNA being a very powerful marker of molecular identification, the size of the bands expected during amplification is 1500bp. From there, we can affirm that our amplification was well carried out; this is evidenced by figure 3, which shows a good alignment of the nucleotide sequences showing conserved and varied areas (Sneath *et al.,* 1973).

The present study sequences showed identity percentages ranging from 99.24% to 100% to those already existing in the NCI database. *Bacillus subtilis* 99.24% similarity, *Pseudomonas monteilii* 100% similarity, *Pseudomonas aeruginosa* 100% similarity, Pantoea dispersed 100% similarity. Table VII shows the correlation between E. value, Maxe score and percentage similarity for the strains. In view of our results, we find that, *Pseudomonas aeruginosa* and *Pantoea dispersa* have a low E. value, but have a very high percentage of similarity. The results were expressed as a percentage of similarity of the strains identified in the database.

Figure 8 shows the multiple alignment of the sequences of the strains identified by the gene encoding 16S rRNA according to different genera. This figure shows a variable part and a non-variable part, and allowed us to build the phlogenetic tree.

The present study results are similar to those found by Ngo-Itsouhou *et al.,* (2020) who working on strains isolated from soils in Brazzaville in the Republic of Congo showed that the multiple alignment of bacteria consisted of a variable zone and a non-variable zone.

Figure 6 shows the phylogenic tree of the ten strains including: *Pantoea dispersa*, *Pseudomonas monteilii*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Lysinibacillus fusiformis*, *Enterococcus faecium*, *Botryllus schlosseri clone*, *Staphylococcus saprophyticus* and newly obtained *Staphylococcus saprophyticus* and *Staphylococcus* sequences from the newly obtained *Staphylococcus saprophyticus* and *Staphylococcus saprophyticus*, and the newly obtained *Staphylococcus saprophyticus* and *Staphylococcus* obtained from the reference GeneBackeri. The phylogenic tree was constructed with MEGA 7 software (Kumar *et al.,* 2016). With regard to this phylogenic tree, we see that it is divided into three groups. A group containing the genus Pseudomonas, a group containing the genus Pantoae and a group containing the genus Bacillus. In view of these results, we notice a close relationship between the different isolates. The present study also find that, the group of the genus Pseudomonas is very close to the group of the genus Bacillus and have a common ancestor.

In view of the results obtained, we allow ourselves to conclude that, this study allowed us to count, isolate and characterize phenotypically but also to identify on the molecular level and to observe the cellulolytic potential of the various microorganisms in the soils. Out of a total of 84 isolates counted and characterized phenotypically, 10 were amplified by the 16S rRNA gene and then sequenced, four (4) of them were submitted in GenBanck and made it possible to distinguish by phylogeny seven genera including: the genus *Bacillus*, genus *Pseudomonas*, genus *pantoea*, genus *Staphylococcus*, genus *Enterococcus*, genus *Lysinibacillus* and genus *Botrylus*. 52 of these isolates exhibited cellulolytic activity. In short, rRNA16S remains after all one of the very powerful tools for molecular identification as well as microbial biodiversity in soils.

Author Contribution

Irène Marie Cécile Mboukou Kimbatsa: Investigation, formal analysis, writing—original draft. Thantique Moutali Lingouangou: Validation, methodology, writing—reviewing. Léa Gwladys Gangoue:—Formal analysis, writing—review and editing. Itsouhou Ngô: Investigation, writing—reviewing. Faly Armel Soloka Mabika: Resources, investigation writing—reviewing. Joseph Goma-Tchimbakala: Validation, formal analysis,

writing—reviewing. Etienne Nguimbi: Conceptualization, methodology, data curation, supervision, writing—reviewing the final version of the manuscript.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval: Not applicable.

Consent to Participate: Not applicable.

Consent to Publish: Not applicable.

Conflict of Interest: The authors declare no competing interests.

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