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Isolation and Characterization of Bacteria Involved in *Daniellia oliveri, Ficus sycomorus*, *Hymenocardia acida* and *Terminalia glaucescens* Leaf Litter Decomposition and their Hydrolytic Enzyme Potentials

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Authors' contributions

This work was carried out in collaboration among all authors. Author GNB designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors STT and AN performed the labaratory analysis, the statistical analysis and correct the manuscript. Author JTM and PM managed the decomposition experience on the field. Author AI mananaged the literature searches and supervised all the study. All authors read and approved the final manuscript.

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ABSTRACT

The effect of microorganisms particularly those of bacteria on litter decomposition in the tropical savannahs of Adamawa Cameroon is poorly investigated. Litter decomposition was conducted in the field on the litter of *Ficus sycomorus, Terminalia glaucescens, Daniellia oliveri,* and *Hymenocardia acida*, and the dynamic of bacterial populationas well as their enzymatic activity during degradation of leaf litters was studied. For this purpose, bacteria were isolated from the

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litterof these plants at the initial time (zero), 12 and 24 weeks of degradation by serial dilution method and spreading onto nutrient agar culture medium. Identification of bacteria was done under photonic microscope after Gram staining and the production of enzymes was carried out on specific media. Results indicate that: 25 bacteria were isolated from distinct phenotypic characteristics on nutrient agar. Based on morphological appearance as observed under light microscope, 24% of isolates were found to be single bacilli; 28% bacilli cluster; 16% were found to be streptobacilli and 32% diplococci. For sugar fermentation test (TSI), 20% of isolates produced gas and acidic reaction, 40% produced acidic butt reaction, while only 20% produced H_2S gas ; 76% of isolates were positive to citrate utilization (SIM) test; 20% of isolates were positive to catalase production test, whereas only 20% of isolates were motile. The aforementioned study of phenotypic, microscopic and biochemical enabled identification of two genera: the genus *Bacillus* and the genus *Micrococcus* representing respectively 68 and 32% of the isolates. The genus *Bacillus* consisted of *Bacillus cereus* (41.17%, with 7 phenotypes), *Bacillus megaterium* (17.64%, with 3 phenotypes), *Bacillus subtilus* (35.29%, with 6 phenotypes) and *Bacillus* sp. (5.88%, with 1 phenotype). The genus *Micrococcus*re grouped 08 phenotypes. The relative abundance of isolates demonstrated that in all leaf litters and soils, there was a great diversity of bacterial isolates at the initial time and then a considerable decrease in this diversity during litters degradation. The genus *Bacillus* was generally dominant at all decomposition periods of different plant species. A total of 64% of bacteria isolates produced at least one enzyme. 36% of the isolates produced amylase, 56% produced cellulase, 40% produced esterase, 48% produced lipase, while 48 % of the isolates produced protease. This strong enzymatic activity of the isolated bacteria suggests their competences in the degradation process of leaf litter, and therefore, the interest in exploiting them in litter degradation units for an efficient production of organic fertilizer.

Keywords: Bacteria; extracellular enzymes; leaf litter decomposition; organic fertilizer; sudano-guinea savannah of Adamawa; Cameroon.

1. INTRODUCTION

In Cameroon especially in the northern part of the country, agricultural activities are important for food security and remain the main socio-economic activity in the region. However agricultural production remains low, partly due to the low quality of inputs, diseases and pests, but above all to the declined soil fertility [1,2,3,4,5]. Therefore, it is important to explore effective and less expensive methods to improve soil fertility in this region. One promising issue is the use of trees particularly their litters to improve soil fertility. Trees improves soil nutrient balance by reducing unproductive nutrient losses from erosion and leaching and by increasing nutrient inputs through nitrogen fixation and increase biological activities by providing biomass and suitable microclimate [6].

Litter fall is a major pathway for the return of organic matter and nutrients from aerial parts of plant communities to the soil surface [7], which fertilizes and protects ground surfaces from the scouring actions of rainfall, by reducing soil erosion [8]. Litter fall measurement has been a standard non-destructive technique for assessing the productivity, phenology, and turnover of biomass in a plant community, where the biomass of the litter and its chemical content (including heavy metals) quantify the annual return of organic matter and nutrient elements to the soil [9].

Ficus sycomorus, Terminalia glaucescens, Daniellia oliveri, and *Hymenocardia acida* are among the most socio-economic important trees species recognized for their importance in restoring soil fertility of Guinean savannah Highlands of the Adamawa (Cameroon) [3,4,10].The litter of these trees species can be used in farming systems to improve soil fertility. It is recognized that leaf litter of these tree species effectively contribute to soil fertility in African savannah areas [11,12].

In general, an increase in soil fertility is positively associated with leaf litter quality, nutrient concentrations leaves tissues, decomposition rates, and nitrogen mineralization [13]. Also nutrient availability plays an important role in the ability of plants to withstand environmental stress. High nutrient levels contribute to maintaining osmotic pressure in leaves, enabling plants to withstand a higher degree of drought stress [14]. Leaves are chemically composed of lignocellulosic substances that have to be broken down by microorganisms to maintain the carbon

cycle [15]. Phylloplane microorganisms are chemoorganotrophic species requiring organic nutrients for growth [16]. Microbial decomposers, namely fungi and bacteria, produce an array of enzymes that transform plant litter into a more suitable food source for stream detritivores. Microbial enzymes, involved in the degradation and transformation of plant cell-wall polysaccharides, have undergone many biotechnological applications [17].

In the natural environment, both bacteria and enzymes they produce play a significant role in the biodegradation of plant litter. Bacteria produce a wide range of cell wall-degrading enzymes which include pectinases, ligninases, cellulases and amylases. These enzymes are essential in the decay of dead plant material and thus assist in recycling carbon compounds in the biosphere. Enzymes facilitate the phase of metabolism in which complex compounds are broken into simple ones and these enzyme activities have been used widely as an index of soil fertility or ecosystem status [18].

Plants are attractive hosts for microorganisms because they provide a variety of nutrients. Plant-associated microorganisms can be considered as pathogens, mutualists, or commensals [19]. With this range of associations, microbial hydrolytic enzymes are essential to harm or benefit the plant. According to Jalgaonwala and Mahajan [20], the order of enzymatic activities of microorganisms from plants is as follows: proteolytic >amylolytic > cellulolytic > esteratic.

Litter degradation freely in the nature takes moreless too long. It is therefore important to degrade the litter under controlled conditions in order to reduce the degradation time, optimize the biomass and use it as organic fertilizer. To achieve this it is crucial to characterize organisms involved in the degradation of litter in order to produce them at large scale for the degradation of the litter.

Effect of soil organism communities and soil microorganisms in the litter decomposition process have been well studied for tropical forest and temperate ecosystems [21,22,23,24,25]. However, little information was available for tropical savannahs particularly those of Adamawa Cameroon. This study was investigated to describe the successive changes in the bacterial community composition as a response of changes occurring in leaf litter decomposition of *Ficus sycomorus*, *Terminalia glaucescens*, *Daniellia oliveri, and Hymenocardia acida*, which are locally abundant and endemic to the soudano-guinea savanna zone, at three time periods (zero, 12 and 24 weeks) of decomposition, and then, screen the ability of isolated bacteria for hydrolytic enzymes production.

2. MATERIAL AND METHODS

2.1StudyArea

Trials were conducted in the Adamawa region (6 - 8°N, 12 - 15°E, altitude 1200 m asl). This geographical situation gives at this region a Sudano-guinea climate type with one dry season (November - March) and a rainy season (April - October). The mean annual rainfall is about 1500 mm, the mean annual temperature is approximately 22°C and the mean relative humidity about 69% [10]. The seasonally arid situation of Adamawa is due to the influence of the harmattan which recalls the harsh climatic conditions of the Sudano-sahelian savanna, while its rainfall and its thermal amplitude recall the humid subequatorial regions [26]. The rock is made up of a metamorphic pedestal composed of granites and Pan-African gneiss cut up by syenites and the volcanic formations of mio-pliocene period, leading to formation of various soil types [27].The area of this region is covered with either the shrub savannah or the tree savannah, dominated by *Daniellia oliveri* and *Lophira lanceolata* [10].

2.2 Leaf Litter Decomposition

Litter decomposition experiment was conducted in Ngaoundere III sub-division within the Ngaoundere University precisely behind the dean office. Fresh fallen leaf litters were collected directly from the Ngaoundere humid savannahs, close to the University, during the maximum leaf fall period (November 2016 - March 2017).

Study was carried out on the field by using litterbag technic according to Bocock and Gilbert [28] method, and consisted of a nylon material with a 2 mm mesh [29]. Bags were of different sizes according to litter type to avoid the compression of the material, and thus creating artificial conditions in the litterbags. In total, one hundred and forty-four (144) litterbags in a split-plot design (4 species x 18 replications x 2 plots) were each filled with 10 ± 0,01 g of the leaf litter

and placed on soil top of the both plots, during 24 weeks (running from April to November 2017). The litterbags were slightly covered with vegetation litter to avoid destruction of litterbags by animals.

2.3 Leaf Litter Sampling

During the course of decomposition, litter was sampled at three time points: t_i = no litter degradation, t_{12} = 12 weeks of litter decomposition, and t_{24} = 24 weeks of litter degradation, because we expected changes in bacteria community composition in line with leaf decomposition.

To achieve this work, a total of 12 leaf litter and two soils samples were collected as follow; for leaf litter, 10 g were randomly sampled in each plant leaf litter decomposition block at the three time points above-mentioned. However, for soils collections, 10 g of composite soils were randomly sampled on the site before the implementation of the litter decomposition experiment, and 10 g others at 24 weeks (t_{24}) of leaf litter decomposition after removing the device.

Each sample was collected in sterile jar maintained in aseptic conditions and marked accordingly, based on the source and date of collection. The collected samples were brought to the laboratory for bacteria isolation and their biochemical characteristic.

2.4Isolation of Bacteria from Samples

Serial dilution techniques were used for the isolation of bacteria. In this technique sample suspension was prepared by adding 5g of each sample to 50ml of sterile saline water (0.85% w/v) and shaken vigorously for at least 20 minutes. Each dilution was then sedimented for 5 minutes. Sterile dilution blanks of 9 mL of saline solution were marked sequentially starting from 10^{-1} to 10^{-4}. 1 ml from each stock suspension was transferred to the 10^{-1} dilution blank using a sterile pipette. 1 ml from the 10^{-1}dilution was transferred to the 10^{-2} tube for each succeeding step then from the 10^{-2} to the 10^{-3}, then from the10^{-3} to the 10^{-4}. From each dilution tube 0,5ml of dilution was transferred into nutrient agar culture media by flooding technic and incubated at 37°C for 24 hours. Nutrient Agar (NA) culture media contained 0,5% peptone,0,3% yeast extract, 0,5% NaCl, 0,25% glucose, 1,5% agar, distilled water and pH was adjusted to 7±0,2 at room temperature. After successful growth of microorganisms the pure cultures of bacteria were subculture inHAlants; incubated at 37°C to achieve vigorous growth [30].

2.5 Assesment of Micromorphological and Biochemical Characteristic of Isolated Bacteria

Cultural characterization of colonies on Nutrient agar medium and Gram stain were performed to observe the colonies and cellular morphologyand gram nature of the bacteria, motility and biochemical characterizationof the strains were also carried out. The biochemical tests of Catalase production; Triple sugar iron, and citrate utilizationwere performed as described by Saha and Santra [30].

2.5.1 Cultural characterization of colonies

Pure colonies of bacteria isolates were characterized according to their shape, texture elevation and color on Nutrient agar medium for cultural characterization [31].

2.5.2 Gram type and micromorphology determination

Gram type determination was conducted on 48 hour old cultures of bacteria isolates colony according to the standard staining technique as described by Ayitso et al. [31]. A thin smear of pure isolates colony was made on a clean glass slide, dried in air and fixed by passing through flame of a burner. The smear was covered with crystal violet, kept for one minute. The slide was washed with water, then covered with Gram iodine and let stand for one minute. The slide was again washed with water. Decolorized with alcohol, was achieved by rocking the slide gently for twenty seconds till the violet colour came off the slide and then washed with water immediately. This later counterstained with safranin for twenty seconds. Washed with water, blot dried and then examined under the oil immersion lens of microscope at 100X. Gram nature and micromorphology of bacteria isolates were then recorded.

2.6 Biochemical Characterization of Isolates

2.6.1 Determination of catalase activity

All isolates obtained were screened for catalase activity after two days of subculture on newly

fresh nutrient agar following the method of Mahon et al. [32] using the slide (drop) method. Positive reactions were evident by immediate effervescence (bubble formation).

2.6.2 Citrate utilization test

Simmons citrate agar (SIM) was used to differentiate bacteria that have the ability to consume citrate as its sole source of carbon and ammonium as sole nitrogen source. Pure colonies of bacteria were inoculated on nutrient agar for twenty four hours. Then fresh pure colonies were streaked using a sterilized inoculating loop on the surface of the Simmons citrate agar slant with a light inoculum. The tubes were then incubated at 30℃ for 48 hours with loose caps [33].

2.6.3 Triple sugariron (TSI) test

Triple sugar iron was used to differentiate the microorganisms based on dextrose, lactose and sucrose fermentation and hydrogen sulfide production. This medium was prepared as agar slope and the pure colonies of bacteria were inoculated by stabbing into the butt using a sterilized straight wire loop and the surface of slope was inoculated by streaking over the entire surface of the slant and then incubated at 30 ºC for 48 hours with loose caps. Gas production was determined by cracking of the medium, formation of hydrogen sulphide was determined by the blackening of the whole buffer or a streak of ring of blackening at the slant butt junction. Glucose fermentation was determined by the yellowing of the butt. The fermentation of lactose or sucrose or both was determined by the yellowing of both the butt and the slant [33].

2.7 Screening of Bacteria Isolates for Extracellular Hydrolytic Activities

In order to detect the production of extracellular hydrolases, different enzymatic agar plate assays were performed as follow:

2.7.1 Determination of extracellular amylase activity

Starch agar medium was used to detect the amylase activity [34]. The assay medium inoculated with each bacteria isolate was incubated at 30℃ for 48 hours. After incubation, the amylolytic activity was detected by flooding the agar plates with Gram's iodine solution (2.0%). The appearance of clear zones around the growing colonies against dark blue was considered as positive and was measured in cm.

2.7.2 Determination of extracellular cellulase (CMCase) activity

For the determination of cellulase activity, colonies were plated on 1% CMC agar plates and the plates were incubated at 30 ^{o}C for 48 hours, after plates were flooded Grams Iodine (0.133 g KI and 0.067 g Iodine dissolved in 20 ml distilled water) and were incubated with the dye for 10-12 minutes and then washed with water. The circular clear zone appearing around the growth colony was measured in cm as cellulase activity [35].

2.7.3 Determination of lipase and esterase activities

Phenol Red Agar method (modified method of Narasimhan and Bhimba, [36]) determination of lipase and esterase activities was done with the help of phenol red agar plate method. The medium contained phenol red (0.01%), 20 or 80 (1%) for lipase or esterase activity respectively, 1.0 g of peptone, 0.5 g of NaCl, 0.01 g of $CaCl_2.5H_2O$, 7 g of $MgSO_4.7H_2O$, 7 g of KH_2PO_4, agar (2%) in 100 ml of distilled water. The pH was adjusted to 7.3 - 7.4 using 0.1 N NaO. After sterilization, the medium was poured into petriplates and after solidification, pure colonies of bacteria were inoculated in the center of the petriplate by the help of sterilized straight wire loop and the plates were incubated at 30℃ for 48 hours. The circular zone of yellow colour appeared around the colony growth was measured in cm as lipase activity.

2.7.4 Determination of extracellular proteolytic activity

The relative activity of protease production was detected for bacteria isolates on milk agar plate, containing basal salt of starch casein amended with 20% of skimmed milk, following the method of Jani et al. [37]. Bacteria were grown in the middle of the milk agar plate and incubated for 48 hours. Zones of casein hydrolysis (clear zones) measured in cm indicated positive results.

2.8 Keys of Identification and Statistical Analysis

Microbial counts were expressed as the number of colony forming units (CFU) per gram of leaf litter/soil. Bacteria including were identified

referring to Bergey's manual [38]. Values were treated with one way analysis of variance (ANOVA) to find out the difference between bacteria loads and Duncan analysis, to find out the significance of difference between the means of microbial load.

Enzymatic activity (EA) of different tested substrates performed in triplicates was examined. The growth diameter was measured and the clear zone representing enzyme activity was calculated by using the formula:

Enzyme Activity (EA) = (Diameter of tested substrate hydrolysis zone - Diameter colony) in cm, [39].

Based on the EA test, organisms were categorized into four groups; showing excellent activity: +++ = (EA > 2), good activity: ++ = (1 ≤ EA ≤ 2), poor activity: + = (EA < 1) and no activity: - = (EA = 0).

3. RESULTS

3.1 Bacteria Loadand Dynamics

The enumeration of bacteria population of leaf litters and the soil of the experimental site before the implementation of the litter decomposition experiment revealed that the number of bacteria degrading leaves was between 0.52 x 10^6 CFU/g and 1.41333 x 10^6 CFU/g, while the number of bacteria in soil was 2.25 x 10^6 CFU/g. The number of bacteria in the experimental soil was statistically higher ($p < 0.0001$) than those found in leaf litters.

At 12 weeks after experimental set up, there was an increase in the bacteria population in all the treatments compared to the initial substrates, the number of bacteria in treatments was between 0.8 x10^6 and 1.97333 x10^6 CFU/g of liter. The number of bacteria in *Ficus sycomorus* leaf litter was statistically higher ($p < 0.0001$) than that of other decomposing leaf litters; *Terminalia glaucescens* litter had the lowest number of bacteria. Compared to the initial state, increased bacteria population was more accentuated in leaf litters of *Ficus sycomorus* and *Hymenocardia acida*.

At the end of the litter decomposition experiment (24 weeks after the implementation of the decomposition device), the enumeration of bacterial population in the decomposed leaf litter and soil under these litters revealed that the number of bacteria in the litters was between 0.28 x10^6 CFU / g and 1.84667 x10^6 CFU/g, while the number of bacteria in soil was 1.36 x 10^6 CFU/g. The number of bacteria in the soil under experimentation was statistically lower ($p < 0.0001$) than in leaf litter of *Ficus sycomorus*. There was a decrease in the bacterial population compared to that after 12 weeks of decomposition (Fig. 1).

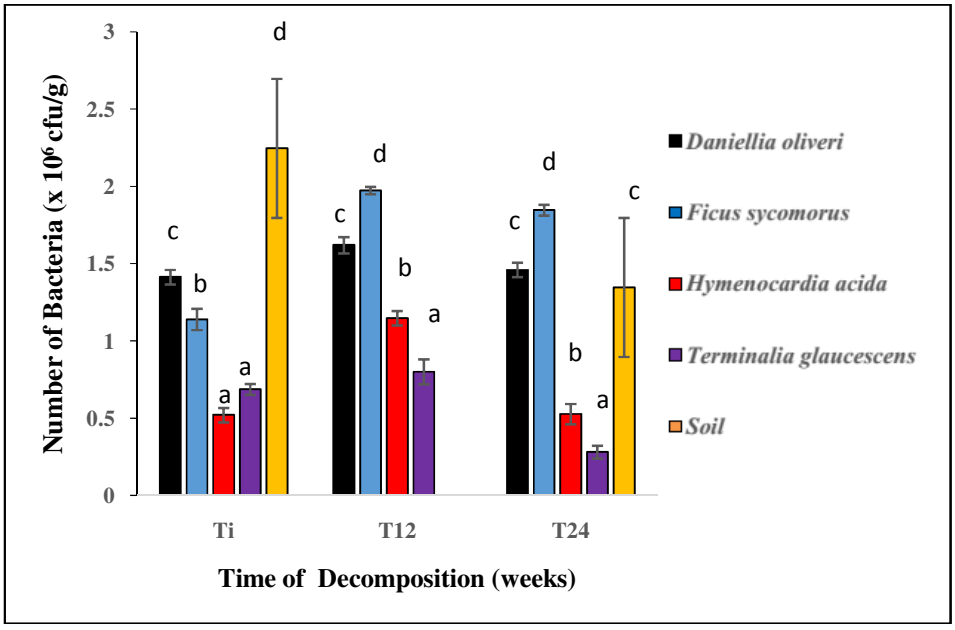

Fig. 1. Bacteria load in different leaf litter and soils at different period of decomposition

The dynamics of the bacteria population revealed in *Daniellia oliveri*there a statistically significant increase (p = 0.0536) in the number of bacteria at 12 weeks of litter decomposition. At 24 weeks of decomposition, there was a slight decrease in the number of bacteria approaching that of initial substrate. In *Ficus sycomorus*, there was a statistically significant increase (p < 0.0001) in the number of bacteria at 12 weeks of litter decomposition. At 24 weeks of decomposition, the number of bacteria remained was statistically higher than that of the initial substrate. In *Hymenocardia acida*, there was a statistically significant increase (p = 0.0002) in the number of bacteria at 12 weeks of decomposition of litter, but at 24 weeks of decomposition, there was a significant decrease in the number of bacteria approaching that of the initial substrate. In *Terminalia glaucescens*, no significant increase in the number of bacteria at 12 weeks of litter decomposition was observed. At 24 weeks of decomposition, the number of bacteria significantly decreased (p < 0.0014) compared to the previous periods.

The dynamics of bacteria population in the soil of the experimental site revealed a statistically significant decrease (p = 0.0106) in the number of bacteria in soil under decomposition of the litters, at 24 weeks of decomposition (Fig. 2).

3.2 Characterization and Identification of Bacterial Isolates

In this study, bacteria strains were isolated in Nutrient agar culture media; and preamble the cultural behaviors of isolates in this medium, bacterial isolates were selected as presenting distinct phenotypic characteristics. Details of the colony features of the bacteria are noted (Table 1). The colonies of isolates had concentric (12%), rough (8%) or smooth (80%) texture ; transluscent (20%), white (24%), whitish-cream (8%), white-cream (12%), whitish (12%), blue-green (4%), yellow-brown (4%), orange (4%), pink (4%) or red (8%) in colour ; They were circular (48%), Irregular (28%), Irregular and spreading (8%) and spreading or lobate (16%) in shape, flat (48%) or convex (52%) elevation.

Gram stain determination showed that all of the isolates were gram positive bacteria. Based on morphological appearance as observed under light microscope 24% of isolates were found to be single bacilli cells, 28% cluster of bacilli cells, 16% were found to be streptobacilli cells and 32% diplococci cells (Table 1). Different biochemical tests were also performed for all isolates to know their biochemical characteristics. It was observed concerning sugar fermentation (TSI) test that 20% of isolates produced gas and acidic reaction and 40% produced acidic butt

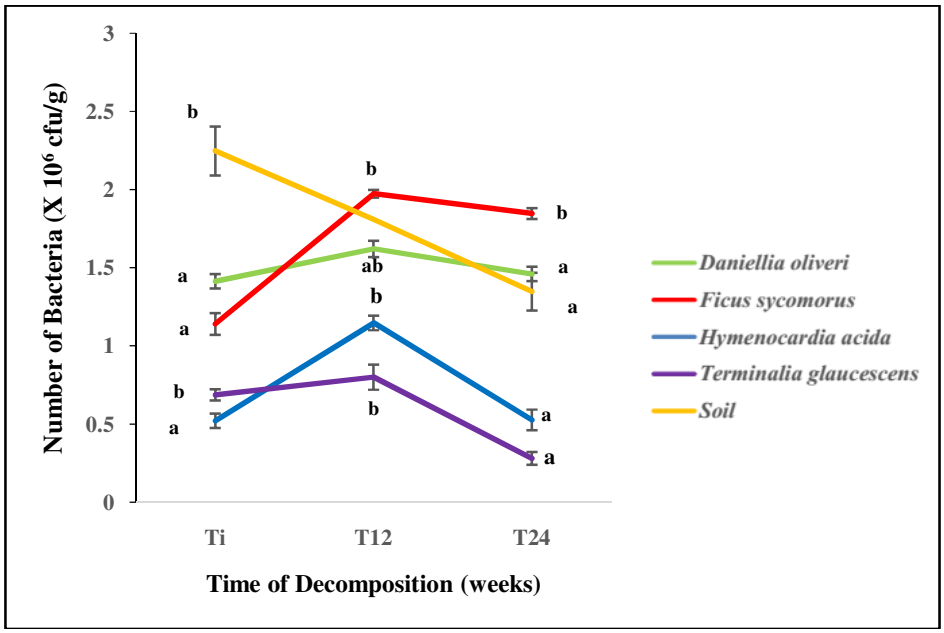

Fig. 2. Dynamic of bacteria population in different litters and soils during the course of decomposition

test, 20% produced H_2S gas (Fig. 5). However 76% of isolates were positive to citrate utilization (SIM) test (Fig. 6), 20% of isolates were positive to catalase production test, while 20% of isolates were positive to motility test (Table 1).

The aforementioned phenotypic, microscopic and biochemical characteristics have enable the identification of 25 isolates belonging to two genera; the genus *Bacillus* and the genus *Micrococcus* representing respectively 68 and 32% of the isolates. The genus *Bacillus* consisted of *Bacillus cereus* 41.17% (Fig. 4A, with 7 phenotypes), *Bacillus megaterium* 17.64% (Fig. 4B, with 3 phenotypes), *Bacillus subtilus* 35.29% (Fig. 4C, with 6 phenotypes) and *Bacillus* sp. 5.88% (Fig. 4D, with 1 phenotype), and the genus *Micrococcus* (Fig. 4E) had 08 phenotypes.

The relative abundance of these isolates demonstrated that in all leaf litters and soils, there was a great diversity of bacterial isolates at the initial time, and then a considerable decrease in this diversity during the litter degradation. *Daniellia oliveri* leaf litter was rich with 10 isolates, while that of *Terminalia glaucescens* was poor with 4 bacterial isolates at the initial time (Fig. 3).

In *Daniellia oliveri* litter, the genus *Bacillus* was dominant (representing 70% of the genera) at the initial time, then the two genera present (*Bacillus* and *Micrococcus*) tended to be equal at 12 and 24 weeks of litter degradation. In *Ficus sycomorus* litter, the dominance of genera was the equal at initial time, but the genus *Bacillus* became very dominant at 12 and 24 weeks of litter degradation. Concerning other litters and soils, a very strong dominance of the *Bacillus* genus (greater than 87%) was observed reaching 100% at 24 weeks of degradation in litter of *Terminalia glaucescens* and in soil. Only the isolates *Bacillus subtilus* (4) and *Bacillus cereus* (2) were common to all litters and were present up to 24 weeks of decomposition (Fig. 3).

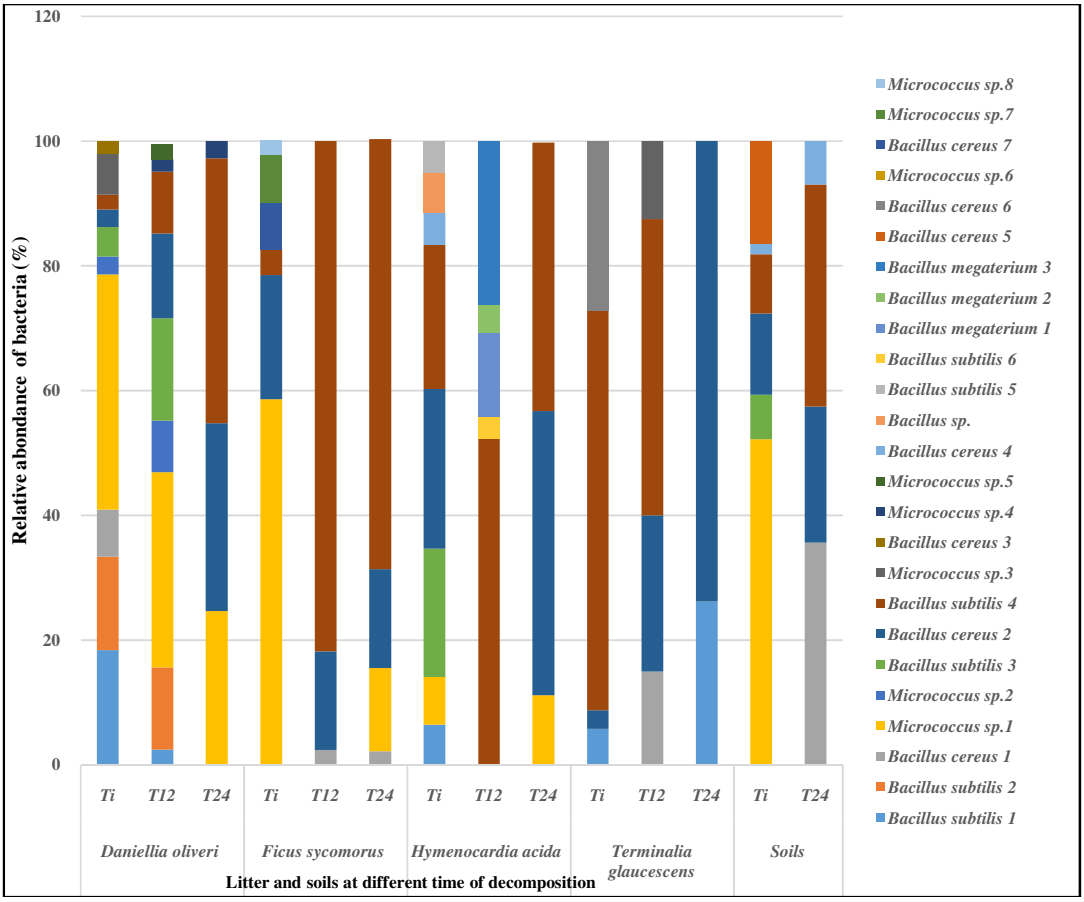

Fig. 3. Occurrence of the predominant bacterial genera, represented in percentage, in leaf litters and soils at different period of litter decomposition

Table 1. Characterization of bacteria isolated from Litters(based on descriptions of Saha&Santra, 2014 and Ayitso&Onyango, 2016)

isolates	Litter / Soil	Texture	Colour	Shape	Elevation	Gram stain	Bacilli	Bacilli cluster	Streptobacilli	Diplococci	SIM	TSI	H2S	Catalase	Motility	identification
A	DO, HA, TG	smooth	transluscent	Irregular and spreading	flat	+	+	-	-	-	+	Gasacidic	-	-	+	*Bacillus subtilis 1*
B	DO	smooth	white-creamy	circular	convex	+	+	-	-	-	+	Acidicbutt	-	+	+	*Bacillus subtilis 2*
C	DO,S, TG,FS	smooth	transluscent	circular	flat	+	-	+	-	-	+	Gasacidic	-	+	-	*Bacillus cereus 1*
D	DO,HA,S,FS	smooth	blue-green	circular	convex	+	-	-	-	+	+	-	-	+	-	*Micrococcus sp.1*
E	DO	smooth	transluscent	circular	convex	+	-	-	-	+	+	Gasacidic	-	-	+	*Micrococcus sp.2*
F	DO,HA,S	smooth	white-creamy	Irregular and spreading	flat	+	+	-	-	-	+	Gasacidic	-	-	-	*Bacillus subtilis 3*
G	DO,HA,,S, TG,FS	concentric	white	circular	flat	+	-	+	-	-	+	Acidicbutt	-	-	-	*Bacillus cereus 2*
H	DO,HA,S, TG,FS	smooth	white	circular	convex	+	+	-	-	-	+	-	+	+	-	*Bacillus subtilis 4*
K	DO	rough	transluscent	circular	convex	+	-	-	-	+	+	-	-	+	-	*Micrococcus sp.3*
L	DO	smooth	transluscent	lobate	convex	+	-	+	-	-	+	Acidicbutt	-	-	-	*Bacillus cereus 3*
M	DO	smooth	red	irregular	flat	+	-	-	-	+	-	Acidicbutt	-	-	-	*Micrococcus sp.4*
N	DO	smooth	red	circular	convex	+	-	-	-	+	+	Gasacidic	-	+	-	*Micrococcus sp.5*
T	HA	smooth	Yellow-brown	circular	convex	+	-	+	-	-	+	Acidicbutt	+	-	+	*Bacillus cereus 4*
U	HA	concentric	white	lobate	flat	+	-	-	+	-	-	Acidicbutt	+	+	+	*Bacillus sp.*
W	HA	smooth	whitish	irregular	convex	+	+	-	-	-	+	Acidicbutt	-	+	-	*Bacillus subtilis 5*
Y	HA	smooth	whitish	irregular	flat	+	+	-	-	-	+	Acidicbutt	-	-	-	*Bacillus subtilis 6*
Z	HA	smooth	white-creamy	lobate	flat	+	-	-	+	-	+	-	-	-	-	*Bacillus megaterium 1*
a	HA	smooth	whitish-creamy	irregular	convex	+	-	-	+	-	-	-	-	-	-	*Bacillus megaterium 2*
b	HA	smooth	whitish-creamy	circular	flat	+	-	-	+	-	+	-	+	-	-	*Bacillus megaterium 3*
d	S	smooth	whitish	irregular	flat	+	-	+	-	-	+	-	-	-	-	*Bacillus cereus 5*
i	TG	concentric	white	irregular	flat	+	-	+	-		-	-	-	-	-	*Bacillus cereus 6*
k	TG	rough	white	circular	convex	+	-		-	+	+	-	-	-	-	*Micrococcus sp.6*
l	FS	smooth	white	lobate	flat	+	-	+	-	-	+	Acidicbutt	-	+	-	*Bacillus cereus 7*
m	FS	smooth	orange	circular	convex	+	-	-	-	+	-	-	+	+	-	*Micrococcus sp.7*
n	FS	smooth	pink	irregular	convex	+	-	-	-	+	-	Acidicbutt	-	+	-	*Micrococcus sp.8*

DO*: Daniellia oliveri;* FS*: Ficussycomorus;* HA*: Hymenocardia acida;* TG*:Terminalia glaucescens* and S: Soil*; -* :negative result; *+:* positive result

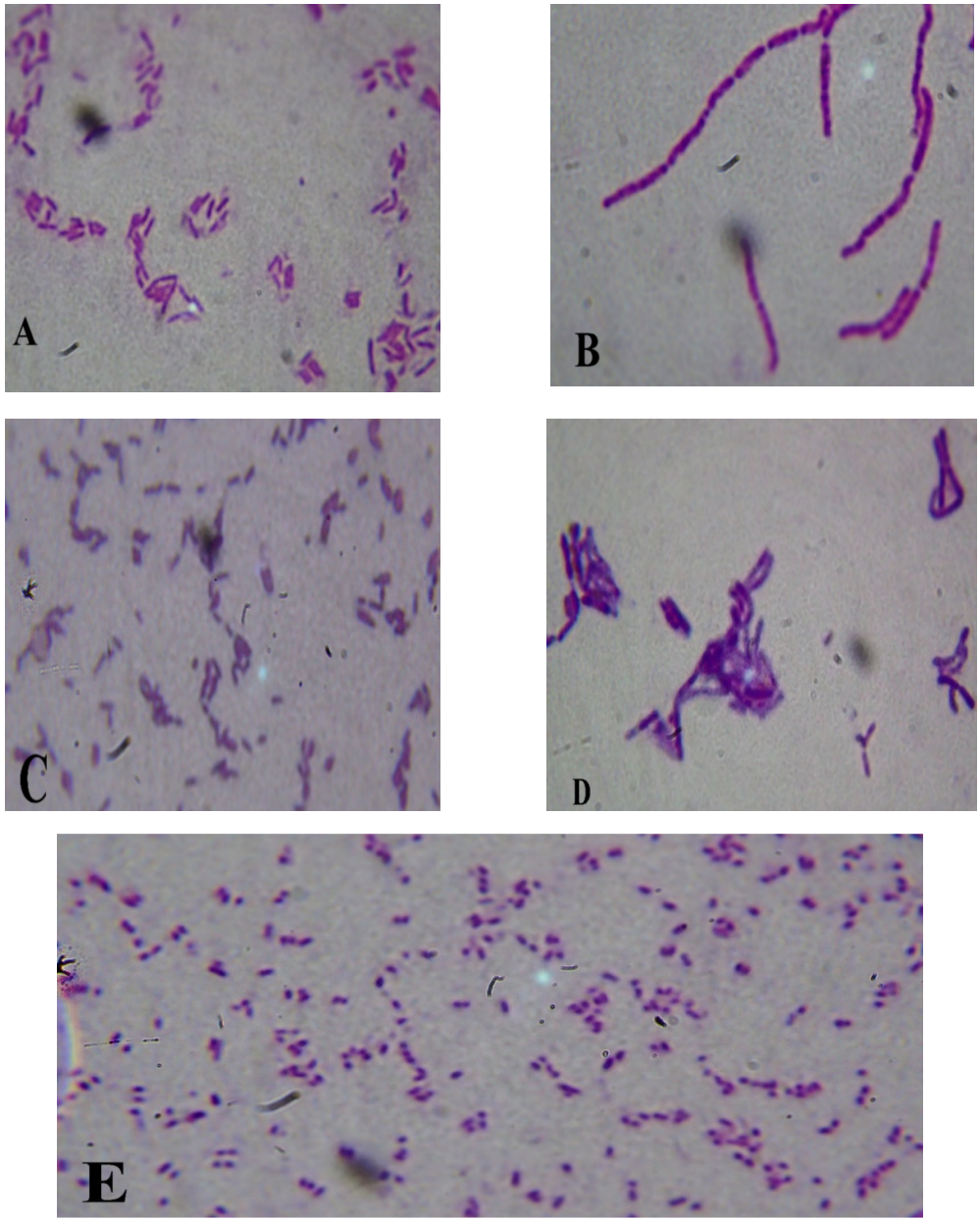

Fig. 4.SomeMicromorphology of the bacteria isolates.(A), *Bacillus cereus*, showing cluster of bacilli cells; (B), *Bacillus megaterium*, showing streptobacilli cells; (C), *Bacillus subtilis* showing single bacilli cells; (D), *Bacillus* sp., showing aggregate of streptobacilli cells, (E), *Micrococcus* sp. ; showing *Diplococcus* cells (cocci in pair)

3.3 Enzymes Activities of Isolated Bacteria

A total of 64% of the isolates produced at least one enzyme on specific culture media. 36% of the isolates produced amylase (Fig. 7A) with 11.11% having excellent activity, 22.22% with good activity and 66.67% with low amylase production. Furthermore, 56% of the isolates were suitable for the production of cellulase (Fig. 7B) by hydrolysis of carboxy methyl cellulose with 64.28% having excellent activity, 14.28% with good activity and 21.43% with weak activity. Likewise, 40% of the isolates had esterase (Fig. 7C) activity with 1% having excellent activity, 80% having good activity and 1% having weak activity. Lipase production was expressed by 48% of the isolates with 25% with excellent

activity, 66.67% good activity and 8.33% having low activity. At the end 48% of the isolates produced protease (Fig. 7D) with 91.67% having excellent activity and 8.33% having weak activity (Table 2).

4. DISCUSSION

Microorganisms such as bacteria, archaea, fungi, and protozoans are very important in all processes related to decomposition of leaf litter. The microbial components of soil are entirely responsible for the decomposition of organic matter [40]. The roles of microorganisms as summarized by Hoff et al. [41] include extracellular and intracellular decomposition of complex nutrient sources, transportation of simple nutrients across cell membranes for metabolic processes and detoxification of compound that could inhibit microbial growth. Statmets [42] reported that microbial decomposition of leaf litters allows carbon,

Table 2. Extracellular enzyme produced by isolated bacteria

Isolates	Identification	Amylase	CarboxyMethyl cellulase	Esterase	Lipase	Protease
A	*Bacillus subtilis 1*	+++	+	-	+++	-
B	*Bacillus subtilis 2*	-	+++	-	-	-
C	*Bacillus cereus 1*	-	++	-	++	+++
D	*Micrococcus sp.1*	+	+++	++	++	+++
E	*Micrococcus sp.2*	-	-	-	-	+++
F	*Bacillus subtilis 3*	-	+	-	-	-
G	*Bacillus cereus 2*	-	+	++	+	+++
H	*Bacillus subtilis 4*	-	+++	++	++	++
K	*Micrococcus sp.3*	+	++	+	++	+++
L	*Bacillus cereus 3*	-	-	-	-	-
M	*Micrococcus sp.4*	-	-	-	-	-
N	*Micrococcus sp.5*	-	-	-	-	-
T	*Bacillus cereus 4*	+	+++	++	+++	+++
U	*Bacillus sp.*	++	+++	++	++	+++
W	*Bacillus subtilis 5*	-	-	-	-	-
Y	*Bacillus subtilis 6*	-	-	-	-	-
Z	*Bacillus megaterium 1*	++	+++	++	++	+++
a	*Bacillus megaterium 2*	-	-	-	-	-
b	*Bacillus megaterium 3*	+	+++	++	++	+++
d	*Bacillus cereus 5*	+	-	-	-	-
i	*Bacillus cereus 6*	-	-	-	-	-
k	*Micrococcus sp.6*	-	-	-	-	-
l	*Bacillus cereus 7*	-	-	-	-	-
m	*Micrococcus sp.7*	+	+++	+++	+++	+++
n	*Micrococcus sp.8*	+	+++	++	++	+++

Excellent activity: +++; Good activity: ++; Poor activity: +; and No activity: -

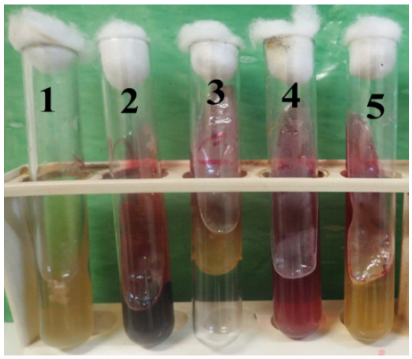

Fig. 5. Triple sugar iron (TSI) slants showing negative biochemical tests (tubes 4). Tubes 1and 5 have acidic butt. Tube 3 produced gas. Tube 2 produced H_2S

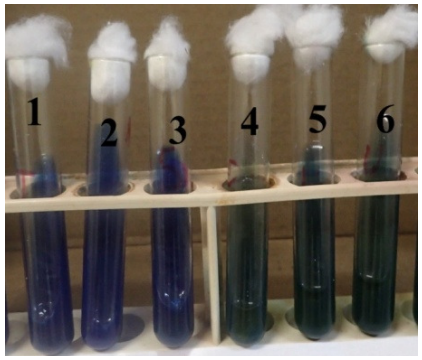

Fig. 6. Citrate utilization test on Simmon's citrate agar slant. Tubes 1, 2 and 3 present positive test for citrate utilization, while tubes 4,5, and 6 show a negative biochemical test

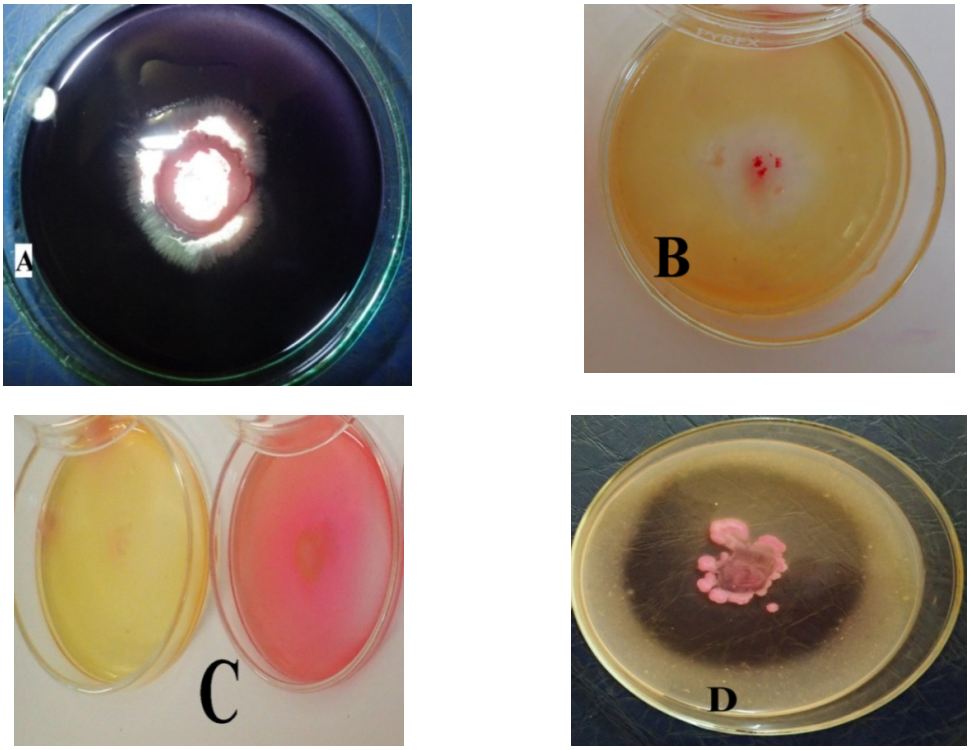

Fig. 7. Enzymatic activities of different bacteria isolated from leaf litters and soils. (A) showing weak amylase activity; (B) showing good cellulase activity indicated by clear zones around colonies, (C) showing excellent esterase activity (left) compared to no activity (right) and (D) showing excellent protease activity indicated by clear zones around colonies

nitrogen, phosphorus, potassium, calcium, zinc and other minerals to be deposited back into the nutritional bank. The findings of Rigobelis and Nahas [43] revealed that microbial leaf litter results allow the release of nutrients to the soil in the forest. Fungi and bacteria as decomposers of plant litter play broadly similar roles as degraders of organic matter, but are phylogenetically distant and represent fundamentally different life forms and a range of other specific traits.

4.1 Bacteria Load

The presence of bacteria on litters at the initial time and their development during the decomposition of these litters suppose that these bacteria are able to use carbon present in these plants as a carbon source for their development. The microbial faunais characterized by an incredible metabolic and physiological versatility that enables microorganisms to inhabit hostile ecological niches and to exploit carbon and energy sources, compounds unpalatable for higher organisms [44]. Variation in microbial population between litters may be attributed to the differences in the physicochemical properties of the litters and texture of leaves, which directly influence the rate of decomposition [29].

The increase of bacterial population at 12 weeks of litter decomposition supposes a strong activity of microorganisms which can use sugar in plant material as a carbon source for their activities and development, and these bacteria are therefore at the exponential phase of their development .This increase in the bacterial population during the decomposition of leaf litters has also been reported by Rajendran and Kathiresan [45]. On the other hand, the reduction in this population at 24 weeks of litters decomposition suggests the start of the end of the litters decomposition process by bacteria. The sharp decrease of the number of bacteria in soil under the decomposing litters at 24 weeks suggests microorganisms involved in the decomposing litter produced bactericidal compounds. According to Goya et al. [46] and Yu et al. [47], allelopathic compounds such as ester, terpene, phenols and tannin, produced from leaf litter during its decomposition affect the population of soil microbes.

4.2 Characterization and Identification of Isolated Bacteria

The characterization of isolates on nutrient agar medium demonstrated that the colonies of isolates had concentric, rough or smooth texture ;Transluscent, white, whitish-cream, white-cream, whitish, blue-green, yellow-brown, orange, pink or red in color ; They were Circular, Irregular, Irregular and spreading or lobate in shape ; with flat or convex elevation. These cultural characteristics are those generally observed in bacteria; similar a cultural characteristic of bacteria has been indicated by Ayitso and Onyango [31]; Saha and Santra [30].

The obtained results showed that all of the bacteria cells had ability to retain the violet dye (gram - positive organisms).Gram-positive bacteria have mostly been isolated from decaying plant matter [48,30].

Gram stain showed single bacilli, bacilli cluster, bacilli chain and diplococcus cells, combined to biochemical characteristics; isolates in this study displayed the typical characteristics of members of the genera *Bacillus* and *Micrococcus*. *Bacillus* genus were the most frequently observed in all leaf litters and soils ; this corroborates the findings results of Bigelow et al. [49] and Rigobelis and Nahas [43], who reported that *Bacillus* spp. are able to survive adverse environmental conditions by producing extremely drought resistant endospores and they could also thrive under any type of vegetation. *Bacillus* spp. were the most abundant bacteria isolated from *E. camaldulensis* and *T. grandis* leaf litters at different periods of decomposition as reported by Ndibe and Onwumere [50]; according to them *Bacillus licheniformis, Bacillus subtilis, Pseudomonas putida, Micrococcus luteus* and *Proteus vulgaris*, were common bacteria isolated from both tree decomposing leaf litter. Rigobelis and Nahas [43] reported that bacteria are the major group of microorganisms responsible for 25 - 30% of the total soil microbial biomass.

4.3 Extracellular Enzyme Produced by Bacteria

The isolated bacteria have variously produced a wide range of hydrolytic enzymes which are competent in the degradation of litter. In fact, more than 2 / 3 of the isolates were able to produce amylase, cellulase, lipase, esterase and protease.

4.3.1 CarboxyMethyl cellulase

Cellulose is one of the most abundant forms of biomass present on the earth and is considered as an inexhaustible source of raw material for various products [51]. A sure way of utilizing cellulase starts with its breakdown into its smaller oligosaccharides or monosaccharides. Cellulases are a class of enzymes that catalyze these reactions [51]. Based on the cleavage performed by enzymes, they are divided into three types: i) Endo which cleaves at random sites of the biopolymer ; ii) Exo which cleave the 2 or 4 units from the edge of the reduced cellulase chain that is formed by the Endo cellulose ; iii) - Glucosidase that hydrolyzes extra cellular products to individual monosaccharide [52]. Cellulases are known to convert cellulose into monomeric or dimeric structure hence carboxymethyl cellulose (CMC) was used as a carbon source, which is a soluble form of cellulose [51,52]. Gram's iodine dye were used to observe the zone of clearance produced by the activity of cellulase. It is widely accepted that the diameter of the zone of clearance indicates the ability of a bacteria to hydrolyze cellulose. Several previous reports have shown that certain microbes are able to utilize cellulose as a source of energy. These microbes produce extracellular cellulase and hence, are known as cellulolytic microorganisms. Bacilli and fungi are most popular class for commercial production as these cellulases have very high economic value [51].

Cellulase-producing bacteria have been isolated from a wide variety of sources such as composting heaps, decaying plant material, feces of ruminants, and hot springs [53]. Bacteria having high growth rate as compared to fungi has the good potential for use in cellulase production [54]. Many aerobic microorganisms, both bacterial and fungal, use the cell free cellulase to digest cellulose [55]. In this study, 56% of isolated bacteria produced cellulolytic enzyme with 64.28% having excellent activity, demonstrating the strong ability of isolates to degrade plants which are mainly made of cellulose. Cellulose degradation is largely an aerobic process and the primary cellulolytic bacterial isolates are *Pseudomonas* sp. [56], *Bacillus subtilis* [57], *Bacillus licheniformis* [58], and *Bacillus brevis* [59]. *Fibrobacter succinogenes* from the rumen is also an important cellulose degrader [60].

Akhtar et al. [61] reported that, cellulase producing *Bacillus* spp. (*Bacillus megaterium*

AS1, B. *subtilis* AS2 and B. *subtilis* AS3) were able to solubilize CMC in laboratory assay and to effectively degrade leaf litter Biomass of *Hevea brasiliensis*, *Eucalyptus marginata*, *Polyalthia longofolia*, *Tectona grandis*, and *Mangifera indica*. Maximum endoglucanase activity was observed in *Cellulomonas*, *Bacillus*, and *Micrococcus* sp. At 40℃ and neutral pH by Immanue et al. [62].

4.3.2 Amylase

Amylolytic enzymes or amylases which are the various starch splitting enzymes are widely distributed in bacteria and fungi. They break down starch to reducing sugars mainly maltose and dextrins. They are categorized into exo-acting, endo-acting and debranching enzyme. Among the amylases, ß-amylase is exo-acting whereas α-amylaseisendo-acting enzyme. In recent years, interest in its large scale microbial production ofα-Amylase has increased dramatically due to its wide spread use in food, textile, baking and detergent industries [63]. In this report, 36% of isolated bacteria produced amylolytic enzyme with more than 33.33% of isolates having good activity. Kathiresan et al. [64], demonstrated that bacteria of the genus *Bacillus* associated with the degradation of leaves in mangroves had a strong amylase activity; and were also competent in the production of lipase and protease.

4.3.3 Protease

Casein or skimmed milk agar plate assays are mainly used for qualitative determinations of protease activity. The hydrolysis zone produced on the casein agar could be related to the amount of protease produced by bacteria. But some exceptions have been reported, such as the protease produced by *Bacillus licheniformis*, which produces very narrow zones of hydrolysis on casein agar plates inspite of large enzyme production by submerged culture [65]. The results showed that 48% of the isolates produced the protease with 91.67% having excellent activity. Saha and Santra, [30] indicated vast application of protease in degradation of waste. Proteases occupy a pivotal position with respect to their applications in both physiological and commercial fields. Proteolytic enzymes catalyze the cleavage of peptide bonds in other proteins, and microorganisms elaborate a large array of intracellular and/or extracellular proteases [66].

4.3.4 Lipase and esterase

Lipolytic enzymes [Esterase (E.C. 3.1.1.1) and Lipases (E.C. 3.1.1.3)] belong to a group of enzymes whose biological function is to catalyze the hydrolysis of triacylglycerols into diacylglycerols, monoacylglycerols, free fatty acids and glycerol [67]. Many attempts were done by researchers to classify lipolytic and esterolytic enzymes [68].

Industrial applications may require specific enzymes such as leather industry that mostly require lipase and dairy industry that requires esterase [69]. Some important lipase producing bacteria genera include *Bacillus*, *Pseudomonas* and *Burkholderia* etc. Lipase/esterase-producing bacteria have been found in diverse habitats such as soil contaminated with oil, dairy waste, industrial wastes, oil seeds and decaying food, compost heaps, coal tips and hot springs [70, 71]. Esterases, which catalyze the cleavage and formation of ester bonds and are known as α/ß-hydrolases. These enzymes are widely distributed in animals, plants, and microorganisms [72] Esterases (EC 3.1.1.1, carboxyl ester hydrolases) hydrolyze esters of short chain carboxylic acids (= 12 carbon atoms) [73]. In this report, 40% of the isolates had esterase activity with 80% having good activity, while lipase production was carried out by 48% of the isolates with 25 % having excellent activity and 66.67% good activity. This strong lipolytic and esterase activity of the isolates suggests their effectiveness in the degradation of fatty acids contained in leaf litters. Two extracellular lipolitic enzymes from *Bacillus subtilis* were characterized by Eggert et al.[73].

5. CONCLUSION

This study aims to investigate the dynamic of bacterial population during degradation of leaf litters of *Ficus sycomorus, Terminalia glaucescens, Daniellia oliveri,* and *Hymenocardia acida* in sudano-guinea savannah of Adamawa, Cameroon as well as their enzymatic activity. 25 bacteria were isolated from distinct phenotypic characteristics on nutrient agar. The study of phenotypic, microscopic and biochemical enabled identification of two genera: the genus *Bacillus* and the genus *Micrococcus* representing respectively 68 and 32% of the isolates. The genus *Bacillus* consisted of *Bacillus cereus* (41.17%, with 7 phenotypes), *Bacillus megaterium* (17.64%, with 3 phenotypes), *Bacillus subtilus* (35.29%, with 6 phenotypes)

and *Bacillus* sp. (5.88%, with 1 phenotype). The genus *Micrococcus* regrouped 08 phenotypes. The relative abundance of isolates demonstrated that in all leaf litters and soils, there was a great diversity of bacterial isolates at the initial time and then a considerable decrease in this diversity during litters degradation. The genus *Bacillus* was generally dominant at all decomposition periods of different plant species. A total of 64% of bacteria isolates produced at least one enzyme. 36% of the isolates produced amylase, 56% produced cellulase, 40% produced esterase, 48% produced lipase, while 48% of the isolates produced protease. These findings suggest important differences in bacteria populations and biochemical activities between plants leaf litter species. Microorganisms present in nature represent a great resource for biotechnological exploration of products and processes, making it possible to discover enzymes with new properties and applications. This work could provide basic data for further investigations on hydrolytic enzymes from bacteria involved in plants leaf litter decomposition and constitute an important step for the production of bacteria inoculum capable of transforming leaf litter biomass and into organic fertilizer.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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