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# Identification and Enzymatic Potential of *Bacillus* Species Isolated from Traditional Cassava Starters: Potential for *Attiéké* Production

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Authors SN and MB designed the study and wrote the protocol. Author CE wrote the first draft of the manuscript. Authors SS and CE managed the literature searches and performed the analysis. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aims:** Microbial enzymatic activities are important in cassava dough fermentation for *attiéké* production. The objective of this study was to describe the molecular identification and the amylolytic, pectinolytic and cellulolytic enzymes potential of four (4) *Bacillus* species involved in cassava mash fermentation for the preparation of *attiéké*.
**Place and Duration of Study:** Laboratory of Biotechnology, UFR Biosciences, University Félix Houphouet-Boigny (Côte d'Ivoire), between February 2016 and April 2016.

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**Methodology:** Four *Bacillus* strains (*Bas* 04, *Bas* 13, *Bas* 58 and *Bas* 66) used for this study were isolated from the traditional cassava starter ‘’*mangnan*’’. By using the PCR amplification of ribosomal 16S genes, *Bacillus* strains were identified. After culture in nutrient agar, strains were suspended in a tryptone salt solution. When we obtain *Bacillus* cells absorbance (turbidity) of 1.00 at 600 nm, 100 µL of this suspension were used to inoculate 5 mL of liquid medium containing peptone (1%), meat extract (1%), NaCl (0.3%) and 1% of starch or Pectin or Carboxylmethyl cellulose for enzymes production. The enzymatic activities were studied at different temperatures ranged from 25°C to 50°C and pHs (4.5, 5.0 and 5.5).
**Results:** *Bacillus thuringiensis* was identified with 99% to 100% of similarity, referring to the NCBI database. The best amylase, cellulase and pectinase activities were obtained with *Bas* 66 (3.80 U/mg), *Bas* 13 (0.45 U/mg) and *Bas* 58 (1.09 U/mg) at different optimum temperatures (25, 40 and 50°C) and pHs (4.5, 5.0 and 5.5), respectively.
**Conclusion:** These enzyme activities are important for cassava dough fermentation, using these *Bacillus* strains, contributing to the softening of the mash thus improving texture and allowing the digestibility of *attiéké*.

*Keywords: Attiéké; Bacillus thuringiensis; cassava; extracellular enzymes; fermentation.*

## 1. INTRODUCTION

*Bacillus* species are Gram positive rods often arranged in pairs or chains with rounded or square ends and usually a single endospore. The endospores are generally oval or sometimes round or cylindrical and are very resistant to adverse conditions [1]. They are ubiquitous and diverse both in the terrestrial and marine ecosystems, and are mostly identified in the fermentation of several foods. In Africa, *Bacillus* are involved in the fermentation of *Prosopis africana* seeds to produce *Okpehe* [2], the African locust bean (*Parkia biglobosa*) to produce *soumbala* [3] and in the fermentation of cassava (*Manihot esculenta* Crantz*)* roots for *foofoo*, *lafun* and *attiéké* production [4,5,6]. In Côte d'Ivoire, *attiéké* is the most consumed cassava product. It is a ready-to-eat steamed granular cassava meal, couscous-like product, with slightly sour taste and whitish or yellowish colour. *Attiéké* is consumed two to three times a day with meat, fish or vegetables [7]. Its production involves the use of traditional starter cultures called *mangnan* by *ébrié* ethnic group in Côte d'Ivoire. This uncontrolled starter constitutes the main source of microorganisms contributing in cassava dough fermentation [8] and improving the texture, colour and flavour of *attiéké* [9]. For *attiéké* production, cassava roots are peeled, chopped to pieces, washed and grated. During the grinding process, the pieces are mixed with 10% (w/w) of *mangnan* and 0.1% (v/w) of palm oil. The inoculated pulp was fermented overnight in covered bins. After fermentation, the cassava paste is filled into jute bags and pressed for several hours. The dewatered paste are squeezed through a sieve to obtain rounded granules that are sun-dried and then cleaned to remove fibers and waste. The dried granules are steamed to produce *attiéké* [10]. Several microorganisms such as lactic acid bacteria, *Bacillus spp*., yeasts and molds have already been reported to play synergistic roles in the cassava fermentation [11,6].

*Bacillus* species were suspected of producing extracellular enzymes such as amylases, pectinases and cellulases. The amylolytic enzymes catalyze the hydrolysis of starch into glucose and dextrin hydrolysates used as carbon (energy) sources in fermentation [12,13]. Pectinases hydrolyzing pectic molecules and cellulolytic enzymes responsible for the breakdown of celluloses contribute together to the soft consistency and texture of cassava mash [14,15]. These three different enzymes produced by *Bacillus spp.* were important because they synergistically take part in quickly softening of the cassava mash thus improving its digestibility when transformed into *attiéké* [16].

This paper describes the molecular identification and the amylolytic, pectinolytic and cellulolytic enzymes potential of four (4) *Bacillus* species involved in cassava mash fermentation for the preparation of an esteemed Ivorian dish called *attiéké*.

## 2. MATERIALS AND METHODS

### 2.1 *Bacillus* Stains Preparation

The four presumptive *Bacillus* strains (*Bas* 04, *Bas* 13, *Bas* 58 and *Bas* 66) used for this study were isolated from the traditional cassava starter

''*mangnan*'' collected from 11 manufacturing units in the District of Abidjan (Koumassi, Abobo, Marcory, Attécoubé, Port-Bouet, Treichville, Adjamé, Yopougon, Cocody, Bingerville and Anyama) and from 3 areas (Bassam, Dabou and Jacqueville) located in peri-urban areas of this District [17]. *Bacillus* strains were selected on the basis of their great ability to produce extracellular enzymes as amylases, pectinases and cellulases in solid medium. For isolation, *Bacillus* strains were prepared after an enrichment step described as follow: 10 g of each sample were diluted in 90 mL of a sterile buffered peptone water and incubated at 30°C for 18 hours. The medium was then heated in a water bath at 80°C for 10 min in order to select bacteria on nutrient agar (Scharlau, Spain). In solid medium, enzymes production was carried out on Petri dishes using the Ouattara et al. [18] method. The medium contained 0.28% of $(NH_4)_2SO_4$; 0.6% of $K_2HPO_4$; 0.01% of $MgSO_4,7H_2O$; 0.2% of $KH_2PO_4$; 0.02% of yeast extract; 2% of agar and 1% of different carbohydrate sources (starch for amylase production, citrus pectin (Sigma) for pectinase production and Carboxylmethyl cellulose for cellulase production).

## 2.2 Molecular Identification of *Bacillus* Isolates

### 2.2.1 Total DNA extraction

DNA was extracted by thermic shock. The *Bacillus* strains were grown during 24 hours at 30°C on a nutrient agar medium. A loopful of pure culture was then suspended in 300 µL of sterile distilled water and successively maintained at - 20°C for 30 min and at 99°C for 15 min. After centrifugation at 13000 g for 10 min, the supernatant was used as the total DNA extract.

### 2.2.2 PCR amplification of ribosomal 16S gene, partial sequencing and sequence analysis

The 500 bp fragment containing the hyper variable 16S rDNA region of each sample was amplified by using two primers: F27 (5'-AGAGTTTGATCCTGGCTCAG-3') and R520 (5'-ACCGCGGCTGCTGGC-3') [19]. The PCR amplification was carried out in a Biometra thermocycler (model Tgradient, Germany). Reactions were performed in a final volume of 50 µL containing 1 µL of DNA extract, 0.25 µL of 5U taq polymerase (Go Taq DNA polymerase, Promega®, USA), 5 µL of 10X buffer, 1 µL (10 mM) of deoxynucleoside triphosphate (dNTPs, BioRad® France) and 2 µL (10 mM) of each primer (Eurogentec, Lyon, France). After an initial denaturation at 95°C for 4 min, reactions were run for 35 cycles, each cycle comprising: denaturation at 95°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 1 min. Finally, an extension at 72°C for 10 min was carried out. The presence and yield of specific PCR products were monitored by agarose 0.8% (w/v) gel electrophoresis at 90 V for 1 hour in Tris-Borate-EDTA buffer 1X and made visible by ethidium bromide staining and UV transillumination. PCR products were purified using the nucleospin extract II kit (Machereye Nagel, Germany) and then, sequenced using the primer F27. The partial 16S rRNA gene sequences determined in the *Microbiologie, Adaptation, Pathogénie* laboratory (Lyon, FRANCE) have been deposited in the National Center for Biotechnology Information (NCBI) database.

## 2.3 Enzymes Production and Microbial Growth

For enzymes (Amylases, Pectinases and Cellulases) production in liquid medium, strains were cultivated on nutrient agar medium during 24 hours at 30°C. After the period of incubation, pure culture was suspended in a tryptone salt solution in order to obtain *Bacillus* cells absorbance (turbidity) of 1.00 at 600 nm. Then, 100 µL of this suspension were used to inoculate 5 mL of liquid medium containing peptone (1%), meat extract (1%), NaCl (0.3%) and 1% of carbon sources: starch for amylase production, Pectin for pectinase production and Carboxylmethyl cellulose for cellulase production [20]. The medium was incubated for 24 hours at 30°C under constant rotary agitation (105 rpm). The growth of strains was spectrophotometrically (Pioway, China) measured (600 nm) at 0; 12 and 24 hours in different production media. After incubation, culture was centrifuged at 15,000 rpm for 30 min and the resulted supernatant use as extracellular enzyme extracts.

## 2.4 Determination of Enzymatic Activities

All the studied enzymes activities were determined in accordance with the optimal conditions (pH and temperatures) of the cassava mash fermentation heaps as well as these of each enzyme.

The amylasic activity was determined by hydrolyzing soluble starch substrate 1% (w/v)

(Sigma, USA) and quantifying the liberated reducing sugars after complexation with 3,5 DNS acid [21]. Reactions were performed in a 100 mM acetate buffer (pH 4.5 to pH 5.5). The different amylasic extracts (100 µL) were mixed with 150 µL of starch substrate and incubated at 25°C (optimum temperature of the studied amylases) and at 30°C (average temperature of fermentation) for 30 min. Spectrophotometric glucose quantification was carried out at 540 nm by reference to a standard glucose solution (1 mg/mL). For cellulase assay, the same protocol was carried out at 30°C and 40°C (optimum temperature of the studied cellulases) by using caboxymethyl cellulose (CMC) as substrate. Concerning pectinase activity, citrus pectin (Sigma) was used as substrate. The enzymatic reaction was measured for 30 min at 30°C and at 50°C which is the optimum temperature of the studied *Bacillus* pectinases. The released amount of galacturonic acid was quantified at 540 nm by referring to a standard curve of a galacturonic acid solution (10 mM). One unit of each specific activity was defined as the amount (mg) of enzyme that hydrolyses 1 µmol of substrate per min under the assay conditions.

### 2.5 Estimation of Proteins Concentration

The proteins concentration was estimated by following the Lowry et al. [22] method. Bovine serum albumin (BSA) was used as the standard protein.

## 3. RESULTS AND DISCUSSION

### 3.1 Electrophoresis of PCR Products

The electrophoretic analysis of amplified ribosomal 16S genes revealed fragment sizes of about 500 bp as compared to molecular markers (Fig. 1).

### 3.2 16S rDNA Sequencing and *Bacillus* Species Identification

The sequencing of the hyper variable 16S rDNA region allowed the identification at genus and species level of *Bacillus* isolates. The sequences obtained from each isolate were aligned with those of the NCBI database and all the studied microorganisms were identified as *Bacillus thuringiensis* with 99% to 100% of similarity (Table 1). The different gene sequences obtained are listed in Appendix 1.

**Table 1. Degree of similarity of rDNA sequences and identification of the scientific name of *Bacillus* species isolated from the traditional *attiéké* starter *mangnans***

| ***Bacillus species*** | **Degree of similarity (%)** | **Identification name** |
|---|---|---|
| Reference | 100 | *Bacillus thuringiensis* |
| *Bas* 04 | 99.58 | *Bacillus thuringiensis* |
| *Bas* 13 | 99.59 | *Bacillus thuringiensis* |
| *Bas* 58 | 100 | *Bacillus thuringiensis* |
| *Bas* 66 | 99.38 | *Bacillus thuringiensis* |

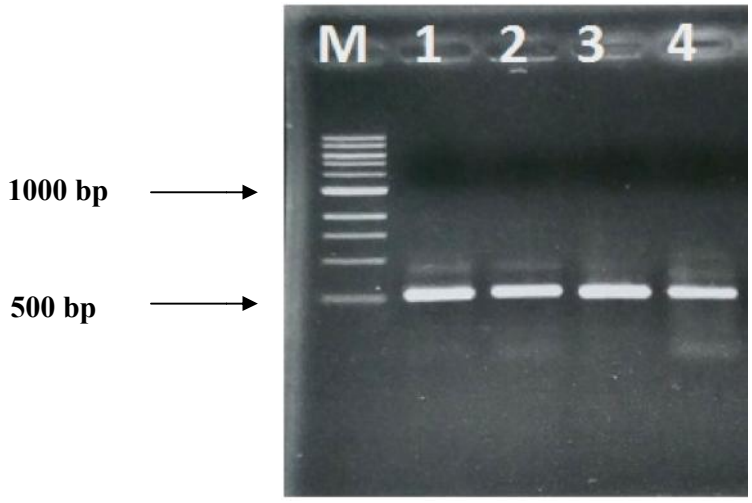



**Fig. 1. Agarose gel electrophoresis of PCR products of four *Bacillus* species isolated from the traditional *attiéké* starter *mangnan***

*Lane 1 : Bas 04 ; Lane 2 : Bas 13 ; Lane 3 : Bas 58 and Lane 4 : Bas 66. Lane M, DNA size markers (GeneRuler DNA ladder mix)*

*Bacillus* are microorganisms involved in cassava fermentation to *attiéké* production. In this study, four *Bacillus* strains isolated from *mangnan* were identified as *Bacillus thuringiensis* by the ribosomal 16S gene sequencing method. In Côte d'Ivoire, *Bacillus thuringiensis* was never specifically identified in *attiéké* fermentation. However, in Indonesia, it was already reported the presence of this *Bacillus species* in a cassava fermented food named 'Tape' [23].

It has been shown that *Bacillus thuringiensis* secretes some proteins that are toxic to immature insects (larvae) and thus, widely used as biopesticide in agriculture [24,25]. However, these proteins are generally safe for humans and animals [26]. When *Bacillus thuringiensis* is consumed in food, it is confined to the gut with its growth being inhibited. Its toxins are also denatured like other proteins in the diet [25]. As *Bacillus thuringiensis* is not dangerous for human health, it can be used for cassava dough fermentation for *attiéké* production. Also, it produces methionine, an essential amino acid which cannot be synthesized by human and animals. The amino acid constitutes an important food additive necessary for physiological balance [27]. Indeed, methionine participates in peptides and proteins synthesis. It is a glutathione precursor, a tripeptide that reduces reactive oxygen species and thus protects cells from oxidative stress [28]. The fulfilled beneficial effects of *Bacillus thuringiensis* above make it suitable for the fermentation of cassava dough in order to improve the textural and nutritional quality of the thereafter produced *attiéké*.

### 3.3 Growth of *Bacillus* Strains in Enzymatic Production Media

The growth of the studied strains in all enzymatic production media increases up to 12 hours. After this time period, growth remains stable or decline slightly. All strains showed optimal growth in the medium containing starch as carbon source (Fig. 2A) followed by those supplemented by cellulose (Fig. 2B) and then pectin (Fig. 2C).

The improved growth of the strains was obtained in starch medium because this polysaccharide could be better assimilated by strains for their growth compared to cellulose and pectin.

### 3.4 Amylolytic Activity

All of the studied *Bacillus* strains produced extracellular amylases with optimum activity of 3.80 U/mg of proteins obtained from *Bas* 66 strain at 25°C (Data not shown) and at pH 5.5 (Fig. 3A).

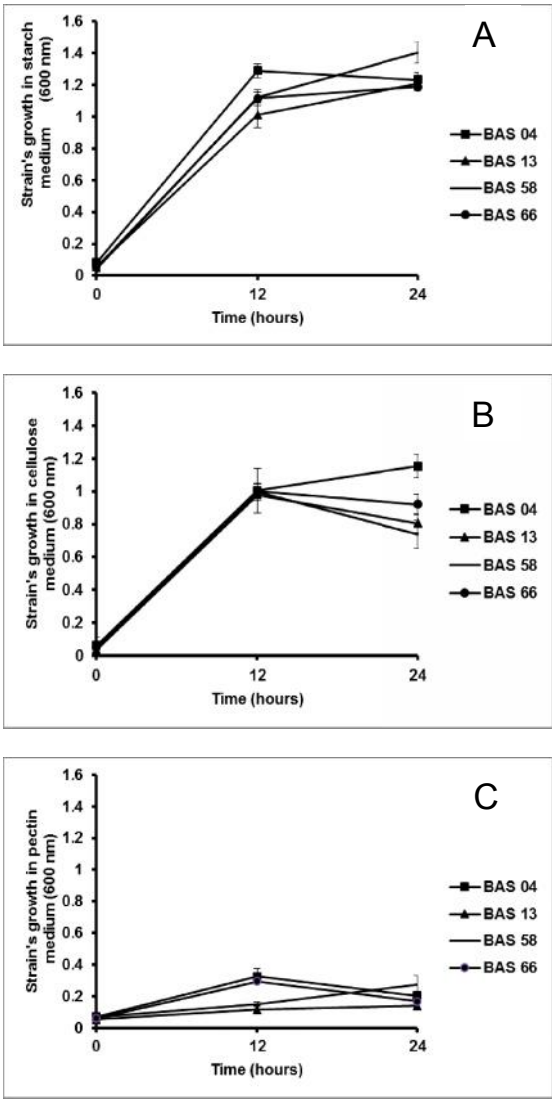



**Fig. 2. Growth of *Bacillus* strains in different enzymatic production media**

*A: Starch medium, B: Cellulose medium, C: Pectin medium*

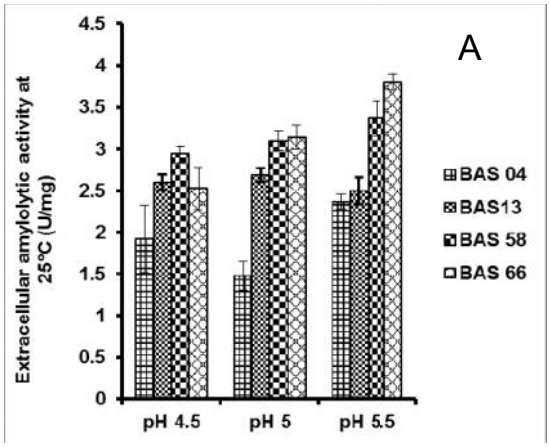
The production of extracellular amylases by *Bacillus spp*. has been demonstrated in several scientific reports [29,30,31,32,23]. For *attiéké* production, it is very important to use *Bacillus spp*. that have the ability to secrete extracellular enzymes for better hydrolysis of the starch in cassava dough. Our study showed that extracellular amylase activities of the fourth strains were higher at 25°C compared with those measured at 30°C (average temperature of

cassava dough fermentation). In this respect, it would be interesting to create the conditions for fermentation at 25°C and pH 5.5 to allow a better activity of secreted amylases from the studied Bacillus species. Feller et al. [33] have also reported about an amylase whose activity at 25°C is higher than those determined at 30 and 35°C thus corroborating our results. However, the present results are different to those of Vidyalakshmi et al. [34] who have reported about another amylase activity from Bacillus spp. that was higher at 35°C compared with those measured at 25°C and at 30°C. It is also important to highlight that, during the fermentation of the cassava dough, the measured pH values decreased from 6.2 to 4.4 while the temperature varied between 28-30°C [6,35]. In view of the foregoing parameters, it could be conclude that all the studied strains are able to produce extracellular amylases throughout the duration (24 h) of the fermentation and then, contribute significantly to cassava starch hydrolysis. Indeed, it was previously reported that microbial strains which secrete amylase quickly, and that have the ability to metabolize starch could be used as starter for the bioconversion of cassava dough and thus accelerate significantly the fermentation process [36]. Besides their beneficial action in fermentation, these cheap Bacillus amylases could be used in medical and pharmaceutical applications for therapeutic and diagnostic tools for managing a quite number of diseases ranging from ordinary problems to gene therapy by correcting the enzyme deficiency and in analytical chemistry industries because of their low cost, ease of production and economic advantages [32,37].

### 3.5 Cellulolytic Activity

The extracellular cellulolytic activities of the four *Bacillus thuringiensis* species were also observed. They were optimally expressed at pH 5.5 and 30°C for *Bas* 13, *Bas* 66 and *Bas* 04 strains (Fig. 4A) and at 40°C and pH 5.5 for *Bas* 58 (Fig. 4B). At 40°C, *Bas* 66 showed its maximum cellulolytic activity in the extracellular medium at pH 4.5.

The variable and interesting extracellular activities observed could suggest a combined action of several cellulases that could be adequately used during cassava dough and similar substrate hydrolysis. So, *Bas* 04, *Bas* 13 and *Bas* 66 deserve attention as ferment for *attiéké* and other cassava based products. The addition of cellulolytic bacteria in the cassava dough involves considerable softening in fiber structure and thus, helps in improving the digestibility of the final product (*attiéké*).The cellulase activity of the studied *Bacillus thuringiensis* species could also be explored in various fields including textile, animal feeds and paper de-inking industries [38].



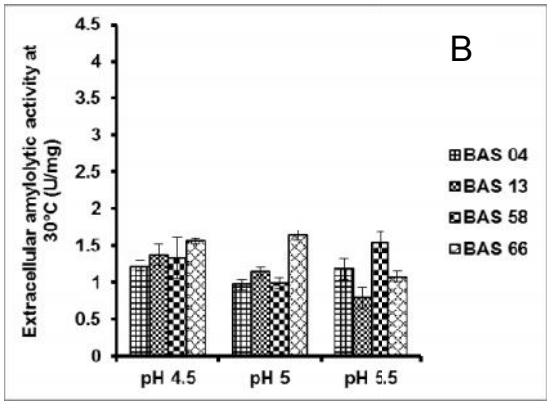




**Fig. 3. Extracellular amylolytic activities at 25°C (A) and 30°C (B) of amylases produced by four *Bacillus* (Bas 04, Bas 13,Bas 58 and Bas 66) strains isolated from the traditional *attiéké* starter *mangnan***

### 3.6 Pectinolytic Activity

The pectinolytic activity was detected in all the studied strains but the optimal activities were measured in the liquid reaction medium at pH 5.5. The optimum pectinase activities were detected at 50°C but the highest (1.098 U/mg of proteins) was observed with *Bacillus thuringiensis* 13 (*Bas* 13). In contrast, the weakest pectinase activity (0.38 U/mg of proteins) was observed with *Bas* 58 at pH 5.0 (Fig. 5B).

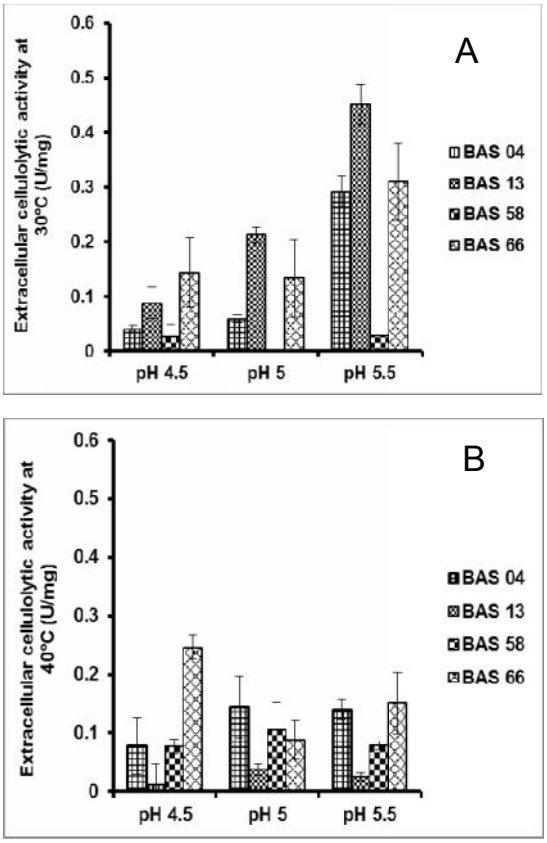



**Fig. 4. Extracellular cellulolytic activities at 30°C (A) and 40°C (B) of cellulases produced by four *Bacillus* (*Bas* 04, *Bas* 13, *Bas* 58 and *Bas* 66) strains isolated from the traditional *attiéké* starter *mangnan***

Beside the production of amylolytic and cellulosic enzymes, the studied *Bacillus* strains can also produce extracellular pectinases. These results were already reported for *Bacillus polymyxa* [39]. In this study, the maximal pectinolytic activity was observed at pH 5.5 which match the range of pH variation during the fermentation process. As the temperature of the fermentation process is closed to 30°C, *Bas* 13 that showed maximal pectinase activity at pH 5.5 and at 30°C could be an interesting starter for the softening of cassava dough and thus, it could more participate in improving the textural quality of the obtained *attiéké*. Extracellular pectinase produced by *Bacillus* species are of main interest in food biotechnology [40]. Indeed, they were several times identified in improving the digestibility of plant organic matter [41]. Also, pectinases play important roles in the metabolism of almost all organisms (plants, animals, fungi, bacteria and viruses) [40]. Given the wide applications of pectinases, the *Bacillus thuringiensis* pectinase produced in this study could have potentials for biotechnological applications in various industries such as the pharmaceutical, agricultural and bioremediation [40].

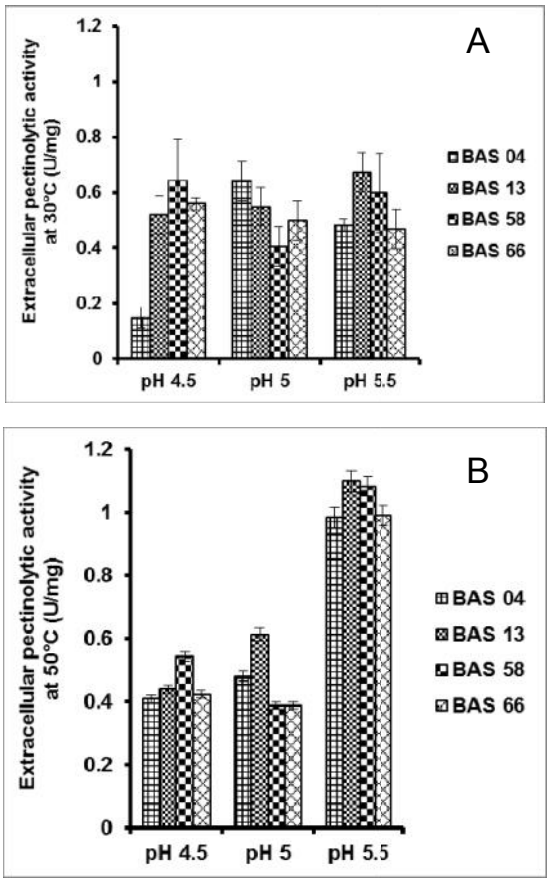



**Fig. 5. Extracellular pectinolytic activities at 30°C (A) and 50°C (B) of pectinases produced by four *Bacillus* (*Bas* 04, *Bas* 13, *Bas* 58 and *Bas* 66) strains isolated from the traditional *attiéké* starter *mangnan***

## 4. CONCLUSION

*Bacillus thuringiensis* isolated from the traditional cassava inoculum *mangnan* can produce many extracellular enzymes such as amylases, cellulases and pectinases. The interesting extracellular activities of these enzymes at 30°C and in pH values ranged from 6.0 to 4.5 were important for cassava mash fermentation to *attiéké* production. The studied *Bacillus thuringiensis* strains were able to produced amylases, cellulases and pectinases which expressed their activities in different pH and temperature conditions. As these conditions match the cassava dough fermentation parameters, *Bas* 04, *Bas* 13, *Bas* 58 and *Bas* 66

could be used together as alternative starters in fermentation for softening and improving the digestibility of *attiéké* and other cassava products. However, the strains must be tested to be non pathogenic.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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## APPENDIX 1

***Bas* 66**

TACTGCAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTA
ACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAA
CATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGC
GTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGA
GGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGG
AATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGT
CGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCT
AACCAGAAAGCCACGGCTAACTACGTGCCAGAGC

***Bas* 58**

TACTGTAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTA
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AACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAAAA

***Bas* 13**

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TAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGAT
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GAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAG
GGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCG
GGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTA
CCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCCGCGGTGAA

***Bas* 04**

ATACTGCAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGT
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ACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCG
CGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGA
GGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGG
AATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGT
CGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCT
AACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCC



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