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Diversity and enzymatic characterization of *Bacillus* species isolated from traditional cassava starters used for *attiéké* production

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ABSTRACT

Fermentation plays an important role in the production of cassava-based foods in West Africa. In Côte d'Ivoire, this step requires the use of a traditional cassava starter. Thus, the objective of this study was to identify and evaluate the enzymatic profile of the different *Bacillus* species present in these traditional cassava starters. Technique based on the analysis of the 16S rDNA sequence was used for the identification of *Bacillus* species. Enzymatic activity of different species identified was carried out with API ZYM system. Based on the 16S r DNA sequence analysis, seven species of *Bacillus* (*Bacillus subtilis, Bacillus cereus, Bacillus pumilus, Bacillus amyloliquefaciens, Bacillus methylotrophicus, Bacillus vallismortis* and *Bacillus toyonensis*) were identified. *B. subtilis* was the most identified species with a frequency of 30% followed by *B. amyloliquefaciens* (18%), *B. methylotrophicus* (10%), *B. cereus* (6%), *B. toyonensis* (6%), *B. vallismortis* (4%) and *B. pumilus* (4%). Many of these species have been able to produce osidases, phosphatases, lipases and proteases. Seventeen enzymes from these different enzyme groups were synthesized by the identified *Bacillus* species. The dominant and enzyme-producing species could be used for the development of a starter culture.

Keywords: *Bacillus* species, enzymes, traditional cassava starter.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a major root crop in the tropics and its starchy roots are significant sources of calories for more than 500 million people worldwide (FAO, 2008). It is the fourth agricultural resource after rice, wheat and maize in the world (Koko et al., 2014). It has important agronomic advantages such as high yields in poor soils, resistance to drought and diseases. Cassava is traditionally processed into a wide variety of fermented products with different local names (*attiéké, gari, fufu, agbelima, chikwangue placali*, *attoukpou*) (Sahouegnon et al., 2014; Yao et al., 2015). The fermentation is controlled by several microorganisms, some of which have positive effects such as product preservation, flavor development, cyanide reduction and changes in functional properties.

In Côte d'Ivoire, the use of a traditional starter is required for the

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fermentation of cassava dough to make some of the different products referred to above. Its production varies according to ethnic group (*Alladjan, Ebrié, Adjoukrou, Abouré*) methods. Traditional starter processing method consists in cooking and fermenting the whole peeled cassava roots for 72 hours (*Alladjan*, *Ebrié*, *Adjoukrou* method). Another method to make the traditional starter is to use the whole unpeeled raw cassava roots and let it ferment (*Abouré* method) (Tetchi et al., 2012). These traditional starters were colonized by a wide variety of microorganisms which constitute the main source of microbial activities during the cassava dough fermentation (Djeni et al., 2008). Several studies have identified the microorganisms (lactic acid bacteria, yeast and moulds, *Bacillus,* coliforms and enterococci) associated to the traditional cassava starters (Kakou et al., 2010; Tetchi et al., 2012; Bouatenin et al., 2013 ; Kakou et al., 2016**)**. Among the microflora, *Bacillus* genus plays an increasingly important role in food and industrial beverage (Grass et al., 2004)**.** Different species of *Bacillus* can produce several important enzymes such as amylase, cellulase, tannase, pectinase and betaglucosidase (Schallmey et al., 2004; Oyeleke et al., 2011). In Côte d'Ivoire, studies have already shown the ability of *Bacillus* spp isolated from the traditional cassava starter to produce enzymes (Bouatenin et al., 2013; Ehon et al., 2015). However, any studies have been carried out on the different species of *Bacillus* colonizing these traditional cassava starters.

Thus, the objective of this research was to identify the different species of *Bacillus* from traditional starter (*Alladjan, Ebrié, Abouré*) used for *attiéké* production and their enzymatic profile.

MATERIALS AND METHODS

Samples collection and isolation of *Bacillus* strains

The traditional cassava starters (*Alladjan* starters, *Ebrié* starters, *Abouré* starters) were collected in four cities of Côte d'Ivoire. The *Ebrié* starter were collected in Abidjan, Bouaké and Abengourou, the *Abouré* starters in Bonoua and the *Alladjan* starters in Abidjan. For the isolation of *Bacillus* strains, 10 g of each starter was homogenized with 90 mL of sterile buffer peptone water. Serial dilutions of the different samples were spread on a Plate Count Agar containing 1% starch. For preliminary identification, bacterial colonies were isolated and characterized by their morphological properties and appearance. Fifty strains of *Bacillus* were isolated from the different traditional cassava starters for the species identification.

Isolation of chromosomal DNA

Chromosomal DNA was prepared from overnight culture on agar Mossel. Isolation and purification were conducted with a kit (Instagen Matrix Bio-Rad, USA) according to the manufacturer's instructions.

16S rDNA amplification

To amplify the 16S rDNA gene, a primer pair (Table 1) hybridizing to two conserved regions was used for the identification of *Bacillus* strains. PCR mixture consisted of 0.2 mM of each primer (16R1522 and 16F27), 20 µL of 1X Master Mix(5PRIME Hot MasterMix 2,5X DOMINIQUE Dutscher, France), 1 µL of DNA and H_2O in a final volume of 50 µL.

Table 1: Primers used for gene amplification.

Primer name	Tm (°C)	%GC	Oligonucleotide (5'--- 3')
16R27	57,3	50	AGAGTTTGATCCTGGCTCAG
16F1522	44,7	60	AAGGAGGTGATCCAGCCGCA

Amplification conditions consisted of 94 °C initial denaturation for 2 min, 35 cycles of 94 °C for 1 min, 58 °C for 30 seconds (hybridization), 65 °C for 2 min (extension) and final extension at 65 °C for 7 min before cooling at 4 °C in PCR thermocycler 2720 Thermalcycler type (AB Applied Biosystems, Syngapore). Ten (10) µL of the amplified products of PCR were analyzed by electrophoresis in 1% of agarose gel stained with ethidium bromide.

Sequencing and phylogenetic analysis

PCR products were purified and quantified by electrophoresis in 08% (w/v) agarose gel. The 16S rDNA obtained was sent to the sequencing platform of Cochin Eurofins MW operon (France). DNA sequences were determined by chain-termination method (Sanger et al., 1977) using automatic ABI 3730Xl sequencing kit 96 capillary DNA Analyzers. The sequences obtained were compared with sequences in the database of NCBI (National Center for Biotechnology Information) using the BLAST program. The taxonomic browser NCBI server (http://www.ncbi.nlm.nih.gov/blast) was helped to find the affiliation of strains.

Enzymatic activities

Enzymatic activity of *Bacillus* strains was performed using API-ZYM system according to the manufacturer's instructions. The results were analyzed with a reading table of API-ZYM system.

RESULTS

Figure 1 shows the electrophoretic profile of the 16S rDNA gene of *Bacillus* strains isolated from different types of traditional cassava starter. After the phylogenetic analyses based on the alignment of the nucleotide sequences, the percentages of homology were between 95 and 99%. *Bacillus subtilis* was the most identified species with a frequency of 30% followed by *B. amyloliquefaciens* (18%) and *B. methylotrophicus* (10%) (Figure 2). The frequency of identification of *B. toyonensis* and *B. cereus* was 6% each. *B. vallismortis* and *B. pumilus* species with a frequency of 4% were the least identified. The frequency of unidentified species (*Bacillus* spp) was 22%.

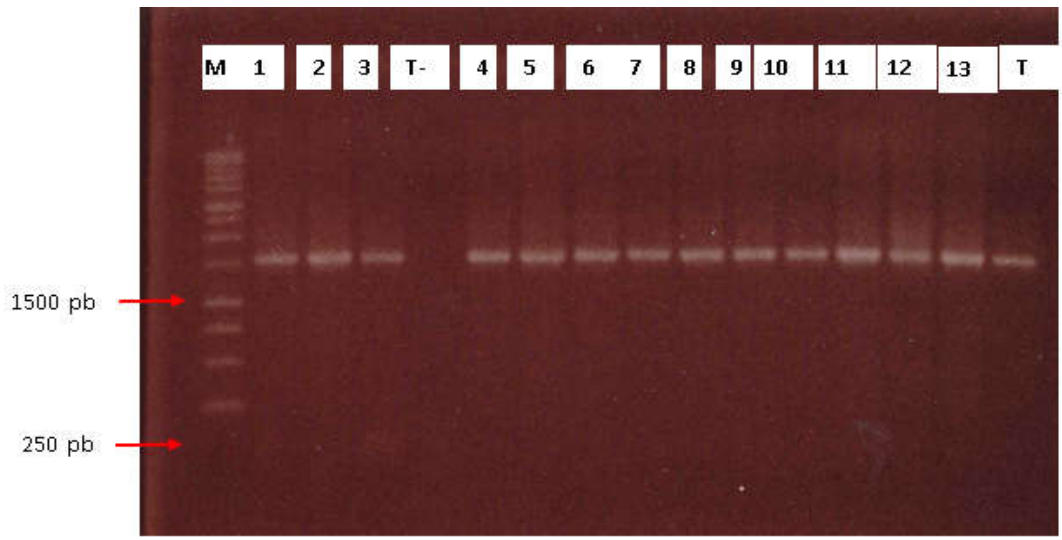
Isolation frequencies of *Bacillus* species by site and type of traditional cassava starter

Table 2 shows the different *Bacillus* species and their isolation frequencies in traditional cassava starter. *B. subtilis* and *B. amyloliquefaciens* were identified in the three types of traditional cassava starter from the five sites investigated. These two species predominated in the *Ebrié* starter of AB-KOUTE site, with frequencies of 12% for *B. subtilis* and 4% for *B. amyloliquefaciens*. *B. toyonensis*, *B. cereus* and *B. methylotrophicus* were isolated from *Ebrié* starter of AB-KOUTE and Abengourou, as well as in the *Alladjan* starter. *B. vallismortis* was isolated in the *Ebrié* and *Alladjan* starter whereas *B. pumilus* was found only in the *Ebrié* starter.

Seven *Bacillus* species isolated in this work were identified in the *Ebrié* starter of Abengourou site.

Enzymes produced by *Bacillus* species isolated from traditional cassava starters

Table 3 shows the distribution groups of enzymes produced by *Bacillus* strains. Phosphatases (alkaline phosphatase, acid phosphatase, naphthol phosphohydrolase) were produced by 71% of the species, followed by lipase producing strains (esterases C4, lipase esterases C8, lipases C14), with a frequency of 66%. Osidases (alpha-galactosidase, beta-galactosidase, beta-glucuronidase, alpha-glucosidase, beta-glucosidase, N-acetyl-beta-glucosaminidase, alpha-mannosidase, alpha-fucosidase) were produced by 32% species. Proteases (leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, alpha-chymotrypsin) were the enzymes least expressed by *Bacillus* species with a frequency of 24%. Table 4 indicates the enzymes produced by the *Bacillus* species isolated from traditional cassava starter. Among the nineteen enzymes sought, seventeen enzymes were produced by at least one or more *Bacillus* species whereas two enzymes, esterase (C4) and alpha-glucosidase, were produced by all *Bacillus* species isolated. The enzymes alpha-fucosidase and α-mannosidase were not produced by any species. A high percentage (90%) of *Bacillus* isolated, produced beta-glucosidase and esterase lipase, 82% produced alkaline phosphatase and 76% produced naptol phosphohydrolase.



M: DNA ladder, Lane 1 to 3 and 4 to 13: amplification of the 1500 bp 16S rDNA from 13 strains of *Bacillus,* T- : negative control; T+ positive control.

Figure 1: Electrophoretic profile of the 16S rDNA gene of *Bacillus* strains from traditional cassava starter.

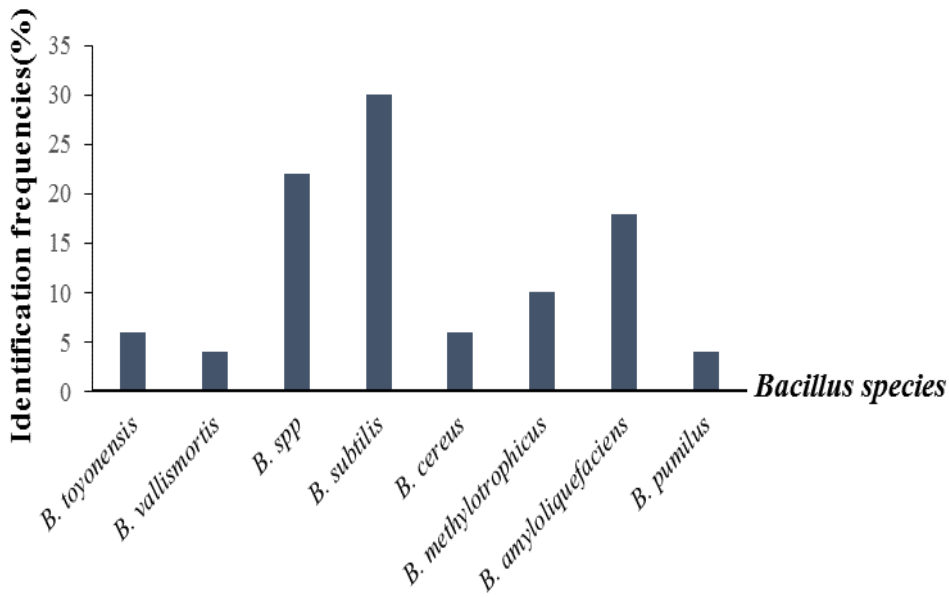


Figure 2: Isolation frequencies of *Bacillus* species from traditional cassava starter.

Table 2: Isolation frequencies of *Bacillus* species by site and type of traditional cassava starter.

	Identification frequencies				
Types of starter	***Ebrié* starter**			***Abouré* starter**	***Alladjan* starter**
Sampling site	**ABENG**	**BOUAKE**	**AB-KOUTE**	**BONOUA**	**AB-NG**
Strains					
B. toyonensis	1(2%)	0	1(2%)	0	1(2%)
B. vallismortis	1(2%)	0	0	0	1(2%)
Bacillus sp	2(4%)	3(6%)	2(4%)	1(2%)	3(6%)
B. subtilis	3(6%)	2(4%)	6(12%)	1(2%)	3(6%)
B. cereus	1(2%)	0	1(2%)	0	1(2%)
B. methylotrophicus	2(4%)	0	1(2%)	0	2(4%)
B. amyloliquefaciens	1(2%)	2(4%)	4(8%)	1(2%)	1(2%)
B. pumilus	1(2%)	1(2%)	0	0	0

N= Number; **F**: Frequency

Table 3: Groups of enzymes produced by *Bacillus* strains.

Types of starter	**Frequencies of *Bacillus* species (%)**					
	***Ebrié* starter**			***Alladjan* starter**	***Abouré* starter**	**Total**
	Sampling site					
Enzyme groups	**AB-KOUTE**	**BOUAKE**	**ABENG**	**AB-NG**	**BONOUA**	
Lipases	21	10	15	17	3	**66**
Phosphatases	20	12	17	17	5	**71**
Osidases	11	4	8	8	1	**32**
Protéases	9	2	5	7	1	**24**

Table 4: Enzymes produced by *Bacillus* species isolated from traditional cassava starters.

	Number and frequency of *Bacillus* strains [N(F)]					
Types of starter	***Ebrié* starter**			***Alladjan* starter**	***Abouré* starter**	
Sampling site	**AB-KOUTE**	**BOUAKE**	**ABENG**	**AB-NG**	**BONOUA**	**Total**
Enzymes						
PAL	10(20)	8(16)	10(20)	10(20)	3(6)	41(82)
EST	15(30)	8(16)	12(24)	12(24)	3(6)	50(100)
ESTLIP	14(28)	8(16)	9(18)	12(24)	2(4)	45(90)
LIP	3(6)	0(0)	2(4)	2(4)	0(0)	7(14)
LEUAR	9(18)	2(4)	8(16)	8(16)	3(6)	30(60)
VALAR	5(10)	0(0)	1(2)	3(6)	0(0)	9(18)
CYSAR	4(8)	0(0)	2(4)	3(6)	0(0)	9(18)
TRYP	2(4)	0(0)	1(2)	2(4)	0(0)	5(10)
α-CHY	3(6)	3(6)	1(2)	1(2)	0(0)	8(16)
PAC	8(16)	8(16)	9(18)	6(12)	2(4)	33(66)
NAPP	12(24)	7(14)	7(14)	10(20)	2(4)	38(76)
α-GAL	5(10)	0(0)	4(8)	3(6)	0(0)	12(24)
β-GAL	7(14)	0(0)	5(10)	5(10)	0(0)	17(34)
β-GLUC	2(4)	1(2)	2(4)	1(2)	0(0)	6(12)
α-GLU	15(30)	8(16)	12(24)	12(24)	3(6)	50(100)
β-GLU	14(28)	8(16)	9(18)	11(22)	3(6)	45(90)
NAβG	1(2)	0(0)	1(2)	1(2)	0(0)	3(6)
α-MAN	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
α-FUC	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)

Enzymes : PAL: Phosphatase alcaline ; EST: Estérase (C4) ; ESTLIP : Estérase lipase (C8) ; LIP: Lipase (C14) ; LEUAR : Leucine arylamidase ; VALAR : Valine arylamidase ; CYSAR : Cystine arylamidase ; TRYP : Trypsine ; α-CHY: Alpha-Chymotrypsine ; PAC : Phosphatase acide ; NAPP : Naphtol phosphohydrolase ; α-GAL : Alpha-galactosidase (mélibiase) ; β-GAL: Béta-galactosidase (lactase) β-GLUC : Béta-glucuronidase (hyaluronidase) ; α-GLU : Alpha-glucosidase (maltase) ; β-GLU : Béta-glucosidase (cellulase) ; NAβG : N-acétyl-béta-glucosaminidase (chitinase) ; α-MAN : Alpha-mannosidase ; α-FUC : Alpha-fucosidase ; **N**: Number; **F**: Frequency.

DISCUSSION

Seven species of *Bacilllus* have been identified from traditional starter of cassava analyzed, namely *B. toyonensis*, *B. vallismortis*, *B. subtilis*, *B. cereus*, *B. methylotrophicus*, *B. amyloliquefaciens* and *B. pumilus*. These species differ from those reported by Assanvo et al. (2002) in their study based on the microflora of the traditional starter of cassava for the production of *attiéké* in Dabou. Indeed, these authors reported three species of *Bacillus*, namely *B. sphaericus*, *B. brevis*, and *B. coangulans.* Contrary to these authors, the species identified in this study have been reported by other authors during the fermentation of cassava roots. *B. subtilis*, *B. cereus*, *B. pumilus* and *B. amyloliquefaciens* were identified by Amoa-Awua and Jakobsen (1995) during the process of the cassava roots fermentation. Bouatenin et al. (2013) identified the presence of *B. amyloliquefaciens* and *B. cereus* from *Adjoukrou* traditional cassava starter. *Bacillus* species can produce α-amylase which can hydrolyze starch and release the sugars for the production of organic acids mainly lactic acid. *B. subtilis* (30%) was the important isolate among the seven species identified in this study. This result corroborates those of Adewumi et al. (2009) during the production of *gari* and Azokpota et al. (2007). *Bacillus* are found to produce some products such as vitamin B3, B12, K and digestive enzymes such as amylase, protease, lipase (Tamehiro et al., 2002).

The enzymatic characterization of the different species in the present study has showed that these could produce osidases, phosphatases, lipases and proteases. The ability of *Bacillus* species to produce osidases during the cassava root fermentation has been reported by several authors including Amoa-Awua and Jakobsen (1995), Bouatenin et al. (2013), Ehon et al. (2015). Indeed, these osidases are involved in the hydrolysis of starch and the release of simple sugars which are essential for the synthesis of organic acids.

Bacillus species have shown overall a good α-glucosidase (100%) and β-glucosidase (94%) synthesis ability. β-glucosidase is the main enzyme responsible for the natural degradation of the cyanogenetic glucosides of cassava into glucose and acetone cyanohydrin (Djoulde et al., 2005). According to Mkpong et al. (1990), 65% of homology was observed between β-glucosidase activity and that of linamarase. Concerning α-glucosidase, it is involved in the hydrolysis of isomaltose resulting from the degradation of starch by α-amylase.

Most of the species could produce phosphatases, contrary to the study of Parvathi et al. (2009) on the species of *Bacillus pumilus* isolated from the aquatic environment. The lipolytic and proteolytic activity of *Bacillus* species have been reported in various studies (Ouoba et al., 2003a; Ouoba et al., 2003b; Mo et al., 2010; May and Al-Allaf, 2011). According to Azokpota et al. (2006), the proteolytic activity of *Bacillus* species enables them to contribute to the release of bioactive peptides which play a very important role in the enzymatic process during fermentation. Moreover, in potein-rich products, the proteolytic activity of the *Bacillus* species would be responsible of the pH increase (Steinkraus, 1984). According to NDir et al. (1997), the lipolytic activity of *Bacillus* spp has an impact on the organoleptic quality of the products.

Conclusion

Seven species of *Bacillus* (*Bacillus subtilis, Bacillus cereus, Bacillus pumilus, Bacillus amyloliquefaciens, Bacillus methylotrophicus, Bacillus vallismortis* and *Bacillus toyonensis*) were identified from the three types of traditional starter. *Bacillus subtilis* was the most present among the seven species. Several species of *Bacillus* have shown a capacity to produce various enzymes notably osidases, phosphatases, lipases and proteases. Among the osidases, β-glucosidase and α-glucosidase were the most produced by the species. All species were able to produce esterase (C4) and alpha-glucosidase enzymes. It should also be noted that some species have been able to produce up to 15 enzymes.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

ZBAB Collected the traditional cassava starter samples; ACK, OK and TDY contributed to the performing of the experiments and writing of the manuscript; NRK and MK revised critically for important intellectual content. All authors read and approved the final manuscript.

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