European Scientific Journal March 2015 edition vol.11, No.9 ISSN: 1857 – 7881 (Print) e - ISSN 1857- 7431

PRELIMINARY TECHNOLOGICAL PROPERTIES ASSESSMENT OF *BACILLUS* SPP. ISOLATED FROM TRADITIONAL CASSAVA STARTERS USED FOR ATTIEKE PRODUCTION

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Abstract

This study was carried out to select *Bacillus* strains as potential microbial starters for cassava dough fermentation into *attiéké* (a fermented and steamed granular cassava, couscous-like product) regarding their enzymes (amylase, pectinase, cellulase, phytase, tannase and beta-glucosidase) production. For this purpose, 42 presumptive *Bacillus* spp were isolated from traditional cassava starters and screened *in vitro*. All the selected strains produced amylase, pectinase, cellulase while 37 (88.09%) produced phytase and 27 (64.28%) were able to produce beta-glucosidase. Regarding these technological properties mainly production of all these enzymes, only 13 *Bacillus* strains (30.95%) could be used as potential microbial starters in association with lactic acid bacteria for the controlled fermentation of cassava dough in order to improve and standardize the organoleptic quality of *attiéké* by softening and detoxification actions.

Keywords: Attiéké, Bacillus, enzyme, fermentation, cassava

Introduction

Cassava (*Manihot esculenta* Crantz) is the third agricultural resource after rice and maize as a source of calories in tropical countries (FAO, 2008). Cassava is traditionnally processed into a wide variety of fermented products with different local names (fufu, agbelima, chikwangue, farhina) (Longe, 1980 ; Hahn, 1989; Amoa-Awua, 1996). In Côte d'Ivoire, the most popular food derived from fermented cassava is *attiéké* (Djeni *et al*., 2008). Recently,

this staple food has become very popular as well it is currently exported in Europe, USA and in Asia by the African diaspora (Kacou, 2000). Cassava fermentation during *attiéké* production requires the use of a tradtional starter called "mangnan" whose preparation varies according to ethnic groups. 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). *Bacillus* spp, lactic acid bacteria, yeasts and moulds are microorganisms which could play an important role in the cassava fermentation (Assanvo *et al*., 2006). The positive effects of these microorganisms include product preservation, flavor development and cyanide reduction (Akindahunsi *et al*., 1999). There are several enzymes (amylase, cellulase, tannase, pectinase and beta-glucosidase) of importance produced by different species of *Bacillus* (Schallmey *et al*., 2004).

Amylase improves digestibility while pectinase and cellulase contribute to soften quickly cassava dough (Amoa-Awua and Jakobsen, 1995; Bouatenin *et al*., 2012). As concern beta-glucosidase, this enzyme breaks down cyanogenic glucosides and contributes significantly to the detoxification of cassava during *attiéké* fermentation (Bouatenin *et al*.,2013). In addition, *Bacillus* spp play an important role in inhibiting rot and therefore induce a better conservation of the fermented food by production of antimicrobial compounds (Jacques, 2009, 2011; Savadogo *et al*., 2011). Some species of *Bacillus* could also produce lactic acid (Rosenberg *et al*., 2005; Ouyang *et al*., 2013) which gives to *attiéké* an acidulous taste. However, *attiéké* processing technology is characterized by empiric steps which are very difficult to control (Sotomey *et al*., 2001). Moreover, differences in the traditional starters used are the basis of the different organoleptic qualities obtained for various types of *attiéké* sold on the market (Desmazeaud, 1996; Wesby, 1991).

In the present study, the biochemical characterization and the enzymatic profile (amylase, pectinase, cellulase, phytase and beta glucosidase) of *Bacillus* spp. originated from traditional starter were investigated in order to develop a suitable industrial starter culture in combination with lactic acid bacteria for the standardization of *attiéké* production.

Material and methods

Samples collection and isolation of *Bacillus* spp.

Approximately 100 g of traditional cassava starters (Adjoukrou starters) were collected in the big areas of *attiéké* manufacturing units from eleven (11) areas in the District of Abidjan (Koumassi, Abobo, Marcory Attécoubé, Port-Bouet, Treichville, Adjamé, Yopougon, Cocody, Bingerville

and Anyama) and 3 areas in peri-urban areas of this District mainly Bassam, Dabou and Jacqueville. Traditional cassava were prepared in each processing area with boiled cassava roots packed in specific fermenting bag and left to ferment during three days by microbial flora contaminated the fermenting bag. All collected samples were mixed and 10 g were transferred in 90 mL of sterile buffered peptone water. After homogenization, the mixture was incubated at 30 °C during 18 h for an enrichment. Then the medium was treated at 80 °C for 10 min to select bacteria with sporulation capacity. The bacteria were isolated by continual streaking on nutrient agar supplemented with 0.1 % nystatin to inhibit the growth of fungal organisms. Five presumptives colonies were randomly picked from the agar plates and identified based on morphological and biochimical characteristics.

Morphological and biochemical characterization

Morphological and biochemical characterization of isolates were perfomed by Gram staining method and catalase test. For mobility test, each colony was sub-cultured in brain-heart-broth and the medium was then incubated at 30°C for 4 h. After incubation, the mobility was determinated by using of an optical microscope.

The isolate identified as *Bacillus* spp. were stored at - 80° C in vials containing nutrient broth added with 20 % (v/v) glycerol (Merck).

The ability of these strains to ferment different carbohydrate sources (raffinose: indigestible sugar, cassava starch and glucose) was studied. The agar medium was prepared with 1 % yeast extract; 2 % peptone casein, 0.005 % bromocresol purple, 1.7 % agar and 1 % of various carbohydrates sources. Then, the agar-medium were inoculated with bacterial colonies and incubated at 30 ° C for 48 h in anaerobic condition. The capacity of the strains to ferment each carbohydrate source is assessed by change of medium color comparatively to the negative control.

Production of enzymes (pectinase, amylase, tannase, cellulase and phytase)

The screening of different enzymes was carried out using the method described by Ouattara *et al*. (2008). Thus, the mineral medium containing (0.28 % $(NH4)_2SO_4$, 0.6 % K_2HPO_4, 0.01 % $MgSO_4$ $7H_2O$, 0.2 % KH_2PO_4, 0.5 % glucose) was supplemented with 0.02 % yeast extract, 2 % agar and 1 % different carbohydrate sources (pectin, cassava starch, tannic acid, carboxyl methyl cellulose, phytic acid) as only carbohydrate source related to different enzymatic activities. The medium was then adjusted to pH 5.6 and four wells (0.5 cm diameter and 2 to 3 mm deep) were performed aseptically in agar medium. The wells were then subsequently loaded with 7 µL of bacterial suspension (density of 1 at 600 nm). The different Petri

dishes were incubated at 30° C for 48 h. After incubation, the solid medium was flooded with a solution of iodine and potassium iodide to reveal clear zone around the wells, indicating the presence of an enzymatic activity.

Production of beta-glucosidase

The capacity of *Bacillus* to produce beta glucosidase was carried out by the method described by Weagant *et al*. (2001) using 4-nitrophenyl-β -D-glucopyranoside as substrate. Briefly an aliquot (0.75 mL) of bacterial suspension (Mc Farland standard turbidity N°3 in tryptone-salt) was cultivated in 0.25 mL of medium containing: 0.1 g of 4-nitrophenyl-β-D-glucopyranoside in 100 mL of NaH_2PO_4 solution (0.666 M) adjusted to pH 6 and sterilized although MF- Millipore MCE Membrane (0.2 microns). Then the medium was incubated at 30 ° C for 12 h. After incubation, the bacterial growth was stopped by adding 1 mL of sodium carbonate solution (1M). The appearance of yellow coloration indicates beta-glucosidase activity.

Results and Discussion

Morphological and biochemical characterization

Forty-two (42) regular rods strains with Gram positive, catalase positive, mostly peritrichous mobility were selected as presumptive *Bacillus.* Among them, 36 (85.71 %) had the ability to ferment glucose into acid product without gas production, 3 isolates (7.14 %) fermented glucose with gas production while 3 strains (7.14 %) had not the ability to ferment glucose. None strains were able to ferment the cassava starch and raffinose.

The presence of *Bacillus spp* in traditional cassava starters used for *attiéké* production was indicated by several authors. Indeed, *B.circulans , B. lentus B. sphaericus, B. brevis, B. coagulans, B. amyloliquefacien* and *B. cereus* were identified as dominant species (Coulin *et al*.,2006; Assanvo *et al*., 2006 ; Bouatenin *et al*.,2013). Acidification of medium from glucose is in agreement with results of Sembene (2002) which showed the ability of *Bacillus* to ferment glucose. Also, Combet *et al*. (1995) reported that the strains of *Bacillus* could ferment the monosaccharides with CO_2 production under some culture conditions. Despite this interessant capacity, Bouatenin *et al*. (2012) have cleary demonstrated that, lactic acid bacteria are most responsible of acidification during cassava fermentation. Indeed, the acid quantity produced by *Bacillus* spp during "in vitro" fermentation ranged from 0.0045 to 0.047 % after 24 h. While lactic acid bacteria produced twice in the same conditions (Bouatenin *et al.,* 2012). *Bacillus substilis* produce high concentrations of lactate which acidifies the retting juice (Asiedu, 1992).

Production of pectinase, amylase, tannase, cellulase and phytase

The 42 presumptive *Bacillus* spp. showed different production levels of the studied enzymes indicated by the presence of halos diameters ranging from 0.8 to 3.5 cm around the wells (Fig:1).

Figure 1: Enzyme production (amylase, pectinase, cellulase and phytase) by the selected *Bacillus* strains. The agar wells were inoculated with the bacterial suspension. The appearance of clear zones after 48 h of incubation at 30°C around the wells indicates enzymatic activity.

Three strains (7.14%) produced highly amylase, 17 (40.48%) for pectinase, 7 (16.66%) for cellulase and 16 (38.09%) for phytase. However none tannase activity was synthesized (Table1).

Table1: Screening of Amylase, Cellulase, Pectinase and Tannase productions.

	Enzyme activities of Bacillus strains (%)				
Halos diameters (cm)	α-Amylase	Pectinase	Cellulase	Phytase	Tannase
0	0	0	0	5 (*11.90*)	0
0.8 – 1.5	21 21 (*50*)	13 (*30.95*)	18 (*42.86*)	10 (*23.81*)	0
1.6 – 2.5	18 18 (*42.86*)	12 (*28.57*)	17 (*40.48*)	11 (*26.19*)	0
2.6 – 3.5	33 3 (7.14)	17 (*40.48*)	7 (*16.66*)	16 (*38.09*)	0

In parentheses are indicated percentages.

The ability of *Bacillus* spp. to produce different hydrolytic enzymes is an interesting technological property. The presence of amylase activity during cassava fermentation was mentioned by Oyewole and Odunfa (1992) and Cordeiro *et al*. (2003). Strains with amylolytic activity are able to

hydrolyze the starch contained in cassava dough to produce sugar used as energy source for the growth of differents microorganisms involving in cassava dough fermentation (Ketiku and Oyenuga, 1972). In our study, none strains had fermented cassava starch. Indeed to degrade starch in fermentable sugar for acid production, the strains should possess in addition to alpha-amylase more other enzymes namely β- amylase, Amyloglucosidase or glucoamylase, isoamylase and pullulanase (Adeyanju *et al*.,2007) .

Several studies revealed that the rigidity of vegetable tissue is due to pectins and cellulose (Okafor *et al.*,1984; Bombily, 1995; Alkorta *et al.*, 1998; Caffall and Mohnen, 2009). *Bacillus* spp. is responsible for the break down of cassava texture through cellulase activity (Bas 4 ; Bas 67 ; Bas 10 ; Bas 97; Bas 99...) (Amoa-Awua and Jakobsen, 1995). Moreover, the occurrence of *Bacillus* spp. producing pectinase in the microflora of traditional *attiéké* starters is of considerable interest since published information on the involvement of *Bacillus* spp. in attiéké processing is very limited. Thus, these microorganisms (Bas 4 ; Bas 22 ; Bas 18 ; Bas 14 ; Bas 57 ; Bas 102 ; Bas 67 ; Bas 58 ; Bas 101 ; Bas 66 ; Bas 97; Bas 99...) would break down the structures of cellular partition (pectin) of cassava tubers and thus contribute to soften quickly cassava dough during fermentation (Bouatenin *et al*., 2012).

Production of beta-glucosidase

The production of beta-glucosidase by the 42 *Bacillus* strains were also expressed at differents level. Indeed, the great producers strains induced important coloration of the culture medium, while the most low-producing strains induced a lesser visual color (Fig:2).

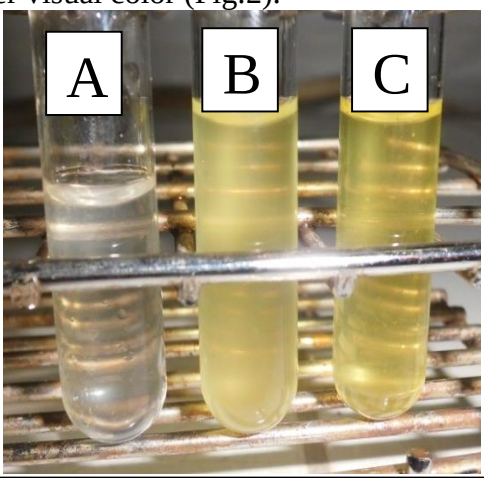

Figure 2: Production of beta glucosidase by the selected *Bacillus* strains: A: negative, B: low production and C : great production.

Tryptone-salt containing inoculums, was added with 4-nitrophenyl-β-D-glucopyranoside and was cultivated during 12 h at 30° C. The beta glucosidase activity was revealed by Na_2CO_3 (1 M).

On all strains tested, 15 (35.7%) *Bacillus* strains had not produced the beta glucosidase, while 3 (7.14%) were weak producer, 11 (26.19%) were middle producer and 13 (30.95%) showed a most important capacity to produce beta-glucosidase.

The presence of antinutritional factors (acid tannic, phytate and cyanogenic glucoside) in cassava will be harmful for human health (Koua, 2013) and the reduction of antinutritional factors in foods involve the use of microbial strains that have the ability to produce tannase, phytase and beta-glucosidase during processing. The ability of strains to produce beta-glucosidase is important because cassava contains various amounts of cyanogenic glucosides. Bacteria with ß-glucosidase activity (Bas 22 ; Bas 102 ; Bas 67 ; Bas 58 ; Bas 101 ; Bas 66; Bas 99...) may probably able to hydrolyse linamarin into glucose and acetone cyanohydrin and then, use the glucose for their metabolism. These strains were able of breaking down cyanogenic glucosides and contributed significantly to the detoxification of cassava during *attiéké* fermentation (Bouatenin *et al*.,2013).

Table 2 presents the top 20 isolated microbial strains based on their technological properties. Among the 42 tested isolates, 13 strains (Bas 4, Bas 22, Bas18, Bas 12, Bas 14, Bas 57, Bas 102, Bas 67, Bas 58, Bas 101, Bas 66, Bas 97 and Bas 99) were able to produce all the enzymes.

Table2 : Top 20 of isolated Bacillus strains showing starters potentialities

Strains	Amylase	Pectinase	Cellulase	Phytase	β- glucosidase
Bas 4	+++	+++	+++	+++	++
Bas 22	++	+++	+	+++	+++
Bas 18	++	+++	++	++	++
Bas 12	+	++	+	+++	++
Bas 13	+++	+++	++	++	-
Bas 14	++	+++	++	++	+
Bas 105	+	++	++	+++	-
Bas 69	++	+++	+++	-	+++
Bas 57	++	+++	++	++	++
Bas 102	++	+++	++	+++	+++
Bas 67	++	+++	+++	+++	+++
Bas 58	++	+++	++	++	+++
Bas 101	+	+++	+++	+++	+++
Bas 66	++	+++	++	++	+++
Bas 90	++	+++	+	+++	-
Bas 107	++	++	++	+++	-
Bas 84	++	++	++	+	-
Bas 86	+	+++	++	+++	-
Bas 97	++	++	+++	+++	++
Bas 99	+++	+++	+++	+++	+++

+ :low producer ; ++ :medium producer ; +++ :great producer ; - : negative reaction

For potentials starters cultures by the control of the fermentation process of cassava dough into *attiéké*, 13 strains (Bas 4, Bas 22, Bas18, Bas 12, Bas 14, Bas 57, Bas 102, Bas 67, Bas 58, Bas 101, Bas 66, Bas 97 and Bas 99) could be used in association with lactic acid bacteria.

Conclusion

The results of the present work indicate importance of *Bacillus* during the fermentation of cassava dough, initiated by a traditional starter for *attiéké* production. According to their performance for enzymes production, 13 presumptive *Bacillus* spp. were preselected, they will undergo other analyzes and identification of these microorganisms, after which some strains could be to use for a future pilot plant fermentation in association with lactic acid bacteria for better a acidificication of cassava dough for *attiéké* production. Starter cultures can improved quality and safety, as compared to spontaneous and uncontrolled traditional fermentation.

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