Turkish Journal of Agriculture - Food Science and Technology, 4(12): 1077-1084, 2016

Turkish Journal of Agriculture - Food Science and Technology

www.agrifoodscience.com,
Turkish Science and Technology

Towards Added Value Attieke Production in Côte d'Ivoire Using *Bacillus* spp. as Starters

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ARTICLEINFO

Article history:
Received 05 April 2016
Accepted 27 October 2016
Available online, ISSN: 2148-127X

Keywords:

Bacillus spp.
Technological properties
Attiéké
Standardization
Cassava

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ABSTRACT

In Côte d'Ivoire, the most fermented cassava food product is "attiéké". Various microorganisms involved in this fermentation process. *Bacillus* spp. are well-known for their multi-potential enzymatic activities. In this study, *Bacillus* spp. strains were studied for their ability of growing in environmental stress as follow: NaCl (2 to 9%) and lactic acid (0.1 to 1%). The growth of the studied strains was inhibited at 5% (1 strain), 7% (2 strains) and 8% (7 strains) for NaCl and beyond 0.25% for lactic acid. The ability of the isolated *Bacillus* strains to ferment cassava dough for "attiéké" production was also tested. The results of sensory tests showed that "attiéké" produced with *Bacillus* spp. strains was quite similar to "attiéké" control (traditional "attiéké") except for the brilliance and granulation for which the control obtained the highest scores. The present research indicated that cassava dough fermentation, initiated by the inoculation of *Bacillus* strains associated with or without lactic acid bacteria should be useful to improve and standardize the quality of "attiéké" produced in Côte d'Ivoire.

Introduction

Cassava (*Manihot esculenta* Crantz) is among the most important root crops in the world by providing food for one billion people (Bokanga, 2001; FAO, 2008).Cassava roots are often subjected to fermentation processing before consumption and various fermented cassava products are available; these are: "gari", "foofoo", "lafun", "attiéké", etc (Obadina et al., 2009; Coulin et al., 2006). In Côte d'Ivoire, the most popular fermented cassava food is "attiéké" defined as steamed semolina (Assanvo et al., 2002; Dje et al., 2008). The food "attiéké" is consumed two to three times a day in combination with meat, fish or vegetables and this food is one of the main generating of monetary incomes for producers (Toka et al., 2008; Djeni et al., 2011). According to Aboua et *al*. (1990), the total consumption of Ivorian population is about 28,000 to 34,000 tons of "attiéké" per year which represents the equivalent of 40,000 to 50,000 tons of fresh cassava. Despite this economical and nutritional importance, , the preparation of "attiéké" by women is still an informal activity mainly based on their traditional experience and not on a rational knowledge (Essers et al., 1993; Fortin et al., 1998; Mosso et al., 2000). The processing of cassava roots into "attiéké" involves the following steps: peeling, cutting, washing and grating. During grating step, the cassava dough is mixed with 10% of a traditionally prepared microorganisms inoculum called ''*mangnan*'' and 0.1% of palm oil. This inoculum was previously prepared by the storage of boiled cassava roots for three days of natural fermentation in unwashed jute bag. The inoculated cassava pulp is then fermented overnight in covered bins. Afterwards, the fermented pulp is filled into bags and pressed for several hours in order to drain the liquid phase. The remaining press cake is removed from the bags and squeezed through a sieve to obtain granules that are sun-dried and then cleaned to take out fibers and wastes. The dried granules are finally steamed in order to produce "attiéké" (Coulin, 2004). It is important underlying that the traditional inoculum used for "attiéké" production consists of a wide variety of microorganisms (lactic acid bacteria, *Bacillus* spp., yeasts and moulds) which constitutes the main source of microbial activities during the cassava dough fermentation (Coulin et al., 2006; Djeni et al., 2008). Among these microorganisms, *Bacillus* spp. produces different enzymes that detoxify and soften cassava dough (Bouatenin et al., 2012). Moreover *Bacillus* spp. can grow in slightly acidic medium and resist to other environmental stress factors (Coulin et al., 2006; Kumar et al., 2013). Some species of *Bacillus* could also produce lactic acid (Rosenberg et al., 2005; Ouyang et al., 2013) which may give to "attiéké" an acidulous taste. Therefore, an extensive description of the

microbial diversity of cassava dough appears as prerequisite for the development of starter cultures to improve the indigenous processing. This description should include the biochemical identification of the representative microorganisms (Miambi et al., 2003). This would justify the scientific efforts going towards the development of starters containing high concentrations of living microorganisms, in order to ensure the regularity and stability of the final product (Yao et al., 2013). Thus, the aim of this study is to search out *Bacillus* strains possessing interesting technological properties which could be used as starter cultures in order to control and standardize the fermentation of cassava dough for producing "attiéké" with good quality.

Material and Methods

Samples Collection, Bacillus Identification and Enzymes Production

Traditional cassava starters ("*mangnan*") were 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. *Bacillus* spp. strains were isolated after an enrichment step described as follow: 10 g of different samples were diluted in 90 mL of sterile buffer peptone water and incubated at 30°C during 18 h. The medium was then heat in water bath at 80°C for 10 min in order to select spore forming bacteria on nutrient agar (Scharlau). Concerning *Bacillus* spp. identification, morphological and biochemical characterization of isolates were performed by Gram staining and catalase tests. For motility test, each colony was sub-cultured in brain-heart-broth medium at 30°C for 4 h. For enzymes production such as amylase, pectinase, cellulase and phytase, the tests were carried out using the method described by Ouattara et al.(2008); while for production of β–glucosidase enzymes, the method described by Weagant et al. (2001) was performed.

Effects of Environmental Factors Stress on The Growth Parameters of Bacillus Spp.

Effect of temperature: Different strains were purified on nutrient agar (Scharlau) and inoculated in nutrient both containing 1% of glucose (Scharlau). Then, 100 µL of pre-culture (OD_{600} = 1) was used to inoculate 5 mL of the same broth. The medium was incubated at various temperatures (30°C; 37°C; 45°C and 55°C) for 18 h. After incubation, the optical density (OD) was recorded at 600 nm to determine the optimum growth temperature.

Effect of NaCl, Lactic acid and pH: The response of *Bacillus* spp. strains to the osmotic and acid stress induced by NaCl and lactic acid was carried out using the method described by Sow (2004). The broth medium containing 2% of glucose, 1% of yeast extract and 1% of casein pepton (Oxoid) was supplemented with different concentrations of NaCl (2; 3; 4; 5; 7 and 9%) and lactic acid (0.1; 0.2; 0.25; 0.75 and 1%). A pre-culture was made in medium broth and was incubated at 37°C for 16 h. 100 µL of the pre-culture (OD= 1 at 600 nm) were used to inoculate 5 mL of the medium mentioned above. Then, a medium was incubated at 37°C for 18 h and the bacterial growth was recorded by reading the OD at 600 nm. To evaluate the influence of pH on microbial growth, the same method mentioned above was performed for different pH values: 3; 4; 5; 7; 9 and 11.

Lactic Acid Production

For lactic acid production, the *Bacillus spp.* strains were pre-cultured in nutrient broth (Scharlau) containing 1% of glucose (Scharlau). Two reference strains of lactic acid bacteria: *lactobacillus plantarum* W1582 and W 1366 (Centre for Industrial Microbiology, Gembloux, Belgium) were tested to compare their performance in Man Rogosa and Sharpe (MRS) medium (Scharlau) at 37°C for 16 h and pH 7. After incubation, 100 µL of the pre-culture (OD= 1 at 600 nm) were used to inoculate 5 mL of the broth medium (pH=7) mentioned above. The mixture obtained was centrifuged for 15 min at 4,000 rpm and the supernatant was titrated with a solution of NaOH (0.1 N), using phenolphthalein as indicator. The total titratable acidity was calculated as a percentage of lactic acid.

Laboratory-Scale of "Attiéké" Production

Following the previous studies of the environmental stress and lactic acid production, 5 potential *Bacillus* strains were selected for cassava dough fermentation into "attiéké" production.

Inoculum preparation: One hundred and fifty grams (150 g) of cassava mash were inoculated with 10^8 CFU/g of *Bacillus* spp. strains. The resulting mixture was incubated at 30°C for 24 h. Concerning the preparation of traditional "attiéké" (control), the traditional inoculum (*mangnan*) was used.

Fermentation of cassava dough: After grinding of cassava roots, the dough was portioned into six different sterile plastic bags. The different portions of cassava dough were inoculated with the starter culture of *Bacillus* spp. and incubated at 30°C for 12 h. After incubation, the fermented cassava dough was pressed with a manual screw press, to remove water. The press cake, was sieved on a synthetic sieve, in order to remove thick fibers and pieces of pulp insufficiently ground. The powdery mass was then granulated during 20 minutes into grains similar to semolina. The grains were dried in the sun and the cassava semolina was steamed to obtain "attiéké".

Total Titratable Acidity

Total titratable acidity was determined according to the method described by Nout et al. (1989). Ten (10) grams of each sample (fermented cassava dough and "attiéké") were diluted in 90 mL of distilled water and filtered through a Whatman filter paper. 10 mL of the filtrate was titrated with NaOH (0.1 N) using phenolphthalein as indicator; the total titratable acidity is given by the following formula:

$$TTA = \frac{V_b \times N_b \times 0.09 \times 100}{V_f}$$

TTA = Total titratable acidity (%)
V_b = Volume of the basic solution (mL);
N_b = Normality of basic solution;
0.09 = Conversion factor for lactic acid;
V_f = Volume of filtrate (mL).

pH Determination

The determination of pH was carried out according to the protocol described by Nout et al. (1989). Ten (10) grams of each sample were mixed with 20 mL of sterile distilled water. The mixture obtained was filtered through a Whatman filter paper. The pH was then recorded by using a pH meter (Hanna HI 2223).

Sensory Analysis

Sensorial quality attributes of the different "attiéké" samples were evaluated using distinguishing parameters such as, colour, brilliance, graininess, presence of fibers, aroma, texture, odor, sourness and sweetness. Ten (10) trained panelists were chosen to determine the general acceptability of "attiéké" produced. A sensorial profile was done by a 9- point hedonic rating scale based on the different degrees of acceptability of the product. This profile was conducted with scores ranging by '9' (having excellent characteristic) to '1' (very weak characteristic) for the attributes description.

Statistical analysis

Experiments were conducted in triplicate and the results were expressed as mean ± SD. The results were also subjected to analysis of variance (ANOVA) and differences between means were assessed by Duncan multiple range tests at the significance defined at $P \leq 0.05$, using SPSS 18.0 software and Excel 2007.

Results

Bacillus Identification and Enzymes Production

A total of 141 *Bacillus* strains were isolated from the traditional "mangnan". Among these, 10 strains characterized by interesting enzymatic properties were subjected to the effects of environmental stress and the production of lactic acid. The enzymatic properties of *Bacillus* strains used for cassava dough fermentation are summarized in Table 1.

Effect of Temperature

The growth curves of the 10 *Bacillus* strains subjected to various incubation temperatures are presented in Figure 1. The selected *Bacillus* strains had their optimum growth at 37°C, unlike the *Bacillus* strains Bas 13 and 103, which had their optimum growth at 45°C. The stabilization of the growth curve was observed at 55°C. Concerning *Bacillus* strain Bas 58, a decrease of its growth rate was observed at 45°C.

Effect of NaCl Concentration

The growth of *Bacillus* strains decreased with the increasing of NaCl concentration in the medium. For 5% of NaCl concentration, none growth was observed for the strain Bas 13. Furthermore the growth of Bas 99 and 102 was not observed for 7% of NaCl concentration. Also, for the other *Bacillus* strains (Bas 4; 18; 57; 58; 66; 97and107), their growth was inhibited at 8% of NaCl concentration (Figure 2).

Effect of Lactic Acid Concentration

The increasing of lactic acid concentration (0.1 to 1%) caused a decrease of *Bacillus* spp. growth rate, followed by inhibition. The growth of *Bacillus* (Bas 57 and 58) was inhibited for 0.25% of lactic acid concentration, whereas the growth of the other isolates, was inhibited for a concentration grater than 0.25% (0.25% to 1%) (Figure 3).

Effect of pH

For pH values between 3 and 4, it was observed a slowdown of microbial growth. Beyond pH 4, an increasing of growth rate was observed up to a maximum of pH 7 for *Bacillus* strains (Bas 18; 57; 58; 66; 97; 99; 102 and 107). In addition, Bas 4 and 13 reached their maximum growth for pH values of 8 and 9. Beyond the values of pH 7, 8 and 9, a decline of the microbial growth was observed for all the tested strains (Figure 4).

Table 1 Enzymatic properties of isolated *Bacillus spp*. strains

Strains	Amylase	Pectinase	Cellulase	Phytase	β- glucosidase
Bas 4	+++	+++	+++	+++	++
Bas 18	++	+++	++	++	++
Bas 13	+++	+++	++	++	-
Bas 57	++	+++	++	++	++
Bas 102	++	+++	++	+++	+++
Bas 58	++	+++	++	++	+++
Bas 66	++	+++	++	++	+++
Bas 107	++	++	++	+++	-
Bas 97	++	++	+++	+++	++
Bas 99	+++	+++	+++	+++	+++

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

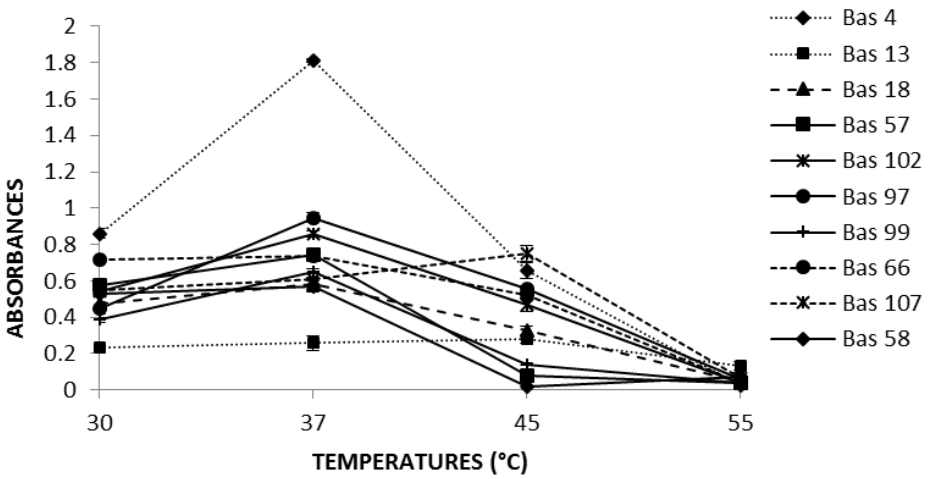

Figure 1 Effect of temperature on *Bacillus* strains growth. The observation is a mean of 3 replicate experiments (n=3)

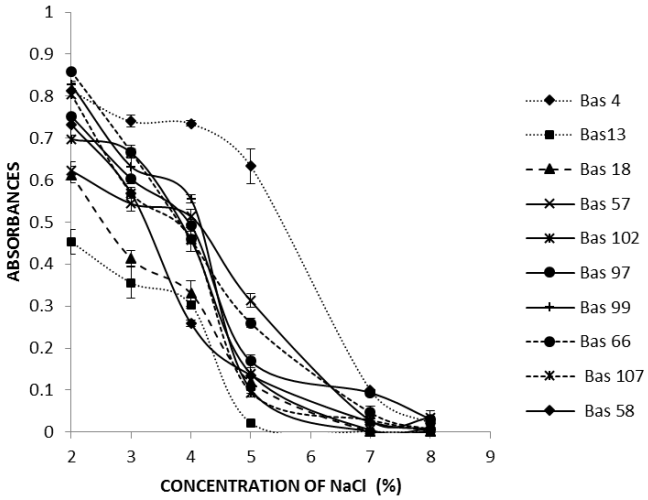

Figure 2 Effect of NaCl concentration on *Bacillus* strains growth. The observation is a mean of 3 replicate experiments (n=3)

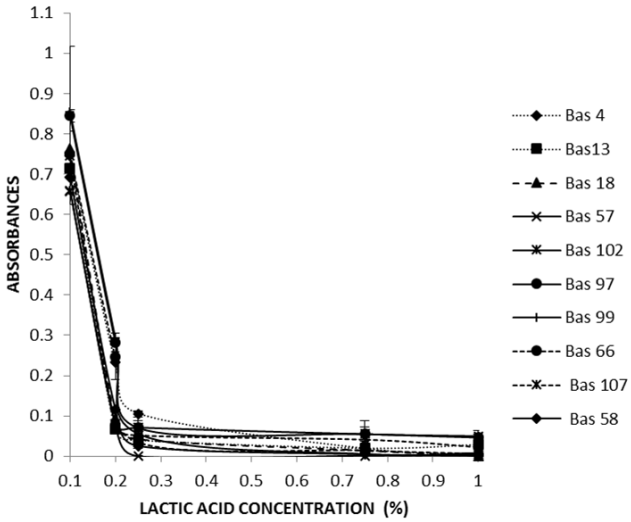

Figure 3 Effect of lactic acid concentration on *Bacillus* strains growth. The observation is a mean of 3 replicate experiments (n=3)

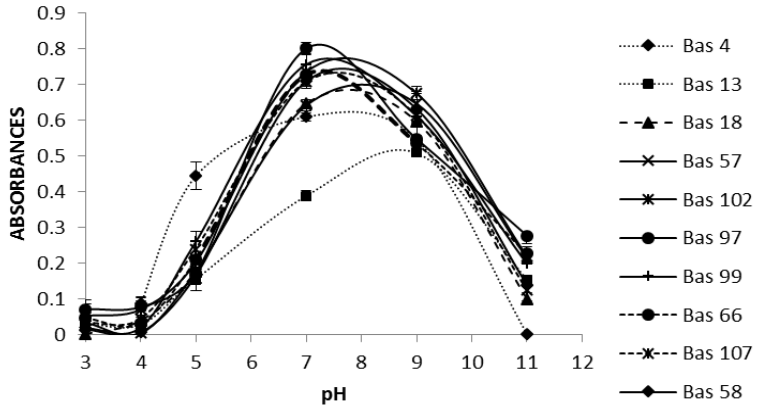

Figure 4 Effect of pH variation on *Bacillus* strains growth. The observation is a mean of 3 replicate experiments (n=3)

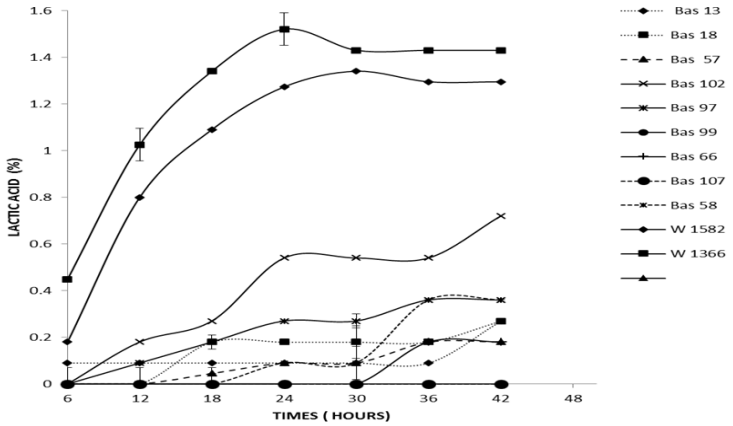

Figure 5 Lactic acid production of microbial strains. The observation is a mean of 3 replicate experiments (n=3)

Lactic Acid Production

Two strains of lactic acid bacteria (*lactobacillus plantarum* W1582 and W 1366) produced a great quantity of lactic acid in comparison with *Bacillus* strains. Only Bas 102 produced 0.18 to 0.54% of lactic acid during 24 h of incubation (Figure 5).

Total Titratable Acidity and pH

The different pH values for fermented cassava pulps inoculated with *Bacillus* spp. strains decreased after 12 hours. Also, the pH values of different samples of "attiéké" obtained with *Bacillus* spp. strains (Bas 4, Bas 58, Bas 66, Bas 97 and Bas 102) had a lower pH compared to the control (Table 2).

Sensory Test

Sensory test for "attiéké" produced by using the different *Bacillus* strains (Bas 4, Bas 58, Bas 66, Bas 97 and Bas 102) as starters was judged slightly similar to the control. Nevertheless the control one had a better brilliance, contained less fibers and more rounded grains. For the brilliance, "attiéké" control obtained the highest score of 3.95 compared to the scores (2.9 to 3.5) of "attiéké" produced with *Bacillus* spp. The tested "attiéké" control had the best graininess and acidulous taste scores (6.95 and 3.95) and contained less fibers (score of 2.95 against scores ranged from 3.2 to 4.05 for "attiéké" produced with *Bacillus* strains) (Figure 6).

Discussion

The presence of *Bacillus* spp. strains in fermented cassava dough was reported by several studies (Assanvo et al., 2002; Coulin et al., 2006; Bouatenin et al., 2012). These bacterial species are very important because they produce different enzymes which contribute to the softness and detoxification of cassava dough (Bouatenin et al., 2012). Concerning their growth at different temperatures, all strains had their optimal growth at 37°C, except the strains Bas 13 and Bas 107 which had their optimum growth at 45°C. This result is not surprising, because the studied *Bacillus* spp. strains have been isolated from "mangnan", prepared at ambient temperature (28 to 35°C) (Assanvo et al., 2002). According to Dje et al. (2008), the optimum temperature of cassava dough during fermentation for "attiéké" production is 35°C. Therefore, the *Bacillus* strains could easily evolved in fermented cassava dough for the production of "attiéké". The growth of *Bacillus* strains was inhibited for 5% (1 strain), 7% (2 strains) and 8% (7 stains) of NaCl concentrations. Indeed, the presence of NaCl in the medium constitutes an osmotic stress (Obilie et al., 2003) and this enables *Bacillus* spp. strains to synthesize several organic compounds such as betain; trehalose; glycin; camitine; proline for maintaining their vital functions in order to protect themselves (Sow, 2004).

Concerning the influence of lactic acid concentration, the growth of all the strains was inhibited beyond 0.25%

of lactic acid, contrary to the studies conducted by Coulin et al. (2006), where the strains of *Bacillus* spp. had their growth for more than 0.3% of lactic acid. From this point of view, our isolated strains would be able to show metabolic activities in a fermentation medium with lactic acid concentration lower than 0.25%.

For lactic acid production, *Lactobacillus plantarum*, is well-known to produce a great quantity of lactic acid in a fermentation medium. Indeed, Oyewole (1992), reported that *Lactobacillus plantarum* strain is associated with high acid lactic production during cassava fermentation for "foofoo" production. Concerning "attiéké" production, the lactic acid bacteria play also an important role in acidification by lactic acid production (Coulin et al., 2006). But, the capacity to produce lactic acid was also observed for *Bacillus* strains in fermentation medium (Penga et al., 2013; Ouyang et al., 2013). According to Poudel et al. (2015), *Bacillus* spp. can produce lactic acid as observed with *Bacillus thermoamylovorans* which has ability to ferment starch for lactic acid production. The lactic acid production by *Bacillus* spp. would be useful during of the fermentation of cassava dough, because the of the acidification step which determines the final acidulous taste of "attiéké". For instance, the *Bacillus* strains Bas 102 produced a great quantity of lactic acid (0.18 to 0.54%) showing higher performance than the *Bacillus* spp strains. studied by Bouatenin et al. (2012). For the *in vitro* test of cassava dough fermentation, the concentration of *Bacillus* (10^8 CFU/g) was used according to the maximum level of *Bacillus* contained in the traditional starter (mangnan) (Assanvo et al., 2006). This traditional starter (mangnan) had a better acidification rate of cassava dough compared to that obtained with *Bacillus* strains, because traditional starter contains several microorganisms which are mostly lactic acid bacteria. According to Sotomey et al. (2001), the pH value of traditional cassava dough after 12 h of fermentation is 4.61. In this study, the pH value of traditional cassava dough for the same duration of fermentation was 4.13. In addition, our results showed that the pH value obtained after 12 h of cassava dough fermented with the selected *Bacillus* strains was slightly lower than that obtained with traditional starter (mangnan) for the same time.

Table2 pH values and total titratable acidity of cassava dough and "attiéké"

Sample	pH	Total titratable acidity (%)
Pulp after grating (pulp without strains tested)	$6.37^{d} \pm 0.14$	$0.18^{a} \pm 0.00$
Pulp after 12 h fermentation with Bas 4	$4.20^{ab} \pm 0.03$	$0.52^{b} \pm 0.14$
Pulp after 12 h fermentation with Bas 58	$4.32^{bc} \pm 0.12$	$0.5^{b} \pm 0.21$
Pulp after 12 h fermentation with Bas 66	$4.2^{ab} \pm 0.04$	$0.52^{b} \pm 0.19$
Pulp after 12 h fermentation with Bas 97	$4.18^{ab} \pm 0.11$	$0.48^{b} \pm 0.10$
Pulp after 12 h fermentation with Bas102	$4.4^{c} \pm 0.03$	$0.40^{ab} \pm 0.19$
Control pulp after 12 h fermentation	$4.13^{a} \pm 0.11$	$0.34^{ab} \pm 0.03$
Attiéké obtained with Bas 4	$4.3^{bc} \pm 0.025$	$0.021^{ab} \pm 0.001$
Attiéké obtained with Bas 58	$4.35^{c} \pm 0.015$	$0.024^{b} \pm 0.006$
Attiéké obtained with Bas 66	$4.3^{bc} \pm 0.00$	$0.016^{a} \pm 0.003$
Attiéké obtained with Bas 97	$4.27^{b} \pm 0.05$	$0.019^{ab} \pm 0.001$
Attiéké obtained with Bas102	$4.29^{bc} \pm 0.045$	$0.019^{ab} \pm 0.001$
Control attiéké	$4.06^{a} \pm 0.05$	$0.025^{b} \pm 0.004$

Data are represented as Means ± SD (3 replicates). Means in the column with no common letter differ significantly (P<0.05) for each sample.

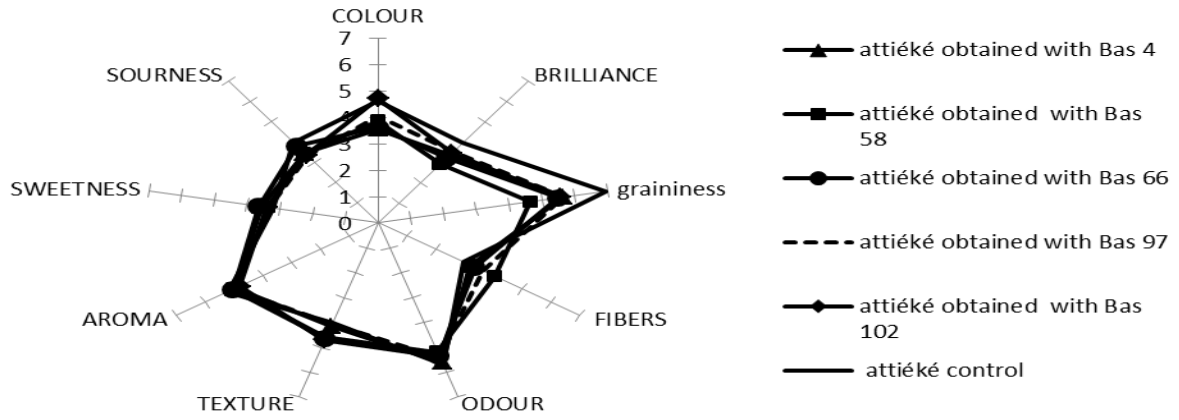

Figure 6 Sensory parameters of different "attiéké" samples

The pH values of the different "attiéké" samples ranged from 4.27 to 4.35 compared to the control (pH= 4.06). These results are closed to those of Djeni et al. (2014) ranging from 4.13 to 4.29 for traditional "attiéké" of different towns of Côte d'Ivoire (Abidjan, Dabou and Jacqueville).

From these points of comparison, our results were satisfying, because the sensory test performed by panelists revealed a slightly difference between "attiéké" produced with *Bacillus* spp. strains and "attiéké" control (traditional "attiéké"). From a general point of view, "attiéké" produced by *Bacillus* spp. strains was judged acceptable. Furthermore, it is important noting that the synergistic role of *Bacillus* spp, yeasts and moulds is important for more synthesis of enzymes in order to soften cassava dough inoculated with the traditional starter (*mangnan*).

Conclusion

The *Bacillus* spp. strains isolated from "*mangnan*" are able to produce lactic acid which is useful for a good fermentation of cassava dough. The production of "attiéké", by using this type of starter (*Bacillus* spp. strains) highlighted satisfactory results. Therefore, a biotechnological application would be possible, to control and improve the fermentation of cassava dough, in order to obtain a good quality of "attiéké" in Côte d'Ivoire. Before this biotechnological step it would be interesting to quantify enzymatic production (cellulases and pectinases) of *Bacillus* spp. strains, for a better softening control of the cassava dough.

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