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SCREENING OF LACTIC ACID BACTERIA AS POTENTIAL STARTER FOR THE PRODUCTION OF *ATTIÉKÉ*, A FERMENTED CASSAVA FOOD

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Abstract: *The purpose of this paper is to resolve unpredictable quality of Attiéké by the selection of Lactic acid bacteria (the predominant microorganism of cassava fermentation) as microbial starter culture. Thus, 237 lactic acid bacteria were isolated from traditional cassava starter and their acidification activity was evaluated using MRS-soluble starch broth. Also, their abilities to produce α-amylase, pectinase, cellulase, phytase, tannase and β-glucosidase were studied. Among the isolated strains, 126 were lactococci and 111were lactobacilli. The first acidifying group (34.24 % of isolates) decreases the pH of 1 unit after 6 h of fermentation and the second acidifying group (20.10 %) after 12 h of fermentation. The latter group (45.66 %) consists of isolates which did not decrease the pH of 1 unit less than 12 h of fermentation. Among faster acidifying LAB, 22.67 % were able to produce amylase, 2.67 % showed pectinase production capacity, 5.33 % cellulase and 8 % were able to produce β-glucosidase. These 17 isolates could be preselected as potential starter for cassava dough fermentation into Attiéké production.*

Keywords: *Lactic acid bacteria, acid production, enzyme production, cassava fermentation, Attiéké.*

1. Introduction

Cassava (*Manihot esculenta Crantz*) is an important staple food grown in tropical countries. It has important agronomic advantages such as high yields in poor soils, resistance to drought and diseases, storability in the soil after maturity and high yield of starch, in comparison with over starchy crops such as yam [1]. However, cassava is faced with two major problems, namely rapid postharvest deterioration [2] and toxicity due to cyanogenic glucosides [3, 4].

To avoid these problems, cassava roots are processed by several ways. Over 90 % of the cassava processed in Africa is used for human nutrition as fermented products [5]. So fermentation is the most popular method used for cassava process. More, the fermentation process has been shown to be a suitable method to enhance the safety, organoleptic and nutritional quality of many cassava-derived foods [6-7].

In Côte d'Ivoire, *Attiéké* is the most fermented cassava product that is widely consumed [8]. It is also known in neighbouring countries [9]. *Attiéké* is a steamed granular cassava meal ready-to-eat, couscous-like product, with slightly sour taste and whitish colour [10]. *Attiéké* is a typically Ivorian food which was originally prepared and consumed exclusively by ethnic groups living in the lagoon area such as *Ebrié*, *Adjoukrou*,

Alladjan, Avikam... this food, a simple dish as only a product of self-consumption, *Attiéké* has now acquired the statute of annuity product, nourishing big urban markets. Thus, there has been a change from family production to commercial production stimulated by the increasing urban demand and the formation of small cooperatives [11].

Attiéké is produced by women according to a traditional technology requiring several hard operations [12]. To obtain *Attiéké*, cassava roots are peeled, cut into pieces and washed. Before grinding, 10% of a traditional starter, and 0.1% of palm oil are added and the pieces are ground to a fine paste, which is placed in large bowls. The dough is left to ferment for about 15 to 24 h at ambient temperature. After fermentation, the dough is placed in a jute bag and pressed continuously by a hand press. The pressed cake is then passed through sieve for sifting. The grains are formed by shaking and rotating the powder in a large bowl. Then, the grains are sun-dried. After drying, fibres and dirt are removed by sprinkling the grains, which the grains are poured into the sieve for steaming with boiling water.

Traditional starter used for *Attiéké* production is obtained after 2 to 3 days of spontaneous fermentation of cassava roots (raw or cooked), thus colonized by wide variety of microorganisms. This starter is the main source of microorganisms necessary for the fermentation of cassava dough during *Attiéké* process. Among microorganisms isolated of traditional starters, Lactic Acid Bacteria (LAB) are predominant [13-14]. LAB are mainly responsible for the acidification of cassava dough [15-16-17]. LAB can further contribute to the reduction of cyanogenic glucosides [18-2], to development of taste, flavor, and texture of fermented food [19].

In fact, quality of *Attiéké*, like fermented food in Africa, is unpredictable because of unregulated conditions and sensory fluctuations. Characteristic of each *Attiéké* and the difference between them are probably due to the difference in their traditional starters used to conduct the fermentation [10]. Various investigations on the microbiology and biochemistry of cassava fermentation and *Attiéké* production have been done. To date, investigations aimed to standardize *attiéké* process. It is in this context that our study was performed to select LAB with suitable technological properties as potential starter to control the cassava dough fermentation into *Attiéké*.

2. Matherial and methods

2.1. Material

The biological material used in this study was constituted by already traditional cassava (*Manihot esculenta Crantz*) starter, which was obtained after 2 to 3 days of spontaneous fermentation of boiled cassava roots.

2.2. Sampling

Traditional starters were taken in small scale attiéké production in fourteen processing zones (Yopougon, Adjamé, Treichville, Marcory, Koumassi, Cocody, Port-Bouët, Abobo, Attécoubé, Dabou, Jacqueville, Anyama, Bingerville and grand Bassam). On each processing zone, 3 producers were randomly selected and about 100 g of the traditional ferment by producer were taken. Samples were collected by scraping the surface of traditional inocula. Then, samples were put in plastic bags (Stomacher) and immediately transported to the laboratory for microbial analysis.

2.3. Isolation of lactic acid bacteria

All samples were mixed and divided into 3 parts. Preparation of stock solutions, 10 g of each part were aseptically homogenized

in a stomacher with 90 mL of sterile peptone buffered water (Oxoid, Basingstoke, United Kingdom). Stock solutions were incubated at 30 °C for 18 h. Isolation of LAB were carried out using plates of De Man Rogosa and Sharp agar (MRS), Mayeux, Sandine Eliker agar (MSE), Bile Esculin Azide agar (BEA) and M17 agar (all from OXOID, Basingstoke, Hampshire, UK). These media were used as describe previously by Kostinek et al. [17] to obtain the widest possible species variety of LAB associated to the fermentation of cassava. These different media were supplemented with 0.1 % of nystatin to inhibit fungal growth. Plates were streaked and incubated anaerobically at 30 °C for 48 h.

After incubation, presumptive LAB were identified as Gram positive, oxidase negative and catalase negative and 5 colonies were randomly picked from agar plates. After picking, strains were grown in the same type of medium from which they were isolated, and streaked out repeatedly to check for purity. Stock cultures of the isolates were stored in MRS broth containing 20 % of glycerol at -80 °C.

2.4. Fermentative type and acidification capacity of isolates

Fermentative type of LAB was determined by the ability of strains to produce gas from glucose by using MRS agar supplemented with 0,005 % of bromocresol. Medium was put in tube and sterilized for 15 min at 121°C. After cooling, each strain was cultivated at 30 °C for 48 h. A negative control was carried out in the same conditions but not inoculated with strains. The ability of each strain to produce acid is assessed by the change of the medium colour comparatively to the negative control. Presence of gas at the bottom of the tube indicates heterofermentative LAB, otherwise (absence of gas) the strain is homofermentative LAB.

2.5. Study of rate of acidification of isolates

Fermentation was carried out with each isolate by using a modified MRS broth with 2 % of soluble starch as substrate. Broth was distributed in tubes (5 mL by tube) and autoclaved at 121°C for 20 min. Inoculum of each strain was obtained as following: a loopful of each isolate collected from a MRS agar plate was cultured in 5 mL of MRS broth and incubated at 30°C for 18h; 100 µL of microbial culture (OD_{600nm} = 1) transferred in 5 mL of MRS-soluble starch broth and incubated at 30 °C for 24 h. Every 3 h, biomass was measured by turbidity using a spectrophotometer. Also, the pH was measured with pH-meter (Hanna) and titratable acidity by titration with NaOH (0.1 N) using phenolphthalein as indicator.

2.6. Screening of enzymatic activities

Screening of α-amylase producing LAB

The ability of the isolates to produce amylase was performed as described by Sanni et al. [20]. Many spots of each isolate were made with an inoculation needle on modified MRS agar without glucose but with 2 % of soluble starch (w/v) as the only carbon source. The plates were incubated at 30°C for 48 h in an anaerobic jar. After incubation, the culture plates were flooded with Lugol's iodine and a colourless area around the growth indicated a positive test.

Screening of pectinase, cellulase, phytase and tannase producing LAB

The ability of the isolates to produce pectinase, cellulase, phytase and tannase was detected as described by Sanni et al. [20], but with slight modification. Thus, the soluble starch was replaced by pectin, carboxy-methyl-cellulose, phytic acid and

tannic acid as only carbon source for research of pectinase, cellulase, phytase and tannase, respectively. For phytase and tannase media, 3 % of agar was used. All plates were incubated at 30 °C for 48 h in an anaerobic jar. After incubation, Lugol's iodine was used for the revelation of pectinase, cellulase and phytase production. For tannase production, appearance of clear zone around the growth attests a positive test.

Screening of β-glucosidase producing LAB

The screening of β-glucosidase activity was tested according to the method of Weagant et al. [21] using 4-nitrophenyl-β-D-glucopyranoside, a linamarin analogue, as substrate. The medium for testing β-glucosidase activity was prepared by adding 0.1 g of 4-nitrophenyl- β-D-glucopyranoside (Merck, Darmstadt, Germany) to 100 mL NaH_2PO_4 (0.666 M; pH 6) (Merck, Darmstadt, Germany) and filter-sterilized (millipore filter of 0.2 microns). The test culture was grown on MRS for 24 h at 30 °C. Colonies were picked from the plates using a sterile loop and were emulsified in trypton-salt (McFarland Turbidity Standard No. 3). Thereafter, 0.75 mL of culture was added to 0.25 mL of the test medium and was incubated at 30 °C for 12 h. After incubation, 1 mL of sodium carbonate (1 M) was added to the medium and the change of colour (from colourless to yellow) indicated a production of β-glucosidase.

3. Results and discussion

Results of LAB isolation are given in Table 1. In our study, different media were used to isolate the widest possible species of LAB associated with fermentation of cassava. A total of 237 strains characterized as Gram positive, Oxydase negative and Catalase negative were isolated. Among them, 97 were isolated from MRS, 25 from MSE, 70 from BEA and 45 from M17. The results show that number of lactococci (126) with a percentage of 53.16 % was high than number of rods (111) with 46.84 %. The high number of cocci has been previously reported by Coulin et al. [14] who found a highest growth of cocci in these traditional inocula as well at the start of fermentation of cassava dough in *Attiéké* process. However, several investigations showed that lactobacilli (*Lactobacillus plantarum*) were the most predominant LAB in cassava fermentation [3-13-16-17]. This is probably a result of differences in geographic regions, products and the practiced production technologies in the different studies. In our case, the high number of cocci can be explained by three most of these media wich were specific of cocci. Thus, BEA is a medium specific for *Enteroccocus*, MSE for *Leuconostoc* and M17 for *Steptococcus*. This study also revealed those traditional starters were dominated by homofermentative LAB (89.03%) which convert carbohydrates exclusively into lactic acid. But the finding of heterofermentative LAB confirms that spontaneous fermentation of cassava is heterofermentative type. More, heterofermentative LAB produce also acetic acid and other volatiles compounds which contribute to aroma and flavour of fermented products by formation of lactic acid, acetoin, acetaldehyde, diacetyl, peptides and amino acid which are precursors of aromas [22].

Homofermentative Cocci (41 strains) isolated on BEA agar may be *Enterococcus. Enterococcus* strains are often isolated from cassava fermentation. These microorganisms play an important role in conservation and bacteriological quality of food and their nutritional and organoleptic properties [23]. However,

these are markers of fecal contamination (*Enterococcus faecalis* and *Enterococcus faecium*) and are considered as hygienic indicator in the manufacturing process of foods. Thus, their use as starter is controversial [23].

None heterofermentative cocci was isolated in MSE medium. It is may be that there is no *Leuconostoc* in our samples because the occurrence of *Leuconostoc* is high at the beginning of fermentation while our samples were collected at the end of fermentation i.e the third day during the spontaneous fermentation of cassava roots into traditional starters.

Table 1

Lactic acid bacteria isolated from traditional starters of *Attiéké*

Morphology of bacteria			Media of isolation				Total
			MRS	MSE	BEA	M17	
Cocci	Fermentative type	Homofermentative	47	10	41	21	126
		Heterofermentative	2	0	3	2	
Rod	Fermentative type	Homofermentative	38	14	21	19	111
		Heterofermentative	10	1	5	3	
TOTAL			97	25	70	45	237

MRS: De Man, Rogosa and Sharpe; MSE: Mayeux Sandine Eliker: BEA: Bile Esculine Azide

Among 237 presumptive LAB isolated, 18 failed to grow upon further propagation. Thus, 219 strains were used for acid production. The acidification activity was measured by the change in pH (ΔpH) during time and change of pH was calculates as $\Delta pH = pH_{at\ time} - pH_{zero\ time}$. The initial pH of MRS-soluble starch broth used to evaluate the acidifying activity of isolates was 6.2. For this study, the variation of pH of 1 unit (ΔpH=1) was taken as reference to evaluate acidifying activity of isolates. Thus the isolates were clustered into 3 groups (Table 2). At 6 h of fermentation, 75 (34.24 %) isolates of LAB were able to decrease the pH of 1 unit. They were clustered in Group 1, the faster acidifying LAB, with pH value ranging between 3.93 and 5.20. The second group, the medium acidifying LAB that registered 44 isolates (20.10 %) decreased the pH of 1 unit at 12 h of fermentation with pH values between 4.79 and 5.18. Finally, the third group (slow acidifying LAB), registered isolates which couldn't decrease the pH of 1 unit in less than 12 h of fermentation. This cluster with mean of pH of 5.33 and mean titratable acidity of 0.35 % was constituted by 100 isolates (45.66 %). A good pH reduction is important to reduce the levels of contaminating microorganisms present on the raw materials, utensils and the environment which can compete with the starters for nutrients [24]. In this study, fast acidifying LAB showed a high property of acidification and confirmed their major role of acidification in *Attiéké* production. They acidify cassava dough by production of organic acids which lowered the pH. Some isolates were able to produce lactic acid at rate of 0.72 % and decrease the pH to 3.93 at 6 h of fermentation. Our results demonstrated also that acidifying activity of LAB in cassava dough takes place mainly at the beginning of fermentation (6-12 h). In this time, majority of LAB (54.33 %) which is clustered in faster and medium acidifying LAB produced high amount of lactic acid. After 12 h of fermentation, decreasing of pH is weak. LAB remaining contribute to maintain the environment acid by the weak production of lactic acid after 12 h of fermentation.

Table 2

Characteristics of isolates according to their rates of acidification

	Fast acidifying LAB	Medium acidifying LAB	Slow acidifying LAB
	ΔpH>1 at 6 h	ΔpH>1 at 12 h	ΔpH<1 after 12 h
Number of isolates (%)	75 (34.24 %)	44 (20.10 %)	100 (45.66 %)
Mean of pH	5.01±0.2	5.14±0.12	5.33±0.27
pH min	3.93	4.79	4.76
pH max	5.20	5.18	6.2
Mean of TA (%)	0.400±0.104	0.377±0.067	0.351±0.08
TA min (%)	0.275	0.228	0.171
TA max (%)	0.720	0.570	0.581

ΔpH: variation of pH; TA: Titratable Acidity

For the screening of their enzymes activities, only the 75 fast acid producer strains were tested for amylase, pectinase, cellulase, phytase and tannase production. All isolates were able to grow in different media of enzymes activities. However, after revelation, none isolate showed phytase and tannase activities because of absence of colorless area around the spot. Contrariwise, 17 were able to produce amylase, in which 6 were able to produce β-glucosidase (LAB 4, LAB 16, LAB 68, LAB 113, LAB 127 and LAB 251). Four strains were able to produce cellulase (LAB 16, LAB 171, LAB 210, and LAB 245) while 2 strains (LAB 72 and 114) were able to produce pectinase (Table 3).

Table 3

Enzymes production by lactic acid bacteria

Enzymes	Number of isolates tested	Number of LAB producing enzymes	Percentage
Amylase	75	17	22.67
Pectinase	75	2	2.67
Cellulase	75	4	5.33
Tannase	75	0	0
Phytase	75	0	0
β-glucosidase	75	6	8

Furthermore, to be selected as pure microbial starter for cassava dough fermentation, enzymes synthesis is required. Ability of isolates to produce amylase is important because cassava roots are composed of more than 80 % starch. The hydrolysis of starch must provide simple sugars easily metabolizable into lactic acid, which is expected to increase the content of this acid in cassava dough. In addition, this facilitates the digestibility of starchy food. However, production of amylase was a rare trait among LAB.

Thus, only 22.67 % of our isolate were able to produce amylase. As to Kostinek et al. [17] and Edward et al. [25], none amylolytic LAB was found during their studies for the selection of LAB strains as potential pure microbial starter for cassava dough fermentation into *Gari*.

The breaking down of the coarse texture of the cassava roots was critical for the softening of cassava tissue, which is necessary for the quality improvement of cassava product [26]. This action is achieved by microorganisms which produce pectinase and cellulase in cassava dough. Cassava roots itself contains linamarase which hydrolysis linamarin when the plant cells are disrupted. However, the endogenous linamarase of cassava is insufficient for a complete detoxification of cassava [27]. So, microbial β-glucosidase activities are also necessary to detoxify the cassava dough by the hydrolysis of linamarin present in the plant cells.

Among 75 LAB isolated with important acid production, 17 were preselected as potential starter for *Attiéké* production on the basis of their biochemical properties. Biochemical properties considered were fast acid production, α-amylase, β-glucosidase, cellulase, and pectinase activities. The results of the preselected starter are shown in Table 4.

Table 4

Preselected LAB as starters

Code of isolates	α-amylase	β-glucosidase	Cellulase	Pectinase
LAB 4	+	+	-	-
LAB 16	+	+	+	-
LAB 19	+	-	-	-
LAB 20	+	-	-	-
LAB 21	+	-	-	-
LAB 34	+	-	-	-
LAB 68	+	+	-	-
LAB 69	+	-	-	-
LAB 72	+	-	-	+
LAB 113	+	+	-	-
LAB 114	+	-	-	+
LAB 127	+	+	-	-
LAB 140	+	-	-	-
LAB 171	+	-	+	-
LAB 210	+	-	+	-
LAB 245	+	-	+	-
LAB 251	+	+	-	-

4. Conclusion

This is an essential preliminary work to select pure microbial starter for the control of cassava dough fermentation into Attiéké. In regarding to interesting technological properties (acidification activity and enzymes synthesis), 17 presumptive LAB strains have been preselected for further studies. Thus, the next step will be a more detailed analysis and identification of these microorganisms. Their mixer in pilot scale fermentation of cassava dough is also necessary to finally select the ones which can induce an optimal and constant desirable physico-chemical and organoleptic quality of *Attiéké* properties.

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