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BIOCHEMICAL CHARACTERIZATION OF MICROBIAL POPULATIONS INVOLVED IN LOH-DJIBOUA COCOA'S FERMENTATION IN COTE D'IVOIRE

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Abstract: *Comprehensive report on biochemistry characteristics of microorganisms involved in Loh-Djiboua cocoa fermentation is made in order to highlight diversities that occur among them. Microbial growth monitored showed that Yeasts, LAB and Bacillus were initially present, while AAB emerged after 12 h. Throughout the process the AAB population load was higher than other microorganisms with rates between (7.76 - 7.39) log CFU / g of beans while yeast, LAB, AAB and Bacillus reached a maximum of load (7.28 - 8.82) log CFU/g of beans, under fermentative conditions of temperature (28 – 36 °C) with a peak at 45 °C (24 - 60 h) and pH ranged between 4.5 – 8. The 24 best producing yeasts pectolytic enzymes resulting from 267 isolates presented four fermentatives profiles. Among the 173 Bacillus isolates were obtained 45.08% pectinolytic activity streams with high, medium and low production level, 37.05% citrate lyase activity strains and 70.02% power acidifying strains. Homofermentative type (92.83%) largely dominated the heterofermentative type (7.72%) among the 210 strains of LAB isolated. All LAB isolated showed ability to ferment glucose while only 2 not ferment fructose and 9 not ferment sucrose. 76.19% proved to be able to assimilate citrate. The 166 isolates of AAB with clear acidifying power were constituted into 2 genera mainly Acetobacter (80.76%) and Gluconobacter (19.24%). This study emphasized the clear dominance of AAB population mainly constituted of Acetobacter during the growth dynamic of microbial groups involved in Loh-Djiboua cocoa fermentation and showed the wide variety in activities and population among these different groups.*

Keywords: *indigenous microflora, biochemistry characteristics, growth dynamic, Ivorian beans fermentation*

1. Introduction

Fermentation of cocoa beans is the first step in chocolate-making chain, followed by drying and roasting. This step which is crucial for ensuring desired characteristics cocoa flavor and aroma, is one of the most important process leading to commercial cocoa and chocolate quality [1, 2]
During the cocoa beans fermentation, the mucilaginous sugary pulp surrounding the bean is removed by the action of various microbial species indigenously present in the cocoa beans such as yeasts, lactic acid bacteria (LAB), acetic acid bacteria (AAB) and *Bacillus* sp [3]. Microbial fermentation induces numerous chemical reactions leading to a deep modification of the biochemical characteristics of beans [4]. Early in this process, yeasts are favored under anaerobic conditions, high sugar

content and low pH. They produce ethanol and present pectinolytic activity. The reduced oxygen availability, combined with increased temperature and pH, favor the development of LAB, which consume citric acid and ferment sugar to lactic acid, acetic acid, ethanol and mannitol. The increased aeration, due to pectin degradation, promotes the development of AAB, which oxidize ethanol into acetic acid, in a highly exothermic process [3, 4, 6]. Ethanol and acetic acid diffuse deep into the beans and combine with the temperature, trigger the activation of endogenous enzymes, mainly proteolytic enzymes but also aminopeptidase, invertase, polyphenol oxidase and glycosidase [7]. This leads in serial reactions responsible for final quality of the fermented beans and chocolate [3].

Although the entire physiological role of these microbial groups involved in cocoa fermentation is not well-defined, it's clear that any fermentation cannot be correctly processed without the micro flora. Several studies have investigated the microbial diversity of spontaneous cocoa pulp fermentation in different countries such as Ghana [8], Brazil [9], Malaysia and Trinidad [10], Côte d'Ivoire [4], but very few studies are focus on the diversity of this indigenous microflora in each area of cocoa producing countries.

Geographic location is known to have an influence on the composition and characteristic of microbial consortium responsible for cocoa fermentation [3] since changes in the local climatic conditions influence the sequence of microorganisms involved in this fermentation. In Côte d'Ivoire, there are different cocoa producing regions with certainly different qualities. The characterization of the microbial flora involved in cocoa fermentation in each region may allow well knowing their diversity and establishing their cartography.

The region of Loh-Djiboua (5° 40′ N 5° 30′ W), a part of main growing areas of cocoa in Côte d'Ivoire which recorded for the 2013-2014 campaign a production of 110.000 tons of cocoa beans [11], has been so far no studies concerning microbial diversity involved in cocoa fermentation in this western region.

Yet these studies could be used to carry out the cartography of Ivorian cocoa microbial flora. The aim of study is to establish a comprehensive report on the biochemistry characteristic and to investigate some functional properties of the microbial groups involved in cocoa fermentation in Loh-Djiboua. This could help us to control this process and improve quality of product.

2. Matherials and methods

2.1. Fermentation conditions and sampling:

Spontaneous cocoa bean heap fermentation was performed at the CNF (National Floristic Center) of the University Félix Houphouet Boigny.

Cocoa pods constituted of mixed genotypes (Foraster, Trinitario and Criollo cultivars) were harvested from Loh-Djiboua (geographic coordonates 5° 40′ N 5° 30′ W), a western province of Côte d'Ivoire. Beans were removed from pods and mass about 50 kg fermented traditionally by heap fermentation on banana leaves for six days.

Samples of fermenting cocoa bean about 200 g were taken directly at 15 cm depth on the fermenting mass according to a fixed time schedule, notably at the start (0 h) and each 12 h of fermentation. Each sample was transferred into sterile plastic bag for microbiological analysis. The pH and temperature were also regularly recorded at the same depth on the fermenting heap, with pH-meter and

thermometer (Hanna instruments, Europe, Romania).

2.2. Isolation and Enumeration

Fermenting samples (25 g) were aseptically mixed with 225 ml of 0.1% saline-peptone water (Oxoid, Basingstoke, United Kingdom) in a Stomacher bag (Seward, Worthington, United Kingdom) and manually shaken for 5 min to give an uniform suspension of the cocoa pulp material. After appropriate dilution in sterile saline, 0.1 ml samples from each dilution were spread inoculated onto duplicate plates of different agar media.

Yeasts were enumerated on Malt Yeast Peptone Glucose agar [12, 13]. LAB were isolated by plating the fresh fermented samples on three selective agar media; MRS, MSE and M17 agar (all from OXOID, Basingstoke, Hampshire, UK), supplemented with 50 Pg/mL of nystatin to inhibit fungal growth. AAB were enumerated on potato medium [14]. Nutrient agar (Merck) supplemented with 0.1 % nystatin to inhibit the growth of fungal was used as a general medium for *Bacillus* sp. The culture was incubated at 30°C for 48 h.

Following incubation, the number of colony-forming units (expressed as CFU per gram cocoa pulp bean mass) was recorded, and each colony type was morphologically characterized and counted. Yeasts, LAB and AAB were identified according conventional biochemical identifications keys. Identification of *Bacillus* sp isolates were done using conventional colonial morphology, Gram staining and biochemical reactions according to Bergey's manual of systems bacteriology. Isolated strains of LAB were stored in MRS buffer medium, AAB in Luria Bertani medium, Yeasts and *Bacillus* sp on the same isolation medium at -80 °C supplemented with 20 % (v/v) glycerol in Eppendorf tubes for further studies.

2.3. Biochemical characterization of microbial from Loh-Djiboua's cocoa beans fermentation

2.3.1. Yeasts population

Yeasts strains isolated were analyzed for their ability to produce pectinolytic enzymes and catabolize carbohydrates containing in cocoa pulp

Pectinolytic strains were screened according to [4, 15] methods. The carbon metabolism of pectinolytic yeasts strain study was carried out by[16] method. The carbohydrates tested were D-glucose, maltose, D-fructose, sucrose, lactose, and D-galactose. Cultures were incubated at 30 ºC for 48 h to 3 weeks. The presence of gas in Durham tubes indicates that the isolates ferment carbohydrates.

2.3.2. LAB population

LAB strains isolated were analyzed for their ability to produce acid by catabolizing the main sugar and for their ability to catabolize citric acid containing in cocoa pulp.

The carbon metabolism of bacterial strains was evaluated by [17] method with slight modification. This study was performed in a modified MRS medium containing the appropriate carbohydrate at 2 % as sole carbon source, 1.7 % agar and supplemented with 0.005 % of bromocresol purple. The carbohydrates tested were glucose, fructose and sucrose that are known to be the sugars contained in the cocoa pulp [18]. Each strain was cultivated by central sting in the medium and then incubated at 30 °C for 72 hour in anaerobic conditions. The capacity of strains to metabolize the carbon source is assessed by the presence of colony in the tube and the change of medium color due to pH lowering, comparatively to the negative control. The fermentative type

was determined by ability of strains to produce gas from carbon source. Indeed, the presence of gas at the bottom of the tube accompanied by yellow zone indicates heterofermentative LAB type while homofermentative LAB form are characterized by the absence of gas on the tube presenting yellow zone.
LAB strains presenting citrate lyase activity were also screened according to [19] method. After incubation, the colonies which appear blue on the medium are those able to metabolize citrate.

2.3.3. AAB population

AAB strains isolated were analyzed for their ability to produce acid in solid medium and by identification of their main genera involved in Loh-Djiboua's cocoa beans fermentation.
Acidification capacity in solid medium of bacterial strains was evaluated as described by [6] method. Acid production in solid medium was monitored by formation of yellow zone around the spot. Acidification capacity of strains was evaluated by measuring of the yellow zone diameter. The isolates were identified up to genera (*Acetobacter and Gluconobacter*) using Over oxidation capacity test of [6] method.

2.3.4. Bacillus sp population

Bacillus sp strains isolated were screened for their ability to produce pectinolytic enzymes, acid in liquid medium and to catabolize citric acid containing in cocoa pulp. Pectinolytic activity of strains were screened according to [4, 14] method. *Bacillus* sp strains presenting citrate lyase activity were also screened by [19] method.
Acidify capacity of *Bacillus* sp strains was evaluated by [20] method. A negative control was prepared in the same conditions and not inoculated with the microbial culture. Acid production was monitored by formation of yellow area in the tube with or not production of gas and acidify capacity was analyzed by evaluating in a visual scale.

3. Results and discussion

3.1. Change in pH, temperature and dynamic of the microorganism during cocoa bean fermentation

Analysis of heap spontaneous fermentation condition indicates that the pH of fermentation heap ranged from 4.5 at the beginning to 8.0 at the end of the fermentation process, while the temperature ranged from 28 to 36 °C with a peak at 45 °C within 24 -60 h (Figures 1A and 1 B). The same profile of temperature and pH variation has been regularly recorded in cocoa fermenting mass in Côte d'Ivoire [4, 6] and other countries [3 ,5] indicating that the increase of both parameters constitutes an inherent property of cocoa fermentation worldwide.
Moreover, pH continuously increasing during the fermentation became alkaline at the end of the process. Several authors [5, 6, 21] also reported an alkaline pH (8.5, 7.9 respectively) at the end of spontaneous cocoa fermentation in Côte d'Ivoire. This result confirms the particularity of fermentation conditions of Ivorian cocoa since alkaline pH has not been yet reported in other country [3, 5, 22]. We could not explain the reason for why, sometimes the pH became alkaline in cocoa fermentation but we find that Loh-Djiboua cocoa fermentation conditions remain the same as those of the country. So this particularity of fermentation of Loh-djiboua cocoa beans provided an ecosystem that selected for successional growth of various species of yeasts, LAB, AAB and *Bacillus*
The dynamic of microorganism's population obtained from numeration during fermentation is shown in Figure 2. In general, simultaneous growth of Yeasts, LAB, AAB and *Bacillus* took place with an

undetectable level of bacterial load for AAB at the beginning (Figure 2) since no colony corresponding to AB was identified on the plate at this time, in contrast to the other microorganisms.

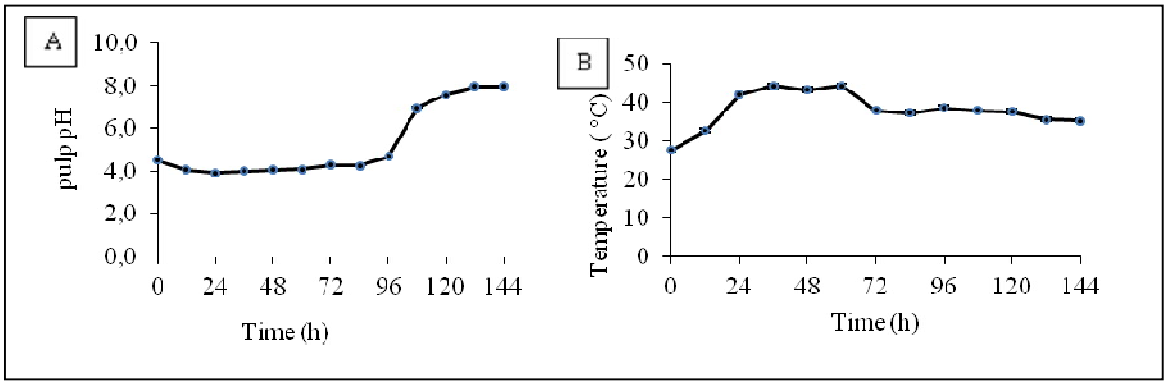

Fig. 1. Evolution of pH (A) and temperature (B) during Lôh-Djiboua's cocoa beans fermentation. Error bar indicate standard deviations between three replicates

These microorganisms are present throughout the fermentation process with some particularities. Indeed, populations of yeasts, LAB and *Bacillus* sp are present from the beginning of the process (5.56 to 5.96) log CFU/g of beans, unlike AAB population which appears after 12 h of fermentation.

Then, the microbial population of yeasts, LAB and AAB rapidly increased to their maxima (7.58 to 8.82) log CFU/g of beans, at 12 h and 72 h of time respectively and subsequently decreased in two stages for LAB and for yeasts, gradually until reaching undetectable levels at the end of fermentation.

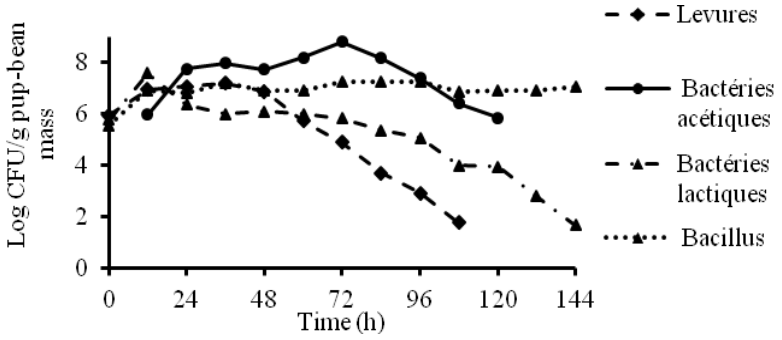

Fig. 2: Microbial growth dynamic during Lôh-Djiboua cocoa beans fermentation

Bacillus sp unlike the other microbial genera, recording a different growth dynamic insofar after a rapid increase over the first 12 h, these bacteria recorded a slow increase until the end of fermentation with high values around 7.70 log (CFU / g of beans). Moreover, we can also noticed that AAB population recorded the highest load than that of other microorganisms between 24 and 96 h of fermentation with rates ranged (7.76 - 7.39 log CFU / g of beans) comparatively to the other

microbial types. The presence of these microorganisms from the beginning on the fermentation heaps would be due to an accidental inoculation of fermentation heaps by a variety of microorganisms from the pod surface, knives, labors hands, fermentary [23, 24] while the lack of AAB observed during the same time, can be explained by their undetectable low rates. Furthermore difference in growth dynamics recorded by *Bacillus* sp strains isolated is due to their inherent nature which allowed them able to withstand drastic environmental conditions and show great adaptability. Finally regarding AAB, it also should be noted that the same growth pattern was observed with maximum population in Mexico [1] and in Ghana [8]. But on the other hand, as AAB population isolated was consistently larger than the other microbial groups, this differs from most other fermentations where yeasts or LAB were the dominants microorganisms in Bahia [24] and in Ghana [25]. This could, in certain extent, explain differences observed in cocoa quality between these countries and be a particularity of Loh Djiboua cocoa fermentation leading to well fermented beans.

3.2. Biochemical characterization of yeast strains isolated

Search of pectinolytic activity of yeasts isolated from Loh-Djiboua cocoa fermentation revealed that only 10.48% of the 267 yeasts isolated were able to produce pectinolytic enzymes. Although the proportion of yeast strains pectinolytic activity is lower than that reported by [2] with 17.56%, the study has revealed a wide level of production with large, medium and small producers. This result suggested that some of yeasts isolates from Loh-Djiboua cocoa fermentation would participate in aeration of the fermentation heaps by producing these pectinolytic enzymes. Aeration conditions necessary for growth of AAB are in part created by pectinolytic enzymes which break down the pectin responsible for the viscosity and stickiness of cocoa pulp [4, 22]. So this wide level of pectinolytic activity obtained by yeast strains during Loh-Djiboua cocoa fermentation enable strengthen the elimination of the pulp and thereby accelerate this process. This ensuring thus permanently quality commercial of Loh-Djiboua cocoa's beans.Hence, the best pectinolytic yeasts isolated (24 strains) based on halo diameter were further studied for their specificity. Their carbon metabolism was analyzed. The strains showed a large carbon metabolism profile (3) with capacity to ferment glucose, fructose, sucrose and galactose among the six carbohydrates tested (Table 1).

Table 1:

Fermentative profile of pectinolytic Yeasts strains isolated from Lôh-Djiboua

Profiles	Sugars						Number of isolates
	Glucose	Fructose	Sucrose	Maltose	Lactose	Galactose	
1	+	+	+	-	-	-	**14**
2	+	+	-	-	-	-	**8**
3	+	+	-	-	-	+	**2**

NB: (+): Gaz production (-): not Gaz production

This suggested a great diversity of species among yeast streams isolated. It is known that, yeasts present one of the wide microbial diversity in which strains can behave very differently. This difference in carbon metabolism is due to the transport system associated to the membrane which exerts selection of carbohydrate to enter into the yeast cells [26]. Generally, the pulp of cocoa fermentation contains mainly

glucose, fructose and sucrose which are to be fermented into ethanol and at term, into acetic acid [3]. These reactions are essential for specific colour, flavour and aroma of the cocoa beans and chocolate [27]. From this point of view, with their large carbon metabolism profile, the best pectinolytic yeasts isolates should keep entirely their enzyme production capacity due to their adaptability and contributed to the maintain of the quality of Loh-Djiboua cocoa beans.

3.3. Biochemical characterization of AAB strains isolated

The study of biochemical characteristics of AAB revealed that 166 AAB strains isolated are grouped into two mainly genera Acetobacter (134 strains, 80.72%) and Gluconobacter (32 strains, 19.24%) with a clear dominance of Acetobacter genus. And it is known that Acetobacter genus can degrade the acetic acid produced into CO_2 and H_2O when there is a high level of dissolved oxygen and no ethanol in the medium [28] which is not desirable. However, this dominance would be an advantage for Loh Djiboua cocoa fermentation, since it may enable to obtain quality beans and chocolate due to ability of Acetobacter genus to degrade lactic acid into acetoin and CO_2 [1, 29, 30]. Indeed, lactic acid product degradation could help reduced the load of lactic acid beans after fermentation thereby contributing to the achievement of quality beans while the presence of acetoin, a chocolate precursor aroma, would provide a good quality chocolate. In addition, the study of the acidification capacity of these acetic bacteria strains revealed between the 166 isolates a large diversity in production level of acetic acid with strong (14 strains, 8.33%) medium (97 strains, 57.73%) and low (56 strains, 33.33%) producers in which medium producers load is the most important. This large diversity of production level associated with the dominance of acetic acid bacteria population on the other microbial strains of fermentation could lead to an important production of acetic acid even if the best acidification strains isolated from Loh Djiboua cocoa fermentation showed less important capacity of acetic acid production [6]. Acidification is one of the most relevant properties in cocoa fermentation since it influences greatly the quality of fermented bean and chocolate [3]. The production of acetic acid during cocoa fermentation allows the development of chocolate flavor and aroma [31, 32]. Hence, the strong acidification of Loh-Djiboua cocoa beans, which is a desired parameter for improvement of fermentation, should ensure the production of high quality beans market.

3.4. Biochemical characterization of LAB strains isolated

Regarding LAB study, biochemical and morphological identification of strains isolated, revealed the presence of Lactococci (33 strains) and Lactobacilli (177 strains) in Ivorian cocoa spontaneous fermentation. Evaluation of LAB strains fermentative type further showed that all the Lactococci stains isolated were homofermentative whereas for Lactobacilli strains isolated with 162 strains (91.73%) homofermentative and 15 strains heterofermentative. In spite of this fermentative type diversity observed on these two main groups of Lactococci and Lactobacilli strains isolated, the LAB population is characterized by a large dominance of homofermentative type (162 strains, 92.83%) on the heterofermentative type (14 strains, 7.17%) as it is reported about in Ghana [8] Nigeria [13] and Cote d'Ivoire [21]. Homofermentative LAB strains are known to convert sugars almost exclusively into lactic acid and then produce more lactic acid than

heterofermentative strains [33]. As AAB and particularly Acetobacter genus remain in a high percentage of the total microbial population in this region, high concentrations of lactic acid product will be transformed [1, 29, 30] leading to well fermented beans and quality chocolate. Concerning main sugars carbon metabolism, among 210 LAB strains isolated all were able to metabolize glucose (100%) unlike fructose (99.04%) and sucrose (95.75%). These results are consistent [21] highlighting among these strains a wide diversity in their capacity to metabolize these main sugars. Indeed during fermentation, glucose and fructose were used by LAB to be converted into lactic acid, acetic acid, ethanol, and mannitol. Sucrose inversion took place in the beans due to cotyledon invertase activity [7] and/or induced acid hydrolysis as a result of acetic acid penetration into the beans upon fermentation. Glucose was preferentially fermented above fructose following sucrose hydrolysis [8]. In light of this particularity, growth and thus activity of these strains isolated during fermentation should not be limited by a possible depletion in one of these carbon sources.

One the other hand, 76.19% of the LAB strains isolates presented the capacity to catabolize citric acid. Citrate metabolism constitutes, an important and particular property, since LAB is not usually able to utilize citric acid as carbon source [21]. This finding confirms the results concerning LAB strains isolated from Agboville in Cote d'Ivoire [21]. Citric acid is the compound responsible for the initial pH of cocoa pulp before fermentation processing [9, 24, 34]. The degradation of this acid in the first stage of fermentation allows the raise of the pH favorable for the development of many bacterial groups [3].

3.5. Biochemical characterization of Bacillus sp strains isolated

Biochemical characterization of *Bacillus* sp strains isolated from Loh-Djiboua cocoa fermentation showed that among 170 isolates 44.32% of strains presented pectinolytic activity with strong (19.23%), medium (19.23%) and low (61.53%) producers. 37.05% of the isolates were able to metabolize citric acid while about 70% of them expressed acidification capacity.

These results suggested that *Bacillus* strains involved in cocoa fermentation are able to express a wide diversity of useful activities for successful cocoa fermentation as they are able to survive under fermentation conditions. This emphasis the important role that *Bacillus* sp may play with these capacities in the remaining time of Cocoa fermentation as no useful role has been clearly attribute to them in cocoa fermentation [35], ensuring to maintain the merchantability of Loh-Djiboua cocoa beans and derivate chocolat.

4. Conclusions

The current emergence sequence of microorganisms with yeasts, LAB, AAB and *Bacillus* has been observed during Loh-Djiboua cocoa fermentation with a clear dominance of AAB population mainly constituted of *Acetobacter* on other microbial groups. Biochemical characterization of each microbial group emphasized the wide variety in activities and population among these different groups. However, other biochemical or molecular methods are necessary to better apprehend this diversity.

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