

Characterization and Enzyme Activities of Microorganisms from a Traditional Cassava Starter Used for the Production of Adjoukrou Attieke (Cote d'Ivoire)

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Abstract: Fourteen strains responsible for the fermentation of cassava dough during Adjoukrou attieke production were investigated for enzyme activities in relation with the food properties to elucidate their possible roles during the process. Selected strains were phenotypically characterized and α -amylase, β -glucosidase and pectinase activities were measured using synthetic substrates. The influence of pH and temperature on enzyme activities and microbial growth were also tested. The results showed that the yeast strain *Candida tropicalis* LVX14 had the best amylase activity (144 ± 3.3 U mL⁻¹) at 30°C and pH 5. Lactic acid bacteria, particularly *Leuconostoc. mes. ssp. mesenteroide/dextranicum* LABX2 showed the highest β -glucosidase activity with 69.4 ± 13.29 U mL⁻¹ at 35°C for pH 6 while *Bacillus amyloliquefaciens* BX5 and *Rhizopus oryzae* MX4 showed responsibilities in the softening by producing pectinase activities of 0.215 ± 0.02 U mL⁻¹ at 35°C and pH 6 and 30°C at pH 5, respectively. This study allowed selecting four strains with particular metabolic characteristic and state condition of fermentation at 30°C for a pH between 5 and 6. The selected strains showed an interesting combination of enzyme activities, suggesting their possible usefulness as a mixed starter culture in a controlled attieke fermentation process.

Key words: α -amylase, β -glucosidase, fermenting microorganisms, pectinase, traditional cassava starter

INTRODUCTION

Cassava, the enlarged root of *Manihot esculenta* Crantz is an important staple food for about 80% of Cote d'Ivoire's estimated population especially those living in the Southern. It has important agronomic advantages such as high yields in poor soils, resistance to drought and diseases, storability in the soil after maturity and a comparatively high yield of starch, in comparison with other starchy sources such as yams (Kouadio *et al.*, 1991). However, it has two important deficiencies. The first is that many varieties contain the toxic cyanogenic glycosides, linamarin and (to a lesser extent) lotaustralin which have fatal consequences when consumed in unprocessed foods (Okafor *et al.*, 1998). Even when cassava foods are processed, small quantities of the glucosides are often left and these give rise to diseases such as goitre when the foods containing these traces are consumed continuously in diets low in protein (Okafor *et al.*, 1998). The second deficiency of cassava is that it is very poor protein, containing only

about 1% (Cooke and Coursey, 1981). Cassava may be processed by boiling, roasting, drying or by fermentation, depending on the variety (Kouadio *et al.*, 1991). The most popular processing method however especially for the varieties which are high in the cyanogenic glucosides is by fermentation. One of the most popular fermented foods derived from cassava in Cote d'Ivoire is Attieke. Attieke (steamed cassava fermented semolina) is one such fermented cassava product and is of significant importance for an increasing number of people in Cote d'Ivoire (Assanvo *et al.*, 2006) and other countries in the world. In Cote d'Ivoire, Aboua estimated the consumption of attieke between 28,000 and 34,000 tons per year, the equivalent of 40-50 tons of fresh cassava. The popularity of attieke to urban dwellers is associated with its cheapness, lower bulk (as compared to other cassava product) and its characteristic of ready to eat food. In Cote d'Ivoire, there are several types of attieke linked to the ethnic groups who produce them. Three of these groups (Adjoukrou, Ebrie and Alladjan) were chosen for attieke production and consumption

studies because they are produced and consumed on a large scale in urban areas, particularly in Abidjan. A consumer survey on these three types of attieke, in three municipalities in Abidjan (Koumassi, Cocody and Yopougon) showed that Adjoukrou attieke is highly appreciated by most consumers (Assanvo *et al.*, 2006). Moreover, Djeni *et al.* (2011) established characteristics of each of the three attieke type and the differences between them, probably due to the difference in their traditional starters used to conduct the fermentation. However, attieke is given less attention with respect to process optimization and quality standardization. The production laborious and time consuming covers a combination of steps among which roots peeling, grating, fermentation, pressing, granulation, sundrying and steaming. It is one of the few products whose fermentation is not spontaneous but involves the use of an inoculum. This inoculum is obtained after 2-3 days of spontaneous fermentation of cassava roots, thus colonized by a wide variety of microorganisms which constitutes the main source of microbial activities during the cassava dough fermentation (Djeni *et al.*, 2008). Many different methods exist for the processing of the traditional starter. The main purpose for using this inoculum is to shorten the fermentation time to 15 h compared with other fermented products like gari (dish of fermented and roasted cassava root) or chickwangue (heavy type of dough with a light elastic texture made out 100% of cassava) where the fermentation lasts for 2-5 days (Heuberger, 2005) and to improve the texture and flavour of the final product.

Earlier studies by Bouatenin on the screening of microorganisms involved in the fermentation of cassava dough during attieke production allowed the preselection of 42 microbial strains (Bacillus, lactic acid bacteria, yeasts and moulds) with particular metabolic activities (amylase, linamarase, pectinase, high rate of acidification) from the three main attieke traditional starters.

In the present research, the characterization and the enzymatic profiles of some interesting microorganisms originated from Adjoukrou traditional starter were investigated, to better understand their role during the cassava dough fermentation process as well as to identify particular properties that may be relevant to use of these organisms in starter cultures.

MATERIALS AND METHODS

Microbial strains used: The microorganisms (lactic acid bacteria, *Bacilli* sp., yeasts and moulds) used in this study were pre-selected from a set of 115 isolates from Adjoukrou traditional attieke starters according to earlier study of Bouatenin based on the qualitative production

of amylase, linamarase and pectinase enzymes by these microorganisms. Among these strains, some were characterized by their particular capacities such as production of amylase, linamarase, pectinase and high rate of acidification. LAB were routinely propagated in De Man, Rogosa and Sharpe (MRS) broth medium (Oxoid, Milan, Italy) while yeasts and moulds were propagated in Sabouraud chloramphenicol broth medium (Fluka, Bochemica 89579, Sigma-Aldrich Chemie GmbH, India) and *Bacilli* sp. propagated in Mossel broth (Oxoid LTD, Basingstore, UK) before carrying any assays.

Physiological and biochemical characterization of isolates: LAB isolates were purified by successive streaking on MRS agar media before characterization. The agar plates were incubated anaerobically (BBL Gas Pak, H₂ and CO₂; Becton-dickinson, Cockeysville, MD, USA) for 24 h at 30°C. Bacilli were purified by successive streaking on plates of Plate Count Agar (PCA Oxoid Ltd. Basingstore, UK) containing 2% of soluble starch and incubated at 32°C for 2 days. The Gram positive and catalase negative/positive strains were subjected to the following physiological and biochemical tests: gas (CO₂) formation from glucose, Motility, arginine hydrolysis, growth in 2, 4 and 8% NaCl, growth at 5, 15 and 45°C according to Harrigan and McCance (1990). The fermentation pattern among carbohydrates was determined by using the API 50 CH gallery with the API 50 CHL medium (Biomerieux, France) for LAB and API 50 CHB medium (Biomerieux, France) for Bacilli. Anaerobiosis in the inoculated tubes was obtained by overlaying with sterile paraffine oil. The inoculated galleries were incubated at 30°C and the observations were made after 24 and 48 h. The identification of isolates was facilitated with the use of a computer program, APILAB PLUS, Version 3.2.2. (Biomerieux) and reference to Bergey's Manual of Systematic Bacteriology (Wood and Holzappel, 1995).

Yeasts were characterized for their assimilation patterns using API 20 C AUX gallery (Biomerieux) and identification made with reference to standard descriptions (Beuchat, 1993; Deak, 1993). The description of the moulds through the surface appearance, shape, color and microscopic observation had allowed to provide the type and names of certain species.

Extracellular enzyme activities: For a given enzyme activity, experiments were performed at the same time under identical growth conditions (same growth medium and substrate concentration), allowing comparisons between strains, considering that activity values in the given periods of time can be assimilated to productivity,

more particularly to overall productivity at 24 h (Pirt, 1975). Each experiment was achieved on 250 mL of specific substrate solution inoculated with the selected strains at a rate of 10^6 cells mL^{-1} and incubated for 24 h at 30°C under rotary agitation (105 rpm). For activity measurements, 10 mL of inoculated substrate were taken and centrifuged at 6000 rpm for 40 min. Assays were performed on the supernatants used as enzymatic extract.

α -amylase activity: For the detection of α -amylase activity, the isolates were cultivated on a liquid medium containing 1% of starch, 1% of meat extract, 1% of peptone and 0.3% NaCl. The medium was buffered to pH 6.9 and incubated for 24 h at 30°C . α -amylase activity was determined by means of the DNS method using 1% (w/v) soluble starch (Sigma) as substrate in 0.2 mol L^{-1} citrate-citric acid buffer (pH 6.9). Enzymatic solution (0.1 mL) was mixed with 0.75 mL of substrate and incubated at 37°C for 30 min. Spectrophotometric glucose quantification was carried out at 540 nm according to Vazquez *et al.* (2004). One Unit (U) of amylase was defined as the amount of enzyme that released 1 μmol of glucose per min, under the current assay conditions.

β -glucosidase activity: For the determination of β -glucosidase activity, LAB isolates were cultivated in a MRS broth without glucose while yeasts and moulds were in sabouraud broth without glucose. These media contain 1% of linamarine and 0.9% of NaCl. *Bacillus* sp. were cultivated in a media containing 1% of linamarine, 0.3% of meat extract, 0.5% of peptone and 0.3% yeast extract, 0.3% K_2HPO_4 , 0.1% KH_2PO_4 and 0.9% of NaCl. The media were buffered to pH 7 and incubated for 24 h at 30°C . β -glucosidase activity was measured using p-nitrophenyl- β -D-glucopyranoside (p-NPG) as substrate, according to Gallo. The assay mixture contained 75 μL of p-NPG 2.5 mmol L^{-1} in 0.5 mol L^{-1} of potassium phosphate buffer, pH 7.5 and 50 μL of extracts. After 10 min of incubation at 37°C , the reaction was stopped by heating the mixture at 95°C for 5 min and then the absorbance was measured at 410 nm. The data obtained were converted to μmol of p-NP by reference to a calibration curve. One unit of β -glucosidase activity (μmol) was defined as the amount of enzyme required to release 1 μmol of p-NP per sec under the assay conditions.

Pectinase activity: For the determination of pectinase activity, the isolates were cultivated on a liquid medium containing 1 g of yeast extract in 20 mL distilled water, 5 mL of $(\text{NH}_4)_2\text{SO}_4$ 20%, 5 mL of aqueous glycerol 87%,

250 mL of aqueous solution of polygalacturonic acid 2%, 200 mL of 0.1 mol L^{-1} phosphate buffer at pH 8, 100 mL of distilled water, 1 mL of 1 mol L^{-1} $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ during 24 h at 37°C . The pectinase activity was measured according to Macedo *et al.* (2000) using 0.2% of polygalacturonate synthetic substrate. The assay mixture contained 4 mL of polygalacturonate 0.2 % dissolved in acetate buffer pH 5, 0.1 mol L^{-1} and 1 mL of extract. After incubation at 40°C for 10 min, the reaction was stopped by addition of 1 mL of Dinitrosalicylic Acid (DNSA) (Sigma Aldrich, Steinheim, Germany) and the absorbance was measured spectrophotometrically at 540 nm. The pectinase activity was expressed as International Unit (IU) per mL of reaction medium. One unit of enzyme activity (μmol) was defined as the amount of enzyme required to release 1 μmol of galacturonic acid under the assay conditions.

Effect of temperature and pH on enzymes activities: The effect of pH and temperature was measured only on the strains which had the highest enzymes activities during the essays in the same conditions than those earlier used. Various pH (4-8) and temperatures (25 - 50°C) were tested for 16 h of experiment. The pH was taken with a pH meter (JENWAY 3310, Jenway Ltd. UK). The pH meter was calibrated against standard buffer solutions at pH 4.0 and 8.0. In the same time, tenfold serial dilutions of culture media were prepared for microbial population enumeration. Indeed, enumeration of LAB was carried out using plates of De Man, Rogosa and Sharp agar (MRS, Merck 10660, Merck, Darmstadt, Germany) which were incubated anaerobically at 30°C for 3 days. Yeasts and moulds were enumerated on plates of Sabouraud chloramphenicol agar (Fluka, Bochemica 89579, Sigma-Aldrich Chemie GmbH, India) incubated at 30°C for 4 days. *Bacillus* were enumerated on plates of Plate Count Agar (PCA Oxoid Ltd. Basingstore, UK) with 2% soluble starch and incubated at 32°C for 2 days.

Statistical analysis: The data obtained were subjected to analysis of variance (Statistica, 99 Edition Alabama, USA) and mean differences determined by Duncan's multiple range tests at a significance level ($p < 0.05$). Principal Component Analysis (PCA) was used to establish relationship among variables represented by different isolates compounds and to classify the strains according the enzyme activities. Only factors with eigenvalues > 1 were retained and Varimax rotation was used as rotation type. PCA was performed using XLSTAT Software Program version 2012/05/01 copyright Addinsoft 1995-2012.

RESULTS

Identification of selected microorganisms: The presumptive LAB and *Bacilli* sp. were divided into three groups based on their several morphological, physiological and biochemical characters. LAB showed a characteristic cell morphology appearing as cocci in pairs or short chains. The *Bacilli* sp. appeared as a combination of long and short rods. Within LAB, only 1 group (group I) was obtained within which strains did not show growth at 45°C but grew at 5 and 15°C and produced CO₂ from glucose. They did not produce NH₃ from arginine and mobility. However, *Bacilli* sp. showed 2 groups (group II and III) among which strains had abilities to grow at 15 and 45°C but not at 5°C. Strains of both *Bacilli* groups differed by their for NH₃ production from arginine and growth in the presence of 8% NaCl. The presumptive isolates belonging to the above groups were further characterized using the API 50 CH System. Sugar fermentation profiles in the API 50 CH indicated that the isolates of LAB and *Bacilli* sp. could be assigned to 3 groups corresponding to individual species. In general, all the strains fermented glucose, fructose, cellobiose, trehalose, maltose, salicin and ribose. None of these fermented erythritol, D-arabinose, L-xylose, adonitol, L-sorbose, dulcitol, inositol, inuline, melizitoze, glycogen, xylitol, Dtagatose, D-fucose, L-fucose D-arabitol and 2-ketogluconate.

Strains of group II differed from those of group I by their ability to ferment galactose, D mannose, rhamnose, sorbitol, glucerol and N-Acetyl glucosamin. In contrast to strains of groups I and II, strains of group III fermented fewer carbohydrates. According to to Bergey's Manual of Systematic Bacteriology and the APILAB Plus Software, all LAB strains were identified as *Leuconostoc. mes. ssp. mesenteroide/dextranicum* (group I) with a percentage of identification (id%) in the range of 86-99%, strains of group II as *Bacillus amyloliquefaciens* (group II) with a strong id (96%) and strains of group III as *Bacillus cereus* at 93.7% id (Table 1).

After a preliminary morphological screening, the yeast isolates were selected and examined through the API 20 C AUX System. The results indicated that isolates corresponded to *Candida tropicalis* at 99 id% (Table 1). Three species of moulds (*Chrysonila* sp., *Apergillus flavus* and *Rhizopus oryzae*) were also identified according to the morphological and cultural characteristics.

Enzyme activities: Almost all strains tested in this study possessed at least one of the tested enzyme activities at a detectable level. As observed for α -amylase activity, all isolates possessed an activity against potato starch but with important variations (Fig. 1a). As shown in this Fig. 1, single culture fermentations of isolates lead to high α -amylase activities with the yeasts strains *Candida tropicalis* LVX1 *Candida tropicalis* LVX8 and *Candida tropicalis* LVX14 with the highest activity (144 ± 3.3 U mL⁻¹) with *C. tropicalis* LVX14. Among the other microorganisms, the highest amylase activity (70 ± 3 U mL⁻¹) was recorded after 24 h of culture and this activity represented the half of this of yeasts.

Large differences in production of extracellular β -glucosidase were found within the microorganisms tested with the highest activities obtained with the LAB species (Fig. 1b). *Leuconostoc. mes. ssp. mesenteroide/dextranicum* LABX11 and *Leuconostoc. mes. ssp. mesenteroide/dextranicum* LABX21 pure cultures registered their maximum β -glucosidase activities after 20 h of culture with respective values of 54.7 ± 3.11 and 43.1 ± 5.9 U mL⁻¹ but these activities were lower than this of le strain *Leuconostoc. mes. ssp. mesenteroide/dextranicum* LABX2 which showed an activity of 68.09 ± 11.45 U mL⁻¹ only after 12 h of culture. Whereas, in the other cultures, this activity was detected until the end of the fermentation but with significant differences compared to the LAB activity.

In all treatments, measures of pectinase activity indicates that this activity was the highest in the pure culture of *Bacillus amyloliquefaciens* BX5 and mould *Rhizopus oryzae* MX4. There was no significant

Table 1: Identification of selected microorganisms after galleries API

Microorganisms	Codes	Presumptive species	Percentage of identification	No. of species	Total No. of species
LAB	LABX1	<i>Leuconostoc. mes. ssp.</i>	86-99.0	5	5
	LABX2	<i>mesenteroides/dextranicum</i>	-	-	-
	LABX3	-	-	-	-
	LABX11	-	-	-	-
	LABX21	-	-	-	-
Bacillus	BX 3	<i>Bacillus cereus</i>	93.7	1	3
	BX5	<i>Bacillus amyloliquefaciens</i>	96.0	2	-
	BX8	-	-	-	-
Yeasts	LVX1	<i>Candida tropicalis</i>	99.0	3	3
	LVX8	-	-	-	-
	LVX14	-	-	-	-

Isolates of Adjoukrou starter

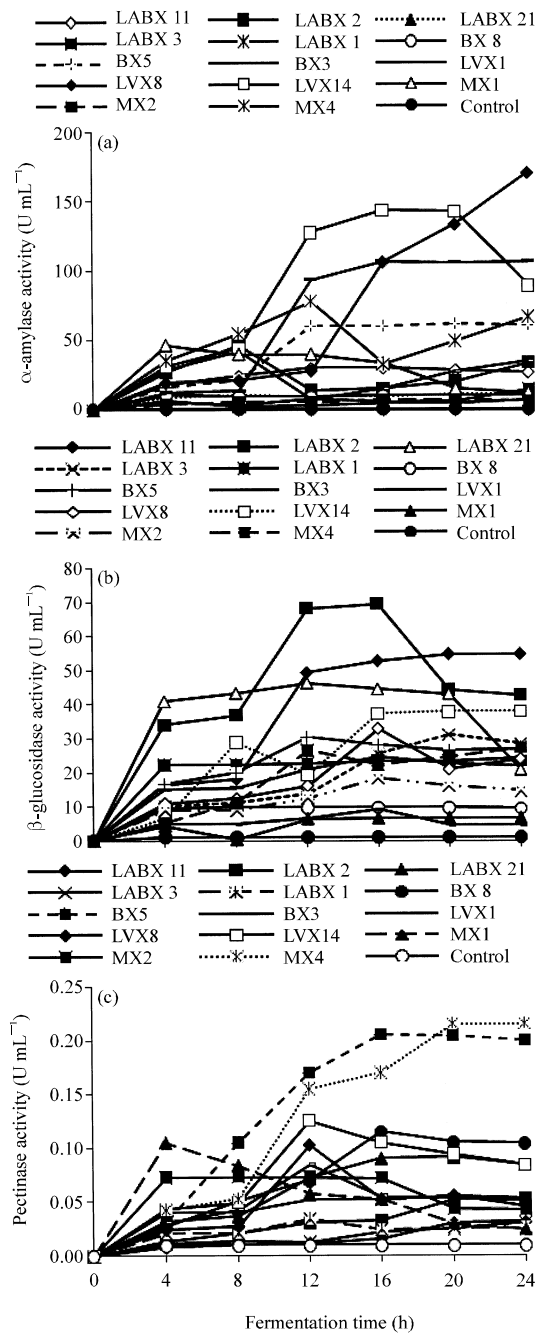


Fig. 1: a) α -amylase activity; b) β -glucosidase activity and c) pectinase activity of selected strains from starter cassava traditional Adjoukrou

difference between both strains whose pectinase activities progressively increased throughout the fermentation (Fig. 1c) to reach respective values of values of 0.21 ± 0.01 and 0.215 ± 0.02 U mL⁻¹ after 24 h de fermentation. The other tested microorganisms (yeasts

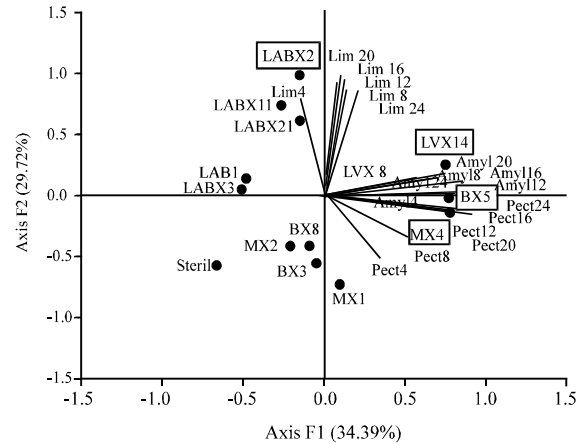


Fig. 2: Principal Components Analysis (PCA) of enzymatic activities (α -amylase, β -glucosidase and pectinase). Selected strains originating from a traditional cassava starter Adjoukrou. Amyl 4-24: α -amylase activity of 4-24 h; Lim 4-24: Linamarase (β -glucosidase) activity 4-24 h; Pect 4-24: Pectinase activity of 4-24 h; LABX2: *Leuconostoc. mes. mesenteroide/dextranicum*, BX5: *Bacillus amyloliqueficiens*, MX4: *Rhizopus oryzae*, LVX14: *Candida tropicalis*

and LAB) registered the low values of pectinase activities ranged between 0.05 and 0.1 U mL⁻¹ throughout the experiments (Fig. 1c).

In an attempt to simplify the interpretation of the data and classify the strains according to their performance and select probable starter cultures for a controlled fermentation, principal component analysis of the matrix of correlation coefficients was computed from data of enzyme activities. In this study, the first two principal components (axis F1 and F2) explained 64.11% of the variation in the data with the first component (PC1) accounting for >34.39% of the variation and the second component (PC2) accounting for 29.72% of variation. Figure 2 shows the representation of the different enzymatic activities and strains tested according to the first and second dimensions. As it could be expected, amylase activity is highly correlated and represented very near to the yeast specie *Candida tropicalis* LVX14. In the same way, a strong correlation between the pectinase activity and the strains *Bacillus amyloliqueficiens* BX5 and *Rhizopus oryzae* MX4 was observed while LAB strains, particularly *Leuconostoc. mes. ssp. mesenteroide/dextranicum* LABX2 was represented the nearest of β -glucosidase activity on the positive side of the second dimension 2 (Fig. 2). Thus, these four strains (*Candida tropicalis* LVX14, *Bacillus amyloliqueficiens*

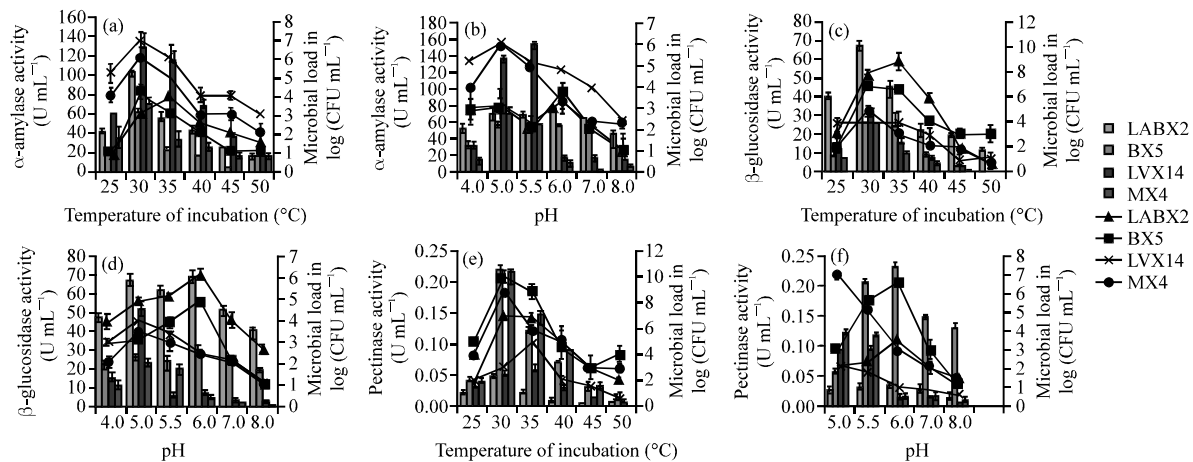


Fig. 3: Effect of temperature and pH on microbial growth and enzyme activities of isolates from Adjoukrou traditional cassava; a) α -amylase activity U mL^{-1} vs. temperature; b) α -amylase activity U mL^{-1} vs. pH; c) β -glucosidase activity (U mL^{-1}) vs. temperature; d) β -glucosidase activity (U mL^{-1}) vs. pH; e) pectinase activity (U mL^{-1}) vs. temperature; f) pectinase activity (U mL^{-1}) vs. pH

BX5, *Rhizopus oryzae* MX4 and *Leuconostoc. mes. ssp. mesenteroide/dextranicum* LABX2) were selected to constitute starter cultures and the effect of pH and temperature was tested on their enzyme activities.

Effect of temperature and pH on enzyme activities and microbial growth: The effect of pH and temperature was further investigated on selected strains enzyme activities and growth. Results showed that enzymatic activities were widely distributed over a range of pH (4 and 8.0) and temperature (25 and 50°C) values. All tested strains (*Candida tropicalis* LVX14, *Leuconostoc. mes. ssp. mesenteroide/dextranicum* LABX2, *Bacillus amyloliquefaciens* BX5 and *Rhizopus oryzae* MX4) showed enzyme activity whatever the temperature or pH applied. In general, maximal hydrolysis of any of substrates occurred at temperatures near 30°C. Enzyme activities were rapidly reduced as the temperature increased above the optimum (30°C). Indeed, the lowest activities were observed at 25°C and temperatures over 45°C (Fig. 3).

Concerning the pH, activities were the highest at pH ranged between 5 and 6. As seen in Fig. 3, *Candida tropicalis* LVX14 displays two peaks of α -amylase activity, respectively at pH 5 and 5.5 with a maximal microbial load of 6.3×10^7 and 10^7 CFU mL^{-1} , respectively at pH 5 and 30°C. However, β -glucosidase activity was the highest at pH 6 with the strains *Leuconostoc. mes. ssp. mesenteroide/dextranicum* LABX2 which growth was maximal at 35°C for the same pH. In the same time However, dual pH optima of pectinase activity were observed at pH 5.5 and 6 for strain *Bacillus amyloliquefaciens* BX5. Its maximum

microbial load is observed at pH 6 and 30°C. *Candida tropicalis* LVX14 and moulds were many at pH 5 and 30°C. Liberation of pectinase activities was most effective at lower pH values with a peak in activity typically occurring at pH 5.5-6.0 for a temperature of 30°C. Indeed, maximum activities of approximately 0.22 units were seen for strains *Bacillus amyloliquefaciens* BX5 and *Rhizopus oryzae* MX4 in these conditions while the maximal growth was observed at 30°C but at different pH values (pH 6 and 5) (Fig. 3).

DISCUSSION

Fourteen interesting microorganisms (LAB, yeasts, moulds and *Bacillus* sp.) isolated from traditional cassava starters in different villages of the department of Dabou (Cote d'Ivoire) were selected for their ability to efficiently produce hydrolytic enzymes involved in the fermentation of cassava during attieke production. According to their general characteristics and metabolic patterns these microorganisms were presumptively identified (Hammes and Vogel, 1995). The presumptive LAB, yeasts, Moulds and *Bacilli* sp. were divided into different groups based on their several morphological, physiological and biochemical characters. The APILAB Plus Software proposed an identification of the LAB strains as *Leuconostoc. mes. ssp. mesenteroide/dextranicum* (group I) with a percentage of identification (id%) in the range of 86-99%, the *Bacillus* strains as *Bacillus amyloliquefaciens* (group II) with a strong id% (96%) and as *Bacillus cereus* with a 93.7%id (group III). All yeasts were identified as *Candida tropicalis* with a 99% id. Similar identifications were achieved by Hammes and Vogel

(1995). For all isolates, enzyme profiles made apparent a greater variability between strains belonging to the same group, allowing a higher degree of strain differentiation. Indeed, amylase strains of LAB, yeasts, bacillus and moulds were repeatedly isolated from various Africa fermented foods (Johansson *et al.*, 1995; Olasupo *et al.*, 1996). The evolution of microbial enzyme activities clearly showed an interesting enough profile of yeasts amylase activity particularly for the strains *Candida tropicalis* LVX14 and *Candida tropicalis* LVX1. The presence of amylase activity during cassava fermentation was mentioned by Oyewole and Odunfa (1992) and the researchers attributes this activity to yeasts. The specie *Candida tropicalis* LVX14 displayed the highest amylase activity ($144 \pm 3.3 \text{ U mL}^{-1}$) obtained after 16 h of fermentation. Unlike yeasts, a low amylase activity was observed with the other isolates (lactic bacteria, mould and Bacillus). This observation was also reported by Djoulde during their study cassava fermentation. Similarly, Amoa-Awua *et al.* (1997) also showed the ability of some *Bacillus* sp. (*Bacillus subtilis*, *Bacillus pumilus*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis* and *Bacillus cereus*) to produce amylase activity but at a lower rate than yeasts. These microorganisms are able to hydrolyze starch with α -amylase to produce organic acids mainly lactic acid improving the taste of the finished product. Starch was fermented according to a now well-established pattern for different LAB (Guyot *et al.*, 2000; Calderon *et al.*, 2001) and characterized by a fast starch hydrolysis, a transient reducing sugar accumulation and a growth linked amylase production. However, for isolated microorganisms, yields and specific rates of amylase production were markedly higher than those reported for the previously characterized reference strains (Guyot *et al.*, 2000; Sanni *et al.*, 2002).

Under the same growth conditions, β -glucosidase activities obtained with LAB strains were higher than those of yeasts, Bacilli and Moulds. It was interesting to note that all LAB strains (LABX1, LABX2, LABX3, LABX11 and LABX21) isolated from Adjoukrou traditional cassava starter belonged to cluster of *Leuconostoc. mes. ssp. mesenteroide/dextranicum* species. β -glucosidase activities of these strains were accelerated and reached the highest levels during the fermentation. The high levels of β -glucosidase activities detected among *Leuconostoc. mes. ssp. mesenteroide/dextranicum* strains could be a useful technological trait used to improve the nutritional value of products. The ability of *Leuc. mesenteroides* to produce β -glucosidase was earlier reported in several studies (Obilie *et al.*, 2004) similarly, Zotta *et al.* (2006). Though not all β -glucosidases are linamarase, this result was

interpreted as probable linamarase activity of the isolates. Okafor and Ejiofor (1985) found that *L. mesenteroides* only produced linamarase when linamarin was present. Therefore, most probably, these isolates were capable of breaking down cyanogenic glucosides and contributed significantly to the detoxification of cassava during attieke fermentation with the highest activity produced by strains *Leuconostoc. mes. ssp. mesenteroide/dextranicum* LABX2.

Almost all other microorganisms (yeasts, moulds and *Bacillus* sp.) investigated have proven effective enough in the production of β -glucosidase activity thus contributing with the LAB species to the detoxification of cassava dough during attieke production. Moreover, β -glucosidase yeasts, moulds and *Bacillus* sp. was earlier reported (Brimer *et al.*, 1995; Lei *et al.*, 1999). β -glucosidases have also been studied in different bacterial groups including LAB isolated from meat (Coombs and Brenchley, 2001), wine (Spano *et al.*, 2005) and soy-bean (Choi *et al.*, 2002).

Another important property investigated concerned the production pectinase enzymes. Possession of pectinase activity was critical to the ability of isolates to disintegrate cassava tissue. Possession of pectinase activity was also sufficient to cause the breakdown of the texture of cassava dough as shown by Amoa-Awua and Jakobsen (1995) and Djoulde. Among all the isolates, *Bacillus amyloliquefaciens* BX5 and *Rhizopus oryzae* MX4 displayed the highest activities (0.24 U mL^{-1}). (Gobbetti *et al.*, 1996a, b) found that moulds and Bacilli exhibited a broad range of pectinase activities on synthetic substrates. These strains can secrete depolymerases capable to divide the polymer in oligomers of small sizes assimilated by microorganisms. This observation gave an indication that isolates from adjoukrou traditional cassava starters were likely to be the agents responsible for the softening of cassava dough. This result is consistent with earlier studies (Amoa-Awua and Jakobsen, 1995). The other microorganisms (yeasts, moulds and LAB), at a lesser extent showed also ability to produced pectinase enzymes during fermentation thus contributing to yield a smooth textured dough. The contribution of yeasts to the modification of cassava texture in comparison to the moulds has been assigned a secondary role by Amoa-Awua *et al.* (1997). Tudor and Board (1993), in their review, list some yeasts such as *C. tropicalis*, *C. krusei* and several species of *Zygosaccharomyces* as food spoilage yeasts but in the research by Amoa-Awua *et al.* (1997), yeasts have been shown to play a positive role in cassava fermentation by contributing to the modification of the texture of the product.

During sourdough fermentation, enzymatic events depend on several factors, such as pH, fermentation time, temperature and activities of microorganisms. This research has examined the influence of potentially inhibitory parameters such as pH and temperature both singularly on the enzyme activities and growth of the four most efficient strains (*Leuconostoc. mes. mesenteroide/dextranicum* LABX2, *Bacillus amyloliquefaciens* BX5, *Candida tropicalis* LVX14 and *Rhizopus oryzae* MX4). *Bacillus amyloliquefaciens* BX5 and *Rhizopus oryzae* MX4 species had high pectinase activities. In addition, the high levels of linamarase (β -glucosidase) and amylolytic activities were detected respectively in *Leuconostoc. mes. ssp. mesenteroide/dextranicum* LABX2 and *Candida tropicalis* LVX14 strains. Most of the selected strains studied had their highest enzyme activity at pH values between 5.0 and 6.0 and at 30°C and their maximal growth occurred between 30 and 35°C for pH value of 6 excepted *Candida tropicalis* LVX14 which optimal growth occurred at pH 5. According to Spier *et al.* (2006), temperature of 30°C coupled to a humidity of 90% allows to a more intense production of α -amylase for starch hydrolysis and the production fermentable sugars for ulterior acidification. Also, Pandey *et al.* (2005) achieve higher yields of α -amylase at temperatures between 30 and 37°C. However, conditions for obtaining maximal β -glucosidase activity by *Leuconostoc. mes. mesenteroide/dextranicum* LABX2 were also estimated at 30°C but at pH 6. In these conditions the degradation of cyanide compounds is important so that below or above this range, the activity is greatly reduced. Although, β -glucosidase activity was best at 30°C, the microbial growth of this LAB was maximal at 35°C. These observations are reinforced by the fact that β -glucosidase has its optimum activity at pH between 5.5 and 6 at a temperature of 37 \pm 2°C (Cooke *et al.*, 1978).

The effect of external factors notably pH and temperature on the pectinase activity of isolates indicates that *Rhizopus oryzae* MX4 produced some strong activities at 30°C and at pH 5. In these conditions, this strain recorded a total activity of 22 \pm 0.006 U mL⁻¹ with a maximum load of 10⁶ CFU mL⁻¹. These results are in agreement with those of Singh and Rao (2002). According to them the genera *Aspergillus*, *Rhizopus* and *Geotrichum* have the ability to produce pectinolytic activity at an optimum pH of 5-30°C. In contrast, *Bacillus amyloliquefaciens* BX5 its highest pectinolytic activity at 30°C for a pH value of 6 with maximal microbial load at 35°C. These findings are consistent with those Avallone *et al.* (2002) who identified some *Bacillus* such *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus*

cereus and *Bacillus mycoide* able to get their polygalacturonase activity at pH 6 to temperature between 30 and 40°C. According to Gainvors *et al.* (2004), various microorganisms synthesize polygalacturonases active at acidic pH of 4-6 in different incubation temperatures. This indicates that the phenomena at the origin of the softening are acid-tolerant and optimum temperature variations. Strong pectinase activity of *Bacillus amyloliquefaciens* BX5 and mould *Rhizopus oryzae* MX4 selected in the above conditions indicates that their use in co-culture for the softening of cassava dough during fermentation will be very useful.

Efficient amylolysis, pectinolysis and detoxication were chosen as the main criteria of starter strains selection because in a starchy food matrix, it is expected to increase the availability of energy sources for other associated non-amylolytic microorganisms, to contribute to a rapid pH and cyanide compounds decrease and to impart favourable rheological properties. However, complementary functional properties have to be considered for fermented food production, either from different types of starchy materials or in combination with legumes.

CONCLUSION

This study, carried out with a limited number of selected strains has shown that some activities which may contribute to the nutritional improvement of fermented foods will be expressed at the strain level within a same species. Moreover, it still remains to be determined if such starter cultures would be able to simultaneously express these different metabolic activities in complex food matrixes, since it has been reported that some can undergo catabolic repression by glucose, such as raffinose fermentation (Prevost *et al.*, 1993) and amylase production (Guyot and Morlon-Guyot, 2001). The biochemical traits of these selected strains however were mostly investigated *in vitro*. Therefore, to assess the performance of the strains in establishing themselves as dominant starter cultures in actual fermentations and to validate whether these biochemical traits are indeed expressed and have a positive impact on product quality, these strains will then be used in pilot plant fermentations where their performance will be evaluated.

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