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# Excretion of β-glucosidase and pectinase by microorganisms isolated from cassava traditional ferments used for attieke production in Côte d'Ivoire

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ABSTRACT

This research work aimed at studying production kinetic of β-glucosidase and pectinase by microbial strains isolated from two traditional cassava ferments; with a view of their potential use as starter cultures. The study was carried out on 230 strains and 28 were selected on the basis of their ability to hydrolyze limanarin and pectin. Results showed that the excretion of β-glucosidase and pectinase by the preselected microorganisms is regulated by a repression mechanism. Only *Lactobacillus plantarum LBZ46* produces β-glucosidase whose activity is modulated by inactivation. *Lactobacillus plantarum LBZ46* and *Lactobacillus plantarum LBY9* were able to excrete β-glucosidase greater than 50 ± 5 EU/mL.The optimum temperature for β-glucosidase excretion and biomass production was 35 °C for *Lactobacillus plantarum LABY9* from the Alladjan ferment. With regard to *Lactobacillus plantarum LABZ46* from ferment Ebrie, the optimum temperature for β-glucosidase production was 30 °C, while the optimum temperature for biomass production was 35 °C.The optimum pH of β-glucosidase excretion and biomass production ranges from pH 5 to pH6. Regarding the pectinolytic excretion, two strains of *Bacillus* and one strain of mould were able to excrete an amount of pectinase equivalent to 0.2 ± 0.06 EU/mL. It is of the *Bacillus amyloliquefaciens BY4, Bacillus subtilis BZ15* and *Rhizopus oryzae MZ4*. Considering the diversity of production kinetics of β-glucosidase and pectinase, the use of these isolates for a controlled fermentation of cassava dough would be optimal in co-culture for the production of attieke Alladjan and Ebrie in Côte d'Ivoire.

## 1. Introduction

Attieke is the major fermented plant food in Côte d'Ivoire. It is a steamed granular cassava (Manihot esculenta Crantz) meal ready-to-eat, couscous-like product, with slightly sour taste and whitish colour (Djeni et al., 2011). The largest amounts of attieke are prepared by three ethnic groups (Adjoukrou, Alladjan and Ebrie) at the origin of attieke production and which supply the big city of Abidjan (Assanvo et al., 2006). But, increasingly, the production and consumption of attieke has spread throughout the Ivorian territory and in the West African sub-region, with population migrations (Sotomey et al., 2001, Djéni et al., 2011). Also, the large export market potential of attieke cannot be underestimated as the size of the West African ethnic population overseas keep increasing. It is also exported to Europe as a dehydrated product without any established specifications, contrarily to gari, a similar product in Ghana and Nigeria. Recent data on attieke production and consumption estimated its consumption 450 000 tons per year (CNRA, 2003) for a cassava production estimated at 2.9 million tonnes and represents 50% of the food crop (FAOSTAT, 2010). But, the cassava used for the preparation of attieke has two important deficiencies. Firstly, the bitter varieties contain the toxic cyanogenic glucosides linamarin and (to a lesser extent) lotaustralin and secondly, it is very poor in protein, containing only about 1% (Sanni et al., 2002). In addition, the attieke production is 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). Initiation of a spontaneous process takes a relatively long time, with a high risk for failure. Failure of fermentation processes can result in spoilage and/or the survival of pathogens, thereby creating unexpected health risks in food products (Holzapfel, 2002). Thus, from both a hygiene and safety point of view, the use of starter cultures for fermentation is recommended, as it would lead to a rapid acidification of the product and thus inhibit the growth of spoilage and pathogenic

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bacteria (Holzapfel, 2002). Also, microorganisms play an important role in inhibiting rot and therefore induce a better conservation of the fermented food by production of antimicrobial compounds (Jacques, 2011). Some species of Bacillus could also produce lactic acid (Rosenberg et al., 2005; Ouyang et al., 2013) which gives to attieke an acidulous taste. In addition, in the industrial utilization, microorganisms are considered the best choice for enzyme productions (β-Glucosidase and pectinase). Microorganisms producters beta-glucosidase, this enzyme breaks down cyanogenic glucosides and contributes significantly to the detoxification of cassava during attieke fermentation and microorganisms pectinases activities break down of the coarse texture of cassava dough Bouatenin et al. (2013). So, the selection of microorganisms, with such interesting activities could to the improvement and the optimization of the traditional fermentation of cassava into attiéké. However, attieke processing technology is characterized by empiric steps which are very difficult to control (Sotomey et al., 2001). Moreover, differences in the traditional starters used are the basis of the different organoleptic qualities obtained for various types of attieke in Côte d'Ivoire (Djeni et al., 2011).

Therefore, the present work aims to make a kinetic study of the production of β-glucosidase and pectinase for the determination of optimal conditions and growth factors of prescreened pectinolytic and glucosidic strains for later use for control and optimization of the cassava fermentation process in attieke (Alladjan and Ebrie) in Côte d'Ivoire.

## 2. Materials and methods

### 2.1. Microbial strains used

In this study, two hundred and thirty strains (230) were isolated (lactic acid bacteria, bacilli sp., yeasts and moulds) from Alladjan and Ebrie traditional attieke starters. Among these isolates, 28 were selected according to previous study of Bouatenin et al. (2012), based on the qualitative production of linamarase and pectinase enzymes. These strains used, have been characterized and identified by morphological, biochemical as well as molecular approaches (Bouatenin et al., 2013; Djeni et al., 2015).

### 2.2. Evaluation of enzyme activity of isolates

#### 2.2.1. Preparation of inocula and seeding of culture media

Inoculum preparation was performed according to the method described by Aka (2009). Colonies of lactic acid bacteria, Bacillus, yeast, and moulds were plated individually into 5 ml of specific broths (MRS broth for lactic acid bacteria, nutrient broth for bacilli, and sabouraud broth for yeast and mould). The whole was incubated at 35 °C for 48 h. The deposit obtained in the lower part of each flask was recovered by decantation and then introduced into previously sterilized centrifuge tubes. These tubes were then centrifuged at 5000 rpm for 5 min. After centrifugation, the pellet was collected and the microbial density was determined from a densimate (Bio Merieux, France) against a blank consisting of sterile broth uninoculated. The concentration of inocula obtained varies between 5 and 7 Mac Farland (McF). Inocula concentrations were converted to $10^6$ CFU/mL to perform the various enzymatic analyzes.

#### 2.2.2. 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. Each experiment was achieved in 250 ml liquid meduim inoculated with a pure strain at a rate of $10^6$ cells $ml^{-1}$ and incubated for 24 h at 30 °C under rotary agitation (105 rpm). Every 4 h, 10 ml of the culture medium are removed and centrifuged at 6000 rpm/40 min. The resulting supernatant was dialysed at an appropriate concentration at 4 °C. The dialysate constitutes the crude enzyme preparation. Thus, β-glucosidase activity was measured using *p*-nitrophenyl-β- D-glucopyranoside (*p*-NPG) as substrate, according to (Gallo, 2004). 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 second under the assay conditions.

#### 2.2.3. 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 $(NH_4)_2SO_4$ 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 and 1 ml of 1 mol $l^{-1}$ $MgSO_4 \cdot 7H_2O$.Each experiment was achieved in 250 ml liquid meduim inoculated with a pure strain at a rate of $10^6$ cells $ml^{-1}$ and incubated for 24 h at 30 °C under rotary agitation (105 rpm). Every 4 h, 10 ml of the culture medium are removed and centrifuged at 6000 rpm/40 min. The resulting supernatant was dialysed at an appropriate concentration at 4 °C. The dialysate constitutes the crude enzyme preparation. Thus, 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.

#### 2.2.4. Effect of temperature and pH on enzymes activities and microbial population

The effect of pH and temperature was measured only on the strains which had the kinetic highest production enzymes activities during the essays in the same conditions than those previously used. These were five (05) species (Lactobacillus plantarum LBZ46, Lactobacillus plantarumY9, Bacillus amyloliquefaciens BY4, Bacillus subtilis BZ15 and Rhizopus oryzae MZ4). The influence of pH and temperature on the activities of these preselected strains was carried out under the same conditions as described above but at a pH range of 4.5–8 in phosphate buffer. The temperature conditions were carried out at 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C at pH 5. At each pH or temperature step, regardless of the type of activity, 1 mL of the medium was collected for enumeration by the conventional method of dilution and plating on MRS Agar for LAB, mossel for Bacilli, and Sabouraud for yeasts and moulds.

### 2.3. 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 ($P < 0.05$). This software also made it possible to calculate the averages and standard deviations of the parameters analyzed.

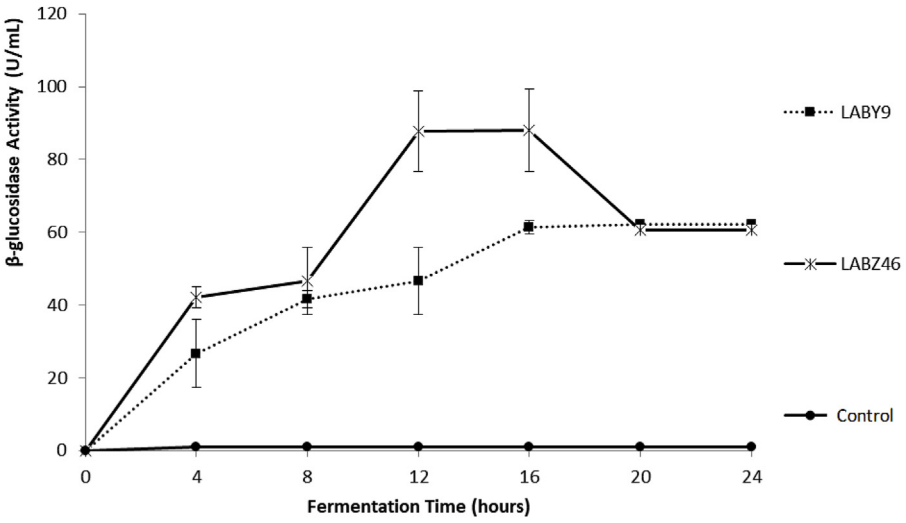


**Fig. 1.** Regulation of the secretion of β-glucosidase by two strains of lactic acid bacteria selected from the traditional ferments Alladjan and Ebrie.
**LABY9:** *Lactobacillus plantarum* LABY9 selected as a microbial component of potential starter Alladjan.
**LABZ46:** *Lactobacillus plantarum* LABZ46 selected as a microbial component of potential starter Ebrie.

## 3. Results and discussion

To determine the culture conditions for optimal production of the enzymes (β glucosidase and pectinase) responsible for the hydrolysis of these substrates, a kinetic study of excretion was performed. Among the two hundred and thirty (230) strains of microorganisms (lactic bacteria, yeasts, moulds and *Bacillus* sp. twenty-eight (28) were selected on the basis of their ability to hydrolyze limanarin and pectin. Of the 28 preselected strains, the secretion of β-glucosidases is mainly ensured by strains of lactic acid bacteria. The excretion of β-glucosidase by the preselected microorganisms is regulated by a repression mechanism. Only *Lactobacillus plantarum LBZ46* produces β-glucosidase whose activity is modulated by inactivation. *Lactobacillus plantarum LBZ46* and *Lactobacillus plantarum LBY9* were able to excrete β-glucosidase greater than 50 ± 5 EU/mL. An example of the kinetics of β-glucosidase excretions is presented in Fig. 1. The low proportion of microorganisms secreting β-glucosidase was explained by the presence of strains whose enzyme is membrane. The Secretion of this enzyme was regulated by a catabolic repression mechanism and in some strains the activity of the secreted enzyme was modulated by partial inactivation.This mechanism of regulation of β-glucosidase excretion and modulation of the activity of certain forms by glucose or cellobiose have been observed in previous studies (Stoppok et al., 1982; Nazir et al., 2009). On the other hand, the secretions of pectinases are the work of some strains of *Bacillus* and moulds. Of the 28 strains of microorganisms preselected from the ferments, two strains of *Bacillus* and one strain of mould were able to excrete an amount of pectinase equivalent to 0.2 ± 0.06 EU/mL. It is of the *Bacillus amyloliquefaciens BY4*, *Bacillus subtilis BZ15* and *Rhizopus oryzae MZ4* (Fig. 2). Gobbetti et al. (1996) found that mould and Bacillus exhibit a wide range of pectinolytic activities on synthetic substrates.The regulatory mechanism was repressive and the enzyme activity secreted by these strains were modulated by inactivation. According to Sakai et al. (1993), pectinase production was regulated by induction and repression processes, an induction linked to the presence of substrates, in this case pectin, repression by carbon sources readily available by microorganisms. During the different monocultures realized, it was also clearly demonstrated that the excretion of hydrolytic enzymes by the selected strains of the different traditional ferments was influenced by several factors, in particular the pH, the fermentation time, the temperature and the activities of microorganisms. Thus, the effects of temperature and pH on β glucosidase excretion and biomass production by each lactic acid bacteria selected, were evaluated. *Lactobacillus plantarum LABY9* and *Lactobacillus plantarum LABZ46* were selected respectively as a microbial component of potential starter Alladjan and Ebrié for their high β-glucosidase activity.The overall results clearly show that the optimum temperature for β-glucosidase excretion and biomass production was 35 °C for *Lactobacillus plantarum LABY9* from the Alladjan ferment (Fig. 3A). With regard to *Lactobacillus plantarum LABZ46* from ferment Ebrie, the optimum temperature for β-glucosidase production was 30 °C, while the optimum temperature for biomass production was 35 °C (Fig. 3C). The optimum pH of β-glucosidase excretion and biomass production ranges from pH 5 to pH6. The amounts of β-glucosidase and biomass produced in the optimum pH range of 62.2 ± 2.28 EU/mL to 70.7 ± 3.2 EU/mL; 1 ± $4.10^5$ CFU/mL at 1.2 ± $9.10^9$ CFU/mL, respectively (Fig. 3B and 3D). These works were consistent with that of Cooke et al. (1978) conducted during the research of lactic acid bacteria with linamarasic activity for the fermentation of cassava dough. For these authors the linamarase activity was optimal at pH between 5.5 and 6 at a temperature of 37 °C ± 2. Regarding the effect of pH and temperature on the pectinolytic activity of the isolates studied, the mould strain (*Rhizopus oryzae MZ4*) and the two Bacillus strains (*Bacillus amyloliquefaciens BY4* and *Bacillus subtilus BZ15*) were selected for their best pectinolytic activity.The mould (*Rhizopus oryzae MZ4*) excretes best pectinolytic activity with maximum microbial loads at 30 °C for pH 5 (Fig. 4A and B). These results were in agreement with those of Singh and Rao (2002) who investigated the polygalacturonase properties of moulds in various synthetic culture media. According to the latter, the genera *Aspergillus, Rhizopus* and *Geotrichum* have the ability to produce pectinolytic activity at an optimum pH of 5 at 30 °C. On the other hand, the pectinolytic activities of Bacillus (*Bacillus amyloliquefaciens BY4* and *Bacillus subtilus BZ15*) were elevated at 30 °C at pH 6 with maximum loadings at 35 °C at the same pH (Fig. 4A, B, 4C, 4D).These results were consistent with those of Avallone et al. (2002). Indeed, these authors have identified certain *Bacillus* such as *Bacillus subtilis, Bacillus pumulus, Bacillus amyloliquefaciens, Bacillus licheniformis, Bacillus cereus* and *Bacillus mycoides* capable of obtaining their polygalcuronase activity at pH 6 at a temperature between 30 and 40 °C in coffe fermentation.The high activity of the strains under the conditions described, could be explained by the fact that the depolymerases contain lyases and polygalacturonases. Lyases and polygalacturonases act at acidic pH or near neutral pH with their preferred substrate which is polygalacturonic acid.These polygalacturonases secreted by the microorganisms will act by hydrolysis mechanisms increasing the production of these enzymes at pH between 5 and 6 and at a temperature between 30 and 35 °C. Indeed, various microorganisms synthesize active polygalacturonases at acid pH between 4 and 6 at different incubation temperatures (Grainvors et al., 1994).

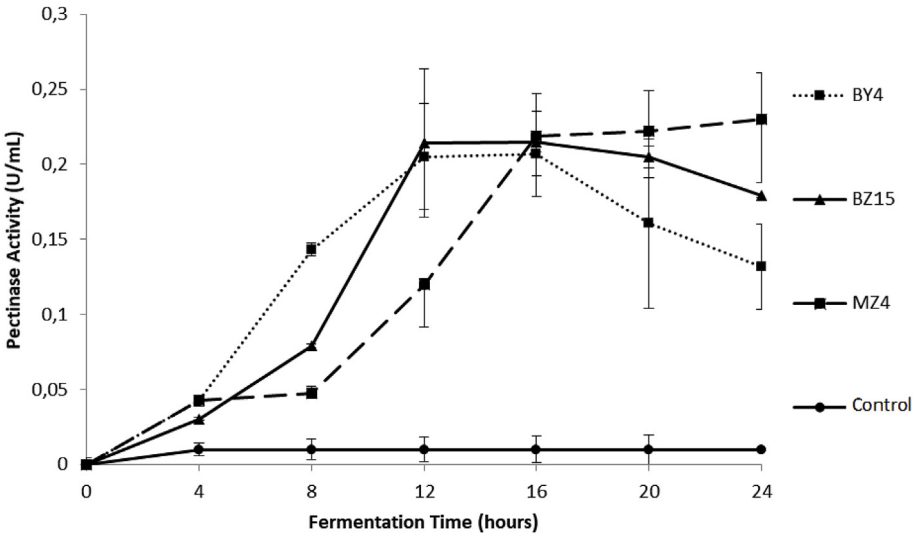


**Fig. 2.** Regulation of the secretion of pectinases by two strains of Bacillus and a selected mould of the traditional cassava ferments Alladjan and Ebrie.
**BY4:** *Bacillus amyloliquefaciens* BY4 selected as a microbial component of potential starter Alladjan
**BZ15:** *Bacillus subtilus* BZ15 selected as a microbial component of potential starter Ebrie.
**MZ4:** *Rhizopus oryzae* MZ4 selected as a microbial component of potential starter Ebrie.

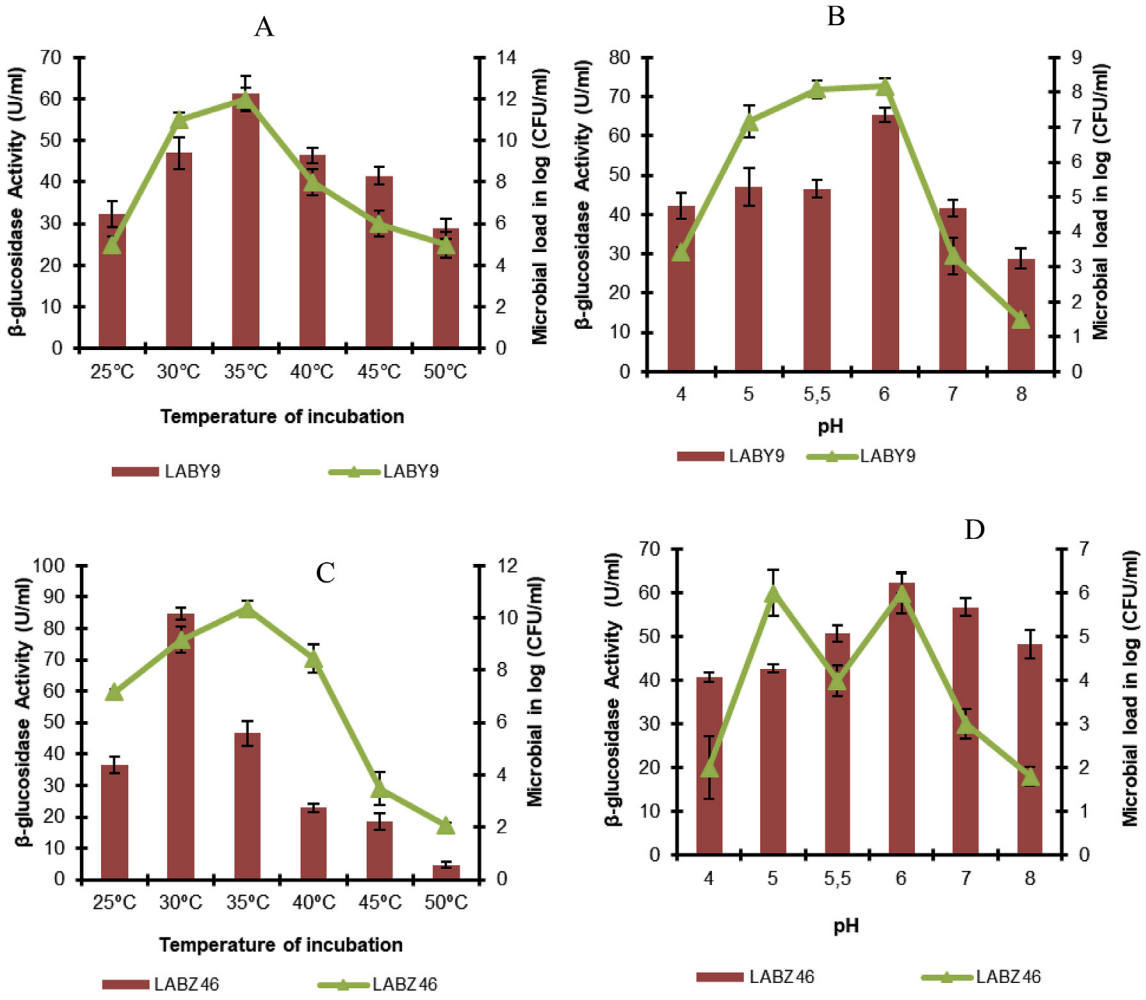


**Fig. 3.** Influence of temperature and pH on β-glucosidase excretion and on biomass production by selected microorganism species of Alladjan ferment (A and B), Ebrie (C and D).
**LABY9:** *Lactobacillus plantarum* LABY9 selected as a microbial component of potential starter Alladjan
**LABZ46:** *Lactobacillus plantarum* LABZ46 selected as a microbial component of potential starter Ebrie.

## 4. Conclusion

This study aimed at the establishment of two (2) types selected starters specific to each type of attieke in Côte d 'Ivoire (Alladjan attieke and Ebrie attieke). From this study, *Lactobacillus plantarum LABY9* and *Bacillus amyloliquefaciens BY4* were selected as the microbial component of potential starter Alladjan. While *Lactobacillus plantarum LABZ46, Rhizopus oryzae MZ4* and *Bacillus subtilus BZ15* were selected

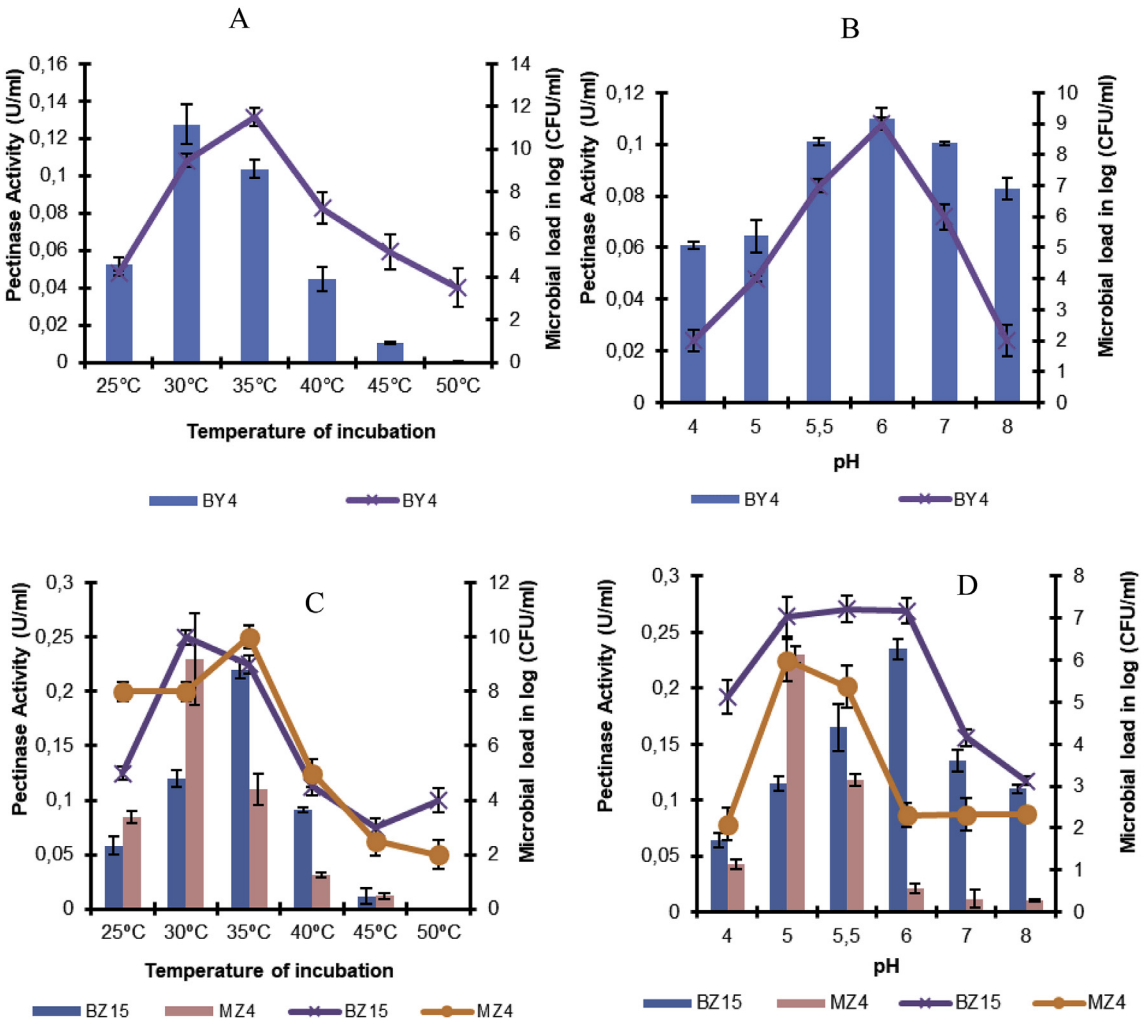


**Fig. 4.** Influence of temperature and pH on pectinase excretion and on biomass production by selected microorganism species of Alladjan ferment (A and B), Ebrie (C and D).
**BY4:** *Bacillus amyloliquefaciens* BY4 selected as a microbial component of potential starter Alladjan
**BZ15:** *Bacillus subtilus* BZ15 selected as a microbial component of potential starter Ebrie
**MZ4:** *Rhizopus oryzae* MZ4 selected as a microbial component of potential starter Ebrie.

as the microbial component of the potential Ebrie starter. The influence of temperature and pH on the growth and enzymatic activity of these strains indicated optimal activity at 30 °C and pH between 5 and 6, regardless of the enzyme and strain studied. However, microbial loads of lactic and bacilli were highest at 35 °C for pH 6. Therefore, for to evaluate the performance of these strains in order to establish a scientific basis as dominant lactic ferments for fermentation of products to validate whether these biochemical characteristics are well expressed, these strains must be used to conduct the fermentation of cassava dough for the production of attieke.

## Conflict of interest

Authors have no conflict of interest regarding the publication of paper.

## Acknowledgements

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101217.

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