

Full Length Research Paper

Screening of microorganisms producing polygalacturonase (PG) in microbiota of fermented cassava

Sonagnon H. S. Kouhondé,^{1,2,3*} Marius K. Somda,² Innocent Y. Bokossa,³ Lamine S. Baba-Moussa,⁴ Frank Delvigne,¹ Alfred S. Traore² and Philippe Thonart¹

¹University of Liège, Faculty of Agricultural Sciences of Gembloux (FUSAGx) Bio Industry Unit, Centre Wallon de Biologie Industrielle (CWBI) Passage of Deportees 2, 5030 Gembloux, Belgique.

²University of Ouagadougou, UFR-SVT, Research Centre for Biologicals Food and Nutrition Sciences (CRSBAN), Laboratory of Microbiology and Biotechnology, 03 BP 7131 Ouagadougou 03, Burkina-Faso.

³University of Abomey-Calavi, Faculty of Sciences and Technology, Laboratory of Microbiology and Food Technology 01 BP 4521 Cotonou 01, Bénin.

⁴University of Abomey-Calavi, Faculty of Sciences and Technology, Laboratory of Biology and Molecular Typing in Microbiology.

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Polygalacturonases (PGs) are known for their application in the clarification and extraction of fruit juice and wine. In this study, PG activity was investigated in 125 strains of microorganisms comprising of bacteria and yeast previously isolated from the microbiota of fermented cassava. In order to investigate PG activity, these purified strains were grown on agar media containing apple pectin or polygalacturonic acid (PGA) at 0.5 to 1%. A screening test performed at the end of cultivation revealed PG activity in each producing strain, allowed the detection of *Saccharomyces cerevisiae* KSY4, *Saccharomyces cerevisiae* KSY5, *Saccharomyces cerevisiae* KSY9, *Bacillus* sp. KSB30 and *Bacillus* sp. KSB32, which were identified using phenotypic test. Screening assays carried out on Petri dishes showed limitations, the depolymerization reactions were carried out using two substrates (pectin and PGA) at different concentrations with each extract from strain isolated which exhibited PG activity. The results show that at pH 5 at 30°C/25 min, *Bacillus* sp. KSB32 exhibited a PG production of 2.33 U/ml on pectin (5 g/l) versus 1.35 U/ml on PGA. An increase in PG production, reaching 3.24 U/ml was concomitant with the pectin concentration. These results conclude that PG activity is function of nature and substrate concentration.

Keywords: Cassava, *Bacillus* sp. KSB32, *Saccharomyces cerevisiae* KSY4, fermentation, polygalacturonase (PG).

INTRODUCTION

Polygalacturonases (PGs) are enzymes used in industries for many applications. In agro-industries they are used for the clarification of juices, coffee fermentation, and extraction of oils or retting of fibers (VidhyaSagar et al. 2013). However, some undesirable activities have been observed during the production of PG using a commercialized *Apergillus* species (Bekhouche et al. 2006; Segla et al. 2009). This is one of the reasons motivating research into PG in other species

such as *Saccharomyces*, *Rhodotorula*, *Candida*, *Trichoderma viride* (BITRS-1001) and *Bacillus* (Alimardani-theuil et al. 2011; Juwon and Ogunmolu Emmanuel 2011; Rehman et al. 2013). Hence, the detection of PG activity in microorganisms has been extensively studied; the most widely known studies were carried out on the mould species, *Aspergillus* (Sandri et al. 2011; Anisa et al. 2013; Esawy et al. 2013; Fontana et al. 2005) and yeasts (Gainvors et al. 2000; Alimardani-theuil et al. 2011). Among the more recent studies, the optimisation of strain performance for enzyme production was greatly improved using a physical mutagenic agent (UV irradiation), chemicals (colchicine), (Akbar et al. 2013)

*Corresponding author: Email: kouhondes@hotmail.fr

and the Surface Methodology Response (SMR), (Gonçalves et al. 2012). Substrates such as corn flour and wheat have also been tested in order to optimize production (Palaniyappan et al. 2009). These studies highlight the interest in manipulating the genome of microorganisms to optimize production rather than altering environmental parameters in order to induce significant enzyme production. However, very few studies have been focused on PG activity in lactic acid bacteria. Some authors (Sakellaris et al. 1988; Karam and Belarbi 2005; Bekhouche et al. 2006) identified *Lactobacillus* species as potentially producing PG. However, all these authors note that PG production by this species is low (1U/ml of 48h culture at 30°C) (Bekhouche et al. 2006), although the literature mentions high proportions (30 U/ml) which were obtained from the same species by other authors (Sakellaris et al. 1988).

The microflora of cassava consists of many species including, *Penicillium*, *Lactobacillus*, *Weisella*, *Saccharomyces*, *Pichia*, *Kluyveromyces*, *Candida* etc. (Huch et al. 2008; Segla et al. 2009), and the objective of this work is to detect microorganisms that develop PG activity in this flora.

MATERIALS AND METHODS

Microbial and Plant Material

The microbial material employed in this study concerned of 125 strains of bacteria and yeasts isolated from the microbiota of fermented cassava during work undertaken at Laboratory of Microbiology and Biotechnology of CRSBAN (University of Ouagadougou). The plant material including cassava roots, has freshly harvested in the province of Banfora approximately 450km from Ouagadougou capital of Burkina Faso.

Composition of Media used for Microbial Screening

Two types of media were used: a medium containing apple pectin (Sigma 76282) and a second medium in which the pectin was substituted by PGA (Sigma P3850). The bacteria media composed per litre: peptone 10 g; meat extract 5 g; yeast extract 5 g; K₂HPO₄ 5 g; Na₂HPO₄ 3 g; sodium acetate 5 g; diammonium hydrogen citrate 2 g; CaCO₃ 4 g; (NH₄)₂SO₄ 2 g; Tween80 1 ml; MnSO₄ 0.1 g; MgSO₄ 0.1 g; pectin / PGA 0.5-2% and glucose 0.1-0.5%; the yeast medium comprised of: peptone 10 g; yeast extract 10 g; pectin / PGA 0.5-2% and 20 g of glucose.

Sterilization of the pectin or PGA was performed in a water bath at 65°C for 15 min before being incubated for 24 h at 30°C (method developed by (Campos 1993; Ciza 2001)); this operation (heating - incubation) was repeated three times.

Highlighting the Polygalacturonase Activity on Petri Dishes

Inoculation of the media screen was carried out from the liquid culture of each strain. 2 µl of culture of each strain was taken and placed onto an agar media of pectin (0.5%) containing 0.25% glucose. Cultures were incubated at 30°C for 72 h for the bacteria and yeasts; a solution of potassium iodide (8 g I₂ and 4 g of KI in 1 liter of water) was then spread over the whole surface of the cultures. Pectin hydrolysis is shown by the appearance of a halo around the strains identifying a positive test response. The diameter of the halo surrounding each PG producing strain is proportional to the amount of PG synthesized in the medium.

Production of Polygalacturonase Extracts by Strains

Preparation of Pre-cultures

Pre-cultures were grown in flasks containing YPD medium (1% yeast extract, 1% peptone and 2% glucose) for the yeast and Nutrient Broth for the bacteria. They were inoculated from a fresh subculture; incubation was carried out using an orbital shaker at 30°C for 24 h.

Culture

Cultures were inoculated with a pre-culture volume equivalent to 10% of the volume of the culture medium (the composition of the culture medium is the same as that of the screening medium, but without agar and glucose). Incubations were performed at 30°C for 48 h with orbital shaking.

Determination of Certain Growth Parameters during Polygalacturonase Production in Flasks

Optical Density (OD) and pH of the Cultures

The optical density of the cultures was followed during the fermentation, which lasted 48 hours, and for the pre-cultures of each strain. OD was determined using a spectrophotometer (VWR V1200) at 600 nm; the change in pH throughout the whole process was followed using a pH meter (Hanna pH 209). As soon as the electrode was introduced into the culture, the pH increased immediately (Juwon et al. 2012).

Biomass

The dry matter method was used. At the end of the fermentation process, 5 ml of culture of each strain was taken and filtered through Wattman paper of 45µm

diameter. The cake culture was rinsed with distilled water and then oven dried at 105°C. To negate the mass of filter paper, the wet mass was found and determined after 24 h oven drying, followed by cooling the drying solids in jars for 15 minutes. The biomass (mg/ml culture) is the mass of completely dehydrated microorganisms in a culture (JuwonOgunmolund Emmanuel 2011).

Determination of Polygalacturonase Produced by each strain

After 48 h of fermentation, the cultures were centrifuged at 10,000 g for 15 min and the supernatant containing the crude enzyme was collected and stored at 4°C for the implementation of enzymatic reactions (Rehman et al. 2013).

Implementation of Depolymerization Reactions

The enzymatic extract from each strain was mixed with solutions of pectin or PGA, whose pH was adjusted to 5, at concentrations ranging from 5, 10 to 20 g/l. The reaction took place in a water bath at 30°C for 60 min; the enzyme reaction was stopped by being placed into another bath previously set at 100°C for 10 min.

Determination and calculation of polygalacturonase activity

The PG activity assays were performed using the DNS method (3,5-dinitrosalicylic acid) and the absorbance was read using a spectrophotometer at 540 nm (Rehman et al. 2013). Indeed, after the action of PG on pectin substrates an increase in PGA degradation products were observed into the medium. Galacturonic acid was one of the most predominant molecules; therefore, D galacturonic acid (Sigma, 73960) was chosen as a standard. One unit activity of PG is defined as the amount of enzyme required to liberate 1 µmol of galacturonic acid per minute, depending on the specific conditions (pH and temperature) of the reaction.

PG activity (U / ml) = $\Delta OD \cdot d1 \cdot (1/p) \cdot (1/v \cdot t)$

ΔOD : optical density difference between the analyzed sample and the control

p: slope of the calibration curve

d1: dilution factor of the enzyme extract

v: volume of the enzyme extract

t: incubation time

Phenotypic characterization of strains producing polygalacturonase

The PG-producing microorganisms, isolated and purified on suitable media as described in previous research (Amoa-Awua et al. 1996; Louembe et al. 2002; Darman

et al. 2005), were observed under a microscope. Before proceeding to microscopic observation, the colonies were freshly sub-cultured using a sterile platinum loop, a colony of each strain was deposited onto a slide, along with a drop of sterile water, to observe their morphology using a microscopy (at x40, Zeiss Primostar, Germany). Bacterial colonies were stained using the Gram method and the color of the walls was observed under the microscope at x100 to distinguish them. These strains underwent the catalase test and were again cultured for 48 h to determine their profile; their fermentative capacity to degrade sugars was determined using the API 20CAux kit for yeasts and API 50CHB for bacteria strains. The results were noted after the visual observation of galleries and analyzed in the database ApiwebTM (BioMerieux).

STATISTICAL ANALYSIS

The values representing mean ± Standard Deviation (SD), values were considered statistically significant if the P value was less than or equal 0.05. Comparisons among the different groups were carried out by ANOVA tests using Minitab (statistical package; V16.0).

RESULTS AND DISCUSSION

Evolution of growth parameters during fermentation

The evolution of growth parameters during the production of polygalacturonase (PG) by different strains was monitored during the experiments, which lasted 48 hours. Before the beginning of PG production, the pH of the pre-cultures was adjusted to 5.0 for solutions of pectin and polygalacturonic acid. After culturing for 24 h, an increase in pH was noted both in the medium containing pectin as well as the medium supplemented with PGA (Figure 2). The increase in pH was observed for all strains in both media except KSB32 strain, where a decrease in pH from 7 to 6.2 was observed between 24 h and 48 h, while the pH was 5.3 for the same species in pectin medium. This observation was made by Mokemiabeka et al. (2011) during the fermentation of cassava leaves. The noted increase in pH during cultivation (pectin and PGA) is due to the production of NH₄⁺ in the medium.

In addition, other parameters allowed monitoring of the process; the growth of the strains was followed throughout PG production by measuring the optical density of the cultures (Figure 3). With an optical density of the pre-cultures between 0.5 and 1.0 at the start of cultures, we noted a decrease in the OD of the solutions containing pectin, whereas with acid solutions containing PGA an increase in OD was observed. This variation showed that strains degrade PGA more easily than pectin. However, the difference in growth observed ($\geq \Delta OD$ about 1.5) between 24 h and 48 h in cultures containing pectin was very high for all strains. Conversely, for cultures containing PGA there was a small increase in the OD after the same culture duration; hence, 48h into the fer-

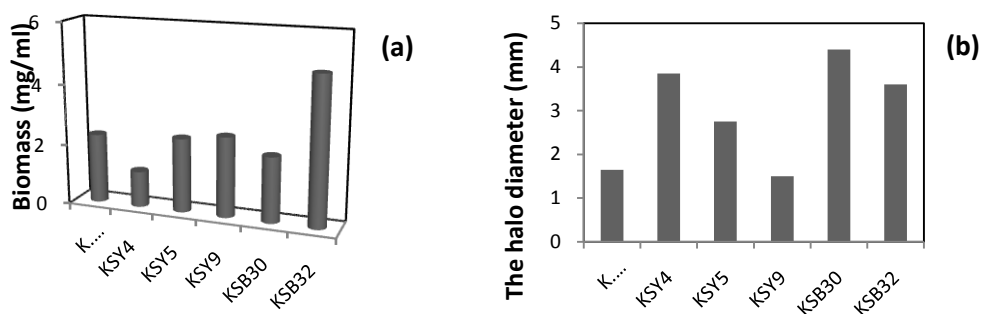


Figure 1. Change in crop biomass in PGA medium 5 g/l after 48 h at 30°C (a) and the halo diameter of each strain in pectin media 5 g/l after 72 h at 30°C (b).

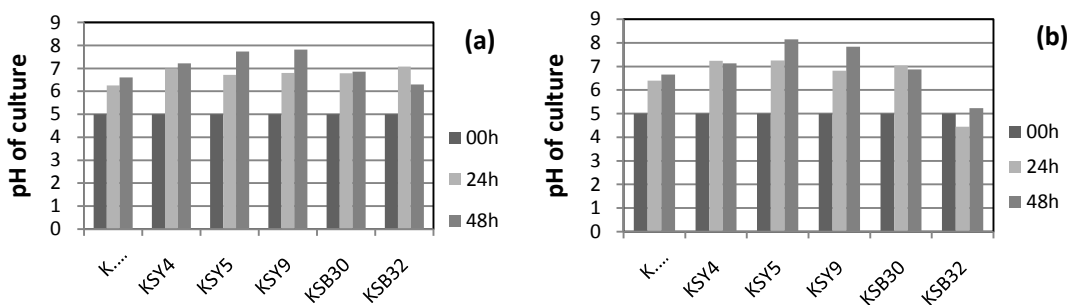


Figure 2. Evolution of pH of the culture medium of each strain on pectin (a) and PGA (b) medium respectively 5 g/l, 30°C for 48 h.

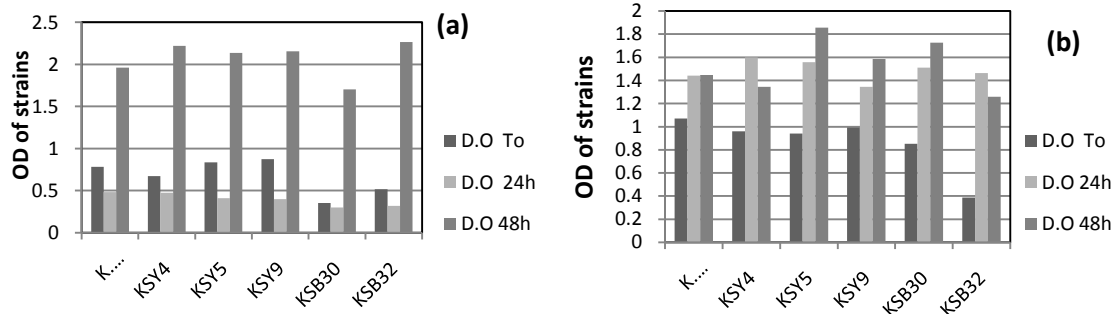


Figure 3. Evolution of OD for 6 strains in a pectin medium (a) and PGA medium (b) respectively, 5 g/l, pH 5 and 30°C for 48 h

mentation, the pectin substrate became more assimilable by the strains. In addition, the difference in OD observed between 24 and 48 h in the medium containing pectin, could be due to the complex structure of the apple pectin molecule (highly methylated), as in these experiments the substrates were used as only carbon source. This increase in OD is related to the large amount of PG produced by strains in a medium containing pectin. For more complex molecules, the microorganism will produce sufficient enzyme to degrade the substrate. Note the

high OD values recorded at the end of the cultures indicate the smooth production by each strain.

Detection of Polygalacturonase activity in some Strains isolated from fermented cassava

Among the media used for the detection of PG in isolates, we observed with pectin that the diameter of the halo obtained after the screening test was larger and easier



Photo: Detection of polygalacturonase activity on a pectin medium.

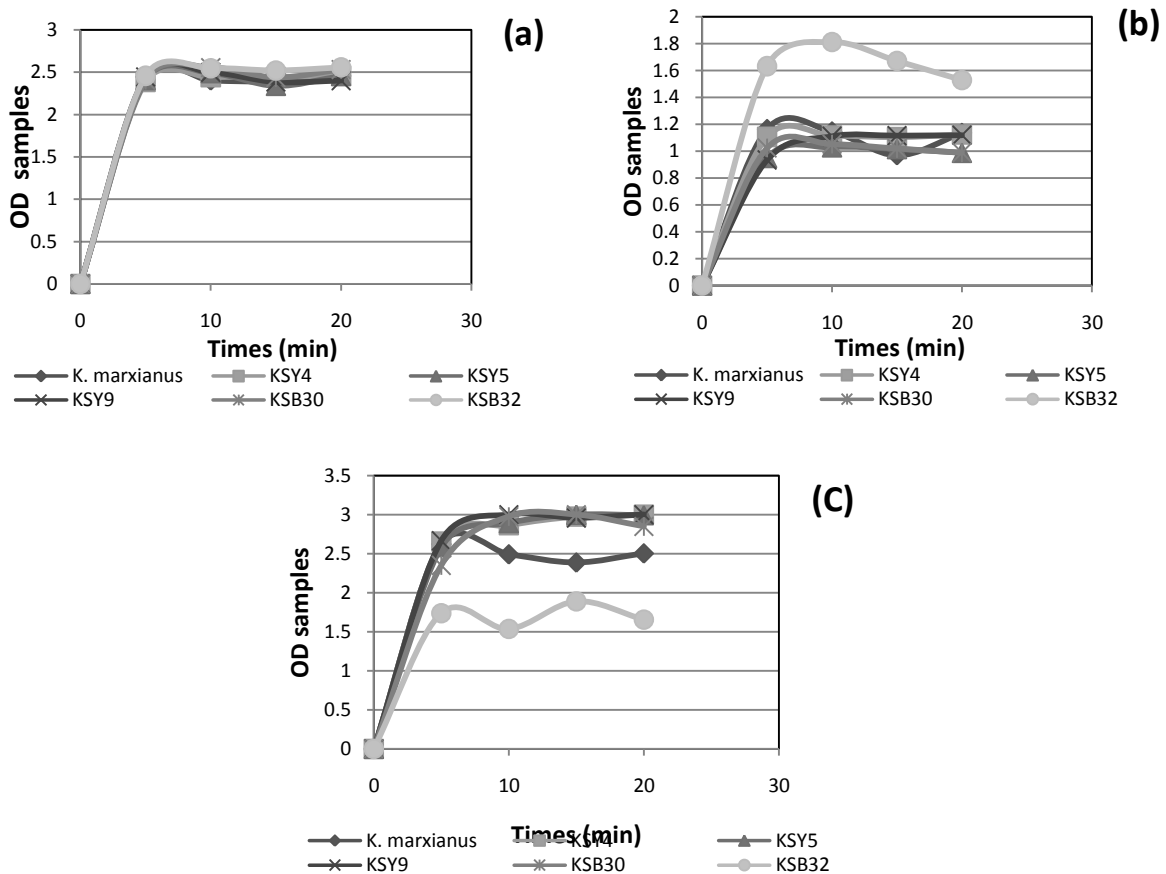


Figure 4. The OD of sample showed kinetic degradation of pectin substrates (a) and polygalacturonic acid (b) 10 g/l and polygalacturonic acid 20 g/l (c), respectively, at pH 5, 20 min at 30°C for reaction with the enzyme extracts of producing strains.

to see than on the medium containing PGA; these observations were also made by Campos 1993 and Ciza 2001. This difference is related to the structure of these two molecules. Furthermore the monitoring of substrates degradation kinetics (Figure 4) showed that all strains are of the same profile in a solution containing 10 g/l of pectin. By against the KSB32 strain was alone in exhibited better growth with polygalacturonic acid at the same concentration.

PG production by microorganisms is the subject of much controversy. Some authors argue that all species of bacteria don't produce PG (Amoa-Awua et al. 1996; Kaur et al. 2004; Segla et al. 2009; Alimardani-theuil et al. 2011). Nevertheless, PG could be detected in bacteria by other authors and the results of their work showed the possibility of obtaining PG from these microorganisms (Sakellaris et al. 1988; Karam and Belarbi 2005; Bekhouche et al., 2006).

Table 1. Change in PG (U/ml) activity producing by strains after 25 min of incubation according to the pectin concentration at pH 5, 30°C.

[Pectin] g/l	<i>K. marxianus</i>	KSY4	KSY5	KSY9	KSB30	KSB32
5	1.52±0.05a	2.13±0.05b	1.44±0.01	1.39±0.04	2.47±0.00c	2.34±0.06d
10	3.10±0.00	3.34±0.01	3.15±0.02	3.12±0.02	3.30±0.01	3.24±0.00

Value represented are in format means±SD. *K. marxianus* (yeast, used as control), KSY4, KSY5 and KSY9 are yeasts; KSB30 and KSB32 are bacteria; (b), (c) et (d) significant compared to control (a) (P≤0.05)

Table 2. Change in PG (U/ml) activity producing by strains after 25 min incubation according to the polygalacturonic acid (PGA) concentration at pH 5, 30°C.

[PGA] g/l	<i>K. marxianus</i>	KSY4	KSY5	KSY9	KSB30	KSB32
5	0.63±0.01	0.81±0.01	0.65±0.03	0.56±0.02	0.63±0.05	1.35±0.08
10	0.94±0.00	0.88±0.02	0.82±0.00	0.91±0.01	0.60±0.02	1.98±0.02
20	3.61±0.07a	3.93±0.00b	3.93±0.23	3.93±0.14	3.83±0.11c	3.22±0.16d

Value represented are in format means±SD. *K. marxianus* (yeast, used as control), KSY4, KSY5 and KSY9 are yeasts; KSB30 and KSB32 are bacteria; (b), (c) et (d) significant compared to control (a) (P≤0.05).

It was shown in previous work that PG production is sometimes accompanied by the production of linamarase (Campos 1993).

However, none of the bacteria strains isolated from the flora of agbelima, derived from the fermentation of cassava roots (Amoa-Awua et al. 1996), produced PG; while these microorganisms are linamarase producers.

Polygalacturonase activity of each PG Producer Strain

PG activities were initially followed by measuring the halo diameter (Figure. 1b) using the photo detection test (see photo). Besides this test, depolymerization reactions were implemented between the various solutions and enzyme samples of each strain at 30°C for 25min, and was used to assess the production of PG by the detected strains (Tables 1 & 2). The amount of PG recorded in all strains, using the same substrate, showed that any increase in substrate concentration was followed by an increase in PG (Tables 1 & 2) activity. In addition, at the same concentration (10 g/l) of substrate, higher values of PG activity were recorded with pectin. We deduce that PG activity is strongly influenced by the structure and concentration of the substrate.

The species cultivated in this study, at a concentration of 5g/l, showed that the bacteria produced PG like yeasts. Thus, for the KSB32 strain, an equivalent of 2.339 U/ml was obtained versus an average production of 1.624U/ml for yeast (Table 1) obtained under the same conditions. This places the KSB32 strain among the most productive, which justifies the amount of biomass obtained for KSB32 in the late cultures (Figure. 1a).

Morphological Identification and phenotypic strain Characterization

Microbial PG-producing by colonies was observed by mi-

croscopy (at x40, Zeiss Primostar, Germany) revealed that strains KSY4, KSY5 and KSY9 are rounded cells with a distinct nucleus, grouped or not. The biochemical sugar uptake test performed using API 20C Aux (BioMerieux, France) identified KSY4 as belonging to the species *Saccharomyces cerevisiae* KSY4; KSY5 and KSY9 were identified as belonging to *Saccharomyces cerevisiae* KSY5 and *Saccharomyces cerevisiae* KSY9, respectively. The physiological characteristics of bacteria (KSB30 and KSB32) showed that both strains are Gram-positive, catalase positive and oxydase negative. Examination of their shape and arrangement using a microscope at x 40 showed KSB30 cells arranged in clusters and KSB32 cells were rod-shaped, rounded, sometimes mobile, isolated or arranged in clusters. The fermentation profile of each of these strains was then determined using an API50 CHB (BioMérieux, France) kit before being identified as belonging to the *Bacillus sp. KSB30* and *Bacillus sp. KSB32*, respectively. Both species also degrade other sugars, e.g., arabinose, cellobiose, sucrose and starch. However, KSB30 strain is capable of hydrolysing ribose and xylose. Given their ability to ferment at least one sugar of five carbon atoms such as xylose, arabinose and ribose, these strains would be considered optional heterofermentative bacteria.

This study shows that the flora of retting cassava contains microorganisms which produce pectinolytic enzymes. From a microbial consortium initially consisting of 125 strains, we were able to detect 5 strains which produced polygalacturonase (PG) with 3 yeasts (*Saccharomyces cerevisiae* KSY4, *Saccharomyces cerevisiae* KSY5, *Saccharomyces cerevisiae* KSY9) and 2 bacteria (*Bacillus sp. KSB30* and *Bacillus sp. KSB32*), identified using phenotypic tests.

After screening tests on Petri dishes, cultures performed in liquid medium allowed us to assess the abil-

ity of each strain to produce PG. Thus PGA and pectin media were used at concentrations of 5 to 20 g/l at pH 5 and incubated at 30°C for 25 min.

This experiment confirmed that these strains actually produce PG and shows us that the substrate concentration and nature greatly influence the activity of PG strains.

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