

Original article

Microbiology

Growth and α -amylase production by strains of *Lactobacillus plantarum* and *Rhizopus oryzae* cultures in cassava starch medium

Francois-Xavier ETOA¹, Roger D. DJOULDE^{2*}, Jean-Justin ESSIA NGANG¹¹Department of Biochemistry University of Yaoundé I- Cameroon²Institute of Agricultural Research for Development of Cameroun, Maroua, P.O Box: 33 Maroua- Cameroon

*Corresponding author: Tel. (237) 986-17-64, Email: djoulde@gmail.com

ABSTRACT

A cassava starch medium was used to analyse the dynamics of batch growth and α -amylase production of strains of *Lactobacillus plantarum* and *Rhizopus oryzae* isolated from cassava dried chips. The strains displayed a growth of 0.5h^{-1} and 0.55h^{-1} , a biomass yield on cassava starch of 0.49g/g and 0.5g/g , a maximum specific cassava starch uptake rate ($\mu_{\text{m}}/\text{Yx/s}$) of $2.98\text{mmol g}^{-1}\text{h}^{-1}$ and $3.21\text{mmol g}^{-1}\text{h}^{-1}$, a maximum oxygen uptake rate of $14.7\text{mmol g}^{-1}\text{h}^{-1}$ and $12.94\text{mmol g}^{-1}\text{h}^{-1}$, an oxygen efficiency of growth of 0.565 and 0.58 , a carbon recovery of 1.035 and 0.97 , and an energy recovery of 1.0 and 0.95 respectively. Enough α -amylase enzymes were synthesized, and cassava starch hydrolysis was not a limiting factor for growth of both strains. These results suggest the possibility to use these strains for developing a dual starter culture for cassava fermentation, and α -amylase production from cassava starch medium.

Keywords: Cassava, α -amylase, *Lactobacillus plantarum*, *Rhizopus oryzae*, starter culture

RESUME

Un milieu à base d'amidon de manioc a été utilisé pour évaluer la dynamique de la croissance et de production d' α -amylase des souches de *Lactobacillus plantarum* et de *Rhizopus oryzae* isolés des cossettes de manioc séché. Les résultats obtenus ont montré respectivement un taux de croissance de $0,5\text{h}^{-1}$ et de $0,55\text{h}^{-1}$, la productivité en biomasse (Qx) sur amidon de manioc respective de $0,49\text{g/g}$ et $0,5\text{g/g}$. Le taux spécifique de dégradation d'amidon de manioc ($\mu_{\text{m}}/\text{Yx/s}$) obtenu est de $2,98$ du $\text{mmol g}^{-1}\text{h}^{-1}$ et $3,21\text{mmol g}^{-1}\text{h}^{-1}$, alors que la demande spécifique en oxygène ($q\text{O}_2$) étaient respectivement de $14,7$ du $\text{mmol g}^{-1}\text{h}^{-1}$ et $12,94\text{mmol g}^{-1}\text{h}^{-1}$. L'efficacité d'utilisation d'oxygène pour la croissance de $0,56$ et de $0,58$. La chaleur spécifique de fermentation (qf) était respectivement de $1,035$ et de $0,97$ et les rapports chaleur spécifique sur demande spécifique en oxygène en fonction du taux de dilution de $1,0$ et de $0,95$. L'hydrolyse de l'amidon de manioc n'était pas un facteur limitant pour la croissance des deux microorganismes. Les résultats obtenus ici suggèrent la possibilité d'utiliser ces souches pour développer un ferment mixte destiné à la fermentation du manioc, et la production d' α -amylase à partir du milieu amidon de manioc.

Mots clés: Manioc, α -amylase, *Lactobacillus plantarum*, *Rhizopus oryzae*, ferment

INTRODUCTION

Cassava (*Manihot esculenta crantz*) and cassava-based products are amongst important food crops in Africa and are major source of starch for millions of peoples in the tropics [1]. It is established that during cassava fermentation, certain lactic acid bacteria and Yeast predominate [2]. It has also been reported that some of those micro-organisms are able to produce amylases [4]. These amylases could convert starch into monosaccharides and later to lactic acid, alcohol,

aldehydes, and many other components which are able to contribute to improve organoleptic qualities of cassava based products [2, 3]. Due to high concentration of starch in cassava (more than 80% of dry matter) [5], the selection of micro-organisms capable of metabolising starch is essential for developing cassava based "babies foods" leading, to increase nutritional and economical value of this crop. This paper aims at describing growth kinetics and amylase production by strains of *Lactobacillus plantarum* and

Rhizopus oryzae isolated from fermented cassava dried chips with potential to further utilization as starter culture for cassava retting with the scope of energy foods and sugar production and production of biomass from Cassava starch based media.

MATERIAL AND METHODS

Isolation and identification of microorganisms

About 1 Kg of fermented cassava chips were collected and divided in 100g parts. Each part was mixed with about 900 ml of sterile 0.1 % peptone water to form a solution. Serial dilutions were then made from solution and plated. The lactic acid bacteria were isolated on de Man Rogosa Sharpe (MRS) agar as described by Sharpe *et al.*, [6] mould were plated on Potatoes Dextrose Agar (PDA). All isolations were carried out at 30°C for 24 to 48 hours. Isolates were picked randomly from MRS and PDA plates, subcultures have been provided in 10ml tubes. Cells were washed two times in physiological water by centrifugation at 2500 rpm for 10 minutes. Isolates were then subjected to physiological and biochemical tests [7] and identification method based mainly on following: Microscopic and macroscopic examination, mobility and spores, catalase test, Gram stained, growth temperatures, homofermentative and heterofermentative character, the thiamine requirement for growth, fermentation of different carbon sources (API 50 CH N° 5030, strip, biomerieux Charbonière, Bains France). *Lactobacilli spp.* was taxonomically classified following the discriminatory schemes of Kandler and Wiess [8], Hammes *et al.* [9], and Larpent et Larpent-Gouraud, [10]. Moulds and Yeast were identified according the "Bergey's manual" [7].

Screening for α -amylase producers

This was as described by Kéléké *et al.* [11]. Soluble starch replaces glucose in MRS and Potatoes Dextrose Agar (PDA). 1% Chloramphenicol at 0.05g/l, as bacteria inhibitors was added to PDA-starch medium. Before being inoculated, 2 ml of a solution of aniline bleu (25g/l) were added to both media and sterilised at 120°C for 20 minutes. Plates were then inoculated and incubated at 30°C for 24-48 hours. Plates were then stained with a solution containing 0.33% of

iodine and 0.66% of potassium iodine. Halos were finally screened.

Culture conditions

Strains were cultured in a 7 liter Bioreactor (Biolafite France) at 30°C and agitated at 200 rpm. The used medium was cassava based medium described by Monica *et al.* [14] contained per liter, Cassava starch, 20g; MgSO₄.7H₂O, 0.6g; MnSO₄, 0.3g. The pH was adjusted to 6.0 by adding NaOH (5 M). Inoculation at 10% V/V was performed with a 18 hours precultured stater of previously isolated amyolytic strains.

Amylase assay

One hundred millilitre of cultured medium were collected every 24 h and centrifuged at 2500rpm. The supernatant was then removed aseptically. The α -amylase activity was measured by incubation 0.1 ml of appropriately diluted supernatant with 0.8 ml of a solution containing 2% of soluble starch in 0.1 mol/l citrate buffer (pH 5.5) at 55°C. The reaction was stopped by adding 0.1ml of 1 mol/l H₂SO₄. After incubation, residual starch contents were determined with a spectrophotometer (Spectronic Geneysis) after different periods at 620 nm by adding 0.1 ml of the reaction mixture to 2.4 ml of an iodine solution (containing 30 g/l of KI and 3g/l of I₂) and diluted with distilled water to 4%. The α -amylase unit is defined as the amount of α -amylase that permits the hydrolysis of 10mg of starch in 30 min under the conditions described above.

Biomass assay

The biomass was followed by the classical numeration on agar medium method. Samples were taken at 24 h intervals under aseptic conditions. One millilitre of each sample was placed into 9ml of sterile 0.1% peptone water to form a solution. Serial dilutions were then made from the solution and plated. *Lactobacillus* counts were on de Man Rogosa Sharpe (MRS) agar incubated under anaerobic conditions (BBL Gas Pak). *Rhizopus oryzae* count were determined on Potatoes Dextrose Agar (PDA) containing 1% Chloramphenicol at 0.05g/l. *Lactobacillus plantarum* were incubated at 30°C for 24 h and *Rhizopus oryzae* at the same temperature for 4-5 days.

Additional measured parameters

Carbon and reduce oxygen (energy) balances were evaluated as describe by Solomon, and Erickson [12]; and Stouthamer and Verselved [13].

The oxygen efficiency of growth or energetic Yield coefficient (η_o) has been used to characterize energy flow during microbial growth.

RESULTS AND DISCUSSION

Characteristics of micro-organisms

Characteristics of isolated micro-organisms are presented in table 1.

Table 1: Phenotypic characteristics of isolated Strains.

Source of isolation		Cassava fermented and dry chips	
Isolation medium		MRS (Man Rogosa Sharp)	PDA (Plate count-agar)
Morphology		Gram + Bacilli	
Motility		Non mobile	
Catalase		-	
Lactic acid		L(+)	DL
Growth at 15°C		-	+
Growth at 25°C		±	+
Growth at 45°C		-	+
Enzymes	α-amylase	+	+
	β-glucosidase	-	+
	Pectine méthylesterase	+	-
	Pectate lyase	+	+
	Pectin lyase	+	+
Carbon Sources	Esculin	+	+
	Amygdalin	+	+
	Arabinose	-	-
	Cellobiose	+	+
	Galactose	-	+
	Glucose	+	+
	Lactose	+	+
	Maltose	+	-
	Mannitol	-	-
	Sucrose	+	-
	Tréhalose	-	+
	Raffinose	+	-
	Rhamnose	+	-
	Ribose	-	+
Xylose	+	+	
Orientation	<i>Lactobacillus plantarum</i>	<i>Rhizopus oryzae</i>	

NB: Biochemical tests (carbon sources) are given according to API 50 CH pattern of sugar fermentation.

Effect of medium composition on growth of microorganisms.

In the initial stages of this study, several different media were tested with both microorganisms. The type of starch employed had a striking effect on both the rate and extent of growth (Fig 1 and 2)

Cassava starch proved to be a better substrate for *Rhizopus oryzae* than other starch use in this study. Growth on starch medium yielded a characteristic growth curve with an initial phase gradually accelerated until a rapid exponential phase was attended for the two strains (Fig 1 and 2).

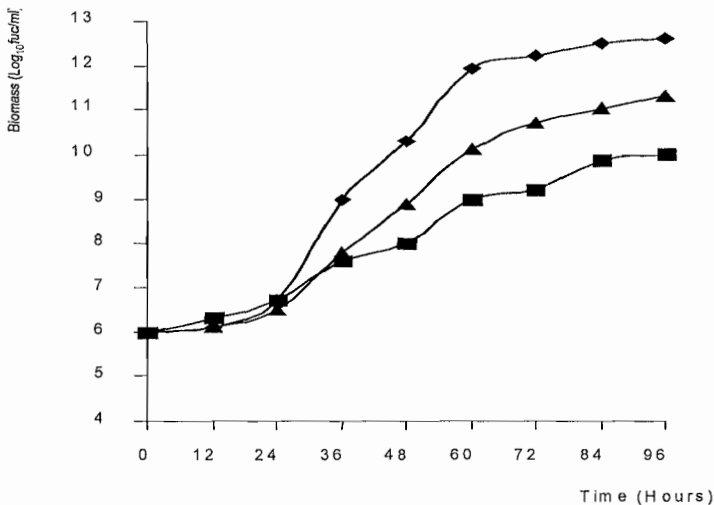


Figure 1: Effect of different Media [Cassava starch (◇), Potatoes Starch (■) and Sigma Starch (▲)] on growth of *Rhizopus oryzae* isolated from cassava dried chip.

Growth kinetics on Cassava starch based medium showed for strains, a monoxic growth and a very short adaptation phases (about ¼ generation which could be correlated to adaptation for the new carbon sources). The onset of the stationary phase coincides with the exhaustion of the cassava starch from the medium. The respiratory quotient during the exponential growth phase was between 1.10 and 1.16 for the two strains. Typical macroscopic growth parameters for the strains tested are indicated in Table 2. Carbon and redox (energy) balances were calculated using the concepts of C-mol and reductase degree, considering biomass and carbon dioxide to be the only fermentation products and assuming a standard biomass composition [12, 13]. Both balances fell within 95% confidence intervals. The oxygen efficiency of growth or energetic Yield coefficient (η_o) has been used to characterise energy flow during microbial growth, because it is approximately equal to the fraction of substrate energy incorporated into biomass.

Yield of biomass of *Rhizopus oryzae* were higher than those obtained with *Lactobacillus plantarum* in the same medium and this could be related to the growth conditions used in the reactor, particularly the pH [6] which favours mould, and the absence of some nutrients such as vitamins, aminoacids..., which are necessary for bacteria growth as pointed out by Antier *et al.* [15], and Fiecher [16], the deficiency or excess trace element or vitamins influence microbial growth.

The metabolic pattern observed confirms that the starch fermenting yeast belongs to the so-called Crabtree-negative or aerobic respiring microorganisms. When oxygen is supplied in excess, these organisms could display a purely oxidative metabolism irrespective of the growth rate [17]. This behaviour may result from an efficient balance between the rate of sugar transport into the cell, the transport rate of glycolytic intermediates into the mitochondria (respiratory capacity) and the rate of consumption of these intermediates in anabolic flux prevents an overflow reaction at the point of pyruvate, thus avoiding ethanol formation [16, 18].

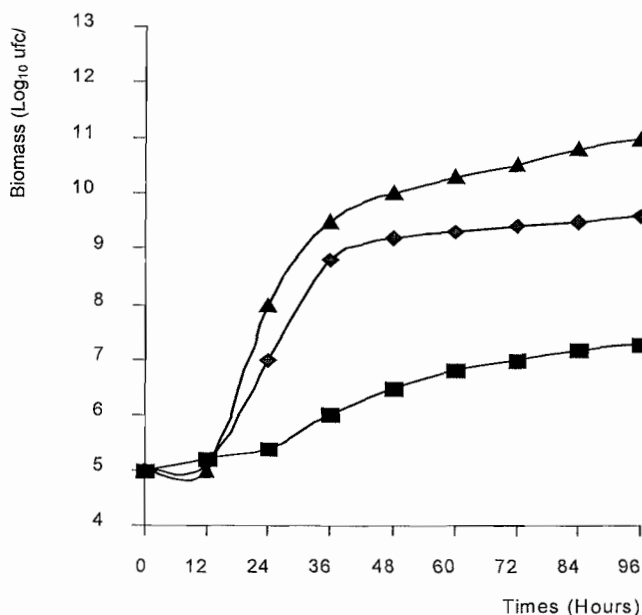


Figure 2: Effect of different types Cassava starch (◇), Potatoes Starch (■) and Sigma Starch (▲) Medium on growth of *Lactobacillus plantarum* isolated from cassava dried.

For the strains tested, the average specific rate of cassava starch consumption during the exponential growth phase was respectively $3.21 \text{ mmol g}^{-1} \text{ h}^{-1}$ for *Rhizopus oryzae* and $2.98 \text{ mmol g}^{-1} \text{ h}^{-1}$ for *Lactobacillus plantarum* (Table 2). This was equivalent to the uptake of $6.42 \text{ mmol g}^{-1} \text{ h}^{-1}$, and $5.96 \text{ mmol g}^{-1} \text{ h}^{-1}$ respectively, of which 2.2 mmol and 2.6 mmol have theoretically been catabolized through respiration. Comparing these results with data on the maximum biomass yields, and metabolic fluxes of some common Crabtree-negative microorganisms, noted in literature [19], we find a similar kinetic and stoichiometric potential for aerobic metabolism in these microorganisms. These microorganisms had a similar or higher capacity for cassava starch conversion than the maximum rates observed for starch consumption in aerobic batch cultures. This situation could also be observed in other Crabtree-negative microorganisms [20]. It thus looked likely that in this case, sugar transport was not the rate-limiting step in growth. The

discrepancy between the data remains unclear as some reports have shown a high rate of ethanol formation in batch cultures of *Saccharomyces diastaticus* and *Rhizopus oryzae*, in cassava starch based medium [21] he noted that about 30% of starch was converted into ethanol, which resulted in low yield values of biomass on this sugar. Several hours of deceleration phase, associated with maximum ethanol productivity, and diauxic type growth have also been observed. Bales and Castillo [22] reported a decrease in the biomass yield with increasing whey concentration with *Candida pseudotropicalis* 744. This suggested that cultures were not carbon-limited. Although data on dissolved oxygen were not available for those cultures, the use of media with high cassava starch concentration and culture systems with insufficient oxygen transfer rates, such as shake flasks or poorly aerated fermenters, supported the view that they were oxygen-limited.

Table 2: Aerobic batch culture parameters of *Lactobacillus plantarum* and *Rhizopus oryzae* grown in Cassava based synthetic medium.

(μ_m (h⁻¹): Maximum specific growth rate calculated from dry weight measurements, Yx/s (gg⁻¹): Overall yield of Biomass on cassava starch, q_s: Maximum specific cassava starch uptake rate (μ_m /Yx/s), q_{o2}: Maximum oxygen uptake rate (average of experimental measurements over the exponential growth phase), η_o : Oxygen efficiency of Growth, CR: Carbon recovery, ER: Energy recovery)

Strain	μ_m (h ⁻¹)	Yx/s (gg ⁻¹)	q _s (mmol g ⁻¹ h ⁻¹)	q _{o2} (mmol g ⁻¹ h ⁻¹)	η_o	CR	ER
<i>L. plantarum</i>	0,50±0,05	0,49±0,08	2,98±0,72	14,7 ±1,26	0,565±0,023	1,035±0,05	1,0±0,061
<i>R. oryzae</i>	0,55±0,07	0,5±0,025	3,210±1,02	12,94 ±1,27	0,58±0,037	0,97±0,029	0,95±0,036

Dynamic of α -amylase formation

A common feature of α -amylase formation could be observed in both cultures of *Rhizopus oryzae* and *Lactobacillus plantarum* (Fig 3).

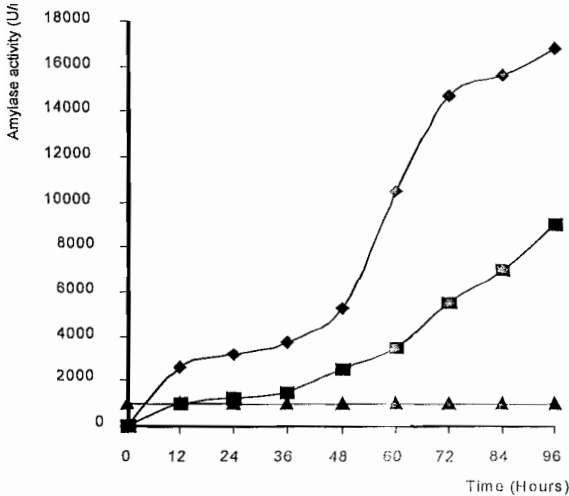


Figure 3: Evolution of α -amylase activity of *Lactobacillus plantarum* (\diamond) and *Rhizopus oryzae* (\blacksquare) during batch culture in cassava starch medium.

During the exponential growth phase the specific activity is constant and increase 1,3-1,8 fold during the brief deceleration phase (Table 3). Net enzyme increased with the exhaustion of the biomass. Total activity remained constant at least 24 hours after beginning of stationary phase in the

case of *Lactobacillus plantarum* when it began with the exponential phase for *Rhizopus oryzae*, indicating that that enzyme was stable and no degradation occurred when cells entered this phase. We could also notice that, so long as exponential growth was maintained, the rate of enzyme synthesis remained constant and the specific activity is unchanged both for *Lactobacillus plantarum* and *Rhizopus oryzae*. The medium cassava starch content was initially around 1% in the media and change to 0.01% after 84 hours of fermentation for both microorganisms (Fig. 5), this indicated bio transformation of cassava starch during batch culture. Submerged fermentation tended to reduce carbohydrate, protein and mineral contents of cassava roots [23]. Fermentation also caused a reduction in starch content, while the total soluble and reducing sugar levels were increased during the first 24 hours and 36 hours respectively; sugars are reduced during the latter periods of fermentation due to utilization by microorganisms and the conversion of sugars into organic acids (data not reported). The rate of cassava starch hydrolysis is greater than the uptake rate, leading to 0.0045mg/ml residual starch, during the 48 hours of fermentation for *Lactobacillus plantarum* and 0.0015mg/ml after the same time for *Rhizopus oryzae*, thus hydrolysis of starch are not a limiting factor. The presence of amylase microorganisms in lactic fermentation of cassava has already been reported [24]. Regez et al., [25] also isolated numerous *Lactobacillus plantarum* strains but did not report any amylolytic strains.

Table 3: α -amylase levels of different yeasts during batch cultures in cassava starch medium.

	α -amylase activity (U/ml)		R
	Exponential growth phase ^a	Stationary growth phase	
<i>Lactobacillus plantarum</i>	4300 \pm 390	7400 \pm 90	1,72
<i>Rhizopus oryzae</i>	9800 \pm 102	15800 \pm 570	1,61

^aAverage of experimental measurements over the exponential growth period.

R: Ratio of stationary to exponential activity.

Studies have been reported on the solid state fermentation of cassava [26, 27, 28]. In this study, the spectrum of microorganisms implicated in the fermentation of grated cassava root for the production of gari was similar to those we found with lactic acid bacteria and yeasts dominating the latter periods of the process. Moulds found were

Rhizopus spp., *Mucor spp.*, *Penicellium spp.* and *Fusarium spp.* In the same way, a succession trend was established among the lactic acid bacteria, with *Lactobacillus plantarum* being predominated during the last 36 hours of submerged fermentation [28].

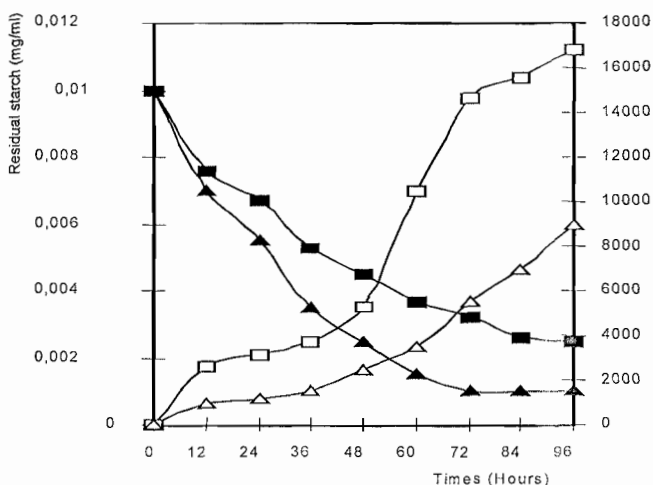


Figure 4: Biomass evolution of *Lactobacillus plantarum* (■) and *Rhizopus oryzae* (▲) and α -amylase production by *Lactobacillus plantarum* (□) and *Rhizopus oryzae* (Δ) during batch culture in cassava starch medium

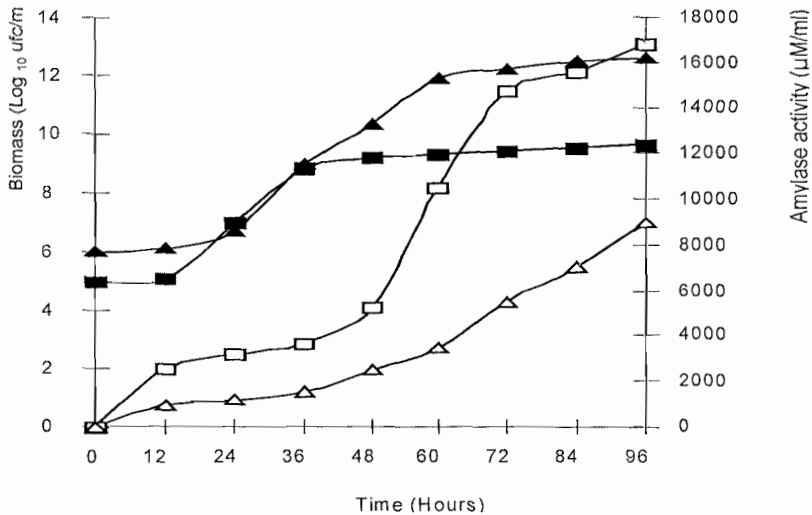


Figure 5: Starch degradation by strains of *Lactobacillus plantarum* (■) and *Rhizopus oryzae* (▲), and α-amylase production by *Lactobacillus plantarum* (□) and *Rhizopus oryzae* (Δ) during batch culture in cassava starch medium.

In this work natural amylolytic strains of *Lactobacillus plantarum*, and *Rhizopus oryzae*, have been isolated from cassava dried chips. Those strains are of particular interest, not only for their taxonomy, but also for their capacity to develop rapidly and massively in cassava starch based media. Further investigation could be conducted in the view of developing a dual starter culture for cassava fermentation since those two strains had hydrolytic action on cassava starch, the different microorganisms could have specific and complementary roles during cassava fermentation.

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