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Isolation and physiological study of an amylolytic strain of *Lactobacillus plantarum*

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Summary. An amylolytic lactic acid bacterium identified as *Lactobacillus plantarum* was isolated from cassava roots (*Manihot esculenta* var. Ngansa) during retting. The amylolytic enzyme synthesized was an extracellular α-amylase with an optimum pH of 5.0 and an optimum temperature of 55° C. Cultured on starch, the strain displayed a growth rate of 0.43 h^{-1}, a biomass yield of 0.19 $g \cdot g^{-1}$ and a lactate yield of 0.81 $g \cdot g^{-1}$. The growth kinetics were similar on starch and glucose. Sufficient enzyme was synthesized and starch hydrolysis was not a limiting factor for growth. Biosynthesis of the enzyme was observed when the glucose concentration was less than 6.7 $g \cdot l^{-1}$ and reached up to 4 $IU \cdot ml^{-1}$ at the end of the fermentation.

Introduction

The lactic microflora plays an important role in the preparation of traditional foods based on fermented cassava (gari, chikwangue, foo-foo) (Regez et al. 1988; Okafor et al. 1984). However, the function of this microflora in the preservation of foods, the detoxification of cyanide compounds and the improvement of organoleptic qualities has not yet been perfectly elucidated. Artisanal methods are still used for manufacturing these foods (Muchnik and Vinck 1984). As the fermentation stage occurs naturally with epiphytic lactic microflora, the quality of the food products is not very well standardized. The mass inoculation of cassava roots with one or several selected strains would enable better control of natural fermentation and to orientate it towards the production of a better-quality, standardized product, hence the interest of better knowledge of the nature and function of this epiphytic microflora.

As cassava consists mainly of starch (over 80% dry matter; Ketiku and Oyenuga 1970), the selection of a lactic acid bacterium capable of metabolizing starch appears to be particularly important. It should be noted that the capacity of lactic acid bacteria to convert starch into lactic acid is not a frequent characteristics. Only species of *Streptococcus bovis, S. equinus, Lactobacillus amylophilus, L. amylovorus, L. acidophilus, L. cellobiosus,* (and others isolated from animal digestive tracts and plant wastes) have been described in the literature as amylolytic lactic acid bacteria (Cotta 1988; Champ et al. 1983; Nakaruma and Crowell 1979; Nakaruma 1981; Sen and Chakrabarty 1986; Sneath 1986). There is practically no information on the physiology of these microorganisms.

The present article describes the isolation and identificaton of a new amylolytic lactic acid bacterium from cassava roots during retting. The physiology of the bacterium and the properties of the amylase produced were also investigated.

Materials and methods

Origin of the plant material. Cassava roots (*Manihot esculenta* var. Ngansa) were harvested in the Brazzaville area 15 months after planting.

Isolation and identification of strains. Peeled roots were immersed in rain-water. Sampling was carried out 4 days after fermentation by random selection of six roots cut into 0.5-cm cubes and mixed under sterile conditions. Sixty grams was sampled and diluted in 540 ml sterile peptone solution. Decimal dilutions (0.1 ml) were spread on JP2 medium (see below) in Petri dishes. After incubation for 48 h at 30° C, dishes were exposed to iodine vapour to detect starch hydrolysis areas. Strains isolated were then purified by three successive transfers on JP2 medium and cultures were routinely checked for purity by microscopic observation. Microorganism identification was based on the following examinations: (1) configuration of the lactic acid produced using an enzymatic method (Ivorec-Szylit and Szylit 1965) with dehydrogenase L and D (Boehringer, Mannheim, FRG), (2) homolactic or heterolactic character, determined by acetic acid or ethanol production; (3) absence of catalase, (4) microscopic and macroscopic examination of morphology, mobility and spores, (5) Gram stain, (6) arginine deamination, (7) growth at 15° and 45° C, (8) fermentation of different carbon sources (API 50CH no. 5030 strips, Biomérieux, Charbonnieres les Bains, France). Evaluation of results and

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identification of the different strains were carried out using Bergey's Manual (Sneath 1986).

Strains and culture media. Three strains were used as reference: *L. plantarum* (Lacto Labo, Dange, St. Romain, France), *S. equinus* CNCM 103233 and *L. amylophilus* CNCM 102988T.

The JP2 medium contained ($g \cdot l^{-1}$): M66 universal peptone, 2.5; soya peptone obtained by papain digestion, 5; casein peptone obtained by pancreatic digestion, 2.5; yeast extract, 5; meat extract, 2.5; $MgSO_4 \cdot 7H_2O$, 0.1; NaCl, 3; $(NH_4)_2SO_4$, 2; K_2HPO_4, 0.2; Prolabo (Paris, France) soluble starch, 3; Tween 80, 0.4 ml. The pH was adjusted to 6.75 before sterilisation.

Physiological studies were performed using an MRS basal medium (de Man et al. 1960) by changing the carbon sources as follows: (a) 5% glucose, (b) 5% starch and (c) 3% of starch plus 2% glucose.

Culture conditions. Strains were cultured in a 2-l bioreactor (Biolafitte, Poissy, France) at 30° C and agitated at 200 rpm. The pH was adjusted to 6.0 by the addition of NaOH 5 N. Inoculation at 10% v/v was performed with a 20-h pre-culture in the same medium used for fermentation.

Analytical methods. The biomass concentration was determined by measurement of optical density (OD) at 540 nm related to the dry weight measured after two washing and centrifugation cycles and drying at 105° C for 24 h. For starch cultures, hydrolysis of residual starch was performed using a mixture of amylases (thermamyl + dextrosyme supplied by Novo). The dry weight and OD were then determined as above. Lactic acid, glucose, acetic acid and ethanol concentrations in the supernatant were assayed by HPLC. Compounds were separated by using an Aminex HPX 87H column (BioRad, Richmond, Va., USA) with a 0.8 $ml \cdot min^{-1}$ flow (pump LDC 3200) of 0.006 M H_2SO_4 at 65° C. Analyses were carried out by a refractive index detector (PU 4026 Philips, Eindhoven). Total sugars in media containing starch were also determined with anthrone using the method of Dubois et al. (1956).

Determination of amylase activity. Enzymatic extracts were prepared by ultrafiltration of culture supernatant using a Diaflo PM 10 membrane (Amicon, Danvers, USA). Enzymatic activities were then assayed by adding 0.1 ml enzymatic extract to 0.8 ml of a solution containing 1.2% of soluble starch (Prolabo) in 0.1 M phosphate buffer, pH 6.0. The reaction was stopped by addition of 0.1 ml of 5 M NaOH. After incubation for 10 min at 40° C, the increase in reducing power was determined using the method of Miller (1959). One enzyme unit is defined as the amount of enzyme that releases one micromole of reducing power equivalent per minute under the conditions described.

Characteristics of the enzyme. The effect of pH on the enzyme activity was studied in a 2.5–8.0 pH range using a 0.1 M citrate-phosphate or 0.1 M TRIS-HCL buffer. Enzymatic activity was measured under standard conditions (see above), except for variation of the buffer. The enzymatic activity profile according to temperature was determined within a 10–80° C temperature range under standard conditions.

Results

Isolation and identification of L. plantarum A6

Seven amylolytic microorganisms were isolated on JP2 medium from retted cassava roots. Two of them were selected for their capacity to produce lactic acid from starch (revealed by HPLC). Morphological, physiological and biochemical characteristics of these two microorganisms are presented Table 1. The ability of these cultures to utilize 49 different carbohydrates was studied using API 50CH no. 5030 strips. The results were compared, using a computer, with the percentage positive reactions of different *Lactobacillus* spp. as per API: 99.9% of similarity with *L. plantarum* was observed and hence these cultures were identified as strains of *L. plantarum*. The two strains A6 and A43, which displayed precisely the same sugar degradation profiles, are probably similar.

The amylolytic activities on JP2 medium of *L. plantarum* A6, *S. equinus* and *L. amylophilus* indicated that the starch hydrolysis zone was largest for *L. plantarum* A6. It was therefore selected for further studies.

L. plantarum A6 growth kinetics

Figures 1, 2 and 3, represent growth kinetics, sugar consumption, lactic acid and amylolytic enzyme production by *L. plantarum* A6 cultured on different media as indicated in Materials and methods. The main fermentation parameters are shown in Table 2. It was observed (Table 2) that the growth of *L. plantarum* A6 on glucose MRS medium was fully comparable with that of *L. plantarum* (Lacto Labo). The growth rate ($0.43\ h^{-1}$) and biomass productivity ($0.75\ g \cdot l^{-1} \cdot h^{-1}$) were slightly lower than those of the standard (Lacto Labo) strain, but the biomass and lactate yields were practically identical. The strain thus does not seem to display specific nutritional requirements in comparison to the

Table 1. The characteristic of *Lactobacillus plantarum* strains A6, A43 and Lacto Labo

Strain	A6	A43	Lacto labo
Ratio of D/L-lactic acid	(69/31)	(66/34)	(73/27)
Homolactic	+	+	+
Catalase	−	−	−
Bacterium shape	Short rod	Short rod	Short rod
Gram stain	+	+	+
Spore	−	−	−
Mobility	−	−	−
Deamination of arginine	−	−	−
Growth at 15° C	+	+	+
Growth at 45° C	−	−	−

+, positive; −, negative

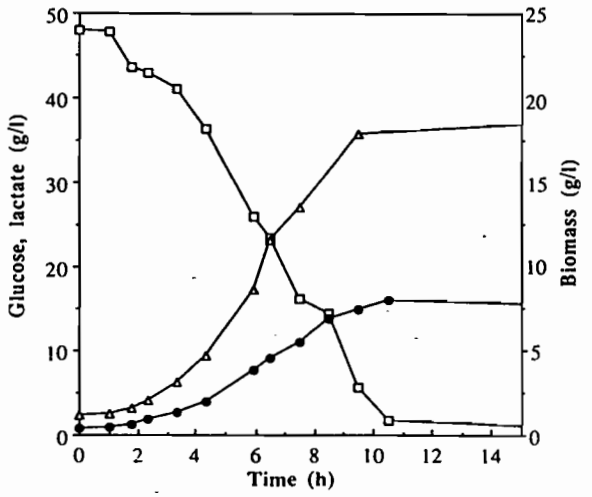

Fig. 1. Fermentation of *Lactobacillus plantarum* A6 on glucose MRS medium at 30° C and pH 6.0: □, glucose; △, lactic acid; ●, biomass

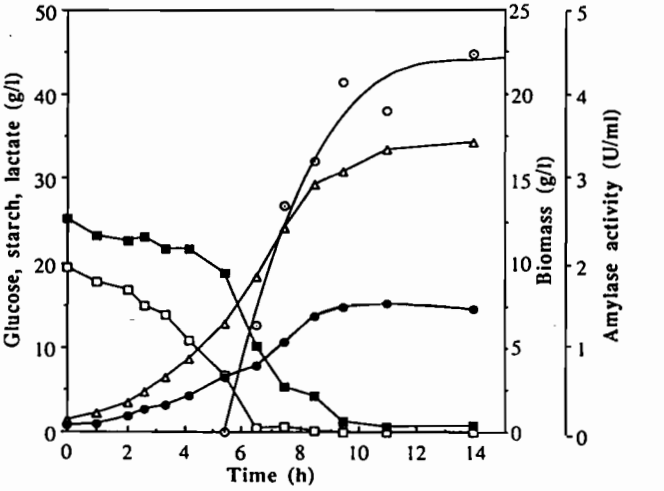

Fig. 3. Fermentation of *L. plantarum* A6 on starch + glucose MRS medium at 30° C and pH 6.0: □, glucose; ■, starch; △, lactic acid; ●, biomass; ○, amylase activity

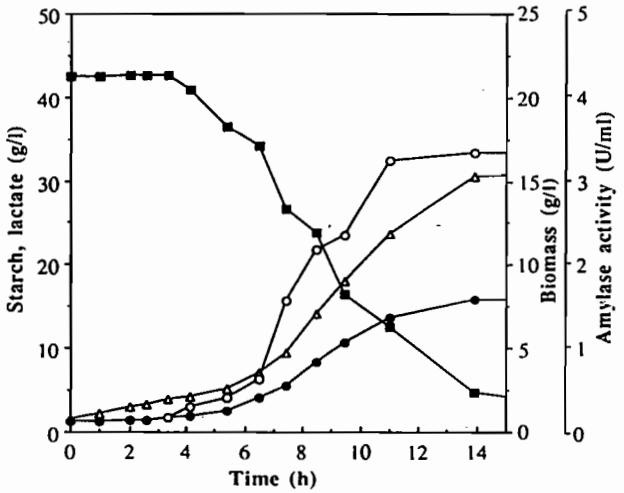

Fig. 2. Fermentation of *L. plantarum* A6 on starch MRS medium at 30° C and pH 6.0: ■, starch; △, lactic acid; ●, biomass; ○, amylase activity

common strain, which makes it possible to envisage mass production.

On starch MRS medium (Fig. 2), the strain exhibited the same kinetic profiles and the same yields. A level of 3.3 $IU \cdot ml^{-1}$ of enzyme had been synthesized at the end of fermentation; nearly 50% was synthesized during the decreasing phase of growth. The rate of starch hydrolysis was greater than the uptake rate, leading to a 3 $g \cdot l^{-1}$ maltose peak during the 7th hour of fermentation (result not shown). Hydrolysis of starch is thus not a limiting factor.

The kinetics on starch + glucose MRS medium (Fig. 3) indicate that the rate of starch breakdown is very low and amounted to about 14% at the end of 4 h fermentation. Also, no α-amylase was formed up to this stage, thereby indicating that the enzyme present in the inoculum was responsible for the starch breakdown up to 4 h of the fermentation. It thus appears that α-amylase was not formed as long as the glucose concentration remained at about 6.7 $g \cdot l^{-1}$. Extremely rapid uptake of starch was then observed and it correlated well with considerable synthesis of amylase. Growth kinetics showed a constant rate and rapid adaptation to change of substrate. Amylase concentration at the end of the fermentation (4.3 $IU \cdot ml^{-1}$) was distinctly higher than that observed on starch MRS medium.

The residual starch concentrations at 14 h in starch MRS and starch + glucose MRS media were different; 4.71 $g \cdot l^{-1}$ in the former as against 0.9 $g \cdot l^{-1}$ in the latter medium. These difference are probably related to initial starch concentration in the media and the presence of readily utilizable glucose in the latter medium. The rate of biomass formation was comparatively slow in starch MRS medium in the initial stages of fermenta-

Table 2. Fermentation parameters of *L. plantarum* (Lacto Labo) and *L. plantarum* A6 cultured on different media at pH 6.0 and 30° C

Strain	Medium	μ (h^{-1})	$Y_{x/s}$ ($g \cdot g^{-1}$)	$Y_{p/s}$ ($g \cdot g^{-1}$)	Productivity ($g \cdot l^{-1} \cdot h^{-1}$)
L. plantarum (Lactolabo)	MRS (glucose, 50 g/l)	0.57 ± 0.03	0.22 ± 0.03	0.75 ± 0.04	1.05
L. plantarum A6	MRS (glucose, 50g/l)	0.43 ± 0.03	0.19 ± 0.02	0.81 ± 0.10	0.78
L. plantarum A6	MRS (starch, 50 g/l)	0.45 ± 0.03	0.23 ± 0.03	0.75 ± 0.10	0.63
L. plantarum A6	MRS (starch 30 g/l + glucose 20 g/l)	0.41 ± 0.06	0.19 ± 0.02	0.74 ± 0.06	0.71

tion. For example, the biomass formation at 4 h was 0.4 and 2 $g \cdot l^{-1}$ in starch MRS and starch + glucose MRS media, respectively. The α-amylase formation was initiated at 2.5 h in starch MRS medium and its rate of formation was very low up to 6 h. In contrast, the enzyme was not detectable up to 6 h in starch + glucose MRS medium. It is interesting to note that the residual starch concentration was 0.9 $g \cdot l^{-1}$ at 23 h in starch MRS medium.

Characterization of the amylolytic enzyme

Comparison of the HPLC profiles after starch hydrolysis by the cell-free extract and commercial amylolytic enzymes (*Aspergillus oryzae* α-amylase, Sigma A0273; potato β-amylase, Sigma A7005 and *A. niger* amyloglucosidase Sigma A3514; Sigma, Poole, Dorset, UK) was performed to characterize the amylolytic activity exhibited by *L. plantarum* A6. Under these conditions, the main products of starch hydrolysis analysed by HPLC were glucose from amyloglucosidase, maltose from β-amylase and a mixture of glucose, maltose and oligosaccharide (retention time of 5.2 min) from α-amylase. The breakdown profiles of starch by the enzyme from *L. plantarum* A6 was similar to that of α-amylase, thereby indicating that the enzyme synthetised by *L. plantarum* A6 is an extracellular α-amylase.

Effect of pH on enzymatic activity

Enzymatic activity was tested within the pH range 2.5–8.0 (Fig. 4). Maximum enzyme activity was observed at pH 5.0. Seventy percent of the activity was still observed at pH 4.0, but at pH 2.5 and 8.0 the enzyme was almost completely inactivated.

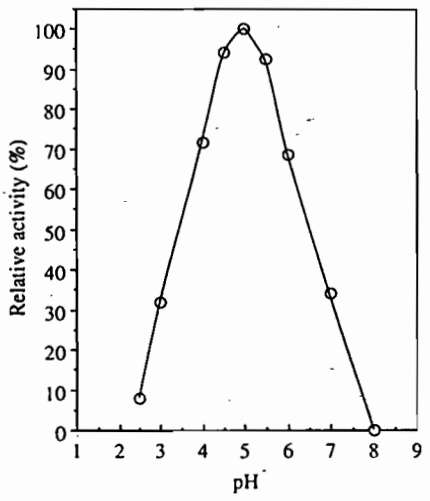

Fig. 4. Influence of pH on the amylase activity of *L. plantarum* A6 at 40° C

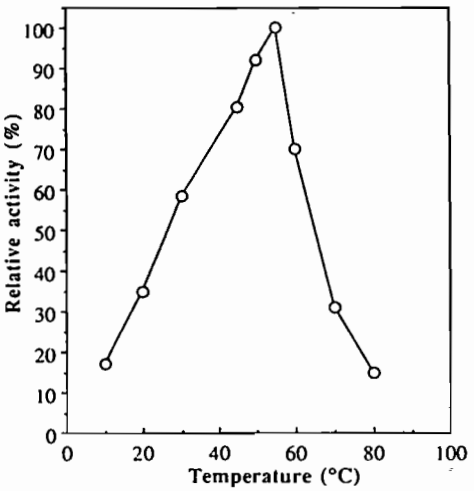

Fig. 5. Influence of temperature on the amylase activity of *L. plantarum* A6 at pH 6.0

Effect of temperature on enzymatic activity

The influence of temperature on the amylase activity tested within the range 10–80° C is presented in Fig. 5. The optimal temperature was 55° C under the conditions studied and 60% activity was still observed at 30° C.

Discussion

The presence of amylase in lactic acid bacteria has been already reported. However, to the best of our knowledge, no author has described any strains of amylolytic *L. plantarum*. Investigating the bacteria microflora of fermented cassava tubers, Regez et al. (1988) isolated numerous *L. plantarum* strains, but no amylolytic strains were reported. Recent studies (Scheirlinck et al. 1989) were carried out to integrate the α-amylase gene of *Bacillus stearothermophillus* in the genome of an *L. plantarum* strain. However, the expression, stability and competitiveness of the transformed strain in a natural medium remain to be verified.

In our work, a natural amylolytic strain of *L. plantarum* was isolated from cassava roots. The enzyme synthesized was identified as an α-amylase on the basis of HPLC analysis. However, a further purification stage would be necessary to ensure that this activity is not associated with other amylases (amyloglucosidase and β-amylase). Nevertheless, it is to be noted that only α-amylase activity has been described in the literature for amylolytic lactic acid bacteria.

Investigation of the properties of the enzyme indicated its extracellular and acidotolerant characteristics. Thus nearly 70% of the enzyme activity remains at pH 4.0, (a state attained after the 3rd day of retting (Okafor et al. 1984). These properties appear to be different from those observed in other amylolytic lactic acid bacteria. Indeed, the extracellular character was observed in *L. cellobiosus* (Sen and Chakrabarty 1986), but the

temperature and pH activity profiles of the enzyme synthesized by this microorganism are quite different to those of *L. plantarum* A6. In contrast, two *Lactobacillus* amylolytic lactic acid bacteria isolated from a chicken crop by Champ et al. (1983) have comparable profiles but the amylase synthesized by these two microorganisms were cell-linked.

The use of this strain can be envisaged in several fields of application. The high biomass yield and the high growth rate on starch mean that the microorganism could be used in starter cultures to improve the conservation and stability of starch-based fermented foods. The particularly high starch/lactic acid conversion yield (80%) also suggests the possibility of industrial lactic acid production from starch residues, though more investigation will be needed to evaluate its commercial viability. Since it has been shown that *L. plantarum* is capable of energy uncoupling (Giraud et al. 1991), this would simplify the procedure proposed by Kurosawa et al. (1988) in which a co-immobilized mixed culture system of *A. awamori* and *S. lactis* was proposed.

The data reported here would thus appear to show that this new lactic acid bacterium is of particular interest not only from a taxonomic point of view but also for its capacity to develop rapidly and massively in starch-based media. Further research will therefore be undertaken to purify the enzyme for better characterization of its biochemical properties, to monitor growth of this bacterium on raw starch and to determine the real improvement in quality and stability of foodstuffs resulting from mass inoculation of cassava tubers with the strain.

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