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Degradation of Raw Starch by a Wild Amylolytic Strain of *Lactobacillus plantarum*

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***Lactobacillus plantarum* A6, isolated from fermented cassava, can break down cassava raw starch that has not been subjected to preliminary physicochemical treatment. When the pH was kept at 6, the microorganism cultured in a bioreactor excreted a high α-amylase activity (60 U/ml). Synthesis of the enzyme occurred during the stationary phase and resulted in full hydrolysis of the cassava starch granules. This gave 41 g of lactic acid from 45 g of raw starch after 3 days of fermentation. Enzymatic attack was evident under scanning electron microscopy in the rougher appearance of the surface of starch granules and in the presence of large cavities in some of them. In contrast, when the pH was not regulated, only a small amount of α-amylase activity was produced (2 U/ml) and no decrease in the starch content of the medium was observed. However, under scanning electron microscopy, some granules displayed a rougher surface, which might have been the result of weak enzymatic attack.**

Lactobacillus plantarum is a lactic acid bacterium common in numerous natural fermentation processes, such as those of silage, cabbage, cucumber, olive, cassava, etc. According to McDonald et al. (15), its ability to maintain a pH gradient between the inside and the outside of the cell in the presence of a large amount of acetate or lactate may explain why the bacterium can withstand acidified media and completes these fermentation processes. It converts low-molecular-weight sugars almost quantitatively into lactic acid, thus contributing to organoleptic qualities and the preservation potential of fermentable products. The bacterium is recommended as a starter culture in many cases for fermentation control and to obtain products of even quality (4, 16, 21, 23, 25, 27).

In the case of silage, the quantity of fermentable sugar is sometimes too small to ensure rapid production of a stabilizing amount of lactic acid. This can be overcome by the addition of other sources of fermentable carbohydrates, such as molasses, whey, or starch combined with α-amylase (26). Scheirlink et al. (22) proposed the use of the strain *L. plantarum* transformed by electroporation with plasmids containing the α-amylase gene of *Bacillus stearothermophilus* and of the endoglucanase of *Clostridium thermocellum*. However, use of such a genetically engineered strain under natural fermentation conditions may raise legal or ecological problems.

A wild strain of *L. plantarum* (strain A6) was recently isolated from retted cassava and selected for its ability to break down soluble starch (8). This was the first description of an *L. plantarum* amylolytic strain, and it was found that it synthesizes large amounts of extracellular α-amylase. A more detailed investigation of this enzymatic activity was carried out because of the original features of the microorganism (9). However, all of these studies were performed with soluble starch, whereas in nature, starch is found in a crystalline insoluble form which makes it less available to enzymatic hydrolysis. Work was therefore carried out to determine the capacity of the strain *L. plantarum* A6 to break down raw starch. This information would be useful in evaluating the potential for the utilization of *L. plantarum* A6 as a starter in certain traditional fermentation processes.

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MATERIALS AND METHODS

Organism. The microorganism used was *L. plantarum* A6 isolated from retted cassava (8). The strain was conserved in glycerol at −80°C.

Medium and culture conditions. The basal liquid nutrient medium contained 10 g of soy peptone obtained by papain digestion, 0.5 g of K_2HPO_4, 0.5 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $MnSO_4 \cdot H_2O$, 0.5 g of $CaCl_2 \cdot 2H_2O$, and 1,000 ml of distilled water. The medium was sterilized at 121°C for 20 min, cooled to about 30°C, and mixed with the starch. The latter was obtained from roots of cassava plants (*Manihot esculenta* cv. Ngansa) harvested in the Brazzaville region 15 months after planting. Two types of starch were prepared. (i) Dry-heated starch was prepared as follows. The roots were peeled, diced, frozen at −80°C, and freeze-dried for 48 h. The flour obtained by grinding and sieving, with a 5% moisture content, was autoclaved (121°C, 20 min). (ii) Raw starch was prepared as follows. The roots were peeled and ground with a food mixer. The resulting pulp was sieved to remove fibers and homogenized with the basal liquid nutrient medium. The media thus prepared were transferred to a previously autoclaved bioreactor. The strain was cultured in a 2-liter bioreactor (Biolafitte, Poissy, France) at 30°C and agitated at 300 rpm. The pH was adjusted to 6.0 by the addition of 5 N NaOH. Inoculation of 0.01% (vol/vol) dry-heated starch and 10% raw starch was performed with a 20-h preculture. The medium used for the inoculum was identical to that of MRS medium (5) except that glucose was replaced by 2% soluble starch (Prolabo, Paris, France).

Estimation of the bacterial population. The bacterial population was monitored by use of a miniaturized most-probable-number method derived from that developed by Hernandez et al. (12) and adapted for counting lactic acid bacteria. The culture medium was API 50CHL medium (reference 50400; Biomérieux, Craponne, France) complemented with 10 g of glucose per liter. It was placed in 9-ml tubes and autoclaved

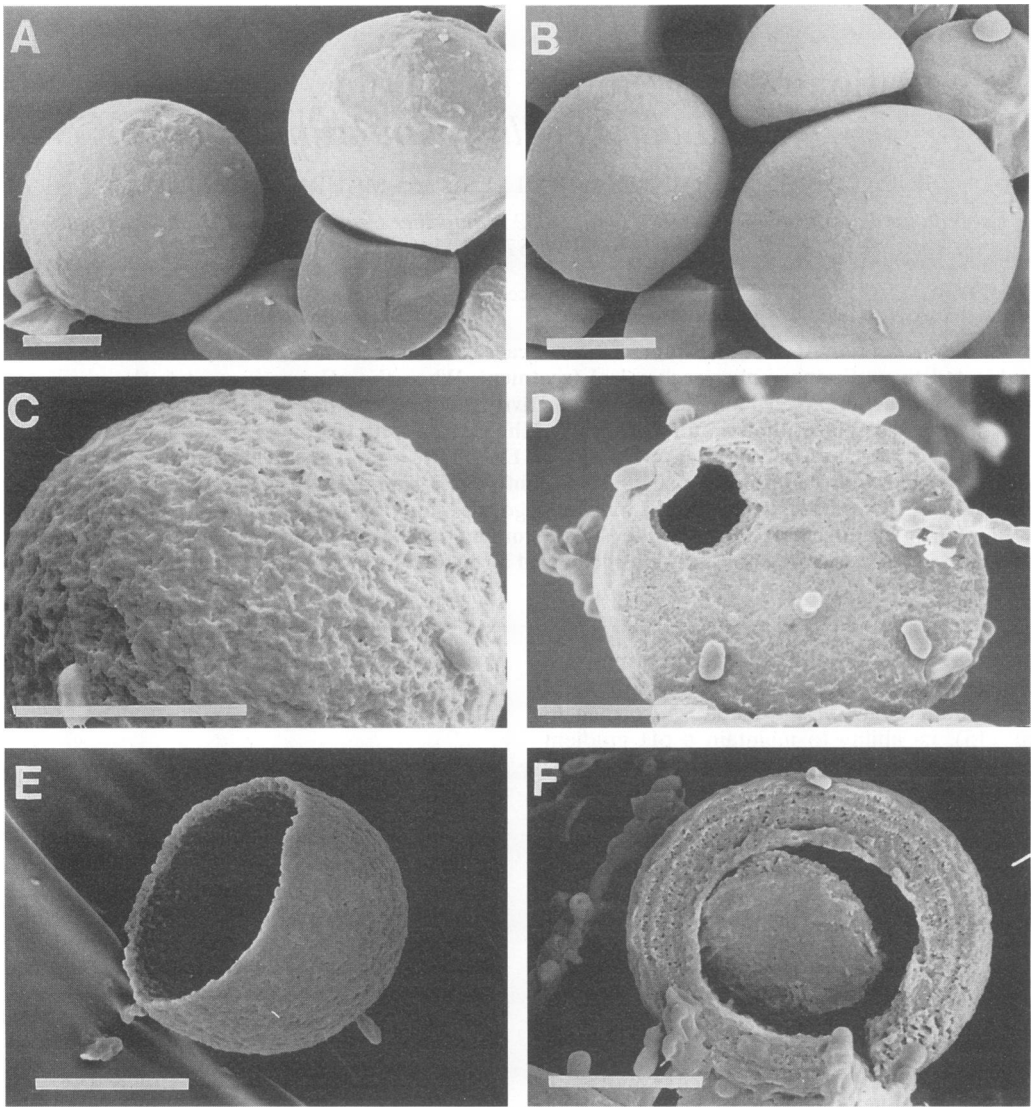

FIG. 1. SEMs showing the effect of *L. plantarum* A6 on cassava starch granules. (A) Dry-heated starch; (B) raw starch; (C, D, E, and F) raw starch granules after fermentation for 2 days with controlled pH. Bars, 5 μm.

(121°C, 20 min). Decimal dilutions of the sample to be counted were performed directly in the medium and placed on sterile microtitration plates (96 wells) at 200 μl per well and 12 wells per dilution. After incubation for 48 h at 30°C, the wells whose color had changed from purple to yellow were counted as positive. A computer program developed by Institut Pasteur, Lille, France (12), was then used to evaluate the most probable number of bacteria according to the number of positive wells.

Analytical methods. Raw starch contents were estimated by measurement of the dry weight obtained after two washing and centrifugation cycles and drying at 105°C for 24 h. Lactic acid was determined in the supernatant by high-performance liquid chromatography (HPLC) using an Aminex HPX 87H column (Bio-Rad Laboratories, Richmond, Calif.) with 0.006 M H_2SO_4 at a flow rate of 0.8 ml/min, at 65°C, and with refractive index detection.

Assay of α-amylase activity. α-Amylase activity was assayed by observing degradation of starch by measurement of its iodine-complexing ability. One enzyme unit is defined as the amount of enzyme that permits the hydrolysis of 10 mg of starch in 30 min under the conditions described by Giraud et al. (9).

Scanning electron microscopy. Sample preparation consisted of four steps: fixation by glutaraldehyde to prevent deformation of the structure, complete dehydration of the material, total removal of ethanol (dehydrating agent) by application of CO_2 at the critical point, and homogeneous gold metallization. The methodology used was similar to that reported by Blaha and Paris (1). SEM examination was performed with a JEOL JSM-6300F microscope (Université de Montpellier II).

RESULTS

Fermentation of *L. plantarum* A6 on dry-heated cassava starch. SEM observation showed that after treatment (freezing to −80°C, freeze-drying, and dry autoclaving), the starch granules had a crystalline appearance very similar to that of raw starch

granules. Although the granule surface was rougher than that of the raw starch granules (Fig. 1A and B), the preparation treatment did not appear to have caused considerable modification to the granule structure. However, the treatment reduced the endogenous microbial population from 10^5 to 10^1 bacteria per g of cassava flour. This reduced contamination risk and growth of *L. plantarum* A6 could be monitored by inoculating starch which had conserved its crystalline structure with a very low concentration of the bacterium (0.01%).

Figure 2 shows growth kinetics, starch degradation, and lactic acid and amylase production by *L. plantarum* A6 cultured in a fermentor on dry-heated starch with the pH set at 6. The initial starch (88 g/liter) was converted entirely into lactic acid (0.91 g/g of starch) after fermentation for 6 days. No latency phase was observed; microorganism growth occurred during the first 12 h of fermentation with a 1.1-h generation time. During this phase, the bacterial population changed from 3×10^5 to 1.5×10^{10} bacteria per ml and subsequently remained stable until the end of fermentation. Production of amylase, which was found in the culture supernatant, occurred at the end of the growth phase (90% of the amylase activity was formed during the first 8 h of the stationary phase). The cells then functioned as resting cells and continued to produce lactic acid until exhaustion of the substrate.

Fermentation of *L. plantarum* A6 on raw cassava starch. The inoculum size used in the trials was increased from 0.01 to 10% to achieve development of *L. plantarum* A6. Two parallel fermentation were performed, one with controlled pH (Fig.3) and one without pH control (Fig. 4). Although the starch had not been sterilized, no development of contaminant microorganisms was observed under optical microscopy, and HPLC did not reveal the production of any metabolite other than lactic acid. In both cases, the growth phase of *L. plantarum* A6 was short, being completed during the first 6 h. The final populations attained comparable levels. However, the differences in amylase activity were quite significant. Maximum amylase activity, 60 U/ml, was obtained with a controlled pH as compared with amylase activity of 2 U/ml with noncontrolled pH. It appeared that the pH should be kept at about 6.0 for bacterial cells to produce sufficient amylase activity to ensure the complete hydrolysis of raw starch granules. As before, amylase activity was produced principally at the end of the growing phase, enabling full conversion of raw starch granules to lactic acid. This gave 41 g of lactic acid from 45 g of raw starch after 3 days of fermentation. In fermentation without pH control, the culture pH rapidly fell below a level that the organism could tolerate. No amylase synthesis occurred during the stationary phase, and no noteworthy disappearance of starch was observed. Fermentation stopped rapidly, and the quantity of lactic acid produced was limited at 8 g/liter after the first day of fermentation.

SEM observation of digested starch granules. Enzymatic attack of raw starch during fermentation with controlled pH was observed by using SEM. The initially smooth granules (Fig. 1B) were rougher after 24 h of fermentation (Fig. 1C), and some displayed large cavities (Fig. 1D). Shell residues resulting from total digestion of the inner part of granules were observed (Fig. 1E). Enzymatic hydrolysis also revealed the lamellar organization of the starch granules (Fig. 1F). However, the progress of degradation was not homogeneous. Smooth granules and entirely digested granules were observed in the same sample. In addition, the proportion of granules displaying enzymatic attack appeared to be fairly small, whatever the stage of fermentation. A decrease in the number of granules was observed as fermentation progressed, with total disappearance occurring after 3 days. No strongly degraded granules and only slight surface erosion of some granules were observed in fermentation without pH control.

DISCUSSION

Numerous bacteria have been described in the literature as being amylolytic, but very few are capable of breaking down raw starch. Dettori-Campus et al. (6) sought the property in over 80 *Bacillus* strains and observed that only strains of *B. stearothermophilus* and *Bacillus amylolyticus* effectively degraded raw starch. Amylase synthesis is a rare characteristic of lactic acid bacteria. The principal strains of this type to be identified are *Streptococcus bovis*, *Streptococcus equinus*, *Lactobacillus amylophilus*, *Lactobacillus amylovorus*, *Lactobacillus acidophilus*, and *Lactobacillus cellobiosus* (11, 14, 17, 18, 24). Others isolated from animal digestive tracts have been described as amylolytic (2, 3). The ability to break down raw starch has been shown only in *S. bovis* 148 isolated from bovine rumen (20) and in *L. amylovorus* isolated from cattle waste-corn fermentation (13). It is shown here for the first time that a wild strain of *L. plantarum* isolated from fermented cassava can break down cassava raw starch. It was also found that pH control was required to enable *L. plantarum* A6 to achieve the complete hydrolysis of raw starch granules.

Lactic acid production causing rapid acidification of the medium to pH 3.6 was observed during fermentation on raw starch without pH control. This resulted mainly from the conversion by the microorganism of low-molecular-weight sugars present initially in the cassava and probably also from the weak hydrolysis of starch granules. Surface erosion of certain granules was detected by SEM. This limiting of the degradation phenomenon is related, first, to the small amylase activity synthesized (1/30 of that with controlled pH) and, second, to the weak activity of the enzyme at such a pH (only 15% relative activity at pH 3.5 in comparison with 100% at pH 5.5) (9). The effect of the pH on amylase synthesis had already been observed for *L. plantarum* A6 in flask cultures (7); initial addition of calcium carbonate to limit acidification of the medium increased the amount of amylase synthesized fivefold. This effect was also reported by Pompeyo et al. (19) for *L. amylovorus* and *L. amylophilus*, where larger amounts of amylase were found in the presence of $CaCO_3$.

Total conversion of starch granules to lactic acid was ob-

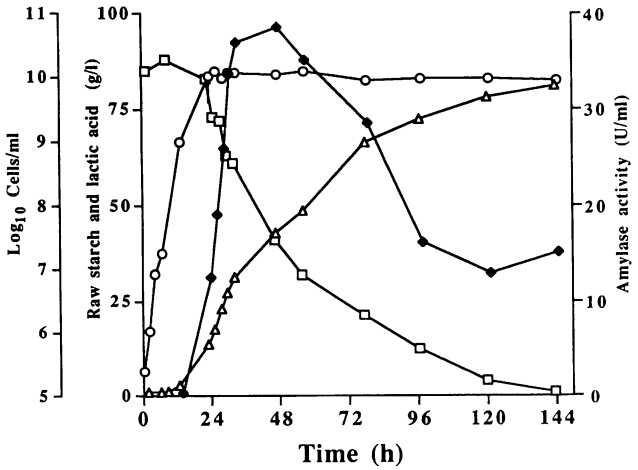

FIG. 2. Fermentation of *L. plantarum* A6 on dry-heated cassava starch at 30°C and pH 6.0. Symbols: □, starch; △, lactic acid; ◆, amylase activity; ○, $\log_{10}$ cells.

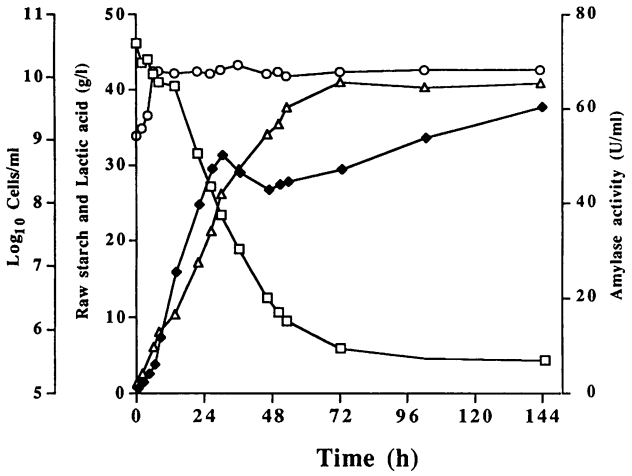

FIG. 3. Fermentation of *L. plantarum* A6 on raw cassava starch at 30°C and pH 6.0. Symbols: □, starch; △, lactic acid; ◆, amylase activity; ○, $\log_{10}$ cells.

served for both dry-heated starch and raw starch during fermentation with pH control. The present results showed that amylase activity was not associated with cell growth, in contrast with previous work (8) in which it was found that enzyme production more closely paralleled growth of the culture. The difference may be due to substrate quality: cassava raw starch was used here instead of the soluble starch of previous experiments or the medium composition. Further studies would be required to clarify this point.

The results indicate that mass inoculation of certain natural fermentation processes with *L. plantarum* A6 will not cause total hydrolysis but simply a weak attack of the starch granules. This was observed in the case of natural cassava fermentation for production of gari, a traditional foodstuff in West Africa. The use of *L. plantarum* A6 resulted in faster acidification and a higher lactic acid content (4 g per 100 g [dry weight]), i.e., an increase of over 25% in comparison with results with a nonamylolytic strain of *L. plantarum* (10).

These encouraging results make it possible to envisage numerous applications of *L. plantarum* A6 as a starter in some traditional food fermentation processes where the quantity of lactic acid produced is sometimes too small to obtain a high-quality product. In addition, the capacity of *L. plantarum* A6 to fully convert starch into lactic acid when fermentation conditions are controlled might be of considerable interest for industrial production of the acid directly from raw starch, especially if a mutant derived from this strain is found to produce nonracemic lactic acid.

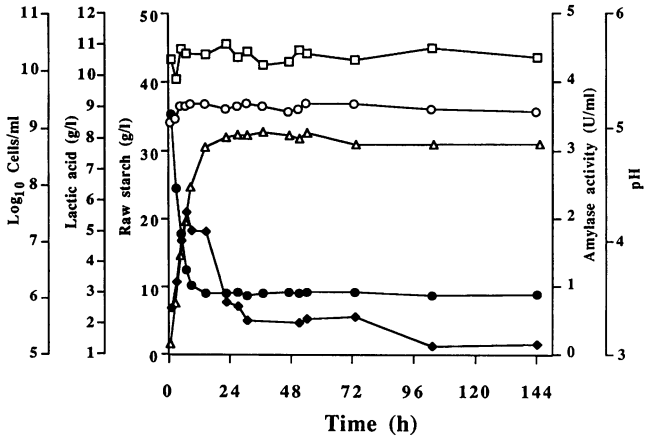

FIG. 4. Fermentation of *L. plantarum* A6 on raw cassava starch at 30°C without pH control. Symbols: □, starch; △, lactic acid; ◆, amylase activity; ○, $\log_{10}$ cells; ●, pH.

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