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Purification and characterization of an extracellular amylase from *Lactobacillus plantarum* strain A6

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E. GIRAUD, L. GOSSELIN, B. MARIN, J.L. PARADA AND M. RAIMBAULT. 1993. Extracellular amylase from *Lactobacillus plantarum* A6 was purified by fractionated precipitation with ammonium sulphate and by anion exchange chromatography. The homogeneity of the purified fraction was tested by polyacrylamide gel electrophoresis and showed multiple amylase forms. A major form had an estimated molecular weight of 50 kDa. It was identified as an α-amylase, with an optimum pH of 5·5, an optimum temperature of 65°C and K_m value of 2·38 g l^{-1} with soluble starch substrate. The enzyme was inhibited by *N*-bromosuccinimide, iodine and acetic acid. The enzyme activation energy was 30·9 kJ mol^{-1}.

INTRODUCTION

Lactic acid bacteria can rarely convert starch into lactic acid. The main such strains identified are *Streptococcus bovis*, *Strep. equinus*, *Lactobacillus amylophilus*, *Lact. amylovorus*, *Lact. acidophilus* and *Lact. cellobiosus* (Nakamura and Crowell 1979; Nakamura 1981; Sen and Chakrabarty 1984; Kandler and Weiss 1986; Hardie 1986). Others, isolated from animal digestive tracts, have been described as amylolytic lactic acid bacteria (Champ *et al.* 1983; Cotta 1988). Practically no information is available about the characteristics of these enzymes. To the best of our knowledge, only the research by Lindgren and Refai (1984) and Sen and Chakrabarty (1986) covered the characterization of an amylolytic activity produced by a lactic acid bacterium.

A wild strain of *Lact. plantarum* (strain A6) was isolated from retted cassava and examined for its ability to break down starch (Giraud *et al.* 1991). The strain possesses particularly interesting characteristics. It is the first time that a *Lact. plantarum* amylolytic strain has been described and it was found that it synthesizes large amounts of extracellular α-amylase and its growth kinetics on glucose or starch-based media are similar.

More detailed investigation of this enzymatic activity was carried out because of the original features of the micro-organism and there has been only a limited amount of research on the amylolytic activities of lactic acid bacteria. The purification and characterization of the extracellular amylolytic activity excreted by *Lact. plantarum* A6 are described here.

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MATERIALS AND METHODS

Organism

Lactobacillus plantarum strain A6 isolated from retted cassava was used (Giraud *et al.* 1991). The strain was preserved in glycerol at −80°C.

Medium and culture conditions

The medium composition was identical to that of MRS medium (De Man *et al.* 1960) except that glucose was replaced by 2% soluble starch (Prolabo, Paris, France) and $CaCl_2$ was added at 0·5 g l^{-1}. The bacterium was cultured in a 2 l bioreactor (LSL-Biolafitte, Saint Germain en Laye, France) at 30°C and agitated at 200 rev min^{-1}. The pH was adjusted to 6·0 by the addition of 5 mol l^{-1} NaOH. The reactor was inoculated with 10% (v/v) of 20-h preculture in the same medium used for fermentation.

Amylase assay

α-Amylase activity was measured by incubating 0·1 ml of appropriately diluted enzyme solution with 0·8 ml of a solution containing 1·2% of Prolabo soluble starch in 0·1 mol l^{-1} citrate-phosphate buffer, pH 5·5 at 55°C. The reaction was stopped by the addition of 0·1 ml of 1 mol l^{-1} H_2SO_4. After incubation, residual starch contents after different lengths of time were determined colorimetrically at 620 nm by adding 0·1 ml of the reaction mixture to 2·4 ml of an iodine solution containing (g l^{-1} in distilled water): KI, 30; I_2, 3, diluted to 4%. 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 above. The protein concentration was estimated using the method described by Bradford (1976) with a Biorad Kit (Cat no. 500-0001, Ivry Sur Seine, France) with bovine serum albumin as standard.

Purification of amylase

Fermentation was stopped after culture for 9 h. After the removal of cells by centrifugation (15 000 *g* for 15 min, 4°C), the supernatant fluid (750 ml) was filtered through a cellulose filter (0·45 μm pore size, HAWP type; Millipore, Saint Quentin les Yvelines, France) to remove cell debris. Powdered ammonium sulphate was then added slowly to the supernatant fluid with constant stirring at 4°C. Most of the amylase activity was precipitated at between 50 and 70% saturation. After ammonium sulphate fractionation, the precipitated protein collected by centrifugation (15 000 *g* for 30 min, 4°C) was resuspended in 50 mmol l^{-1} KH_2PO_4/Na_2HPO_4 buffer pH 6·8 (standard buffer). The enzyme solution was washed, concentrated with a PM-10 Amicon ultrafiltration membrane and loaded onto a DEAE-cellulose column (DE-52; Whatman Laboratory Sales, Hillsboro, Oregon, USA) (25 × 250 mm, flow rate 2·5 ml min^{-1}, 25°C) previously equilibrated with the standard buffer. The enzyme was eluted with a concave sodium chloride gradient (0–1·0 mol l^{-1}) and fractions (5 ml) were collected. The fractions that were enzymatically the most active were pooled, dialysed overnight at 4°C against the standard buffer and used for further studies. They were kept at −30°C. No activity was lost for at least 3 months under such conditions.

Polyacrylamide gel electrophoresis

This was carried out according to the method of Laemmli (1970) with a 10% running gel and 4% stacking gel. Electrophoresis under non-denaturing conditions was performed in the absence of sodium dodecyl sulphate and β-mercaptoethanol in any buffer. Gels were run at a constant 150 V for 1 h at 25°C. Proteins were stained by the silver method of Oakley *et al.* (1980).

Amylase stain

After electrophoresis, the gel was incubated for 1 h at 30°C in 0·1 mol l^{-1} citrate-phosphate buffer, pH 5·5, containing 1% soluble starch. After two washes with distilled water, light lanes (representing starch hydrolysis areas of amylase activity) were detected by immersing the gel in Lugol's solution.

Molecular mass determination

SDS-PAGE electrophoresis was used to determine the approximate molecular mass of amylase. Myosin (200 000), β-galactosidase (116 250), phosphorylase-b (97 400), bovine serum albumin (67 000) and ovalbumin (45 000) served as marker proteins (Biorad, Cat no. 161-0315).

Kinetics

The Michaelis constant (K_m) of the purified enzyme was determined with different concentrations of soluble starch as substrate. Values were obtained from double reciprocal plotting as described by Lineweaver and Burk (1934).

Optimum pH and temperature

The effect of pH on the enzyme activity was studied in a 3–7·5 pH range with 0·1 mol l^{-1} citrate-phosphate buffer at 55°C. The enzymatic activity profile according to temperature was determined within a 10°–80°C temperature range under standard conditions (see above).

RESULTS

Purification of amylase

The results of the purification of amylase produced by the strain *Lact. plantarum* A6 are summarized in Table 1. The first step in purification was conventional $(NH_4)_2SO_4$ frac-

Table 1 Purification of α-amylase of *Lactobacillus plantarum* (strain A6) cultivated in a modified MRS medium containing 2% (w/v) soluble starch and 0·5 g l^{-1} $CaCl_2$ at 30°C

Materials	Volume (ml)	Protein (mg)	Activity (U)	Specific activity U mg^{-1}	Yield (%)	Purification (fold)
Culture filtrate	750	82·5	35 100	425	100	1
$(NH_4)_2SO_4$ (50–70% fraction)	39	18·1	25 935	1433	73·9	3·4
Ultrafiltrate	8·8	10·4	16 016	1540	45·6	3·6
DEAE cellulose (117–130 fractions)	61·8	1·5	12 484	8270	35·6	19·5

tionation. The 50–70% fraction revealed maximum enzyme activity and was selected for further purification by DEAE-cellulose. The elution profile (Fig. 1) displayed only one amylase activity peak. The enzyme was eluted at 0·44–0·52 mol l^{-1} NaCl. The maximum purification level achieved was about 20-fold and the recovery yield was approximately 35%. Enzyme purity and efficiency of the proposed purification procedure were estimated by polyacrylamide gel electrophoresis under non-denaturing conditions. The samples from the different purification steps were loaded onto the gel in such a way as to deposit constant amylasic activity (7 U per lane). The sensitivity of the technique used to visualize protein (silver staining) made it possible to assess the effectiveness of the method used for purifying the enzymatic activity (Fig. 2).

A major band was shown in the purified fraction (lane 3) together with three low intensity sub-bands spaced at regular intervals. Lugol visualization (lane 4) after incubation of the gel with starch showed that the amylasic activity was sited principally at the main band level but was also present at a weaker level in the sub-bands. It was noted that these three bands of minor activity were initially observed in the crude extract and were thus not formed during the purification procedure.

Molecular mass

In SDS-PAGE analysis (Fig. 3), a well-defined band for a molecular weight of 50 kDa and a diffuse band with a molecular weight of close to 150 kDa were observed. The same electrophoretic profiles were found in the presence or absence of β-mercaptoethanol (results not shown).

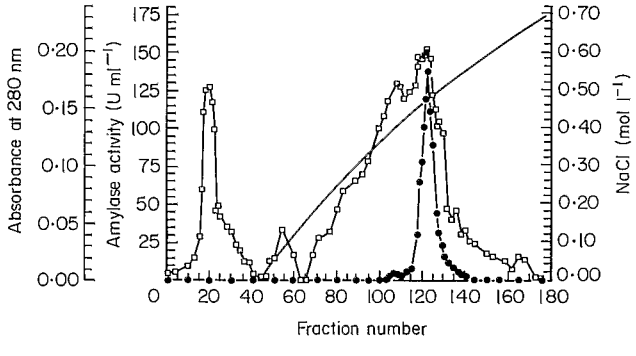

Fig. 1 Elution profile of *Lactobacillus plantarum* α-amylase on DEAE-cellulose column chromatography. The ultrafiltered enzyme solution obtained after ammonium sulphate precipitation was applied to the column. The column was equilibrated with phosphate buffer (pH 6·8) and the sample eluted with an NaCl gradient (0–1·0 mol l^{-1}) in phosphate buffer. Fractions (5 ml) were collected. □, Absorbance at 280 nm; ●, amylase activity; —, NaCl concentration

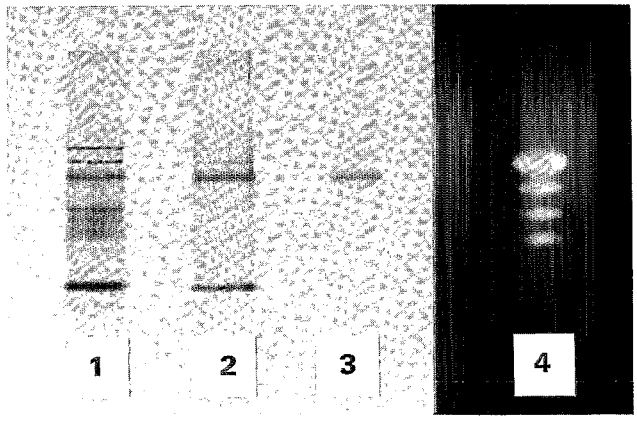

Fig. 2 Polyacrylamide gel electrophoresis of active fractions obtained from each purification step. Lane 1, Cell-free extract; lane 2, after ammonium sulphate precipitation; lane 3, after DEAE-cellulose chromatography; lane 4, after DEAE-cellulose chromatography, the bands supposed to be α-amylase were assumed by their starch-hydrolysing activity

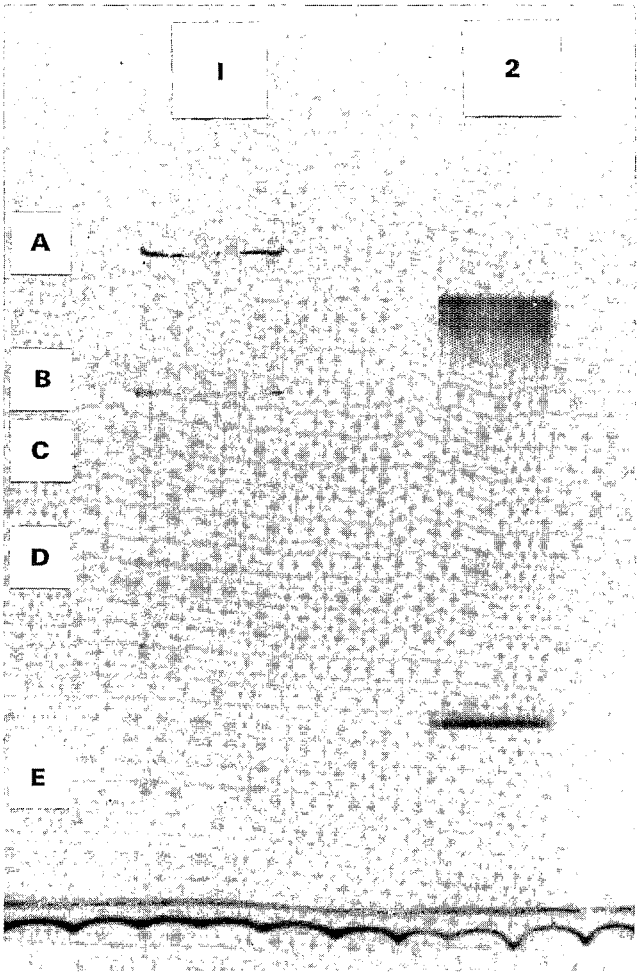

Fig. 3 SDS-polyacrylamide gel electrophoresis of purified α-amylase. Lane 1, Standard proteins (Biorad): A, myosin (MW 200 000); B, β-galactosidase (MW 116 250); C, phosphorylase b (MW 92 500); D, bovine serum albumin (MW 66 200); E, ovalbumin (MW 45 000). Lane 2, Purified α-amylase

Starch hydrolysis

The mode of action of the purified enzyme on soluble starch was examined by simultaneously measuring the decrease in iodine-staining power and the production of reducing sugars from starch using the method of Miller (1959). Starch hydrolysis by the enzyme (Fig. 4) was accompanied by a rapid reduction in the iodine-staining capacity of the substrate with correspondingly slow release of reducing sugar. Such a pattern is typical of an endo-attacking enzyme cleaving 1,4-glucosidic linkages.

Effects of pH on enzyme activity

The enzyme acts preferentially at an acid pH with an optimum at pH 5·5 (Fig. 5). Activity loss at pH levels of

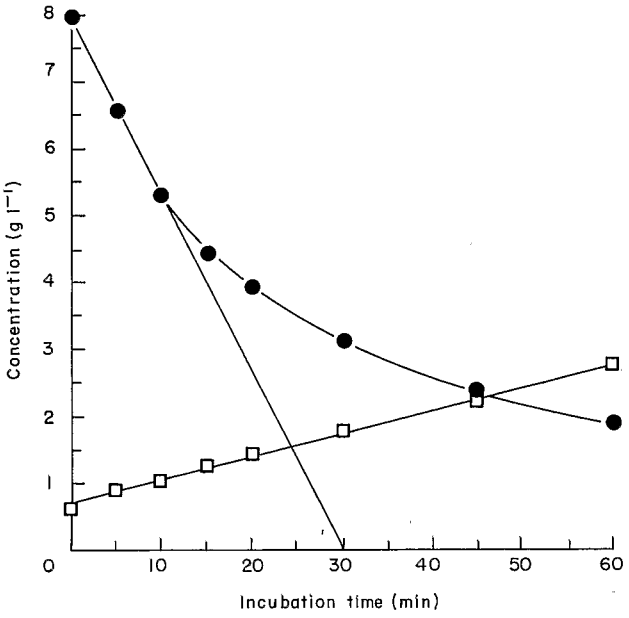

Fig. 4 Reduction in iodine-staining and the release of reducing power from starch by the purified enzyme at pH 5·5 and 55°C. ●, Concentration of starch determined by iodine staining; □, concentration of reducing power

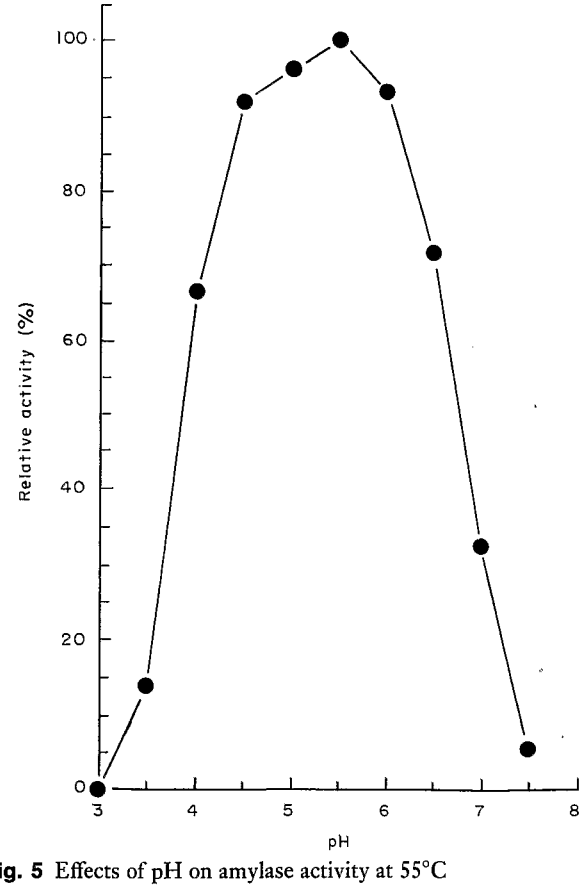

Fig. 5 Effects of pH on amylase activity at 55°C

4·5–6 did not exceed 10–15%. Activity falls rapidly outside this range, decreasing to zero close to pH 3 and 8. Activity is almost totally retained for 5 d at ambient temperature. However, at extreme pH levels, and especially at pH 3·8, the enzyme was unstable and 80% loss of activity was observed over a 5 d period.

Effects of temperature on enzyme activity

The influence of temperature on the amylase activity in 0·1 mol l^{-1} citrate-phosphate buffer, pH 5·5, tested within the 10°–80°C range is shown in Fig. 6. The optimal temperature was 65°C. The activation energy of the enzyme was 30·9 kJ mol^{-1} as calculated from the Arrhenius plot.

Michaelis constant (K_m)

The enzyme K_m value was determined with soluble starch as substrate and was found to be 2·38 g l^{-1} at 30°C and pH 5·5 from the Lineweaver–Burk plot.

Action of cations

The effect of cations at 0·01 mol l^{-1} were all tested as chloride or sulphate, as these two ions have no effect on enzymatic activity. The results are displayed in Table 2 and show that the enzyme was strongly inhibited by mercury (100% at the concentration used). No other significant activation or inhibition was observed with the other cations.

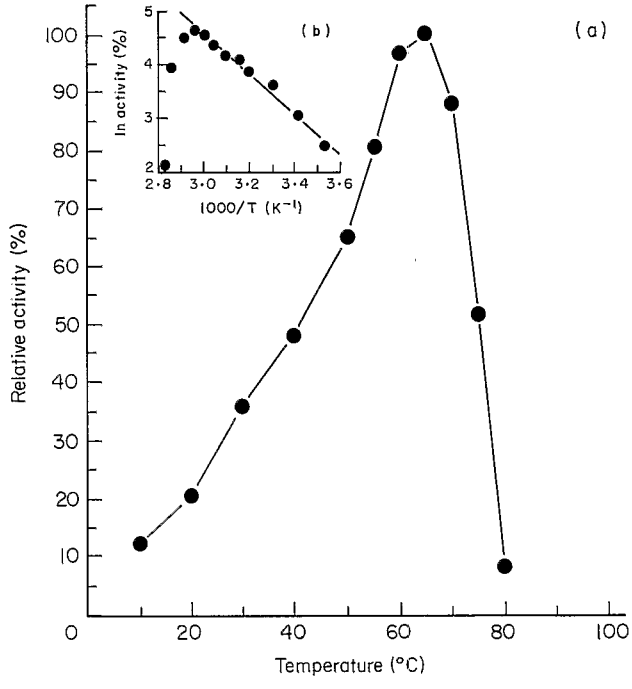

Fig. 6 Effects of temperature on amylase activity at pH 5·5. (a) Relative activity *vs* temperature; (b) Arrhenius plot

Table 2 Influence of cations (0·01 mol l^{-1}) on amylase activity

Cation	% activity
$BaCl_2$, $2H_2O$	103
$CuSO_4$	109
$MgSO_4$, $7H_2O$	104
$CoCl_2$, $6H_2O$	100
$NiCl_2$, $6H_2O$	109
$HgCl_2$	0
$ZnSO_4$, $7H_2O$	107
$FeSO_4$, $7H_2O$	93
$CaCl_2$, $2H_2O$	95
KCl	114
$FeCl_3$	115
CdN_2O_6, $4H_2O$	98
$SnCl_2$, $2H_2O$	89

Action of other effectors

The other effectors tested at concentrations of 10^{-2} or 10^{-3} mol l^{-1} are shown in Table 3. Strong inhibition by *N*-bromosuccinimide and partial inhibition with 4-dimethylaminobenzaldehyde were found, indicating the

Table 3 Influence of effectors on amylase activity

Effector	Concentration (mol l^{-1})	% activity
Amino-5-tetrazole	10^{-2}	112
4-Dimethylaminobenzaldehyde	10^{-2}	70
Iminodiacetic acid	10^{-2}	118
Woodward reagent	10^{-2}	102
EDTA	10^{-2}	91
N-acetylacetone	10^{-2}	100
N-ethylmaleimide	10^{-2}	90
2, 5-Dimethoxytetrahydrofuran	10^{-2}	111
Trifluoroacetic anhydride	10^{-2}	89
Acetic acid	10^{-2}	82
Iodine	10^{-3}	6
Iodine/tyrosine	$10^{-3}/5 \times 10^{-3}$	79
N-bromosuccinimide	10^{-3}	0
N-bromosuccinimide/tryptophan	$10^{-3}/5 \times 10^{-3}$	63

presence of a tryptophan group at the active site. Inhibition by iodine suggests the action of a phenol group and probably tyrosine. The simultaneous addition of amino acid (tryptophan with *N*-bromosuccinimide in the first case and tyrosine with iodine in the second case) limited the extent of the observed inhibition. The decrease in activity measured after the addition of acetic acid suggests the action of an amino acid with an OH group (serine and/or threonine).

DISCUSSION

The purification procedure described above makes it possible to obtain, in only two stages, a protein fraction containing most of the amylasic activity of *Lact. plantarum* A6 enriched by a factor of nearly 20. Testing the homogeneity of the fraction by electrophoresis under native conditions revealed a major protein and three others that were quantitatively unimportant. However, all the proteins detected in the purified fraction possessed an amylasic activity. It was considered that these procedures were sufficient for purifying the extracellular amylasic activity of *Lact. plantarum* A6. SDS-PAGE analysis of the purified fraction resulted in distribution between a clearly defined band (50 kDA) and a diffuse band with a molecular weight of close to 150 kDa.

Several hypotheses can be put forward to account for these many amylase forms. The one we find most satisfactory is the suggestion that the purified extract consists of a population of aggregates of a 50 kDa amylase. This interpretation is based on the fact that most of the bacterial amylases described have a molecular weight of this order (Fogarty 1983). This type of aggregation of purified enzyme was observed in *Bacillus subtilis* amylase (Robyt and Ackerman 1973), with zinc as the factor inducing clumping. The factor remains to be defined in our case.

Further experimental studies would be required to support this hypothesis. The amount of enzyme isolated was not large enough for further investigation. Immunological characterization would probabably determine the type of relation between the different amylase forms observed and thus confirm the hypothesis.

With regard to the characteristics of the lactic acid bacterial amylases described in the literature, the properties of the enzyme synthesized by *Lact. plantarum* A6 are different. The enzyme from a *Leuconostoc* spp. studied by Lindgren and Refai (1984) had a pH optimum of 6·0 and a temperature optimum of 40°C. Two active enzyme fractions were clearly separable by isoelectric focusing. The enzyme isolated from *Lact. cellobiosus* (Sen and Chakrabarty 1986) had a molecular weight of 22·5–24 kDa, a pH optimum from 6·3 to 7·9 and a temperature optimum of 40–50°C. However, compared with the characteristics of the other bacterial amylases, the properties of amylase of *Lact. plantarum* A6 are very similar to those of *B. subtilis* (Fischer and Stein 1960; Welker and Campbell 1967; Robyt and Ackerman 1973; Fogarty 1983), i.e. extracellular enzyme, identical optimum pH, identical optimum temperature, presence of tyrosyl phenolic groups at the active site and presence of multiple forms (aggregates).

The exceptional capacity of *Lact. plantarum* A6 to break down starch led us to consider the acquisition of this feature. Might it result from the transfer of genetic material between *B. subtilis* and *Lact. plantarum*, as both are microorganisms in the natural microflora of fermented cassava (Nwanko *et al.* 1989) and their amylasic activities we found to be very similar? Closer investigation would enable us to answer this question.

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