2ème Atelier international / *2nd International Workshop*
Voies alimentaires d'amélioration des situations nutritionnelles
Food-based approaches for a healthy nutrition
Ouagadougou, 23-28 / 11 / 2003

Amylolytic lactic acid bacteria from *pozol*: a natural potential to produce complementary foods?

Díaz-Ruíz[1] Gloria, Guyot[2] Jean-Pierre, Ruíz-Terán[1] Francisco, Morlon-Guyot[2] Juliette, Wacher[1*] Carmen

[1] Departamento de Alimentos y Biotecnología, Facultad de Química, UNAM, 04510 México, D. F. México.
[2] UR106 "Nutrition, Alimentation, Sociétés", IRD, BP 64501, 34394 Montpellier cedex 5, France.

Corresponding author: wacher@servidor.unam.mx*

- Abstract -

Pozol is a maize acid beverage of Mayan origin, consumed in Southeastern Mexico. It is obtained from natural fermentation of nixtamal (heat and alkali treated maize) dough but nixtamalisation is not widely used in Africa. Hence, it is of interest to compare *pozol* with African maize-based fermented foods. As starch is the main carbohydrate of nixtamal, the participation of amylolytic lactic acid bacteria (ALAB) has been found to be important. In contrast with other starchy fermented foods from Africa, in which *Lactobacillus* is the main ALAB, *Streptococcus bovis* is the dominant species during *pozol* fermentation. The purpose of the present study was to compare *S. bovis* main physiol*ogi*cal characteristics with those of ALAB isolated from African fermented foods, as well as to evaluate its potential use to increase the energy density of nixtamal-based gruels to develop a new complementary food for young children.

S. bovis strain 25124 produces an α-amylase which is cell-associated, with optimum temperature of 30 - 37°C and neutral pH, but unstable at low pH values; whereas amylases from *Lactobacillus plantarum* A6 from *gari* and *Lactobacillus fermentum ogi* strains have optimum activity at lower pH values and higher temperatures. It showed extremely low amylase yield relative to biomass [139 U (g cell dry weight)$^{-1}$] and specific rate of amylase production [130.7 U (g cell dry weight)$^{-1}$ h^{-1}], when compared with *L. plantarum* A6 and *L. fermentum Ogi*. In contrast, it showed a high specific growth rate (0.94 h^{-1}) and an efficient energy conversion yield to bacterial cell biomass [0.31 g biomass (g substrate)$^{-1}$]. These would confer the strain competitive advantage and are the possible reasons for its dominance during fermentation.
Because of its physiol*ogi*cal characteristics, *S. bovis* has a potential as a starter culture to ferment nixtamal dough; however, because of its instability at low pH values, it would only be active at the initial stages of fermentation. In contrast, *Lactobacillus fermentum ogi* strains amylase would be active in the acidified substrate.

Key words: *Pozol* – nixtamal – Lactic acid bacteria – Amylase – Complementary foods.

- Résumé -

Les bactéries lactiques amylolytiques du *pozol* ont-elles un potentiel naturel pour produire des aliments de complément ?

Le *pozol* est une boisson acidulée d'origine Maya consommée au Sud-Est du Mexique. Elle est obtenue par fermentation naturelle du nixtamal (maïs cuit à la chaux), mais la technique de la nixtamalisation (cuisson alcaline) est très peu répandue en Afrique. Ainsi, une comparaison entre le *pozol* et les aliments fermentés africains à base de maïs présente un certain intérêt. Les bactéries lactiques sont responsables de l'acidification de la pâte et, l'amidon étant le principal glucide du nixtamal, les bactéries lactiques amylolytiques (BLA) ont un rôle déterminant. Contrairement à de nombreux aliments amylacés fermentés africains pour lesquels les principales BLA appartiennent au genre *Lactobacillus*, *Streptococcus bovis* est l'espèce qui domine pendant la fermentation du *pozol*. L'objectif de ce travail est de déterminer les principales caractéristiques physiologiques de *Streptococcus bovis* pour les comparer avec celles d'autres BLA isolées d'aliments fermentés africains (*Lactobacillus*), et d'évaluer son utilisation potentielle pour augmenter la densité énergétique de bouillies préparées à partir de nixtamal, en vue de développer un nouvel aliment de complément du jeune enfant.

La souche *S. bovis* 25124 a été cultivée à 30°C en milieu MRS à base d'amidon. Les concentrations en biomasse, en sucres totaux et réducteurs, en acide lactique, glucose, maltose, oligoholosides (du maltotriose au maltoheptaose), l'activité amylolytique et le pH ont été déterminés dans le milieu de fermentation prélevé toutes les 30 min. La stabilité de l'amylase dans des extraits bruts de *S. bovis* a été déterminée à différents pH (3 à 7,5) et températures (15 à 60°C). *S. bovis* 25124 a un rendement cellulaire en amylase [139 U (g poids sec cellulaire)$^{-1}$] et une vitesse spécifique de production d'amylase [130.7 U (g poids sec cellulaire)$^{-1}$ h^{-1}] extrêmement bas. En revanche, elle présente un taux spécifique de croissance (0.94 h^{-1}) et un rendement cellulaire relatif au substrat consommé [0.31 g biomasse (g substrat)$^{-1}$] élevés. Ces dernières caractéristiques conféreraient à la souche un avantage compétitif et pourraient expliquer sa dominance pendant la fermentation. Son amylase est associée à la cellule et présente des optima de température entre 30-37°C et de pH à la neutralité, mais est instable aux bas pH. C'est une α-amylase qui produit à partir de l'amidon du maltotetraose (G4) comme principal maltooligoside. De par ces caractéristiques, *S. bovis* pourrait être utilisé comme culture starter pour fermenter des pâtes de maïs nixtamalisées. Son amylase devrait être capable d'hydrolyser et de diminuer la viscosité de bouillies produites à partir de nixtamal. Toutefois, en raison de son instabilité aux bas pH, elle ne pourrait être active que pendant les premières heures de la fermentation. En revanche, l'α-amylase de *Lactobacillus fermentum ogi* E1 est active à des pH plus bas, permettant d'envisager un couplage des deux souches pour hydrolyser l'amidon et augmenter la densité énergétique de bouillies destinées aux jeunes enfants.

Mots-clés: *Pozol* – Nixtamal – Bactéries lactiques – Amylase – Aliments de complément.

INTRODUCTION

Pozol is a maize acid beverage of Mayan origin, consumed in Southeastern Mexico[1]. To prepare it maize grains are boiled in lime-water during approximately 90 min, then washed with water and hand-rubbed to remove the pericarp. These grains are called nixtamal. They are coarsely ground and kneaded to obtain a dough, which is shaped into balls, wrapped in banana leaves and left to ferment at ambient temperature. The acidified dough is suspended in water to obtain a refreshing beverage[2].

The concentration of mono- and disaccharides from maize is drastically reduced during nixtamalization, so that starch is then the main carbohydrate available for fermentation[1]. In contrast, other methods to prepare maize before fermentation, as soaking (as in *ogi* and *kenkey)* and germination (kaffir beer), would increase the concentration of sugars[3]. Hence, it is of interest to compare *pozol* with African maize fermented foods.

A natural fermentation occurs and lactic acid bacteria are responsible for acidification of the dough[4]. As starch is the main carbohydrate of nixtamal, the participation of amylolytic lactic acid bacteria (ALAB) in this fermentation has been found to be important. In contrast with other starchy fermented foods from Africa in which *Lactobacillus* is the main ALAB[5,6], *Streptococcus bovis* is the dominant species during *pozol* fermentation[7].

Infant growth is crucial during the first months and up to 2 years of age, hence special attention to their feeding is needed during that time. Breastfeeding is considered the best food during the first months, but from 4 to 6 months children require complementary foods. This is a critical step, as adequate foods need to be used in order to prevent them from becoming malnourished[8]. It is common to use cereal-based gruels or porridges as complementary foods in underdeveloped countries. These are accessible and easy to prepare, but their high starch content makes them extremely viscous after cooking and they do not provide the required energy density.

Fermentation with amylolytic lactic acid bacteria isolated from *pozol* would be an option to reduce their viscosity and increase their energy density.

The purpose of the present study was to determine *Streptococcus bovis* main physiological characteristics, to compare them with those of ALAB isolated from African fermented foods (*Lactobacillus*), as well as to evaluate its potential use to increase the energy density of nixtamal-based gruels to develop a new complementary food for young children.

MATERIALS AND METHODS

Streptococcus bovis strain 25124, isolated from *pozol* by our group[7] was grown on MRS-starch medium (with 20 g $liter^{-1}$ soluble potato starch instead of glucose) at 30°C. Cultures were grown in 1-liter flasks with 800 ml medium. They were inoculated with 25 ml of 12 h precultures grown in the same medium at 30°C. The fermentation broth was sampled every 30 min. Concentration of biomass was determined by measuring optical density at 600 nm and converted to dry weight, measured after two washing and centrifugation cycles and drying at 80°C until constant weight.

Cells were removed by filtration through 0.22 μm pore size membranes. Lactic was quantified on a Perkin Elmer 250 liquid chromatograph with hydrogen loaded ion-exchange column (BioRad Aminex HPX-87H, 300 by 7.8 mm) and a Perkin Elmer 30 refractive index detector. The mobile phase was 0.01 N H_2SO_4 with a flow rate of 0.6 ml min^{-1} at 50°C. Hydrolysis products from starch were determined by HPLC, using a

Prodigy 5 ODS 2 C_{18} column (250 by 4.6 mm; Phenomenex) with a flow rate of 0.8 ml min^{-1} at 35°C and the refractive index detector. Glucose and maltose were determined by using a commercial enzymatic test (product 1113950, Boehringer, Mannheim, Germany). Total and reducing sugars concentrations were determined by the methods of Dubois et al.[9] and Miller[10], respectively. Residual starch concentration was determined by measuring the iodine-starch complex color[11.]

To measure cell-bound amylase activity, 10 ml cultures were centrifuged (27,200 x g during 15 min at 4°C); the pellets were washed and suspended in 0.1 M phosphate buffer (pH 6.8). The activity was assayed at pH 6.8 and 37°C by measuring the iodine-complexing ability of starch[5]. One enzyme unit was defined as the amount of enzyme hydrolysing 10 mg of starch in 30 min.

To prepare crude extracts, bacteria were grown in MRS-starch medium for 4 hours. Cultures were centrifuged (10 min at 15,300 x *g* and 4°C) and the cell pellets were washed with water. Cells were treated with 4 mg ml^{-1} lysozyme in TS buffer (50 mM Tris/HCl, pH 8, 25% w/v sucrose) for 1 h at 37°C. Protoplasts were sonicated a variable number of cycles with intermediate cooling using a Sonic dismembrator 550 (Fisher). Triton X-100 (Sigma) at final concentration of 1% was added to the disrupted cells and they were agitated at low temperature. Cell debris was removed by centrifugation (15 min at 27200 *g* and 4°C). Crude extract amylase activity was quantified in the crude extract by measurement of the iodine complexing ability of starch at pH and temperature optima as previously described by Agati *et al*.[5]. The effect of pH on crude extract activity was studied over the pH range 4.0 - 7.5 with 0.1 M citrate-phosphate buffer and over pH 7.5 - 9.0 with 0.1 M Tris-HCl buffer. The same buffers were used to determine pH stability, pH 3 value was also considered. One volume of crude extract amylase was dialysed against water, diluted in an equal volume of the above buffers and the mixture was incubated during 25h at 30°C. After this time residual activities of samples were determined under standard conditions, $CaCl_2$ was added at a final concentration of 50 mM.

The effect of temperature was studied between 10 and 65°C at pH 6.8. Stability of α-amylase was estimated by incubating crude extracts at 15, 25, 30, 37, 45, 50, 55 and 60°C at pH 6.8 for 60 min. $CaCl_2$ was added at a final concentration of 50 mM. Samples were cooled in an ice-water bath after incubation and the residual activities were determined.

RESULTS AND DISCUSSION

Characteristics of *S. bovis* 25124 amylase

Optimum pH and temperature of *S. bovis* 25124 amylase were between 6.6 and 6.75 and 36 - 40°C. The maximum stability of this amylase was observed at pH 6.5. It was more stable at pH values between 7.0 - 9.0 than at acidic pH values, with a sharp decrease in activity at the pH range 5.0 to 6.0. Complete enzyme inactivation was found at pH 4. This amylase was sensitive to 1 h incubation at temperatures above 30°C, losing most (70%) of its activity at 45°C. The amylase of *L. plantarum* A6 isolated from retted cassava[6] has optimum pH 5.5 and 65°C**.** Conditions for amylase from *L. fermentum* strains isolated from *ogi* are 4.5 - 5 and 35 - 45°C[5]. Amylase of *pozol* strain has optimum temperature close to the ambient temperature that prevails during maize processing into *pozol*, whereas optimum pH relates more with the conditions that develop during the first 6 h of fermentation (table 1). Maltotriose, maltotetraose, maltoheptaose and maltohexaose were obtained from starch hydrolysis by *S. bovis* amylase crude extract, and this mode of action is similar to that of an α-

amylase. It is interesting to observe that it is well adapted to initial pH conditions which prevail in nixtamal due to alkaline cooking, but their instability at acidic pH will probably limit its action in the course of fermentation.

Table 1: Characteristics of amylases from ALAB isolated from starchy fermented foods.

Strain	Temperature for optimum activity	pH for optimum activity	Stability to temperature	Stability to pH	Food
Streptococcus bovis 25124	36-40°C	6.5-6.75	< 40°C	> 5	*Pozol*
Lactobacillus plantarum A6	65°C	5.5	Not available	>4.5	Retted cassava[6]
Lactobacillus fermentum Ogi	35-45°C	4.5-5.0	Not available	Not available	*Ogi*[5]

Comparison of growth parameters on starch of *S. bovis* from *pozol* and of *Lactobacillus* sp. isolated from African starchy fermented foods

Amylolytic lactic acid bacteria from other starchy fermented foods[5,6] have been identified as members of the genus *Lactobacillus*; however, the predominant ALAB in *pozol* was found to be related to *Streptococcus bovis.* It is possible that nixtamalization of maize selects for this bacterium, which is thermoduric and capable of growing in the presence of 6.5% NaCl and at pH 9.6. A comparison of kinetic data of this microorganism with species of *Lactobacillus* isolated from retted cassava and *ogi*, are presented in table 2. Metabolic quotients and $Y_{lac/s}$ are in the range of classical values reported for other ALAB, which are between 1.1-3.1 g (g cell dry weight)$^{-1}$ h^{-1} for q_{lac}, 2.3-3.5 g (g cell dry weight)$^{-1}$ h^{-1} for q_s and 0.67-0.84 (g g^{-1}) for $Y_{lac/s}$. All other parameters were different. *S. bovis* is characterized by a low yield of lactic acid relative to biomass ($Y_{lac/x}$), extremely low of both amylase yield relative to biomass ($Y_{amy/x}$) and specific rate of amylase production (q_{amy}), and by high specific growth rate and biomass yield ($Y_{x/s}$). These last characteristics are very different from those described for ALAB such as amylolytic strains of *L. plantarum* and *L. fermentum*.

The specific growth rate determined for the *pozol S. bovis* strain (0.94 h^{-1}) could give it a competitive advantage over the other non-amylolytic LAB species of *pozol*. Though substrate consumption and lactate production parameters are in the classical range observed for other ALAB (table 2), relatively high value of $Y_{x/s}$ indicates efficient energy conversion into biomass, which would also confer an additional competitive advantage, in spite of a very low efficiency in starch hydrolysis and total sugar consumption. Nevertheless, other parameters would need to be considered, as those related to tolerance at low pH of *S. bovis* strains and their amylase. In particular, taking into account low pH instability of the amylase, competitive advantages should be effective only during the first hours of *pozol* fermentation, when pH is still high enough, and progressively counterbalanced by the inhibitory effect due to acidification. Amylases of some ALAB isolated from African fermented foods, as *L. fermentum* isolated from *ogi*, have optimum low pH values[5] and would be active throughout the fermentation.

Table 2: Yields and metabolic quotients for *Streptococcus bovis* strain 25124, *Lactobacillus plantarum* A6 and *Lactobacillus fermentum Ogi* E1.

Parameter	Value obtained for		
	S bovis 25124 [a]	*L. plantarum* A6[12]	*L. fermentum Ogi* E1[13]
Total sugar consumed (%)	25.5	Nd	78
Y x/s (g g $^{-1}$)	0.31	Nd (0.18)	0.1
Y lac/s (g g $^{-1}$)	0.78	Nd (0.84)	0.33
Ylac/x (g g $^{-1}$)	2.56	Nd (4.7)	3.3
Yamy/x (U g $^{-1}$)	139	2300 (5700)	5400
μ (h $^{-1}$)	0.94	0.43 (0.41)	0.35
qlac [g (g cell dry weight) $^{-1}$ h $^{-1}$]	2.4	3.1 (3.0)	1.1
qs [g (g cell dry weight) $^{-1}$ h $^{-1}$]	3.0	Nd (2.3)	3.5
qamy [U (g cell dry weight) $^{-1}$ h $^{-1}$]	130.7	989 (2337)	1890

Y x/s (growth yield relative to substrate);
Y lac/s (lactic acid yield relative to substrate);
Ylac/x (lactic acid yield relative to biomass);
Yamy/x (amylase yield relative to biomass);
μ (maximum specific growth rate);
qlac (specific rate of lactic acid production);
qs (specific rate of substrate consumption);
qamy (specific rate of amylase production).
( ) Fermentations at controlled pH (6.0);
Nd = not determined;
a. This work.

CONCLUSION

Because of its physiological characteristics, *S. bovis* has a potential as a starter culture to ferment nixtamal dough. Its amylase should be able to hydrolyze starch and decrease the viscosity produced by nixtamal´s starch; however, because of its instability at low pH values, it would only be active at the initial stages of fermentation.

S. bovis, in mixed culture with low pH resistant amylase producing ALAB, have a potential use to increase the energy density of gruels for young children.

ALAB isolated from African starchy fermented foods, such as *ogi* and *gari*, belong to a different genus (*Lactobacillus*); characteristics of their amylases are also different (in some cases resistant to low pH values), so they would remain active longer during the acidification stage of the substrate.

Differences in substrate preparation (nixtamalization vs soaking) seem to have a profound effect on the kind of microbiota that is established, as well as on the characteristics of their amylases.

REFERENCES

1. Wacher C, Cañas A, Bárzana E, Lappe P, Ulloa M, Owens JD. Microbiology of indian and mestizo *pozol* fermentations. Food Microbiol 2000;17:251-56.
2. Ulloa M, Herrera T, Taboada J. Mexican *pozol*. In: Steinkraus KH, ed. Handbook of indigenous fermented foods. New York: Marcel Dekker, 1983:226-33.
3. Steinkraus KH, ed. Handbook of indigenous fermented foods. New York: Marcel Dekker, 1996.

4. Wacher MC, Cañas A, Cook PE, Bárzana E, Owens JD. Sources of microorganisms in *pozol*, a traditional Mexican fermented maize dough. World J Microbiol Biotechnol 1993;9:226-74.

5. Agati V, Guyot JP, Morlon-Guyot J, Talamond P, Hounhouigan DJ. Isolation and characterization of new amylolytic strains of *Lactobacillus fermentum* from fermented maize doughs (*mawe* and *ogi*) from Benin. J Appl Microbiol 1998;85:512-20.

6. Giraud E, Brauman A, Keleke S, Lelong B, Raimbault M. Isolation and physiological study of an amylolytic strain of *Lactobacillus plantarum*. Appl Microbiol Biotechnol 1991;36:379-83.

7. Díaz-Ruiz G, Guyot JP, Ruiz-Terán F, Morlon-Guyot J, Wacher C. Microbial and physiological characterization of weakly amylolytic but fast-growing lactic acid bacteria: a functional role in supporting microbial diversity in *pozol*, a Mexican fermented maize beverage. Appl Environ Microbiol 2003;69(8):4367-74.

8. WHO. Complementary feeding of young children in developing countries: a review of current scientific knowledge. Geneva: WHO, 1998.

9. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28:350-6.

10. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal Chem 1959;31:426-8.

11. Nakamura LK. *Lactobacillus amylovorus*, a new starch-hydrolyzing species from cattle waste-corn fermentations. Int J Syst Bacteriol 1981;31:56-63.

12. Guyot JP, Calderón M, Morlon-Guyot J. Effect of pH control on lactic acid fermentation of starch by *Lactobacillus manihotivorans* LMG 18010T. J Appl Microbiol 2000;88(1):176-82.

13. Calderón M, Loiseau G, Guyot JP. Nutritional requirements and simplified cultivation medium to study growth and energetics of a sourdough lactic acid bacterium *Lactobacillus fermentum Ogi* E1 during heterolactic fermentation of starch. J Appl Microbiol 2001;90:508-16.