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Lactic acid production from mussel processing wastes with an amylolytic bacterial strain

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The aim of this work was to study the capability of amylolytic lactic acid bacteria to hydrolyse the glycogen in mussel processing wastes (MPW) for lactic acid production. From four strains tested, Lactobacillus plantarum *A6 was selected. Cultures of this strain on MRS medium with starch or mussel glycogen, showed similar levels of amylase production for both polysaccharides. With glycogen, sugar consumption rate was lower than with soluble starch and, although the micro-organism produced higher yields of enzyme, a fraction of approximately 20% of the total sugars remained undegraded. However, the different substrates did not seem to induce the production of different types of amylase and the values of the apparent K_m for starch and for glycogen were close, revealing a similar affinity for both substrates. Therefore, the differences in hydrolysis and consumption of the substrate seem to be related with the higher grade of branching of glycogen. Culture in MPW showed a decrease in pH unfavourable for amylolytic activity and uncoupling between lactic acid and biomass production. By controlling pH and increasing the initial protein concentration to avoid uncoupling, similar lactic acid production and higher yields than obtained with starch were reached with MPW.* © *1999 Elsevier Science Inc. All rights reserved.*

Keywords: Amylolytic lactic acid bacteria; mussel processing wastes; lactic acid production; glycogen; amylase production; *Lactobacillus plantarum* A6

Introduction

Mussel processing wastes (MPW) are an underexploited liquid by-product generated in large volume ($\approx 60{,}000\ m^3\ year^{-1}$) in the industrial steam treatment of mussels, that contain glycogen as main component ($\approx 10\ g\ l^{-1}$).[1] This waste, with a COD $\approx 25\ g\ l^{-1}$, represents an important eutrophication factor of the Galician Rías Baixas estuaries (NW of Spain). The aim of this work was to develop a new procedure for both the treatment and economical exploitation of MPW, based on its use as a substrate for amylolytic lactic acid bacteria cultures. This new procedure would not require previous steps of saccharification, desalination or concentration necessary for other bioproductions developed on MPW.[1–4] It would be directed not only to the stabilisation of these effluents, reducing pathogenic microorganisms, but also to production of potentially economically interesting products such as lactic acid or amylases, jointly with biomass with a possible probiotic activity for animal feeding.

Lactic acid bacteria are involved in the production of many traditional fermented foods and are therefore so considered as GRAS (generally recognized as safe) organisms. Several lactic bacterial strains with amylolytic activity have been described, most of them isolated from natural fermentation processes,[5,6] traditional fermented foods[7–9] or animal digestive tracts.[10,11] These amylolytic strains are able to degrade different types of

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starch (corn,[6] potato,[12] cassava raw starch[8]). Amylases produced by stains of *Lactobacillus amylovorus* and *Lactobacillus amylophilus* were shown to hydrolyse other polysaccharides such as amylose, amylopectin or glycogen.[13] Nevertheless, apart from the mentioned work, and to the best of our knowledge, no other previous work has been done with amylolytic lactic acid bacteria using glycogen as substrate.

In this work several lactic acid bacteria strains were screened to select the most suitable for use in MPW. The specificity for glycogen of the amylase produced by the selected strain, *Lactobacillus plantarum* A6, was studied, as well as the effect of culture conditions, i.e. protein content and pH, on the bacterial growth, amylase production and activity, and lactic acid production.

Materials and methods

Microorganism and inoculum preparation

The lactic acid bacteria assayed were: *Lactobacillus plantarum* A6 (LMG 18053) isolated from retted cassava in the Congo,[14] *Lactobacillus manihotivorans* (LMG 18010T)[9] and *Lactobacillus plantarum* R10101/2, isolated from cassava starch fermentations in Colombia, and *Pediococcus* sp. VA403 isolated from cow rumen. All were maintained in glycerol at −80°C and kept in the ORSTOM collection in the LBMT at Montpellier.

Culture media and conditions

MPW medium was obtained directly from the effluents of mussel cooking industries, after a previous step of acidification and decantation,[15] its essential composition was: total sugars, 10.33 g l^{-1}; reducing sugars, 0.09 g l^{-1}; total nitrogen (of protein nature ~95%), 1.6 g l^{-1}; total phosphorus, 0.09 g l^{-1}; sodium chloride, 18 g l^{-1}. The pH was adjusted to 6.0. A classical medium for lactic bacteria culture MRS,[16] modified in that glucose was replaced by soluble starch (Prolabo, France) or glycogen obtained from MPW (94.25% purity), was used as reference medium.

Batch cultures were conducted aerobically at 30°C and 150 rev. min^{-1}, either in 250-ml Erlenmeyer flasks with 100 ml of medium and orbital agitation, or in a 2-l bioreactor (LSL-Biolafitte, France). Fermentations at regulated pH were maintained at 6.0 with 5 N NaOH. Inoculation at 5% (v/v) was performed with a 20-h pre-culture in the same medium.

Analytical methods

Sampling was done taking aliquots in sterile conditions in which, after appropriately diluting, biomass concentration was determined by measurement of optical density at 600 nm and related to the dry weights obtained at 105°C for 24 h, after two centrifugation (10,000 rev. min^{-1} for 15 min) and washing cycles. The rest of the analytical determinations were carried out in the supernatant.

Starch was determined colorimetrically at 620 nm by adding 0.1 ml of the sample to 2.4 ml of an iodine solution containing (g l^{-1} in distilled water): KI, 30; I_2, 3; diluted to 4%. Using glucose as standard, total sugars were estimated by the phenol–sulphuric method of Strickland and Parsons.[17] This method was also used for residual glycogen and starch determination, after precipitation of their aqueous solutions with the double volume of absolute ethanol and redissolution of the pellet obtained by centrifugation (5,000 rev. min^{-1} per 10 min) in a convenient volume of distilled water. Reducing sugars were estimated by the 3,5-dinitrosalicylic method.[18]

Glucose, maltose, ethanol and lactic, citric and acetic acid concentrations were analyzed by HPLC, after membrane filtration (Whatman, 0.45 μm) of samples, using an Aminex HPX 87H column (Biorad, Laboratories, Richmond, CA, USA) with a 0.8 ml min^{-1} flow of H_2SO_4 6 mM at 65°C and a refractive index detector.

Amylolytic activity was measured incubating 0.1 ml of appropriately diluted enzyme with 0.8 ml of a solution containing 1.25% of soluble starch (Prolabo) in 0.1 M citrate–phosphate buffer (pH 5.5) at 55°C. The reaction was stopped in 10 min by addition of 0.1 ml of H_2SO_4 1 M and the residual starch determined by the iodine method described above. One enzyme unit was defined as the amount of enzyme that allows the hydrolysis of 10 mg of starch in 30 min under the conditions described above.

Enzyme partial purification

The enzyme was produced in glycogen MRS medium, in bioreactor culture using the conditions described above. After 20 h of incubation, cells were removed by centrifugation (10,000 rev. min^{-1} for 15 min at 4°C) and the supernatant filtered through a 0.45-μm cellulose filter. Ammonium sulphate was then added slowly, with constant agitation at 4°C, up to 70% of saturation. Precipitated proteins were recovered by centrifugation (15,000 rev. min^{-1} for 30 min at 4°C) and redissolved in 30 ml of KH_2PO_4/Na_2HPO_4 50 mM buffer (pH 6.8). The enzyme solution was concentrated by ultrafiltration with a PM-10 Amicon membrane, the retentate desalted by means of a final diafiltration step, to a final volume of 7 ml.

Kinetics

The apparent Michaelis constant (K_m) of the concentrated enzyme was determined with different concentrations of soluble starch (Prolabo, France) and glycogen obtained from MPW (94.25% purity), at 25 and 15 min, respectively, at 55°C and pH 5.5. Values were adjusted to the Michaelis–Menten equation and parameters obtained by the Quasi-Newton minimisation method.

Results and discussion

Screening of different lactic acid bacteria strains for their amylolytic capability

To compare the amylolytic aptitude of four available strains, cultivation was conducted in flasks, in an MPW medium, that contained 12.3 g l^{-1} glycogen, and on an MRS medium in which glucose was replaced by 8 g l^{-1} soluble starch.

The results obtained with the different strains grown on MPW, compared with MRS–starch (*Figure 1*), showed in all cases a lower use of the carbon source, lower growth and lower, and more variable, enzymatic activity levels. The fact that growth stopped with MPW medium before reaching a complete exhaustion of

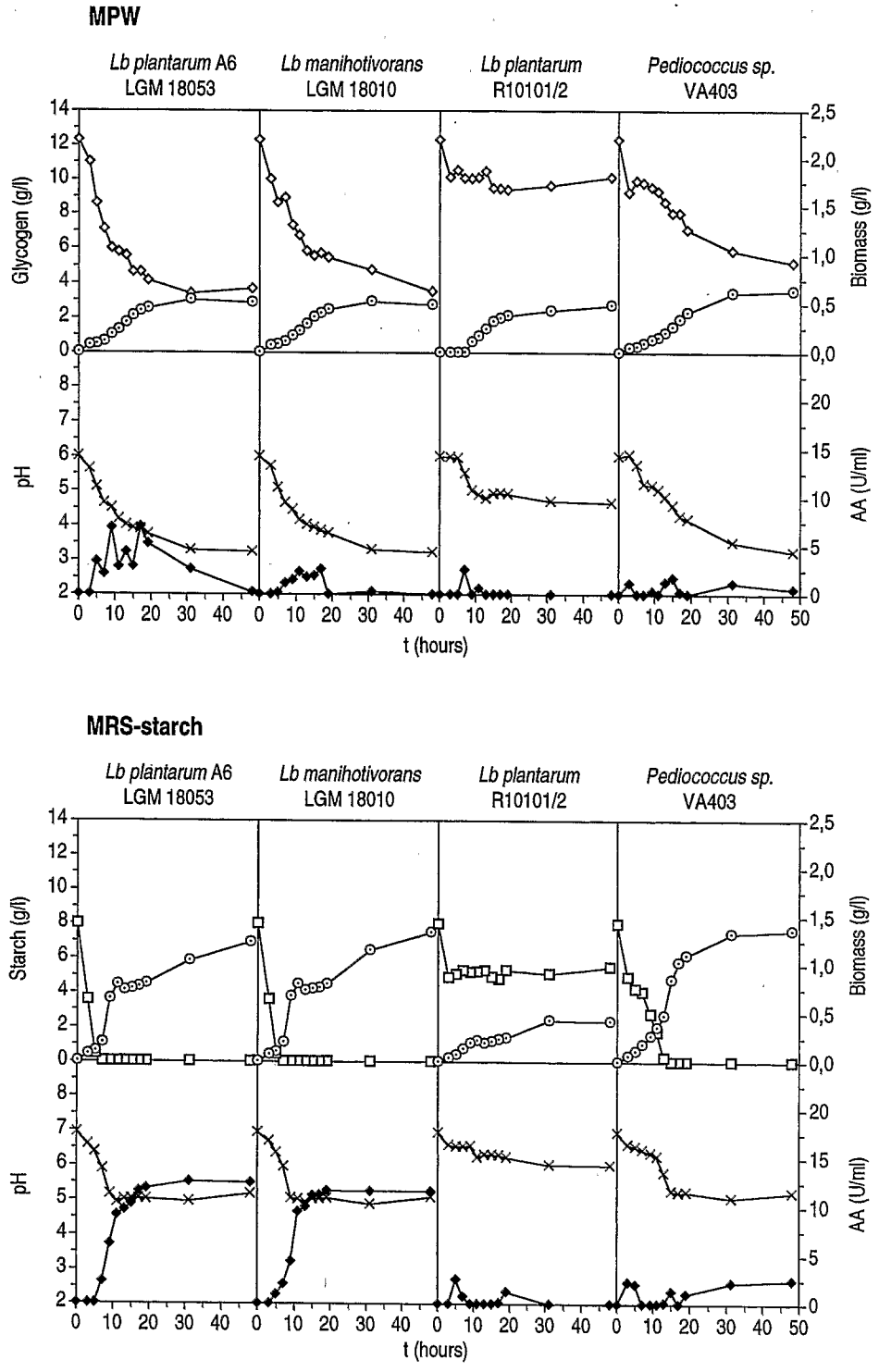

Figure 1 Screening of four lactic acid bacterial strains on MPW (above) and on MRS–starch (below). ◇, Glycogen; □, starch; ⊙, biomass; ♦, amylolytic activity (AA); ×, pH

glycogen may be due to the decrease in pH < 4.0, that disfavours amylolytic activity[19] and growth.[20]

Meanwhile, in cultures on MRS–starch, probably because it is a more buffered medium, pH kept in values above 4.0. With the exception of the strain R10101/2, which has a low amylolytic activity, in the other cases a total consumption of the carbon source occurred. In particular with *L. plantarum* A6 and *L. manihotivorans*, starch degradation took place in the first 7 h of culture. *Pediococcus* sp. VA403, was able to degrade starch totally and to reach levels of growth similar to the other amylolytic strains tested. Nevertheless amylolytic activity was not detectable in high amount in the free cell supernatant, suggesting that

amylolytic activity might be, in this strain, bound to cell membrane.

Comparison between kinetics of Lactobacillus plantarum A6 on MRS with glucose, starch or glycogen, and MPW

From the two most amylolytic strains: *L. plantarum* A6 and *L. manihotivorans*, the first one was selected for its higher levels of amylase activity on MPW. With this strain, kinetics of cultures on MRS medium with 1% (w/v) of different sources of carbon: glucose, soluble starch and mussel glycogen — obtained from MPW — were analyzed, and the results compared with the ones obtained in MPW medium (*Figure 2*).

No enzyme production took place with MRS–glucose (only a residual activity due to inoculum was detected) suggesting that amylase biosynthesis may be repressed by glucose.[14] From the results obtained on MRS–glycogen medium it is evident that the strain is able to hydrolyse the glycogen from MPW and achieve an amylolytic activity similar to that obtained in MRS–starch. In all the MRS based media pH stabilized at values over 4.0, favourable for enzyme activity.

In MRS–starch medium, culture kinetics showed a fast hydrolysis of starch with a high increase in reducing sugars (5.48 g l^{-1} at 4 h) and a rapid sugar consumption (0.97 g l^{-1} h^{-1}). Meanwhile, in MRS–glycogen medium the slope of sugar consumption was lower (0.76 g l^{-1} h^{-1}). Due to a slower breakdown of glycogen, reducing sugars seemed to be assimilated at the same time they were liberated, resulting in a lower level in the medium. Although glycogen disappeared totally, approximately 20% of total sugars remained not assimilated by the strain. This fraction probably constitutes a mussel glycogen limit dextrin to the amylases produced by *L. plantarum* A6.

Lactic acid production, in MRS–glucose, MRS–starch and MRS–glycogen media was coupled with biomass production (*Figure 2*). Culture in MRS–glucose medium showed a rapid uptake of glucose that was transformed directly to lactic acid, reaching higher acid and lower biomass productions than with MRS–starch. In MRS–glycogen both, biomass and lactic acid production were lower, due to reduced utilization of the substrate.

Concerning the decrease in lactic acid associated with acetic acid increase during the stationary phase

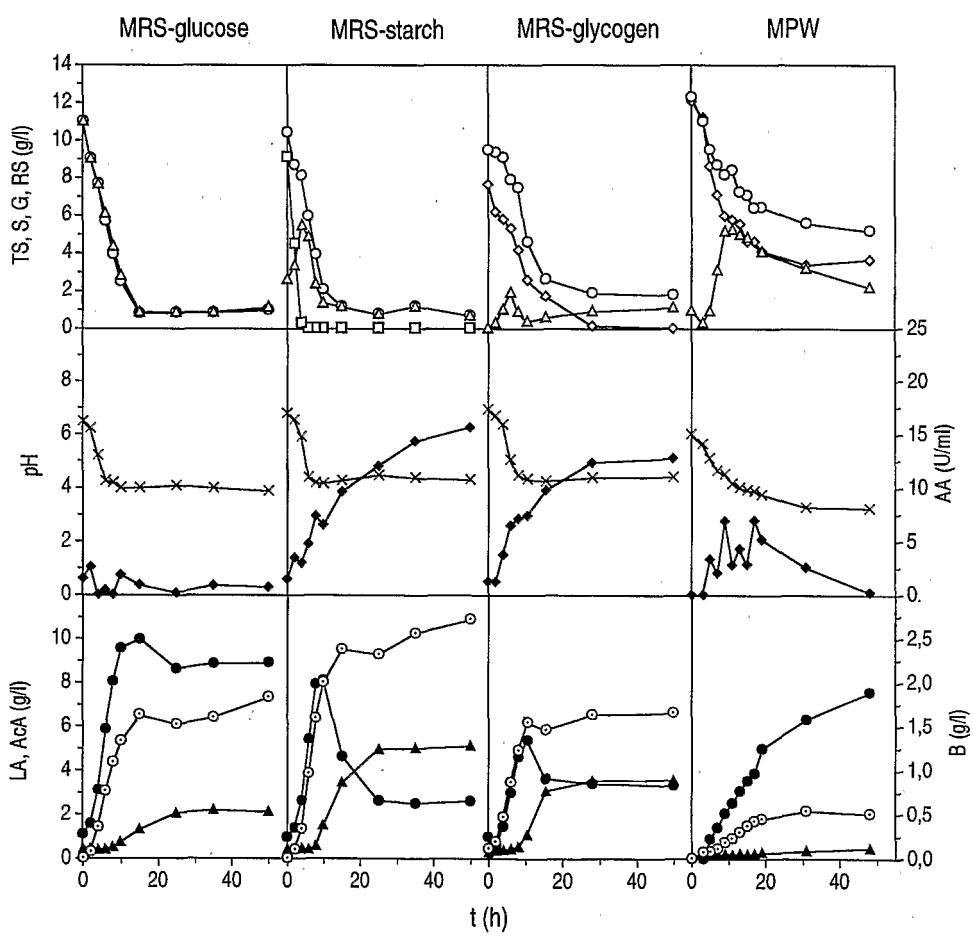

Figure 2 Fermentation of *L. plantarum* A6 on MRS–glucose, MRS–starch, MRS–glycogen and MPW medium. ○, Total sugars (TS); □, starch (S); ◇, glycogen (G), △, reducing sugars (RS); ×, pH; ♦, amylolytic activity (AA); ●, lactic acid (LA); ▲, acetic acid (AcA); ⊙, biomass (B)

(*Figure 2*), it is known that some lactic acid bacteria are able to use lactic acid as an energy source. After the exhaustion of glucose, lactic acid can be converted in acetic acid in the presence of oxygen by *Lactobacillus plantarum* taking the pyruvate oxidase pathway.[21] No oxidation of lactate was observed in cultures of the strain *L. plantarum* A6 under nitrogen atmosphere with any of the carbon sources tested (not shown data). Here, in aerobic conditions, it seems that the associated metabolism of lactic and acetic acids is related to the polymeric substrate (starch or glycogen) because it is not so clearly observed when glucose is the carbon substrate. On MPW medium no lactate decrease occurred during stationary phase because there still are available sugars.

In MPW cultures, the high level of reducing sugars remaining in the medium could be due to a limitation of growth caused by some nutrient deficiency, e.g. proteins. The production of lactic acid was maintained at the expense of these reducing sugars, although growth had stopped, showing uncoupling between growth and energy production.

Table 1 shows the maximal productions and yields for the assayed media. It should be noted that in MRS–glycogen, although productions are lower, the yields of amylolytic activity (both in consumed sugar and produced biomass) and lactic acid are higher than with MRS–starch.

It can be concluded that the amylases produced by the strain were capable of hydrolysing glycogen although at lower rates than starch. This may diminish inhibition or repression phenomena by reducing sugars and may be the reason why higher yields of enzyme with respect to biomass are produced in media with glycogen. The limited growth with MPW medium could be due to an unbalanced nutrient composition as well as to the poor buffering capacity of the medium, leading to lower final pH. Nutrient deficiency and/or low pH might explain the observed uncoupling between growth and energy.

Kinetic parameters on starch and glycogen of the amylase produced

The amylase activities of the supernatants from 20-h-old cultures in MRS–starch and MRS–glycogen, in a reactor with controlled pH at 6.0, were studied on both substrates: soluble starch and glycogen from MPW. For hydrolysis studies, enzyme concentration was adjusted to 20.6 U ml^{-1}, substrate concentration 10 g l^{-1} (pH 5.5) and temperature 55°C.

Amylolysis was followed by three different methods: (i) measuring the decrease in iodine-staining; (ii) the total ethanol precipitable residual carbohydrates; and (iii) reducing sugars formed.

The amylolytic patterns (*Figure 3*) for starch and glycogen degradation by the enzymes produced in MRS–starch and MRS–glycogen were similar, and correspond to the α-amylase produced by *L. plantarum* A6, characterized by Giraud *et al.*[19] Thus, the different substrates in the culture medium did not seem to induce the production of different types of amylases. In both cases, the maximum level of reducing sugars produced with starch was higher than with glycogen.

For the comparison of the hydrolysis of soluble starch and glycogen, the iodine method, that was valid for determining the amylolytic activity on starch (forming a blue coloured complex with amylose), was not valid for glycogen as the brown colour produced (as with amylopectin) was not sufficiently intense for accurate measurement (data not shown). As for the measure of residual total carbohydrates, differences were found in the precipitation of both polysaccharides with absolute ethanol: while mussel glycogen precipitates totally (in fact it was obtained from the effluent by precipitation with ethanol), only 80% precipitated for the commercial soluble starch. This may lead to errors in result comparison, and so this method was rejected. The determination of reducing sugars formed seemed to be the most reliable method, and so was used for direct comparison of the results of hydrolytic activities on starch and glycogen, and for the determination of the kinetic parameters.

The determination of the kinetic parameters was done with the enzyme from MRS–glycogen culture supernatant, partially purified following the described methodology. *Table 2* shows the data obtained in the different steps of concentration of the amylase. It was possible to concentrate to 3.1 times specific activity, with a yield of 83.6% of total activity.

The apparent K_m of the concentrated enzyme for soluble starch and glycogen from MPW, in both cases at 55°C and pH 5.5, were found to be 4.50 and 5.22 g l^{-1}, respectively. Both values, although slightly lower

Table 1 Culture parameters of *Lactobacillus plantarum* A6 on medium with glucose (MRS), starch (MRS–starch), mussel glycogen (MRS–glycogen) and on mussel processing wastes (MPW)

Medium	μ (h^{-1})	X max (g l^{-1})	AA max (U ml^{-1})	P max (g l^{-1})	Y x/s (g g^{-1})	Y aa/s (g g^{-1})	Y p/s (g g^{-1})	Y aa/x (g g^{-1})
MRS	0.39	1.83	–	9.97	0.17	–	0.87	–
MRS–starch	0.44	2.72	15.7	8.10	0.27	1.41	0.86	5.42
MRS–glycogen	0.38	1.66	12.8	5.37	0.20	1.54	0.94	7.55
MPW	0.20	0.54	7.0	7.56	0.08	1.14	0.89	12.56

Temperature 30°C. μ: growth rate; X max: maximal biomass production; AA max: maximal amylolytic activity; P max: maximal lactic acid production; Y x/s: biomass/substrate yield; Y p/s: lactic acid/substrate yield; Y aa/x: amylolytic activity/biomass yield

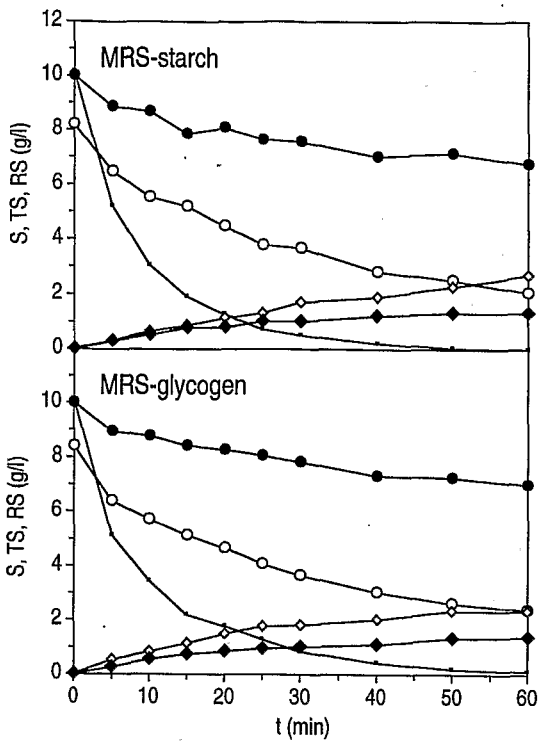

Figure 3 Kinetics of degradation of starch (black symbols) and glycogen (white symbols), at 55°C and pH 5.5, by post-incubates of *L. plantarum* A6 cultured in MRS–starch and with MRS–glycogen media, measured by colour disappearance of the starch–iodine complex (■), total sugars precipitable with ethanol (●,○) and reducing sugars formed (◆,◇)

for starch, are very close, which confirms the capability of the enzyme to hydrolyse glycogen from MPW with similar affinity to starch. The different maximum level of reducing sugars produced with the same concentration of substrate (*Figure 3*) is therefore not due to different enzyme affinity but may be related to the different grade of ramification of glycogen and starch. As reported by Fogarty,[22] α-1,6 glucosidic linkages in branched polymers like glycogen can not be attacked by α-amylases. Moreover, formation of enzyme substrate complexes appears to be restricted by the presence of branch (α-1,6) linkages, involving slow hydrolysis of specific bonds near the branch points of the α-limiting dextrin.

The more limited use of glycogen in MPW than in MRS, by *L. plantarum* A6, seemed to be due to inadequate pH for enzyme activity or to nutritional limitations in MPW. Therefore, we studied whether pH conditions or medium composition could be improved.

pH regulation by addition of $CaCO_3$

Addition of 5.6% (p/v) calcium carbonate, a classical buffering agent in lactic fermentation,[12] to the MPW medium was done in order to maintain adequate pH values for the enzymatic activity. Additionally calcium has a specific effect on enzyme stability to extremes pH and temperatures, treatment with urea or exposure to proteases.[22]

The results (*Figure 4*) showed that addition of $CaCO_3$ allowed the pH to be maintained at values above 5.0 extending amylolytic activity and promoting higher use of glycogen towards lactic acid production. Nevertheless biomass and lactic acid production continued to be uncoupled. The low levels of amylase activity and biomass obtained, similar to control culture, compared with the results obtained in MRS–glycogen (*Figure 2*), suggest that some nutritional deficiencies in MPW could explain the observed uncoupling.

Effect of nitrogen concentration. Addition of phosphorus and different levels of protein to MPW

In comparison with MRS medium, the concentrations of phosphorus and nitrogen in MPW are substantially lower. Therefore, the MPW medium was supplemented with KH_2PO_4 up to the same levels of MRS (0.47 g l^{-1} total P), and three different initial concentrations of total proteins were tested: 10, 15 and 20 g l^{-1}. The supplement consisted in a mixture (2:2:1) of casein peptone (Casitone, DIFCO, USA), meat extract (DIFCO, USA) and yeast extract (LABOSI, France). The results (*Figure 5*) showed small effect of protein supplementation on amylolytic activity and on lactic acid production, but in a more relevant way on biomass production. Increasing protein content to 15 g l^{-1} appeared to be sufficient to increase biomass production. Phosphorus and protein supplementation seemed to restore coupling between growth and lactate production. Nevertheless, even with the same levels of phosphorus and protein as MRS, pH decreased in supplemented MPW to lower values. Therefore, with MPW, a

Table 2 Concentration of the amylase produced in MRS–glycogen with controlled pH 6.0 (see Materials and Methods Section)

Steps	Volume (ml)	Proteins (mg)	AA (U)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Supernatant	760	102.7	11,392	111	100.0	1.0
Precipitate	30	32.2	11,109	345	97.5	3.1
Ultrafiltrate	7	27.5	9527	346	83.6	3.1

AA: amylolytic activity

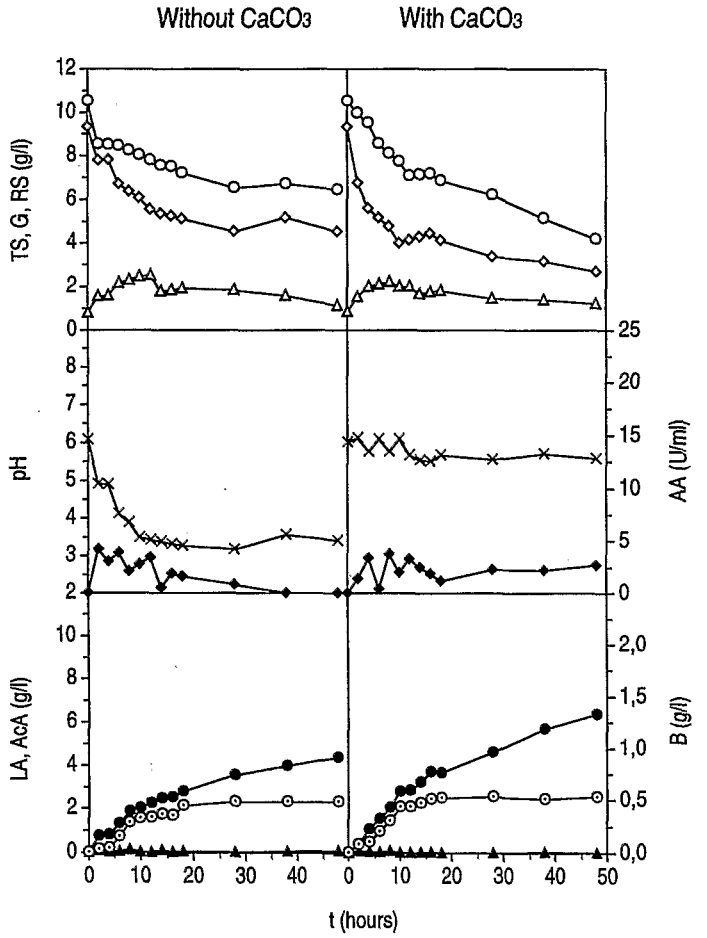

Figure 4 Culture kinetics of *L. plantarum* A6 on MPW with and without 5.6% $CaCO_3$. ○, Total sugars (TS); ◇, glycogen (S); △, reducing sugars (RS); ×, pH; ♦, amylolytic activity (AA); ●, lactic acid (LA); ▲, acetic acid (AcA); ⊙, biomass (B)

less buffered medium, pH should be controlled for the maintenance of enzyme activity.

pH-controlled culture in supplemented mpw medium

A culture in a bioreactor, with MPW supplemented with P and protein to the levels established in the preceding culture (0.47 g l^{-1} total P and 15 g l^{-1} protein), was conducted with pH maintained at 6.0. The results (*Figure 6*) showed that with pH control nearly total hydrolysis of glycogen was reached in 24 h of culture, leaving only the fractions that could not be hydrolyzed by the amylase produced. Growth, and associated amylase and lactic acid production, stopped at approximately 15 h of culture. The maximal values of biomass, amylolytic activity and lactic acid production, and their respective yields (w/w) are shown in *Table 3*. With regard to the yields of lactic acid on glycogen, as expected, pH control and supplementation of MPW allowed better substrate conversion efficiencies than for experiments performed at non-regulated pH in unsupplemented MPW (*Table 1*).

The amylolytic activity obtained is higher than with MRS–glycogen without pH control (12.8 U ml^{-1} at 30 h) and with pH control (17.6 U ml^{-1} at 20 h, obtained in the culture for enzyme purification). Therefore, pH control seems to be more important in MPW with a bigger improvement of enzyme production and yields, than in MRS–glycogen.

Conclusions

This work demonstrates the aptitude of amylolytic lactic acid bacteria to hydrolyse the glycogen in mussel processing effluents and opens a new way for the exploitation of these wastes.

The α-amylase producing strain *Lactobacillus plantarum* A6 assayed, was able to use glycogen from MPW although at a slower rate than soluble starch. The lower level of reducing sugars in the medium with glycogen may diminish enzyme repression and explain why higher yields of enzyme were achieved. However, the different substrates used in the medium did not seem to condition the production of different types of amylase and the values of the apparent K_m for starch and for glycogen were very close. Therefore, the dif-

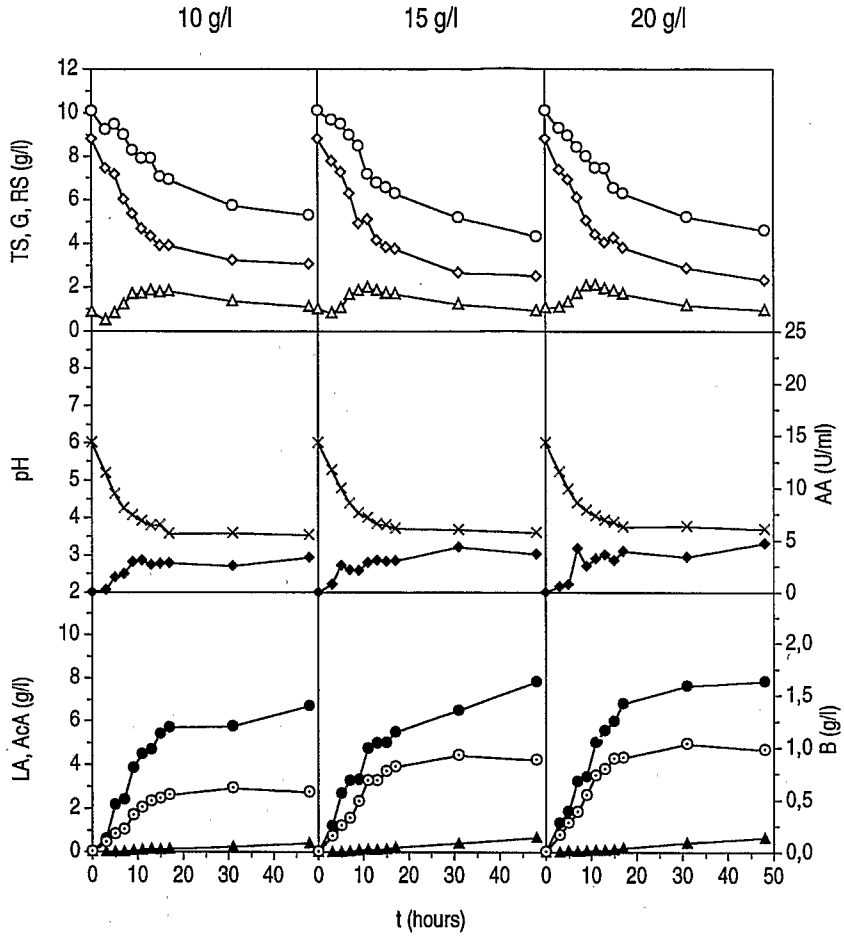

Figure 5 Time course of *L. plantarum* A6 culture on MPW supplemented with KH_2PO_4 up to 0.47 g P l^{-1} and three levels of initial protein: 10, 15 and 20 g l^{-1}. ○, Total sugars (TS); ◇, glycogen (S); △, reducing sugars (RS); ×, pH; ♦, amylolytic activity (AA); ●, lactic acid (LA); ▲, acetic acid (AcA); ⊙, biomass (B)

ferences in the use of the substrate (with a fraction approximately 20% of the total sugars from mussel glycogen that remained undegraded) seem to be associated more with the higher grade of ramification of glycogen than with enzyme affinity for this substrate.

Culture on MPW showed a decrease in pH unfavourable for amylolytic activity and an uncoupling between lactic acid and biomass production. The control of pH to maintain enzyme activity and the increase of initial protein concentration to avoid uncoupling allowed similar lactic acid production and higher yields than with soluble starch at non-controlled pH, to be reached with MPW.

Therefore, the direct use of the effluents would only require protein and phosphorus supplementation and pH control. The deficit of protein could be partially corrected using the effluents directly, without the previous step of clarification[15] in which approximately 2 g l^{-1} protein were recovered by acid precipitation. Both the levels of maximal production and yields of lactic acid and biomass, obtained in short term cultures, could be interesting from an economical point of view,

Table 3 Culture parameters of *Lactobacillus plantarum* A6 on mussel processing wastes (MPW), supplemented of protein and phosphate, and controlled pH=6.0

μ (h^{-1})	X max (g l^{-1})	AA max (U ml^{-1})	P max (g l^{-1})	Y x/s (g g^{-1})	Y aa/s (U g^{-1})	Y p/s (g g^{-1})	Y aa/x (U g^{-1})
0.43	1.55	24.0	8.41	0.24	3.56	0.98	14.65

Temperature 30°C. μ: growth rate; X max: maximal biomass production; AA max: maximal amylolytic activity; P max: maximal lactic acid production; Y x/s: biomass/substrate yield; Y p/s: lactic acid/substrate yield; Y aa/x: amylolytic activity/biomass yield

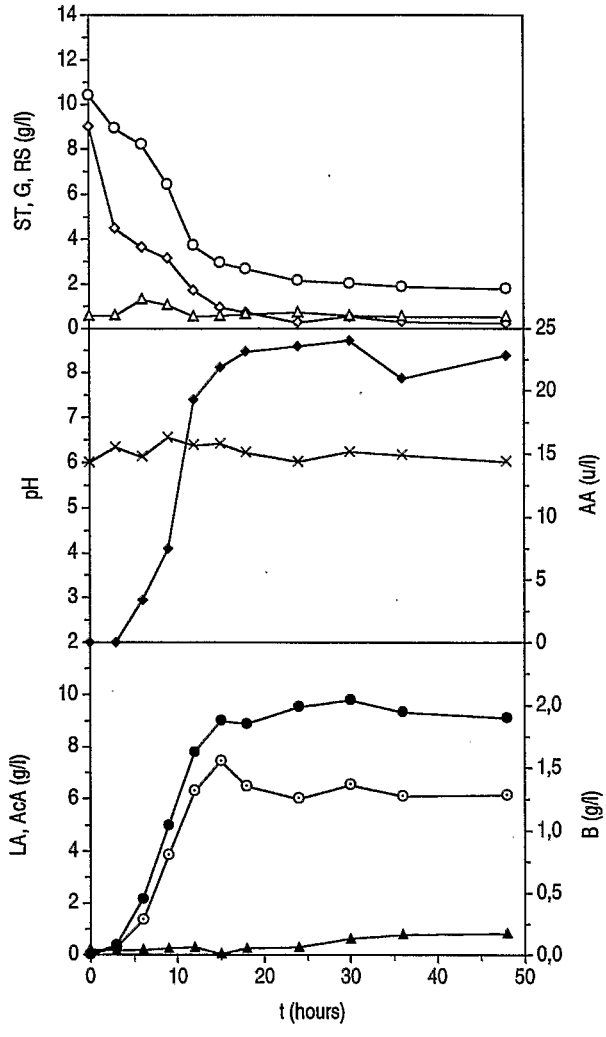

Figure 6 Culture of *L. plantarum* A6 on supplemented MPW medium (15 g l^{-1} protein and 0.47 g l^{-1} total P) in a reactor with pH controlled at 6.0. ○, Total sugars (TS); ◇, glycogen (S); △, reducing sugars (RS); ×, pH; ♦, amylolytic activity (AA); ●, lactic acid (LA); ▲, acetic acid (AcA); ⊙, biomass (B)

as compared with those obtained industrially by fermentation of other carbohydrates.[23]

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References

1. Murado, M. A., González, M. P., and Pastrana, L. Mussel processing wastes as a fermentation substrate. In: *Fisheries Processing: Biotechnological Applications* (Martin, A. M., Ed.). Chapman and Hall, London, 1994, 311–343
2. Pastrana, L., González, M. P., and Murado, M. A. Production of gibberellic acid from mussel processing wastes in submerged batch culture. *Bioresource Technol.* 1993, **45,** 213–221
3. Pintado, J., González, M. P., and Murado, M. A. Interactions between pretreatment of the medium and nutrient concentration of mussel processing effluents for citric acid production. *Enzyme Microb. Technol.* 1997, **20,** 544–549
4. Murado, M. A., González, M. P., Torrado, A., and Pastrana, L. M. Amylase production by solid state culture of *Aspergillus oryzae* on polyurethane foams. Some mechanistic approaches from an empirical model. *Process Biochem.* 1997, **32,** 35–42
5. Sen, S. and Chakrabarty, S. L. Amylase from *Lactobacillus cellobiosus* D-39 isolated from vegetable wastes: characteristics of immobilized enzyme and whole cell. *Enzyme Microb. Technol.* 1987, **9,** 112–116
6. Nakamura, L. K. *Lactobacillus amylovorus,* a new starch hydrolysing species from cattle waste-corn fermentations. *Int. J. Syst. Bacteriol.* 1981, **31,** 56–63
7. Lindgren, S. and Refai, O. Amylolytic lactic acid bacteria in fish silage. *J. Appl. Bacteriol.* 1984, **57,** 221–228
8. Giraud, E., Champailler, A., and Raimbault, M. Degradation of raw starch by a wild amylolytic strain of *Lactobacillus plantarum. Appl. Environ. Microbiol.* 1994, **60,** 4319–4323
9. Morlon-Guyot, J., Guyot, J. P., Pot, B., Jacobe de Hault, I., and Raimbault, M. *Lactobacillus manihotivorans* sp. nov., a new starch-hydrolyzing lactic acid bacterium isolated from cassava sour starch fermentation. *Int. J. Sys. Bacteriol.* 1998, **48,** 1101–1109
10. Champ, M., Szylit, O., Raibaud, P., and Aït-Abdelkader, N. Amylase production by three *Lactobacillus* strains isolated from chicken crop. *J. Appl. Bacteriol.* 1983, **55,** 487–493
11. Cotta, M. A. Amylolytic activity of selected species of ruminal bacteria. *Appl. Environ. Microbiol.* 1988, **54,** 772–776
12. Chatterjee, M., Chakrabarty, S. L., Chattopadhyay, B. D., and Mandal, R. K. Production of lactic acid by direct fermentation of starchy wastes by an amylase-producing *Lactobacillus. Biotechnol. Lett.* 1997, **19,** 873–874
13. Pompeyo, C. C., Gómez, M. S., Gasparian, S., and Morlon-Guyot, J. Comparison of amylolytic properties of *Lactobacillus amylovorus* and of *Lactobacillus amylophilus. Appl. Microbiol. Biotechnol.* 1993, **40,** 266–269
14. Giraud, E., Brauman, A., Keleké, S., Lelong, B., and Raimbault, M. Isolation and physiological study of an amylolytic strain of *Lactobacillus plantarum. Appl. Microbiol. Biotechnol.* 1991, **36,** 379–383
15. Murado, M. A., González, M. P., Pastrana, L., Siso, M. I. G., Mirón, J., and Montemayor, M. I. Enhancement of the bioproduction potential of an amylaceous effluent. *Bioresource Technol.* 1993, **43,** 155–163
16. Man, J. C., Rogosa, M., and Sharpe, M. E. A medium for the cultivation of *Lactobacilli. J. Appl. Bacteriol.* 1993, **23,** 130–135
17. Strickland, J. D. H. and Parsons T. R. A practical handbook of sea water analysis. *Bull. Fish. Res. Board Can.* 1968, **167,** 57–62
18. Bernfeld, P. Enzymes of starch degradation and synthesis. *Adv. Enzymol.* 1951, **12,** 379–427
19. Giraud, E., Gosselin, B., Marin, B., Parada, J. L., and Raimbault, M. Purification and characterization of an extracellular amylase from *Lactobacillus plantarum* strain A6. *J. Appl. Bacteriol.* 1993, **75,** 276–282
20. Giraud, E., Lelong, B., and Raimbault, M. Influence of pH and initial lactate concentration on the growth of *Lactobacillus plantarum. Appl. Microbiol. Biotechnol.* 1991b, **36,** 96–99
21. Divies, C., Frey, L., Hubert, J. C., and Roissart, H. Métabolisme d'autres substrats carbonés par les bactéries lactiques. In: *Bactéries Lactiques,* Vol. 1 (Roissart, H. and Luquet, F. M., Eds.). Lorica, Uriage, 1994, 291–307
22. Fogarty, W. M. Microbial amylases. In: *Microbial Enzymes and Biotechnology* (Fogarty, W. M., Ed.). Applied Science Publishers, London, 1983, 1–92
23. Miall, L. M. Organic acids. In: *Primary Products of Metabolism. Economic Microbiology,* Vol. 2 (Rose, A. H., Ed.). Academic Press, London, 1978, 95–98