Appl Biochem Biotechnol (2009) 152:262–274
DOI 10.1007/s12010-008-8252-x

Minerals and Organic Nitrogen Present in Grape Marc Hydrolyzates Enhance Xylose Consumption by *Lactobacillus pentosus*

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Received: 23 December 2007 / Accepted: 7 April 2008 /
Published online: 26 June 2008

Abstract This work deals with the nutritional evaluation of grape marc hydrolyzates as fermentation medium for *Lactobacillus pentosus*. Usually, the fermentation of xylose and arabinose in the presence of glucose remains a primary obstacle for economical biomass conversion. The few microorganisms that can grow simultaneously on both pentose and hexose sugars contained in lignocellulosic feedstocks typically grow slowly and demonstrate marginal yields and productivities. Moreover, lignocellulosic hydrolyzates contain phenolic compounds and other components originated by the degradation of sugars that can inhibit lactic acid fermentation. However, in this case, grape marc hydrolyzates not only did not need a detoxification stage, but it also improved the xylose consumption by *Lactobacillus pentosus* with a faster and more efficient conversion of hemicellulosic sugars compared with synthetic media. After analysis of grape marc hydrolyzates, it was observed that minerals such as K (2,707 mg/L), Ca (3,681 mg/L), and Mg (198.5 mg/L) are present in higher concentration than those found in the general medium of *Lactobacillus* (1,705 mg/L of K, 58.3 mg/L of Ca, and 27.0 mg/L of Mg). Moreover, grape marc hydrolyzates contain an additional source of nitrogen (9.2 g/L) which, together with their elevated mineral concentration, improved lactic acid fermentation compared with synthetic media.

Keywords Grape marc · Hydrolyzates · Nutritional components · *Lactobacillus pentosus* · Fermentation

Introduction

Lignocellulosic feedstocks are largely composed of cellulose, hemicelluloses, and lignin. Lactic acid bacteria can efficiently ferment glucose-containing solutions coming from cellulose solubilization. Nevertheless, the conversion of the pentoses liberated during the

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fractionation of the hemicellulose fraction, mainly xylose and arabinose, has been proven to be more difficult [1]. Because the cost of culture medium components can represent more than 20% of all process costs, an economical biomass conversion process critically depends on the rapid and efficient conversion of essentially all the sugars present in both the cellulose and hemicellulose fractions [1]. In consequence, fermentation of xylose and arabinose in the presence of glucose remains as a primary obstacle for economical biomass conversion. The few microorganisms that can grow simultaneously on both pentose and hexose sugars contained in lignocellulosic feedstocks typically grow slowly and demonstrate marginal yields and productivities [1].

Usually, in many bacteria, pentoses such as xylose must be transported before they can be isomerized and phosphorylated by intracellular enzymes to D-xylulose-5-P (D-xylose isomerase and D-xylulose kinase in the case of xylose [2]), and finally metabolized through the phosphoketolase pathway (Fig. 2). *Lactobacillus pentosus* is able to efficiently transport and metabolize D-xylose, although the gene(s) encoding the D-xylose transport function could not be identified in the *xyl* gene cluster [2]. Chaillou et al. [2] also demonstrated that the transport of D-xylose is the rate controlling step in the metabolism and growth of *L. pentosus*. Arabinose, the second most abundant pentose-sugar in this kind of materials, is usually metabolized by bacteria in the L-form but, in certain cases, D-arabinose can be used as a carbon and energy source by D-arabinose conversion to D-ribulose and posterior transformation to D-ribulose-5-phosphate, an intermediate in the pentose phosphate pathway. The enzymes involved in these reactions belong to the L-fucose and ribitol catabolism pathways [3]. D-arabinose is transported into the cell by an L-fucose permease and isomerized to D-ribulose with the L-fucose isomerase (also called D-arabinose isomerase). D-ribulose, which is an intermediate and inducer of the ribitol catabolic pathway, is then phosphorilated to D-ribulose-5-phosphate by the D-ribulokinase of the ribitol pathway.

Several works deal with the utilization of genetically engineered lactic acid bacteria to enhance pentose consumption [4, 5]. However, in a previous work [6] we have found that *L. pentosus* is able to use a wide range of carbon sources such as glucose, xylose, and arabinose in fermentations performed using hemicellulosic sugars from agricultural residues. However, scarce studies have reported the influence of hemicellulosic hydrolyzate components on this fermentation. In this way, Chaillou et al. [2] found that low concentrations of D-mannose, a minority sugar in these materials, enhance the growth of *L. pentosus* on D-xylose. Garde et al. [7] indicate that the presence of glucose in the fermentation media, at concentrations higher than 5 g/L, represses the bioconversion of xylose into lactic acid by *Lactobacillus xylosus*. In addition, lactic acid bacteria are generally recognized as nutritionally fastidious [8] as they need nutritional media with very high requirements, the general media for lactic acid bacteria being composed of peptone, yeast extract, and minerals such as Mg, Mn, and Fe [8, 9], although corn steep liquor and yeast extract have been also proposed as *Lactobacilli* medium [8, 10].

Hemicellulosic grape marc hydrolyzates are rich in organic nitrogen, minerals, and phenolic compounds that can affect negatively or positively the consumption of xylose during the lactic acid fermentation by *L. pentosus*. As a first approach a synthetic media was prepared using the most common sugars found in these hydrolyzates, but the lactic acid fermentation by *L. pentosus* rendered poor yields and global volumetric productivities. As a consequence, this work examines the influence of different isomeric and minor sugars, as well as other components (including organic nitrogen, minerals, and phenolic compounds) present in the hemicellulosic hydrolyzates from distilled grape marc, to see their influence during the lactic acid fermentation by *Lactobacillus pentosus.*

Materials and Methods

Raw Material Samples of grape marc were dried at room temperature and milled to a particle size suitable for hydrolysis treatment (approximately 1 mm). To carry out the chemical characterization of the raw material, grape marc was submitted to a quantitative acid hydrolysis [10], given the following composition: cellulose, 10.8%; hemicelluloses, 11.2% (xylan, 7.5%; araban, 2.2%; and acetyl groups, 1.6%); lignin, 50.9%; extracts, 14.7%; and others, 12.5%.

Grape Marc Hemicellulosic Hydrolyzate The grape marc hemicellulosic hydrolyzate was prepared by means of an acid hydrolysis of grape marc carried out in autoclave at 130 °C with 3% sulfuric acid for 30 min using a liquid/solid ratio of 8 g/g, and posterior solids elimination by filtration.

Microorganism Lactobacillus pentosus CECT-4023T (ATCC-8041) was obtained from the Spanish collection of type cultures (Valencia, Spain). The strain was grown on plates using the complete media proposed by Mercier et al. [9] at 31 °C for 24 h. The microorganism was kept at −80 °C until use. Inocula were prepared by cell recovery from plates with 5 mL of sterile water. Biomass in inocula was measured by optical density at 600 nm and adjusted by water elimination by means of centrifugation and posterior resuspension with an adequate volume of the culture medium (hydrolyzate or synthetic) to reach a final concentration of 2.0 g/L.

Lactic Acid Fermentation Grape marc hemicellulosic hydrolyzate was neutralized with $CaCO_3$ to a final pH of 6.5, and the precipitated $CaSO_4$ was separated from the supernatant by filtration. The clarified liquors were supplemented with 10 g/L of yeast extract and 10 g/L of corn steep liquor (CSL), sterilized in autoclave (100°C/h), and inoculated with a 5% volume of the inoculum suspension. Moreover, lactic acid fermentations were also carried out employing synthetic hemicellulosic sugar solutions supplemented with 10 g/L of yeast extract and 10 g/L of corn steep liquor. In selected experiments, fermentations carried out with synthetic sugars were additionally supplemented with minerals (0.015 g/L $MnSO_4$ H_2O; 5.1 g/L K_2HPO_4; 0.045 g/L $NaOOCCH_3$; 16.3 g/L $CaSO_4$; 2.2 g/L $MgSO_4$ $7H_2O$), extra organic nitrogen (extra addition of 10 g/L yeast extract), or minerals plus extra organic nitrogen. Fermentations were carried out in orbital shakers at 150 rpm and 31 °C in 250-mL Erlenmeyer flasks with a final volume of 100 mL, in the presence of 10 g/L $CaCO_3$. Samples (2 mL) were taken at given fermentation times and centrifuged at 5,000 rpm for 10 min. The supernatants were stored for glucose, xylose, arabinose, lactic acid, and acetic acid analysis.

Analytical Methods

Glucose, xylose, arabinose, lactic acid, and acetic acid were measured by a Hewlett Packard high-performance liquid chromatographic system (HPLC) (Agilent, model 1100, Palo Alto, CA). The system consisted of an HP-1050 Intelligent Auto Sampler, an HP-1047A Refractive Index detector, and an HP-1050 pump. Separation was achieved at 50 °C using an Aminex HPX-87H (Bio-Rad) ion exclusion column eluted with 0.6 ml/min of 0.003 M sulfuric acid. The separation of hemicellulosic sugars (xylose, galactose, mannose, fucose, and rhamnose) in hydrolyzates was carried out by high-performance anion exchange

chromatography with Pulsed Amperometric Detection (HPAEC-PAD) on a Dionex equipment. Five microliters of the sample previously filtered by a 0.45-μm membrane (Sartorius, Germany) was injected and eluted at the flow ratio of 1 mL/min on a CarboPac PA-1 (4×250 mm) in combination with a CarboPac PA-1 guard column (4×50 mm) maintained at 30 °C. Although the CarboPac PA-1 column allows the separation of monosaccharides and oligosaccharides, no oligosaccharides were observed. The chromatographic separation of monosaccharides was completed in 20 min. To fulfill the chromatographic separation, a gradient of eluents (deionized water, 200 mM sodium hydroxide, and 2 M sodium acetate in 200 mM sodium hydroxide) was used. All eluents were degassed using helium.

The nitrogen content of the fermentation media was analyzed using a Thermo Finningan Flash Elemental Analyzer 1112 series (San Jose, CA), whereas minerals were analyzed using an Atomic Absorption Spectrometer 220 Fast Sequential VARIAN (Palo Alto, CA) after mineralization of the liquid medium by means of digestion with HNO_3 65% in a Microwave Labstation MarsXpress, CEM Corporation (Matthews, USA).

Phenolic compounds were extracted with ethyl acetate following the methodology proposed by Cruz et al. [11].

Results and Discussion

Influence of Isomeric Sugars on the Lactic Acid Fermentation of *Lactobacillus pentosus*

In a preliminary set of experiments, *Lactobacillus pentosus* was employed to assess the effects of sugars present in media made from raw distilled grape marc hydrolyzates or synthetic sugars simulating the same composition. Table 1 lists data concerning batch fermentations into lactic acid, observing a different behavior depending on the carbon source employed. In this way, *L. pentosus* was able to metabolize all the starting glucose and arabinose and 92.1% of the initial xylose in only 12 h when using hemicellulosic sugars from distilled grape marc (Fig. 1a). However, when a mixture of commercial sugars consisting of D-xylose, D-arabinose, and D-glucose was employed as the carbon source, xylose was scarcely consumed (43.9%), whereas no arabinose consumption was observed after 18 h (Fig. 1b). Because of this, lactic acid productivity using synthetic sugars was almost two times lower than when using grape marc hydrolyzates.

Lochner et al. [12] found that a thermophilic *Lactobacillus* utilized L-(+)-arabinose but not D-(−)-arabinose, stressing the importance of the isomer of the sugars tested. On the basis of this work, a new set of experiments was carried out replacing this sugar by the L-form. The results summarized in Table 1 and Fig. 1c indicate a complete consumption of L-arabinose, although D-xylose was scarcely consumed, with 32% of the starting sugar remaining after 30 h of fermentation. From these data, it can be supposed that hemicellulosic sugars from grape marc hydrolyzates are composed by L-arabinose instead of D-arabinose. The ability to use D-arabinose as a carbon source has been described for *Klebsiella aerogenes* [13] and *Escherichia coli* [3] mutants, involving the activity of L-fucose and ribitol catabolic pathway enzymes. In this case, *L. pentosus* does not have the enzymes to metabolize D-arabinose, but it has L-arabinose isomerase, which transforms L-arabinose into L-ribulose, following the scheme showed in Fig. 2. Figure 2 also shows the *L. pentosus* pentose phosphate pathway for xylose and glucose consumption. As has been demonstrated previously [14], *L. pentosus* is a facultative heterofermentative microorganism,

Table 1 Fermentative parameters of fermentations carried out with synthetic sugars or hemicellulosic sugars from grape marc.

Carbon source				
	Grape marc hydrolyzates	Synthetic xylose, glucose, and D-arabinose	Synthetic xylose, glucose, and L-arabinose	Grape marc hydrolyzates without phenolic compounds
Time (h)	12	18	30	12
Xylose $_{t=0}$ (g/L)	7.6	7.6	8.1	8.3
Xylose $_{t=\text{final}}$ (g/L)	0.6	4.1	2.6	0.9
Xylose consumed (%)	92.1	45.9	68.1	89.3
Glucose $_{t=0}$ (g/L)	2.8	3.1	2.9	3.0
Glucose $_{t=\text{final}}$ (g/L)	0.0	0.0	0.0	0.0
Glucose consumed (%)	100.0	100.0	100.0	100.0
Arabinose $_{t=0}$ (g/L)	1.9	2.5	2.7	2.4
Arabinose $_{t=\text{final}}$ (g/L)	0.0	2.5	0.0	0.0
Arabinose consumed (%)	100.0	0.0	100.0	100.0
Lactic acid $_{t=0}$ (g/L)	2.5	1.0	0.9	2.4
Lactic acid $_{t=\text{final}}$ (g/L)	9.0	6.0	6.5	9.5
Acetic acid $_{t=0}$ (g/L)	2.4	2.0	2.0	2.2
Acetic acid $_{t=\text{final}}$ (g/L)	5.4	3.9	5.3	5.5
Q_P (g/L h)[a]	0.54	0.28	0.19	0.59
$Y_{P/S}$ (g/g)[a]	0.55	0.77	0.50	0.55
Theoretical yield (%)[b]	68.9	97.7	71.4	69.1

Data correspond to the time of maximum lactic acid production

[a] Nomenclature: Q_P, global volumetric productivity of lactic acid; $Y_{P/S}$, sugars to lactic acid yield (g lactic acid produced) /(g xylose consumed + g glucose consumed + g arabinose consumed).

[b] (g lactic acid produced × 100)/((g xylose consumed × 0.6) + (g glucose consumed) + (g arabinose consumed × 0.6)).

degrading hexoses (glucose) via the Embden–Meyerhoff–Parmas pathway (EMP-P) and pentoses (xylose and arabinose) via the phosphoketolase pathway (PK-P).

Influence of Organic Nitrogen, Minerals, and Phenolic Compounds on the Lactic acid Fermentation of *Lactobacillus pentosus*

In spite of the improvements achieved in the previous experiments, when fermentations of hemicellosic sugars from grape marc (Fig. 1a) were compared with fermentations carried out with commercial D-xylose, D-glucose, and L-arabinose (Fig. 1c), a lower productivity of lactic acid and a lower xylose consumption were observed in the last case (Table 1). This may be caused by the fact that hydrolyzates from grape marc contain other components such as organic nitrogen, minerals, and phenolic compounds that could stimulate xylose consumption.

Several studies have reported the presence of phenolic compounds in hemicellulosic hydrolyzates obtained from different feedstocks [11, 15]. To study their influence on lactic acid fermentation by *L. pentosus*, these phenolic compounds were removed from hemicellulosic hydrolyzates by extraction with ethyl acetate [15]. Using this methodology, 98–99% of the initial phenolic compounds indirectly measured as the reduction in the absorbance of the medium at 279 nm were removed. Figure 3 shows the lactic acid profile as well as the sugar consumption for fermentation media prepared using hemicellulosic

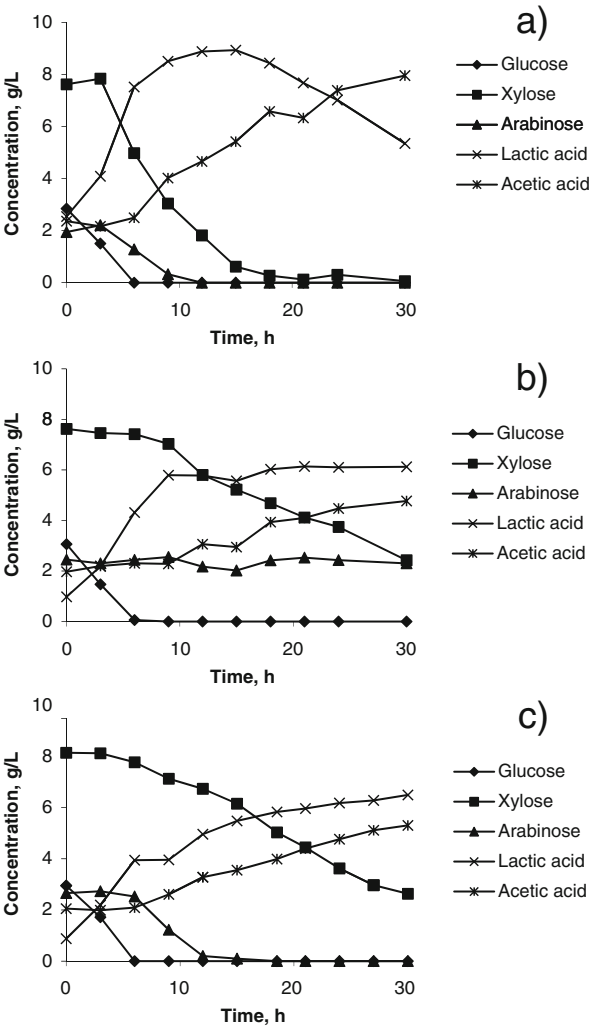

Fig. 1 (a) Fermentation carried out with the hemicellulosic hydrolyzate from grape marc; (b) fermentation carried out with glucose, xylose, and D-arabinose; (c) fermentation carried out with glucose, xylose, and L-arabinose. All media were supplemented with 10 g/L of yeast extract and 10 g/L of corn steep liquor. *Diamond* (glucose), *square* (xylose), *triangle* (arabinose), *x* (lactic acid), and *asterisk* (acetic acid)

hydrolyzates after the extraction of phenolic compounds. It can be observed that the absence of phenolic compounds did not change the kinetics of product formation and sugar consumption comparing with Fig. 1a. Moreover, Table 1 shows the fermentative parameters of fermentations using grape marc hydrolyzates with presence or absence of phenolic compounds, observing similar product yields (0.55 g/g), theoretical yields (68.9–69.1%, respectively), and productivities (0.54–0.59 g/L h, respectively) in both cases. Consequently, it can be assumed that the ethyl acetate extractable phenolic compounds did not influence *L. pentosus* metabolism and, particularly, the xylose consumption under these operational conditions.

Furthermore, additional analysis of the nitrogen content reveals that grape marc hydrolyzates supplemented with 10 g/L of yeast extract and 10 g/L of CSL contain an extra amount of nitrogen (9.2 g/L) comparing to the commercial media employed in previous experiments. Consequently, the possible benefits derived from the utilization of

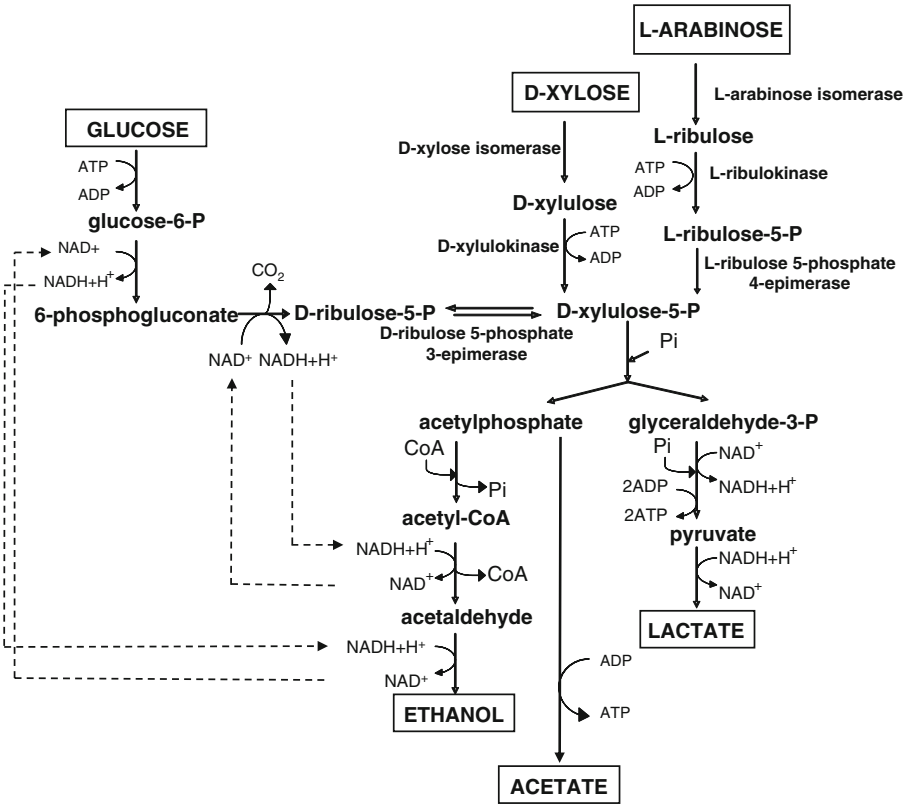

Fig. 2 Pentose phosphate pathway for the metabolism of glucose, xylose, and L-arabinose by *Lactobacillus pentosus*

higher nitrogen concentrations were tested in a new set of experiments. Figure 4 compares two fermentations carried out in synthetic media made with D-glucose, D-xylose, and L-arabinose, supplemented with 10 g/L of yeast extract (Fig. 4a) or 20 g/L of yeast extract (Fig. 4b) plus 10 g/L of CSL, and shows that in the fermentation carried out in the presence of the higher concentration of yeast extract, xylose consumption has improved significantly (from 45.7% to 65.6%). As a result, lactic acid production improved by 53%. These data are presented in Table 2 for both fermentations at the time of maximum lactic acid production.

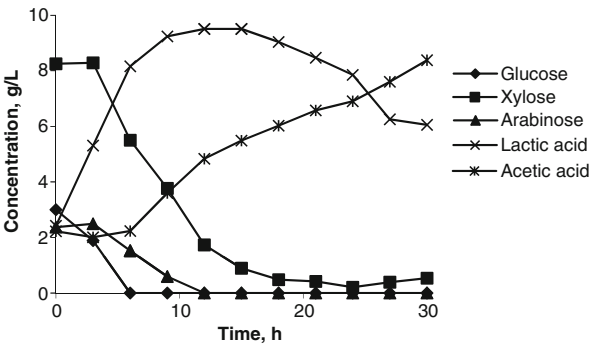

Fig. 3 Fermentation carried out using the hemicellulosic hydrolyzate from grape marc after phenolic compound extraction with ethyl acetate, and supplementation with 10 g/L of yeast extract and 10 g/L of corn steep liquor. *Diamond* (glucose), *square* (xylose), *triangle* (arabinose), *x* (lactic acid); *asterisk* (acetic acid)

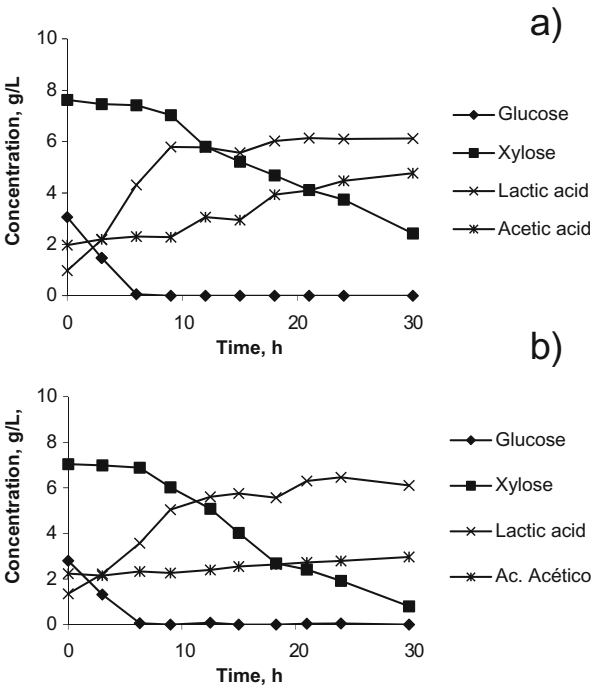

Fig. 4 (a) Fermentation carried out using synthetic sugars (glucose and xylose), and 10 g/L of yeast extract and 10 g/L of corn steep liquor as nutrients; (b) fermentation carried out using synthetic sugars (glucose and xylose) supplemented with 20 g/L of yeast extract and 10 g/L of corn steep liquid as nutrients. *Diamond* (glucose), *square* (xylose), *x* (lactic acid); *asterisk* (acetic acid)

Table 3 shows the composition of hydrolyzates and synthetic medium. It can be observed that hemicellulosic hydrolyzates from grape marc are rich in K, Ca, and Mg. Among the mentioned minerals, Ca and Mg are found in much higher concentration in grape marc hydrolyzates than in synthetic medium. Moreover, Table 3 shows the mineral composition of the general medium of *Lactobacillus* proposed by Mercier. It can be observed that the nutritional supplements employed in this work for synthetic media elaboration (10 g/L of both yeast extract and corn steep liquor) provide a similar mineral composition to the one proposed by Mercier, except for Na concentration. Based on these data, additional experiments were carried out using commercial xylose and glucose in the presence of the same concentrations of the major metals detected in grape marc (Mn, K, Na, Ca, and Mg), as described in "Materials and Methods". The fermentation profiles plotted in Fig. 5 reveal that minerals improved the consumption of xylose although, after 30 h of fermentation, acetic acid was obtained at higher concentration than in experiments carried out in the absence of minerals. Fermentation parameters (Table 2) at the time of maximum lactic acid production indicate that minerals have improved lactic acid productivity (0.37 g/L h) and yields, comparing to fermentations carried out in the absence of minerals (0.17 g/L h).

Summing up the results presented in Figs. 4b and 5, it can be said that metals and extra organic nitrogen addition has improved xylose consumption in the synthetic medium, although the percentage of xylose consumed was still lower (43.1%) than when using hemicellulosic sugars from grape marc (92.1%). On the other hand, some authors have found that mannose can improve the growth of *L. pentosus*. For instance, Chaillou et al. [2] investigated the influence of low D-mannose addition (0.5 mM) on the growth of *L. pentosus* on D-xylose, finding that this microorganism grew approximately 1.9-fold faster when D-mannose was present (generation time of 240 min in the presence of mannose

Table 2 Fermentative parameters of fermentations carried out with xylose (around 8 g/L) and glucose (around 3 g/L) as carbon sources, under different nutritional conditions.

	Control[c]	Extra addition of 10 g/L of yeast extract	Extra addition of minerals[d]
Time (h)	18	21	12.5
Xylose $_{t=0}$ (g/L)	7.7	7.0	6.8
Xylose $_{t=\text{final}}$ (g/L)	4.2	2.4	3.9
Xylose consumed (%)	45.7	65.6	43.1
Glucose $_{t=0}$ (g/L)	3.1	2.8	3.2
Glucose $_{t=\text{final}}$ (g/L)	0.0	0.0	0.0
Glucose consumed (%)	100.0	100.0	100.0
Lactic acid $_{t=0}$ (g/L)	1.1	1.4	0.9
Lactic acid $_{t=\text{final}}$ (g/L)	4.1	6.3	5.6
Acetic acid $_{t=0}$ (g/L)	2.1	2.2	1.2
Acetic acid $_{t=\text{final}}$ (g/L)	4.1	2.7	4.3
Q_P (g/L h)[a]	0.17	0.24	0.37
$Y_{P/S}$ (g/g)[a]	0.46	0.67	0.77
Theoretical yield (%)[b]	58.3	88.8	95.0

Data correspond to the time of maximal lactic acid production

[a] Nomenclature: Q_P, global volumetric productivity of lactic acid; $Y_{P/S}$, sugars to lactic acid yield (g lactic acid produced)/(g xylose consumed + g glucose consumed).

[b] (g lactic acid produced × 100)/((g xylose consumed × 0.6) + (g glucose consumed))

[c] Control consisted on xylose and glucose as carbon source, and 10 g/L of yeast extract plus 10 g/L of corn steep liquor as nutrients

[d] Minerals consisted on (0.015 g/L $MnSO_4$; 5.1 g/L K_2HPO_4; 0.045 g/L $NaOOCCH_3$; 16.3 g/L $CaSO_4$; 2.2 g/L $MgSO_4$)

versus 455 min in its absence). This increased growth rate in the medium containing mannose was paralleled by an increased rate of PEP-dependent mannose phosphorylation, they found a linear relationship between the doubling times of various *L. pentosus* strains on D-xylose and the levels of PEP-dependent phosphorylation of D-mannose. These authors also observed the absence of D-xylose transport and growth in two mutants of *L. pentosus* defective in the phosphoenolpyruvate–mannose phosphotransferase system, concluding that

Table 3 Composition of metals in the hydrolyzates and in synthetic medium (units are in mg/L).

Medium	Mn	K	Na	Ca	Mg	Al	Ni	Cu	Zn	Fe
Hydrolyzates	10.5	2,707	16.3	3,681	198.5	1.2	<1.0	<1.0	<1.0	<1.0
Hydrolyzates with CSL + YE	4.8	3,331	45.7	3,840	278.4	<1.0	<1.0	<1.0	<1.0	1.8
Synthetic medium[a]	<0.5	1,056	31.9	56.8	61.8	<1.0	<1.0	1.7	<1.0	2.2
Mercier medium[b]	<0.5	1,705	1,356	58.3	27.0	11.3	<1.0	<1.0	<1.0	<1.0

[a] Synthetic medium employed in this work composed by hemicellulosic sugars (glucose, xylose, and arabinose) in the same concentrations than those found in the hydrolyzate, supplemented with 10 g/L of yeast extract (YE) and 10 g/L of corn steep liquor (CSL).

[b] Synthetic medium composed by hemicellulosic sugars (glucose, xylose, and arabinose) in the same concentration than that found in hydrolysates, and supplemented with the nutrients proposed by Mercier et al. [9].

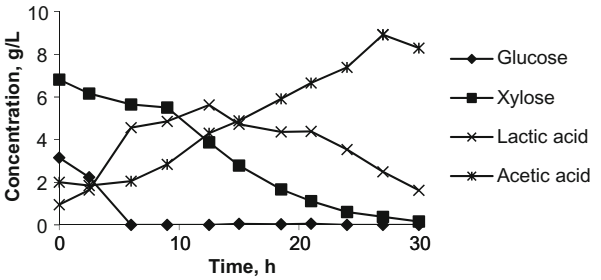

Fig. 5 Fermentation carried out using synthetic sugars (glucose and xylose) supplemented with yeast extract (10 g/L), corn steep liquor (10 g/L), and minerals consisting of: Mn, K, Na, Ca, and Mg, at the concentrations described in "Materials and Methods." *Diamond* (glucose), *square* (xylose), *x* (lactic acid), *asterisk* (acetic acid)

D-xylose transport in this microorganism seems to be caused by a mechanism of facilitated diffusion of D-xylose via the EIIMan protein complex of the phosphoenolpyruvate (PEP)–D-mannose phosphotransferase system (PTS). All these observations also suggested that the transport of D-xylose could be the rate-limiting step in the growth of *L. pentosus* on D-xylose. Therefore, the highest rate of xylose consumption found using grape marc hemicellulosic sugars could be explained for the presence in these hydrolyzates of other minor sugars, particularly mannose. For this reason, more accurate analyses of hemicellulosic sugars contained in grape marc hydrolyzates were carried out using high-performance anionic exchanged chromatography with a Pulse Ampreometric Detection (HPAEC-PAD). The results indicate that the peak attributed to xylose in those chromatograms obtained with the interaction ION-300 column and refraction index detection was actually composed of 50.3% xylose, 22.8% galactose, 16.5% mannose, and 2.4% fructose. Figure 6 shows the chromatogram of hydrolyzates obtained after PAD analysis.

A set of experiments was accordingly carried out using the above sugar composition. The synthetic medium consisted of 4.7 g/L of D-xylose, 3 g/L of D-glucose, 1.3 g/L of

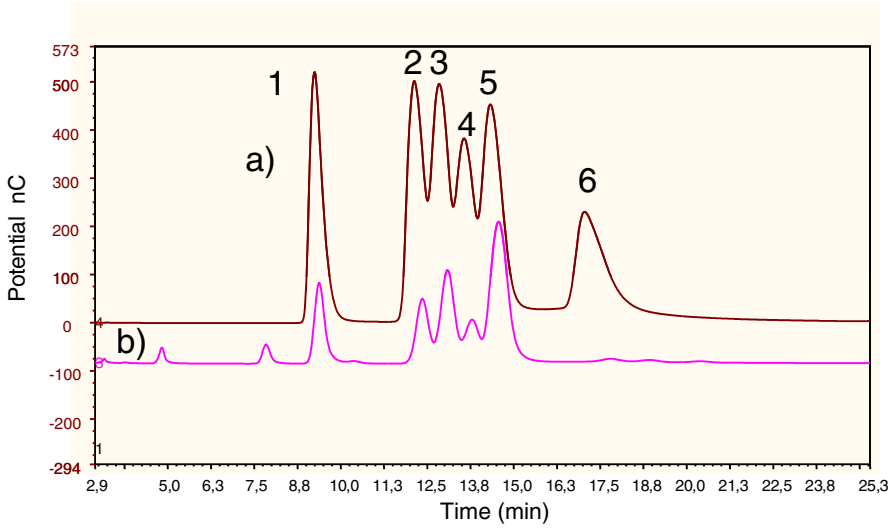

Fig. 6 (*a*) PAD chromatogram of the control synthetic medium containing arabinose (*1*), galactose (*2*), glucose (*3*), mannose (*4*), xylose (*5*), fructose (*6*); (*b*) PAD chromatogram of the hemicellulosic grape marc hydrolyzate

D-mannose, 2 g/L of L-arabinose, 1.8 g/L of D-galactose, and 0.22 g/L of D-fructose, as well as 10 g/L of yeast extract and 10 g/L of corn steep liquor. Because of the fact that xylose, galactose, mannose, and fructose have the same retention time in HPLC analyses, these sugars were quantified together as sugars with the same retention time (SSRT). Figure 7a shows the kinetics of lactic acid production and sugar consumptions, observing that the SSRT consumption is still lower than that observed when using hemicellulosic hydrolyzates (Fig. 1b). Consequently, a final set of experiments was carried out using the previous medium supplemented with minerals at the same concentrations as before (Fig. 7b), and these minerals and extra 10 g/L of yeast extract (Fig. 7c). Table 4 shows the fermentation parameters determined in these three experiments with different compositions. The best results were achieved when the medium was supplemented with both, minerals and extra yeast extract, followed by that supplemented only with minerals. Under these conditions, the SSRT consumption was 92.3%, and 90.5%, respectively. The results achieved using the most sophisticated complex synthetic medium (lactic acid=8.9 g/L; Q_P=0.44 g/L h;

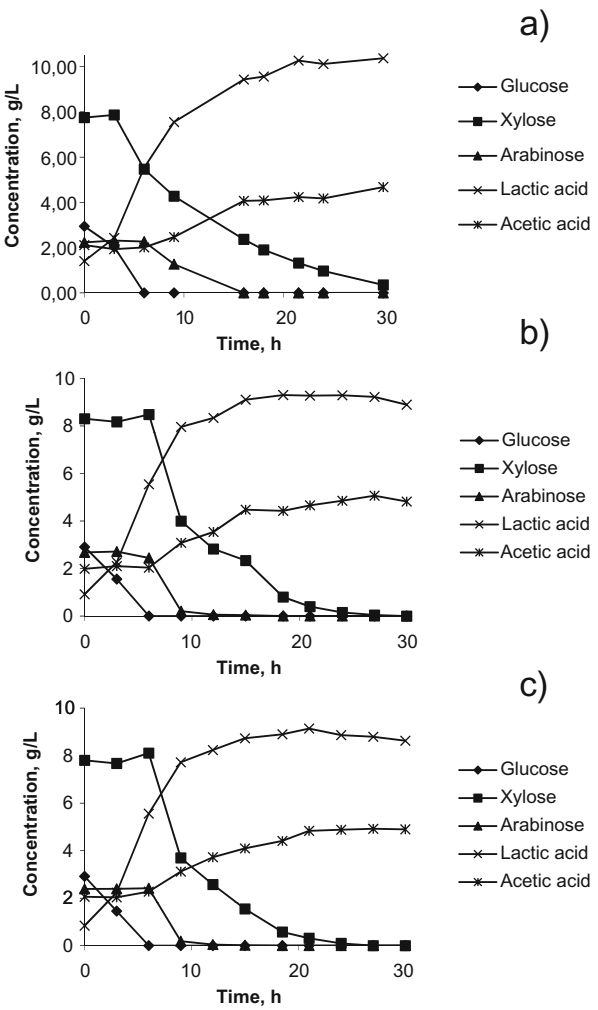

Fig. 7 a) Fermentation carried out using L-arabinose, glucose, and SSRT sugars (xylose, mannose, galactose, and fructose) as well as 10 g/L of yeast extract and 10 g/L of corn steep liquor as nutrients; (b) fermentation carried out in the same conditions as in Fig. 7a with the extra addition of minerals (Mn, K, Na, Ca, and Mg) at the concentrations described in "Materials and Methods"; c) fermentation carried out in the same conditions as in Fig. 7b, with the extra addition of 10 g/L of yeast extract. *Diamond* (glucose); *square* (SSRT); *triangle* (arabinose); *x* (lactic acid); *asterisk* (acetic acid)

Table 4 Fermentative parameters of fermentations carried out using synthetic sugars consisting on xylose, arabinose, galactose, glucose, mannose, and fructose as carbon sources.

	Control[d]	Extra addition of minerals[e]	Extra addition of minerals and organic nitrogen[f]
Time (h)	21.5	18.5	18.5
SSRT $_{t=0}$ (g/L)[a]	7.8	8.3	7.8
SSRT $_{t=\text{final}}$ (g/L)[a]	1.3	0.8	0.6
SSRT consumed (%)[a]	83.0	90.5	92.3
Glucose $_{t=0}$ (g/L)	2.9	2.9	2.9
Glucose $_{t=\text{final}}$ (g/L)	0.0	0.0	0.0
Glucose consumed (%)	100.0	100.0	100.0
Arabinose $_{t=0}$ (g/L)	2.2	2.7	2.4
Arabinose $_{t=\text{final}}$ (g/L)	0.0	0.0	0.0
Arabinose consumed (%)	100.0	100.0	100.0
Lactic acid $_{t=0}$ (g/L)	1.4	0.9	0.8
Lactic acid $_{t=\text{final}}$ (g/L)	10.3	9.3	8.9
Acetic acid $_{t=0}$ (g/L)	2.1	2.0	2.0
Acetic acid $_{t=\text{final}}$ (g/L)	4.2	4.4	4.4
Q_P (g/L h)[b]	0.41	0.45	0.44
$Y_{P/S}$ (g/g)[b]	0.76	0.64	0.64
Theoretical yield (%)[c]	94.0	80.6	80.9

Data correspond to the time of maximum lactic acid production.

[a] SSRT is composed by xylose, galactose, mannose, and fructose

[b] Nomenclature: Q_P, global volumetric productivity of lactic acid; $Y_{P/S}$, sugars to lactic acid yield (g lactic acid produced)/(g pentoses (xylose and arabinose) consumed + g hexoses (glucose, galactose, mannose, and fructose) consumed).

[c] (g lactic acid produced × 100)/((g pentoses (xylose and arabinose) consumed × 0.6) + (g of hexoses (glucose, galactose, mannose, and fructose) consumed))

[d] Control with 10 g/l of yeast extract and 10 g/L of corn steep liquor, without addition of extra minerals neither extra amount of organic nitrogen

[e] Minerals consisted of (0.015 g/L $MnSO_4$; 5.1 g/L K_2HPO_4; 0.045 g/L $NaOOCCH_3$; 16.3 g/L $CaSO_4$; 2.2 g/L $MgSO_4$)

[f] The addition of organic nitrogen consisted of 20 g/L of total yeast extract

$Y_{P/S}$=0.64 g/g, and theoretical yield=80.9%) compare favorably with the data reported for the batch production of lactic acid in hemicellulosic grape marc hydrolyzates (SSRT consumption=92.1%; lactic acid=9.0 g/L; Q_P=0.54 g/L h; $Y_{P/S}$=0.55 g/g, and theoretical yield=68.9%). Moreover, as it has been reported in a previous work [6], *L. pentosus* can efficiently ferment xylose in the presence of glucose. In summary, the final synthetic medium defined in this work, with the complex mixture of sugars, and extra mineral and yeast extract supplementation, makes it possible to obtain similar results to the ones achieved with hydrolyzates from grape marc.

Conclusions

Grape marc is an agroindustrial residue which, after acid hydrolysis, can be efficiently employed as nutritional media for *L. pentosus*. Hemicellulosic hydrolyzates from grape marc are rich in nitrogen and minerals such as Mg, Ca, and K that improve pentose

consumption compared to synthetic media. Moreover, it was observed that although grape marc hydrolyzates were composed by glucose, arabinose, and xylose as well as other sugars (galactose, mannose, and fructose), the fermentation of xylose and arabinose in the presence of hexoses was not an obstacle for an economical *biomass* conversion.

Acknowledgments We are grateful to the following institutions for the financial support of this work: Spanish Ministry of Science and Technology (MCYT) (project CT Q2006-02241/PPQ), which has partial financial support from the FEDER funds of the European Union, Xunta de Galicia (project PGIDIT05BTF38301PR), the "Ramón y Cajal" and "Isidro Parga Pondal" programs financed by the MCYT and Xunta de Galicia of Spain, respectively, and the Universidad Autónoma de Tamaulipas for the partial support of Mr. Óscar Manuel Portilla Rivera.

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