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ARTICLE

Development of Cost-Effective Media to Increase the Economic Potential for Larger-Scale Bioproduction of Natural Food Additives by *Lactobacillus rhamnosus*, *Debaryomyces hansenii*, and *Aspergillus niger*

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Yeast extract (YE) is the most common nitrogen source in a variety of bioprocesses in spite of the high cost. Therefore, the use of YE in culture media is one of the major technical hurdles to be overcome for the development of low-cost fermentation routes, making the search for alternative-cheaper nitrogen sources particularly desired. The aim of the current study is to develop cost-effective media based on corn steep liquor (CSL) and locally available vinasses in order to increase the economic potential for larger-scale bioproduction. Three microorganisms were evaluated: *Lactobacillus rhamnosus*, *Debaryomyces hansenii*, and *Aspergillus niger*. The amino acid profile and protein concentration was relevant for the xylitol and citric acid production by *D. hansenii* and *A. niger*, respectively. Metals also played an important role for citric acid production, meanwhile, *D. hansenii* showed a strong dependence with the initial amount of Mg^{2+}. Under the best conditions, 28.8 g lactic acid/L (Q_{LA} = 0.800 g/L·h, $Y_{LA/S}$ = 0.95 g/g), 35.3 g xylitol/L ($Q_{xylitol}$ = 0.380 g/L·h, $Y_{xylitol/S}$ = 0.69 g/g), and 13.9 g citric acid/L (Q_{CA} = 0.146 g/L·h, $Y_{CA/S}$ = 0.63 g/g) were obtained. The economic efficiency ($E_{p/€}$) parameter identify vinasses as a lower cost and more effective nutrient source in comparison to CSL.

KEYWORDS: Vinasses; corn steep liquor; yeast extract; lactic acid; xylitol; citric acid

INTRODUCTION

The production of economically viable food additives such as lactic acid (LA), xylitol, and citric acid (CA) is still limited by a number of factors including price of nutrients and efficient fermentation of sugars. Most studies reported on LA, xylitol, and CA production by bacteria, yeast, and fungus, respectively, were performed in media containing expensive nutrients which are not economically feasible. Yeast extract (YE) is the most commonly used nutrient source in laboratory-scale fermentations (*1*). For example, Mercier et al. (*2*) recommended the following nutrients for LA production by bacteria: 5 g YE/L, 10 g peptone/L, 5 g sodium acetate/L, 2 g sodium citrate/L, 2 g K_2HPO_4/L, 0.58 g $MgSO_4 \cdot 7H_2O$/L, 0.12 g $MnSO_4 \cdot H_2O$/L, and 0.05 g $FeSO_4 \cdot 7H_2O$/L. Xylitol is usually produced in fermentation media containing the following nutrients: 3 g YE/L, 3 g malt extract/L, and 5 g peptone/L (*3*), whereas, CA by *Aspergillus niger* is produced using fermentation broths made of nutrients, including 5 g YE/L and 10 g peptone/L (*4*). Most of these media contain YE, probably due to the purine and pyrimidine bases and B vitamins that lead to good yields (*5*, *6*). However, the high cost of YE impairs the economics of fermentation because YE is estimated to account for about 38% of the total cost of LA (*7*). Consequently, it is economically interesting to find low-cost media to replace the traditional nutrients employed in these processes.

It has been suggested that for commercial production of biochemical(s), economical and commercially available media, such as industrial or agricultural byproducts, must be investigated to reduce production costs (*8*). These commercial media include cheese whey permeate, molasses, casamino acids, soybean hydrolyzate, and ram horn protein hydrolyzates after acid hydrolysis, although acid hydrolyzed nutrients result in low LA production performance compared with nutrients hydrolyzed with enzymes, and corn steep liquor (CSL) (*9*). A breakdown of the medium cost indicates that CSL, a byproduct of the corn wet-milling industry, is a cost-effective medium for fermentation (*10*). Recently, some attempts have been made with vinasses, the main liquid wastes of the alcohol industry from the distillation process of lees and low-quality wines, as economic nutrients for some biotechnological processes (*11*, *12*). Vinasses are acidic effluents with high BOD, COD, and solid concentration (*13*). The physical−chemical composition of the vinasses is very complex. The main characteristics are a pH between 3 and 6; a COD that can be higher than 30000 mg/L; organic matter between 900 and 35000 mg/L; potassium that can be higher than 2500 mg/L; phenolic components in

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quantitites up to 1000 mg/L; and discharge temperatures of 90 °C (*14*). Their release to surface waters cause significant environmental problems being necessary to apply efficient treatments prior to vinasse disposal in the environment, in fact, current legislation requires wineries to treat their effluents from now on (*15*). Consequently, taking into account that vinasses have a high organic content, including acids, carbohydrates, phenols, and unsaturated compounds, a most profitable application could be their use as economic nutrients for biotechnological processes. The price of YE is estimated in 7.3 $/kg, CSL costs only 0.07 $/kg, meanwhile, vinasses employed in this study are a byproduct of wineries (*12*).

Lactic acid has a wide range of applications as acidifier, flavoring, pH buffering agent or inhibitor of bacterial spoilage in a wide variety of processed foods (*6*). In contrast with other food acid additives, it has a mild acidic taste. It is nonvolatile, odorless, and it is classified as GRAS (generally regarded as safe) by the FDA in the U.S. (*16*). Furthermore, the applications of LA include textile-finishing operations, intermediates for the production of emulsifying agents, manufacture of cosmetics and pharmaceuticals, and production of polylactic acid-based degradable plastics (*17*). Xylitol is a five-carbon polyol with high sweetening power. It has physical and chemical properties favorable for food technological applications, including sugar-free confections, yogurts, jams, bakery products, and drinks. Furthermore, xylitol has anticaries properties, is tolerated by diabetics, and has been recommended for parenteral nutrition (*18*). Finally, CA, the most important organic acid produced in tonnage by fermentation, is widely used in the food, beverage, pharmaceutical, and cosmetic industries and finds applications in a variety of other industries, from textiles to electroplating (*19*).

The aim of the current study is to develop a fermentation process based on the substitution of expensive nutrient supplements with cheaper renewable low-cost products: CSL or vinasses. First of all, metals, proteins, and amino acids were quantified in all fermentation broth. The influence of the sterilization process on the fermentation media composition was also assessed. Finally, the effect of these supplementations on the kinetics of LA, xylitol, and CA by *L. rhamnosus*, *D. hansenii*, and *A. niger*, respectively, was studied.

MATERIALS AND METHODS

Vinasses Sampling, Storage, Tartaric Acid Recovery, and Characterization. Vinasses from the campaign of 2007, obtained after the distillation of lees, were kindly supplied by the certified brand of origin of Valdeorras (Ourense), Spain, and stored at 4 °C. Vinasses were used directly as nutrient or after tartaric acid (TA) recovery. TA is found precipitated in lees as potassium bitartrate and calcium tartrate and can be recovered in two steps: the first one involves the dissolution of the tartrate salts with HCl, whereas during the second step the TA is selectively precipitated to calcium tartrate (*20*). The removed substances during the second stage, without the TA recovered by filtration, remain in stream B. **Figure 1** shows the flow diagram of the process.

Vinasses were oven-dried to constant weight at 102 °C to determine the percentage of solids. Ashes in vinasses were oven-dried to constant weight at 550 °C. Nitrogen and carbon percentages were analyzed using a Thermo Finningan Flash Elemental Analyzer 1112 series, San Jose, CA (U.S.A.), meanwhile, Fe^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, Al^{3+}, and Cu^{2+} were analyzed in ashes using an Atomic Absorption Spectrometer 220 Fast Sequential, VARIAN, Palo Alto, CA (U.S.A.). Previously, 0.15 g ashes were digested with 5 mL of HNO_3 65%, 1 mL of H_2O_2 30%, and 0.5 mL of HF 40% in a Microwave Labstation MLS 1200 MEGA, MILESTONE, Bergamo (Italy).

Microorganisms and Inoculum Preparation. *Lactobacillus rhamnosus* CECT-288 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. (*2*), which contains 20 g glucose/L, 5 g YE/L, 10 g peptone/L, 5 g sodium acetate/L, 2 g sodium citrate/L, 2 g K_2HPO_4/L, 0.58 g $MgSO_4 \cdot 7H_2O$/L, 0.12 g $MnSO_4 \cdot H_2O$/L, 0.05 g $FeSO_4 \cdot 7H_2O$/L, and 10 g agar/L at 37 °C for 24 h. Inocula were prepared by solubilization of cells from plates with 5 mL sterile water. Biomass in inocula was measured by optical density at 600 nm and adjusted to the desired value (7.4 g dry cells/L) by dilution with water.

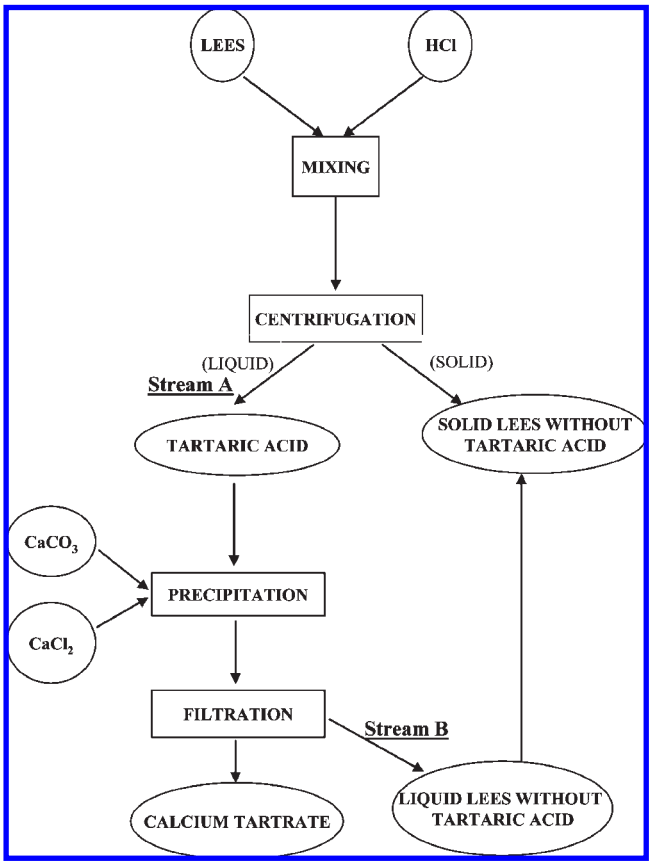

Figure 1. Flow diagram of the tartaric acid recovery.

Debaryomyces hansenii NRRL Y-7426 was kindly provided by the National Center for Agricultural Utilization Research (Peoria, Illinois). Freeze-dried cells were grown on a basal medium containing 30 g commercial xylose/L, 3 g yeast extract/L, 3 g malt extract/L, and 5 g peptone/L. The microorganism was maintained in agar slant tubes containing a medium formulated with the same components and concentrations as the previous one plus 20 g/L agar. Inocula were prepared by solubilization of cells with sterile water and underwent growth during 24 h in the previous medium without agar. Biomass in inocula was measured by optical density at 600 nm and adjusted by dilution with water to reach a final concentration in the inocula of 2.3 g/L. Inoculum was added to fermentation broth to reach a final concentration of 0.12 g/L.

Aspergillus niger CECT-2090 (ATCC 9142, NRRL 599), obtained from the Spanish Collection of type Cultures (Valencia, Spain), was used in this work. The microorganism was grown on potato dextrose agar slants (Scharlau, Barcelona, Spain) at 33 °C for 5 days. Spore suspensions containing about $0.78-1.09 \times 10^3$ CFU/mL were prepared by adding 3.0 mL of sterile distilled water to the slant and shaking vigorously for 1 min and subsequently used as inoculum.

Lactic Acid Fermentation. Experiments were carried out in 250 mL Erlenmeyer flasks with a final volume of 100 mL using media containing 30 g glucose/L. **Table 1** shows the nutrients employed in media 1 to 10. Medium 1 was formulated with 30 g CSL/L as the only nutrient to evaluate if CSL is enough to cover the nutritional requirements of *L. rhamnosus*. Media 2−10 were formulated using 20, 30, or 40 g/L of vinasses without TA recovery, vinasses obtained after TA recovery, and vinasses obtained after TA recovery plus the removed substances contained in stream B. The amount of stream B obtained after treating 20, 30, or 40 g/L was 15.3, 29.0, and 38.8 mL, respectively. Medium 25 was formulated with the nutrients

Table 1. Nutrients Employed in Fermentation Broths

fermentation			
L. rhamnosus	*D. hansenii*	*A. niger*	nutrients
medium 1	medium 11	medium 21	CSL (30 g/L)
medium 2	medium 12		vinasses after TA extraction (20 g/L) + stream B (15.3 mL)
medium 3	medium 13	medium 22	vinasses after TA extraction (30 g/L) + stream B (29.0 mL)
medium 4	medium 14		vinasses after TA extraction (40 g/L) + stream B (38.8 mL)
medium 5	medium 15		vinasses after TA extraction (20 g/L)
medium 6	medium 16	medium 23	vinasses after TA extraction (30 g/L)
medium 7	medium 17		vinasses after TA extraction (40 g/L)
medium 8	medium 18		vinasses before TA extraction (20 g/L)
medium 9	medium 19	medium 24	vinasses before TA extraction (30 g/L)
medium 10	medium 20	medium 24	vinasses before TA extraction (40 g/L)
medium 25			mercier
	medium 26		yeast extract (3 g/L) + malt extract (3 g/L) + peptone (5 g/L)
		medium 27	NH_4NO_3 (25 g/L), $MgSO_4 \cdot 7H_2O$ (2.5 g/L), $CuSO_4$ 0.04 (g/L)

proposed by Mercier et al. (*2*). Fermentation media were sterilized in autoclave at 100 °C during 60 min. After sterilization, calcium carbonate (30 g/L) was added to neutralize the LA produced. After inoculation (5 mL), fermentations were carried out in orbital shakers (New Brunswick, Edison, NJ) at 120 rpm and 37 °C. Samples (2 mL) were taken at given fermentation times and centrifuged at 6000 rpm for 3 min. The supernatants were stored for glucose and LA analyses.

Xylitol Fermentation. Shake flask fermentation experiments were carried out under microaerophilic conditions in 250 mL Erlenmeyer flasks containing 100 mL of culture media. These culture media were prepared with pure xylose (60 g/L) and supplemented with the nutrients indicated in **Table 1** for media 11−20. The nutrients were similar to the previous experiments (CSL or vinasses). Additionally, medium 26 was formulated with 3 g yeast extract/L, 3 g malt extract/L and 5 g peptone/L (*3*). After sterilization (autoclave at 100 °C during 60 min), 30 g/L of calcium carbonate was added to the culture medium to keep the pH in the vicinity of 6. After inoculation (5 mL), fermentations were carried out in orbital shakers (New Brunswick, Edison, NJ) at 100 rpm and 31 °C for 174 h. Samples (2 mL) were taken at given fermentation times and centrifuged at 6000 rpm for 3 min. The supernatants were stored for xylose and xylitol analyses.

Citric Acid Fermentation. Fermentations were carried out using 250 mL Erlenmeyer flasks with 50 mL of fermentation broth containing glucose (10 g/L), sucrose (6.5 g/L), fructose (14 g/L), and the nutrients of **Table 1**: medium 21 with 30 g CSL/L, media 22−24 with 30 g vinasses/L, and medium 27 with NH_4NO_3 (25 g/L), $MgSO_4 \cdot 7H_2O$ (2.5 g/L), and $CuSO_4$ 0.04 (g/L) (*21*). After sterilization in autoclave at 100 °C for 60 min, calcium carbonate was added in excess amount (30 g/L) to guarantee the neutralization of all the CA produced, supposing a conversion of 1 mol of CA per mol of sugar. Each flask was inoculated with 5 mL of the spore inoculum suspension, and incubated at 31 °C in an orbital shaker (New Brunswick, Edison, NJ, USA) at 200 rpm. Samples (2 mL) were taken at given fermentation times and centrifuged at 6,000 rpm for 3 min. The supernatants were stored for glucose, sucrose, fructose, and citric acid analyses.

Experimental data for LA, xylitol, and CA were carried out in duplicate, and means are reported. The volumetric productivities (Q_P) were calculated for the fermentation times (each one indicated in the text) corresponding to the highest value of product concentrations.

Analytical Methods. Glucose, xylose, sucrose, fructose, lactic acid, xylitol, and citric acid during fermentations were measured using a Hewlett-Packard high-performance liquid chromatographic system (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 Interaction ION-300 (Transgenomic, Inc., U.S.A.) column eluted with 0.6 mL/min of 0.003 M sulfuric acid.

Quantification of Protein. The total protein content was determined by quantification of the total nitrogen using the Havilah et al. (*22*) method, applied to digests obtained by the classic procedure of Kjeldahl.

To measure the soluble protein concentration, we used the bicinchonic acid method using "Pierce BCA Protein Assay Kit" by Thermo ScientiWc (catalogue number: 23225). This assay allows the colorimetric detection and quantization of soluble protein using a unique reagent containing bicinchoninic acid (*23*). Responsible for the color formation with BCA are the macromolecular protein structure, the number of peptide bonds and the amino acids cysteine, cystine, tryptophan and tyrosine (*24*). The kit enables the measurement of the protein concentration with a broad range (20−2000 μg/mL). The preparation was performed according to the instructions of the "Pierce BCA Protein Assay Kit". A series of dilutions of known concentration were prepared from bovine serum albumin (BSA) following the dilution scheme for standard test tube protocol. The colorimetric detection was performed using the Cintra 6 UV/visible spectrophotometer, at 562 nm wavelength. The protein concentration was determined based on the standard curve. The protein concentration was then correlated with the weight of each sample to get the concentration.

Table 2. Chromatographic Conditions Used in the Determination of Amino Acids

time (min)	flow (mL/min)	% AccQ.Tag	% acetonitrile	% water
0	1.00	100	0	0
2.5	1.00	99	1	0
17	1.00	96	4	0
22	1.00	95	5	0
24	1.00	91	9	0
31.5	1.00	83	17	0
36	1.00	83	17	0
42	1.00	0	60	40
44	1.00	100	0	0
45	1.00	100	0	0

Amino Acid Analysis. Chemicals and chromatographic instrumentation: AccQ.Fluor reagent kit (AQC, borate buffer) and AccQ.Tag Eluent A concentrate were acquired from Waters (Milford, MA). Acetonitrile (MeCN), disodium ethylenediaminetetraacetic acid (EDTA), phosphoric acid, sodium acetate trihydrate, and sodium azide were from Baker (Phillipsburg, PA); triethylamine (TEA) was purchased from Aldrich (Milwaukee, WI). Amino acid standards and taurine and hydroxyproline were from Sigma (St. Louis, MO). HPLC systems used were a Waters system Alliance 2695 with a 2475 scanning fluorescence detector. Empower 2 was used to control system operation and results management.

Derivatization of standards and samples and chromatographic analysis: 10 μL of sample was buffered to pH 8.8 (AccQ.Flour borate buffer) to yield a total volume of 100 μL. Derivatization was initiated by the addition of 20 μL of AccQ-Fluor reagent (3 mg/mL in MeCN). Reaction of the AQC with all primary and secondary amines was rapid and excess reagent was hydrolyzed within 1 min. Completion of hydrolysis of any tyrosine phenol modification was accelerated by heating for 10 min at 55 °C.

Separations were carried out using a Water AccQ-Tag column (3.9 × 150 mm with a 4 μm particle size) with a flow-rate of 1.0 mL/min and performed at 37 °C. The chromatographic conditions used are described in **Table 2**; AccQ.Tag was used as eluent A, acetonitrile was used as eluent B and water as eluent C. Detection was accomplished by fluorescence with excitation at 250 nm and emission at 395 nm. Amino acids were identified

Table 3. Carbon and Nitrogen Contents (Milligrams per g of Dried Vinasses) and Concentration of Minerals in Vinasses (Expressed as Milligrams of Metal per Kilogram of Dried Ash)[a]

	vinasses and stream B before addition to fermentation broth			composition of the fermentation broth								
				20 g/L of vinasses			30 g/L of vinasses			40 g/L of vinasses		
	VbTAe	VaTAe	SB	VaTAe	VaTAe+SB	VbTAe	VaTAe	VaTAe+SB	VbTAe	VaTAe	VaTAe+SB	VbTAe
C (mg/g)	60.1	494.9	22.5	9.9	13.3	13.9	14.8	21.4	26.5	19.8	28.5	35.3
N (mg/g)	3.2	52.1	12.5	1.0	3.0	0.7	1.6	5.2	1.4	2.1	6.9	1.9
Fe^{2+} (mg/kg)	61.0	596.2	1.5	11.9	12.2	14.2	17.9	18.3	26.9	23.8	24.4	35.9
Mn^{2+} (mg/kg)	8.1	28.9	3.2	0.6	1.1	1.9	0.9	1.8	3.6	1.2	2.4	4.8
Zn^{2+} (mg/kg)	3.5	14.7	0.9	0.3	0.4	0.8	0.4	0.7	1.6	0.6	0.9	2.1
Ca^{2+} (mg/kg)	945.8	1,930.4	10,838.3	38.6	1,694.7	219.4	57.9	3,205.4	417.1	77.2	4,277.1	556.1
Mg^{2+} (mg/kg)	122.8	518.8	96.0	10.4	25.0	28.5	15.6	43.5	54.1	20.8	58.0	72.2
Al^{3+} (mg/kg)	128.0	1,862.1	25.0	37.2	41.1	29.7	55.9	63.1	56.5	74.5	84.2	75.3
Cu^{2+} (mg/kg)	12.7	167.9	0.6	3.4	3.5	2.9	5.0	5.2	5.6	6.7	7.0	7.4

[a] VbTAe, vinasses before TA extraction; VaTAe, vinasses after TA extraction; SB, stream B; VaTAe+SB, vinasses after TA extraction plus stream B.

Table 4. Concentration of Proteins in Fermentation Broths Before and After Sterilization

	before sterilization		after sterilization			
	proteins (g/L)		proteins (g/L)		increment (%)	
nutrients	BCA[a]	Havilah[b]	BCA[a]	Havilah[b]	BCA[a]	Havilah[b]
vinasses before TA extraction	11.4	11.71				
stream B	5.4	5.3				
CSL (30 g/L)	2.8	3.1	3.2	3.8	14.1	22.6
vinasses after TA extraction (20 g/L) + stream B (15.28 mL)	1.7	4.1	2.6	4.3	50.6	4.9
vinasses after TA extraction (30 g/L) + stream B (29.04 mL)	2.7	6.8	4.7	7.5	77.0	10.3
vinasses after TA extraction (40 g/L) + stream B (38.75 mL)	3.3	9.1	5.3	9.8	59.8	7.7
vinasses after TA extraction (20 g/L)	0.91	3.9	2.2	4.1	145.3	5.1
vinasses after TA extraction (30 g/L)	1.2	5.8	3.5	6.3	187.7	8.6
vinasses after TA extraction (40 g/L)	1.7	7.0	4.2	7.9	146.0	12.9
vinasses before TA extraction (20 g/L)	3.1	3.3	4.2	4.6	34.4	39.4
vinasses before TA extraction (30 g/L)	5.2	5.7	6.2	6.6	18.8	15.8
vinasses before TA extraction (40 g/L)	6.7	7.3	8.2	8.5	22.6	16.4
YE (3 g/L); malt extract (3 g/L); peptone (5 g/L)	2.2	2.7	2.5	3.8	10.4	40.7

[a] Bicinchonic acid method. [b] Havilah's method.

by retention time using an amino acid standard to which taurine and hydroxyproline were added.

RESULTS AND DISCUSSION

Vinasses Characterization. The compositional analyses of the vinasses provide insight into the fate of elemental composition (C and N) and metals. These results (**Table 3**) indicate the composition of vinasses, before (VbTAe) and after (VaTAe) TA extraction, as well as the composition of the stream B (SB) generated after TA extraction, and the mixture of VaTAe and SB. **Table 3** also shows the concentration of C, N, and metals corresponding to fermentation broth prepared using 20, 30, and 40 g vinasses/L. Before TA extraction, the determination of its elemental composition resulted to be the following: C, 60.1 mg/g; N, 3.2 mg/g; meanwhile, the determination of metals showed the contents (in mg/kg) of Ca^{2+}, 945.8; Al^{3+}, 128.0; Fe^{2+}, 61.0; Mg^{2+}, 122.8; Cu^{2+}, 12.7; Mn^{2+}, 8.1; and Zn^{2+}, 3.5. All these values except Ca^{2+}, which precipitates in form of calcium tartrate during the processes described in **Figure 1** increase considerably after the TA extraction, with the following values for VaTAe: C, 494.9 mg/g; N, 52.1 mg/g; and metals (in mg/kg) Ca^{2+}, 1,930.4; Al^{3+}, 1862.1; Fe^{2+}, 596.2; Mg^{2+}, 518.8; Cu^{2+}, 167.9; Mn^{2+}, 28.9; and Zn^{2+}, 14.7.

Most of these metals are usually part of the culture media recommended for the cultivation of several microorganisms, including *L. rhamonsus*, *D. hansenii*, and *A. niger*. For example, Mercier et al. (*2*) elaborated a fermentation broth containing Na^{+}, K^{+}, Mg^{2+}, Mn^{2+}, and Fe^{2+} for the fermentation of different *Lactobacillus* strains, meanwhile Zhou et al. (*25*) recommended the use of K^{+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, and Mn^{2+} for the LA fermentation by *Rhizopus oryzae* ATCC 52311. In relation to the xylitol production, there is limited information on the effect of metallic cations on the activity of enzymes involved in the metabolism of xylose. Girio et al. (*26*) showed that the ions Ca^{2+}, Mg^{2+}, and Mn^{2+} did not affect the activity of the enzyme XDH, but Zn^{2+}, Cd^{2+}, and Co^{2+} inhibit strongly the activity of this enzyme. Finally, in relation with the CA production, the essential metals for the growth of *Aspergillus niger* are Fe^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, and Co^{2+}, the levels of most of them being fundamental to increase the yields of CA in poor broths (*27*); meanwhile, Tran et al. (*28*) reported that supplementation of Fe^{2+} between 1 and 10 ppm in the cultivation medium based on pineapple waste in solid-state fermentation by *A. niger* increased CA concentration by 22% when compared with the control.

Influence of the Sterilization Process on the Protein and Amino Acids Content. Table 4 shows the total and soluble protein content of the fermentation media before and after sterilization using the Havilah and the bicinchonic acid (BCA) methods, respectively. As expected, the Havilah method provides higher values of protein since the BCA method permits determination of protein in solution, and this does not allow determination of protein present in the complex materials.

Vinasses with TA presented the higher values of soluble proteins, probably because proteins are partially solubilized

Table 5. Amino Acids Profile (in ppm) for Streams and Fermentation Broths Before Sterilization[a]

	streams to be added to fermentation broths			composition of fermentation broth after nutrients addition										
						20 g vinasses/L			30 g vinasses/L			40 g vinasses/L		
amino Acid (mg/kg)	vinasses	SB	CSL*	YEMEP	CSL**	VaTAe	VaTAe+SB	VbTAe	VaTAe	VaTAe+SB	VbTAe	VaTAe	VaTAe+SB	VbTAe
alanine	106.2	82.3	1605.2	162.9	66.8	16.3	26.3	34.5	17.3	37.7	45.8	18.8	48.1	65.8
arginine	27.0	24.1	194.9	68.9	9.0	5.6	8.6	12.2	4.6	11.3	11.8	4.1	12.9	16.6
aspartic acid	76.9	60.7	463.5	73.2	18.7	6.1	13.7	22.3	5.7	20.6	31.5	4.8	28.6	45.1
glutamic acid	95.3	82.0	462.1	246.4	20.3	4.5	21.1	27.8	9.9	31.9	39.8	8.4	40.3	58.7
glycine	51.4	41.4	570.5	69.5	18.9	8.5	13.6	21.2	8.5	21.2	23.0	9.3	23.0	32.0
hidroxiproline	7.2	n.d.	n.d.	4.5	n.d.	n.d.	n.d.	5.8	n.d.	n.d.	n.d.	n.d.	n.d.	6.1
histidine	8.6	7.5	307.9	44.9	13.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.5
isoleucine	40.5	31.2	200.8	80.0	8. 8	6.8	12.8	12.4	8.8	16.6	16.9	9.6	21.0	24.4
leucine	73.8	54.2	762.5	169.4	24.6	3.2	14.8	21.1	8.1	21.4	30.9	9.5	29.2	44.9
lysine	110.6	73.5	344.0	117.0	23.2	9.0	23.4	31.6	13.2	33.9	48.4	16.3	42.8	69.3
methionine	59.9	50.5	187.2	37.7	27.0	11.3	26.2	26.7	22.2	23.5	28.4	16.4	31.5	40.1
phenylalanine	40.3	29.0	374.7	108.9	11.4	10.7	8.1	11.9	4.1	11.6	17.2	5.6	15.9	24.7
proline	386.2	266.9	5311.6	74.7	238.9	44.2	81.1	115.3	54.1	117.0	179.9	60.6	152.5	254.2
serine	42.7	33.6	546.5	115.8	23.0	11.4	15.0	24.7	8.5	18.8	20.4	7.9	18.6	26.1
threonine	22.5	30.5	938.3	273.4	30.6	7.5	12.7	10.6	8.2	13.2	11.3	6.2	18.2	17.7
valine	49.7	41.5	430.3	113.2	17.8	17.3	13.7	20.0	9.2	18.7	21.2	8.7	23.8	31.1
total content	1,198.8	908.9	12,700.0	1,760.4	543.3	162.4	291.1	398.1	182.4	397.4	526.5	186.2	506.4	764.3

[a] YEMEP: YE (3 g/L); malt extract (3 g/L); peptone (5 g/L); n.d. = no detected; CSL: composition of commercial CSL (*) or media containing 30 g CSL/L (**); SB, stream B; VaTAe, vinasses after TA extraction; VaTAe+SB, vinasses after TA extraction plus stream B; VbTAe, vinasses before TA extraction.

Table 6. Amino Acids Profile (in ppm) for Fermentation Broths after Sterilization[a]

			20 g vinasses/L			30 g vinasses/L			40 g vinasses/L		
amino acid (mg/kg)	YEMEP	CSL	VaTAe	VaTAe+SB	VbTAe	VaTAe	VaTAe+SB	VbTAe	VaTAe	VaTAe+SB	VbTAe
alanine	156.4	65.8	16.4	26.0	35.0	23.0	49.0	57.6	25.4	60.1	66.9
arginine	66.4	9.5	5.4	7.0	11.0	8.3	15.0	13.7	4.8	13.5	17.1
aspartic acid	72.3	21.8	19.6	24.7	27.0	41.5	52.2	42.5	49. 5	63.0	52.8
glutamic acid	238.5	20.4	5.4	18.2	26.0	10.5	33.4	44.5	8.1	40.7	55.6
glycine	68.2	18.9	9.5	12.7	19.5	14.0	27.8	26.5	10.7	28.4	32.8
hidroxiproline	n.d.	3.5	n.d.	n.d.	n.d.	5.5	5.3	n.d.	n.d.	5.3	n.d.
histidine	34.9	9.5	n.d.	n.d.	n.d.	n.d.	6.2	n.d.	n.d.	n.d.	6.0
isoleucine	92.6	13.0	4.8	9.2	12.1	6.5	17.1	20.3	7.1	20.1	24.6
leucine	162.7	28.0	6.9	15.5	21.7	10.1	28.9	37.5	11.9	35.0	45.9
lysine	211.9	24.7	11.6	26.0	32.9	17.5	47.2	57.4	18.9	58.0	63.6
methionine	72.1	25.6	15.4	21.0	18.1	20.0	26.0	19.4	18.5	26.8	22.6
phenylalanine	107.8	14.0	3.5	8.8	12.5	5.4	16.7	20.8	6.6	19.3	25.5
proline	66.4	235.7	42.1	82.5	121.2	62.7	143.9	213.5	73.8	179.6	262.5
serine	114.2	22.9	8.0	10.7	27.0	14.1	34.1	22.8	7.1	20.	27.6
threonine	260.7	30.6	4.6	9.6	12.8	9.2	15.8	19.6	7.4	16.6	25.3
valine	110.1	17.8	9.5	12.9	16.5	12.2	23.1	25.1	10.5	26.9	32.3
total content	1,835.2	561.7	162.7	284.8	393.3	260.5	541.7	621.2	210.8	613.3	761.1

[a] YEMEP: YE (3 g/L); malt extract (3 g/L); peptone (5 g/L); n.d. = no detected; VaTAe, vinasses after TA extraction; VaTAe+SB, vinasses after TA extraction plus stream B; VbTAe, vinasses before TA extraction.

during the TA recovery, as it can be observed in stream B, where 5.4 g soluble proteins/L were quantified. It is notorious, that the concentration values are higher than the values detected in media prepared using commercial nutrients such as YE, yeast malt, and peptone (2.2 g soluble protein/L and 2.7 g total protein/L) or CSL (2.8 g soluble protein/L and 3.1 g total protein/L). In all cases, the protein content increased with the amount of vinasses. Furthermore, a positive effect of sterilization was observed, increasing the concentration of proteins (total and soluble) in the fermentation broth. Higher increments (in the range of 145.3−187.7%) were observed using vinasses after TA extraction.

Tables 5 and **6** show the amino acid profiles before and after sterilization, respectively. This process hardly influenced the concentrations detected. All fermentation media contain considerable amounts of the essential amino acids, comparing favorably with the FAO food protein standard. Most of the essential and nonessential amino acids are present in amounts above or close to reported values of biomass to be used as food/feed supplement (*29*).

Evaluation of CSL as Economic Nutrient. CSL is an excellent source of nitrogen for most microorganisms because it is high in amino acids and polypeptides with considerable amounts of B-complex vitamins (*30*). Besides *L. rhamnosus*, *D. hansenii*, and *A. niger*, CSL has been used as economic nutrient of other microorganisms to produce different metabolites because increased product yields have been reported in various processes when CSL was used to replace conventional nutrient sources (*31*, *32*), possibly due to a more suitable protein profile of this complex substrate (*33*). CSL has been employed as an inexpensive source of essential microbial nutrients for a variety of purposes, including ethanol by *Zymomonas mobiliz* (*34*, *35*)) or *Pichia stipitis* ((*36*), butanol by *Clostridium beijerinckii* (*37*), succinic acid by *Anaerobiospirillum succiniciproducens* (*38*), acetate by *Clostridium thermoaceticum* (*39*), arabinase by *Fusarium oxysporum* (*40*), or

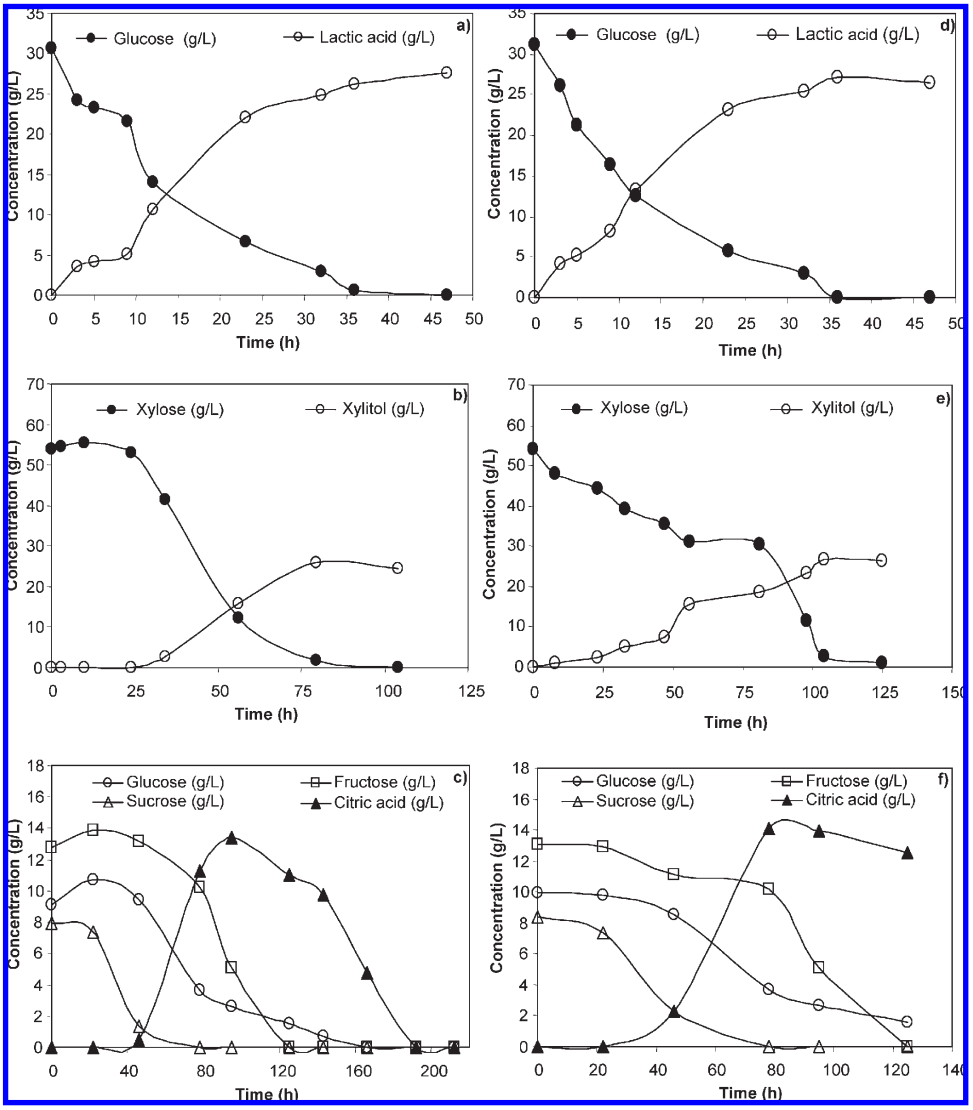

Figure 2. Course with time for the production of (a) lactic acid by *Lactobacillus rhamnosus*, (b) xylitol by *Debaryomyces hansenii*, and (c) citric acid by *Aspergillus niger*, during fermentations carried out with corn steep liquor (30 g/L) as the only nutrient or (d) lactic acid by *Lactobacillus rhamnosus*, (e) xylitol by *Debaryomyces hansenii*, and (f) citric acid by *Aspergillus niger*, during fermentations carried out with fully supplemented media. Results represent the average of two independent experiments. Standard deviations were below 2.6% of the mean.

glucose oxidase by *A. niger* (*9*). Recently, it has also been employed for the high-density spore production of a *B. cereus* aquaculture biological agent (*33*).

Taking into account that CSL is a reach source of nutrients, containing vitamins, minerals, carbohydrates, amino acids, polypeptides, proteins, and metals (*41*), a preliminary set of experiments was carried out to check the possibility of using 30 g CSL/L as unique nitrogen source by *L. rhamnosus* (medium 1 of **Table 1**), *D. hansenii* (medium 11 of **Table 1**), and *A. niger* (medium 21 of **Table 1**).

Lactic acid bacteria are nutritionally fastidious, requiring various amino acid and vitamins for growth. The selection of the right type of the nitrogen source appears to be very important. In this way, CSL has long been proved to be an inexpensive alternative to much more expensive materials, such as YE and peptone (*30*). For that reason, CSL has been employed in the production of lactic acid by *L. rhamnosus* (*6*, *11*, *30*, *42*), *L. penstosus* (*43*), although according to Demirci et al.(*44*) in the production of LA, CSL can negatively affect the product purity, and vitamin supplements may be required to achieve reasonable bioconversion. LA was also produced by *Enterococcus faecalis* using CSL as inexpensive nutrient (*1*). In this case, the addition of 30 g CSL/L supplemented with a minimal amount of YE supported both cell growth and LA fermentation. The experimental results (see **Figure 2** and **Table 3**) showed complete glucose consumption after 36 h by *L. rhamnosus*, with a maximal LA concentration of 27.6 g/L, corresponding to a volumetric productivity (Q_{LA}) of 0.726 g/L·h and product yield ($Y_{LA/S}$) of 0.90 g/g. Consequently, CSL can successfully replace the role of YE in LA fermentation. These results improve those obtained by Bustos et al. (*11*), which observed a poor conversion of glucose into LA by *L. rhamnosus* using only 10 g CSL/L as the only nutrient.

D. hansenii showed a similar favorable bioconversion pattern, with a remarkable maximum xylitol concentration of 25.9 g/L after a long fermentation time of 80 h ($Q_{xylitol}$ = 0.326 g/L·h, $Y_{xylitol/S}$ = 0.50 g/g). Carvalheiro et al. (*45*) studied the effects of several complex nutrients, such as malt extract, peptone, casamino acids, YE, and CSL, on the bioconversion of nondetoxified brewery spent grain (BSG) hydrolyzate to xylitol by *D. hansenii* observing the best results ($Q_{xylitol}$ = 0.36 g/L·h, $Y_{xylitol/S}$ = 0.55 g/g) when using YE to supplement BSG. CSL did not equal these

Table 7. Stoichiometric Parameters, Productivities, and Yields for Bioconversion Assays Carried Out Using Different Microorganisms (Results Calculated for Fermentation Times Leading to Maximum Product Concentration) Using the Following Nutrients: (a) CSL, (b) Vinasses, and (c) Commercial Nutrients[a]

	medium	microorganism	sugar	product	S_0 (g/L)	SC (g/L)	P_{max} (g/L)	T_f (h)	Q_P (g/L·h)	$Y_{P/S}$ (g/g)	$E_{p/€}$ (g/€)
(a)	M1	*L. rhamnosus*	glucose	LA	30.7	30.7	27.6	36	0.726	0.90	13.0
	M11	*D. hansenii*	xylose	xylitol	53.9	52.0	25.9	80	0.326	0.50	12.2
	M21	*A. niger*	G+S+F	CA	29.8	22.1	13.4	95	0.141	0.61	6.5
(b)	M2	*L. rhamnosus*	glucose	LA	31.2	30.4	28.8	36	0.800	0.95	144.0
	M3	*L. rhamnosus*	glucose	LA	26.6	26.6	22.3	23	0.925	0.80	74.4
	M4	*L. rhamnosus*	glucose	LA	28.2	28.2	22.6	23	0.924	0.75	56.5
	M5	*L. rhamnosus*	glucose	LA	29.7	23.7	22.2	36	0.594	0.90	106.9
	M6	*L. rhamnosus*	glucose	LA	27.1	27.1	22.2	32	0.681	0.81	73.9
	M7	*L. rhamnosus*	glucose	LA	27.0	27.0	21.5	23	0.910	0.77	53.6
	M8	*L. rhamnosus*	glucose	LA	31.0	31.0	26.9	49	0.528	0.84	129.5
	M9	*L. rhamnosus*	glucose	LA	27.8	27.8	21.1	36	0.575	0.74	71.7
	M10	*L. rhamnosus*	glucose	LA	27.4	27.4	20.7	32	0.619	0.73	51.6
	M12	*D. hansenii*	xylose	xylitol	50.6	27.3	5.5	146	0.037	0.20	27.3
	M13	*D. hansenii*	xylose	xylitol	55.1	49.0	22.7	152	0.149	0.46	75.6
	M14	*D. hansenii*	xylose	xylitol	58.8	57.9	22.6	152	0.149	0.40	56.6
	M15	*D. hansenii*	xylose	xylitol	52.1	22.9	3.8	174	0.122	0.13	18.9
	M16	*D. hansenii*	xylose	xylitol	55.4	53.7	13.0	118	0.110	0.32	43.3
	M17	*D. hansenii*	xylose	xylitol	54.7	53.2	11.4	118	0.096	0.26	28.5
	M18	*D. hansenii*	xylose	xylitol	51.1	38.9	17.0	146	0.116	0.44	85.0
	M19	*D. hansenii*	xylose	xylitol	56.6	55.9	35.3	93	0.380	0.69	117.7
	M20	*D. hansenii*	xylose	xylitol	60.3	54.1	32.9	93	0.354	0.61	82.3
	M22	*A. niger*	G+S+F	CA	31.5	31.5	13.3	125	0.106	0.48	44.2
	M23	*A. niger*	G+S+F	CA	31.2	25.8	8.3	95	0.088	0.32	27.8
	M24	*A. niger*	G+S+F	CA	29.2	27.2	13.9	95	0.146	0.63	43.3
(c)	M25	*L. rhamnosus*	glucose	LA	31.2	31.2	27.2	36	0.760	0.87	3.7
	M26	*D. hansenii*	xylose	xylitol	54.2	51.6	26.8	104	0.258	0.52	23.3
	M27	*A. niger*	G+S+F	CA	31.3	17.5	14.1	78	0.181	0.45	12.6

[a] G+S+F = glucose (10 g/L), sucrose (6.5 g/L), and fructose (14 g/L). S_0 = initial sugars concentration; SC = sugars consumed at the end of the fermentation; P_{max} = maximum product concentration; T_f = time to achieve the maximum product concentration; Q_P = product volumetric productivity; $Y_{P/S}$ = product yield; $E_{p/€}$ = economic efficiency.

results ($Q_{xylitol}$ = 0.23 g/L·h, $Y_{xylitol/S}$ = 0.43 g/g). In a different study (*46*) it was reported that the combination of 3 g YE/L with CSL increases slightly the xylitol yield, with similar productivity than using 6 g YE/L, as the only nutrients, in fermentations carried out by *D. hansenii* using BSG dilute-acid hydrolyzate as the carbon source. CSL was more effective when working with hydrolyzates. For example, media containing up to 80 g/L xylose, obtained from *Eucalyptus* wood hydrolysis, were efficiently fermented by *D. hansenii* when hydrolyzates were detoxified by charcoal adsorption and supplemented with CSL (*47*).

Nevertheless, the use of CSL as supplemental nutrient by *A. niger*, resulted in incomplete sugars (glucose, sucrose, and fructose) conversion, 25.8% of the initial sugars remaining without being consumed even after prolonged fermentation times (95 h). Sucrose was the first sugar to be consumed, followed by glucose and fructose, thus confirming the behavior observed by Hossain et al. (*48*) in synthetic broth containing a mixture of these sugars and Rivas et al. (*19*) in orange peel hydrolyzates. After total sucrose depletion (78 h), glucose and fructose started to be consumed. A total of 29.1% of the glucose and 39.8% of the fructose, respectively, remained at the end of the fermentation. The maximum CA concentration was only 13.4 g/L, leading to Q_{CA} of only 0.141 g/L·h and $Y_{CA/S}$ of 0.61 g/g. Lofty et al. (*49*) studding different variables (pretreated beet molasses, CSL, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $FeCl_3$, $ZnSO_4$, and $MnCl_2 \cdot H_2O$) found CSL as the most significant variable that increased CA production by *A. niger*, using a basal medium made up of 274.4 g pretreated beet molasses/L, 7.25 g CSL/L, 0.5 g KH_2PO_4/L, 0.15 g $MgSO_4 \cdot 7H_2O$/L, 0.01 g $FeCl_3$/L, 0.0006 g $ZnSO_4$/L, and 0.0006 g $MnCl_2 \cdot H_2O$/L.

Evaluation of Vinasses as Economic Nutrients. According to Wee et al. (*1*), raw materials for industrial LA production need to have several characteristics such as low cost, low levels of contaminants, rapid fermentation rate, high LA yields, little or no byproduct formation, and year round availability. Thus, studies on alternative low-cost media for LA fermentation are needed because of its industrial feasibility and from economic considerations. Lower-value products could be obtained using cheaper raw materials, including vinasses. For that reason, a set of experiments was carried out to check the possibility of obtaining suitable fermentation media using vinasses as unique supplementation factor for the performance of *Lactobacillus rhamnosus* cells grown on synthetic glucose (media 2−9 of **Table 1**). In this study, 20, 30, or 40 g vinasses/L were evaluated using three types of vinasses: vinasses without TA recovery, vinasses obtained after TA recovery, and vinasses obtained after TA recovery plus the removed substances contained in stream B. The experimental results (see **Table 7** and **Figure 3**) showed complete glucose consumption at the end of fermentation (23−49 h), with a maximal LA concentration of 28.8 g/L after 36 h (Q_{LA} = 0.800 g/L·h, $Y_{LA/S}$ = 0.95 g/g) using 20 g VaTAe+SB/L. The global volumetric productivity increased up to 0.924 g/L·h when this concentration was increased up to 40 g/L. In spite of the higher amount of proteins (see **Table 3**) and total amount of amino acids (see **Table 6**), the worst results were achieved when vinasses were used without TA extraction, with global volumetric productivities in the vicinity of 0.528−0.619 g/L·h. These results are particularly interesting because vinasses could be revalued by a dual-use; on one hand, TA can be recovered to be used in enological applications, meanwhile, the

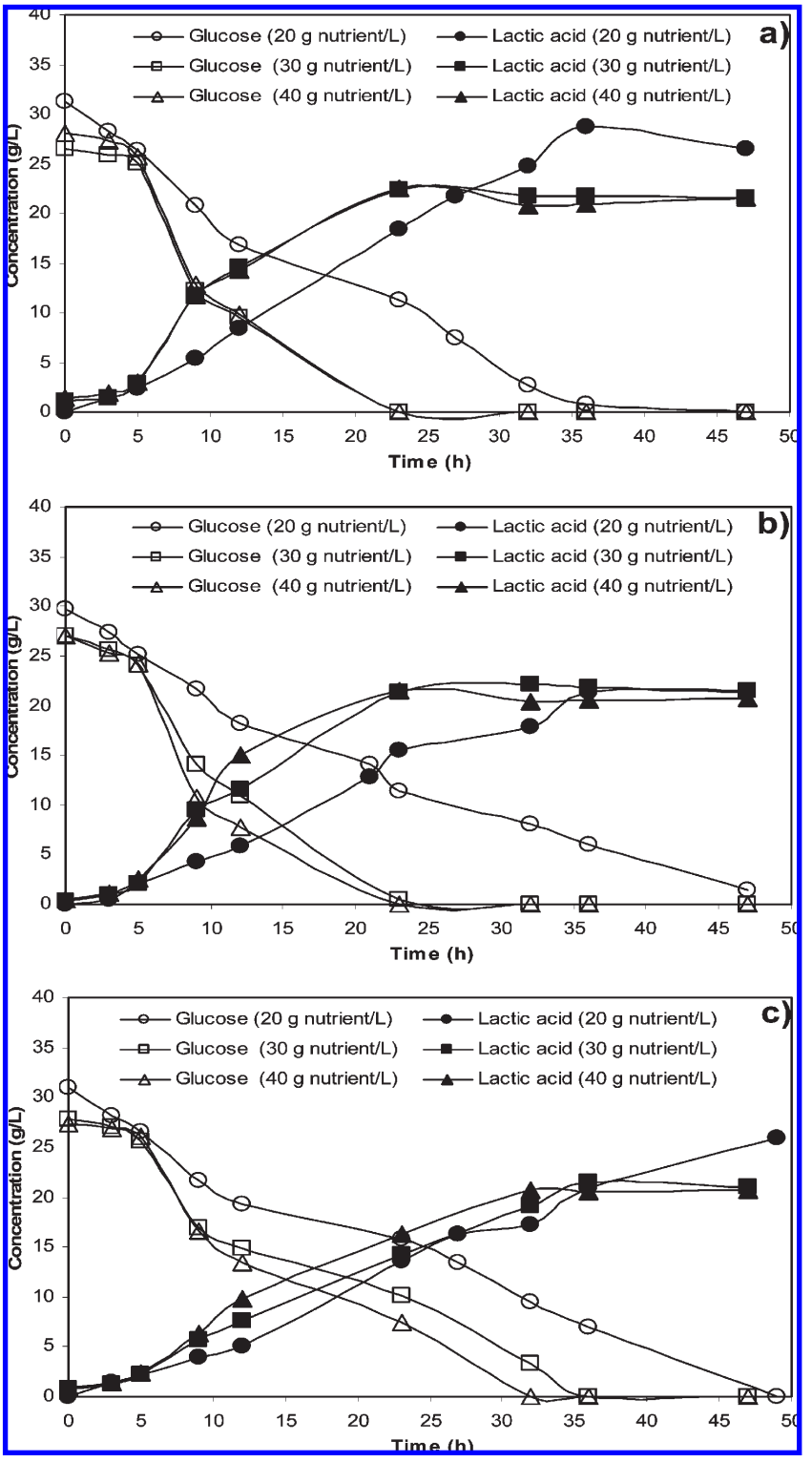

Figure 3. Course with time for the production of lactic acid by *Lactobacillus rhamnosus* during fermentations carried out with (a) vinasses obtained after TA recovery plus the removed substances contained in stream B (VaTAe+SB), (b) vinasses obtained after TA recovery (VaTAe), and (c) vinasses without TA recovery (VbTAe). Results represent the average of two independent experiments. Standard deviations were below 2.9% of the mean.

remaining residue could be used as economic nutrient. These results are similar to those achieved using CSL as the unique supplementation source in medium 1 of **Table 1** (LA = 27.6 g/L, Q_{LA} = 0.726 g/L·h, and $Y_{LA/S}$ of 0.90 g/g).

Conversely, the best results obtained in experiments conducted using *D. hansenii* and *A. niger* were obtained when the fermentation media were prepared using vinasses before TA extraction. **Figure 4** shows a favorable bioconversion pattern for *D. hansenii*, with a remarkable maximum concentration of 35.3 g xylitol/L after 93 h ($Q_{xylitol}$ = 0.380 g/L·h, $Y_{xylitol/S}$ = 0.69 g/g) in medium 19 of **Table 1** (30 g VbTAe/L), increasing significantly the value achieved with CSL (P = 25.9 g/L, $Q_{xylitol}$ = 0.326 g/L·h, $Y_{xylitol/S}$ = 0.50 g/g). *Aspergilus niger* showed a similar behavior (**Table 7** and **Figure 5**), with a maximum CA concentration of 13.9 g CA/L obtained after 95 h (Q_{CA} = 0.146 g/L·h, $Y_{CA/S}$ = 0.63 g/g), using 30 g VbTAe/L. These results are also similar to the

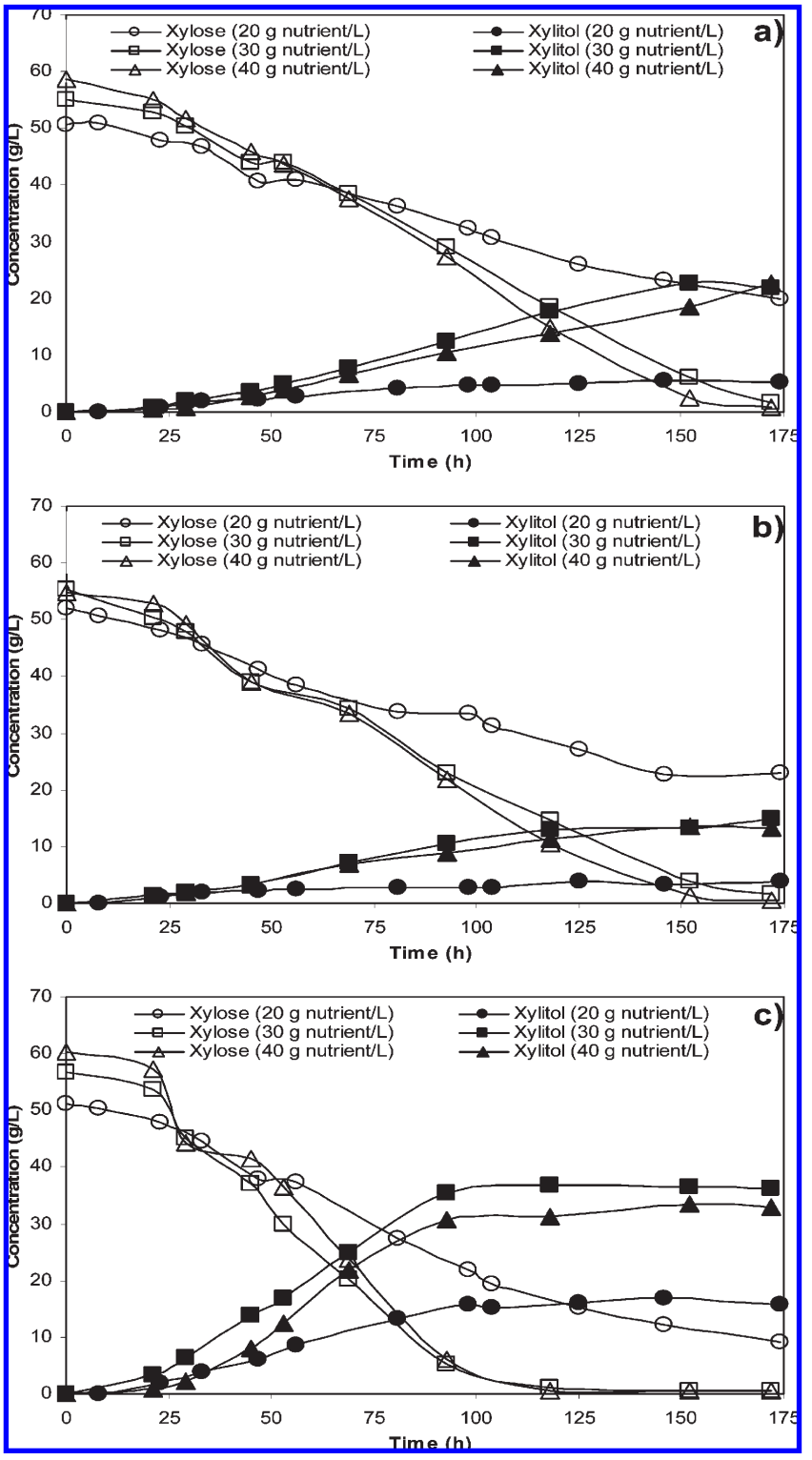

Figure 4. Course with time for the production of xylitol by *Debaryomyces hansenii* during fermentations carried out with (a) vinasses obtained after TA recovery plus the removed substances contained in stream B (VaTAe+SB), (b) vinasses obtained after TA recovery (VaTAe), (c) vinasses without TA recovery (VbTAe). Results represent the average of two independent experiments. Standard deviations were below 2.7% of the mean.

ones observed with CSL in medium 21 of **Table 1** (CA = 13.4 g/L, Q_{CA} = 0.141 g/L·h, and $Y_{CA/S}$ of 0.61 g/g).

LA was produced in all cases with reasonable values, between 20.7 g LA/L (medium 10 of **Table 1**) and 28.8 g LA/L (medium 2 of **Table 1**). CA oscillated between 8.3 g CA/L (medium 23 of **Table 1**) and 13.9 g CA/L (medium 24 of **Table 1**). Nevertheless, *D. hansenii* provided some poor results such as 3.2 g xylitol/L (medium 15 of **Table 1**) or 5.5 g xylitol/L (medium 12 of **Table 1**) and some good results, including 35.3 g xylitol/L (medium 19 of **Table 1**). The big differences observed suggest that *D. hansenii* has some minimal nutritional requirements. For that reason, several adjustments were made between initial substances analyzed and products obtained, finding that xylitol and citric acid production are directly influenced by the concentration and nature of the carbon source, in particular, the initial amino acids content appear to be the most influencing one. Thus, the amount of total

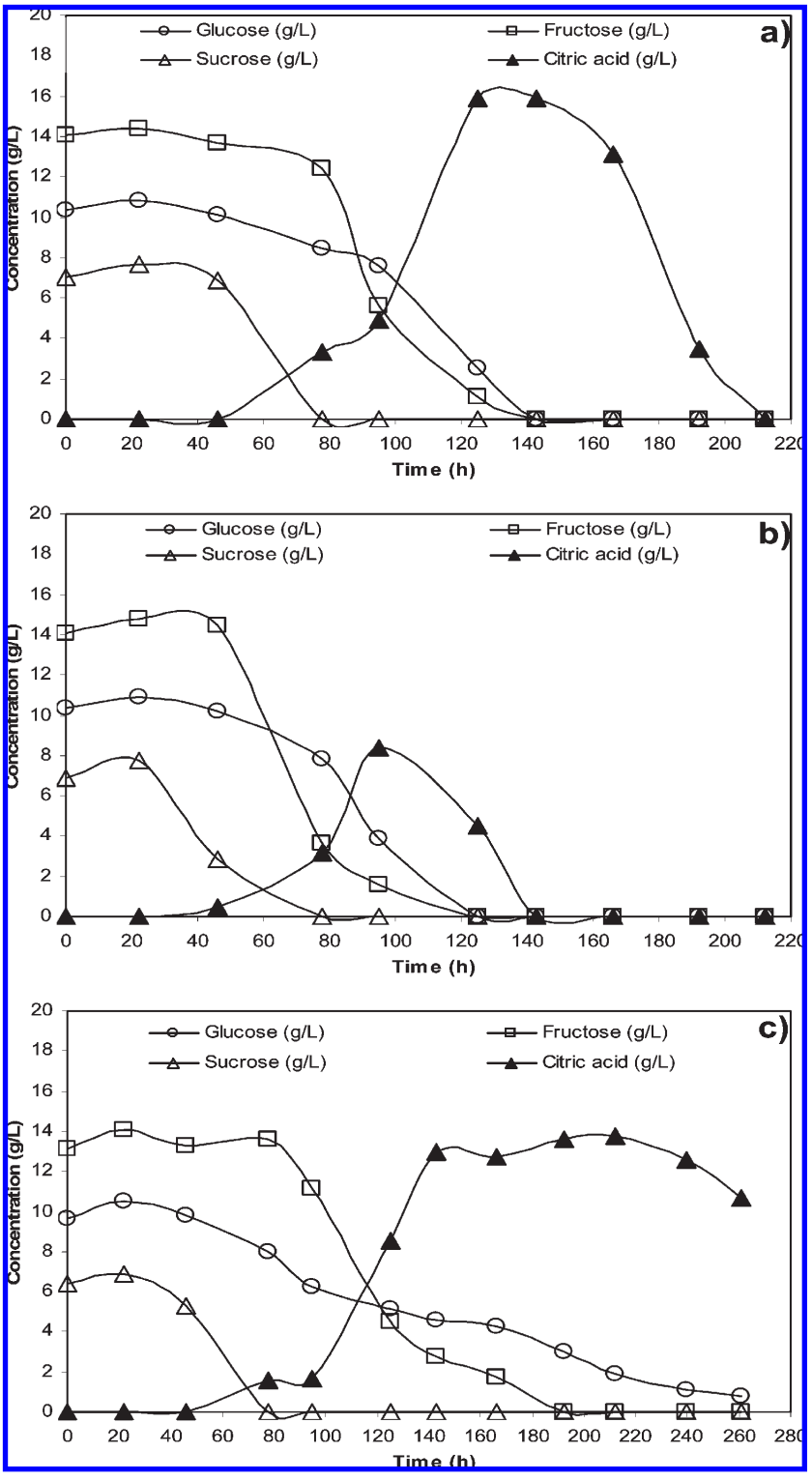

Figure 5. Course with time for the production of citric acid by *Aspergillus niger* during fermentations carried out with 30 g/L of (a) vinasses obtained after TA recovery plus the removed substances contained in stream B (VaTAe+SB), (b) vinasses obtained after TA recovery (VaTAe), (c) vinasses without TA recovery (VbTAe). Results represent the average of two independent experiments. Standard deviations were below 2.4% of the mean.

amino acids at the beginning of the fermentation was represented versus the final concentration of product achieved in each case for the three microorganisms (see **Figure 6**). Good correlations were observed using *D. hansenii*, [xylitol (g/L)] = −2.4676 + 0.0487 * [amino acid (ppm)] ($R^2 = 0.86$), and *A. niger* [CA (g/L)] = 4.1764 + 0.0162 * [amino acid (ppm)] ($R^2 = 0.99$). Furthermore, **Figures 7** and **8** represent the dependence of xylitol and citric acid, respectively, with the total content of C (in mg/g), proteins (in g/L), and amino acids (in ppm) reflecting the importance of the amount and nature of the carbon source.

Finally, in relation with the metals analyzed, *D. hansenii* showed a strong dependence of the xylitol with the initial amount of Mg^{2+}. For example, only 3.8 g xylitol/L were produced starting with 10.4 mg Mg^{2+}/kg (medium 15 of **Table 1**), meanwhile increasing the amount of Mg^{2+} up to 54.1 (medium 19 of **Table 1**) and 72.2 mg/kg (medium 20 of **Table 1**), the final

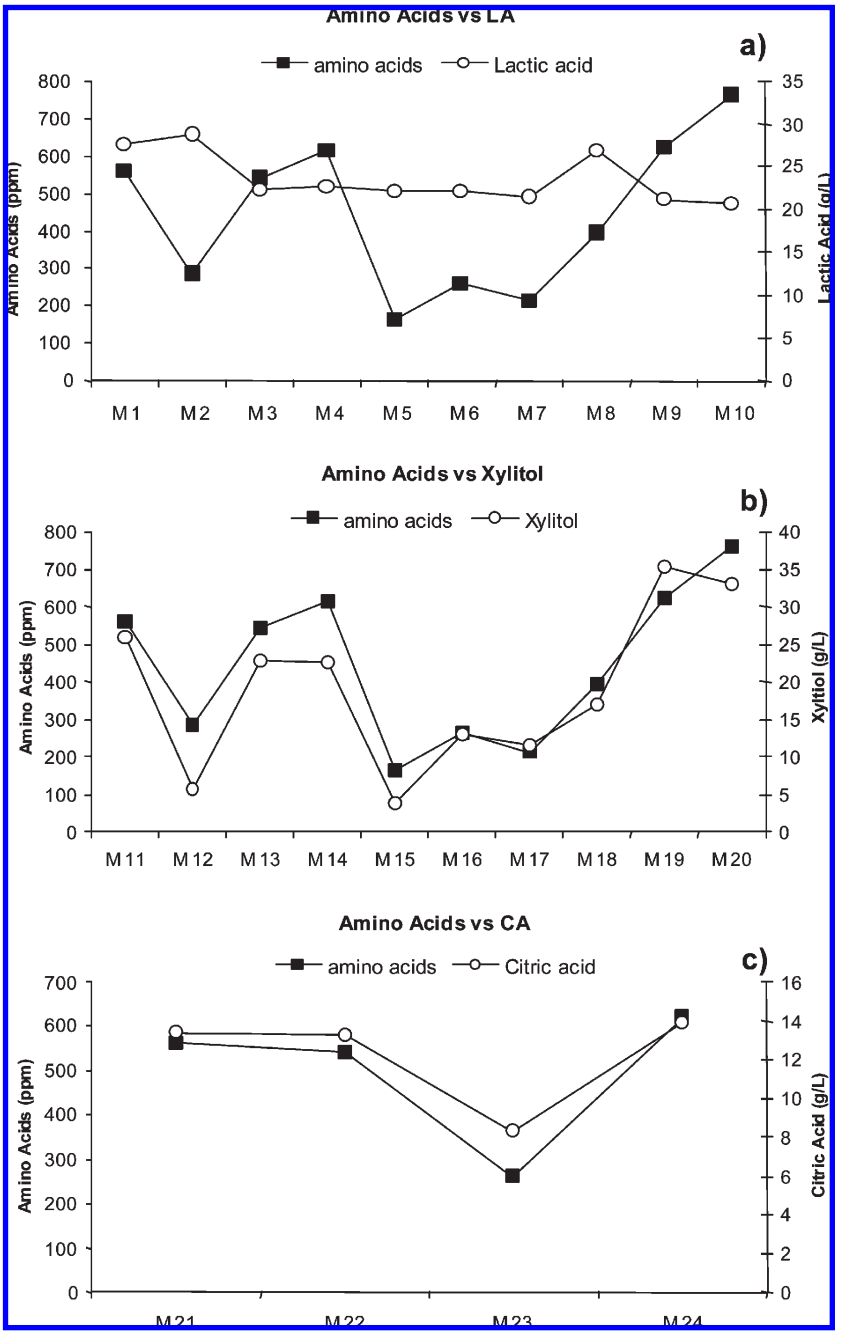

Figure 6. Total initial amino acid content (ppm) versus product (a) lactic acid, (b) xylitol, and (c) citric acid achieved at the end of fermentations.

concentration of xylitol was over 30 g/L, which is in agreement with Mahler and Guebel (*50*) that observed a positive effect of Mg^{2+} redirecting xylose fermentation by *Pichia stipitis* toward xylitol. On the other hand, *A. niger* showed a strong dependence in all the metals considered, with the exception of Al^{3+} and Ca^{2+}. The last one is not representative because Ca^{2+} is added as $CaCO_3$ and $CaCl_2$ to precipitate tartaric acid. Soccol et al. (*51*) also indicated that trace metal ions have a significant impact on CA accumulation by *A. niger* and, in particular, divalent metal ions such as Zn^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+}, and Mg^{2+}, being crucial to take into account the interdependence of medium constituents.

Economy of the Nutrients. On the basis of cost and availability on a large scale production with an industrial process in mind, the suitability of low-cost media (CSL and vinasses) was compared with the main nutrients of the traditional fermentation medium proposed by Mercier et al. (*2*) for the lactic acid production by *L. rhamnosus* (medium 25), 3 g yeast extract/L, 3 g malt extract/L, and 5 g peptone/L for the xylitol production by *D. hansenii* (medium 26) (*3*), and NH_4NO_3 (25 g/L), $MgSO_4 \cdot 7H_2O$ (2.5 g/L), and $CuSO_4$ 0.04 (g/L) for the citric acid production by *A. niger* (medium 27) (*21*). Three fermentation runs were carried out using these commercial nutrients. **Figure 2**d−f shows the time course of these fermentation assays.

In the fully supplemented medium 25, glucose was rapidly consumed by *L. rhamnosus* and converted to lactic acid, reaching 27.2 g/L after 36 h, which represents a volumetric productivity $Q_{LA} = 0.760$ g/L·h and a product yield of $Y_{LA/S} = 0.87$ g/g. These values are similar to those achieved using 30 g CSL/L (medium 1) and 20 g VaTAe+SB/L (medium 2). The maximum xylitol concentration, volumetric productivity, and xylitol yield using medium 26 (26.8 g/L, 0.258 g/L·h and 0.52 g/g respectively) were also similar for fermentations using 30 g CSL/L (medium 11). On the contrary, the fermentations carried out with media 19 and

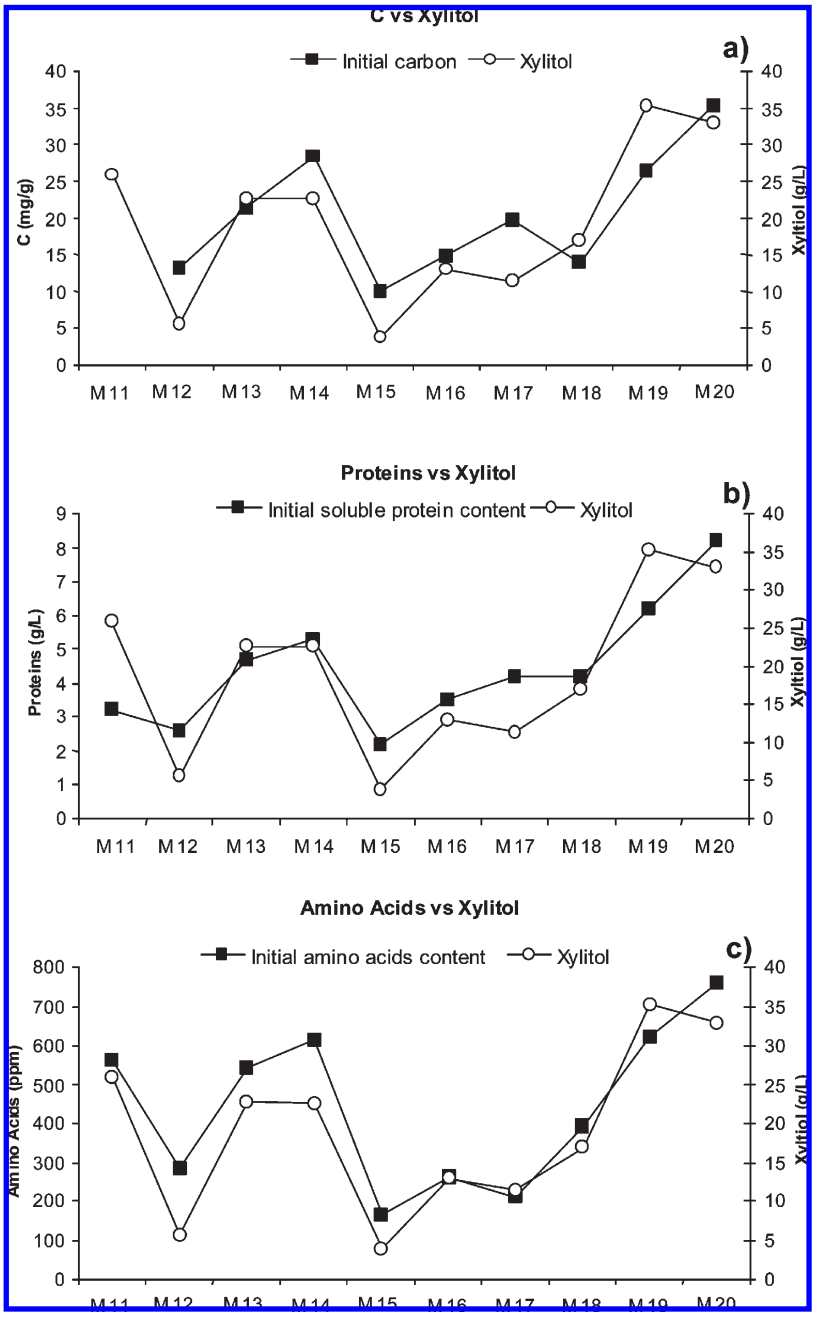

Figure 7. Xylitol produced versus (a) initial carbon (mg/g), (b) initial protein content (g/L), and (c) total initial amino acid content (ppm).

20 of **Table 1** (30−40 g VbTAe/L respectively) the xylitol concentration increased up to 35.3−32.9 g/L after 93 h ($Q_{xylitol}$ = 0.380−0.354 g/L·h, $Y_{xylitol/S}$ = 0.69−0.61 g/g, respectively). Finally, *A. niger* showed a similar behavior (14.1 g CA/L, Q_{CA} = 0.181 g/L·h, $Y_{CA/S}$ = 0.45 g/g), than using 30 g CSL/L (medium 21), 30 g VaTAe+SB/L (medium 22), or 30 g VbTAe/L (medium 24).

Consequently, the approach described in this work with vinasses allows similar or better results than the ones achieved with the fully supplementation media. Taking these results into account, the numerical values of the economic efficiency ($E_{p/€}$) at the end of the fermentations, defined as grams of product (LA, xylitol, or CA) per cost unit of nutrients (*52*) were calculated (**Table 7**) to select the cheapest media that allows the highest product concentration to be obtained. **Table 8** shows the cost of each nutrient. The price for vinasses was assumed to be 10 €/kg, considering the price of vinasses as byproduct (75.23 €/Tm) according to Deurwaarder et al. (*53*), and the costs corresponding to transport, conservation, and profits derived of commercialization.

For the LA production, the economic efficiency ($E_{p/€}$) parameter showed that it was more profitable to use vinasses (media 2−10) than CSL (medium 1, $E_{p/€}$ = 144.0 g/€) and much more than the Mercier medium (medium 25, $E_{p/€}$ = 144.0 g/€) because, using vinasses, higher lactic acid was produced per euro of nutrients. For example, the higher value of $E_{p/€}$ = 144.0 g/€ achieved in medium 2 indicates than 144.0 g of LA can be produced pero 1 € of nutrients. The $E_{p/€}$ parameters calculated during the xylitol production reveal that vinasses are more profitable (media 12−20) than using commercial nutrients (medium 26, $E_{p/€}$ = 23.3 g/€) and much more than using CSL (medium 11, $E_{p/€}$ = 12.2 g/€), achieving the higher value of $E_{p/€}$ = 117.7 g/€ in medium 19. This tendency was similar during the production of citric acid. When vinasses were used (media 22, 24

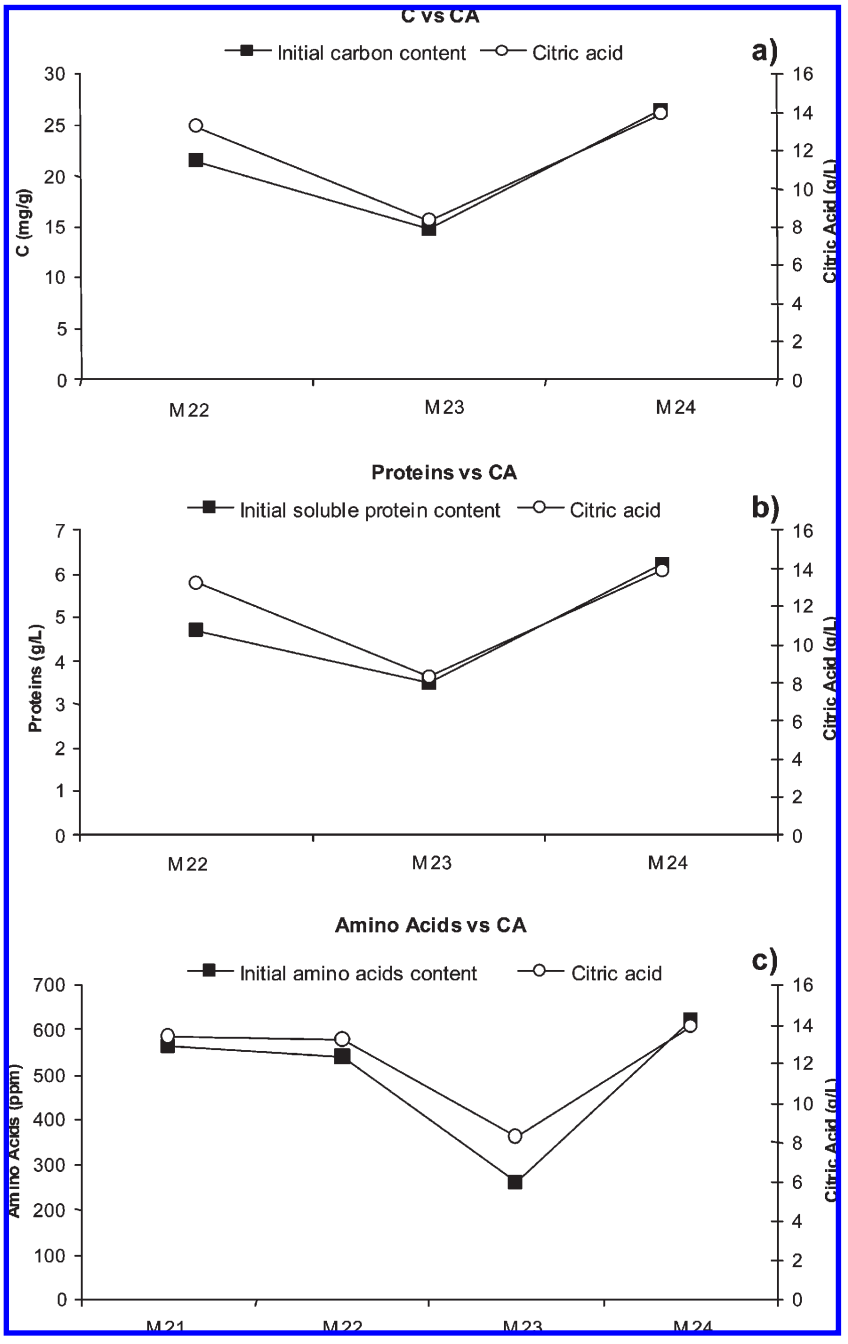

Figure 8. Citric acid produced versus (a) initial carbon (mg/g), (b) initial protein content (g/L), and (c) total initial amino acid content (ppm).

Table 8. Prices of Nutrients Employed

nutrient	cost (€/kg)[a]
CSL	70.8
YE	81.6
YM	136.4
Pept.	99.6
Mercier	140.2
NH_4NO_3	40.7
$MgSO_4 \cdot 7H_2O$	37.5
$CuSO_4$	84.5
vinasses	10

[a] Costs are the average of the regular price obtained from different companies.

and 23), the parameters were 44.2, 43.3, and 27.8 g/€, respectively, compared with $E_{p/€}$ = 12.26 g/€ using commercial nutrients (medium 27), and $E_{p/€}$ = 6.5 g/€ using CSL. The economic efficiency ($E_{p/€}$) parameters showed that it was more profitable to use vinasses than CSL or commercial nutrients.

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