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Formulation of Low-Cost Fermentative Media for Lactic Acid Production with *Lactobacillus rhamnosus* Using Vinification Lees as Nutrients

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Lees coming from different steps in white wine and red wine vinification were characterized under physicochemical analyses to determine the content in carbon, nitrogen, ashes, solids in suspension, organic compounds, and minerals. Due to the hydrolytic activity of *Lactobacillus* strains, lees without autolysis treatments were used directly as the unique nutrient or in combination with corn steep liquor to carry out the glucose to lactic acid fermentation with *Lactobacillus rhamnosus* CECT-288. Time courses of glucose and lactic acid were modeled according to reported models. Using 20 g/L of lees coming from the white wine technology and re-collected after the second decanting step before distillation, as the only nutrient, the values achieved ($P = 105.5$ g/L, $Q_P = 2.470$ g/L·h) were even higher than those obtained with the costly MRS broth ($P = 104.3$ g/L, $Q_P = 2.251$ g/L·h).

KEYWORDS: Lees; corn steep liquor; autolysis; lactic acid; *Lactobacillus rhamnosus*

INTRODUCTION

Viticulture, a subsector of great importance in many countries, generates a huge amount of microbial biomass (lees) that should be managed to avoid harmful effects on the environment. Yeasts proliferated during the fermentation of the must in wine die when nutrients are depleted and settle at the bottom of the barrels together with other microorganisms, suspended solids, colloids, and organic matter to give the lees fraction. Except in the cases of aging wine on lees to enhance the finished wine's body and flavor, when in touch with wine, lees may transmit undesirable flavors. Usually, lees are processed with other muds in costly treatment plants.

Products obtained by biotechnological procedures are preferred by industry and consumers for food-related applications, but fermentation technologies must be cost competitive with chemical synthesis to carry out the biotechnological process at an industrial scale. The nutrients used traditionally in most of the fermentative media, particularly yeast extract and peptone, are very expensive, accounting for almost 30% of the total cost of the process (*1*). Because of this, the search for alternative, financially competitive nutrient sources is particularly interesting. Considering that lees are basically dead yeasts, this waste fraction represents a potential source of nutrients, particularly after being subjected to autolysis.

Autolysis is the hydrolysis of cellular components by hydrolytic yeast enzymes. The main events that occur during this process are breakdown of cell membranes, release of hydrolytic enzymes, liberation of intracellular constituents, and hydrolysis of intracellular biopolymers into products of a low molecular weight (*2*). Literature studies have considered the use of yeast autolysates as nutrients in wheat fermentations (*3*) and alcoholic production by recombinant *Escherichia coli* (*4*). Autolysates of brewery yeast biomass have been used for growth of *Lactobacillus plantarum* in whey (*5*) or *Bacillus thuringiensis kurstaki* (*6*). Lactic acid fermentation has been carried out using lactic acid bacteria autolysates (*7*, *8*) or spent yeasts coming from the fermentation of xylose to xylitol with *Debaryomyces hansenii* (*9*). Most cases dealing with yeast autolysates are based on the utilization of sophisticated and expensive treatments, such as sedimentation with chitosan, enzymatic or chemical processing, and protein solubilization (*2*, *5*, *10*).

Yeast extract, the water soluble portion of autolyzed yeast cells, is the nitrogen source that gave the highest productivities during lactic acid fermentation (*11*), the main contributions being purine and pyrimidine bases as well as B-vitamins (*12*).

Corn steep liquor (CSL) is an inexpensive source of essential microbial nutrients already used for the ethanol production by *Zymomonas mobilis* (*13*, *14*) or *Pichia stipitis* (*15*), succinic acid by *Anaerobiospirillum succiniciproducens* (*16*), or arabinanase by *Fusarium oxysporum* (*17*).

The main purpose of this study is to develop a profitable technology for the benefit of microbial biomass (lees), transforming them into cheap nutrients for fermentation media. After characterization, lees, without autolysis treatments, were directly used alone or in combination with CSL to formulate cheap fermentative media, which were assayed with *Lactobacillus rhamnosus* for lactic acid production, a food industry acidifier.

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Table 1. Percentage of Solids (Grams per 100 g of Wet Lees) and Ashes in Lees and Carbon and Nitrogen Contents (Grams per 100 g of Dried Lees)[a]

lees	solids	ashes	C	N
lees from pressed bagasse without distillation	19.9 ± 0.4a	8.2 ± 0.3a	7.5 ± 0.2a	0.5 ± 0.1a
white lees, first decanting step, without distillation	34.2 ± 1.0b	28.1 ± 0.2b	10.9 ± 0.1b	0.7 ± 0.2ab
white lees, second decanting step, without distillation	31.1 ± 0.3c	11.7 ± 0.2c	11.3 ± 0.4be	1.0 ± 0.1bc
white lees after distillation	17.2 ± 0.1d	23.4 ± 0.4d	6.4 ± 0.3c	0.4 ± 0.0a
red lees, first decanting step, without distillation	16.7 ± 0.4d	6.1 ± 0.5e	7.5 ± 0.2a	0.5 ± 0.1a
red lees, second decanting step, without distillation	10.7 ± 0.3e	10.8 ± 0.3 f	5.0 ± 0.2d	0.4 ± 0.1a
red lees after distillation	44.7 ± 0.5 f	7.9 ± 0.2a	11.7 ± 0.1e	1.2 ± 0.3c

[a] Different letters in the same column indicate significant difference ($p < 0.05$) between treatments. Data indicate the mean values of four replications and their standard deviations.

Table 2. Concentration of Organic Compounds in Wet Lees (Grams per Liter)[a]

lees	glucose	ethanol	lactic acid	acetic acid
lees from pressed bagasse, no distillation	0.4 ± 0.1a	61.9 ± 1.4a	4.3 ± 0.4ac	1.5 ± 0.2ac
white lees, first decanting step, no distillation	1.4 ± 0.2b	80.9 ± 3.5b	5.0 ± 0.1ab	2.4 ± 0.3b
white lees, second decanting step, no distillation	0 ± 0.0c	55.9 ± 0.8c	5.2 ± 0.5ab	1.6 ± 0.2abc
white lees after distillation	0 ± 0.0c	8.5 ± 0.3d	5.9 ± 0.3b	2.3 ± 0.4ab
red lees, first decanting step, no distillation	0.1 ± 0.1c	74.5 ± 2.2e	3.3 ± 0.2c	1.3 ± 0.1c
red lees, second decanting step, no distillation	0 ± 0.0c	63.5 ± 1.5a	11.4 ± 0.8d	6.6 ± 0.5d
red lees after distillation	0 ± 0.0c	5.0 ± 0.3d	21.2 ± 0.6e	10.8 ± 0.4e

[a] Different letters in the same column indicate significant difference ($p < 0.05$) between treatments. Data indicate the mean values of four replications and their standard deviations.

Table 3. Concentration of Minerals in Lees (Expressed as Milligrams of Metal per Kilogram of Dried Ash)[a]

lees	Cu	Mg	Fe	Mn	Ca	Al	Zn
lees from pressed bagasse, no distillation	2331 ± 27a	718 ± 15a	1954 ± 24a	291 ± 11a	4977 ± 47a	929 ± 26a	634 ± 39a
white lees, first decanting step, no distillation	1752 ± 12b	1057 ± 17b	1731 ± 5b	94 ± 3b	2950 ± 15b	3039 ± 104b	172 ± 11b
white lees, second decanting step, no distillation	3513 ± 29c	261 ± 12c	1745 ± 15b	117 ± 11b	3080 ± 5b	1350 ± 34c	109 ± 7c
white lees after distillation	1796 ± 34be	702 ± 22a	1559 ± 19c	103 ± 5b	3210 ± 46b	ND[b]	79 ± 11c
red lees, first decanting step, no distillation	10977 ± 52d	7720 ± 70d	7658 ± 56d	916 ± 14c	20455 ± 123c	5261 ± 47d	5621 ± 34d
red lees, second decanting step, no distillation	1838 ± 11e	6045 ± 40e	3667 ± 62e	553 ± 8d	13890 ± 220d	1180 ± 11e	196 ± 9be
red lees after distillation	3650 ± 19f	1359 ± 21f	5777 ± 137	281 ± 5a	10830 ± 76a	ND	247 ± 4e

[a] Different letters in the same column indicate significant difference ($p < 0.05$) between treatments. Data indicate the mean values of four replications and their standard deviations. [b] Not determined.

Table 4. Results Obtained by Regression of Lactic Acid and Glucose Concentration Data in Controls (MRS Broth or 10 g/L CSL)[a]

	lactic acid production					glucose consumption		
lees	P_0 (g/L)	P_{max} (g/L)	P_r (h^{-1})	r^2	*F* value	$Y_{P/S}$ (g/g)	r^2	*F* value
control (MRS broth) (**Figure 1a**)	3.2	103.0	0.160	0.992	422.1**	0.97	0.997	2486.1*
control (10 g/L CSL) (**Figure 1b**)	4.5	58.0	0.090	0.993	497.3**	0.78	0.969	114.51

[a] P_0 = initial lactic acid concentration (g/L); P_{max} = maximum concentration of lactic acid (g/L); P_r = ratio between initial volumetric rate of product formation (r_p) and initial product concentration P_0 (h^{-1}); $Y_{P/S}$ = product yield (g/g); r^2 = determination coefficient; *F* value = *F*-test statistical parameter. *, significance level > 95%; **, significance level > 99%.

The time courses of glucose consumption and lactic acid production were modeled according to reported models (*18*).

MATERIALS AND METHODS

Lees Sampling and Storage. Lees from the campaign of 2002 were kindly supplied by Cooperativa Vitivinícola do Ribeiro (Ourense, Spain) and stored at 4 °C. In white wine making technology the unfermented grape juice is extracted from the tanks and ferments separated from the grains and skin grape. This wet solid residue is pressed to obtain a lower quality wine called press wine and some lees coming from the pressed bagasse (noted in the text as "lees from pressed bagasse without distillation"). The wine rests in tanks or barrels, decanting the particles in suspension to the bottom. The number of decanting steps depends on the kind of wine, the amount of lees decreasing in each step. We took lees from the first and second decanting steps (noted in the text as "lees from the first or second decanting step without distillation"). These lees can also be mixed and distilled in order to recover ethanol and aromatic flavors used for the production of aromatic spirit liquors, giving lees less useful to winerys (noted in the text as "white lees after distillation").

To obtain red wines the grapes ferment with the juice, grains, and skins in the same tanks. Consequently, there is no wine from pressed bagasse in this step. Lees studied in this work came from the first and second decanting steps (noted in the text as "lees from the first or second decanting step without distillation") and from a mixture of lees and further distillation (noted in the text as "red lees after distillation").

Inoculum Preparation. *L. rhamnosus* CECT-288 was obtained from the Spanish Collection of Type Cultures (Valencia, Spain). The strain was grown on plates using the complete medium proposed by Mercier et al. (*18*), which contains 20 g of glucose/L, 5 g of yeast extract/L, 10 g of peptone/L, 5 g of sodium acetate/L, 2 g of sodium citrate/L, 2 g

Table 5. Results Obtained by Regression of Lactic Acid and Glucose Concentration Data in Experiments Carried out with 10 g/L of Lees and 10 g/L of CSL[a]

	lactic acid production					glucose consumption		
lees	P_0 (g/L)	P_{max} (g/L)	P_r (h^{-1})	r^2	*F* value	$Y_{P/S}$ (g/g)	r^2	*F* value
lees from pressed bagasse without distillation (**Figure 2a**)	4.8	92.8	0.115	0.998	1404.4**	0.89	0.991	529.2*
white lees, first decanting step, no distillation (**Figure 2b**)	3.8	92.0	0.132	0.998	2346.2**	0.92	0.993	594.0*
white lees, second decanting step, no distillation (**Figure 2c**)	4.2	93.3	0.148	0.998	2201.1**	0.89	0.997	1262.6*
white lees after distillation (**Figure 2d**)	4.7	94.7	0.120	0.998	1929.8**	0.92	0.994	879.4*
red lees, first decanting step, no distillation (**Figure 3a**)	5.5	93.1	0.102	0.996	910.7**	0.88	0.985	268.1*
red lees, second decanting step, no distillation (**Figure 3b**)	6.2	96.6	0.096	0.997	987.8 **	0.92	0.986	337.1*
red lees after distillation (**Figure 3c**)	6.3	103.2	0.091	0.996	763.8**	0.97	0.993	1263.7*

[a] P_0 = initial lactic acid concentration (g/L); P_{max} = maximum concentration of lactic acid (g/L); P_r = ratio between initial volumetric rate of product formation (r_p) and initial product concentration P_0 (h^{-1}); $Y_{P/S}$ = product yield (g/g); r^2 = determination coefficient; *F* value = *F*-test statistical parameter. *, significance level > 95%; **, significance level > 99%.

of K_2HPO_4/L, 0.58 g of $MgSO_4 \cdot 7H_2O$/L, 0.12 g of $MnSO_4 \cdot H_2O$/L, 0.05 g of $FeSO_4 \cdot 7H_2O$/L, and 10 g of agar/L at 37 °C for 24 h. Inocula were prepared by solubilization of cells from plates with 5 mL of sterile water. Biomass in inocula was measured by optical density at 600 nm and adjusted by dilution with water to reach a final concentration in the culture media of 7.4 g of dry cells/L.

Lactic Acid Fermentation. Experiments were carried out in 250 mL Erlenmeyer flasks with a final volume of 100 mL using different media containing ~100−110 g of glucose/L. A positive control (the richest medium proposed in the literature) was used using the complete medium proposed by Mercier et al. (*18*) (noted in the text as "MRS broth"). A negative control was performed with CSL (10 g/L) as the only nutrient (this negative control was used to demonstrate that CSL is not enough to cover the nutritional requirements of *L. rhamnosus*). Two sets of experiments were carried out using 10 g/L of lees plus 10 g/L of CSL or 20 g/L of lees. In all cases calcium carbonate (100 g/L) was added to neutralize the lactic acid produced. After inoculation (5 mL), fermentations were carried out in orbital shakers at 200 rpm. Samples (2 mL) were taken at given fermentation times and centrifuged at 6000 rpm for 3 min. The supernatants were stored for glucose and lactic acid analyses. Experimental data were measured in triplicate, and means are reported. Standard deviations were below 2.5% of the mean. The volumetric productivities Q_P were calculated for the fermentation times (each one indicated in the text) corresponding to the highest values of lactic acid concentrations.

Analytical Methods. Organic compounds in lees (glucose, ethanol, lactic acid, and acetic acid) as well as glucose consumed and lactic acid produced were measured by a high-performance liquid chromatograph (Agilent, model 1100, Palo Alto, CA), with RI detection using a Transgenomic ION-300 column (Transgenomic Inc., San Jose, CA) eluted with 0.02 M H_2SO_4 at a flow rate of 0.4 mL/min.

Solids in suspension in lees were oven-dried to constant weight at 102 °C. Ashes in lees were oven-dried to constant weight at 550 °C.

Nitrogen and carbon percentages in lees were analyzed using a Thermo Finningan 1112 series flash elemental analyzer (San Jose, CA).

Cu, Mg, Fe, Mn, Ca, Al, and Zn were analyzed in ashes using a 220 Fast Sequential atomic absorption spectrometer from Varian (Palo Alto, CA). Previously, 0.15 g of ashes was 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).

Statistical Analysis. Data were analyzed using Statgraphics 5 software (Manugistics, Inc., Rockville, MD). A multifactorial analysis of variance was carried out. Differences among mean values were established using the least significant difference (LSD) multiple-range test and were considered to be significant when $p < 0.05$.

Fitting of Data. Experimental data were fitted to proposed models using commercial software (Solver of Microsoft Excel 2002) by nonlinear regression using the least-squares method. Lactic acid production was mathematically modeled following the equation proposed by Mercier et al. (*18*)

$$\frac{dP}{dt} = P_r P\left(1 - \frac{P}{P_{max}}\right) \quad (1)$$

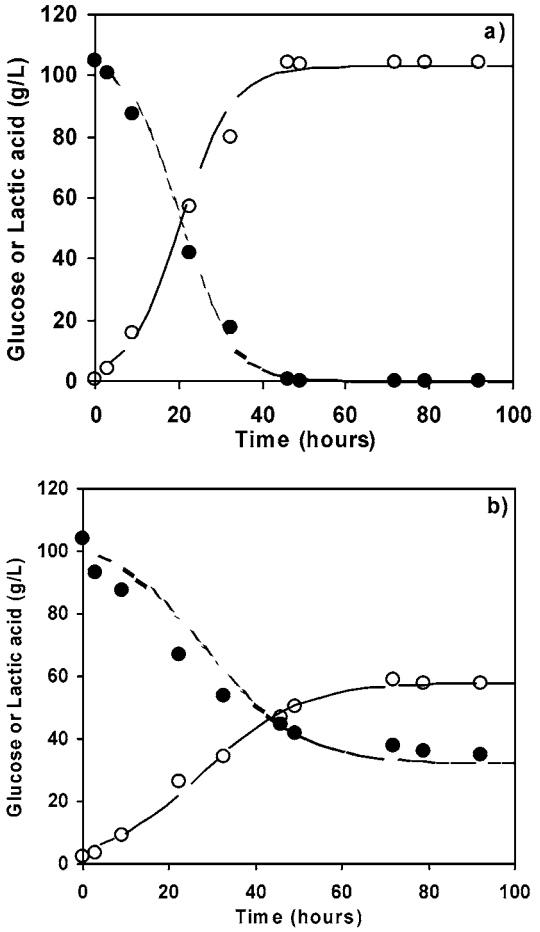

Figure 1. Experimental data and calculated time courses of lactic acid (○) and glucose concentrations (●) during fermentations carried out with (**a**) MRS broth (positive control) or (**b**) 10 g/L of CSL (negative control). Results represent the average of three independent experiments. Standard deviations were below 2.2% of the mean.

where t is time, P is lactic acid concentration, P_{max} is maximum concentration of lactic acid, and P_r is the ratio between the initial volumetric rate of product formation (r_p) and the initial product concentration P_0. Equation 1 can be directly solved to give the expression (*2*)

$$P = \frac{P_0 P_{max}\, e^{P_r t}}{P_{max} - P_0 + P_0\, e^{P_r t}} \quad (2)$$

From the series of experimental data lactic acid concentration/time, the model parameters P_0, P_{max}, and P_r can be calculated for each fermentation medium.

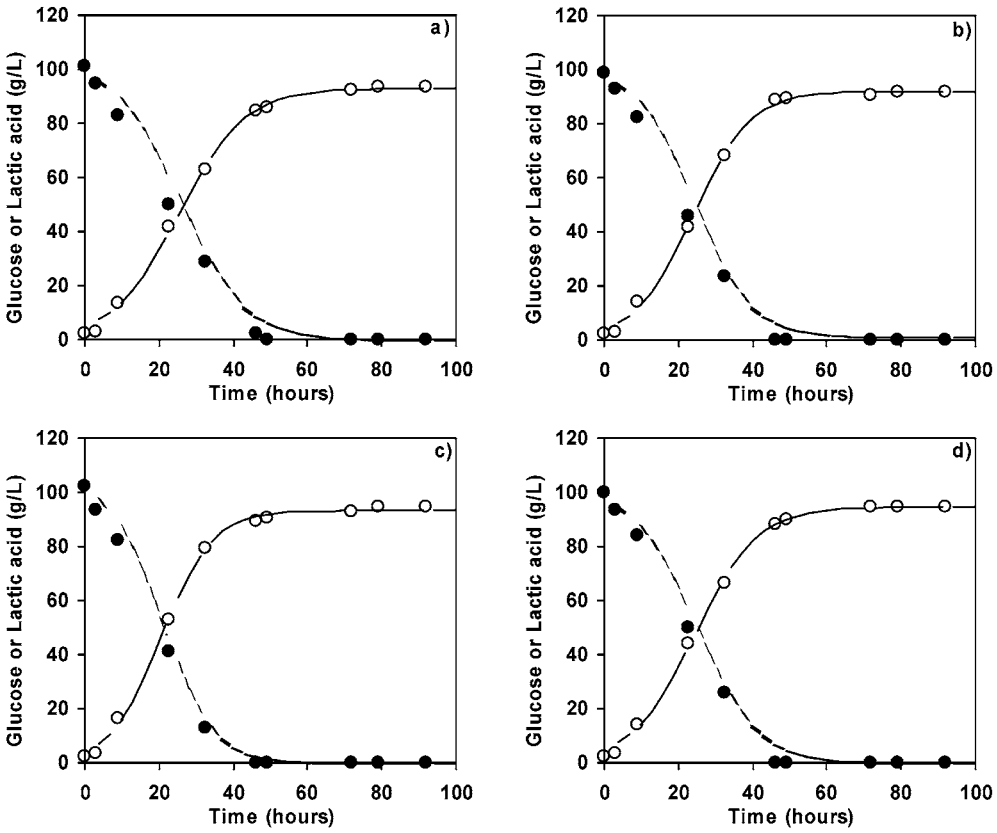

Figure 2. Experimental data and calculated time courses of lactic acid (○) and glucose concentrations (●) during fermentations carried out with 10 g/L of white lees and 10 g/L of CSL: (**a**) lees from pressed bagasse without distillation; (**b**) white lees from the first decanting step without distillation; (**c**) white lees from the second decanting step without distillation; (**d**) white lees after distillation. Results represent the average of three independent experiments. Standard deviations were below 2.4% of the mean.

Glucose consumption by *L. rhamnosus* can be interpreted by the equation

$$S = S_0 - \frac{1}{Y_{P/S}}(P - P_0) \quad (3)$$

where $Y_{P/S}$ is the product yield, P and P_0 are the final and initial lactic acid concentrations, respectively (g/L), and finally S and S_0 are the final and initial glucose concentrations (g/L), respectively. The model parameter $Y_{P/S}$ was calculated for each fermentation medium from the series of experimental data glucose concentration/time and the regression parameters of eq 2.

RESULTS AND DISCUSSION

Lees Characterization. Solids, ashes, nitrogen, and carbon contents are noted in **Table 1**. Solids content is higher in the first decanting step than in the second one because most of the dead yeasts and solids in suspension (other microorganisms, colloids, organic matter, etc.) are removed in this step. The higher content (44.7%) was reached in red lees distilled and re-collected after centrifugation. Ashes oscillate in the range of 6.1−28.1%.

Carbon and nitrogen contents are significantly lower than those reported by Rivas et al. (*9*) for spent yeasts coming from the xylitol production (42.2−46.2 and 5.7−6.3%, respectively) and also used as a cheap nutrient for the lactic acid production, which can be explained because in lees are present not only spent yeasts but also pips, earth, grape skins, etc. Ziegler (*19*) found a higher nitrogen percentage in lees (3−6%).

Organic Compounds. Glucose, ethanol, lactic acid, and acetic acid concentrations are reported in **Table 2**. Glucose concentration was <1.4 g/L in all cases, indicating that sugars were consumed during the fermentation and transformed into ethanol. For that reason, lees not distilled show ethanol concentrations >55 g/L. Lees can be distilled in wineries to recover ethanol and aromatic flavors further used to produce aromatic spirits liquors. Solanes et al. (*20*) recovered 4−8 L of 96° ethanol, 8−12 kg of calcium tartrate, and 8−10 kg of protein cake containing 30−40% of crude protein from 100 kg of fresh lees. In our case, ethanol concentrations after distillation dropped to 8.5 and 5.0 g/L in lees from white wine and red wine vinifications, respectively.

Lactic acid concentrations are relatively high in lees obtained from the red wine making technology. This is usual because red wines are submitted to malolactic fermentations to decrease wine acidity and to give the wines better flavor and stability. During malolactic fermentations malic acid is decarboxylated into lactic acid with bacteria, mainly *Oenococcus oeni* (*21*). The rate of malolactic fermentation and, consequently, the amount of final lactic acid are related to the amounts of polyphenolic compounds such as gallic, caffeic, ferulic, and *p*-coumaric acids, catechin, and quercetin (*22*).

A similar tendency was observed for acetic acid, with higher values in lees obtained from the red wine making technology. Acetic acid bacteria (*Gluconobacter oxydans*, *Acetobacter pasteurianus*, and *Acetobacter aceti*) are present at stages of wine making from the mature grape through vinification to conservation. Low levels of *A. aceti* remain in the wine, exhibiting rapid proliferation on short exposure of the wine to air, causing significant increases in the concentration of acetic acid (*23*). Higher temperatures of wine storage and higher wine

pH favor the development and metabolism of *A. aceti*. Because red wines are made with contact between the must and grape skins, the possibility of finding these bacteria is higher, increasing consequently the amount of acetic acid in wine.

Minerals. Table 3 shows the concentrations of minerals expressed as milligrams of Cu, Mg, Fe, Mn, Ca, Al, and Zn per kilogram of dried ashes in lees. The results show significantly higher metal concentrations for those lees from the red wine making technology. This is related to the process technology where must is in contact with grape skins, which have high metals concentrations. The sulfurization process, which is carried out with calcium sulfate, can provide high Cu concentrations. The amount of Ca can be explained for corrections made in the red wine with $CaCO_3$ due to the drop of pH caused for the acetic acid formation.

Kristl et al. (*24*) found the following average concentrations: 300 mg of Cu/kg of dried lees, 15 mg of Mn/kg, 35 mg of Zn/kg, 0.6 mg of Pb/kg, 24.4 mg of Cd/kg, and 1.0 mg of Cr/kg. These values are slightly lower than the average of our values expressed also as milligrams of metal per kilogram of dried lees: 335 mg of Cu/kg, 36 mg of Mn/kg, and 73.4 mg of Zn/kg.

Positive and Negative Controls. With an industrial process in mind, and on the basis of cost and availability on a large-scale production, the suitability of low-cost media was assessed using *L. rhamnosus* for glucose to lactic acid fermentation. Lees and CSL were chosen as sources of nutritional factors (including proteins, vitamins, and micronutrients) needed by *Lactobacillus* strains. Yeast extract and peptone, the main nutrients of the traditional fermentation medium proposed by Mercier et al. (*18*), reach prices as high as 7.3 and 10.3 $/kg, respectively (*13*). Comparatively, CSL has a price of only 0.07 $/kg (*13*); meanwhile, lees employed in this study were a byproduct of wineries. On the basis of these economic considerations and bearing in mind the harmful effect of lees, the possibility of replacing all of the costly nutrients of the Mercier medium by lees and CSL was assessed.

To establish a reference, two fermentation runs were carried out using the fully supplemented medium MRS broth (positive control) and a medium supplemented with only 10 g/L of CSL (negative control) together with the fermentations cited below. **Figure 1** shows the experimental data as well as the results calculated by eqs 2 and 3 using the regression parameters listed in **Table 4**. Both cases show a kinetic pattern fairly described by the mathematical models with $r^2 > 0.969$ for glucose consumption and lactic acid production. In the fully supplemented medium, glucose was rapidly consumed and converted to lactic acid, reaching 104.3 g/L after 46 h, which represents an experimental volumetric productivity, Q_P, of 2.251 g/L·h and a product yield calculated by regression of data $Y_{P/S} = 0.97$ g/g, as indicated in **Table 4**. On the contrary, the fermentation carried out with CSL as the only nutrient shows a poor conversion of glucose into lactic acid, with a higher lactic acid concentration of 58.6 g/L after 72 h (experimental $Q_P = 0.784$ g/L·h and calculated $Y_{P/S} = 0.78$ g/g).

Evaluation of Lees and CSL for Medium Supplementation. Taking into account the above results, it can be easily concluded that CSL as the only nutrient is not enough to cover the nutritional needs of *L. rhamnosus*; it is necessary to provide additional minerals, as well as carbon and nitrogen amounts. The new experiments were carried out in order to assess the possible improvements derived from supplementing the medium formulated with just 10 g/L of CSL with 10 g/L of lees obtained from the white or red wine making technologies. The main difference between these two processes is that red wines are made by skin fermentation with stem contact, which represents a much higher polymeric phenols content (*25*). Phenolic compounds play a very important role in enology owing to their contribution to wine sensory properties of color, flavor, astringency and bitterness, enzymatic or nonenzymatic browning, haze formation, and aging behavior. These positive contributions to human health, in particular to diseases, make enologists interested in producing wines rich in bioactive phenolic compounds (*26*). Nevertheless, phenolic compounds have a strong inhibitory effect in fermentations carried out with bacteria and yeasts (*27*); consequently, high concentrations of lees from

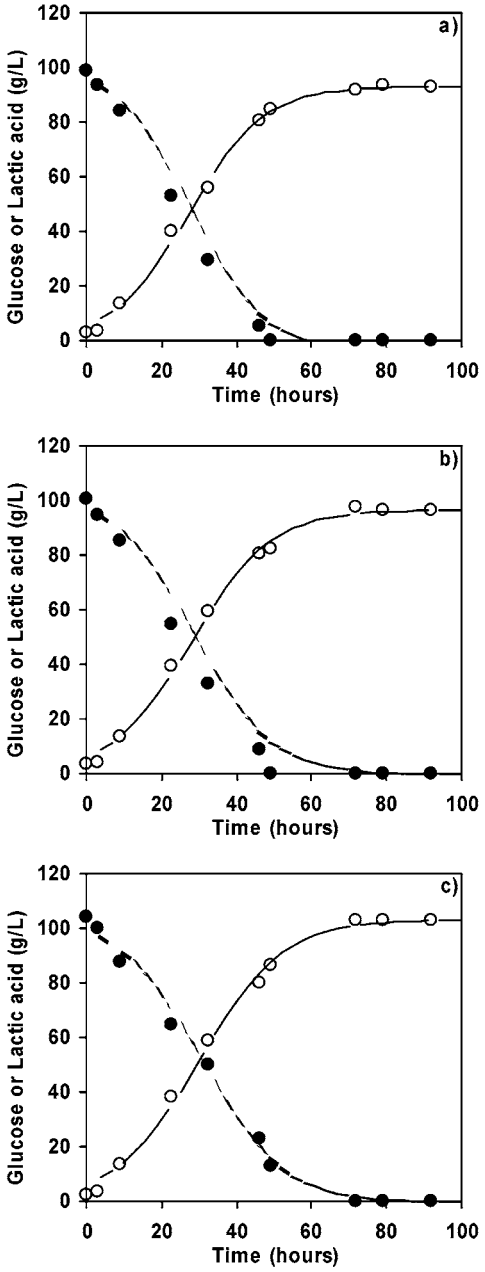

Figure 3. Experimental data and calculated time courses of lactic acid (○) and glucose concentrations (●) during fermentations carried out with 10 g/L of red lees and 10 g/L of CSL: (**a**) red lees from the first decanting step without distillation; (**b**) red lees from the second decanting step without distillation; (**c**) red lees after distillation. Results represent the average of three independent experiments. Standard deviations were below 1.8% of the mean.

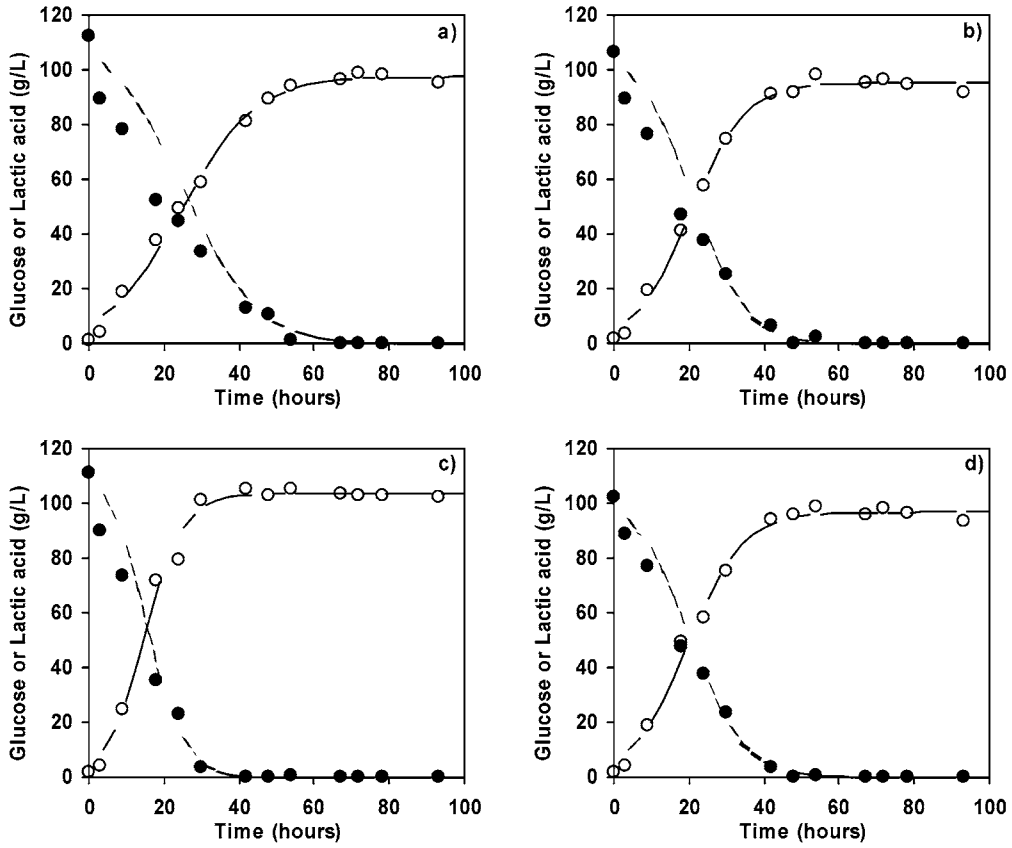

Figure 4. Experimental data and calculated time courses of lactic acid (○) and glucose concentrations (●) during fermentations carried out with 20 g/L of white lees: (**a**) lees from pressed bagasse without distillation; (**b**) white lees from the first decanting step without distillation; (**c**) white lees from the second decanting step without distillation; (**d**) white lees after distillation. Results represent the average of three independent experiments. Standard deviations were below 2.1% of the mean.

Table 6. Results Obtained by Regression of Lactic Acid and Glucose Concentration Data in Experiments Carried out with 20 g/L of Lees[a]

	lactic acid production					glucose consumption		
lees	P_0 (g/L)	P_{max} (g/L)	P_r (h^{-1})	r^2	F value	$Y_{P/S}$ (g/g)	r^2	F value
lees from pressed bagasse without distillation (**Figure 4a**)	7.2	97.4	0.103	0.993	475.7**	0.86	0.964	96.6
white lees, first decanting step, no distillation (**Figure 4b**)	5.1	95.2	0.141	0.995	1267.2**	0.88	0.981	240.8*
white lees, second decanting step, no distillation (**Figure 4c**)	4.8	103.4	0.200	0.992	488.0**	0.91	0.985	349.1*
white lees after distillation (**Figure 4d**)	5.8	96.8	0.139	0.993	580.9**	0.93	0.989	583.8*
red lees, first decanting step, no distillation (**Figure 5a**)	7.6	83.3	0.085	0.988	391.4**	0.82	0.972	217.6*
red lees, seconed decanting step, no distillation (**Figure 5b**)	9.2	90.2	0.074	0.988	351.2**	0.81	0.969	149.0
red lees after distillation (**Figure 5c**)	9.6	75.2	0.065	0.971	118.7**	0.82	0.971	269.0*

[a] P_0 = initial lactic acid concentration (g/L); P_{max} = maximum concentration of lactic acid (g/L); P_r = ratio between initial volumetric rate of product formation (r_p) and initial product concentration P_0 (h^{-1}); $Y_{P/S}$ = product yield (g/g); r^2 = determination coefficient; F value = F-test statistical parameter. *, significance level > 95%; **, significance level > 99%.

the red wine making technology can inhibit lactic acid formation. To evaluate the influence of these phenolic compounds in lees **Table 5** shows the kinetic parameters for lactic acid production and glucose consumption; meanwhile, **Figures 2** and **3** show the kinetic patterns for lactic acid production using all of the lees considered, as well as the glucose consumed.

From a comparison of both figures it can be observed that lees from the red wine production (**Figure 3**) show a slower scope for lactic acid production than lees from the white wine production (**Figure 2**). This can also be observed by comparing the P_r values listed in **Table 5**, which are always higher in lees from the white wine making technology. This behavior can be attributed to the presence of these phenolic compounds that inhibit slightly the cellular growth, a certain period of adaptation of the microorganism to the fermentation broth being necessary, although at the end of the fermentation (after 72 h) most of the glucose was also depleted and similar lactic acid values were achieved, reaching concentrations in the range of 90.7−102.0 g/L. This is close to the 104.0 g/L of lactic acid obtained with the Mercier medium, proving that CSL and lees are useful cheap nutrients for lactic acid production with *L. rhamnosus*.

With regard to the regression parameters listed in **Table 5**, the most remarkable finding was that the product yields calculated for all assays were similar, oscillating in the range of 0.88−0.97 g/g, confirming that at final times all of the lees employed showed a similar behavior.

It can also be emphasized from **Table 5** that P_{max} and $Y_{P/S}$ were higher for distilled lees (from both wine making technologies). This is outstanding because these lees are useless in wineries; meanwhile, no distilled lees can still be used to obtain byproducts such as ethanol and aromatic flavors further used to produce aromatic spirit liquors.

Evaluation of Lees as a Unique Nutrient for Medium Supplementation. The excellent behavior observed in *L. rhamnosus* during the lactic acid production in fermentation broths containing lees as nutrients could be attributed to the fact that *Lactobacillus* strains possess high hydrolyzing activities toward substrates containing proline and alanylprolyl-*p*-nitroanilide (*28*), breaking the wall of the cells contained in lees without additional treatments. This is an important advantage because the lees employed in the present work were used directly as nutrients, which contrasts with costly autolysis treatments used in other works. For example, Guilloux-Benatier and Chassagne (*2*) needed the following steps to break the cell walls: wash the yeast cells; suspend the cells in a buffer containing ethanol (12%), DL-malic acid (3 g/L), acetic acid (0.1 g/L), potassium sulfate (0.1 g/L), and magnesium sulfate heptahydrate (0.03 g/L); adjust the pH with potassium hydroxide; and finally carry out the autolysis of the cell walls at 30 °C for 2 weeks.

On the basis of the proteolytic activity shown by *L. rhamnosus* with lees, the possibility of replacing CSL was considered, using lees as the unique nutrient. **Figures 4** and **5** as well as **Table 6** show another set of experiments carried out using 20 g/L of lees as the only nutrient. **Figure 4** shows the experimental and calculated results for both glucose consumption and lactic acid production using lees from the production of white wine; **Figure 5** shows the same results for lees from the production of red wine. The corresponding fitting parameters are included in **Table 6**.

The behavior observed in **Figure 4** indicates that lees from the white wine making technology can be used as the unique nutrient for the glucose to lactic acid production with *L. rhamnosus*, in particular, the lees re-collected after the second decanting step before distillation. In this case were achieved values even higher than those obtained with the complete medium proposed by Mercier et al. (*18*). After 42 h, the lactic acid concentration was 105.5 g/L and the global volumetric productivity $Q_P = 2.470$ g/L·h; the calculated product yield was 0.91 g/g. All of the lees obtained during the white wine making technology were used successfully with lactic acid concentrations >96.5 g/L after 72 h and calculated products yields >0.86 g/g.

On the contrary, lees from the red wine making technology showed slower conversions. After 42 h, the lactic acid concentration oscillated between 52.6 and 63.1 g/L, and after 72 h, no more than 86.3 g/L was achieved. The calculated product yield was only 0.81−0.82 g/g. These results indicate that although lees from the red wine making technology can be used as a source of cheap nutrients, the phenolic compounds released during this process hinder slightly the fermentation, and extraction with organic solvents should be required to improve these results.

It can also be mentioned that using 20 g/L of distilled lees, the P_{max} and $Y_{P/S}$ results are not so satisfactory as those observed with no distilled lees. This could be explained if we realize that during distillation at high temperatures, some inhibitory compounds can be produced. Contrarily to the behavior observed with just 10 g/L, when we use a higher concentration (20 g/L), the amount of inhibitory compounds is important to inhibit *L. rhamnosus* growth. The amount and kind of lees to be used must be decided on the basis of economic balances and company strategies, taking into account the higher lactic acid concentrations obtained using lees re-collected after the first decanting step; these lees could be distilled to obtain other subprodusts.

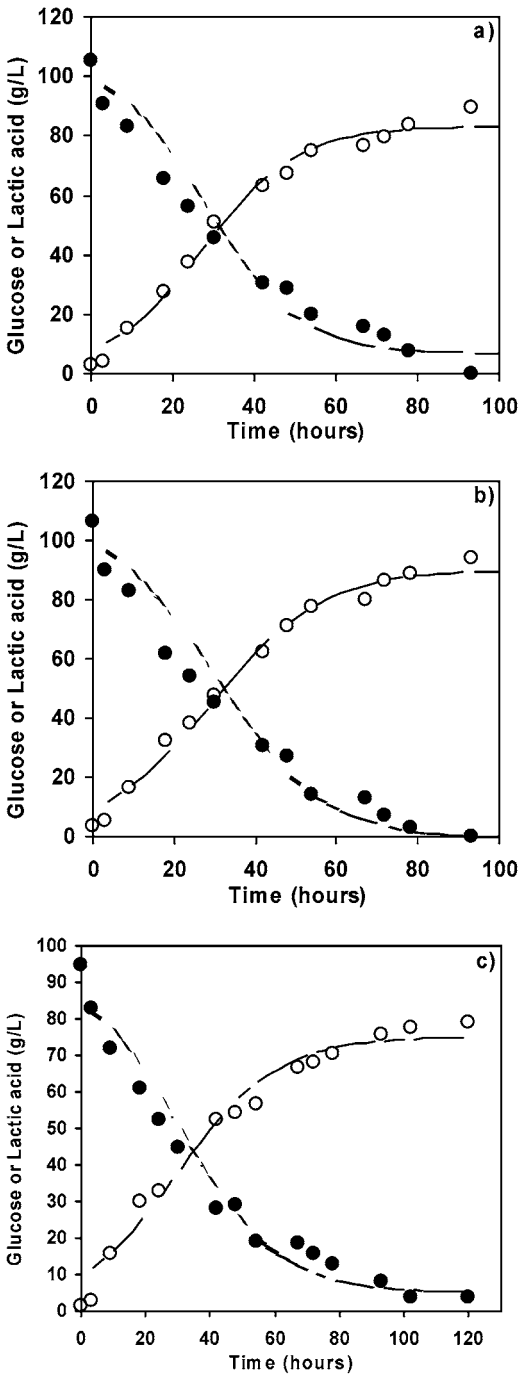

Figure 5. Experimental data and calculated time courses of lactic acid (○) and glucose concentrations (●) during fermentations carried out with 20 g/L of red lees: (**a**) red lees from the first decanting step without distillation; (**b**) red lees from the second decanting step without distillation; (**c**) red lees after distillation. Results represent the average of three independent experiments. Standard deviations were below 2.3% of the mean.

CONCLUSIONS

L. rhamnosus CECT-288 showed a good performance for glucose to lactic acid fermentation using the costly MRS broth, which includes among others yeast extract and peptone. Its ability was hindered when this fermentation medium was replaced by CSL as the only nutrient.

When the MRS broth was replaced by cheap nutrients, using 10 g of vinification lees/L and 10 g of CSL/L as sources of nutritional factors, using lees from different steps from the white

and red wine making technologies, before or after distillation, in all cases, fermentations were carried out effectively with similar high yields and high productivities of lactic acid.

The best results, even higher than those obtained with the MRS broth, were obtained using 20 g/L of lees from the white wine making technology and re-collected after the second decanting step before distillation as the only nutrient. On the contrary, the results indicated that although lees from the red wine making technology could be used as a source of cheap nutrients, the phenolic compounds released due to the skin fermentation with stem contact during the red wine production hindered slightly the fermentation, and extraction with organic solvents should be required to improve these results.

The high productivities achieved in all cases showed that this *Lactobacillus* has proteolytic activity as no autolysis treatments were required to break the cell walls of yeasts in lees.

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