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Evaluation of Biosurfactant Production from Various Agricultural Residues by *Lactobacillus pentosus*

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The cost of biosurfactant production may be significantly decreased by using inexpensive carbon substrates like agricultural residues. However, scarce information can be found in the literature about the utilization of lignocellulosic residues for obtaining biosurfactants. Usually agricultural residues are field burned, producing various toxic compounds to the atmosphere; so, as an interesting alternative to the traditional field burning of this kind of residue, this work proposes the utilization of agricultural wastes (barley bran, trimming vine shoots, corn cobs, and *Eucalyptus globulus* chips) for simultaneous lactic acid and biosurfactant production. Previous to this biotechnological process, lignocellulosic residues were hydrolyzed, using H_2SO_4, under selected conditions and neutralized with $CaCO_3$. Following, *Lactobacillus pentosus* was employed for the fermentation of hemicellulosic hydrolyzates after nutrient supplementation. Biosurfactants were measured by taking into account the surface tension reduction. The highest value of reduction (21.3 units) was found when using hemicellulosic sugar hydrolyzates obtained from trimming vine shoots, corresponding to 0.71 g of biosurfactant per g of biomass and 25.6 g of lactic acid/L. On the contrary, barley bran husk hydrolyzates only produced 0.28 g of biosurfactant per g of biomass and 33.2 g of lactic acid/L. The differences between biosurfactant production can be attributed to the different compositions of the hydrolyzates.

KEYWORDS: Lactic acid fermentation; biosurfactants; agroindustrial wastes; *Lactobacillus pentosus*

INTRODUCTION

The interest in biosurfactants has increased considerably in recent years, as they are potential candidates for many commercial applications. In the food industry, biosurfactants are used as emulsifiers, mainly in bakeries, where they play an important role in the rheological characteristics of flour and meat products. Moreover, biosurfactants can be employed to promote the biodegradation of hydrocarbon bioremediation (*1*−*3*). Biosurfactants have several advantages over chemical surfactants including lower toxicity and higher biodegradability and effectiveness at extreme temperatures or pH values (*4*, *5*). However, in spite of these advantages, biosurfactants must be cost competitive with the chemical synthesis and the utilization of a cheaper carbon source like agricultural residues would be interesting for their production. The culture medium, carbon source, and the growth conditions (pH, temperature, limiting nutrients, and trace elements) can influence the types and yields of biosurfactants (*5*). Various authors have proposed the utilization of oleosus residues, cassava wastewater, potato wastewater, or whey as carbon sources for biosurfactant production (*3*, *6*−*10*), but scarce information can be found about the utilization of lignocellulosic residues for biosurfactant production. Most agricultural wastes are made up mainly of cellulose, hemicelluloses, and lignin, and before fermentation, they have to be fractionated upon chemical and/or enzymatic stages to obtain sugar solutions, which (after nutrient supplementation) could be used as fermentative media for the production of food additives or chemical products (*11*−*13*). Besides, the utilization of lignocellulosic residues for biotechnological purposes like biosurfactant production prevents the field burning of agricultural residues and avoids certain gases such as CO_2, CH_4, and N_2O that are produced to the tropospheric atmosphere, absorbing infrared radiation that brings out the greenhouse effect. Moreover, lignin is one of the main contributors of the total carbon of agricultural wastes. When lignocellulosic residues are field burned, polycyclic aromatic hydrocarbon components such as bezopyrene, catechol, hydroquinone, phenanthrene, and naphthalene are obtained (*14*). Some authors (*15*) have reported that all of these compounds can inhibit DNA synthesis and induce cancerosus tumors in animals and humans.

From an economical point of view, it is necessary to take into account that agricultural residues can be employed for

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Table 1. Composition (%) of the Raw Materials Used in this Study[a]

residue	cellulose	xylan	araban	acetyl groups	lignin
barley bran husks	23.0	26.6	6.1	1.6	21.4
trimming vine shoots	34.1	12.8	0.9	5.3	27.1
corn cobs	31.7	30.9	3.8	3.4	20.3
E. globulus chips	46.3	16.6	0.5	3.6	22.9

[a] The results are expressed as percentage of the initial weight of sample on a dry basis.

Table 2. Conditions Selected for the Acidic Hydrolysis of the Different Lignocellulosic Residues and Concentrations of Sugars Released during the Treatments[a]

residue	H_2SO_4 (%)	time (min)	glucose (g/L)	xylose (g/L)	arabinose (g/L)
barley bran husks	3	15	5.82	40. 7	7.47
trimming vine shoots	3	15	9.18	19.1	2.81
corn cobs	2	15	2.79	36.9	4.10
E. globulus chips	3	60	2.24	19.5	1.25

[a] Temperature, 130 °C; solid/liquid ratio, 8 g/g.

obtaining not only biosurfactants but also lactic acid in the same fermentation process. Lactic acid is a chemical additive with several applications in the food industry as an acidulant or preservative because lactic acid inhibits the growth of pathogens bacteria like *Salmonella* or *Staphylococcus*. Moreover, lactic acid is applied in the elaboration of biodegradable plastic as polymers of lactic acid. In previous works, we have reported the utilization of lignocellulosic materials as substrates for lactic acid production using lactobacilli strains; the microbial biomass as a residue remains during these processes (*11*−*13*). On the other hand, other authors (*16*) found that 15 *Lactobacillus* species produced biosurfactants in the midexponential and stationary growth phases on synthetic media, but no studies can be found about these strains growing on hydrolyzates from lignocellulosic residues for biosurfactant production.

The aim of this work is the evaluation of hemicellulosic hydrolyzates from various agricultural residues (barley bran husks, trimming vine shoots, corn cobs, and *Eucalyptus globulus* chips) as carbon sources for obtaining biosurfactants simultaneously with lactic acid fermentations using *Lactobacillus pentosus*. With this biotechnological process, the biosurfactant production cost could be reduced and the environmental impact created by the field burning of agricultural residues would be decreased.

MATERIALS AND METHODS

Lignocellulosics Hydrolysis. Samples of barley bran husks, trimming vine shoots, corn cobs, and *E. globulus* chips were dried, milled to a particle size less than 1 mm, homogenized in a single lot to avoid compositional differences, and stored until use.

Table 1 shows the composition of lignocellulosic materials employed in this work as carbon sources for lactic acid fermentation and biosurfactants production. On the basis of previous works (*17*−*21*), hydrolyzates of the above raw materials were obtained by thermal treatments in an autoclave at 130 °C with 2−3% H_2SO_4 solutions for 15−60 min, using a liquid/solid ratio of 8 g/g. The conditions employed for the diluted acid hydrolysis as well as the hemicellulosic sugars released are summarized in **Table 2**.

Detoxification of the Hemicellulosic Hydrolyzates *of E. globulus*. On the basis of previous works (*22*), neutralized hydrolyzates obtained from *E. globulus* chips were mixed with 15% weight of charcoal (Probus, Madrid, Spain) and stirred for 1 day at room temperature. The liquors were recovered by filtration.

Microorganism. *L. pentosus* CECT-4023T (ATCC-8041) was obtained from the Spanish Collection of Type Cultures (Valencia, Spain). The strain was grown at 31 °C for 24 h on plates using the complete medium proposed by lactic acid bacteria containing (per liter): 20 g of glucose, 5 g of yeast extract, 10 g of peptone, 5 g of sodium acetate, 2 g of sodium citrate, 2 g of K_2HPO_4, 0.58 g of $MgSO_4 \cdot 7H_2O$, 0.12 g of $MnSO_4 \cdot H_2O$, 0.05 g of $FeSO_4 \cdot 7H_2O$, and 20 g of agar (*23*). Inocula were prepared by preliminary suspension of cells from plates in sterile hemicellulosic hydrolyzates, and the inocula accounted for 10% of the total fermentation volume. The determination of biomass concentration was carried out by optical density measurements at 600 nm (*3*).

Fermentations. Hemicellulosic hydrolyzates from agricultural residues were neutralized with powdered $CaCO_3$ to a final pH of 6.0, and the $CaSO_4$ that precipitated was separated from the supernatant by filtration. The clarified liquors were supplemented with 10 g of yeast extract/L and 10 g of corn steep liquor/L, sterilized at 121 °C for 20 min, and used directly as fermentation media. Experiments were carried out at 31 °C in a 2 L Biostat B batch reactor (Braun, Melsungen, Germany) with 1.0 L of working volume and at 150 rpm. During fermentation, the pH was controlled at 6.0 by the addition of 4 M NaOH. Samples (2 mL) were taken at given fermentation times and centrifuged at 6000 rpm for 3 min. The supernatants were stored for analysis.

Fermentations were carried out in triplicate, and the corresponding results were reported as mean values. Standard deviations were below 2.6% of the mean.

Analytical Methods. Total sugars (glucose, xylose, and arabinose), acetic acid, and lactic acid were measured by a high-performance liquid chromatograph (Agilent, model 1100, Palo Alto, CA) equipped with a RI detector and a column model ION-300 (Transgenomic Inc., San Jose, CA) eluted with 0.02 M H_2SO_4 at a flow rate of 0.4 mL/min.

Surface Tension (ST) Determination. The surface activity of biosurfactants produced by *L. pentosus* was determined by measuring the ST of the samples with the Ring method (*24*) using a Kruss Tensiometer equipped with a 1.9 cm De Noüy platinum ring at room temperature. Tensiometers determine the ST with the help of an optimally wettable ring suspended from a precision balance. In the Ring method, the liquid is raised until contact with the surface is registered. The sample is then lowered again so that the liquid film produced

Table 3. ST of PBS before and after Biosurfactant Extraction, as Well as Others Fermentative Parameters[a]

hydrolyzates	ST_{PBS} (mN/m)	ST_B[b] (mN/m)	F_{CMC}[b]	[BS]	[S]	[BM]	$Y_{BS/BM}$	$Y_{BS/S}$
barley bran husks	72	56 a	1.6 a	2.9	54.0	10.3	0.28	0.05
trimming vine shoots	72	51 b	2.8 b	6.5	31.1	9.1	0.71	0.20
corn cobs	72	54 a	2.3 a	4.7	43.8	8.9	0.53	0.11
E. globulus chips	72	55 a	2.0 a	4.0	23.0	7.4	0.54	0.17

[a] ST of PBS at the beginning of extraction (ST_{PBS}), ST of PBS containing intracellular biosurfactants (ST_B), dilution rate to achieve the CMC (F_{CMC}), total concentration of intracellular biosurfactants (g/L) [BS], concentration of hemicellulosic sugars metabolized during lactic acid fermentation (g/L) [S], biomass concentration (g/L) [BM], g of intracellular biosurfactant per g of biomass ($Y_{BS/BM}$), and g of intracellular biosurfactant per g of sugars consumed ($Y_{BS/S}$). [b] A different letter in each column of ST indicates significant differences as determined by the Tukey test at $p < 0.05$.

beneath the ring is stretched. As the film is stretched, a maximum force is experienced, and the force is measured and used to calculate the ST.

The ST of extracellular biosurfactants was determined by measuring the ST of fermentation media, whereas for determining the intracellular biosurfactant production *L. pentosus* cells were recovered by centrifugation (10000*g*, 5 min, 10 °C), washed twice in demineralized water, and resuspended in phosphate buffer saline (PBS: mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to 7.0) following the methodology proposed by other authors (*3*).

The biosurfactant concentrations (g/L) were determined using a calibration curve: concentration (g/L) = [ST (mN/m) − 76.98]/−8.65. The calibration curve was calculated for a commercial biosurfactant produced by several *Bacilli* (surfactin) using different concentrations of biosurfactant solution, below the critical micelle concentration (CMC) with a known ST (*3*, *24*). In this biosurfactant concentration range, the decrease of ST is linear and it is possible to establish a relationship between the biosurfactant concentration and the ST. Nevertheless, to estimate biosurfactant concentration, it was sometimes necessary to dilute the culture broth to reach the CMC. When a surfactant is added to air/water or oil/water systems at increasing concentrations, a reduction of ST is observed up to a critical level, above which amphiphilic molecules associate readily to form supramolecular structures like micelles and vesicles. This value is known as the CMC.

Statistical Analysis. ST values were subjected to an analysis of variance by using SPSS statistical software package, and significant treatment differences were separated by Tukey's multiple range test at $p < 0.05$.

RESULTS AND DISCUSSION

Biosurfactant Production. The utilization of lignocellulosic residues as carbon source was scarcely studied for biosurfactant production; nevertheless, taking into account that lignocellulosic residues were proposed for several authors to obtain food additives (*11*−*13*), it is expected that these materials could be employed for producing biosurfactants using the accurate microorganism. Biosurfactants can be produced by the microorganism, extracellularly, or associate to the cell membrane.

In this work, the intracellular and extracellular biosurfactant production of *L. pentosus* was evaluated by employing the hemicellulosic sugars obtained from different lignocellulosic residues as carbon sources: barley bran, trimming vine shoots, corn cobs, and *E. globulus* chips. Usually, biosurfactant activities are determined by measuring the changes in ST and interfacial tension. The ST at the air/water and oil/water interfaces can be easily measured with a tensiometer. The ST of distilled water is 72 mN/m, and addition of surfactant lowers this value more than 8 mN/m (*25*). Using the hemicellulosic sugars obtained from *E. globulus* chips, the fermentation broth was necessarily detoxified with activated charcoal to perform an effective bioconversion. In our study, it can be observed that *L. pentosus* did not produce extracellular biosurfactants since there was not a significant reduction in the ST value (data not shown).

For the determination of intracellular biosurfactants, *L. pentosus* cells were recovered and biosurfactants were extracted using PBS. **Table 3** shows the ST of PBS after intracellular biosurfactant extraction. It can be observed that biosurfactants from *L. pentosus* grown on hemicellulosic sugars of trimming vine shoots produced the highest units of reduction in the ST of PBS (21.4 units). This value is close to the data reported by other authors (*16*) for *Lactobacillus casei* ssp. *rhamnosus* GR-1 (19 units) and for *Lactobacillus fermentum* ATCC 23271 (20 units). The maximum surface reduction units found by these authors was achieved using biosurfactants from *L. fermentum* B54 (29 units). Biosurfactants produced by *L. pentosus* grown on trimming vine shoot hemicellulosic sugars decreased significantly the ST of the media as compared with the biosur-

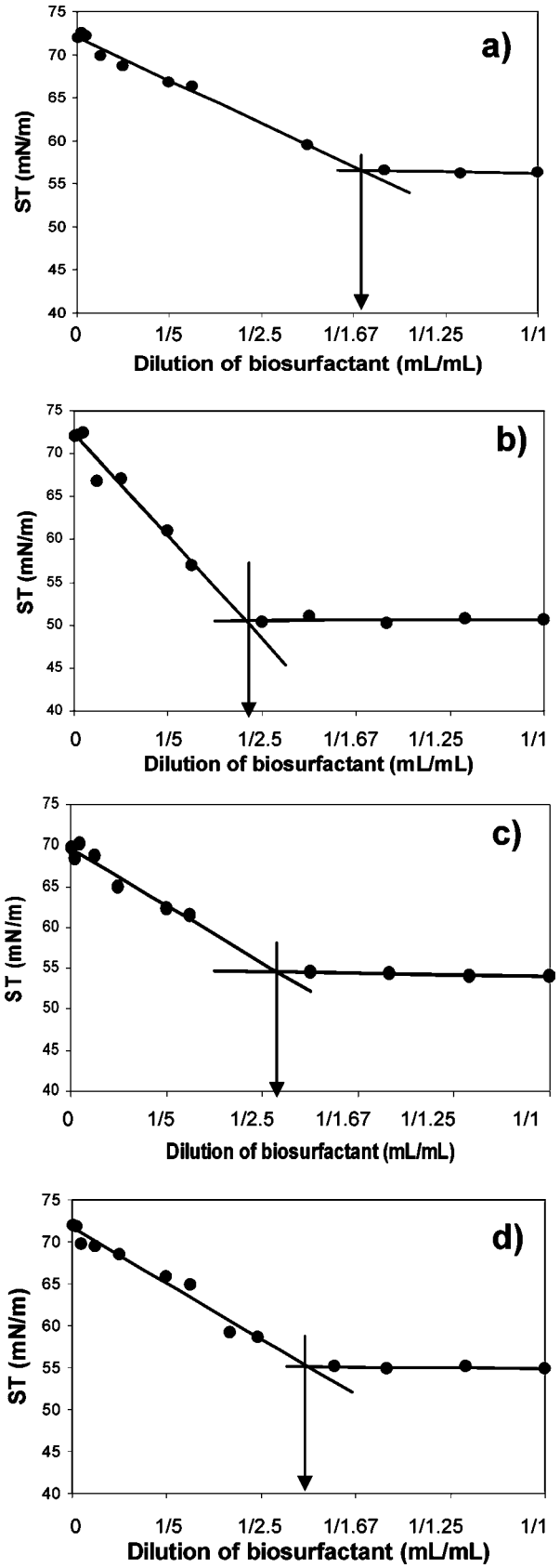

Figure 1. Calculation of the dilution rate for achieving the critical micellar concentration (CMC) of (**a**) barley bran husks, (**b**) trimming vine shoots, (**c**) corn cobs, and (**d**) detoxified *E. globulus* chips hemicellulosic hydrolyzates.

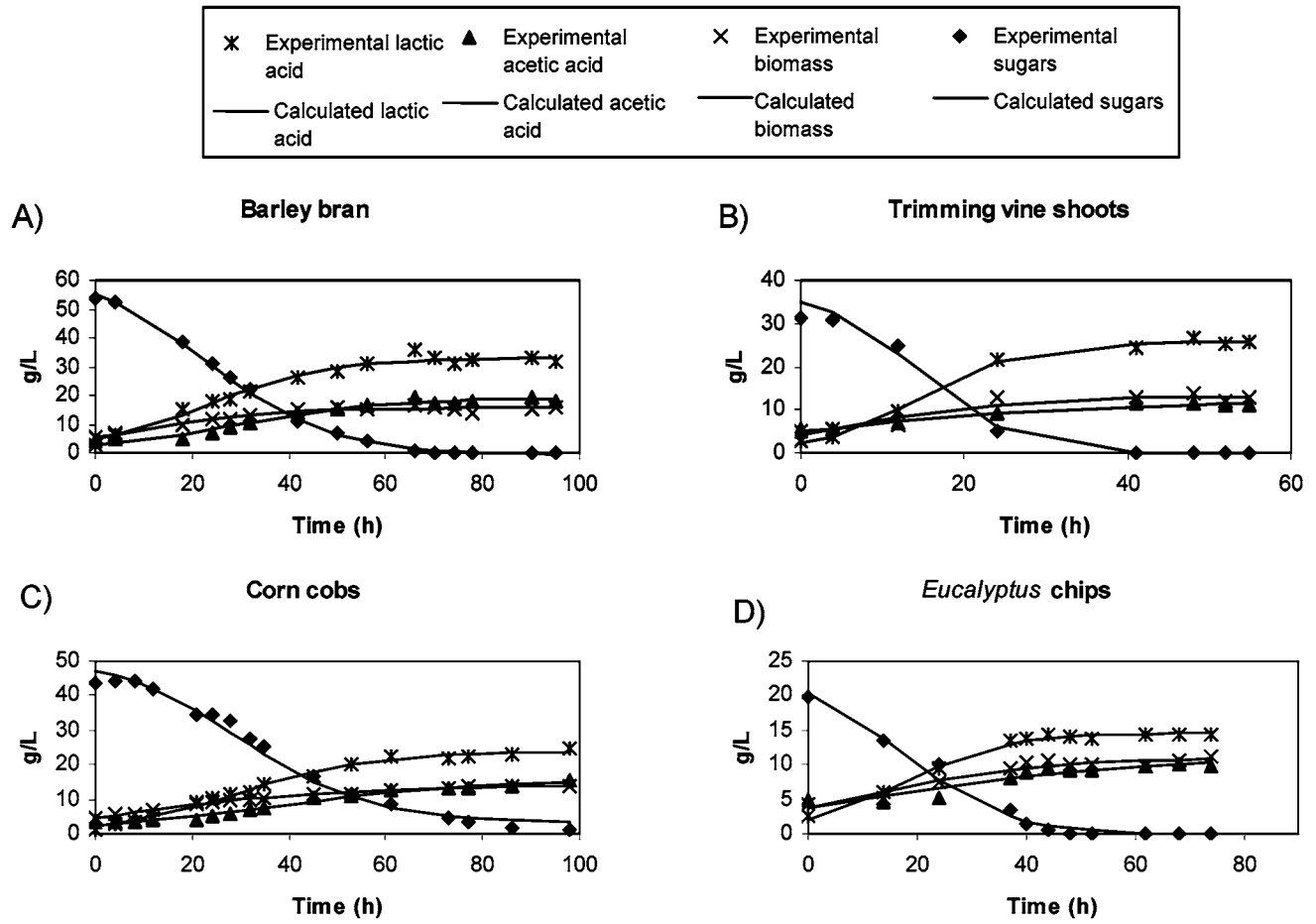

Figure 2. Kinetic modeling of lactic acid (*), acetic acid (▲), and biomass (×) production as well as hemicellulosic sugar consumption during lactic acid fermentation (♦) of (**a**) barley bran husks, (**b**) trimming vine shoots hemicellulosic hydrolyzates, (**c**) corn cobs, and (**d**) detoxified *E. globulus* chips hemicellulosic hydrolyzates using *L. pentosus*.

factants obtained by *L. pentosus* grown on hemicellulosic sugars from barley bran husks, corn cobs, and detoxified *E. globulus* chips (**Table 3**).

CMC is the lowest concentration of biosurfactant that allows us to obtain the highest ST reduction of the media. **Table 3** shows the dilution rates, calculated from **Figure 1**, to reach the CMC for the intracellular biosurfactants obtained in this work. The biosurfactant concentration from *L. pentosus* grown on trimming vine shoots hydrolyzates was 2.8 times above the CMC, whereas the biosurfactant concentration from *L. pentosus* grown on barley bran hydrolyzates was only 1.6 times above the CMC. The highest concentration of biosurfactant produced with trimming vine shoots hydrolyzates as compared with others hydrolyzates can be related with the different hemicellulosic sugar composition of trimming vine shoot as compared to the others agricultural residues.

Nevertheless, total biosurfactant concentrations and CMCs were determined on the basis of previous works using a calibration curve calculated for a commercial biosurfactant (surfactin), produced by several *Bacilli*, using different concentrations of biosurfactant solution, below the CMC, with known ST (*3*, *24*). In all of the cases to estimate the biosurfactant concentration, it was necessary to dilute the PBS-containing intracellular biosurfactants under the CMC (**Figure 1**). **Table 3** shows the total biosurfactant concentration in g/L where trimming vine shoots gave the highest biosurfactant concentration (6.5 g/L) followed by corn cobs (4.7 g/L), detoxified *E. globulus* (4.0 g/L), and barley bran husks hydrolyzates (2.9 g/L).

Table 4. Ratio between Initial Volumetric Rate of Product Formation and Initial Product Concentration (P_r) for Lactic Acid, Acetic Acid, and Biomass and Product Yield ($Y_{P/S}$) Consisting of g of Lactic Acid Produced Per g of Sugars Consumed[a]

	lactic acid		acetic acid		biomass		lactic acid yield	
hydrolyzates	P_r (h^{-1})	R^2	P_r (h^{-1})	R^2	P_r (h^{-1})	R^2	$Y_{P/S}$ (g/g)	R^2
barley bran husks	0.08	0.98	0.06	0.98	0.07	0.95	0.51	0.99
trimming vine shoots	0.18	0.99	0.07	0.97	0.10	0.92	0.67	0.98
corn cobs	0.07	0.99	0.04	0.97	0.05	0.99	0.48	0.98
E. globulus chips	0.11	0.99	0.04	0.95	0.06	0.97	0.61	0.98

[a] The *F* test statistical parameter gave in all of the cases a significance level >99%, except for trimming vine shoots where the P_r of biomass was higher than 96%.

Moreover, **Table 3** shows the g of biosurfactant per g of biomasss ($Y_{BS/BM}$) and the g of biosurfactans per g of sugars consumed ($Y_{BS/BM}$). The maximum $Y_{BS/BM}$ was achieved using trimming vine shoots hydrolyzates (0.70 g/g), whereas the lowest value was obtained employing barley bran husks (0.28 g/g). Corn cobs and *E. globulus* hydrolyzates gave biosurfactants with similar $Y_{BS/S}$.

In relation with the sugar consumption, *L. pentosus* growing on trimming vine shoots produced more biosurfactants per g of sugar consumed than *L. pentosus* grown in barley bran husks, corn cobs, or detoxified *E. globulus* hydrolyzates

(**Table 3**). Several authors have reported that the effectiveness of biosurfactant production is related with the composition of fermentation media (*26*). In this case, trimming vine shoot hydrolyzates present the highest glucose concentration, so it can be supposed that glucose can stimulate the production of biosurfactants reducing the ST of PBS in more units than other sugars.

Lactic Acid Fermentation. It is important to point that during the above biotechnological production of biosurfactants from agricultural residues, lactic acid was produced as the main fermentation product. **Figure 2** shows the kinetic profiles of lactic acid, acetic acid, and biomass obtained during fermentation of agricultural residues by *L. pentosus* as well as the sugar consumption. Total sugars (xylose, glucose, and arabinose), lactic acid, acetic acid, and biomass were mathematically modeled following eq 1 proposed by Mercier (*23*) and applied afterward by other authors during lactic acid fermentations (*3*, *25*):

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

where t is time, P is the product concentration, P_{max} is the maximum product concentration, 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 eq 2:

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

On the other hand, total hemicellulosic sugar consumption can be interpreted by the following 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 product concentrations, respectively (g/L), and finally, S and S_0 are the final and initial hemicellulosic sugar concentrations (g/L).

Table 4 shows the maximum ratio between initial volumetric rate of product formation and initial product concentration, P_r, and the yield expressed as g of lactic acid per g of sugars consumed, $Y_{P/S}$. These fermentation parameters were obtained by applying the above-mentioned kinetic modeling. The maximum value of P_r, 0.18 h^{-1}, was achieved using trimming vine shoot hydrolyzates, followed by detoxified *E. globulus* hydrolyzates (0.11 h^{-1}), barley bran (0.08 h^{-1}), and corn cobs (0.07 h^{-1}), meaning that the faster formation of lactic acid was obtained using trimming vine shoot hydrolyzates as a substrate for *L. pentosus*. The maximum ratio between initial volumetric rate of biomass formation and initial biomass concentration was again achieved using trimming vine shoot hydrolyzates (0.10 h^{-1}), whereas barley bran hydrolyzates, corn cobs, and detoxified *E. globulus* hydrolyzates gave 0.07, 0.05, and 0.06 h^{-1}. Similarly, the maximum ratio between initial volumetric rate of acetic acid formation and initial acetic acid concentration (0.07 h^{-1}) was achieved using trimming vine shoot hydrolyzates, followed by barley bran hydrolyzates (0.08 h^{-1}) and detoxified *E. globulus* and corn cob hydrolyzates (0.04 h^{-1}). Finally, trimming vine shoots produced the highest amount of lactic acid per gram of consumed sugars (0.67 g/g); this fact is in concordance with the g of biosurfactants per g of consumed sugars (0.20 g/g) achieved with trimming vine shoot hydrolyzates, which was higher than using the other agricultural residues.

In conclusion, hemicellulosic sugars from agricultural residues like trimming vine shoots or detoxified *E. globulus* wood hydrolyzates are interesting carbon sources for competitive cost production of biosurfactants. During the fermentation of hemicellulosic hydrolyzates, *L. pentosus* produced lactic acid and intracellular biosurfactants. Among the lignocellulosic residues assayed, trimming vine shoots produced the highest concentration of biosurfactants whereas barley bran husks produced the highest lactic acid concentration. The effectiveness of trimming vine shoots for producing biosurfactants could be related with the highest content of glucose in the hemicellulosic hydrolyzates.

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