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Biorefinery of brewery spent grain to obtain bioproducts with high value-added in the market

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ABSTRACT

The brewery industry is under economic and environmental pressure to minimize residual management costs, particularly brewery spent grain (BSG), which accounts for 80–85% (w/w) of the total by-products generated. BSG is a lignocellulosic material primarily composed of carbohydrates, proteins and lipids. Developing a biorefinery model for conversion of BSG into value-added products is a plausible idea. Previous work optimized the pretreatment of BSG with the ionic liquid [N_{1112OH}][Gly] and further release of fermentable sugar-containing solutions by enzymatic hydrolysis, using an enzymatic cocktail obtained by solid-state fermentation of BSG with *Aspergillus brasiliensis* CECT 2700 and *Trichoderma reesei* CECT 2414. The current work ends the biorefinery process, studying the fermentation of these culture media with two LAB strains, *Lactobacillus pentosus* CECT 4023 and *Lactobacillus plantarum* CECT 221, from which the production of organic acids, bacteriocins, and microbial biosurfactants (mBS) was obtained. In addition to the bacteriocin activity observed, a mass balance of the whole biorefinery process quantified the production of 106.4 g lactic acid and 6.76 g mBS with *L. plantarum* and 116.1 g lactic acid and 4.65 g mBS with *L. pentosus* from 1 kg of dry BSG. Thus, BSG shows a great potential for waste valorization, playing a major role in the implementation of biomass biorefineries in circular bioeconomy.

1. Introduction

The brewing industry produces 137–173 tones of residuals (e.g. spent grain, wort trub, and waste yeast) per each 1000 tones of brewing product, which are increasingly becoming more challenging and expensive to treat [1]. Among them, brewery spent grain (BSG) is a low-cost by-product (€35/ton) that represents approximately 80–85% of the total wastes produced in the industry [2]. Since approximately 0.2 kg of BSG is generated per liter of beer produced, statistically, 37.2 million tons of BSG was produced globally in 2021 from 1.86 billion hectoliters beer, confirming the abundance of this valuable bioresource [2].

BSG is underexploited and mainly used as animal feed because of the high volume, nutrient-rich composition, abundance and availability through the year [2–4]. However, BSG is rich in cellulose and non-cellulosic polysaccharides and can be fractioned to recover the main components in a biorefinery approach for use and transformation into high value-added products [5]. The main inconvenience is that the recalcitrant nature of biomass hampers polysaccharide accessibility for enzymes and microorganisms, making necessary the use of several pretreatment options such as diluted acid, alkali, supercritical CO_2, steam explosion, or organosolv for the conversion of lignocellulosic biomass into value-added products [6]. The selection of the pretreatment depends on the nature of the material to be treated, it being necessary to avoid the degradation of sugars and the consequent appearance of toxic compounds or inhibitors of microbial and biocatalytic reactions [7].

In recent years, treatments based on ionic liquids (IL) have gained relevance, especially bioderived ILs, since their biodegradability gives them an environmental advantage over traditional ILs [8,9]. The

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effectiveness of IL [N_{1112OH}][Gly] has already been demonstrated as a pretreatment of BSG, obtaining a material with low lignin content and high concentration of carbohydrates, which is more susceptible to enzymatic hydrolysis than raw BSG. In addition, this process showed a remarkable recyclability [10].

Within the biological processes for obtaining products with high value-added, the use of lactic acid bacteria (LAB) stands out, due to their consideration as GRAS and immediate applications of them and their metabolites in the food industry [11]. These LAB can produce a wide variety of metabolites with industrial applications such as lactic acid, bacteriocins and microbial biosurfactants [12,13]. The ability of LAB to ferment media formulated from lignocellulosic biomass for the production of these metabolites has already been demonstrated [3,12,14]. The use of agroindustrial wastes to produce bioactive compounds through microbial fermentation as substitutes for culture media ingredients results in lower-cost and environmentally friendly processes [15].

Previous work studied the delignification of BSG with the IL [N_{1112OH}][Gly] and its subsequent hydrolysis using a combination of enzymes obtained by solid state fermentation (SSF) of BSG with *Trichoderma reesei* and *Aspergillus brasiliensis* [16]. The use of these enzymatic liquors as fermentation substrates for producing organic (lactic and acetic) acids, bacteriocins and microbial biosurfactants by LAB could be a promising alternative for valorization of the delignified and hydrolyzed BSG. This approach offers the additional advantage of incorporating these substrates into an efficient production process that could be implemented in the food industry. This study presents, for the first time, the results achieved during the fermentation of these enzymatic liquors using two LAB strains, *Lactobacillus pentosus* CECT 4023 and *L. plantarum* CECT 221, to produce organic acids (lactic and acetic), bacteriocins, and microbial biosurfactants.

2. Material and methods

2.1. Materials

Brewery spent grain (BSG), from the artisanal production of beer, was kindly provided by Letra (Vila Verde, Braga, Portugal). BSG was dried at room temperature, ground in an electric shredder MTD 220E (Saarbrücken, Germany), milled to dust with an IKA® Werke model M 20 mill (Staufen, Germany), sieved to size below 5 mm, homogenized in a single lot and stored at 4 ºC before experimentation.

A carbohydrate-rich material (CRM) was obtained after pretreatment of BSG with the ionic liquid (IL) [N_{1112OH}][Gly] at 90 ºC over 16 h, and a solid loading of 5 wt% after 5 cycles of treatment as described by [17].

2.2. Enzymes

The enzymes were produced by solid-state fermentation (SSF) with the strains *Aspergillus brasiliensis* CECT 2700 and *Trichoderma reesei* CECT 2414, using BSG as substrate following the procedure described in [16]. Briefly, BSG moistened (1.0:2.5 w/v) with a solution of mineral salts (1.3 g/L $(NH_4)_2SO_4$, 5.0 g/L $NaNO_3$, 4.5 g/L KH_2PO_4, and 3 g/L yeast extract) was inoculated with a spores suspension of 1×10^6 spores/g dry BSG and fermented at 30 ºC in a water-saturated atmosphere. Crude extracts were obtained by adding citrate buffer pH 4.8 to SSF media (10 mL/g dry BSG) and incubated for 1 h, 200 rpm at 30 ºC. Solids were separated from the extract by centrifugation at 2755 × *g* for 15 min (Ortoalresa, Consul 21, EBA 20, Hettich Zentrifugen, Germany) and filtered. Subsequently, the enzymatic extracts were analyzed [18].

2.3. Enzymatic hydrolysis

The enzymatic hydrolysis was carried out on CRMs, with the enzymatic cocktails from *A. brasiliensis* and *T. reesei* obtained after SSF. Enzymatic hydrolysis was performed in 100 mL Erlenmeyer flasks at 50 ºC and 150 rpm during 72 h with a solid-liquid ratio 1:60 (w/v), with a mixture of extracts 2.5:0.5 (v/v) *Aspergillus/Trichoderma* as described previously [16]. After the reaction, hydrolyzates were centrifuged at 9503 × *g* for 10 min, to remove solids. The liquid phase was heated for 5 min on a boiling water bath to stop the reaction. Glucose, cellobiose, xylose and arabinose in hydrolyzates were quantified by HPLC as described below.

2.4. Fermentation of hydrolyzates

L. plantarum CECT 221 and *L. pentosus* CECT 4023 strains were tested to produce biomolecules of food interest (lactic and acetic acids, bacteriocins and microbial biosurfactants) using CRM hydrolyzates as substrate. Both strains were incubated at 30ºC and 150 rpm for 24 h into an incubator shaker (Optic Ivymen System, Comecta S.A) in MRS medium to obtain a preculture.

Fermentations were carried out according to [14] with minor modifications. CRM hydrolyzates were supplemented with the nutrients of MRS broth (at the same concentration), except glucose and Tween 80, sterilized in autoclave at 121 ºC for 15 min and cooled to room temperature. Subsequently, 30 mL of hydrolyzate was added into 250 mL sterile Erlenmeyer flasks, inoculated with 10% (v/v) of *L. plantarum* or *L. pentosus* preculture, and incubated at 30ºC and 150 rpm for 72 h. Samples were taken each 24 h to perform the analytical determinations described below.

2.5. Analytical methods

2.5.1. Quantification of organic acids and sugars

The concentration of organic acids (lactic and acetic) and sugars (glucose, xylose, and arabinose) was determined by HPLC (Agilent, model 1200, Palo Alto, CA) equipped with a refractive index detector and an Aminex HPX-87 H ion exclusion column (Bio Rad 300 mm × 7.8 mm, 9 m particles). The elution program was conducted during 23 min at 50 ºC with a flow rate of 0.6 mL/min of 3 mM sulfuric acid [20].

2.5.2. Total antibacterial activity assay

The total antibacterial activity (TAA) produced by *L. plantarum* CECT 221 and *L. pentosus* CECT 4023 as a consequence of the production of bacteriocins and organic acids (lactic and acetic) was determined using a photometric assay [14]. Bacteriocin desorption from the plasmatic membrane of the producer cells (strains CECT 221 or CECT 4023) was carried out adjusting the fermented samples to pH 3.0–3.5 with 5 M HCl and heating them in a boiling water bath for 5 min [3]. The acidified and heated samples were centrifuged at 9503 × *g* for 10 min to obtain cell-free extracts (CFE) containing the bacteriocins and organic acids. The TAA of the CFE was assayed against *Listeria monocytogenes* CECT 934 following the method reported in [21] with minor modifications. Equal volumes of appropriate dilutions of CFE with sterile distilled water and a culture of *L. monocytogenes* CECT 934 (diluted to an absorbance of 0.2 at 700 nm with sterile BHI broth buffered with potassium hydrogen phthalate–NaOH pH 6.0) were added to sterile culture tubes. The control tubes (in triplicate) contained equal volumes of the diluted *L. monocytogenes* CECT 934 culture and sterile distilled water. The samples were incubated for 6 h at 35 ºC and 150 rpm and then, the absorbance of each sample was measured at 700 nm. Bacteriocin concentration was defined as the amount of the antibacterial compound causing 50% growth inhibition (inhibitory dose 50: ID_{50}) compared to control tubes [14] and expressed as antibacterial activity units (AAU) per mL. The ID_{50} values were obtained from dose-response curves as described by Malvido et al., [22].

2.5.3. Microbial biosurfactants (mBS) determination

The fermented samples from *L. plantarum* CECT 221 or *L. pentosus* CECT 4023 cultures were centrifuged at 2755 × *g* for 15 min and the supernatant was used to measure the extracellular mBS in the

supernatant. To extract the cell-bond mBS, the cells were washed twice with distilled water and suspended in phosphate buffer saline (PBS) pH 7.4 at a 1/6 ratio (buffer solution/initial culture volume) with shaking at 150 rpm, 30 ºC for 2 h. This sample was centrifuged and mBS were measured in the supernatant by the Ring method test. The latter method quantified the surface tension (ST) of supernatants at room temperature, by means of a KRÜSS Tensiometer (Hamburg, Germany) coupled with a 1.9 cm DuNoüy platinum ring [23].

The concentration of mBS (g/L) was calculated using the following calibration curve reported in [24]: [mBS (g/L)] = [ST (mN/m) - 76.98]/− 8.65 calculated for a commercial biosurfactant (surfactin) produced by several *Bacilli* strains using different concentrations of BS solutions, below the critical micelle concentration with a known ST.

2.5.4. Mass balance of the biorefinery process

The mass balance proposed for the biorefinery process, to produce biomolecules from BSG, was carried out using 1 kg of dry BSG and both strains *L. plantarum* CECT 221 and *L. pentosus* CECT 4023.

2.6. Statistical analysis

All data were compared by analysis of variance (ANOVA) with Statgraphics Centurion XVI.I software using Tukey's test at a significance level of $P < 0.05$ to determine statistically significant differences.

3. Results and discussion

3.1. Fermentation of hydrolyzates

The scheme for processing BSG into lactic and acetic acids, bacteriocins, and mBS, in a biorefinery concept, is detailed in Fig. 1. It can be observed that BSG was used for two applications. On one side, BSG was employed to produce an enzymatic cocktail, and on the other it was treated with IL [N_{1112}OH][Gly] resulting in a carbohydrate-rich material, that was further hydrolyzed with the combination of enzymes produced by *A. brasiliensis* and *T. reesei*. The hydrolyzate contained 8.18 g glucose/L, 2.77 g xylose/L, 0.46 g arabinose/L, and 4.28 g acetic acid/L. After supplementation with the MRS nutrients (except glucose and Tween 80), the hydrolyzate was fermented with *L. plantarum* CECT 221 and *L. pentosus* CECT 4023 strains during 72 h.

The supplementation with the nutrients of MRS broth, using the same concentration as in the complex medium, except glucose and Tween 80, was based on a previous research conducted by our research team. In this way, significant increases were oberved in glucose and fructose consumption, as well as the production of bacteriocins and lactic acid by *L. plantarum* CECT 211 in culture media prepared with enzymatic hydrolyzates of pretreated chestnut burs and supplemented with MRS nutrients, compared to the unsupplemented media [14]. These findings indicate that MRS nutrients possess properties that promote growth, and production of lactic acid and bacteriocin by *L. plantarum* CECT 211 [14] and other lactic acid bacteria [19]. Consequently, supplementing CRM hydrolyzates with MRS nutrients (including bacteriological peptone, meat extract, yeast extract, dipotassium phosphate, triammonium citrate, sodium acetate, magnesium sulphate, and manganese sulphate) could enhance the availability of energy sources, vitamins, minerals, amino acids, and peptides in the nutrient-enriched CRM. This enhancement could promote the metabolic activity of *L. plantarum* CECT 221 and *L. pentosus* CECT 4023, resulting in improved nutrient consumption and product synthesis.

The kinetics of fermentations with both strains of the MRS nutrients-supplemented hydrolyzate media are shown in Fig. 2. As can be noted, *L. plantarum* (circles in Fig. 2) consumed glucose and arabinose completely within the first 24 h of fermentation. The consumption coincided with the synthesis of acetic acid (from 4.28 to 5.61 g/L) and an abrupt production of lactic acid (7.37 g/L). Interestingly, the lactic acid concentration subsequently decreased to 1.62 g/L by the 72 h of incubation. In contrast, acetic acid concentration exhibited further increments, from 5.61 to 7.98 g/L (24–48 h) and from 7.98 to 8.81 g/L (48–72 h).

In the presence of glucose, *L. plantarum* only consumed small amounts of xylose (0.67 g/L within the first 24 h), the concentration of this carbon source being maintained almost constant until the end of the fermentation.

The culture pH dropped from 5.39 to 4.75 during the first 24 h in accordance with the increase in the productions of lactic and acetic acids. However, after this time, the culture pH exhibited a soft linear increase until reaching a value of 4.85. This slight realkalinization of the fermentation substrate (Fig. 2) could be a result of the higher consumption of lactic acid (4.02 g/L at 24–48 h and 1.73 g/L at 48–72 h) compared to the production of acetic acid (2.37 g/L at 24–48 h and 0.83 g/L at 48–72 h).

This hypothesis is reinforced by the results obtained by other researchers in cultures of LAB. In this way, Lindgren et al. [25] observed that some strains of *L. plantarum* isolated from different commercial silage inoculants were able to degrade lactic acid to produce acetic and formic acids. In addition, Driehuis et al. [26] observed that inoculation of maize silage with *L. buchneri* PW01 led to a decrease in the concentration of lactic acid and an increase in the levels of acetic acid, propionic acid and 1-propanol in comparison with uninoculated silages. Later, Oude-Elferink et al. [27] reported that some strains of *L. buchneri* (LMG 6892 ^{T}, PW01, and PW07) and *L. parabuchneri* LMG 11457 ^{T} can consume lactic acid to produce 0.5 mol of acetic acid, 0.5 mol of 1,2-propanediol and ethanol traces under anaerobic and acidic conditions.

The fermentation kinetics of the MRS nutrient-supplemented hydrolyzate inoculated with *L. pentosus* (triangles in Fig. 2) exhibited a different behavior. Thus, although *L. pentosus* preferentially consumed glucose, the complete depletion of this carbon source was not produced since its concentration dropped from 8.18 to 1.63 g/L (0–24 h) and decreased slightly to 1.19 g/L at the end of the fermentation (Fig. 2). As observed in the *L. plantarum* culture (Fig. 2), the low initial arabinose concentration in the fermentation medium was completely consumed within the first 24 h of incubation by *L. pentosus*. This suggests a rapid assimilation of this sugar by the two lactic acid bacteria.

The level of lactic acid produced by *L. pentosus* within the first 24 h (8.04 g/L) was higher than that produced by *L. plantarum* (see Fig. 2), and the concentration of this organic acid increased slowly until reaching 8.94 g/L at 72 h of incubation (Fig. 2). From a kinetic point of view, it is evident that, the production of lactic acid evolved in parallel with the consumption of sugars in the *L. pentosus* culture. The rapid increase in lactic acid production (from 0 to 8.04 g/L) within the first 24 h coincided with the highest consumption of glucose, xylose, and arabinose. Despite this, *L. pentosus* did not completely consume the glucose and xylose during this period. Subsequently, from 24 to 72 h of fermentation, there was a slight increase in lactic acid production until the end of fermentation (from 8.04 to 8.94 g/L) in parallel with the slow consumption of glucose (from 1.63 to 1.19 g/L) and xylose (from 2.09 to 1.68 g/L).

Surprisingly, the lactic acid consumption noted in the *L. plantarum* culture from the 24 h of fermentation was not observed in the *L. pentosus* culture (Fig. 2), due to the presence of low concentrations of glucose (1.63 g/L) and xylose (2.09 g/L) in the fermentation medium at 24 h of incubation.

Additionally, *L. pentosus* always produced lower concentrations of acetic acid (4.83, 5.37, and 5.45 g/L at 24, 48, and 72 h, respectively) than *L. plantarum* (5.61, 7.98, and 8.81 g/L at 24, 48 and 72 h, respectively).

The culture pH exhibited the most significant decrease within the first 24 h, dropping from 5.39 to 4.73, as observed in the *L. plantarum* culture. This decline was attributed to the highest production of lactic acid (8.04 g/L) coupled with a slow synthesis of acetic acid (0.55 g/L) in this period. Following this, there was a gradual but slight decline in the culture pH until a value of 4.65 at the end of fermentation in parallel to

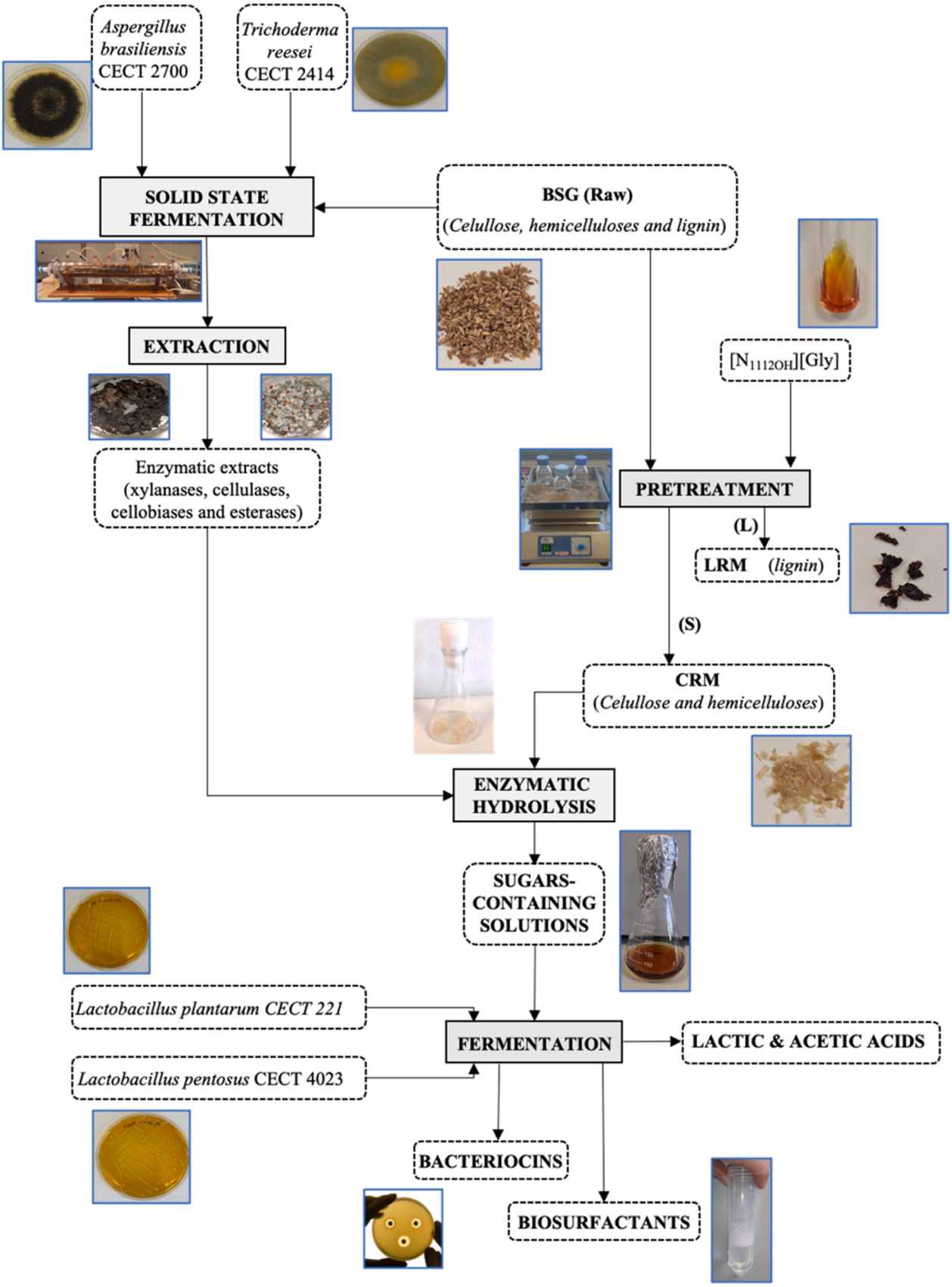

Fig. 1. General flowchart of the integrated BSG biorefinery processing.
BSG: brewery spent grain; CRM: carbohydrate-rich material; LRM: lignin-rich material.

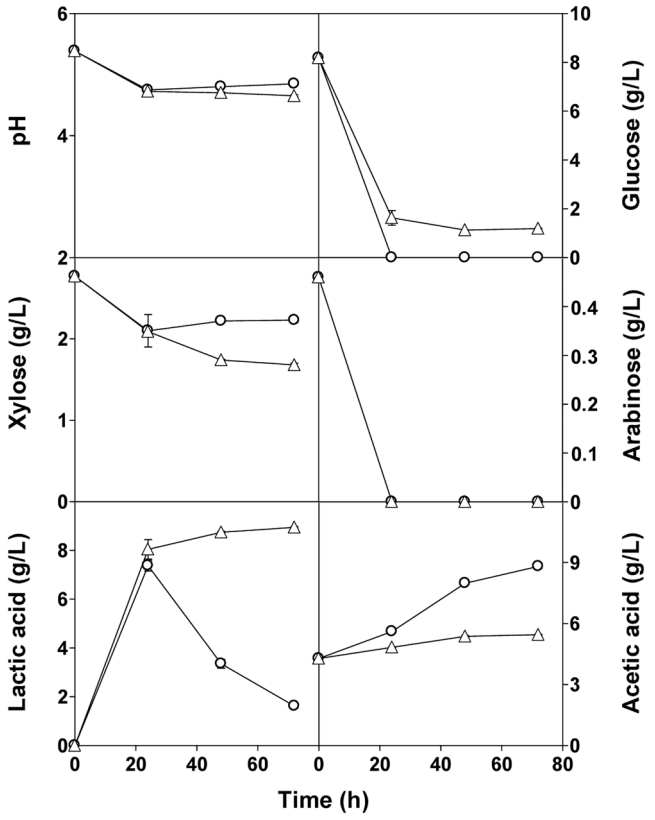

Fig. 2. Fermentation kinetics of nutrient-supplemented hydrolyzate medium with *L. plantarum* CECT 221 (open circles) and *L. pentosus* CECT 4023 (open triangles).

the slight production of lactic (0.90 g/L) and acetic (0.62 g/L) acids in this period (24–72 h).

Thus, the high drop exhibited by the culture pH within the initial 24 h of fermentation in both the *L. plantarum* and *L. pentosus* cultures probably resulted in an unfavorable pH value for biomass and product synthesis, including lactic acid, acetic acid, antibacterial activity, and mBS [22,28] during the 24–72 h of incubation (Fig. 2, Table 2 and Table 3).

To determine if the sugars (glucose, arabinose, and xylose) consumed by both *L. plantarum* and *L. pentosus* supported the production of organic acids (lactic and acetic acids), a mass balance was performed in each fermentation considering the following metabolic reactions [27]:

According to [19], the stoichiometry for production of lactic acid from hexose (glucose, Eq. 1), and lactic acid and acetic acid from pentoses (arabinose and xylose, Eq. 2) can be described as:

$$C_6H_{12}O_6 \rightarrow 2{\cdot}C_3H_6O_3 \quad (1)$$

$$C_5H_{10}O_5 \rightarrow C_3H_6O_3 + C_2H_4O_2 \quad (2)$$

According to [27], the stoichiometry for production of acetic acid and 1,2–propanediol from lactic acid can be described Eqs. 3 and 4, respectively:

$$C_3H_6O_3 + 2{\cdot}NAD^+ + H_2O \rightarrow C_2H_4O_2 + CO_2 + 2{\cdot}(NADH + H^+) \quad (3)$$

$$C_3H_6O_3 + 2{\cdot}(NADH + H^+) \rightarrow C_3H_8O_2 + H_2O + 2{\cdot}NAD^+ \quad (4)$$

Since 1,2–propanediol was not detected in the fermented samples, the Eq. (4) was not considered for the mass balances in the fermentation process.

In the *L. plantarum* culture during the initial 24 h of incubation (Table 1), a positive difference between the theoretical LA concentration [LA_T] (calculated from the concentration of glucose, arabinose and xylose consumed) and the actual concentration of LA detected in the culture medium [LA_{det}] has a positive sign (16.52 mM). This suggests that the total lactic acid concentration potentially produced by the growing strain (Eqs. 1 and 2) was not fully detected in the medium. In contrast, this difference for the acetic acid (AA) has a negative value (−14.62 mM), indicating that the levels of xylose and arabinose consumed were not sufficient to account for the measured concentrations of acetic acid in the culture medium (Eq. 2).

These observations suggest that the undetected lactic acid concentration (16.52 mM) might have been metabolized by *L. plantarum* to produce acetic acid, as noted in [27], and potentially utilized as a carbon source for cell growth [22].

According to Eqs. (2) and (3), the concentration of lactic acid that could have been metabolized to yield the detected concentration of acetic acid (AA) in the medium is calculated as follows:

22.15 mM – 3.06 mM (from arabinose assimilation) – 4.46 mM (from xylose assimilation) = 14.63 mM lactic acid.

Subsequently, the concentration of lactic acid potentially used for cell maintenance [22] can be calculated as follows:

16.52 mM - 14.63 mM = 1.89 mM lactic acid.

The same reasoning could be applied to the results obtained during the 24–72 h of fermentation in the *L. plantarum* culture. In this case, the levels of acetic acid detected in the medium (53.29 mM) could have

Table 1
Concentrations of sugars consumed to support the productions of lactic and acetic acids synthesized during the fermentations with *L. plantarum* and *L. pentosus*.

	***L. plantarum* culture**		***L. pentosus* culture**		***L. plantarum* MRS**	***L. pentosus* MRS**
	0-24 h	**24-72 h**	**0-24 h**	**24-72 h**	**0-24 h**	**0-24 h**
[G]cons (mM)	45.41	-	36.36	2.44	48.51	69.61
Qglu (mM/h)	1.89	-	1.51	0.01	2.02	2.90
[Arab]cons (mM)	3.06	-	3.06	-		
Qarab (mM/h)	0.02	-	0.02	-	-	-
[Xyl]cons (mM)	4.46	-	4.53	2.73		
Qxyl (mM/h)	0.03	-	0.03	-	-	-
[LA]cons (mM)	-	63.83	-	-		
Qla (mM/h)	-	1.33	-	-	-	-
[LA_{det}] (mM)	81.82	-	89.25	9.99	185.38	170.46
QLA (mM/h)	3.41	-	3.72	0.21	7.72	7.10
YLA	0.70	-	0.65	-	0.96	0.88
[AA_{det}] (mM)	22.15	53.29	9.16	10.32	-	5.55
[LA_T]prod (mM)	98.34	-	80.31	7.62	97.02	139.32
[AA_T]prod (mM)	7.53	63.83	7.59	2.73	-	-
[LA_T] - [LA_{det}] (mM)	16.52	-	-8.95	-2.38	-88.36	-31.14
[AA_T] - [AA_{det}] (mM)	-14.62	10.54	-1.57	-7.59		

[G]cons, [Arab]cons and [Xyl]cons: concentrations of glucose, arabinose, and xylose consumed; Qglu, Qarab, Qxyl, Qla: consumption rates of glucose, arabinose and xylose and production rate of lactic acid. Yla: lactic acid yield. [LAdet] and [AAdet]: concentrations of lactic acid and acetic acid detected in the culture medium; [LAT] and [AAT]: theoretical concentrations of lactic acid and acetic acid calculated from the amounts of glucose, arabinose and xylose consumed (Eqs. 1, 2, and 3).

been produced only from the assimilation of lactic acid (based on Eq. 3), considering that glucose, xylose, and arabinose were not detected in the fermentation medium (open circles in Fig. 2). In this case, the molar concentration of lactic acid needed to produce the acetic acid levels detected (53.29 mM) would be 53.29 mM lactic acid (Eq. 3). So, the concentration of lactic acid potentially utilized as a carbon source for cell growth [22] can be calculated as follows:

Total LA consumed (63.83 mM) - LA utilized for AA production (53.29 mM) = 10.54 mM lactic acid utilized for cell growth.

Negative differences between the theoretical molar concentrations of both organic acids and those detected in the medium for both the 0–24 h and 24–72 h periods in the *L. pentosus* culture (Table 1). Consequently, the concentrations detected were higher than the corresponding theoretical values, indicating that the amounts of carbon sources (glucose, arabinose, and xylose) consumed were insufficient to support organic acids production. This suggests that *L. pentosus* used other alternative substrates as carbon sources during the whole fermentation period. Thus, it is likely that the *L. pentosus* strain used the carbohydrates present in the complex nitrogen sources of the MRS broth, mainly in the yeast (17.5% of carbohydrates) and meat (2.9% of carbohydrates) extracts, during the complete fermentation period, corroborating earlier findings [28]. The same trend was also detected to both microorganisms in MRS media, obtaining in the same way negative values between theorical and detected values, wherefore the previous description could be explained by these facts. Additionally, previous studies have highlighted the ability of LAB to utilize amino acids such as arginine and serine present in the complex nitrogen sources of the MRS broth, as nitrogen and carbon sources to obtain energy for cell growth and maintenance [28–30].

On the other hand, focusing on the final bioconversion yield into lactic acid, *L. plantarum* presented better values than *L. pentosus* in CRM (0.70) and MRS (0.96). However, the productivity of this carboxylic acid was lower by *L. plantarum* compared to *L. pentosus,* probably due to the previously explained, being *L. pentosus* able to produce lactic acid from the carbohydrates of meet extract and yeast extracts, since the consumption rate of sugars (glucose, xylose and arabinose) was higher in *L. plantarum* fermentation, what it could not explain this data.

3.2. Total antibacterial activity

Bacteriocins produced by LAB are small, heat-stable peptides that exhibit activity against other bacteria. Moreover, the producer bacteria possess immunity against their own bacteriocins [31]. They can serve as safe food preservatives, meeting the increasing consumer demand for minimally processed foods. Additionally, bacteriocins help maintain the organoleptic properties of food during both processing and storage [32]. Previous work [14] showed that both *L. plantarum* and *L. pentosus* are capable of producing bacteriocins, since the levels of lactic acid produced by both strains were not inhibitory for the growth of *L. monocytogenes* CECT 934 (the target bacterium used in the bacteriocin activity assay).

In this study, both lactic acid and acetic acid were detected in the fermented culture media of *L. plantarum* and *L. pentosus*, but no extracellular mBS (that also could have inhibitory activity) were detected. Thus, all the inhibitory effect of the cell-free supernatants from the cultures of both strains could not be attributed solely to the bacteriocins produced by the two LAB. This is mainly due to the possible complementary inhibitory effect of bacteriocins and organic acids (lactic and acetic) on the growth of the target bacterium [33]. Therefore, the production of total antibacterial activity by *L. plantarum* and *L. pentosus* in the CRM fermentations was also assayed in this work. The results obtained are shown in Table 2. For comparative purposes, a new fermentation (control) for each strain was performed in the commercial MRS broth, which was reported as a standard medium for the growth of these LAB [34]. For both strains, the best results were obtained after 24 h with ID_{50} values of 6.69 ± 0.00 and 6.25 ± 0.19 AAU/mL for *L. plantarum* and *L. pentosus*, respectively in CRM medium. Then, these values decreased slightly with the increase in the fermentation time (Table 2).

Table 2
Total antibacterial activity (ID_{50} in AAU/mL) produced in CRM or MRS cultures by *L. plantarum* CECT 221 and *L. pentosus* CECT 4023.

	Fermentation time		
Strains	**24 h**	**48 h**	**72 h**
L. plantarum (CRM)	6.69 ± 0.00	6.21 ± 0.58	6.25 ± 0.33
L. pentosus (CRM)	6.25 ± 0.19	5.87 ± 0.33	6.03 ± 0.16
L. plantarum (MRS)	4.49 ± 0.00	n.q.	n.q.
L. pentosus (MRS)	5.31 ± 0.00	n.q.	n.q.

n.q.: not quantified

The results obtained showed that *L. plantarum* and *L. pentosus* produced lower levels of antibacterial activity in MRS medium than in CRM medium. This difference could be related to the lower initial glucose concentration in the CRM medium (8.18 g/L) compared to the MRS broth (20 g/L). In the latter medium, *L. plantarum* and *L. pentosus* consumed 8.73 and 12.54 g glucose/L, respectively, remaining relatively higher concentrations of the carbon source in the fermented medium at the end of the fermentation (11.27 and 7.46 g glucose/L, respectively).

Thus, the low initial concentrations of glucose (8.18 g/L), arabinose (0.46 g/L), and xylose (2.77 g/L) in the CRM medium probably limited the growth of the two bacteriocin-producing LAB strains. This growth limitation and the joint consumption of the three carbon sources could produce a stress response by the cells of strains CECT 221 and CECT 4023, stimulating the bacteriocin production, as observed before for *L. amylovorus* DCE 471 [35], *Lactobacillus* spp. [36], and *L. plantarum* CECT-211 [14].

3.3. Production of microbial biosurfactants

Microbial biosurfactants (mBS), produced by different microorganisms (bacteria, yeasts, and fungi) can be excreted extracellularly or attached to the cell membrane [37]. These compounds show exceptional surfactant properties in addition to different bioactivities, including antiinflammatory, antifungal, antiviral, and biostimulant, being therefore applied for microbial-enhanced oil recovery, as pharmaceutical agents, and in other areas of interest [38,39]. However, although mBS are promising, growing this market is very difficult due to the high production cost of these compounds, compared to analogs surfactants from synthetic origin. It is estimated that the cost of mBS production can be up to 12 times more than the corresponding synthetic surfactants [39]. Consequently, mBS only hold around 2.8% of the global surfactants market [40]. The use of agro-industrial wastes to formulate economic culture media for mBS production is a strategy that has been investigated to reduce waste treatment and production costs, making the product even more environmentally friendly [39].

A reduction of 8 mN/m in the surface tension of a medium is considered to be indicative of the presence of mBS [41] In this work, the absence of extracellular mBS was noted in the cultures of *L. plantarum* and *L. pentosus*, since the fermented medium did not exhibit a reduction in surface tension when compared to the unfermented medium. In contrast, the presence of cell-bond mBS was found in both lactic acid bacteria. Table 3 shows the values of the reduction in surface tension caused by the surfactants extracted from the different fermented CRM and MRS samples. For both strains, the maximum reduction in surface tension was observed at 24 h of fermentation, followed by a decrease at 48 h, with a slight stabilization at the end of the fermentation. The highest reduction in surface tension (27.00 ± 0.00 mN/m) was produced by *L. plantarum* compared to that produced by *L. pentosus* (19.50 ± 0.50 mN/m) during the first 24 h of incubation in CRM medium. Consequently, the higher concentration of mBS (2.76 g/L) was obtained with *L. plantarum* at 24 h.

Table 3
Reduction in surface tension (ST_{red}) and concentration of mBS produced ([mBS]) in CRM or MRS cultures for both strains.

	ST_{red} at 24 h (mN/m)	ST_{red} at 48 h (mN/m)	ST_{red} at 72 h (mN/m)	[mBS] at 24 h (g/L)
L. plantarum (CRM)	27.00 ± 0.00	19.67 ± 0.58	19.00 ± 0.33	2.76
L. pentosus (CRM)	19.50 ± 0.50	17.33 ± 0.33	17.67 ± 1.00	1.90
L. plantarum (MRS)	19.50 ± 0.50	n.q.	n.q.	1.90
L. pentosus (MRS)	18.00 ± 0.00	n.q.	n.q.	1.73

n.q.: not quantified

As in the bacteriocin test, control fermentation was performed for each strain in MRS medium for comparative purposes. As observed in Table 3, the reductions in surface tension in this complex culture medium caused by cell-bond mBS were 19.50 ± 0.50 mN/m and 18.00 ± 0.00 mN/m for *L. plantarum* and *L. pentosus*, respectively. These results indicated that the CRM hydrolyzate is a better fermentation substrate to produce mBS than the MRS broth, particularly when *L. plantarum* is used as mBS-producing strain.

3.4. Mass balance of the biorefinery process

The biorefinery process proposed for the pretreatment of BSG consists of three consecutive steps: pretreatment with the ionic liquid (IL) $[N_{1112OH}][Gly]$, enzymatic hydrolysis of the carbohydrate rich fraction to obtain more easily fermentable sugars and the final production of bioactive molecules. Fig. 3 summarizes the mass balance of the process starting with 1 kg of dry BSG. The pretreatment of BSG with the IL allowed to recover 245.0 g of carbohydrate rich material with a considerable increment in the percentage of cellulose (from 27.77 to 45.73%) and hemicellulose (from 24.90 to 40.42%) and a drastic reduction of lignin (from 19.30 to 9.47%). This substantial delignification, along with the morphological structural changes reported in [16], facilitated the enzymatic hydrolysis conversion of polysaccharides into glucose (80.94%) and xylose (70.40%), allowing to recover 89.6 g of glucose, 69.7 g of xylose and 5.4 g of arabinose.

The final step of the biorefinery process involved the efficient conversion of sugars released during the enzymatic hydrolysis to generate various substances, mainly organic acids (lactic and acetic acids), bacteriocins, and microbial biosurfactants. Despite the fermentation conditions were not optimized, high percentages of bioconversion to lactic acid were achieved for both strains: 64.59% with *L. plantarum* and 70.46% with *L. pentosus*. This could be influenced by the absence of inhibitory compounds released during the enzymatic hydrolysis. Therefore, *L. plantarum* and *L. pentosus* produced respectively, 106.4 g and 116.1 g lactic acid from 1 kg of dry BSG after 24 h of fermentation.

Furthermore, the relatively high mBS production by *L. plantarum* (6.76 g) and *L. pentosus* (4.65 g), suggests that the CRM hydrolyzate is a promising low-cost culture medium for the growth and product synthesis of these two LAB strains. Key manufacturers are attentive to delivering cost-effective mBS that will encourage other market players to invest in the market [40]. Indeed, a major challenge of large-scale mBS production is the production cost, which limits the industrial application of these compounds [38]. One alternative to reduce this cost is the use of cheaper culture media since these substrates can represent from 30 to 50% out of the total cost of production [42]. In fact, the price of the cheapest mBs is 34 USD/kg of sophorolipids, which is ten-fold more costly than chemical surfactants such as sodium lauryl sulphate (~1–2 USD/kg) [37]. Despite this, it is expected a strong increment in the coming years for the mBS market, from 16.5 million USD in 2022 to 24.3 million USD by 2032 [37]. These results show the potential of BSG as economic nutrient to produce valuable market products through a green and sustainable route.

4. Conclusions

The results presented here illustrate the possibility of obtaining high value-added products, such as lactic and acetic acids, bacteriocins and

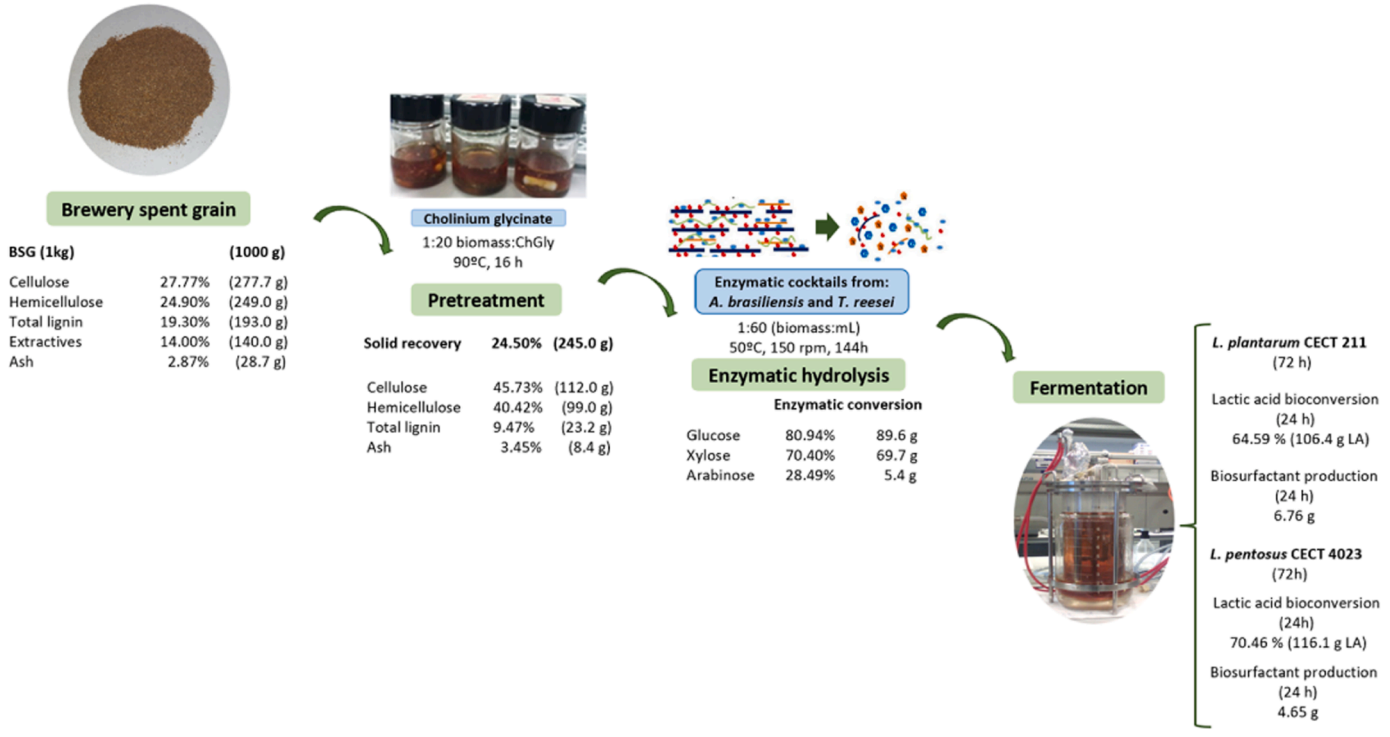

Fig. 3. Mass balance of BSG biorefinery to produce bioactive molecules with *L. plantarum* CECT 221 and *L. pentosus* CECT 4023.

biosurfactants through biotechnological processes, based on a biorefinery model that uses BSG as a substrate. The hydrolyzate obtained was successfully fermented with the *L. plantarum* CECT 221 and *L. pentosus* CECT 4023 to produce the above-mentioned compounds. The production of these biomolecules was slightly higher in the fermentations with *L. plantarum* when utilizing the CRM hydrolyzate as a substrate. This medium proved to be a better substrate for bacteriocin and biosurfactants production by both LAB compared to the commercial MRS broth. This emphasizes the hydrolyzed medium's potential as a low-cost substrate to produce different metabolites. After the shake flask tests, new experiments are being contemplated in a lab scale bioreactor to make a "proof of concept".

CRediT authorship contribution statement

David Outeiriño: Conceptualization, Investigation, Writing – original draft. **Iván Costa-Trigo:** Investigation, Writing – original draft, Writing – review & editing, Methodology. **Aida Ochogavias:** Investigation, Writing – review & editing. **Ricardo Pinheiro de Souza Oliveira:** Funding acquisition, Project administration, Writing – original draft. **Nelson Pérez Guerra:** Funding acquisition, Project administration, Writing – original draft, Writing – review & editing, Methodology. **José Manuel Salgado:** Methodology, Writing – original draft. **José Manuel Domínguez:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

Authors declare that they have no conflict of interest.

Data availability

Data will be made available on request.

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