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Brewers' spent grain and thin stillage as raw materials in L-(+)-lactic acid fermentation

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Brewer's spent grain (BSG) hydrolysates were used for L-(+)-lactic acid (LA) fermentation by *Lactobacillus rhamnosus* ATCC 7469. In this study the effect of the addition of various amounts of thin stillage (TS) in BSG hydrolysate on LA fermentation parameters were evaluated. TS addition significantly increased utilization of glucose by up to 43.0%. In batch fermentation the highest LA concentration and volumetric productivity of 31.0 g/L, and 0.93 g/L/h, respectively, were obtained with the addition of 50% TS. *L. rhamnosus* cell viability also increased with the addition of 50% TS (by 2.4%). TS addition significantly increased free amino nitrogen concentration (by up to 209%) which is important for bacterial growth. A strong positive correlation between free amino nitrogen and LA concentration was noted. Compared with the results obtained in the batch fermentation (50% TS), significantly higher LA concentration, yield and volumetric productivity (54.8, 1.9 and 4.0%, respectively) were achieved in fed-batch fermentation with glucose and TS addition. The results suggest that the combination of the by-products of brewing and bioethanol industries could be suitable for LA production.

Keywords: lactic acid fermentation; brewer's spent grain; stillage; *L. rhamnosus*; by-product utilization

Introduction

Lactic acid (LA) is used in pharmaceutical, food, cosmetic, and other industries *(1)*. In nature, LA occurs as two optical isomers, D-(−)- and L-(+)-LA. L-(+)-LA is the preferred isomer in the food and drug industries, because only this form can be assimilated by humans *(2)*. Currently, nearly all the LA manufactured is based on carbohydrate fermentation, where pure strains of LA-producing microorganisms such as *Lactobacillus* sp. are usually employed. LA bacteria (LAB) are non-spore forming, Gram-positive, aero-tolerant, non-respiring cocci or rods and acid-tolerant bacteria that can ferment sugars to produce LA *(3)*. However, LAB is heterotrophic and most species require amino acids and vitamins owing to their lack of biosynthetic capability *(4)*. In practice, requirements for growth factors are satisfied by the addition of nitrogen sources. Among a variety of nitrogen sources, yeast extract is the best because of its high content of nitrogen compounds, abundant vitamins, pyrimidine and purine bases *(5)*. Yeast extract has been used in many studies as a supplement, but its high cost has made its use in large quantities in industrial processes for LA lactic acid production are economically unfeasible *(6)*. Therefore, as an alternative, cheaper nitrogen sources are of particular interest. Indeed, it has been reported that inexpensive nitrogen sources from inorganic nitrogen-rich materials and agricultural by-products can be used as partial or complete yeast extract replacements *(7)*.

The main by-product of bioethanol production is stillage. Stillage obtained after bioethanol production from starch raw material contains yeast cells and organic materials such as proteins, amino acids and free sugars. Thin stillage (TS) is a supernatant fraction after centrifugation of the whole stillage obtained after ethanol distillation *(8,9)*.

Renewable materials such as lignocellulose and starch from agricultural residues and forestry resources are generally considered to represent an attractive substrate as feedstock for LA production *(2)*. The main problems encountered in the efficient conversion of lignocellulosic biomass to LA are: (a) the resistant nature of biomass and pretreatment problems; (b) the high cost of enzymes and their feedback inhibition; (c) the formation of by-products (ethanol and acetic acid) owing to the heterofermentation of pentose sugars; and (d) carbon catabolite repression caused by the heterogeneity of hydrolysate-sugar composition *(10)*.

Brewers' spent grain (BSG) is the major by-product of beer production *(11)*. Currently, approximately 15–20 kg of BSG is produced per hectoliter of beer, which results in an annual production of over 30 million tonnes of BSG worldwide *(12)*. The main application of BSG has been as an animal feed owing to its high content of protein and fibre or simply as landfill *(13)*. BSG is an abundant, low-cost biomass, rich in carbohydrates (arabinoxylan and cellulose), lignin and protein, and so could provide important ingredients and precursors for the chemical, food and energy industries *(12)*. Possible applications for BSG are in biotechnological, chemical, food and energy processes *(14)*.

In this study BSG hydrolysates were used for LA fermentation by *Lactobacillus rhamnosus*. The aim of this study was to evaluate the effect of TS (5–50%) or TS (5–50%) together with and glucose addition in BSG hydrolysate on the LA fermentation. Parameters include LA concentration, productivity and yield, utilization of glucose and *L. rhamnosus* cell viability. TS was

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investigated as a nitrogen source. Fed-batch fermentation of BSG hydrolysate with TS and glucose addition during fermentation was also evaluated.

Material and methods

Preparation of brewers' spent grain hydrolysate for fermentation

Prior to fermentation, BSG hydrolysis was optimized. Enzyme dosage was performed according to the suppliers recommendation but was later increased according to the results of our investigation. Each enzyme (Termamyl SC®, SAN Super 240 L® and Celluclast 1.5 L®) was evaluated alone. Celluclast® 1.5 L is a preparation (a mixture of hydrolytic enzymes) in which the key enzyme activity is provided by cellulase that hydrolyses (1,4)-β-D-glucosidic linkages in cellulose and other β-D-glucans. Celluclast® is a preparation used for degradation of cellulose into glucose, cellobiose and longer glucose polymers. Termamyl® SC is a heat-stable α-amylase preparation used for degradation of starch into dextrins, maltose and glucose. SAN Super® 240 L is an optimized enzyme preparation that contains amyloglucosidase, α-amylase, protease and glucanases, used for degradation of starch and dextrins into glucose. After optimization of individual enzyme dosage a combination of these three enzymes was used according to the best results obtained for each enzyme.

BSG obtained from a lager beer production was dried at 40°C for 12 h. Dried BSG was finely ground (particle size $<$0.20 mm) in a laboratory DLFU mill from Bühler-Miag (Braunschweig, Germany). For hydrolysate production 50 g of dry BSG was mixed with 300 mL of distilled water and the pH value of the mixture was adjusted to 5.5 with the addition of 10% H_3PO_4, prior to hydrolysis. BSG hydrolysis was performed as previously described *(15)*. The liquid hydrolysate was separated from the solid hydrolysate (liquid to solid ratio 60:40) by centrifugation (4000 rpm for 20 min) and used in LA fermentations. Its pH was adjusted to 6.5 with the addition of 1 M NaOH. After this, the liquid hydrolysate was sterilized at 121°C for 15 min and used as a fermentation medium. Sterilized BSG hydrolysate was used in experiments without and with glucose addition. In experiments with glucose addition, the initial glucose concentration in the hydrolysate was set to 50 g/L prior to the inoculation by addition of a sterile glucose solution (700 g/L).

Thin stillage preparation for fermentation

Distillery stillage used in the experiments was obtained after bioethanol fermentation of potato. TS used in LA fermentation was obtained after centrifugation (4000 rpm for 10 min) of distillery stillage. TS was sterilized at 121°C for 15 min and added in BSG hydrolysate (5, 10, 15, 20, 30, 40 and 50% *v*/v) prior to the inoculation. In fed-batch fermentations, TS was sterilized at 121°C for 15 min and added in BSG hydrolysate to achieve content of 50% (*v*/v) prior to the inoculation.

Microorganism

Lactobacillus rhamnosus ATCC 7469, a homofermentative LA strain, was obtained from American Type Culture Collection (ATCC, Rockville, USA). Stock cultures of *L. rhamnosus* were stored and activated as previously described *(15)*. The Inoculum was prepared by taking 3 mL of the activated culture and transferring it to 60 mL of MRS broth. To reach a high LAB cell number (1×10^9 CFU/mL) the inoculum was incubated for 24 h at 37°C.

LA fermentation

All LA fermentations were performed as batch cultures with shaking (150 rpm, Biosan shaking bath model ES-20, Biosan Ltd, Latvia). The fermentations were performed in 300 mL Erlenmayer flasks with 200 mL of BSG hydrolysate. The fermentation was initiated by the addition of inoculum (5% *v*/v) and incubated at 37°C. The pH was maintained at 6.2 by the addition of a sterile 30% (*w*/*v*) NaOH solution at 4 h intervals. Correction of glucose concentration to initial value (50 g/L) in fed-batch fermentations with TS and glucose addition during fermentation was done when the glucose concentration decreased to ~30 g/L. The correction to initial glucose concentration during fermentation was done by the addition of a sterile glucose solution (700 g/L) combined with TS. The volume of concentrated glucose solution that was needed to achieve initial glucose concentration was calculated and then TS was added so that the combined volume with glucose was $\leq$5% (*v*/v).

Analytical methods

LA concentration was determined by an enzymatic method [L-(+)-LA assay, Megazyme, Wicklow, Ireland]. Prior to LA determination, proteins were removed from samples by precipitation according to the procedure prescribed in LA assay. BSG hydrolysate and TS composition were monitored during fermentation and the following methods were used for analysis: dry matter content was determined by a standard drying method in an oven at 105°C to constant mass *(16)*; protein content was determined by Kjeldahl method as the total nitrogen and multiplied by factor 6.25 *(17)*; and ash content was determined by slow combustion method at 650°C for 2 h *(16)*. Free amino nitrogen (FAN) concentration in BSG hydrolysate and TS was determined by ninhydrin method *(18)*. Metal concentration was determined by inductively coupled plasma mass spectrometry (NexION 300X, PerkinElmeris®, Massachusetts, USA) *(19)*. The number of viable *L. rhamnosus* cells was determined using a pour-plating method. During incubation in Petri plates microaerophilic conditions were maintained using a double MRS medium layer. Samples were incubated for 48 h at 37°C. Total viable cell number was expressed as log CFU/mL.

HPLC analysis

Prior to the high-performance liquid chromatography (HPLC) analysis, proteins were removed and the samples were filtered (0.22 μm). For quantitative analysis of samples, a Dionex Ultimate 3000 (Thermo Scientific, Waltham, MA, USA) HPLC system was used. Sugar determinations were performed using a Thermo Scientific carbohydrate column (Hyper REZ XP Carbohydrate Ca^{2+}, 300 × 7.7 mm, 8 mm) at 80°C. Deionized water was used as the mobile phase with an elution rate of 0.6 mL/min during the analysis. Detection was performed using an RI detector (RefractoMax 520, ERC, Riemerling, Germany). All data acquisition and processing were done using Chromeleon™ 7.2 Chromatography Data System (Thermo Scientific). Calibration curves for quantification of monosaccharides (glucose, arabinose and xylose), disaccharides and trisaccharides were used *(20)*.

Statistical analysis

All of the experiments were performed in triplicate. All values are expressed as means ± standard deviation. The mean values of LA concentration, LA yield, volumetric productivity and cell viability of *L. rhamnosus* were compared by the analysis of variance (one-way ANOVA) followed by Duncan test for mean differences testing (SPSS Statistica 20, IBM Corporation, Armonk, NY, USA). Differences were considered significant at $p < 0.05$. Pearson's correlation coefficient was determined for correlation of FAN and produced LA concentration for $p < 0.01$.

Results and discussion

Analysis of BSG hydrolysate and TS

The chemical composition of BSG hydrolysate and TS is presented in Table 1. BSG hydrolysate and TS contained mostly glucose. During enzymatic hydrolysis the starch present in BSG was completely degraded while 78.6% of initial cellulose content was degraded *(21)*. Before hydrolysis BSG did not contain reducing sugar. Besides glucose, BSG hydrolysate and TS contained arabinose, xylose and various disaccharides and trisaccharides. TS contained a much higher content of disaccharides and trisaccharides than BSG hydrolysate. BSG hydrolysate had higher dry matter content and glucose concentration than TS. TS also had significantly higher total nitrogen, FAN and ash concentration. The chemical composition of stillage varies greatly depending on raw material and various aspects of the bioethanol production process. Although there are seasonal and regional differences in the composition of the particular starch-based raw materials, there are also some similar general composition characteristics relevant for fermentations. The stillage used in this study contained total nitrogen concentration of 0.21 g/L and glucose concentration of 11.2 g/L.

Table 1. Brewers' spent grain (BSG) hydrolysate and thin stillage (TS) chemical composition[a]

Parameter	TS	BSG hydrolysate
Dry matter, %	2.8 ± 0.0	4.8 ± 0.0
Glucose, g/L	11.2 ± 0.2	21.0 ± 0.2
Arabinose (g/L)	0.2 ± 0.0	1.6 ± 0.0
Xylose (g/L)	0.2 ± 0.0	2.0 ± 0.0
Different disaccharides and trisaccharides (g/L)	14.3 ± 0.2	8.0 ± 0.1
Total nitrogen, g/L	1.3 ± 0.0	0.8 ± 0.0
Free amino nitrogen, mg/L	339.3 ± 2.1	55.0 ± 0.1
Ash, percentage dry matter	5.0 ± 0.0	2.0 ± 0.
Zn, mg/kg	4.5 ± 0.0	0.3 ± 0.0
Cu, mg/kg	0.2 ± 0.0	1.8 ± 0.0
Fe, mg/kg	32.2 ± 0. 0	0.6 ± 0.0
Mn, mg/kg	6.9 ± 0.0	1.3 ± 0.0
Co, mg/kg	<0.001 ± 0.001	0.011 ± 0.001
Ca, mg/kg	543.9 ± 1.8	535.8 ± 1.8
Mg, mg/kg	260.7 ± 1.5	236.3 ± 1.4
Na, mg/kg	57.1 ± 0.2	761.8 ± 0.2

[a]Values represent means ± standard deviation calculated from three determinations.

Cibis et al. *(22)* determined slightly higher total nitrogen (0.52–0.81 g/L) and lower reducing sugar (10.5–10.6 g/L) concentration in potato stillage. TS contained significantly higher ash concentration and therefore higher mineral element concentration, especially Zn, Fe, Mn, Ca and Mg, than BSG hydrolysate. Minerals (Mg, Mn and Fe) are essential elements that act as cofactors in enzymatic reactions within LAB *(23)*. Mg, Mn, Ca, Fe and Zn are minerals with a stimulatory effect on LA production and bacterial growth *(24)*. Wang et al. *(2)* reported that Na and K also have a positive effect on LA production by LAB. Based on the chemical composition of these two renewable substrates, TS could be used as a cheap source of nitrogen (especially FAN) and minerals, while BSG could represent a better carbon source.

Effect of TS addition on LA fermentation parameters

The FAN concentration in BSG hydrolysate without and with TS addition is presented in Table 2. Addition of TS significantly increased FAN concentration. The obtained LA concentrations were correlated with corresponding FAN concentrations in BSG hydrolysate. A strong positive correlation between FAN and LA concentration was noted (correlation coefficient value of 0.954).

The addition of various amounts of TS affected LA fermentation parameters such as LA concentration, productivity and yield, glucose utilization, *L. rhamnosus* cell viability and pH (Fig. 1). LA concentration in the LA fermentation of BSG hydrolysate with TS addition is given in Fig. 1(a). Thin stillage addition significantly increased LA concentration (by 8.6% at 5% TS to 21.0% at 50% TS) compared with fermentation without TS addition. The highest LA concentration (11.0 g/L) was obtained with the addition of 50% of TS. The presence of acetic acid was not determined in our preliminary research. *L. rhamnosus* is a homofermentative strain and it does not produce acetic acid under the conditions studied here. Glucose concentration in LA fermentations of BSG hydrolysate with TS addition is reported in Fig. 1(b). With TS addition the glucose utilization increased significantly (by 8.4% at 5% TS to 19.0% at 50% TS). In fermentations with the addition of 50% TS almost all available glucose was utilized. All fermentations with TS, addition lasted 24 h. TS addition increased the metabolic activity of LAB owing to the addition of FAN and minerals. With an increase in TS content in BSG hydrolysate, initial glucose

Table 2. Free amino nitrogen (FAN) concentration in BSG hydrolysate with TS addition[a]

TS content (%) in BSG hydrolysate	FAN concentration (mg/L)
0	55.0 ± 0.1
5	56.8 ± 0.1
10	58.5 ± 0.1
15	75.0 ± 0.4
20	103.3 ± 1.0
30	137.6 ± 1.2
40	148.8 ± 1.2
50	169.8 ± 1.2

[a]Values represent means ± standard deviation calculated from three determinations.

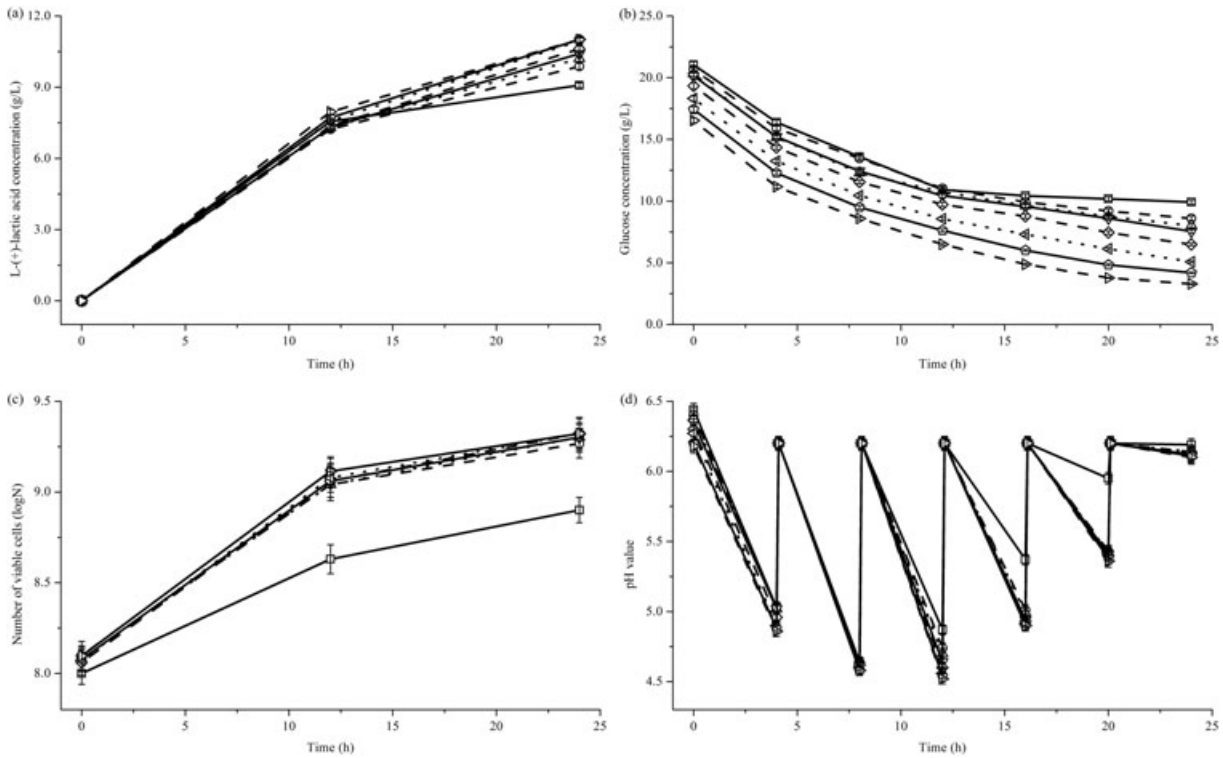

Figure 1. Lactic acid (LA) fermentation of brewer's spent grain (BSG) hydrolysate with thin stillage (TS) addition: (a) LA concentration; (b) glucose concentration; (c) *Lactobacillus rhamnosus* cell viability. Symbols: (□) solid line – without TS; (○), dashed line – 5% TS; (Δ), dotted line – 10% TS; (▼), solid line – 15% TS; (◊), dashed line – 20% TS; (◄), dotted line – 30% TS; (⌂), solid line – 40% TS; (►), dashed line – 50% TS.

concentration decreased. Since TS contained lower concentrations of xylose and arabinose than BSG hydrolysate, with an increase in TS content decrease their concentration. Xylose concentration ranged from 0.70 g/L (50% TS) to 1.96 g/L (0% TS). Arabinose concentration ranged from 0.71 g/L (50% TS) to 1.61 g/L (0% TS). In addition, TS contained higher concentrations of disaccharides and trisaccharides than BSG hydrolysate. With an increase in TS content in BSG hydrolysate, the concentration increased to 8.03 g/L (0% TS) and 11.35 g/L (50% TS). Arabinose, xylose, disaccharides and trisaccharides were not utilized during fermentation. Accordingly, *L. rhamnosus* does not utilize these sugars. *L. rhamnosus* cell viability in the LA fermentations of BSG hydrolysate with TS addition is given in Fig. 1(c). *L. rhamnosus* cell viability was higher in fermentation with TS addition (4.1% at 5% TS to 4.8% at 50% TS). pH values in fermentations of BSG hydrolysate with TS addition are given in Fig. 1(d). TS addition slightly decreased pH value.

LA yield and volumetric productivity in batch LA fermentations of BSG hydrolysate with TS addition are presented in Table 3. LA yield was higher in all fermentations with TS addition (by 82.1% at 5% TS to 83.3% at 50% TS). Addition of TS increased LA yield (by 0.4% at 20% to 1.8% at 50%). The highest yield was achieved after 24 h in all fermentations. Volumetric productivity also increased with an increase in TS content (from 0.4% at 5% to 1.8% at 50%). Significant increase in volumetric productivity was achieved with the addition of 20–50% TS. The highest volumetric productivity of 0.66 g/L/h was achieved with the addition of 50% TS. There is very little information in the literature about LA fermentation of BSG hydrolysate with the addition of simply by-products from bioethanol production. Djukić-Vuković et al. *(24)* achieved LA yield of ~73.4% in LA fermentation on TS by the same *L. rhamnosus* ATCC 7469. The combination of BSG and TS in this work showed a 10% increase in LA yield and three-fold higher LA productivity in comparison with maximal yield and productivity obtained in batch fermentation with TS, as reported by Djukić-Vuković et al. *(25)*. Therefore, BSG and TS could be considered as promising substrates for further

Table 3. L-(+)-Lactic acid (LA) yield and volumetric productivity in batch L-(+)-LA fermentations of BSG hydrolysate with TS addition[a]

TS content (%) in BSG hydrolysate	L-(+)-LA yield (%)[b]	Volumetric productivity (g/L/h)[c]
0	81.8 ± 0.8^{A}	0.63 ± 0.01^{A}
5	82.1 ± 0.8^{A}	0.64 ± 0.01^{AB}
10	82.2 ± 1.0^{A}	0.64 ± 0.01^{AB}
15	82.4 ± 1.2^{A}	0.64 ± 0.01^{AB}
20	82.5 ± 1.1^{A}	0.65 ± 0.01^{BC}
30	82.8 ± 0.9^{A}	0.65 ± 0.01^{BC}
40	83.1 ± 1.2^{A}	0.66 ± 0.01^{C}
50	83.3 ± 1.2^{A}	0.66 ± 0.01^{C}

[a]Values represent means ± standard deviation calculated from three parallel tests. Means written with different capital letter in a column are significantly different ($p < 0.05$).
[b]L-(+)-LA yield was calculated at 24 h of the fermentation.
[c]Volumetric productivity was calculated at 12 h of fermentations.

optimization of batch LA fermentation with glucose addition and fed-batch fermentation with glucose and TS addition during the fermentation.

Effect of TS and glucose addition in batch and fed-batch LA fermentations

In our previous report *(21)* the highest LA yield and volumetric productivity were achieved in fermentation of BSG hydrolysate with an initial reducing sugar concentration of 54 g/L and with pH correction. Accordingly in this study initial reducing sugar concentration in BSG hydrolysate was set to 54 g/L after TS addition and all fermentations were performed with the pH correction. The effect of addition of glucose and various contents of TS on LA fermentation parameters such as LA concentration, its productivity and yield, glucose utilization, *L. rhamnosus* cell viability and pH was assessed (Fig. 2).

LA concentration in the the fermentation of BSG hydrolysate with TS addition is shown in Fig. 2(a). Thin stillage and glucose addition significantly increased LA concentration compared with fermentation without TS addition. The highest LA concentration (31.0 g/L) was obtained with the addition of glucose and 50% TS. Li et al. *(26)* observed a similar trend in LA fermentation by *L. rhamnosus* LA-04-1 on glucose media with the addition of corn steep liquor (CSL). In their research LA production was improved with an increase in CSL concentration. Glucose concentration in LA fermentations of BSG hydrolysate with TS addition is shown in Figure 2b. With TS and glucose addition glucose utilization increased significantly (by 10.5% at 5% TS to 43.0% at 50% TS) compared with the fermentation without TS addition. All batch fermentations with TS and glucose addition lasted 36 hours. Xylose concentration ranged from 0.68 g/L (50% TS) to 1.43 g/L (0% TS) while arabinose concentration ranged from 0.71 g/L (50% TS) to 1.43 g/L (0% TS). The concentration of disaccharides and trisaccharides were in a range of 8.0 g/L (0% TS) to 11.35 g/L (50% TS). Arabinose, xylose, and disaccharides and trisaccharides were not utilized during fermentation. *L. rhamnosus* cell viability in LA fermentations of BSG hydrolysate with TS and glucose addition is reported in Figure 2c. *L. rhamnosus* cell viability was significantly higher in fermentation with the addition of 50% TS (by 2.4%) than in fermentation without TS addition. The pH values in L-(+)-LA fermentations of BSG hydrolysate with TS addition are given in Figure 2d. TS and glucose addition slightly decreased pH value.

LA yield and volumetric productivity in batch and fed-batch LA fermentations of BSG hydrolysate with TS addition are presented in Table 4. LA yield was higher in all fermentations with TS addition (by 82.5% at 5% TS to 86.2% at 50% TS). Addition of 20–50% TS increased significantly LA yield (by 3.2% at 20% TS to 4.8% at 50% TS) compared to the yield obtained in fermentation without the addition. Volumetric productivity was significantly higher when TS was added. The highest volumetric productivity was achieved after 12 h in all fermentations. The volumetric productivity increased with the increase in TS content in BSG hydrolysate (by 6.8% at 5% TS to 39.2% at 50% TS). The highest volumetric productivity of 0.93 g/L/h was achieved with the addition of 50% TS.

Fed-batch fermentation enables control of substrate concentration in an optimal range without inhibition effect, so it could be a promising strategy for intensification of LA production. The LA concentration, glucose concentration, and *L. rhamnosus* cell viability in fed-batch LA fermentations of BSG

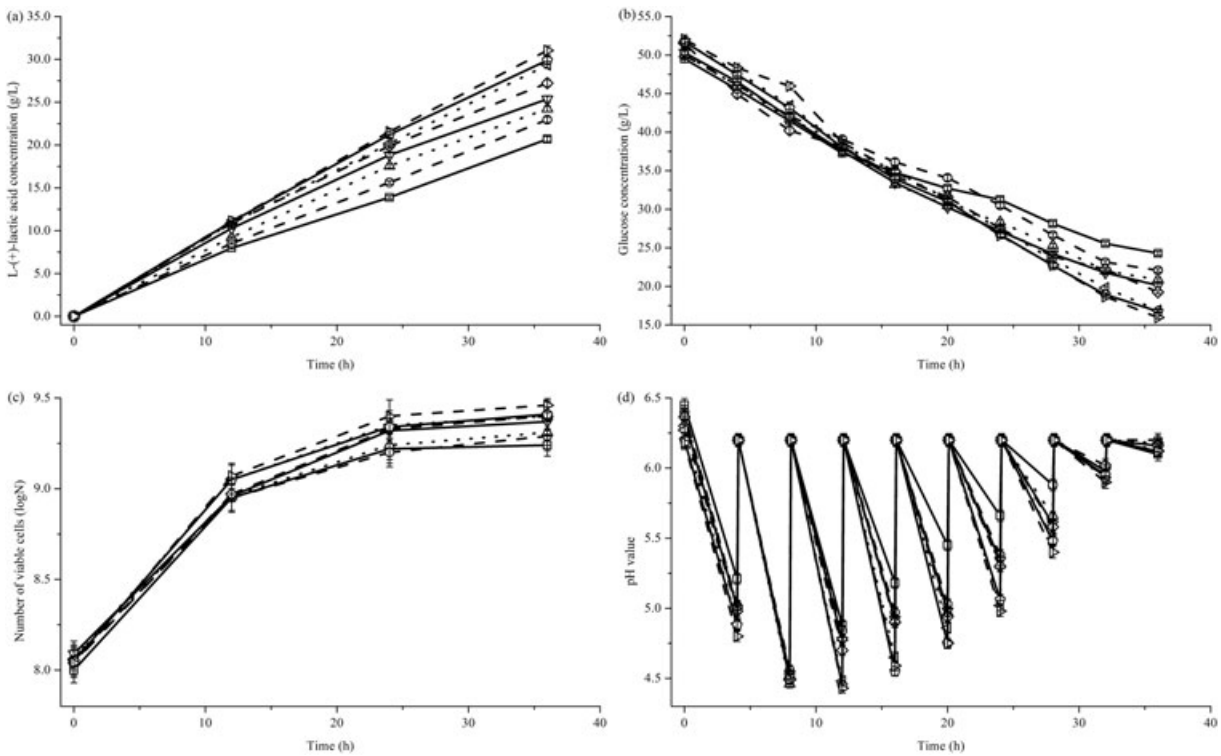

Figure 2. LA fermentation of BSG hydrolysate with TS and glucose addition: (a) LA concentration; (b) glucose concentration; (c) *L. rhamnosus* cell viability. Symbols: (□) solid line – without TS; (○), dashed line – 5% TS; (Δ), dotted line – 10% TS; (▼), solid line – 15% TS; (◊), dashed line – 20% TS; (◄), dotted line – 30% TS; (⌂), solid line – 40% TS; (►), dashed line – 50% TS.

Table 4. L-(+)-LA yield and volumetric productivity in batch and fed-batch L-(+)-LA fermentations of BSG hydrolysate with TS and glucose addition[a]

Thin stillage (TS) content (%) in BSG hydrolysate	L-(+)-LA yield (%)[b]	Volumetric productivity (g/L/h)[c]
Batch fermentation		
0	82.2 ± 1.2^{A}	0.67 ± 0.01^{A}
5	82.5 ± 1.2^{AB}	0.71 ± 0.01^{B}
10	83.2 ± 1.1^{ABC}	0.77 ± 0.02^{C}
15	84.2 ± 0.9^{ABCD}	0.86 ± 0.01^{D}
20	84.9 ± 1.2^{BCD}	0.91 ± 0.01^{E}
30	85.0 ± 1.1^{BCD}	0.91 ± 0.01^{E}
40	85.6 ± 1.3^{CD}	0.92 ± 0.01^{EF}
50	86.2 ± 1.1^{D}	0.93 ± 0.01^{F}
Fed-batch fermentation		
50	87.8 ± 1.2^{E}	0.96 ± 0.01^{G}

[a]Values represent means ± standard deviation calculated from three parallel tests. Means written with different capital letters in a column are significantly different ($p < 0.05$).
[b]L-(+)-LA yield was calculated at 36 h of the fermentation for batch fermentation and at 60 h of fermentation for fed-batch fermentation.
[c]Volumetric productivity was calculated at 12 h of fermentations.

hydrolysate with the addition of glucose and TS during fermentation are presented in Figure 3. The results achieved in batch fermentation showed that it is better to perform feeding of the media at over 30 g/L glucose concentration, to avoid decrease in LA productivity. In fed-batch fermentation total glucose utilization increased significantly by 47.7% compared to the total glucose utilization in batch fermentation with 50% TS.

After 60 h of fed-batch fermentation the highest LA yield (87.8%) and concentration (48.0 g/L) were achieved. The highest volumetric productivity of 0.96 g/L/h was achieved after 12 h of fermentation. Compared to the results obtained in the batch fermentation of BSG hydrolysate (with 5.0% of glucose and 50% TS), significantly higher LA concentration, yield and volumetric productivity (by 54.8%; 1.9%; and 4.0%, respectively) were achieved in a fed-batch LA fermentation with glucose and TS addition (Table 4). Glucose and TS addition during the LA fermentation increased *L. rhamnosus* cell viability by 0.9%. According to the results achieved, fed-batch fermentation could be used to increase the LA fermentation efficiency.

Since xylose, arabinose and di- and trisaccharides were not utilized during LA fermentation future research should be directed towards screening of new strains than can solely or in mixed culture utilize hexose and pentose sugars and oligomers with greater efficiency. However, this will be a challenging task since a great deal of time is needed to screen and isolate homofermentative LA strain that can produce LA with high yields using mixture of sugars. There is little information regarding the homofermentative LA strains that can utilize pentose sugar using PP (pentose phosphate)-pathway and almost all of them are strains obtained using genetic manipulation. Pentose sugars (e.g. xylose and arabinose) are metabolized heterofermentatively via the phosphoketolase (PK)-pathway by the majority of LA producers (especially LAB), which leads to the generation of various by-products, such as acetic acid, ethanol, and carbon dioxide, in

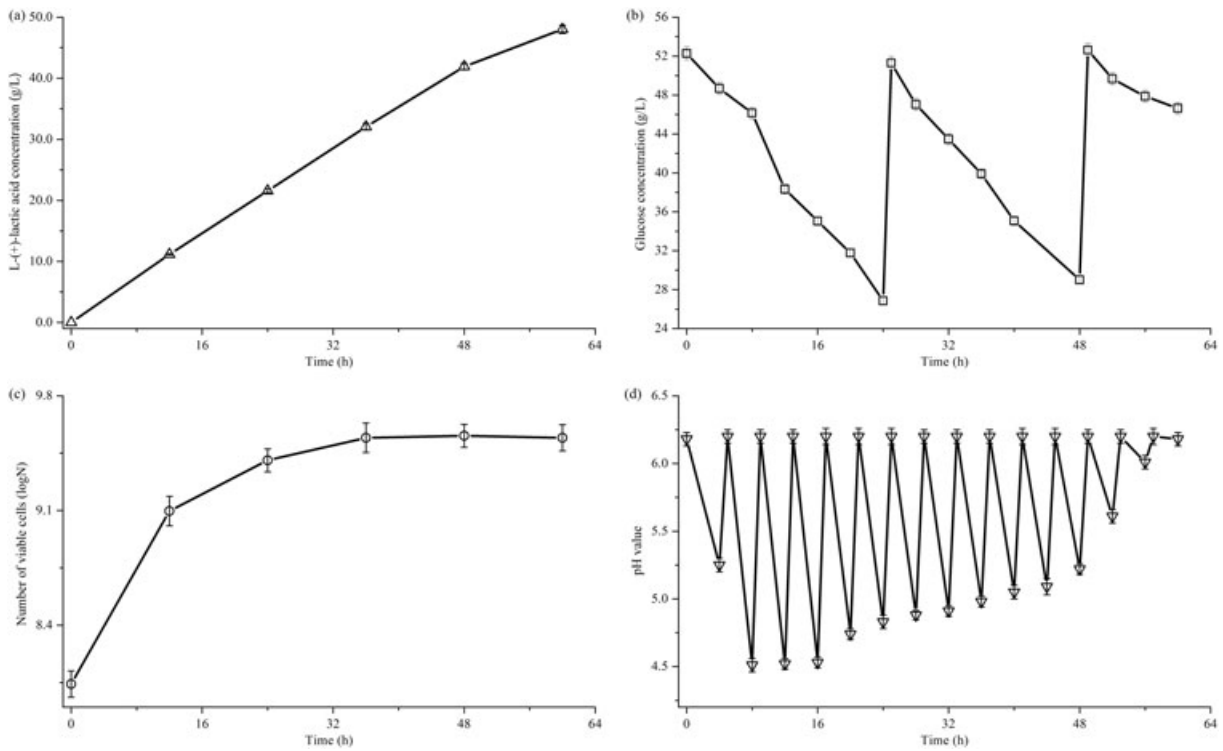

Figure 3. LA fermentation of BSG hydrolysate with TS addition (50%) and with glucose and TS addition during fermentation: (a) LA concentration; (b) glucose concentration; (c) *L. rhamnosus* cell viability. Symbols: (□) – glucose concentration; (○) – Number of viable cells; (Δ) – LA concentration.

addition to LA. This reduces LA yield, with a maximum theoretical yield of 0.6 g/g of consumed pentose and has a negative impact on the separation and purification costs of LA *(27)*. According to Kandler, all LAB except lactobacilli of type I (e.g. *L. delbrueckii*) are able to ferment pentoses, i.e. they are facultative heterofermenters *(28)*.

Li et al. *(29)* used glucose (100 g/L) with the addition of 6.0% of CSL and different salts in LA fermentation by *L. rhamnosus* LA-04-1. LA yield of 80% was achieved which was similar to the yield achieved in this study with the addition of 10% TS (83.2%). However, a higher yield was achieved in this study with the addition of 50% TS in BSG hydrolysate (86.2%).

Rivas et al. *(30)* evaluated the possible application of *Debaryomyces hansenii* spent cells from xylitol production and corn steep liquor in LA production by *L. rhamnosus* CECT-288 in glucose-containing media (100–120 g/L) with the addition of salts. With the addition of 1% of CSL and 1% *D. hansenii* spent cells LA a yield of 82% was achieved, which was similar to that achieved in this study for 5% TS (82.5%).

Yu et al. *(7)* investigated the effect of the addition of CSL, glucose, molasses, Tween 80 and $MnSO_4$ on LA fermentation by *L. rhamnosus* CGMCC 1466. Using 5.6% of CSL in combination with 120 g/L of glucose, 36 g/L of molasses, 2 g/L of Tween 80 and 0.24 g/L of $MnSO_4$, an LA yield of 82% was achieved, which was similar to the LA yield achieved in this study for 5% TS.

It is important to note that TS presents a bioethanol production by-product with a high organic load which could not be discharged as effluent without an adequate pretreatment. Therefore, its utilization to supplement the BSG hydrolysate in media for LA fermentation is an economically and environmentally favourable approach. The high content of nitrogen, particularly of FAN, which mainly came from TS, contributed to the achievement of higher values of all significant LA fermentation parameters.

For industrial scale-up, LA production must be optimized in a pilot-scale plant. Both raw materials must be processed as soon as possible owing to rapid deterioration. Industrial-scale centrifuges are needed for BSG hydrolysate and stillage separation (TS can be separated at the bioethanol plant). Downstream processing depends on funding and available resources. Stillage and BSG have relatively stable chemical composition, and optimizing the process after pilot-scale experiments should maintain product quality as obtained at the laboratory scale, and with the proper purification process (depending on the desired product) the quality would increase. Since stillage and BSG are available in large volumes and are effectively free, using them in LA production would lower the production cost and minimize waste treatment and disposal costs.

Conclusions

TS and BSG as cheap, abundant and renewable substrates for LA fermentation by *L. rhamnosus* ATCC 7469 were studied. The effect of TS addition on performance of LA fermentation was analysed in detail in regard to chemical composition of substrates, media composition during LA fermentation, fermentation mode and important fermentation parameters. TS addition significantly increased LA concentration (by 8.6–21.0%), yield (by 82.1–83.3%) and volumetric productivity (by 0.4–1.8%). TS and glucose addition significantly increased even more LA concentration (11.0–50.0%), yield (by 3.2–4.8%) and volumetric productivity (by 6.8–39.2%) while additional supplementation of media with glucose provided further improvement in LA fermentation parameters, resulting in maximal volumetric productivity of 0.93 g/L/h.

The increase in LA concentration in media correlated with the increase in TS supplementation, reflecting FAN content in fermentation media. In fed-batch fermentation the highest LA concentration, yield and volumetric productivity of 48.02 g/L, 87.8% and 0.96 g/L/h, respectively, were achieved. Overall, in fed-batch mode on the same substrate, significantly higher LA concentration, yield and volumetric productivity (by 54.8, 1.9 and 4.0%, respectively) were achieved.

The study has revealed that the combination of the two industrial waste streams, BSG hydrolysate and TS can make a suitable fermentation media for LA production in batch and fed-batch fermentation although additional supplementation with glucose has to be provided. According to the results obtained, fed-batch fermentation could be used to increase LA fermentation efficiency. TS as nitrogen source and BSG hydrolysate (as a combined source of minerals and carbon) could be attractive substrates for LA fermentation.

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