

Fed-batch L-(+)-lactic acid fermentation of brewer's spent grain hydrolysate

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Brewer's spent grain (BSG) hydrolysates were used for L-(+)-lactic acid (LA) fermentation by *Lactobacillus rhamnosus* ATCC 7469. The aim of this study was to evaluate fed-batch LA fermentation of BSG hydrolysate with the addition of glucose, glucose and yeast extract, and wort during LA fermentation and its effect on fermentation parameters such as LA concentration, its volumetric productivity and yield, and *L. rhamnosus* cell viability. The highest LA yield, volumetric productivity and concentration of 93.3%, 2.0 g/L/h, and 116.1 g/L, respectively, were achieved with glucose and yeast extract addition during fermentation. In fed-batch fermentation with glucose and yeast extract addition significantly higher LA concentration, yield and volumetric productivity (by 194.8; 2.2, and 20.7%, respectively) were achieved compared with batch fermentation. The results indicated that fed-batch fermentation could be used to increase LA fermentation efficiency. Copyright © 2017 The Institute of Brewing & Distilling

Keywords: lactic acid; fed-batch fermentation; brewer's spent grain; *L. rhamnosus*; wort

Introduction

The use of by-products in the food industry results in added value to industry and consumers; the industry benefits from an economic point of view since the disposal represents an additional cost to the producer, while the advantages to consumers include the decreased use of synthetic additives and the high content of valuable bioactive compounds (proteins, vitamins, pigments, antioxidants, antimicrobials, fragrances, etc.) (1). Considerable studies have investigated the conversion of various carbohydrates into high-value-added chemicals. Among these useful chemicals, lactic acid (LA) has gained much attention owing to its wide applications in the pharmaceutical, cosmetics and food industries (2). In nature, there are two optically active forms – L-(+)-, and D-(–)-LA – but since elevated levels of D-(–)-LA are harmful to humans, L-(+)-LA is the preferred isomer in the pharmaceutical and food industries as humans have only L-lactate dehydrogenase (3). Although LA can be produced either by chemical synthesis or by microbial fermentation, an optically pure LA can be manufactured only by fermentation (4). The production of LA biodegradable and biocompatible polymers is driving a current market expansion for LA while food-related applications account for ~85% of the demand for LA (5). Lactic acid producing bacteria (LAB) such as *Lactobacilli* need a large number of nutrients (e.g. amino acids, vitamins) for their growth and LA production (6). Yeast extract is used as a nutrient for bacterial culture media (7) since it contains high contents of nitrogen compounds, purine, pyrimidine bases and vitamins (8).

Beer production includes malting and brewing. Brewing consists of four main steps: (a) wort production that includes mashing and boiling; (b) fermentation; (c) maturation; and (d) filtration and/or stabilization (9). Mashing is the process of mixing malt, and cereal adjuncts if used, with hot water and letting the enzymes to degrade the proteins and starch to yield the soluble malt

extract, wort (10). After this, wort is separated from brewer's spent grain (BSG) and used in beer production.

BSG is a by-product from the brewing process and accounts for 85% of the total by-products generated (11). BSG obtained in all malt brewing is composed mainly of the material from barley husks, with 75% moisture, containing protein (~30% on a dry matter basis) and a high percentage of polysaccharides (~50% on a dry matter basis), namely arabinoxylans (22–23%), cellulose (12–25%), β -glucans (10%) and small amounts of starch (4%) (12). As BSG has high nutrition value, it is usually used as animal feed (11). Possible applications for BSG are in biotechnological, food, energy and chemical processes (13).

Fed-batch culture is a batch culture fed continuously or sequentially with substrate without the removal of fermentation broth, in order to reduce or prevent substrate-associated growth inhibition (14,15). This method may lead to a high cell density because substrate limitation or inhibition can be avoided by maintaining medium substrate concentration at a low level during cultivation (16).

In this study BSG hydrolysate was used for LA fermentation by *Lactobacillus rhamnosus* as a carbon and nitrogen source. The aim of this study was to evaluate fed-batch LA fermentation of BSG hydrolysate with addition of glucose, glucose and yeast extract, and wort and its effect on the fermentation parameters such

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as LA concentration produced, its volumetric productivity and yield, and *L. rhamnosus* cell viability.

Material and methods

Preparation of brewer's spent grain hydrolysate and wort for fermentation

Prior to the LA fermentation the BSG hydrolysis was optimized. Enzyme dosage was added according to the producer's recommendation but was later increased according to the results of our investigation. All commercial enzymes used in BSG hydrolysis (Termamyl SC, SAN Super 240 L, and Celluclast 1.5 L[®]) were kindly provided by Novozymes (A/S Bagsvaerd, Denmark; Table 1). Each enzyme (Termamyl SC[®], SAN Super 240 L[®], and Celluclast 1.5 L[®]) was investigated on its own. After this, a combination of these three enzymes was applied according to the best results obtained for each enzyme.

BSG obtained in a lager beer production was dried at 40°C for 12 h. Dried BSG was finely ground 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 obtained mash was adjusted to 5.5 with the addition of 10% H₃PO₄, prior to the hydrolysis. BSG hydrolysis was optimized in preliminary studies and further performed using the defined procedure. BSG hydrolysis was carried out as previously described (17) using an automated mashing water bath (Glasbläserei, Institut für Gärungs-Gewerbe, Berlin) by sequential addition of the following enzymes: 0.3 mL Termamyl SC (1 h at 90°C), 0.3 mL SAN Super 240 L (1 h at 55°C) and 5.0 mL Celluclast 1.5 L (10 h at 45°C) at 180 rpm. Prior to the addition of Celluclast 1.5 L, pH was adjusted to 5.0 with the addition of 10% H₃PO₄. After enzymatic hydrolysis, the obtained BSG hydrolysate was cooled to 20°C and centrifuged (4000 rpm, 20 min; centrifuge used was a BOECO model C-28A, Hamburg, Germany). The liquid hydrolysate was separated from solid hydrolysate and used in LA fermentations. The pH was adjusted to 6.5 with the addition of 1 M NaOH.

High-gravity wort was prepared by standard 'Congress' method (EBC) for wort production with the only difference being the malt quantity used (100 g instead of 50 g). In LA fermentations with addition of glucose, glucose and yeast extract (1%) and wort, the initial yeast extract concentration in BSG hydrolysate was set to 50 g/L (HiMedia Laboratories Ltd, Mumbai, India) prior to sterilization. The initial reducing sugar concentration in all LA fermentations in BSG hydrolysate was set to 54 g/L by addition of concentrated sterile glucose solution (700 g/L). The concentrated glucose solution was added to minimize the change in the BSG hydrolysate volume.

Microorganism

Lactobacillus rhamnosus ATCC 7469, a homofermentative LA strain, was obtained from American Type Culture Collection (ATCC, Rockville, USA). Stock culture of *L. rhamnosus* was stored and activated as previously described (17). Inoculum was prepared by taking 3 mL of the activated culture and transferring it to 60 mL of MRS broth (HiMedia Laboratories Ltd, Mumbai, India). To reach a high LAB cell number (1 × 10⁹ CFU/mL) the inoculum was statically incubated for 24 h at 37°C.

LA fermentation

All fermentations were performed with shaking (150 rpm, Biosan shaking bath model ES-20, Biosan Ltd., Latvia) in 300 mL Erlenmeyer flasks with 200 mL of BSG hydrolysate. The fermentation was initiated by the addition of inoculum (5% v/v, 1 × 10⁹ CFU/mL) and conducted at 37°C. The pH was maintained at 6.2 (defined as optimal during the preliminary studies) by the addition of a sterile 30% (w/v) NaOH solution in 4 h intervals. Correction of reducing sugar concentration in LA fermentations with the addition of glucose, glucose and yeast extract, or wort was done when the reducing sugar concentration decreased to ~30 g/L.

Analytical methods

All analyses were carried out in triplicate. Results were represented as the mean ± standard deviation. Reducing sugar concentration, calculated as glucose, was determined by 3,5-dinitrosalicylic acid method (18) using a UV-vis spectrometer (UV-1800, Shimadzu, Kyoto, Japan). A calibration curve was made at 570 nm using standard glucose solutions. LA concentration was determined by enzymatic method [L-(+)-LA assay, Megazyme, Wicklow, Ireland]. Prior to the LA determination, proteins were removed from samples by precipitation according to the procedure prescribed in LA assay (Megazyme, Wicklow, Ireland). BSG hydrolysate and wort compositions were monitored during fermentation and the following methods were used for the analysis: dry matter content was determined by a standard drying method in an oven at 105°C to constant mass (19); total nitrogen was determined by Kjeldahl method (20); and ash content was determined by slow combustion method at 650°C for 2 h (19). Free amino nitrogen concentration in BSG hydrolysate and wort was determined by ninhydrin method (21). 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. All chemicals

Table 1. Commercial enzymes used in brewer's spent grain (BSG) hydrolysis

Enzyme	Type	Supplier	Temperature range	pH optimum
Termamyl SC [®]	α -Amylase, 3.2.1.1	Novozymes, Bagsvaerd, Denmark	A/S 80–90°C	5.0–6.0
SAN 240 L [®]	Super Mainly amyloglucosidase, 3.2.1.3, and α -amylases, EC 3.2.1.1	Novozymes, Bagsvaerd, Denmark	A/S 55–65°C	4.5–5.5
Celluclast 1.5 L [®]	Cellulase complex, 3.2.1.4	Novozymes, Bagsvaerd, Denmark	A/S 40–50°C	4.0–6.0

used in experiments were of analytical grade and obtained from Merck KGaA, Darmstadt, Germany.

Statistical analysis

All of the experiments were performed in triplicate. All values are expressed as the mean \pm standard deviation. The mean values of LA concentration, yield and 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, New York, USA). Differences were considered significant at $p < 0.05$.

Results and discussion

Analysis of BSG hydrolysate and wort

The chemical composition of BSG hydrolysate and high-gravity wort is presented in Table 2. BSG hydrolysate had a higher ash content than wort. However, the wort had significantly higher dry matter (5.2-fold), total nitrogen (1.7-fold), free amino nitrogen content (9.6-fold) and reducing sugar concentration (7.3-fold) than BSG hydrolysate.

In our previous research (22) the effects of initial reducing sugar and yeast extract concentration in BSG hydrolysate on LA fermentation parameters were investigated. The highest LA yield and volumetric productivity were achieved in pH controlled fermentation of BSG hydrolysate with an initial reducing sugar concentration of 54 g/L and yeast extract concentration of 50 g/L. Owing to these results, in this study initial reducing sugars and yeast extract concentration in BSG hydrolysate were set to 54 and 50 g/L, respectively.

The LA and reducing sugar concentration, *L. rhamnosus* cell viability and pH values in batch LA fermentation of BSG hydrolysate are presented in Fig. 1. The highest LA concentration was achieved after 36 h of fermentation and was 39.4 g/L (Fig. 1a). Mussatto *et al.* (23) obtained a similar LA concentration (35.5 g/L) in fermentation of BSG hydrolysate with initial reducing sugar concentration of ~50 g/L, however with the addition of MRS broth nutrients (containing 5 g/L of yeast extract, without glucose), and also with pH control. In LA fermentation of BSG hydrolysate (containing 50 g/L of glucose and 12.5 g/L of yeast extract), Liguori *et al.* (24) obtained significantly lower LA concentration (22.2 g/L) in comparison with the results achieved in our previous study (22) for batch fermentation of BSG hydrolysate (containing 54 g/L of glucose) with 10 g/L of yeast extract (35.6 g/L was achieved LA concentration) and also in this

study in batch fermentation of BSG hydrolysate (with 54 g/L of reducing sugar and 50 g/L of yeast extract). During 36 h of fermentation 43.1 g of reducing sugar was utilized (Fig. 1b). Liguori *et al.* (24) achieved similar reducing sugar utilization (37.4 g) in fermentation of BSG hydrolysate with an initial reducing sugar concentration of 50 g/L and yeast extract addition (12.5 g/L). *L. rhamnosus* cell viability increased until the end of fermentation (by 19.7%) and was 9.7 log CFU/mL after 36 h of fermentation. The highest increase in cell viability of LAB was observed in the first 12 h of fermentation (by 13.1%; Fig. 1c). The decrease in pH became faster after the first 4 h of fermentation until 24 h (Fig. 1d).

The LA and reducing sugar concentration, *L. rhamnosus* cell viability and pH values obtained in fed-batch LA fermentations of BSG hydrolysate with addition of glucose, glucose and yeast extract, and wort during fermentation are presented in Figs 2–4.

In fed-batch fermentation with addition of glucose during fermentation higher LA concentration was achieved compared with the batch fermentation without glucose addition (2.75-fold; Fig. 2a). *L. rhamnosus* cell viability after 60 h of fermentation (9.7 log CFU/mL) was the same as the viability obtained in batch fermentation (Fig. 2c). Since viability was the same, glucose addition increased the ability of lactic acid bacteria to synthesize LA to a much greater extent (108.1 g/L). According to the extent of reducing sugar utilization and pH value decrease, LA fermentation was very fast (Fig. 2b and d). The highest reducing sugar consumption was achieved between 12 and 24 h of fermentation and was 25.6 g (Fig. 2b).

In fed-batch fermentation with glucose and yeast extract addition during fermentation much higher LA concentration was achieved compared with the batch fermentation without glucose addition and fed-batch with only glucose addition (by 3.0-fold and 7.4%, respectively) (Fig. 3a). Also higher *L. rhamnosus* cell viability was achieved with yeast extract addition (after 60 h of fermentation 9.9 log CFU/mL; Fig. 3c). The highest reducing sugar consumption was achieved between 12 and 24 h fermentation and was 29.2 g (Fig. 3b). Reducing sugar consumption was higher than in fed-batch fermentation with glucose addition. Yeast extract is considered to be an essential nutrient for *Lactobacillus* strain for efficient LA production (25). Higher LA concentration and *L. rhamnosus* cells viability as well as reducing sugar consumption could be attributed to addition of yeast extract since it contains essential amino acids, vitamins, minerals, proteins and other nutritive compounds necessary for LAB cell metabolism (26).

Since the addition of yeast extract is not economically feasible, the addition of high-gravity wort during fermentation as a carbon and nitrogen source was studied. All malt wort is a rich source of simple sugars, dextrans, β -glucans, pentosans, phosphates, dissolved inorganic ions, proteins, peptides and amino acids, nucleic acid breakdown products, lipids, vitamins, organic acids, bases and phenolic substances (27). In fed-batch fermentation with wort addition during fermentation, much higher LA concentration (2.6-fold) was achieved compared with the batch fermentation without glucose addition (Fig. 4a). High cell viability of *L. rhamnosus* was achieved in this fermentation (after 60 h of fermentation 10 log CFU/mL), similarly to in fed-batch fermentation with glucose and yeast extract addition (Fig. 4c). The highest reducing sugar consumption was achieved between 12 and 24 h of fermentation and was 23.3 g (Fig. 4b).

LA yield and volumetric productivity in batch and fed-batch LA fermentations of BSG hydrolysate are presented in Table 3. After 60 h of fermentation the highest LA yield (91.7–93.3%) and

Table 2. Brewer's spent grain (BSG) hydrolysate and wort chemical composition^a

Parameter	Wort	BSG hydrolysate
Dry matter, %	25.22 \pm 0.04	4.81 \pm 0.03
Reducing sugars, g/L	186.17 \pm 0.18	25.34 \pm 0.18
Total nitrogen, g/L	1.37 \pm 0.01	0.80 \pm 0.01
Free amino nitrogen, mg/L	531.58 \pm 2.70	54.98 \pm 0.13
Ash, % dry matter	0.38 \pm 0.01	1.99 \pm 0.01

^aValues represent means \pm standard deviation calculated from three determinations.

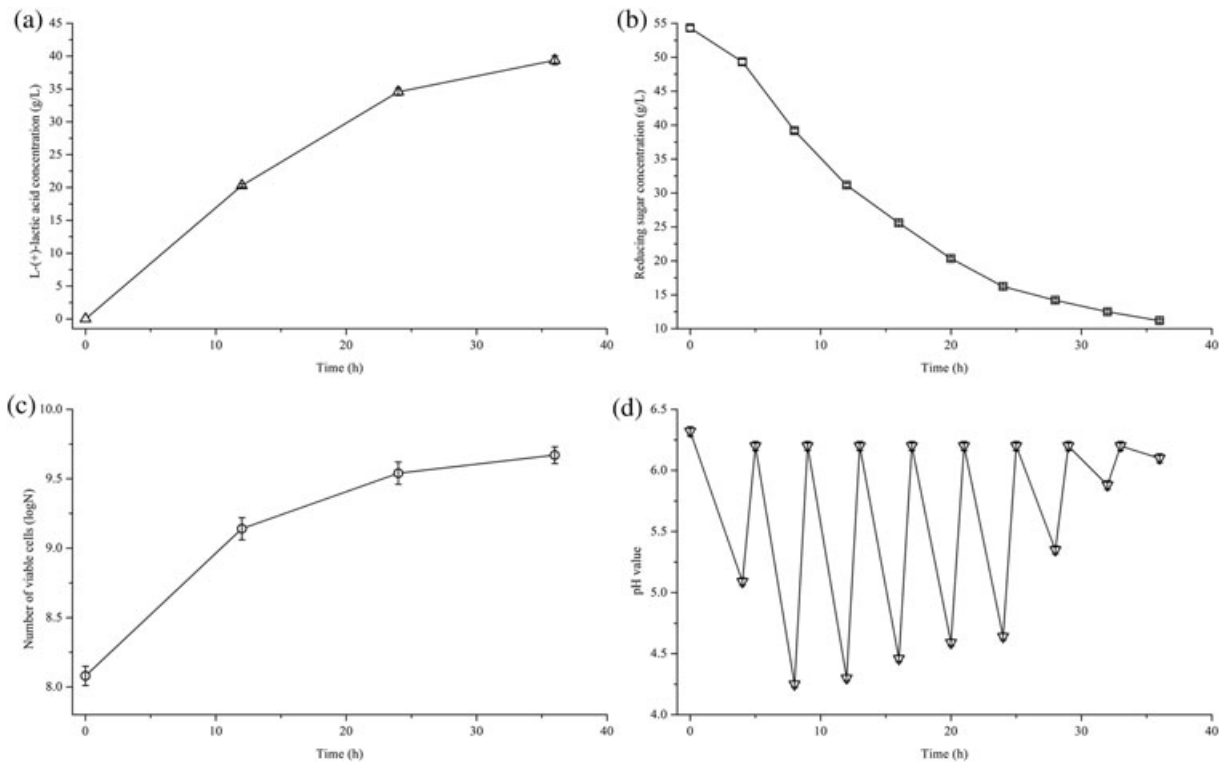


Figure 1. Batch lactic acid (LA) fermentation of brewer's spent grain (BSG) hydrolysate. (a) LA concentration; (b) reducing sugar concentration; (c) *Lactobacillus rhamnosus* cell viability; and (d) pH. Symbols: (Δ) LA concentration; (\square) reducing sugar concentration; (\circ) number of viable cells; (\blacktriangledown) pH

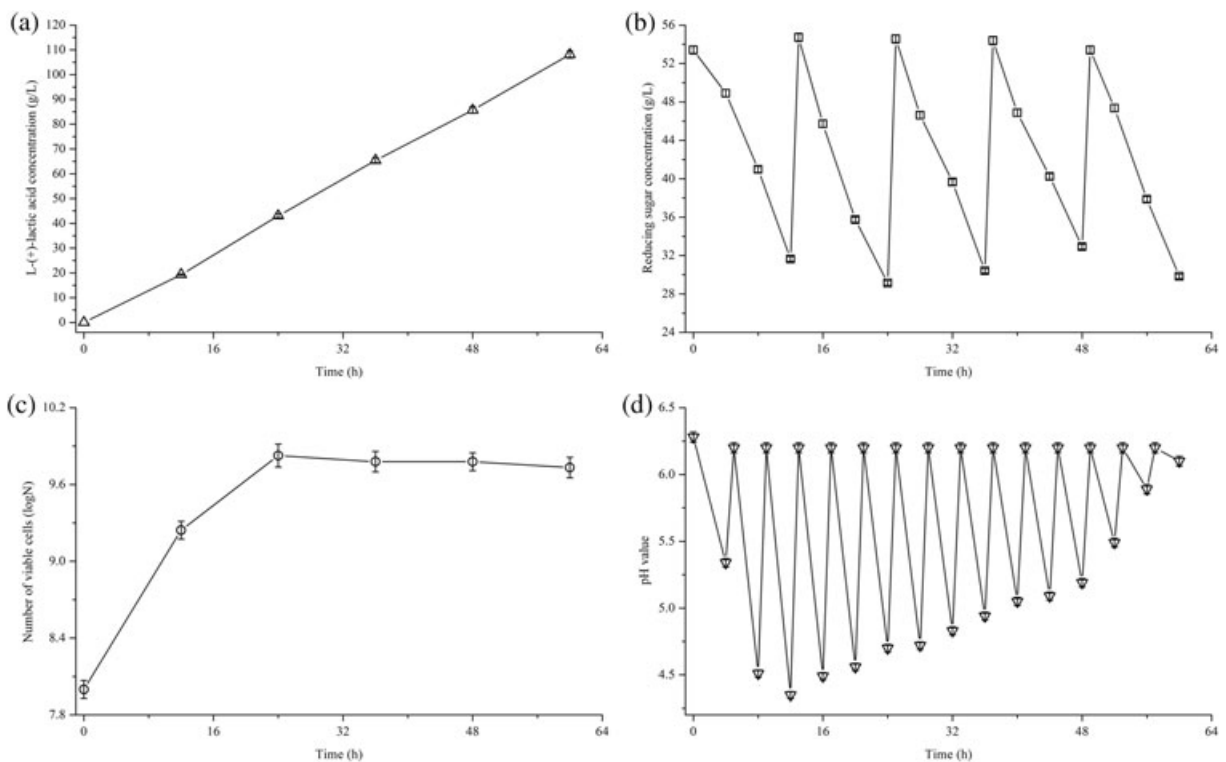


Figure 2. Fed-batch LA fermentation of BSG hydrolysate with glucose addition during fermentation. (a) LA concentration; (b) reducing sugar concentration; (c) *L. rhamnosus* cell viability; (d) pH. Symbols: (Δ) LA concentration; (\square) reducing sugar concentration; (\circ) number of viable cells; (\blacktriangledown) pH

concentration (102.2–116.1 g/L) in all fed-batch fermentations were achieved. The highest volumetric productivity in all fed-batch fermentations was achieved after 36 h of fermentation (1.74–2.04

g/L/h). The highest LA yield and volumetric productivity of 93.3% and 2.04 g/L/h, respectively, were achieved in fermentation with glucose and yeast extract addition during fermentation. Compared

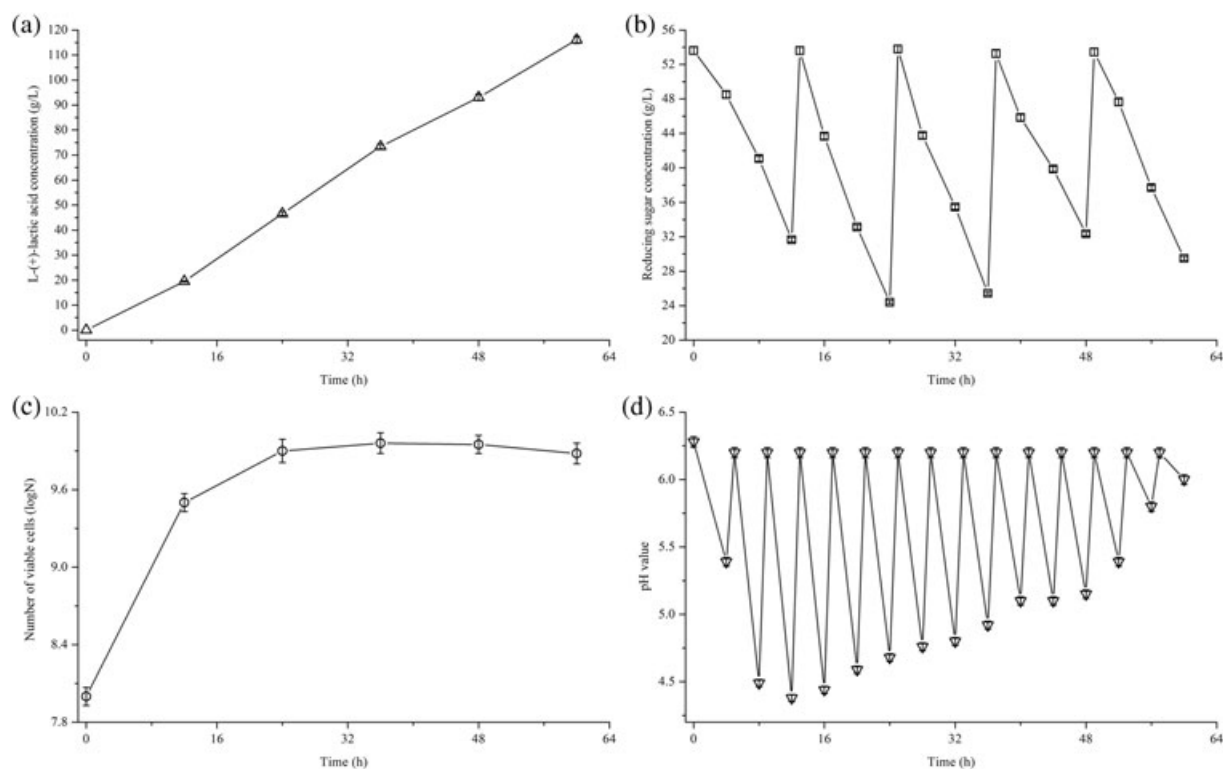


Figure 3. Fed-batch LA fermentation of BSG hydrolysate with glucose and yeast extract addition during fermentation. (a) LA concentration; (b) reducing sugar concentration; (c) *L. rhamnosus* cell viability; (d) pH. (Δ) LA concentration; (□) reducing sugar concentration; (○) number of viable cells; (▼) pH

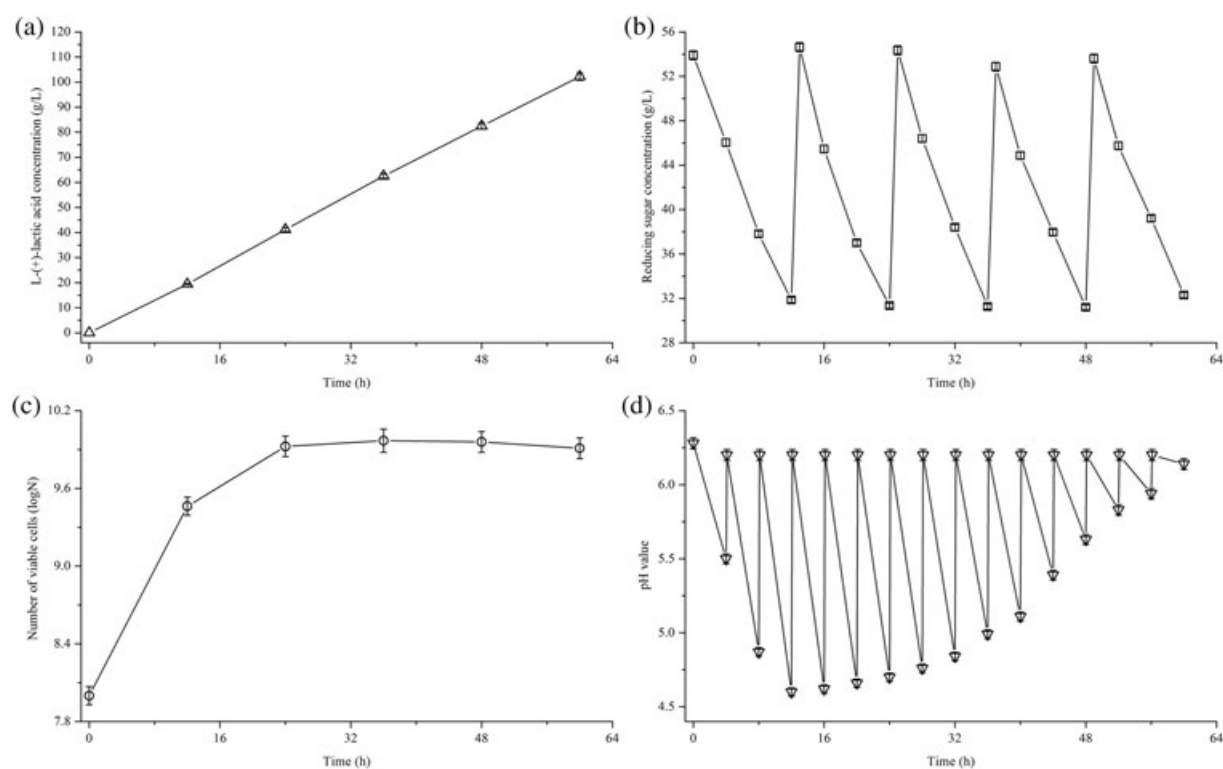


Figure 4. Fed-batch LA fermentation of BSG hydrolysate with wort addition during fermentation. (a) LA concentration; (b) reducing sugar concentration; (c) *L. rhamnosus* cell viability; (d) pH. Symbols: (Δ) LA concentration; (□) reducing sugar concentration; (○) Number of viable cells; (▼) pH

with the results obtained in the batch fermentation a significantly higher LA yield (by 0.3 and 1.6%) and volumetric productivity (by 2.9 and 7.5%) were achieved in fed-batch fermentation with wort

and glucose addition. Glucose and yeast extract addition during fermentation increased LA yield and volumetric productivity even more (by 2.2 and 20.7%, respectively).

Table 3. Lactic acid (LA) yield and volumetric productivity in batch and fed-batch L-(+)-LA fermentations of BSG hydrolysate (with 54 g/L of reducing sugars and 50 g/L of yeast extract) with glucose, glucose and yeast extract, and wort addition during fermentation^a

BSG hydrolysate	L-(+)-LA yield (%) ^b	Volumetric productivity (g/L/h) ^c
Batch fermentation	91.3 ± 1.6 ^a	1.69 ± 0.02 ^a
Fed-batch fermentation, glucose addition	92.8 ± 1.1 ^b	1.82 ± 0.03 ^c
Fed-batch fermentation, glucose and yeast extract addition	93.3 ± 1.1 ^b	2.04 ± 0.03 ^d
Fed-batch, wort addition	91.7 ± 1.0 ^a	1.74 ± 0.02 ^b

^aValues represent means ± standard deviation calculated from three parallel experiments. Means written with different small letter in a column are significantly different ($p < 0.05$).

^bL-(+)-LA yield was calculated at 36 h of the fermentation for batch fermentation and at 60 h of fermentation for fed-batch fermentations.

^cVolumetric productivity was calculated at 12 h of the fermentation for batch fermentation and at 36 h of fermentation for fed-batch fermentations.

Fed-batch processes for high LA production are mostly studied on synthetic and chemically defined media with nitrogen and mineral supplements (16,28,29). Zhang *et al.* (28) investigated possible application of fed-batch fermentation (glucose addition during fermentation) in LA fermentation by *Lactobacillus lactis*-11 on synthetic media with sugar concentration of 38 g/L. LA concentration and volumetric productivity of 96.3 g/L and 1.9 g/L/h were achieved, which were slightly lower compared with the concentration and volumetric productivity achieved in this study in fermentation with glucose and glucose and yeast extract addition. Bai *et al.* (29) investigated the possible application of fed-batch fermentations [glucose solution (1000 g/L) addition during fermentation] for hyper-production of LA by *L. lactis* BME5-18 M in synthetic media with a sugar concentration of 50 g/L. The highest LA volumetric productivity of 2.2 g/L/h was achieved, which was similar to the volumetric productivity achieved in this study in fermentations with glucose and yeast extract addition during fermentation.

Bai *et al.* (16) also investigated possible application of fed-batch fermentations [glucose solution (600 g/L) addition during fermentation] for production of LA by *L. lactis* BME5-18 M in synthetic media. The highest LA volumetric productivity of 1.34 g/L/h was achieved on 50 g/L of glucose, which was lower than the volumetric productivity achieved in this study in fermentation with glucose and yeast extract addition during fermentation. It must be noted that in this study no additional nutrients and/or minerals, except yeast extract, were added to BSG hydrolysate before LA fermentations.

Conclusions

LA fermentation on renewable substrates is a very promising process. Since the cost of fermentation media has a significant impact on economic feasibility of production processes, there is a demand for alternative low cost and abundant substrates. Also implementation of these renewable substrates in the fermentation process removes the environmental impact of uncontrolled deterioration and problems regarding disposal and waste management. BSG was studied as low-cost, abundant and renewable substrate for batch and fed-batch LA fermentation by *L. rhamnosus* ATCC 7469.

Fed-batch LA fermentation of BSG hydrolysate enhanced LA fermentation compared with the batch LA fermentation. In all fed-batch fermentations of BSG hydrolysate higher LA concentration (by 159.4–197.8%), yield (by 0.3–2.2%) and volumetric productivity

were achieved (by 2.9–20.7%) compared with the batch fermentation without glucose addition. After 60 h of fermentation the highest LA yield (91.7–93.3%) and concentration (102.2–116.1 g/L) in all fed-batch fermentations were achieved. The highest volumetric productivity in all fed-batch fermentations was achieved after 36 h of fermentation (1.74–2.04 g/L/h). The highest LA yield and volumetric productivity of 93.3% and 2.04 g/L/h, respectively, were achieved in fermentation with glucose and yeast extract addition during fermentation. In fed-batch fermentation with glucose and yeast extract addition significantly higher LA concentration, yield and volumetric productivity (by 194.8, 2.2, and 20.7%, respectively) were achieved compared with the results obtained in batch fermentation.

The LA concentration, yield and volumetric productivity were comparable to or higher than the results obtained on synthetic and chemically defined media with nitrogen and mineral supplements. The results are in accordance with relevant, up to date research and indicate that fed-batch fermentation could be used to increase LA fermentation efficiency. The study has revealed that BSG hydrolysate obtained using mild and ecofriendly enzyme hydrolysis, as well as with moderate carbon and nitrogen supplementation, is a suitable fermentation media for the growth of *L. rhamnosus* and LA production in batch and fed-batch fermentation.

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