Brewing and malting technology by-products as raw materials in L-(+)-lactic acid fermentation

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ABSTRACT

BACKGROUND: Brewer's spent grain (BSG), brewer's yeast (BY), malt rootlets (MR), and soy lecithin (SL) are valuable and abundant by-products obtained from brewing, malting, and oil industry, respectively. L-(+)-lactic acid (LA) is organic acid that has wide range of application. In this study hydrolysate obtained from BSG and MR mixture (BSGMR), was used in combination with SL and BY extract (BYE) in LA fermentation. The aim was to evaluate the effect and optimize the addition of SL (0.1 and 0.2%) or BYE (10 and 20%) compared to Tween 80 (0.1%) and yeast extract (0.8%), in batch and fed-batch LA fermentation.

RESULTS: Using optimal SL and BYE concentrations (0.19 and 19.12%, respectively) obtained by response surface methodology (RSM) highest LA concentration, yield, and volumetric productivity

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of 28.43 g/L, 93.03%, and 1.04 g/L h^{-1}, respectively, were achieved in batch fermentation, with further increase in fed-batch fermentation (70.17 g/L, 94.57%, and 1.22 g/L h^{-1}, respectively).

CONCLUSION: Achieved results indicated that the combination of industrial by-products, BSG, MR, SL, and BYE can be used as very suitable fermentation media for LA production, and that SL and BYE could be used as cheap replacements for Tween 80 and YE without a decrease in fermentation efficiency.

Keywords: biotechnology; fermentation; waste treatment and waste minimisation; experimental design

INTRODUCTION

Biomass usage and valorization represent interesting research topic today because of increasing demands in fields of chemical and food production. Brewer's spent grain (BSG) is a underutilized lignocellulosic material which constitutes around 85% of all solid by-products generated in the brewing industry.[1] It is mainly containing cellulose, non-cellulosic carbohydrates, proteins and lignin. BSG represents up to 30% (w/w) of the starting malted grain, which makes this a readily available, high volume and low cost by-product within the brewing industry, and a potentially valuable resource for industrial exploitation.[2] The second major by-product of brewing industry that represents 1.5-2.5% of total beer produced is brewer's yeast.[3] The brewer's yeast is an inexpensive nitrogen source and generally recognized as safe (GRAS) that is primarily sold as inexpensive feed.[4] Brewer's yeast (BY) is composed of proteins (including all essential amino acids), carbohydrates, minerals (Ca, P, K, Mg, Fe, among others), B vitamins, lipids, and enzymes

including a biologically active form of chromium known as glucose tolerance factor.[3,5] Tween-80 (polyethylene glycol sorbitan monooleate) is one of the most important non-ionic surfactants. It has been reported that Tween-80 is capable of enhancing the production of lactic acid.[6] Soy lecithin (SL) is a natural mixture of phosphatidylcholine (90%) with other phospholipids and fatty acids and is used as surfactant (emulsifier) in food industry.[7,8] Malt rootlets (MR), cheap and abundant by product of malting industry, are rich in protein, essential amino acids, carbohydrates (cellulose and non-cellulosic), polyphenols, fatty acids, and minerals.[9]

Lactic acid (LA) is used in food, cosmetic, pharmaceutical, and chemical industries. Additionally, LA is used in producing of biodegradable, green, and biocompatible polylactic acid (PLA) polymers, that are widely used as raw materials in packaging (fibers and foams).[10] It is a chiral molecule, which exists as enantiomers L-(+) and D-(-)-LA, and this important organic acid is produced by microbial fermentation and chemical synthesis.[11] Optically pure lactic acid can be acquired through fermentation depending on the strain of lactic acid bacteria (LAB) used, while the chemical synthesis always results in a racemic mixture of lactic acid.[12] Many industries require a high enantiomeric purity of L-(+)-LA for specific applications, especially in the medical, pharmaceutical and food industries, since the D-(-)-LA is considered harmful to humans and can cause decalcification or acidosis.[13] The efficiency and economics of LA fermentation is still a problem from many points of view, and the substrate plays a vital role in the improvement of such a process. 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 due to their abundance.[14] The annual production of 200×10^9 tons of lignocellulosic biomass supports their potential as sustainable platform for successful transition from fossil-based resources to biofuels and bio-based products.[15] Usage of these renewable resources provides

additional benefit regarding that no carbon dioxide is produced as a by-product of fermentation in contrast to oil- and fossil-fuel-based sources.[12]

In this study hydrolysate obtained from BSG and MR mixture was used in L-(+)-LA fermentation by *Lactobacillus rhamnosus* ATCC 7469. The aim of this study was to evaluate the effect of brewer`s yeast extract (BYE) (as cheap substitution for yeast extract (YE)) and SL (as cheap substitute for Tween 80) addition in BSG and MR (BSGMR) hydrolysate on L-(+)-LA fermentation parameters (L-(+)-LA concentration, its yield and productivity, and *L. rhamnosus* cell viability) in batch and fed-batch fermentation. SL and BYE were tested as independent variables using Response surface methodology (RSM). The role of each variable, their interactions and statistical analysis was explained by applying second-degree polynomial model. The data was analyzed statistically and response surface plots were constructed in order to optimize SL and BYE concentration in BSGMR hydrolysate. Optimal SL and BYE concentrations were validated and used in fed-batch fermentation to further increase efficiency of LA fermentation.

MATERIALS AND METHODS

Fermentation media preparation

BSG obtained in a lager beer production was dried at 40ºC for 12 h. MR we obtained dry from malting plant. Dried BSG and MR were finely ground in a laboratory DLFU mill from Bühler-Miag (Braunschweig, Germany). Prior to the LA fermentation mixture of BSG and MR was hydrolysis using the combination of commercial enzyme complexes (Termamyl SC® (±-amylase, EC 3.2.1.1), SAN Super 240L® (mainly amyloglucosidase, EC 3.2.1.3, and ±-amylases, EC 3.2.1.1), and Cellic® CTec2 (cellulase complex, EC 3.2.1.4). For hydrolysate production 40 g of dry BSG and 10 g MR were mixed with 300 mL of distilled water and pH value of the obtained mash was adjusted to 5.5

with the addition of 10% H_3PO_4, prior to the hydrolysis. BSGMR hydrolysis was performed as previously described Pejin et al.[16] using automated mashing water bath (Glasbläserei, Institut für Gärungs Gewerbe, Berlin, Germany). All commercial enzymes used in BSG hydrolysis were kindly provided by Novozymes (A/S Bagsvaerd, Denmark). After enzymatic hydrolysis obtained BSGMR hydrolysate was cooled to 20°C and centrifuged (4000 rpm, 20 min, centrifuge: BOECO model C-28A, Hamburg, Germany). Liquid hydrolysate was separated from solid hydrolysate and used in LA fermentations. Its pH was adjusted to 6.5 with the addition of 1M NaOH. After this, liquid hydrolysate was sterilized at 121°C for 15 min and used as a fermentation medium. Reducing sugar concentration in hydrolysate was 3.5%.

Brewer's yeast extract (BYE) was produced from dried brewer`s yeast (AD BIP-Belgrade Beer Industry, Serbia). For production of BYE method using glass beads previously described by Vieira et al.[17] was used. After cell disintegration obtained mixture was centrifuged (4000 rpm, 15 min) and the supernatant was concentrated at 50°C (approximately 3 h). The obtained BYE was sterilized and used in LA fermentations. Sterilized BYE was added in BSGMR hydrolysate (10 and 20% v/v) prior to the inoculation. SL used in fermentation was commercial (SOJAPROTEIN, Serbia) and was added in BSGMR hydrolysate (0.1 and 0.2 % m/m) before sterilization. Tween 80 used in fermentation was commercial (Sigma Aldrich, Poland) and was added in BSGMR hydrolysate (0.1 % m/m) before sterilization. Yeast extract (HiMedia Laboratories, India) was added in concentration of 0.8% (m/m) (with was increased from standard 0.5% m/m, in MRS broth since sugar concentration in BSG hydrolysate was 3.5 instead of 2.0% in MRS broth) before sterilization. All chemicals used in experiments were of analytical and microbiological grade.

Microorganism

Lactobacillus rhamnosus ATCC 7469, a homofermentative LA strain, was obtained from American Type Culture Collection (ATCC, Rockville, USA). Stock cultures of *L. rhamnosus* was stored and activated as previously described by Pejin et al.[18]. 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 the inoculum was incubated for 24 h at 37°C.

LA fermentation

All LA fermentations were performed with shaking (150 rpm, Biosan shaking bath model ES-20, Biosan Ltd., Latvia). The fermentations were performed in 300 mL rlenm0yer flasks with 200 mL of BSGMR hydrolysate. The fermentation was initiated by the addition of inoculum (5% v/v) and conducted at 37°C. The pH was maintained at 6.20 by the addition of a sterile 30% (w/v) NaOH solution in 4 h intervals.[19] Correction of reducing sugar concentration to the initial value (3.5%) in fed-batch LA fermentations with the addition of glucose and BYE during the fermentation was done every 24 h.

Analytical methods

Reducing sugar concentration, calculated as glucose, was determined by 3.5-dinitrosalicylic acid method[20] using UV/VIS Spectrometer (UV-1800, Shimadzu, Kyoto, Japan). A calibration curve was set at 570 nm using standard glucose solutions. L-(+)-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 L-(+)-LA assay (Megazyme, Wicklow, Ireland). BSGMR hydrolysate, and BYE were monitored for the following quality parameters 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[21]; protein content was determined by Kjeldahl method as the total nitrogen and multiplied by factor[22]; and ash content was determined by slow combustion method at 650 °C for 2 h.[21] Free amino nitrogen (FAN) concentration in BSGMR hydrolysate and BYE was determined by ninhydrin method.[23] A number of viable *L. rhamnosus* cell was determined using a pour-plating method. Microaerophilic conditions were maintained during incubation in Petri plates using 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 used in experiments were of analytical and microbiological grade.

Statistical analysis

The experiments were done in triplicates. All values are expressed as means ± standard deviation. Pearson's correlation coefficient was determined for correlation of free amino nitrogen and produced LA concentration for $p<0.01$. Mean values of LA concentration, yield, and volumetric productivity, and *L. rhamnosus* cell viability 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, U.S.). Differences were considered significant at $p<0.05$. A 3^2 full factorial experimental design (conducted in random order) was applied for the estimation of the regression parameters to fit a second-degree polynomial regression model for a given responses. A polynomial, as given by Eq. (1), quantifies relationships among the estimated response y and a number of independent variables X_i (SL and BYE concentration), ² are regressors associated with the model:

$$y = \beta_0 + \sum_{i=1}^{a} \beta_i X_i + \sum_{i=1}^{a} \sum_{j=1}^{a} \beta_{ij} X_i X_j + \sum_{i=1}^{a} \beta_{ii} X_i^2 \qquad (1)$$

The regressors $^{2}_{0}$, $^{2}_{i}$, $^{2}_{ii}$ and $^{2}_{ij}$ are the model constant, linear coefficient, quadratic and cross-product coefficients, provide a quantitative measure of the significance of linear effects, quadratic of factors and interactions between factors. In the experimental design, low and high factors were coded as −1 and +1; the midpoint was coded as 0. The factors and their coding's are found in Table 1. Four additional assays at the midpoint of the design (0.1% SL, and 10% BYE) were carried out to estimate the error involved in the experiment and also to know if there was any curvature in the responses.

The regression analysis and analysis of variance (ANOVA) with Response surface models were carried, and the proportion of variance explained by the model were given by the multiple correlation coefficient R^2.Values of $p < 0.05$ were considered as statistically significant. All RSM (statistical and graphical) analyses were carried out using the The Design-Expert version 7.0 and SPSS statistica 20.

RESULTS AND DISCUSSION

Analysis of BSGMR hydrolysate and BYE, and FAN concentration in fermentation media

Chemical composition of BSGME hydrolysate BYE chemical composition is presented in Table 2. BSGMR hydrolysate had higher reducing sugar concentration than BYE. However, BYE had significantly higher dry matter (by 1.9 fold), total nitrogen (by 4 fold), free amino nitrogen (by 21 fold), and ash concentration than BSGMR hydrolysate (by 2.3 fold).

Based on chemical composition BYE could be used as a renewable nitrogen source (especially free amino nitrogen (FAN)) and possible source of other nutrients such as vitamin B complex and minerals, while BSG is a better carbon source. Besides carbon source, for efficient LA fermentation nitrogen compounds are also required, preferably in forms that are easily assimilated (amino acids

and FAN).[24] Abundant and inexpensive renewable raw materials rich in FAN can be used as nitrogen sources in cultivation of microalgae and bacteria.[25] FAN concentration in BSGMR hydrolysate was 88.18±0.32 mg/L. Addtion of Tween 80 and SL did not affect FAN concentration, and it was 88.54±1.94 mg/L, as it was expected. YE addition significantly increased FAN concentration (by 4.5 fold) and was 397.94±3.56 mg/L. FAN concentration also significantly increased by addition of BYE (by 3.2 fold at 10% BYE (285.28±2.51 mg/L) to 4.7 fold at 20% BYE (412.25±4.72 mg/L). With the addition of 20% BYE higher concentration of FAN (by 4%) was achieved in comparison with the addition of YE.

Effect of SL and BYE addition on batch LA fermentation using RSM

The effect of the addition of various SL (0, 0.1, and 0.2%) and BYE (0, 10, and 20%) concentrations on LA fermentation parameters (LA concentration, its yield and productivity, and *L. rhamnosus* cell viability) was assessed using 3^2 full factorial experimental design (total of 13 tests). Also for comparison with commercial supplements LA fermentations with the addition of Tween 80 or/and yeast extract were conducted. The dependent variables (responses) analyzed were L-(+)-LA concentration, yield, volumetric productivity, and *L. rhamnosus* cell viability.

Kinetic of lactic acid fermentation, based on LA concentration, reducing sugar consumption and *L. rhamnosus* cell viability, is presented in Figure 1. LA concentration in fermentation of BSGMR hydrolysate with Tween 80, YE, SL and BYE addition is given in Figure 1a. LA concentration increased significantly with the addition of 0.1% Tween 80 (by 6.44%) and SL (by 6.46 at 0.1% SL to 12.82% at 0.2% SL) compared to the fermentation without supplement addition. Also there was no significant difference between LA concentration obtained in fermentations with the addition of Tween 80 (0.1%) and SL (0.1%). Addition of 0.2% SL significantly increased LA concentration by

5.99% compared to the fermentation with the addition of 0.1% Tween 80. LA concentration increased significantly with the addition of 0.8% YE (by 46.45%) or BYE (by 44.58% at 10% BYE to 50.60% at 20% BYE) compared to the fermentation without supplement addition. The addition of 20% BYE also significantly increased LA concentration by 2.83% compared to the fermentation with the addition of 0.8% YE. With the addition of Tween 80 and YE, and SL and BYE, LA concentration increased even further in comparison to the fermentation without supplements addition. The addition of Tween 80 and YE increased LA concentration by 54.69%, while the addition of SL and BYE increased LA concentration by 54.21% (0.1% SL and 10% BYE) to 68.86% (0.2% SL and 20% BYE). The addition of SL and BYE also significantly increased LA concentration by 4.36-9.80% compared to the fermentation with the addition of Tween 80 and YE. Fermentations with Tween 80 or SL addition lasted 24 h, while fermentations with the addition of YE and BYE and combination of Tween 80 and YE, or SL and BYE lasted 36 h. Comparing the results regarding L-(+)-LA concentration, yield, and volumetric productivity there was no significant difference between Tween 80 (0.1%) and SL (0.1%).

The obtained LA concentration was correlated with corresponding FAN concentration in BSGMR hydrolysate without and with supplements addition. A strong positive correlation between FAN and LA concentration (correlation coefficient value of 0.916) was determined. YE and BYE addition as well as Tween 80 and SL addition increased LAB metabolic activity due to the FAN from YE and BYE, and surfactant activity (that facilitate and increase uptake of nutrients) of Tween 80 and SL. The highest LA concentration (28.23 g/L) was obtained with the addition of 0.2% SL and 20% BYE.

Reducing sugar concentration in LA fermentation of BSGMR hydrolysate without and with Tween 80, YE, SL, and BYE addition is given in Figure 1b. Reducing sugar utilization increased

significantly with the addition of 0.1% Tween 80 (by 4.43%) and SL (by 4.80 at 0.1% SL to 10.33% at 0.2% SL) compared to the fermentation without the addition. With the addition of 0.2% SL reducing sugar utilization significantly increased by 5.65% in comparison with the fermentation with addition of Tween 80. Reducing sugar utilization increased significantly with the addition of 0.8% YE (by 37.63%) or BYE (by 36.53% at 10% BYE to 38.01% at 20% BYE) compared to the fermentation without supplements addition. With the addition of Tween 80 and YE, and SL and BYE, reducing sugar utilization increased even further in comparison to the fermentation without supplements addition. The addition of Tween 80 and YE increased LA concentration by 42.80%, while the addition of SL and BYE increased LA concentration by 41.70% (0.1% SL and 10% BYE) to 52.76% (0.2% SL and 20% BYE). SL and BYE addition significantly increased reducing sugar utilization by 2.07-6.98% compared to the fermentation with the addition of Tween 80 and YE.

L. rhamnosus cell viability in batch LA fermentation of BSGMR hydrolysate without and with Tween 80, YE, SL and BYE addition is given in Figure 1c. *L. rhamnosus* cell viability was high in all fermentations (9.3-9.7 log CFU/mL). Significant increase in *L. rhamnosus* cell viability was observed in all fermentations with supplement addition in range from 1.2% (0.1% Tween 80) to 4.62% (0.2% SL and 20% BYE) compared to fermentation without supplements addition. SL (0.1 and 0.2%) and BYE (20%) addition also increased *L. rhamnosus* cell viability by 1.1-1.3% compared to the fermentation with Tween 80 and YE addition.

Higher LA concentration and *L. rhamnosus* cell viability as well as reducing sugar consumption could be explained by SL and BYE addition in BSGMR hydrolysate which reduced surface tension around cell and fermentation media (increasing nutrient uptake) and increased FAN concentration (as well of amino acids and other micronutrients and B vitamins) which are necessary for LAB cell metabolism and improvement of LA fermentation.[26,27]

Data obtained in batch fermentations (highest values of LA concentration, yield and productivity and *L. rhamnosus* cell viability) were fitted into a second order polynomial equation and three-dimensional response plots were generated for every response to understand the interactions between different variables as well as to determine the optimal level of each factor for maximum response (Fig. 1). This gave further insights on the interactions between the two factors tested. The contour plots were indicative of significant interaction between SL and BYE. The highest point on the three-dimensional plots represents the optimum conditions for achieving highest values of the LA fermentation parameters. X_1, and X_2 represented SL, and BYE concentration in fermentation media. Code values for the variable parameters were used to facilitate regression with -1 as the minimum level and +1 as the maximum level. The midpoints (0) were repeated four times as they contribute to the estimation of the quadratic terms in the model (curvature).

Highest LA yield and volumetric productivity achieved in control fermentations with the addition of only Tween 80 or only YE, were 85.10 and 88.82%, and 0.74 and 0.91 g/L h^{-1}, respectively. While the addition of Tween 80 and YE increased LA yield and volumetric productivity to 90.43% and 0.93 g/L h^{-1}, respectively.

Regarding the LA concentration the fitted second-order polynomial equation is shown in Eq. (2) and three-dimensional plot for LA concentration is presented in Figure 2a. Also ANOVA parameters explaining variables significance for LA concentration and yield are presented in Table 3.

$$LA\ concentration = 25.62 + 1.37X_1 + 4.49X_2 + 0.28X_1X_2 - 0.15X_1^2 - 3.34X_2^2 \quad (2)$$

The R^2 value of 0.9997 and adjusted R^2 value of 0.9996 (which was in reasonable agreement with predictor R^2 value of 0.9984) illustrated that the model adequately fitted the data. Also based on the values of coefficients it was determined that SL or BYE, as well as SL and BYE combination

had significant effect on LA concentration. The highest LA concentration of 28.23 g/L was obtained in fermentation with 0.2% SL and 20% BYE.

Three-dimensional plot for LA yield is presented in Figure 2b, and the fitted second-order polynomial equation is shown in Eq. (3):

$$LA\ yield = 90.80 + 1.06X_1 + 3.69X_2 + 0.05X_1X_2 - 0.59X_1^2 - 2.05X_2^2 \quad (3)$$

The R^2 value of 0.9896 and adjusted R^2 value of 0.9821 (which was in reasonable agreement with predictor R^2 value of 0.9056) illustrated that the model adequately fitted the data. Also based on the values of coefficients it was determined that SL or BYE had significant effect on LA yield. Addition of SL and BYE increased LA yield by 1.57 (0.1% SL) to 11.19% (0.2% SL and 20% BYE). The highest LA yield of 92.82% was obtained in fermentation with 0.2% SL and 20% BYE. Compared to fermentation with commercial supplements significant increase in LA yield, in range of 1.98 to 2.64%, was achieved in fermentations with the addition of 20% BYE and 0.1% or 0.2% SL.

ANOVA parameters explaining variables significance for LA volumetric productivity and *L. rhamnosus* cell viability are presented in Table 4. Highest values of volumetric productivity achieved in fermentations without and with the addition of SL and BYE are presented in Figure 2c, and were fitted into second-order polynomial equation shown in Eq. (4):

$$LA\ Vp = 0.93 + 0.053X_1 + 0.12X_2 + 0.008X_1X_2 - 0.016X_1^2 - 0.06X_2^2 \quad (4)$$

Based on the values of R^2 (0.9982) and adjusted R^2 (0.9969) (which was in reasonable agreement with predictor R^2 value of 0.9816) the presented model adequately fitted the data. Value of coefficients suggested that SL or BYE, as well as SL and BYE combination had significant effect on LA volumetric productivity. Addition of SL and BYE increased LA volumetric productivity by 7.25 (0.1% SL) to 49.28% (0.2% SL and 20% BYE). The highest LA volumetric productivity of

1.03 g/L h^{-1} was obtained in fermentation with 0.2% SL and 20% BYE. Compared to fermentation with commercial supplements significant increase in LA volumetric productivity, in range of 4.72 (0.2% SL and 10% BYE) to 10.75% (0.2% SL and 20% BYE), was achieved in fermentations with the addition of SL and BYE.

Highest values of *L. rhamnosus* cell viability achieved in fermentations without and with the addition of SL and BYE are presented in Figure 2d, and were fitted into second-order polynomial equation shown in Eq. (5):

$$L.rhamnosus\ cell\ viability = 9.60 + 0.063X_1 + 0.15X_2 - 0.03X_1^2 - 0.08X_2^2 \quad (5)$$

Based on the values of R^2 (0.9929) and adjusted R^2 (0.9879) (which was in reasonable agreement with predictor R^2 value of 0.9788) the presented model adequately fitted the data. Value of coefficients suggested that SL or BYE had significant effect on LA volumetric productivity.

According to all achieved results for LA batch fermentation parameters and ANOVA Response Surface Quadratic Models analysis, the optimization procedure was conducted using Design-Expert 7 (Numerical and Graphical optimization) and optimized concentrations of SL and BYE were proposed. Concentration of 0.19% SL and 19.12% BYE were proposed as optimal and additional batch fermentation with optimal conditions was conducted for the validation of model. Predicted values of LA concentration, yield, and volumetric productivity, and *L. rhamnosus* cell viability were 28.26 g/L, 92.98%, 1.03 g/L h^{-1} and 9.72 log CFU/mL, respectively.

Kinetic of batch LA fermentation (LA concentration, reducing sugar concentration and *L. rhamnosus* cell viability) of BGSMR hydrolysate with 0.19% SL and 19.12% BYE is presented in Figure 3. LA concentration, yield, volumetric productivity, and *L. rhamnosus* cell obtained in batch fermentations were 28.43 g/L, 93.03%, 1.04 g/L h^{-1} and 9.72 log CFU/mL, respectively, which were in high agreement with the predicted results. Rivas et al.[28] investigated possible application of

Debaryomyces hansenii spent cell from xylitol production and corn steep liquor (CSL) in LA production by *L. rhamnosus* CECT-288 in glucose-containing media with the addition of salts. With the addition of 1% of CSL and 1% of *D. hansenii* spent cells (grown on corncob hydrolysate media), and salts LA yield of 90% was achieved which was similar to the yield achieved in this study in fermentations with the addition of SL and BYE. Volumetric productivity (0.13-0.54 g/L h^{-1}) achieved by Rivas et al.[28] was lower than volumetric productivity achieved in this study.

LA fermentation by *Lactobacillus rhamnosus* HG 09 using hydrolyzed acorn starch (carbon source, 80 g/L reducing sugar), persimmon juice (nitrogen source), and wheat bran hydrolysate (nitrogen source) was investigated by Lu et al.[29]. The highest LA yield achieved (75%) in fermentations with the addition of persimmon juice (1.2%) and wheat bran hydrolysate (2.5%) was lower than LA yield achieved in fermentations with BYE addition.

Baker's yeast cells (their autolysate after sterilization) (BYC) and red lentil flour (RL) were used as cheap nitrogen sources by Altaf et al.[30] in LA fermentation by *Lactobacillus amylophilus* GV6 on starch (from red lentil). With the addition of 1% of BYC and 0.8% RL the highest LA yield of 92% was achieved, which was similar to the LA yield achieved in this study with the addition of 0.2% SL and 10 and 20% of BYE.

Liu et al.[31] investigated effect of five alternative nitrogen sources, malt sprout (MS), corn steep liquor (CSL), NH_4Cl, NH_4NO_3, and diamine citrate (DC), with RSM on L-(+)-LA fermentation by *Lactobacillus plantarum* As.1.3. Achieved L-(+)-LA concentrations (26-30 g/L) and yields (90-93%) were similar to results achieved in this study.

Gao et al. [32] used yeast spent cell slurry (YSCS) (obtained by acid hydrolysis or/and ultrasonic treatment) as nutrient source (solo or in combination with YE) in LA fermentation by *L. rhamnosus* NBRC 3863. Addition of only YSCS resulted in lower LA yield (86%) and volumetric productivity

(0.63 g/L h^{-1}) than achieved in this study. LA yield obtained with the addition of YSCS and 0.3 or 0.5% of YE (89.5-92.1) were comparable with LA yield achieved in fermentations with the addition of BYE without and with SL (88.40-93.03%) addition. LA volumetric productivity achieved with YSCS and 0.3% YE (0.99 g/L h^{-1}) was similar productivity achieved in this study with BYE addition (0.88-0.92 g/L h^{-1}).

Fed-batch fermentation was conducted as it enables control of substrate concentration in an optimal range without inhibition effect, which could intensify LA production. Kinetic of fed-batch LA fermentation (LA concentration, reducing sugar concentration, and *L. rhamnosus* cell viability) of BGSMR hydrolysate with 0.19% SL and 19.12% BYE is presented in Figure 4. After 60 h of fed-batch fermentation the highest LA concentration (70.17 g/L) and yield (94.57%) were achieved. The highest volumetric productivity of 1.22 g/L h^{-1} was achieved after 36 h of fermentation. Compared to the results obtained in the batch fermentation of BSGMR hydrolysate (with 0.19 % SL and 19.12% BYE), significantly higher LA concentration, yield, and volumetric productivity (by 146.82%; 1.68%; and 16.98%, respectively) were achieved in the fed-batch LA fermentation with glucose and BYE addition. Glucose and BYE addition during LA fermentation increased *L. rhamnosus* cell viability by 1.39%. Overall, fed-batch fermentation greatly increased the efficiency of LA fermentation.

Shi et al.[33] used hydrolysate of *Jerusalem artichoke* as fermentation media (with 0.5% YE) for production of L-(+)-LA by *Lactococcus lactis* ATCC 19435 and achieved slightly lower volumetric productivity (0.94 g/L h^{-1}) in fed-batch fermentation than results achieved in this study. Arabic date juice (supplemented with 2% YE, 0.5% K_2HPO_4, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.008% $MnSO_4 \cdot 5H_2O$, and 0.325% sodium acetate$\cdot 3H_2O$) was investigated by Choi et al.[34] as fermentation media in fed-batch fermentations (different feeding strategies) by *L. rhamnosus* KCCM40069.

Authors achieved slightly lower L-(+)-yield (87%) but higher volumetric productivity (1.58 g/L h^{-1}) than results achieved in this study. Nancib et al.[35] used data waste sugar extract (with the addition of 1% YE, 0.1% Tween 80 and various salts) as media in fed-batch LA fermentations by *L. casei* subsp. *rhamnosus* NRRL-B445. LA productivity obtained by these authors, that varied from 0.80 to 1.30 g/L h^{-1} (depending on feeding rates), was slightly lower or similar to the results obtained in this study. Also after 60 h of fermentation Nancib et al.[35] achieved average LA concentrations (range from 56-81 g/L) similar to result from this study.

BSG and MR present abundant lignocellulosic food industry by-products mostly used as animal feed or disposed to landfill. BY and SL have application in human nutrition, BY as sources of essential amino acids and lipids (as well of minerals and vitamin B complex), while SL is source of lipids and emulsifier. All of these raw materials have potential use in production of high-value and platform chemical.[36] The valorization of lignocellulose agro wastes and food industry by-products for production of chemicals such as lactic acid will solve their disposal problem, as well as environmental issues regarding pollution and haze due to their burning or heavy organic load (that could negatively affect water-flows), and simultaneously reduce carbon footprint by diminishing dependence on petrochemical compounds.[26] Their significant polysaccharide content, renewability and unlimited abundance, as well as non-competitiveness with food sources, which is of high importance in this era of exponential food demand, potentiate them as ideal raw materials for LA production.[37] The addition of SL or BYE and their combination had significant positive effect on LA fermentation achieving high values of LA parameters that were comparable or higher than results achieved with commercial supplements, YE and Tween 80. Therefore, SL and BYE could be successfully used as much cheaper replacement (approximately 8 times cheaper) for Tween 80 and YE in LA fermentations without the decline of LA fermentation efficiency. These experiments are

only a stepping stone in our research for improved and cost-efficient LA fermentation on by-product from brewing and malting industry.

CONCLUSION

BSG, MR, SL, and BY were studied as cheap, abundant, and renewable substrates for LA fermentation by *L. rhamnosus* ATCC 7469. Based on optimization, fermentation mode and achieved fermentation parameters the effect of SL and BYE addition on efficiency of LA fermentation was analyzed. In the optimization experiments the addition of SL and BYE in batch fermentations increased LA concentration (by 54.21%-68.86%), yield (by 1.57-11.19%), and volumetric productivity (by 7.25-49.28%). The addition of SL and BYE significantly increased LA concentration (by 4.36-9.80%), yield (1.98 to 2.64%), and volumetric productivity (by 4.72-10.75 compared to the fermentation with the addition of commercial supplements (Tween 80 and YE). Using optimized concentrations of SL (0.19%) and BYE (19.12%), highest LA concentration (28.43 g/L), yield (93.03%), and volumetric productivity (1.04 g/L h^{-1}) were achieved. By using optimized BSGMR hydrolysate in fed-batch fermentation with BYE and glucose addition during fermentation the highest LA concentration, yield, and volumetric productivity of 70.17 g/L, 94.57%, and 1.22 g/L h^{-1}, respectively, were achieved. Compared to the results obtained in the batch fermentation of optimized BSGMR hydrolysate, significantly higher LA concentration, yield, and volumetric productivity (by 146.82%; 1.68%; and 16.98%, respectively) were achieved in the fed-batch LA fermentation. The addition of SL or BYE and their combination had significant positive effect on LA fermentation achieving high values of LA parameters that were comparable or higher than results achieved with commercial supplements, YE and Tween 80. All results suggested that the

combination of industrial by-products, BSGMR hydrolysate, SL, and BYE could make a very suitable fermentation media for cost-efficient batch and fed-batch LA fermentation.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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Table 1. Uncoded and coded values of independent process variables*

Run number	Soy lecithin (X_1, %)	Brewer's yeast extract (X_2, %)
1	0.00 (-1)	20.00 (1)
2	0.00 (-1)	10.00 (0)
3	0.10 (0)	10.00 (0)
4	0.10 (0)	0.00 (-1)
5	0.10 (0)	10.00 (0)
6	0.20 (1)	10.00 (0)
7	0.10 (0)	10.00 (0)
8	0.10 (0)	10.00 (0)
9	0.00 (-1)	0.00 (-1)
10	0.10 (0)	10.00 (0)
11	0.10 (0)	20.00 (1)
12	0.20 (1)	0.00 (-1)
13	0.20 (1)	20.00 (1)

*Values in the bracket represent the coded values.

Table 2. Brewer s spent grain and malt rootlets (BSG R) hydrolysate, and brewer`s yeast extract (BYE) chemical composition*

Parameter	Brewer's spent grain and malt rootlets (BSG R) hydrolysate	Brewer`s yeast extract (BYE)
Dry matter, %	5.04 ± 0.04	9.68 ± 0.06
Reducing sugars, g/L	35.10 ± 0.16	12.67 ± 0.18
Total nitrogen, g/L	1.48 ± 0.01	5.91 ± 0.01
Free amino nitrogen, mg/L	88.18 ± 0.32	1839.83 ± 4.23
Ash, % dry matter	7.09 ± 0.09	16.45 ± 0.38

*Values represent means ± standard deviation calculated from three determinations.

Table 3. ANOVA parameters of the regression model for L-(+)-lactic acid concentration and yield*

Factor	L-(+)-LA concentration					L-(+)-LA yield				
	SS	d.f	MS	F value	p-value	SS	d.f	MS	F value	p-value
X_1	11.15	1	11.15	1830.51	0.0001†	6.76	1	6.76	42.21	0.0003†
X_2	120.87	1	120.57	19791.45	0.0001†	81.77	1	81.77	510.37	0.0001†
X_1X_2	0.30	1	0.30	49.53	0.0003†	0.010	1	0.010	0.062	0.8099
X_1^2	0.064	1	0.64	10.53	0.0142†	0.97	1	0.97	6.05	0.0435†
X_2^2	30.86	1	30.86	5052.78	0.0002†	11.63	1	11.63	72.60	0.0002†
Model SS	169.78	5	33.96	5559.98	0.0001†	106.28	5	21.26	132.66	0.0001†
Residual SS	0.043	7	0.006			1.12	7	0.16		
Total SS	169.82	12				107.40	12			

*SS-sum of squares; d.f.- degrees of freedom; MS-mean of squares;
†Significant effect when $p<0.05$.

Table 4. ANOVA parameters of the regression model for L-(+)-lactic acid volumetric productivity and *Lactobacillus rhamnosus* cell viability*

Factor	L-(+)-LA volumetric productivity					*L. rhamnosus* cell viability				
	SS	d.f.	MS	F value	p-value	SS	d.f.	MS	F value	p-value
X_1	0.017	1	0.017	540.63	0.0001†	0.024	1	0.024	125.48	0.0001†
X_2	0.059	1	0.059	2813.49	0.0001†	0.14	1	0.14	719.62	0.0001†
X_1X_2	0.0002	1	0.0002	7.13	0.0320†	0.000	1	0.000	0.000	1.0000
X_1^2	0.0007	1	0.0007	22.98	0.0020†	0.0002	1	0.0002	12.66	0.0092†
X_2^2	0.010	1	0.010	327.76	0.0001†	0.015	1	0.015	80.26	0.0001†
Model SS	0.12	5	0.024	769.78	0.0001†	0.19	5	0.038	196.44	0.0001†
Residual SS	0.0002	7	0.00003			0.001	7	0.16		
Total SS	12	12				0.19	12			

* SS-sum of squares; d.f.- degrees of freedom; MS-mean of squares;
†Significant effect when $p<0.05$.

Figure captions:

Figure 1. LA batch fermentation of brewer's spent grain and malt rootlets (BSGMR) hydrolysate with the addition of various supplements: (a) LA concentration; (b) reducing sugar concentration; (c) *L. rhamnosus* cell viability. Symbols: (¡) solid line–without supplements addition; (Ë), solid line–0.1% Tween 80; ( ), dashed line–0.1% soy lecithin; (▲), dashed line–0.2% soy lecithin; (▽), solid line–0.8% yeast extract; (Ê), dashed line–10% brewer`s yeast extract; (♦), dashed line–20% brewers`s yeast extract; (◁), solid line–0.1% Tween 80 and 0.8% yeast extract; (▷), dashed line–0.1% soy lecithin and 10% brewer`s yeast extract; (¡ ), dashed line–0.2% soy lecithin and 10% brewer`s yeast extract; (▶), dashed line–0.1% soy lecithin and 20% brewer`s yeast extract; ( ), dashed line–0.2% soy lecithin and 20% brewer`s yeast extract.

Figure 2. Response surface plots representing the effect of independent variables on: (a) L-(+)-concentration; (b) L-(+)-lactic acid yield; (c) L-(+)-lactic acid volumetric productivity; (d) *L. rhamnosus* cell viability.

Figure 3. Batch LA fermentation of brewer's spent grain and malt rootlets (BSGMR) hydrolysate with the addition of 0.19% soy lecithin and 19.12% brewer's yeast extract. Symbols: (¡) L-(+)-lactic acid concentration; ( ) Reducing sugar concentration; (Ë) *L. rhamnosus* cell viability.

Figure 4. Fed-batch LA fermentation of brewer's spent grain and malt rootlets (BSGMR) hydrolysate with the addition of 0.19% soy lecithin and 19.12% brewer's yeast extract. Symbols: (¡) L-(+)-lactic acid concentration; ( ) Reducing sugars concentration; (Ë) *L. rhamnosus* cell viability.

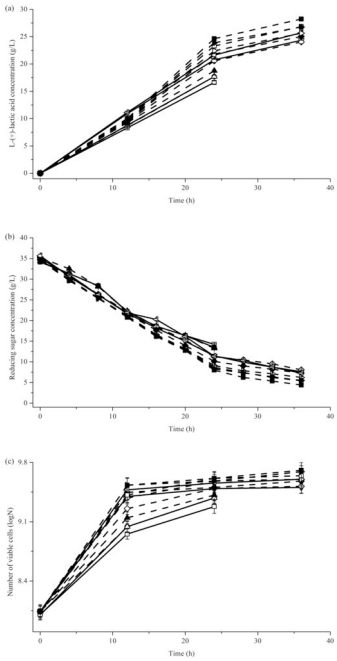
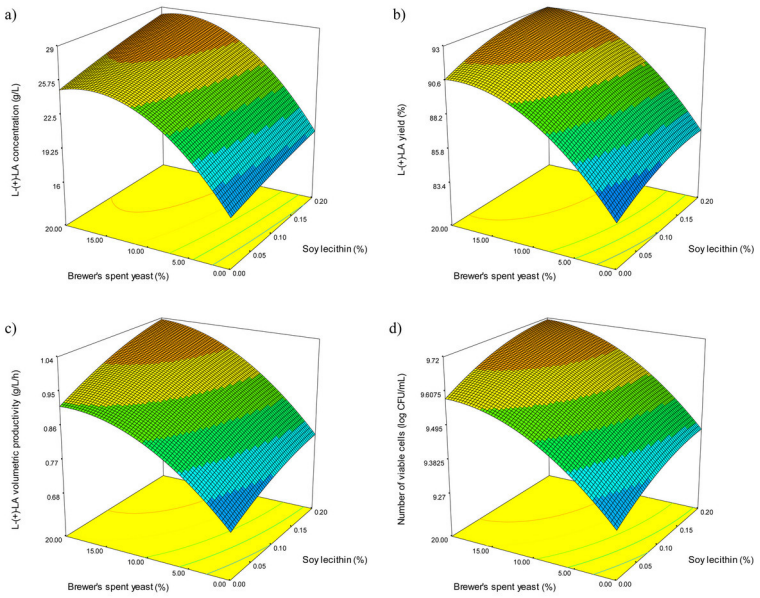


Figure 1.jpg



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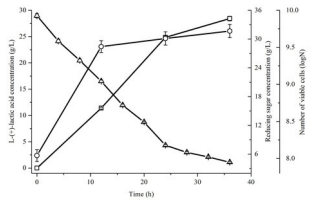

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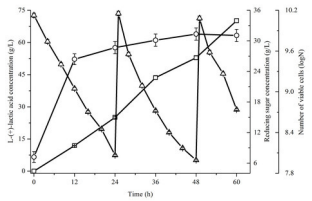

Figure 4.jpg