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THE INFLUENCE OF BREWERS' YEAST ADDITION ON LACTIC ACID FERMENTATION OF BREWERS' SPENT GRAIN HYDROLYSATE BY *LACTOBACILLUS RHAMNOSUS*

UTICAJ DODATKA PISVKOG KVASCA NA MLEČNO-KISELU FERMENTACIJU HIDROLIZATA PIVSKOG TROPA SA *LACTOBACILLUS RHAMNOSUS*

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ABSTRACT

In this study brewers' spent grain (BSG) hydrolysate was produced using optimal conditions. Hydrolysates were used for lactic acid fermentation by Lactobacillus rhamnosusATCC 7469. The aim of this study was to evaluate possibilities of the BSG hydrolysate utilization as a substrate for lactic acid fermentation as well as the effect of dry brewers' yeast (1.0, 3.0, and 5.0 %) addition in hydrolysate on lactic acid fermentation parameters (L-(+)-lactic acid and reducing sugars concentration and number of viable cells-viability). Very high L. rhamnosus ATCC 7469cells viability was achieved in all fermentations (9.26-9.52 log CFU/mLat the end of fermentation). With the increase in brewers' yeast content in hydrolysate, L-(+)-lactic acid concentration increased. The highest L-(+)-lactic acid concentration and yield (89.01 %) were obtained in fermentation of hydrolysate with 5.0 % of brewers' yeast and 5.0 % of reducing sugars.

Key words: *Lactic acid fermentation; brewers' spent grain, brewers' spent yeast.*

REZIME

Mlečna kiselina ima široku primenu u prehrambenoj, farmaceutskoj, hemijskoj, tekstilnoj i industriji polimera. Primena jeftinih i obnovljivih sirovina u proizvodnji mlečne kiseline postupcima fermentacije je osnova ekonomične proizvodnje. U zavisnosti od količine najzastupljeniji sporedni proizvod i proizvodnje piva su: pivski trop i pivski kvasac. Pivski kvasac je relativno jeftin pa se koristi u proizvodnji ekstrakata za primenu u prehrambenoj industriji. Pivski trop čini najveći deo sporednih proizvoda proizvodnje piva (približno 85%). U istraživanju je hidrolizat pivskog tropa proizveden pod optimalnim uslovima. Hidrolizati su korišćeni u mlečno-kiseloj fermentaciji sa Lactobacillus rhamnosus ATCC 7469. Cilj ovog istraživanja je bio da se ispita mogućnost primene hidrolizata pivskog tropa u mlečno - kiseloj fermetnaciji kao i uticaj dodatka suvog pivskog kvasca (1,0; 3,0 i 5,0%) u hidrolizat na parametre mlečno - kisele fermentacije (koncentracijuL - (+) - mlečne kiseline i redukujućih šećera i broj vijabilnih ćelija-vijabilnost). Pri svim ispitivanim sadržajima pivskog kvasca u hidrolizatima u toku mlečno - kisele fermentacije je ostvarena veoma visoka vijabilnost ćelija L. rhamnosus (9,26-9,52 log CFU/ml na kraju fermentacije). S porastom sadržaja pivskog kvasca u hidrolizatu za mlečno-kiselu fermentaciju povećala se i koncentracija L - (+) - mlečne kiseline. Najviša koncentracija kao i prinos L - (+) - mlečne kiseline (89,01%) ostvareni su u fermentaciji hidrolizata pivskog tropa sa 5,0% pivskog kvasca i 5,0% redukujućih šećera.

Ključne reči: *Mlečno-kiselafermentacija, pivskitrop, pivskikvasac.*

INTRODUCTION

In the brewing industry, the major by-products are brewers' spent grain and brewers' spent yeast (*Suwanapong et al., 2013*). In general, since spent yeast in the brewing industry is relatively inexpensive, it is utilized largely in the production of extracts to meet theneeds of food and fermentation industries (*Tanguler and Erten, 2008*). Yeast cells contain plenty of protein, lipid, RNA, vitamins, and minerals. The brewers' yeast is an inexpensive nitrogen source and generally recognized as safe (GRAS) (*Chae et al., 2001*). Large quantities of brewers' spent yeast are obtained after beer fermentation. Brewer's spent grain (BSG) is the most abundant brewing by-product, corresponding to around 85 % of the total by-products generated (*Mussatto, 2014*). The chemical composition of BSG varies according to barley variety, harvest time, malting and mashing conditions, and the quality and type of adjuncts added in the brewing process. In general, BSG is considered as a lignocellulosic material rich in protein and fibre, which account for around 20 and 70 % of its composition, respectively. BSG also contains starch, lipids, amino acids, vitamins, and minerals (*Mussatto et al., 2008*).

Traditionally lactic acid (LA) is used in the food industry as preservative, acidulant, flavoring agent or pH buffer. In the last decades, new technologies of processing LA allow for further fields of applications, such as the production of biodegradable polymers, "green" solvents, and oxygenated-, fine- and commodity-chemicals (*Datta and Henry, 2006*). Fermentation is a dominant route for LA production in industrial facilities and implementation of the processes on renewable and cheap substrates is a base for cost-effective production (*Djukić-Vukovićet et al., 2013*).The annual world market for LA production is expected to reach 830,000 tonnes in 2020 (*Dreschke et al., 2015*). The production of LA from lignocellulosic materials can be performed by sequential steps of chemical and/or mechanical processing (in order to make the cellulose more accessible to the enzymes), enzymatic saccharification (for obtaining solutions containing glucose as main sugar) and finally, the hydrolysate fermentation by

microorganisms, especially *Lactobacillus* species (*Mussatto et al., 2008*).

In this study brewers' spent grain (BSG) hydrolysate was produced using optimal conditions. Hydrolysates were used for lactic acid fermentation by *Lactobacillus rhamnosus*ATCC 7469. The aim of this study was to evaluate possibilities of the BSG hydrolysate utilization as a substrate for lactic acid fermentation as well as the effect of dry brewers' yeast (1.0, 3.0, and 5.0 %) addition in hydrolysate on lactic acid fermentation parameters (L-(+)-lactic acid and reducing sugars concentration and number of viable cells-viability).

MATERIAL AND METHOD

Brewers' spent grain composition

BSG was monitored for the following quality parameters and the following methods were used for analysis: soluble extract, available residual extract and total residual extract (% dry matter) according to MEBAK (2013), protein content (% dry matter) by Kjeldahl method (*AACC, 2008*), starch content after Ewers – polarimetric method (% dry matter) (*International Standard, ISO 10520, 1997*), cellulose content (% dry matter) by Kirschner and Hoffer method. BSG before enzymatic hydrolysis was dried and analyzed. After enzymatic hydrolysis liquid hydrolysate was separated from solid hydrolysate and used in lactic acid fermentation. Solid residue after hydrolysis was dried and analyzed. All BSG analyses were carried out in triplicate. Results were represented as mean ± standard deviation.

Brewers' spent grain hydrolysate preparation

BSG obtained in a lager beer production was dried at 40 ºC 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 were mixed with300 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. BSG hydrolysis was optimized and carried outas described previously by (*Pejin et al., 2014*) using automated mashing water bath (Glasbläserei, Institut für Gärungs Gewerbe, Berlin) by sequential adding of the following enzymes: 0.3 mLTermamyl SC (1 hour at 90 °C), 0.3 mL SAN Super 240 L (1 hour at 55 °C), and 5.0 mL Celluclast 1.5 L (10 hours 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 % H_3PO_4. All commercial enzymes used in BSG hydrolysis (Termamyl SC, SAN Super 240 L, and Celluclast 1.5 L) were kindly provided by Novozymes (Denmark). After enzymatic hydrolysis obtained BSG 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. Liquid hydrolysate was used in LA fermentations. Its pH was adjusted to 6.5 with the addition of 1M NaOH. Brewers' yeast content in hydrolysate was set to 1.0, 3.0, and 5.0 % with the addition of corresponding contents of dry brewers' yeast (AD BIP-Belgrade Beer Industry, Serbia). After this, liquid hydrolysate was sterilized at 121 °C for 15 min and used as a fermentation medium. Two series of experiments were conducted: without reducing sugars concentration correction and with reducing sugars concentration correction to 5.0% in hydrolysate before fermentation. Dry brewers' yeast contained: proteins (minimum 45 g/100g), carbohydrates(27.56 g/100g), minerals (maximum 11 g/100g), lipids (3.0 g/100g), vitamin B1(7.0 mg/100g), vitamin B2 (1.1 mg/100g), vitamin B6 (1.1 mg/100g), vitamin B3 (41 mg/100g), phosphorus (1610 mg/100g), and magnesium (300 mg/100g).

Microorganism

Lactobacillusrhamnosus ATCC 7469, a homofermentative L-(+)-lactic acid strain, was obtained from American Type Culture Collection (ATCC, Rockville, USA). Stock culture of *L.rhamnosus* was stored at -20 °C in 3 mL vials containing de Man Rogosa Sharpe medium (MRS) (Fluka, USA) and 50% (v/v) glycerol as a cryoprotective agent. *L. rhamnosus* culture was activated after storage at -20 °C: 0.2 mL of culture in MRS and glycerol was transffered to 7 mL of MRS broth and incubated for 48 hours at 37 °C. This procedure repeated after 48 hours. Inoculum was prepared by taking 3 mL of the activated culture and transffering it to 60 mL of MRS broth. To reach high lactic acid bacteria cells number inoculum was incubated for 24 hours at 37 °C.

Lactic acid fermentation

All LA fermentations were performed as batch cultures with shaking (150 rpm, Biosan model ES-20, Biosan Ltd., Lithuania). The fermentations were performed in 300 mL Erlenmayer flasks with 200 mL of BSG hydrolysate for 72 h. The fermentation was initiated by the addition of inoculum (5 % v/v). Fermentations were conducted at 37 °C. During fermentations reducing sugars and L-(+)-LA concentration, and number of viable cells were analyzed every 12 h.

Analytical methods

The concentration of reducing sugars, calculated as glucose, was determined by 3,5-dinitrosalicylic acid method using spectrophotometer (*Miller, 1959*). 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 lactic acid determination, proteins were removed from samples by precipitation method (*Methods of Enzymatic BioAnalysis and Food Analysis, 1997*). The number of viable *L.rhamnosus* cells was determined using the pour-plating method during 36 hours. Microaerophilic conditions were maintained during incubation in Petri plates using double MRS medium layer. Samples were incubated for 48 hours at 37 °C. Total viable cells 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. Mean values of treatments were compared by the analysis of variance (one-way ANOVA)followed by Duncan testfor mean differences testing (*IBM SPSS Statistica 20*). Differences were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

BSG composition before and after enzymatic hydrolysis is presented in Table 1.BSG used in this study was produced in high gravity wort production. During hydrolysis soluble and available residual extract content and cellulose content decreased while starch was not determined after the hydrolysis in the solid residual. Protein content just shightly decreased because proteolytic enzymes were not used in the hydrolysis.

BSG hydrolysate contained 2.5 % of reducing sugars. With dry brewers' yeast addition in hydrolysate reducing sugars content increased since brewers' yeast contained carbohydrates (27.56 g/100g). With the increase in brewers' yeast content reducing sugars content increased and was 4.4 % in fermentation with the addition of 5.0 % of dry brewers' yeast.

Table 1. Brewer's spent grain (BSG) composition before and after enzymatic hydrolysis

Parameter	Before enzymatic hydrolysis	After enzymatic hydrolysis
Soluble extract, % dry matter	5.84 ± 0.27	0.23 ± 0.11
Available residual extract, % dry matter	4.47 ± 0.33	1.63 ± 0.14
Total residual extract, % dry matter	10.31 ± 0.37	1.86 ± 0.16
Proteins, % dry matter	26.48 ± 0.35	26.23 ± 0.34
Starch, % dry matter	4.10±0.27	Not determined
Cellulose, % dry matter	15.15 ± 0.38	3.25 ± 0.09

In fermentations without reducing sugars correction L-(+)-LA concentration increased significantly (p<0.05) by 11.01% (1.0 % of brewers' yeast) - 73.46 % (5.0 % of brewers' yeast) (Fig. 1) while in fermentations with reducing sugars correction it increased significantly by 16.14 % (1.0 % of brewers' yeast) - 30.64 % (5.0 % of brewers' yeast) with brewers' yeast addition (Fig. 2). With reducing sugars concentration correction L-(+)-LA concentration increased by 128 % compared to the concentration achieved in fermentation without brewers' yeast addition and reducing sugars concentration correction. L-(+)-LA concentration increased by 198 % when reducing sugars concentration was corrected and 5.0 % of brewers' yeast was added compared to the concentration achieved in fermentation without brewers' yeast addition and reducing sugars concentration correction.

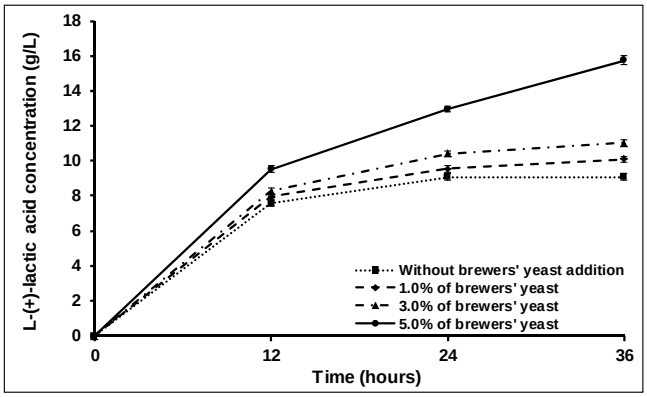

Fig. 1. L-(+)-LA concentration in BSG hydrolysate fermentations without and with brewers' yeast (1.0, 3.0 and 5.0 %) addition and without reducing sugars concentration correction

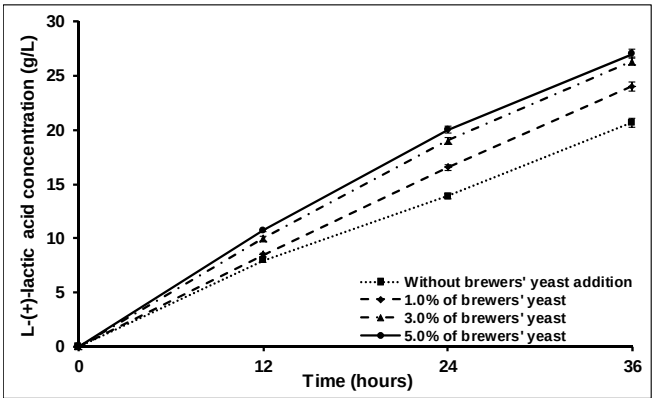

Fig. 2. L-(+)-LA concentration in BSG hydrolysate fermentations without and with brewers' yeast (1.0, 3.0 and 5.0 %) addition and with reducing sugars concentration correction to 5.0 %

In all fermentations *L.rhamnosus* cells viabilitysignificantly (p<0.05) increased until the end of the fermentation. *L.rhamnosus* cells viability was significantly higher (p<0.05) in fermentations with brewers' yeast addition and without reducing sugars correction (2.89 % (1.0 % of brewers' yeast) – 5.00 % (5.0 % of brewers' yeast) than in fermentation without brewers' yeast addition and without reducing sugars correction. *L. rhamnosus* cells viability was also significantly higher (p<0.05) in fermentations with brewers' yeast addition and with reducing sugars concentration correction (1.41 % (1.0 % of brewers' yeast) – 3.03 % (5.0 % of brewers' yeast) compared to the fermentation without brewers' yeast addition and with reducing sugars concentration correction (Figs. 3-4). *L. rhamnosus* requires a complex nutrient composition for its growth because it lacks enzymes to self-synthesize B vitamins and amino acids (*Cui et al., 2012*). However, most *Lactobacillus* require an exogenous nitrogen source of amino acids or peptides to meet cell growth (*Manca de Nadra, 2007*).

Brewers' yeast addition increased L-(+)-LA yieldby 6.85 % (5.0 % of brewers' yeast, without reducing sugars concentration correction) – 9.71 % (5.0 % of brewers' yeast, with reducing sugars concentration correction). The highest L-(+)-lactic yield (89.01 %) was achieved in fermentation of hydrolysate with 5.0% of brewers' yeast and reducing sugars concentration correction (Fig. 5).

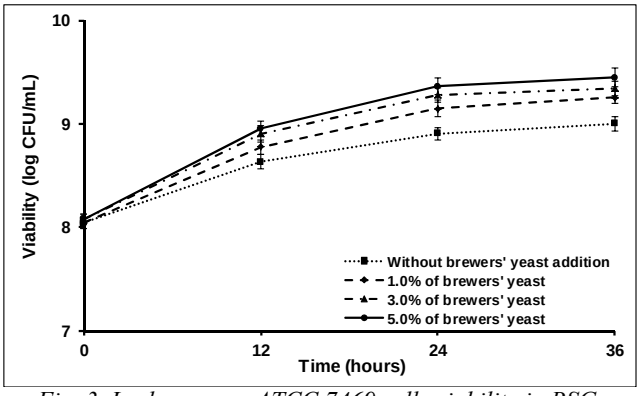

Fig. 3. L. rhamnosus ATCC 7469 cells viability in BSG hydrolysate fermentations without and with brewers' yeast (1.0, 3.0 and 5.0 %) addition and without reducing sugars concentration correction

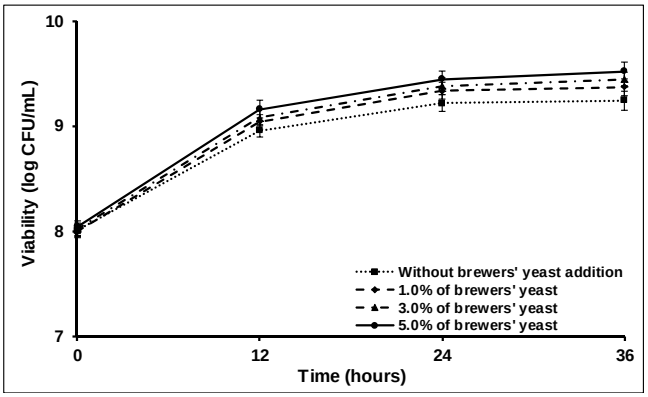

Fig. 4. L. rhamnosus ATCC 7469 cells viability in BSG hydrolysate fermentations without and with brewers' yeast (1.0, 3.0 and 5.0 %) addition and with reducing sugars concentration correction to 5.0 %

Hydrolysates obtained from BSG (*Mussatto et al., 2008*) and other cellulosic materials (*Garde, et al., 2002; Ali et al., 2009*; *Laopaiboon et al., 2010*), required additional nutrients (yeast extract, MRS medium, petone, different salts, etc.) for lactic acid

production by *Lactobacillus* strains. Results obtained in this study showed that brewers' yeast addition significantly increased L-(+)-lactic acid yield and *L. rhamnosus* cells viability.

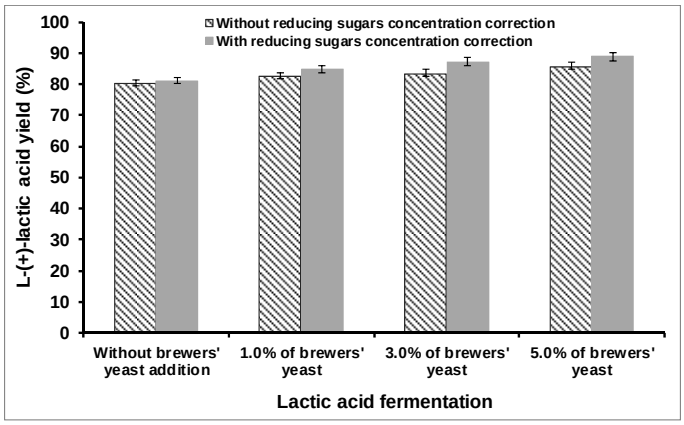

Fig. 5. L-(+)-lactic acid yield in BSG hydrolysate fermentations

CONCLUSION

In fermentations without reducing sugars correction L-(+)-LA concentration increased by 11.01-73.46 % while in fermentations with reducing sugars correction it increased by 16.14-30.64 with brewers' yeast addition. With reducing sugars concentration correction L-(+)-LA concentration increased by 128 % compared to the concentration achieved in fermentation without brewers' yeast addition and reducing sugars concentration correction. L-(+)-LA concentration increased by 198 % when reducing sugars concentration was corrected and 5.0 % of brewers' yeast was added compared to the concentration achieved in fermentation without brewers' yeast addition and reducing sugars concentration correction. Brewers' yeast addition increased L-(+)-LA yieldby 6.85–9.71 %. The highest L-(+)-lactic yield (89.01 %) was achieved in fermentation of hydrolysate with 5.0 % of brewers' yeast and reducing sugars concentration correction.

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