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The influence of calcium-carbonate and yeast extract addition on lactic acid fermentation of brewer's spent grain hydrolysate

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ABSTRACT

Brewer's spent grain (BSG) is the major by-product of the brewing industry, representing around 85% of the total by-products generated. In this study BSG hydrolysate was produced using optimal conditions. Hydrolysates were used for lactic acid (LA) fermentation by *Lactobacillus fermentum* (PL-1) and *Lactobacillus rhamnosus* (ATCC 7469). The aim of this study was to evaluate possibilities of the BSG hydrolysate utilization as a substrate for LA fermentation. The effect of calcium-carbonate (2%) and yeast extract (0.5 to 5%) addition in hydrolysate on LA fermentation were investigated. The LA production by *L. fermentum* and *L. rhamnosus* in BSG hydrolysate was influenced by calcium-carbonate and yeast extract supplementation. *L. fermentum* produced a racemic mixture of L-(+)- and D-(−)-LA while *L. rhamnosus* produced mostly L-(+)-LA (95–98%) in all fermentations. Calcium-carbonate addition increased total LA yield by 13% in *L. fermentum* fermentations and by 17% in *L. rhamnosus* fermentations. Yeast extract addition increased total LA yield by 4–26% in *L. fermentum* fermentations and by 6–8% in *L. rhamnosus* fermentations.

1. Introduction

Lactic acid (LA) is a versatile chemical with a wide range of applications in food, pharmaceutical, cosmetic, textile and polymer industries (Djukić-Vuković et al., 2012). LA is industrially produced either by chemical synthesis or by microbial fermentation. Presently, 90% of the total LA produced annually worldwide is manufactured by LA fermentation and the remainder is produced synthetically by the hydrolysis of lactonitrile (Marques, Santos, Gírio, & Roseiro, 2008). A microbiological method has the advantage that an optically pure LA can be obtained by choosing a strain of LA bacteria, whereas chemical synthesis always results in a racemic mixture of LA (Oh et al., 2005). Pure isomers, L-(+)- and D-(−)-LA, are more valuable than the racemic LD form because each isomer has its own specific industrial application (Abdel-Rahman, Toshiro, & Sonomoto, 2011; Ali, Anjum, & Zahoor, 2009).

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-Vuković, Mojović, Vukašinović-Sekulić, Nikolić, & Pejin, 2013). Food and food-related applications account for approximately 85% of the demand for LA, whereas the nonfood industrial] 15% of the demand (John, Anisha, Madhavan Nampoothiri, & Pandey, 2009). The demand for LA has been estimated to grow yearly at 5–8% (Yadav, Chaudhari, & Kothari, 2011). The annual world market for LA production was expected to reach 367,300 metric tons by the year 2017 (Abdel-Rahman, Toshiro, & Sonomoto, 2013; Ali, Anjum, & Zahoor, 2009). The Global Industry Analyst Inc. announced in January 2011 that the global market for LA is forecast to reach approximately 329,000 metric tons by the year 2015 (Martinez et al., 2013). Traditionally, LA was mostly produced by fermentation of starchy substrates like corn or potato but because of the competition of these substrates with food, lignocelluloses and wastes are currently being studied as a new and promising feedstock (Djukić-Vuković et al., 2015). Brewer's spent grain (BSG) is the most abundant brewing by-product, corresponding to around 85% of the total by-products generated (Mussatto, Moncada, Roberto, & Cardona, 2013). 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 but in general, BSG is considered as a lignocellulosic material rich in protein and fiber, which account for around 20 and 70% of its composition, respectively. BSG also contains starch, lipids, amino acids, vitamins, and minerals (Mussatto, Dragone, & Roberto, 2006; Xiros, Topakas, Katapodis, & Christakopoulos, 2008).

At present, the main market of this low-cost byproduct is for animal feedstuffs, as it is a rich source of protein and fiber (Bohnsack, Ternes, Büsing, & Drotleff, 2011). Its possible applications are in human nutrition (Plessas et al., 2007), as a raw material in biotechnology (Coelho, Rocha, Saraiva, & Coimbra, 2014; Dhillon, Kaur, & Kaur Brar, 2012; Mussatto, Fernandes, Mancilha, & Roberto, 2008; White, Yohannan, & Walker, 2008), energy production (Zanker & Kepplinger, 2002), charcoal production (Sato et al., 2001), paper manufacture

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(Ishiwaki, Murayama, Awayama, Kanauchi, & Sato, 2000), as a brick component (Russ, Mörtel, & Meyer-Pittroff, 2005), and adsorbent (Silva et al., 2004). In biotechnology BSG was used as a substrate for cultivation of microorganisms and enzyme production, additive or yeast carrier in beer fermentation, raw material in production of lactic acid, bioethanol, biogas, phenolic acids, arabitol, xylitol, pullulan, antioxidants, monosaccharides, oligosaccharides, functional proteins and lipids (Kado, Ishii, Takoi, Mitani, & Shinotsuka, 1999; Roukas, 1999; Bránýik, Vicente, Machado Cruz, & Teixeira, 2001; Novik, Wawrzynczyk, Norrlow, & Szwajcer-Dey, 2007; Xiros & Christakopoulos, 2012). 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).

The largest and most diverse genus of LA bacteria is *Lactobacillus*, which includes species with very different biochemical and physiological properties along with special resistance against acidic environment (Martinez et al., 2013). Most LA bacteria require a wide range of growth factors including amino acids, vitamins, fatty acids, purines, and pyrimidines for their growth and biological activity (Kwon, Lee, Lee, Chang, & Chang, 2000). Thus, the substrate composition and nutritional requirements of the strain considerably affect the overall performance of the fermentation (Charalampopoulos, Pandiella, & Webb, 2002). Hydrolysates obtained from BSG (Mussatto et al., 2008) and other cellulosic materials such as wheat straw (Garde, Jonsson, Schmidt, & Ahring, 2002), corn cobs (John et al., 2009), wood (Parajó, Alonso, & Santos, 1996), cassava bagasse, and sugarcane bagasse (John, Madhavan Nampoothiri, & Pandey, 2006; Laopaiboon, Thani, Leelavatcharmas, & Laopaiboon, 2010), required additional nutrients (yeast extract, de Man Rogosa Sharpe medium (MRS), corn steep liquer, petone, different salts, etc.) for lactic acid production by *Lactobacillus* strains. In this study BSG hydrolysate was produced using optimal conditions. Hydrolysates were used for lactic acid fermentation by *Lactobacillus fermentum* and *Lactobacillus rhamnosus*. The aim of this study was to evaluate possibilities of the BSG hydrolysate utilization as a substrate for LA fermentation. The effect of calcium-carbonate (2%) and yeast extract (0.5, 1.0, 2.0, 3.0, 4.0, and 5.0%) addition in hydrolysate on LA fermentation were investigated.

2. Material and methods

2.1. 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 — Mitteleuropäische Brautechnische Analysenkommission (2013), protein content (% dry matter) by Kjeldahl method (AACC — Approved Methods of the American Association of Cereal Chemists, 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 in triplicate. Results were represented as mean ± standard deviation.

2.2. Brewers' spent grain hydrolysis preparation

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 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. BSG hydrolysis was optimized and carried out using automated mashing water bath (Glasbläserei, Institut für Gärungs Gewerbe, Berlin) by sequential adding 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% 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 1 M NaOH. Yeast extract content in hydrolysate was set to 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0% with the addition of corresponding contents of yeast extract (HiMedia Laboratories, India). After this, liquid hydrolysate was sterilized at 121 °C for 15 min and used as a fermentation medium. In experiments with the addition of calcium-carbonate (Merck, Germany), it was added after sterilization at a concentration of 2% (w/v), prior to the inoculation with *Lactobacillus* strains.

2.3. Microorganisms

L. rhamnosus ATCC 7469, a homofermentative L-(+)-lactic acid strain, was obtained from American Type Culture Collection (ATCC, Rockville, USA). *L. fermentum* PL-1, a heterofermentative lactic acid strain, was isolated from cheese and obtained from Department of Biochemical Engineering and Biotechnology, Faculty of Technology and Metallurgy, University of Belgrade. Stock cultures of *L. rhamnosus* and *L. fermentum* were 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. fermentum and *L. rhamnosus* cultures were 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 h at 30 °C for *L. fermentum* or 37 °C for *L. rhamnosus*. This procedure repeated after 48 h. 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 h at 30 °C for *L. fermentum* or 37 °C for *L. rhamnosus*.

2.4. 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 100 mL Erlenmayer flasks with 60 mL of BSG hydrolysate for 72 h. The fermentation was initiated by the addition of inoculum (5% v/v). In experiments with *L. rhamnosus* fermentation were conducted at 37 °C, while *L. fermentum* fermentations were carried out at 30 °C. LA fermentations without the addition of calcium-carbonate were conducted under aerobic conditions obtained by constant shaking. During fermentations, pH value, reducing sugars concentration, L-(+)- and D-(−)-LA concentration, and number of viable cells were analyzed every 24 h.

2.5. 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. LA concentration was determined by enzymatic method (L-(+)-/D-(−)-LA assay, Megazyme, Wicklow, Ireland). Prior to the lactic acid determination, proteins were removed from samples by precipitation method (Methods of Enzymatic BioAnalysis