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UTILIZATION OF THE STILLAGE FROM BIOETHANOL PRODUCTION ON WASTE BREAD FOR LACTIC ACID AND BIOMASS PRODUCTION

KORIŠĆENJE DŽIBRE IZ PROIZVODNJE BIOETANOLA NA OTPADNOM HLEBU ZA PROIZVODNJU MLEČNE KISELINE I BAKTERIJSKE BIOMASE

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ABSTRACT

Lactic acid is an important chemical for food industry and monomer for production of biodegradable polymers. Emerging technologies for sustainable fermentative production of food additives include the utilization of by-products and wastes as substrates. Problem of stillage disposal, as a by-product with significant ecological imprint, is not adequately solved in Serbia and its utilization as a substrate for fermentation could be a promising strategy. Lactobacillus rhamnosus ATCC 7469 was selected for evaluation of optimal inoculum and initial sugar concentration for effective lactic acid and biomass production. Under the selected conditions (5% of inoculum and 55 g L^{-1} of initial sugar concentration) lactic acid yield of 92.3%, volumetric productivity of 1.49 g L^{-1} h^{-1} and number of viable cells of 10^9 CFU ml^{-1} were achieved. High number of viable cells indicates that the fermentation media remained after lactic acid removal could be used as a high-quality animal feed.

Key words: *distillery stillage, lactic acid, biomass, Lactobacillus rhamnosus.*

REZIME

Mlečna kiselina se koristi kao acidulant, konzervans i poboljšivač ukusa u prehrambenoj industriji i kao monomer za proizvodnju biodegradabilnih polimera. Nove tehnologije za održivu fermentacionu proizvodnju aditiva u prehrambenoj industriji baziraju se na korišćenju sporednih i otpadnih proizvoda kao supstrata. Problem odlaganja džibre, kao sporednog proizvoda koji značajno zagađuje životnu sredinu, nije adekvatno rešen u Srbiji pa bi korišćenje džibre za fermentacionu proizvodnju mlečne kiseline moglo biti jedno od rešenja. U ovom istraživanju, ispitivana je proizvodnja mlečne kiseline na džibri od otpadnog hleba pomoću bakterija mlečne kiseline. Najviši prinos ostvaren je u fermentaciji sa Lactobacillus rhamnosus ATCC 7469. Ovaj soj je korišćen za dalji odabir odgovarajuće početne koncentracije inokuluma i šećera za efikasnu proizvodnju mlečne kiseline i biomase. Sa početnom koncentracijom šećera od 55 g L^{-1} i koncentracijom inokuluma od 5% ostvaren je prinos mlečne kiseline od 92,3%, produktivnost od 1,49 g L^{-} h^{-1} i broj ćelija 10^9 CFU mL^{-1}. Prinosi i produktivnosti koji su ostvareni na džibri bez dodatka mineralnih materija i izvora azota su visoki u poređenju sa vrednostima na sličnim otpadnim supstratima dosada objavljenim u literaturi, što ukazuje da bi džibra mogla biti dobar supstrat za industrijsku proizvodnju mlečne kiseline. Zbog velikog broja živih ćelija, zaostali medijum nakon mlečno-kiselinske fermentacije mogao bi se koristiti kao kvalitetna stočna hrana.

Ključne reči: *destilerijska džibra, mlečna kiselina, biomasa, Lactobacillus rhamnosus.*

INTRODUCTION

Biotechnological production of lactic acid is increasing constantly. Research in the field of finding suitable cheap substrates for fermentative lactic acid production is mainly driven by expansion of biodegradable poly-lactic acid market (*Data and Henry, 2006*). It is predicted that lactic acid consumption will continue to grow annually for 9% in Western Europe and 5.5% in Asia (*Malveda et al., 2009*). Industrial by-products and wastes can be utilized as substrates in eco-friendly and cost effective fermentative processes. Stillage is a by-product of bioethanol production on agroindustrial feedstocks. For 1 hL of the produced bioethanol, approximately 13 hL of liquid thin stillage is formed (*Pejin et al., 2009*). Because of complex chemical composition and high BOD values, its storage in the industrial facilities represents important ecological problem and high costs of treatment prior to disposal seriously affect process viability and profitability.

Stillage can be used as animal feed (*Mojović et al, 2010*), soil fertilizer (*Banković-Ilić et al., 2007*) and as a substrate for biogas and butanol production (*Wilkie et al., 2000, Krzywonos et al., 2009*). Because of residual yeast cells stillage could be a good source of soluble and highly degradable proteins, minerals and vitamins (B group) which could support well the growth of lactic acid bacteria (LAB) (*Altaf et al., 2007*). Lactic acid production on corn liquid stillage was reported previously by *Mojović et al., 2011* and on triticale stillage by *Marković et al., 2010*. Selection of microorganisms suitable for effective lactic acid production and safe for use in animal nutrition is important for intended utilization of spent fermentation media as animal feed. *Lactobacillus sp.* and *Rhizopus sp*. are mainly employed in lactic acid fermentations on waste substrates (*Djukić-Vuković et al., 2011a*, *Zhang et al., 2007*). Among *Lactobacillus sp*, the strain *Lactobacillus rhamnosus* was proven as harmless and its positive effects on animal health were verified in numerous reports (*EFSA, 2007, Anadón et al., 2006, Gaggia et al., 2010*).

In this paper a selection of appropriate microorganism for lactic acid and biomass production on the whole distillery stillage remained after bioethanol production on wasted bread was preformed. For improvement of process productivity effect of inoculum and initial sugar concentration was investigated.

MATERIAL AND METHOD

Microorganism

Lactobacillus rhamnosus ATCC 7469, *L. paracasei ssp. paracasei* NRRL B- 4564, *L. casei ssp. casei* NRRL B-441 and *L. pentosus* NRRL- 227 were used for lactic acid fermentation.

Stock cultures of lactic acid bacteria (LAB) were stored at -20 °C in 3 ml vials containing Man Rogosa Sharpe medium (MRS) (Fluka, USA) and 50% (v/v) of glycerol as a cryoprotective agent. The culture was propagated under anaerobic conditions using Anaerocult ® C bags (Merck KGaA, Darmstadt, Germany) at 37°C for 18 h in MRS broth before inoculation to fermentation medium. The fermentation with *L. rhamnosus* ATCC 7469 was performed at temperature of 41°C and the fermentations with *L. paracasei ssp. paracasei* NRRL B- 4564 and *L. casei ssp. casei* NRRL B-441 were performed at temperature of 37°C. The fermentation with *L. pentosus* NRRL- 227 was performed at 30°C.

Stillage

A whole distillery stillage (without separation of solid fraction) remained after bioethanol production on wasted bread was obtained from Reahem Ethanol Plant (Reahem, Srbobran, Serbia). The composition of the stillage is presented in Table 1. Because of efficient previous alcoholic fermentation, the sugar concentration in stillage is low and addition of glucose was necessary. The pH of the stillage was adjusted to 6.5 with 30% solution of NaOH (Sigma-Aldrich, USA), and then sterilized (120 °C/15 min) before fermentation.

Table 1. Chemical comp. of the whole distillery stillage

Chemical composition of stillage	Content
Total reducing sugar (% of dry matter)	9.74
Lipid (% of dry matter)	8.49
Protein (% of dry matter)	58.50
Ash (% of dry matter)	21.47
Dry weight (% of total stillage)	11.55

Lactic acid fermentation

All lactic acid fermentations were performed with shaking (150 rpm, KS 4000i control, IKA®, Werke GmbH & Co. KG, Staufen, Germany). The flasks of 500 ml volume with 250 ml of the fermentation media were used for fermentations under anaerobic conditions maintained by Anaerocult ® C bags (Merck KGaA, Darmstadt, Germany) in the gas pack system. During the fermentation: pH, sugar consumption, lactic acid concentration and a number of living cells were analyzed.

In the first set of experiments the sugar concentration of fermentation media was set at 25 g L^{-1} with addition of sterile 70% glucose solution to sterilized stillage. For screening of the most productive microorganism in lactic acid fermentations, prepared fermentation media was inoculated with 2% (v/v) of inoculum of each investigated strain.

In the next set of experiments effect of inoculum concentrations was investigated. Fermentation media with 25 g L^{-1} initial sugar concentration was inoculated with 2% (v/v), 5% (v/v) and 10% (v/v) of *L. rhamnosus* ATCC 7469 culture (which was previously selected as the most promising one). Also, the effect of different initial sugar concentrations on lactic acid and biomass production in batch fermentations was studied. Initial sugar concentrations of 25 g L^{-1}, 55 g L^{-1}, 75 g L^{-1} and 85 g L^{-1} was set with addition of sterile 70% glucose solution. A selected inoculum concentration of 5% (v/v) was used for inoculation and pH of the fermentation media was controlled at four hour intervals with 30% NaOH solution. All experiments were performed in triplicates and represented values are means.

Analytical methods

The concentration of reducing sugars, calculated as glucose, was estimated by 3, 5-dinitrosalicylic acid method using spectrophotometer, Ultraspec 3300 pro, Biochrom LTD, UK (*Miller, 1959*). Lactic acid concentration was determined by enzymatic method (L-/ D-Lactic acid assay, Megazyme®, Wicklow, Ireland) after deproteinisation according to procedure prescribed in assay. Number of viable *L. rhamnosus* ATCC 7469 cells was estimated using pour plate technique on MRS agar, after incubation at 37°C for 48 h. The chemicals used in experiments were analytical grade.

RESULTS AND DISCUSION

Selection of lactic acid bacteria for fermentations on stillage

The important parameters of lactic acid fermentations with different lactic acid bacteria are presented in Table 2. It could be seen that the highest lactic acid concentration, yield, productivity and biomass production is obtained by *Lactobacillus rhamnosus* ATCC 7469. The differences between numbers of viable cells achieved with different species are not high implying that lactic acid production is more affected by metabolism of investigated strains. Their growth was intense on the stillage but the effectiveness of sugar conversion to lactic acid was not proportional to the number of viable cells present in fermentation media. *L. rhamnosus* ATCC 7469 is a homofermentative strain and a high yield of 0.81 g g^{-1} suggested that this strain should be selected for further optimisation of lactic acid production on the stillage. *Lactobacillus paracasei ssp. paracasei* NRRL B- 4564, *Lactobacillus casei ssp. casei* NRRL B-441 and *Lactobacillus pentosus* NRRL- 227 are a facultative heterofermentative strains (*Zhu et al., 2007*, *Mojović et al., 2011*) and this resulted in lower yields and productivities in the fermentations. It is important to note that effectiveness of lactic acid production could be influenced with the presence of solid fractions of stillage and its suitability for particular species. In our previous work on corn liquid stillage, the best producer of lactic acid was *Lactobacillus paracasei ssp. paracasei* NRRL B- 4564 strain (*Mojović et al., 2011)*.

Table 2. Parameters of lactic acid fermentation on distillery stillage with different lactic acid bacteria

Species of lactic acid bacteria	Lactic acid concentration (g L^{-1})	Number of cells (CFU ml^{-1})	Lactic acid yield[a] (g g^{-1})	Lactic acid productivity[b] (g L^{-1} h^{-1})
Lactobacillus rhamnosus ATCC 7469	20.2	8.2×10^{8}	0.81	0.28
Lactobacillus paracasei ssp. paracasei NRRL B- 4564	16.6	7.0×10^{7}	0.66	0.23
Lactobacillus casei ssp. casei NRRL B-441	13.7	1.0×10^{8}	0.55	0.19
Lactobacillus pentosus NRRL-227	10.1	2.1×10^{8}	0.40	0.14

[a] Initial sugar concentration 25 g L^{-1}
[b] Fermentation lasted for 72 hours

The effect of inoculum concentration and initial sugar concentration on lactic acid and biomass production

The effect of different inoculum concentrations on lactic acid production is presented in Figure 1. The highest lactic acid concentration is achieved in fermentation with 5% (v/v) of inoculum concentration. The kinetics in all samples was relatively similar but the conversion of sugars to lactic acid and yield were the

highest in samples with 5% (v/v) of inoculum. Several studies on whey permeate have shown that inoculum concentrations higher than 5% (v/v) had no significant influence on lactic acid concentration and viable cell number, however it was reported that the higher initial inoculum concentration could reduce the fermentation time (*Panesar et al., 2010*, *Cui et al., 2010*). In this study, inoculation with 5% (v/v) and 10% (v/v) of inoculum resulted in faster lactic acid production during the first 24 hours of fermentation time. However, at the end of fermentation time, a lower lactic acid concentration was achieved with 10% (v/v) of inoculum than with 2% (v/v) of inoculum (Fig.1.).

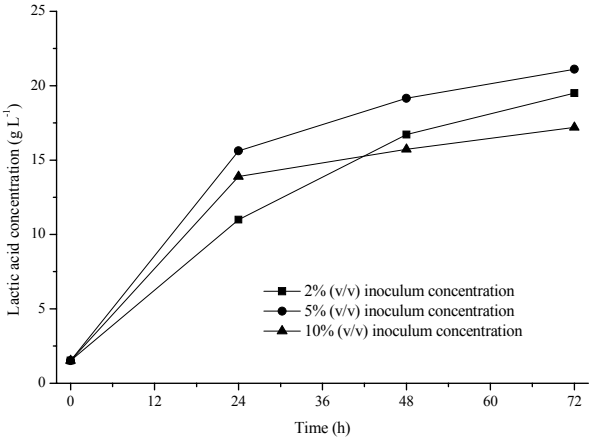

Fig.1. Effect of inoculum concentration on lactic acid production in fermentation on distillery stillage

The effect of different initial sugar concentrations on lactic acid production and a time course of sugar consumption are presented at Figure 2. If we compare the fermentations preformed with 5% (v/v) of inoculum (and 25 g L^{-1} initial sugar concentration) presented in Figure 1. to the fermentation with the same initial sugar concentration presented at Figure 2, we can observe reduction in time required to approach maximal lactic acid concentration. At 34th hour of fermentation with 25 g L^{-1} sugar concentration, presented at Fig. 2, the lactic acid concentration came up to 20.85 g L^{-1}.

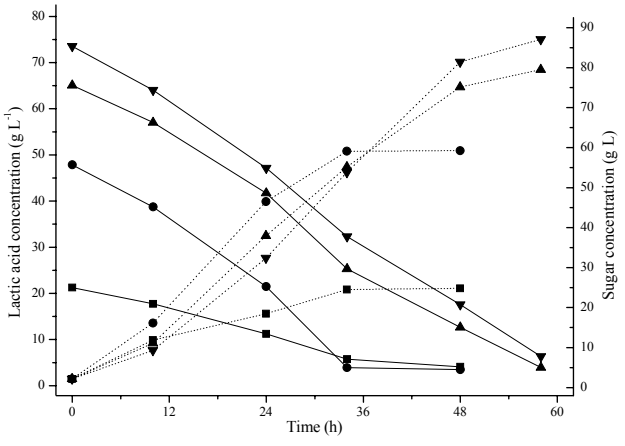

Fig. 2. Effect of different initial sugar concentrations on lactic acid production and sugar consumption in fermentation on distillery stillage. Symbols: ■-initial sugar concentration 25.03 g L^{-1}, ●- initial sugar concentration 55.72 g L^{-1}, ▲- initial sugar concentration 75.58 g L^{-1}, ▼- initial sugar concentration 85.31 g L^{-1}; solid line- sugar consumption, dot line- lactic acid production

In this fermentation pH was maintained by addition of 30% NaOH solution which induced reduction in fermentation time, although without effect on lactic acid yield. Control of pH during the fermentation is important, especially in fermentations with higher initial sugar concentration because of higher productivities and inhibition with produced lactic acid (*Gonçalves et al., 1997*). The important parameters of fermentation such as initial sugar concentration, lactic acid concentration and yield, productivity and number of viable cells achieved in fermentations with different initial sugar concentrations are listed in Table 3.

Table 3. Important parameters of lactic acid fermentations with different initial sugar concentrations

Sample No	Initial sugar concentration (g L^{-1})	Lactic acid concentration (g L^{-1})	Number of cells (10^8CFU ml^1)	Lactic acid yield (g g^{-1})	Volumetric productivity (g L^{-1} h^{-1})
1[a]	25.03	20.85	9.50	0.82	0.61
2[a]	55.72	50.83	10.0	0.92	1.49
3[b]	75.58	68.60	7.20	0.91	1.18
4[b]	85.31	75.06	10.0	0.88	1.29

[a] The parameters were calculated at 34 h of fermentation time;

[b] The parameters were calculated at 58 h of fermentation time when all fermentable sugars were consumed.

The kinetics of sugar consumption and lactic acid production was similar for all samples (Fig. 2), but the highest lactic acid yield was obtained in the samples with initial sugar concentration of 55.72 g L^{-1} (Tab. 3). The highest lactic acid concentration of 75.06 g L^{-1} was achieved in the sample with 85.31 g L^{-1} of initial sugar (sample 4). The lower yield and productivity noticed in this sample (compared to samples 2, with lower sugar content) could be due to substrate inhibition. *Hujanen et al.* (2001) observed similar effect of increase in lactic acid concentration and decline in yields when the initial sugar concentration rose from 80 to 120 g L^{-1} in fermentation on chemically defined media with malt sprout. Also, *Liu et al.*, (2005) reported a significant decline in yield with increase of initial sugar concentrations in lactic acid fermentation by *Rhizopus sp.* In fermentation by *Lactobacillus lactis*, the highest productivity was achieved with initial sugar concentration of 30 g L^{-1}, and it progressively declined by raising the concentrations up to 90 g L^{-1} (*Bai et al., 2003*).

Our previous work had shown that lactic acid fermentation could be performed on triticale, corn and wasted bread liquid stillage with yields of 0.50 g g^{-1}, 0.76 g g^{-1} and 0.80 g g^{-1}, respectively (*Djukić-Vuković et al., 2011b, Djukić Vuković et al., 2011c*). A yield of approximately 0.92 g g^{-1} and lactic acid concentration of 50.83 g L^{-1} achieved in this study on the whole wasted bread stillage were higher than those previously obtained on just a liquid part of the stillage. Solid fraction of the stillage is evidentially a source of important nutrients for lactic acid production. In addition, utilization of the whole stillage, without separation, is economically more favourable approach. Lactic acid concentrations and yields obtained and reported on some other waste substrates were also lower than this obtained on the whole stillage. *Coelho et al.*, (2010) reported a maximal lactic acid concentration of 41.65 g L^{-1} after 48 h of fermentation of cassava wastewater with initial sugar concentration of 50 g L^{-1}.

The number of viable cells at the end of fermentation time was high in all samples and in samples with 55 g L^{-1} and 85 g L^{-1} initial sugar concentration it was over 10^9 CFU ml^{-1} (Tab. 3.). The biomass production and number of viable cells at the end of fermentation is a crucial parameter for use of spent fermentation media in animal nutrition. Acceptability of stillage from bioethanol production for animal feed has been valorised. It has been used in animal diets as wet or dried distillers' grains in

animal diets both in Europe and USA (*Mojović et al., 2011*). According to European Regulation (*EC, 2008*), a minimum content of viable bacterial cells (CFU kg^{-1}) in a complete feedingstuff is the one of the major specifications for animal feed enriched with probiotics and it should be about 10^9 CFU kg^{-1} in complete feed (*Anadón et al., 2006, EFSA, 2010, Busch et al., 2004*). This criterion is satisfied in the spent fermented stillage studied here, which opens the possibilities for further investigation of nutritional qualities and its utilization as a high-value animal feed.

CONCLUSION

The lactic acid and biomass production on stillage as a cheap and waste substrate might be a strategy for recovery of bioethanol production process value. The most effective lactic acid fermentation of the stillage was achieved by *L. rhamnosus* ATCC 7469 with initial sugar concentration of 55 g L^{-1}, 5% (v/v) of inoculum and with pH control in four hour intervals. The yield, productivity and lactic acid concentration was the highest and the number of viable cells was above 10^9 CFU ml^{-1} at the end of this fermentation. These results verified that the whole stillage from bioethanol production on wasted bread could be a good substrate for lactic acid production without mineral or nitrogen supplementation. After lactic acid fermentation, the stillage was enriched with *L. rhamnosus* cells which have the beneficial effects on animal health and this bacterial biomass rich spent fermentation media could bring additional value to the process.

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