Biblid: 1821-4487 (2013) 17; 2; p 64-67
UDK: 591.133.1:636.0

Original Scientific Paper
Originalni naučni rad

EVALUATION OF THE RESIDUE OF LACTIC ACID FERMENTATION ON STILLAGE AS AN ANIMAL FEED
ISPITIVANJE KVALITETA DŽIBRE NAKON MLEČNO-KISELINSKE FERMENTACIJE KAO HRANE ZA ŽIVOTINJE

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ABSTRACT

Lactic acid is a significant chemical for the food industry. Fermentative lactic acid production on wastes could significantly improve the economy and sustainability of the process. In this study, lactic acid production was performed by L. rhamnosus ATCC 7469 on a stillage from bioethanol production on waste bread. Under optimal conditions, in fed-batch fermentation lactic acid productivity of 1.80 g L^{-1} h^{-1} has been achieved with a cell number of above 10^9 CFU mL^{-1}. L. rhamnosus has shown high survival rate of over 85% in the presence of beef bile and at low pH value of MRS broth. The residuals after the fermentation were chemically analysed and their composition corresponded well with the values recommended for the feed. The digestible energy was 17452.25 kJ kg^{-1}, while metabolisable energy was 17360.83 kJ kg^{-1}. The high values of energy parameters indicated that an integrated process for lactic acid and feedstuff production could be a good strategy.

Key words: *lactic acid fermentation, stillage, animal food, probiotics, fed-batch fermentation.*

REZIME

Mlečna kiselina je važna supstanca za prehrambenu industriju. Proizvodnja mlečne kiseline fermentacijom na otpadnim sirovinama može značajno da poveća ekonomičnost i održivost procesa. U ovom radu je ispitivana proizvodnja mlečne kiseline pomoću L. rhamnosus ATCC 7469 na džibri iz proizvodnje bioetanola na otpadnom hlebu. Pod optimalnim uslovima, u dolivnom postupku je postignuta produktivnost mlečne kiseline od 1.80 g L-1 h-1 sa više od 109 CFU mL-1 bakterija. L. rhamnosus je pokazao visok stepen preživljavanja od preko 85% u prisustvu žučnih soli i pri niskoj pH vrednosti MRS bujona. Ostatak nakon mlečno-kiselinske fermentacije džibre je hemijski analiziran i pokazano je da dobijene vrednosti odgovaraju preporučenim. Svarljiva energija je iznosila 17452,25 kJ kg-1, dok je metabolička energija bila 17360,83 kJ kg-1. Visoke vrednosti energetskih parametara hraniva ukazuju da integrisani proces proizvodnje mlečne kiseline i stočne hrane na džibri predstavlja povoljnu strategiju.

Ključne reči: *mlečno-kiselinska fermentacija, džibra, stočna hrana, probiotici, dolivna fermentacija.*

INTRODUCTION

Lactic acid (LA) is a substance broadly used in food, pharmaceutical and chemical industry. Traditionally, LA is produced by fermentation of starch and sugar substrates by lactic acid bacteria, but new technologies for effective utilization of wastes and by-products of food and agro-industry are currently in research focus. Stillage, distillery wastewater from bioethanol production process has been mostly used as a feedstuff in dried (dried distillers' grains with soluble-DDGS) or wet form (wet distillers' grains-WDG) (*Mojović et al., 2012*), although the alternative strategies for utilization as a fertilizer and substrate for methane, hydrogen and acetic acid production were also discussed in literature (*Wilkie et al, 2000*).

Because of high energy requirements of drying for production of DDGS and susceptibility of wet stillage to contamination, a concept of fermented liquid stillage was introduced in animal diet (*Canibe and Jensen, 2012*). Positive effects of fermented animal feed include lower pH value and consequently prolonged shelf life of feed, prevalence of desired lactic acid bacteria to enteropathogens and often improved digestibility in fermented feed (*Canibe and Jensen, 2012*, *Scheuermann, 1993, Busch et al., 2004*). Therefore, for evaluation of feed quality the content of proteins, lipids, easily assimilative carbohydrates and fibres should be determined as well as digestibility which is used for calculation of energy value of the feed (*Semenčenko et al., 2012*).

The utilization of lactic acid bacteria as probiotics in animal diet has been extensively studied during the last decade because of strict regulations on application of antibiotics in animal nutrition (*Canibe and Jensen, 2012*, *Jensen and Mikkelsen, 1998*). Application of probiotics with proven efficiency against enteropatogens could improve animal health and act beneficially on growth of pigs and cattle (*Gaggia et al., 2010*, *Canibe et al., 2007*). The main criterion for probiotic additives is high number of viable bacterial cells in feedstuff (10^6-10^9 CFU kg^{-1}) (*European Food Safety Authority (EFSA), (2010*) if high survival rate of bacteria through intestinal tract was documented (*Anadón et al., 2006*).

In our previous studies wasted bread stillage was studied as a substrate for production of LA in batch processes (*Djukić-Vuković et al., 2012b*). Batch fermentation with *Lactobacillus rhamnosus* ATCC 7469 was optimised in order to achieve both high lactic acid concentration and high number of viable cells at the end of fermentation (*Djukić-Vuković et al., 2012a, Djukić-Vuković et al., 2012b*). To our knowledge, the wasted bread stillage used in this study was not previously studied for the production of lactic acid and/or animal food. In this study, fed-batch lactic acid fermentation on a whole wasted bread stillage was performed in order to improve the productivity of the process and avoid substrate inhibition. Dry residues after lactic acid fermentation were evaluated as a feedstuff on the basis of chemical characterization and the assessment of food energy parameters.

MATERIAL AND METHOD

The stillage remained after bioethanol production on wasted bread obtained from Reahem Ethanol Plant (Reahem, Srbobran, Serbia) was prepared for fermentation as described in our previous study (*Djukić-Vuković et al., 2012b*). *L. rhamnosus* ATCC 7469 strain was used for lactic acid fermentation of the media. The culture was propagated under microaerophilic conditions using Anaerocult ® C bags (Merck KGaA, Darmstadt, Germany) at 37 °C for 18 h in MRS broth before inoculation to fermentation medium. In fed-batch fermentation experiments, feeding solution which consisted of sterile stillage enriched with glucose at concentration of 140 g L^{-1} was supplied after decline of sugar concentration of the medium below 20 g L^{-1} with aim to maintain the concentration around 50 g L^{-1}. The feeding was performed until the fermentation flask was filled up to 70 % of the complete volume. The fermentations were preformed in 1000 mL flasks with initially 400 mL of the fermentation media under previously optimised conditions (41°C, gas-pack system with Anaerocult ® C bags, shaking of 90 rpm, without $CaCO_3$) (*Djukić-Vuković et al., 2012a*). In fermentation media, pH was adjusted to 6.5 in all samples and maintained at 6.5 by addition of 30 % solution of NaOH in four hour intervals. During the fermentation: pH, sugar consumption, lactic acid concentration and a number of living cells were analyzed. The content of reducing sugars was determined by 3,5-dinitrosalicylic acid, with standard curve set at 505 nm (*Miller, 1959*). Lactic acid concentration was determined by enzymatic method (L-/ D-Lactic acid assay, Megazyme®, Wicklow, Ireland) after deproteinization of the sample 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 for 48 h at 37°C, under microaerophilic conditions.

The survival of *L. rhamnosus* ATCC 7469 under the low pH value and in the presence of beef bile was studied according to procedure of *Yavuzdurmaz (2007)*. Briefly, the overnight culture of *L. rhamnosus* ATCC 7469 was inoculated (inoculum concentration of 5% (v/v)) in three different flasks with 75 ml of MRS broth. The first flask with MRS broth of pH =6.5 was used as a control, in the second flask the pH value was set at 2.5 by addition of 2M HCl solution, and in the third flask a beef bile solution was added in order to adjust the concentration of beef bile to 0.3% in MRS broth. The flasks were incubated at 37°C for 4 hours. The number of viable cells was determined at 0h point and after 1h, 2h, 3h and 4 hours of inoculation.

For comparison and assessment of the quality of fermented stillage, the wasted bread stillage and remains after fed-batch lactic acid fermentation were separately centrifuged (1500 rpm, 5 min, Sigma® 2-16, Shropshire, England) and solids were dried in an oven at 37°C for 12h. Prepared in this way, dry residues of unfermented and fermented stillage were further analyzed by methodology of *Semenčenko et al. (2012)* to determine the chemical characterization of feed quality. The content of dry matter, protein, oil, ash, nitrogen free extract (NFE), crude fibers, neutral detergent fibres (NDF), acid detergent fibres (ADF), acid detergent lignin (ADL), cellulose and hemicelluloses in dry residue of unfermented and fermented stillage were determined as previously reported in the study of Semenčenko et al. (2012). Additionally, digestibility and energy parameters of digestible (DE) and metabolisable energy (ME) were obtained as described in the study of *Semenčenko et al. (2012)*.

All chemicals used in experiments were of analytical grade. The measurements 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 Tukey test (Origin® 8, OriginLab Corporation). Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Kinetics of lactic acid and *L. rhamnosus* ATCC 7469 biomass production and consumption of sugar in fed-batch fermentation were presented in Fig. 1. The fastest growth of *L. rhamnosus* ATCC 7469 was noticed at the beginning of fermentation, before the first addition of fresh substrate (Fig.1). The number of viable cells was above 5×10^9 CFU mL^{-1} and such a high number of cells remained almost constant until the end of fermentation, after 54 hours. The maximal lactic acid concentration of 97 g L^{-1}, with lactic acid yield of approximately 87% and productivity of 1.80 g L^{-1} h^{-1} were attained at the end of fed-batch fermentation. The rate of sugar to lactic acid conversion declined after each addition of fresh substrate, so the sugar consumption was slower and every new cycle lasted longer. However, in the fed-batch process 47.6 % higher lactic acid concentration was obtained in comparison to batch process on the same substrate (*Djukić-Vuković et al., 2012b*). With the fed-batch strategy, a problem of substrate inhibition has been overcome and high productivity was achieved. In the processes on complex substrates like stillage, the extraction of product is usually the most expensive phase and high concentration of product in media is critical for good profitability. Previously, fed-batch strategy has been used for lactic acid production on other, mainly synthetic substrates *(Ding and Tan, 2006, John et al., 2007)*.

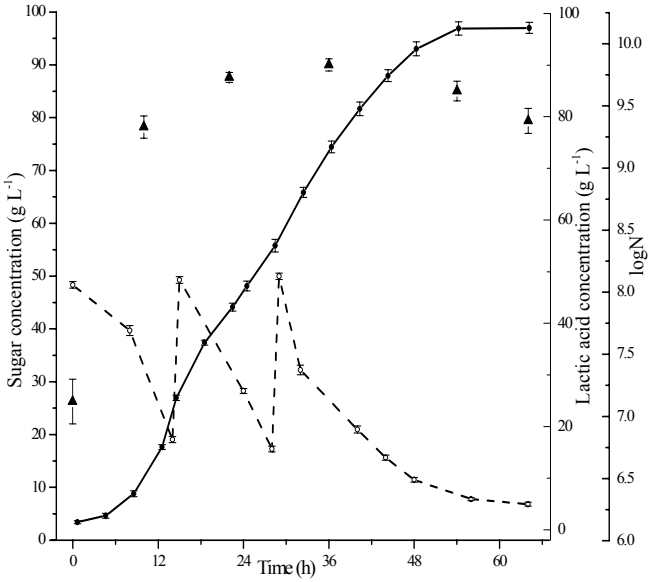

Fig. 1. Time course of fed-batch fermentation on distillery stillage. Symbols: ▲- number of viable cells, solid line - lactic acid concentration, dashed line – concentration of reducing sugars. Vertical bars represent ± standard deviation of three measurements

Very high lactic acid concentration of 180 g L^{-1} was reported by *Ding and Tan (2006)* in the fed-batch fermentation of synthetic media but with exponential addition of glucose and yeast extract. In the process presented in this paper media was not supplemented with external nitrogen sources since the stillage was rich in proteins and contained more than 58% of proteins, dry matter basis (*Djukić-Vuković et al., 2012b*). In a fed-batch fermentation of deproteinized whey with a mixed culture of *L. casei* and *Lactococcus lactis*, a lower yield of 0.77 g g^{-1} and lower lactic acid concentration of 46 g L^{-1} were achieved (*Roukas and Kotzekidou, 1998*).

High number of viable cells at the end of fermentation indicated a potential of spent fermentation media for use as a probiotic feed. Beneficial effects of probiotics depend on their survival through harsh gastric conditions of low pH in stomach and in the presence of bile in the intestine. Many studies confirmed that probiotic characteristics are species/strain specific and should be proven for every strain (*Shah, 2000*, *Gaggia et al., 2010*). In Fig. 2. the survival of *L. rhamnosus* ATCC 7469 in the presence of beef bile and under the low pH value of 2.5 is presented. The highest decrease in the number of cells was noticed instantly after addition of beef bile into the MRS broth with *L. rhamnosus* (Fig.2). The cell number was rising during the first two hours and after the second hour of incubation it started to decrease. The low pH value of 2.5 affected the number of bacterial cells less than the presence of beef bile, although similar curve pattern was noticed in both samples after the second hour of incubation.

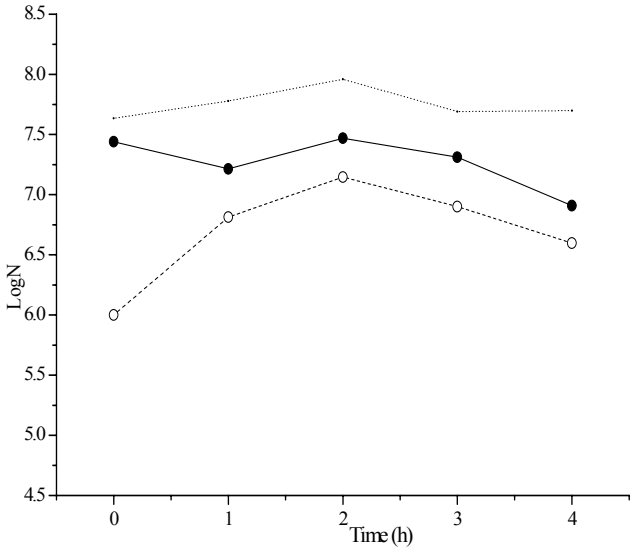

Fig. 2. Number of viable cells of L. rhamnosus ATCC 7469 during the 4 hour incubation in simulated gastric conditions. Symbols: dot line - MRS broth with optimal pH value of 6.5 for LA bacteria; solid line solid symbols – MRS broth with pH value set at 2.5; dashed line, open symbols – MRS broth with 0.3% beef bile

Generally, after four hours of incubation the survival rate was above 85% in both samples. The viable cell number of 8.1×10^6 CFU mL^{-1} was detected in the sample with low pH, and 4×10^6 CFU mL^{-1} in the sample with 0.3% beef bile added. The numbers of bacteria after *in vitro* simulated exposure to gastric environment were very high, especially if we have in mind that total number of *Lactobacillus* sp. and *Enterococcus* sp. cells in a healthy animal intestine counts up to 10^5 - 10^8 CFU g^{-1} (*Anadón et al., 2006*). Also, the survival rate of *L. rhamnosus* ATCC 7469 was higher than the values reported for *L. bulgaricus*, *L. johnsonii* B-2178, *L. gasseri* B-14168 and *L. salivarius* B-1950 strains in a study of *Yavuzdurmaz (2007)* without supplementation by external proteins. Based on the observation of *Yavuzdurmaz (2007)* even higher survival rate of *L. rhamnosus* ATCC 7469 could be expected if it is used as a feed additive due to buffering effect of proteins present in stillage (*Djukić-Vuković et al., 2012b*). Ability of strain *L. rhamnosus* ATCC 7469 to adhere *in vitro* to Caco-2 cell line was reported in literature which is very important characteristic for potential probiotics (*Tuomola and Salminen*, *1998*).

After the fermentation, dried spent fermentation media was chemically characterized and compared with unfermented stillage, prepared in same way. Composition of dry stillage before fermentation and dry residue after lactic acid fermentation is presented in Table 1. As a result of lactic acid fermentation of wasted bread stillage, the content of proteins declined and the content of oil significantly increased (Tab 1). This could be a result of *L. rhamnosus* capability to produce conjugated fatty acids (*Kishino et al., 2002*). The content of proteins in dry residue after LA fermentation was higher than in corn DDGS, while in unfermented stillage it was even higher than the recommended value reported in Book of Regulations for Feed Quality of Republic of Serbia (*Serbian Government, 2010*). The content of proteins was lower after the fermentation but it was still very high and satisfactory compared to the values suggested in the literature. Also, the content of NFE which represents easily assimilative carbohydrates for the growth of lactic acid bacteria was significantly lower after the fermentation. This result corresponds well with the study of *Canibe et al. (2012)*. They noticed that fermentation of forage resulted in lower content of assimilative sugars; however the digestibility of feed was improved. The digestibility of the stillage used in this work was slightly improved by fermentation and it amounted more than 96% (Tab 1.), which is higher than digestibility of corn DDGS (*Semenčenko et al., 2012*). The improvement in digestibility was also documented by *Scheuermann (1993)* when feed was inoculated with probiotic biomass.

Table 1. Chemical characterization of dry stillage before and dry residue of the stillage after LA fermentation

Composition	Dry stillage before LA fermentation (g kg^{-1} DM)	Dry residue after LA fermentation (g kg^{-1} DM)
Dry weight (% of total stillage)	89.17±0.18	89.45±0.22
Protein	402.40±1.27	386.52±2.97
Oil	49.20±0.71	61.15±1.58
Ash	3.50±0.62	6.04±1.13
NFE	493.50 ±3.11	469.00±2.85
Crude fibers	20.40±2.12	23.10±1.86
NDF	60.55±6.58	62.20±6.55
ADF	20.40±2.10	23.10±1.90
ADL	3.40±0.30	4.62±0.71
Hemicelluloses	40.15±4.45	38.90±4.92
Cellulose	17.00±2.40	18.46±2.88
Digestibility	964.45±1.34	966.95±0.51
DE (kcal kg^{-1})	4206.49±0.99	4175.18±0.79
ME (kcal kg^{-1})	4183.56±1.1	4153.31±2.4

Content of fibres was not significantly affected by fermentation, probably because *L. rhamnosus* ATCC 7469 did not possess cellulolytic activity. Total content of crude fibres, as well as the content of different fibre components (ADF, NDF, ADL) were much lower than the values reported for corn DDGS by *Semenčenko et al. (2012)*. It was expected because wasted bread is lower in content of cellulose and other fibres than corn. Because of low content of fibres and high content of proteins and NFE, the digestibility of stillage was very high in comparison to corn DDGS. The highest achieved digestibility of corn DDGS was 81% (*Semenčenko et al., 2012*).

Energy parameters of digestible and metabolisable energy revealed that fermented stillage was lower in energy than unfermented stillage but still high in DE and ME values of more than 16720.00 kJ kg^{-1} (Tab. 1.). In the samples of corn DDGS of different hybrids the highest metabolisable energy was 16158.62 ± 5.1 kJ kg^{-1}, while digestible energy was 17222.85 ± 21.2 kJ kg^{-1} (*Semenčenko et al., 2012*).

Dry residue of wasted bread stillage after lactic acid fermentation corresponded in all investigated parameters with regulations for high quality feed, primary for monogastric animals (*Serbian Government, 2010).* The high number of viable cells in feed is important criteria for animal food enriched with probiotics. The viable cell number of approximately 10^9 CFU mL^{-1} bacteria in complete feedstuff is recommended for the use of probiotic enriched animal feed (*Djukić-Vuković et al., 2012b*). The number of bacteria at the end of fed-fermentation was above 5×10^9 CFU ml^{-1} which implies that fermented stillage is a good quality animal feed with high content of nitrogen and high energy value, enriched with lactic acid bacteria.

CONCLUSION

The wasted bread stillage was studied as a substrate for parallel production of lactic acid in fed-batch fermentation and animal feed enriched with biomass of *L. rhamnosus* ATCC 7469. The high values of lactic acid concentration (97.1 g L^{-1}), yield (87%) and productivity (1.80 g L^{-1} h^{-1}) were obtained after 54 h of intense fermentation with a viable cell number of above 5×10^9 CFU mL^{-1} at the end of fermentation process. *L. rhamnosus* ATCC 7469 strain has shown high survival rate of over 88% at low pH of 2.5 and 85.7% in the presence of 0.3% beef bile in media. The residues after lactic acid fermentation of the stillage were dried and chemically analyzed in order to evaluate composition and compare it with Regulation currently valid for animal feed in Republic of Serbia. It was found that lactic acid fermented stillage was completely in accordance with recommended values for feed. It was very rich in proteins and NFE and poor in fibres. The digestibility of more than 96% and DE and ME of over 4000 kcal kg^{-1} DM basis qualify fermented stillage as a high energy quality feed primary for monogastric animals.

ACKNOWLEDGEMENTS: Research presented in this paper was funded by Ministry of Education, Science and Technological development, Republic of Serbia, project number TR 31017.

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Received: 28.02.2013. Accepted: 25.03.2013.