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POTATO STILLAGE AND SUGAR BEET MOLASSES AS A SUBSTRATE FOR PRODUCTION OF LACTIC ACID AND PROBIOTIC BIOMASS

KROMPIROVA DŽIBRA I MELASA ŠEĆERNE REPE KAO SUPSTRAT ZA PROIZVODNJU MLEČNE KISELINE I PROBIOTSKE BIOMASE

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ABSTRACT

Distillery stillage is abundant industrial waste with a great potential for utilization as a substrate in production of valuable bio-based products. Processing of distillery stillage on cost effective way can significantly decrease the price of bioethanol as alternative fuel and provide a considerable benefit to the environment. In this paper, combined potato stillage and sugar beet molasses substrate is evaluated for integrated lactic acid and probiotic biomass production by Lactobacillus rhamnosus ATCC 7469. Waste substrate based on potato stillage and sugar beet molasses enabled lactic acid and biomass production with maximal volumetric lactic acid productivity of 1.11 g L^{-1} h^{-1} and maximal number of viable L. rhamnosus ATCC 7469 cells of 1.1×10^{9} CFU mL^{-1}. Residue after removal of lactic acid could be recommended as probiotics and betaine-enriched animal feed.

***Key words**: lactic acid, potato stillage, molasses, probiotics, animal feed.*

REZIME

Destilerijska džibra je lako dostupan industrijski otpad koji nastaje u proizvodnji bioetanola, a zbog svog kompleksnog sastava može biti potencijalno dobar supstrat u mnogim biotehnološkim procesima. Imajući u vidu potrebu za povećanjem postojećih i izgradnjom novih kapaciteta za proizvodnju bioetanola, iskorišćavanje otpadne džibre može biti značajno sa aspekta povećanja konkurentnosti bioetanola kao alternativnog goriva, a može imati i pozitivan uticaj na životnu sredinu. U radu je ispitivana mogućnost iskorišćavanja krompirove džibre i melase šećerne repe kao supstrata za paralelnu proizvodnju mlečne kiseline i bakterijske biomase, pomoću probiotskog soja Lactobacillus rhamnosus ATCC 7469. U šaržnoj fermentaciji na ovom kombinovanom supstratu bez dodatnog obogaćivanja vitaminima, mineralnim materijama ili izvorima azota je postignuta maksimalna produktivnost od 1,11 g L^{-1} h^{-1}, maksimalan broj vijabilnih ćelija od $1,1\times10^{9}$ CFU mL^{-1} i koncentracija mlečne kiseline od 18,08 g L^{-1}. Krompirova džibra se može koristiti kao supstrat za rast probiotske biomase i može biti adekvatna zamena za skupe, najčešće korišćene organske izvore azota, kao što su kvaščev ekstrakt ili pepton. Usled nemogućnosti L. rhamnosus ATCC 7469 da podjednako dobro metaboliše sve šećere u melasi šećerne repe za efikasniju proizvodnju mlečne kiseline treba razmotriti druge izvore ugljenika ili izvršiti selekciju Lactobacillus sp. koji efikasno fermentiše saharozu iz melase. Zbog značajnog rasta bakterijske biomase tokom procesa, fermentisani medijum koji zaostaje nakon ekstrakcije mlečne kiseline predstavlja vredan sporedni proizvod fermentacije bogat probiotskom biomasom i betainom koji se može koristiti kao visoko kvalitetna hrana za životinje.

***Ključne reči:** mlečna kiselina, krompirova džibra, melasa, probiotici, hrana za životinje.*

INTRODUCTION

Lactic acid (LA) is widely used in food, pharmaceutical, cosmetics and chemical industries. Recently, LA has been selected as one of the most promising chemical building blocks derived from sugar since its application range is constantly increasing (*Choi et al., 2015*). A considerable amount of produced LA has been used in manufacturing of polylactic acid (PLA), a suitable substitute for petrochemical derived products. Although LA could be produced either by chemical synthesis or microbial fermentation, about 90 % of LA produced globally is obtained by fermentation routes. Microbial fermentation is more favorable since desired L (+) or D (-) isomer of LA could be obtained from various raw materials using selected lactic acid bacteria (LAB) (*Cui et al., 2011).* Utilization of low cost raw materials which are rich in sugars, nitrogen compounds, minerals and other growth factors is essential for economically sustainable LA production. Lignocellulosic materials are inexpensive and abundant substrate however complexity of required pretreatment methods still limits their commercial use for LA production (*Abdel-Rahman et al., 2011*). On the other hand, agro-industrial materials which contain starch or fermentable sugars and other valuable nutrients like protein sources, vitamins and minerals (e.g. potato residues, date juice, wheat bran and soybean hydrolysates) *(Martinez et al., 2013)* are simpler for managing and preferred for LA production. The utilization of distillery stillage and sugar beet molasses, as replacement for expensive nitrogen and carbon sources, for integrated LA and biomass production is an innovative and cost-effective approach studied in this paper.

Distillery stillage is main by-product of bioethanol production. A large amount of liquid stillage remained after distillation of bioethanol is significant source of water and land pollution since it has low pH value and high content of organic constituents (*Mojović et al., 2012)*. Depending on the stillage origin and its chemical composition, the stillage could be used as a substrate in another production processes or must be subjected to adequate treatment methods prior to its discharge to waterflows or land area *(Đukić-Vuković et al., 2016).*

Sugar beet molasses is a by-product of sugar beet processing which has high carbohydrate content, but also it is valuable source of many micronutrients, like vitamins and minerals. Due to its nutritive value molasses is used in many food and non-food processes (*Krulj et al., 2014*).

In the present study, the possibility of potato stillage and sugar beet molasses utilization for integrated LA and biomass production was evaluated. Besides production of valuable products, this approach could be a possible solution for a large environmental problem of distillery stillage disposal.

MATERIAL AND METHOD

Substrate preparation

The stillage remained after bioethanol production on wasted potato was obtained from Reahem Ethanol Plant (Reahem, Srbobran, Serbia) and sugar beet molasses was obtained from Alpis Ethanol Plant (Alpis, Kovin, Serbia). The concentration of reducing sugars in the stillage (originally about 15 g L^{-1}) was set at approximately 55 g L^{-1} with addition of sugar beet molasses. This prepared substrate was used for LA fermentation and is referred to as stillage/molasses substrate. After adjustment of pH in the substrate to 6.5 with 30 % NaOH (Sigma-Aldrich, USA) medium was sterilized at 121 °C for 20 minutes.

Microorganism

Lactobacillus rhamnosus ATCC 7469, a homofermentative L (+) lactic acid strain, used in this experiment was obtained from American Type Culture Collection (ATCC, Rockville, USA). The culture was propagated under anaerobic conditions at 37 °C for 18 h in Man Rogosa Sharpe broth (MRS) (Fluka, USA). This prepared culture was used as an overnight seed culture for initiation of LA fermentation.

Lactic acid fermentation

LA fermentations were performed as batch cultures with shaking (100 rpm, KS 4000i control, IKA®, Werke GmbH & Co. KG, Staufen, Germany) at temperature of 41 °C after inoculation with 5 % (v/v) overnight seed culture. The fermentations were performed in 500 mL flasks with 200 mL of the fermentation media under microaerophilic conditions for 48 h. During the fermentation, pH was adjusted to 6.5 by addition of 30 % NaOH solution in four hours intervals. Samples were aseptically withdrawn and the substrate consumption, LA concentration and a number of living cells were further analyzed.

Analytical methods

The total nitrogen in the stillage and molasses was estimated by Kjeldahl method *(AOAC, 2000)*. In order to determine concentration of reducing sugars, the sample solution was hydrolyzed in HCl at 100 °C for 10 min and then neutralized with NaOH solution. The concentration of reducing sugars was estimated by 3, 5-dinitrosalicylic acid method (*Miller, 1959*). A calibration curve was set at 505 nm using standard sucrose solutions. The concentration of LA was determined by enzymatic method (L-/ D-Lactic acid assay, Megazyme®, Wicklow, Ireland) after deproteinization of the sample. A number of viable cells was estimated using pour plate technique on MRS agar after incubation for 48 h at 37 °C. All chemicals used in experiments were analytical grade.

Statistical analysis. The experiments were done in triplicate. All values are expressed as means ± standard deviation.

RESULTS AN DISCUSSION

The carbon and nitrogen content of the stillage/molasses substrate and MRS broth as a standard media for cultivation of LAB is presented in Fig. 1.

The nitrogen content in potato stillage (1.8 g L^{-1}) mainly originated from the residual yeast biomass, but also from the substrates used for the ethanol fermentation. Compared with nitrogen content in MRS broth which is commonly used for cultivation of LAB (Fig. 1), it can be concluded that the potato stillage can be used as a source of nitrogen for growth of fastidious LAB instead of yeast extract, peptone and other expensive organic nitrogen sources.

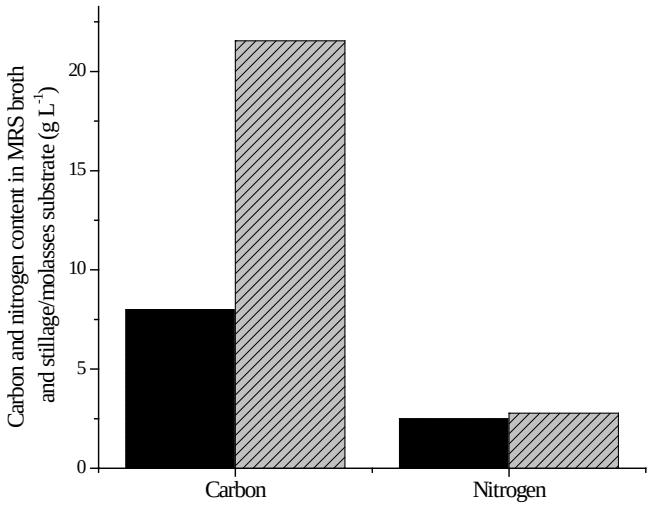

Fig. 1. Comparison of carbon (from sugars) and nitrogen content in MRS broth and stillage/molasses substrate. Symbols: black bars — MRS broth, grey bars — stillage/molasses substrate. Assumptions: Yeast extract, meat extract and peptone from MRS broth contain on average 10 % of nitrogen; Sugars from MRS broth, potato stillage and molasses contain on average 40 % of carbon

Based on literature data, nitrogen content of distillery stillage originated from various substrates is highly variable and can range from 0.65 g L^{-1} in cassava stillage *(Wilkie et al., 2000)* to 10.81 g L^{-1} in wasted bread stillage *(Djukić-Vuković et al., 2013)*. Distillery stillage is a complex nutrient source which besides nitrogen contains valuable products of yeast fermentation, complex of B vitamins, minerals and other growth supporting compounds (*Mojović et al., 2012)*. However, due to effective bioethanol fermentation, potato stillage is destitute source of fermentable sugars and for effective LA production it must be complemented with other carbon source. In contrast to the stillage, sugar beet molasses is an abundant source of sugar, mainly sucrose, but it also contains a lesser amount of glucose and fructose. Molasses has relatively low nitrogen content that can be assimilated by microorganisms since a considerable part of nitrogen compounds represents betaine (*Jevtić-Mučibabić et al., 2011*) and for LA fermentation it requires nitrogen supplementation *(Kotzamanidis et al., 2002)*. Based on chemical composition of potato stillage and sugar beet molasses, these two by-products combined together could be a promising substrate for growth of LAB and LA production. Kinetics of LA and biomass production as well as total sugar consumption in batch fermentation on this combined substrate is presented in Fig. 2, while the values of significant parameters of LA fermentation are presented in Table 1. The highest LA productivity of 1.11 g L^{-1} h^{-1} was achieved in 12th hour of fermentation, after that time the rates of sugar consumption and LA production were notably lower and this resulted in a large residual sugar concentration and lower volumetric productivity at the end of fermentation. The presence of different sugars in the fermentation media, as well as relatively low content of glucose (% of total sugar content: sucrose 64.11, glucose 33.83 and fructose 2.05) corresponds well with the amount of produced LA during the first phase of the fermentation. Many studies on different substrates have shown that *L. rhamnosus* is able to convert glucose to LA with very high efficiency (*Lu et al., 2010; Li et al., 2010; Djukić-Vuković et al., 2013*).

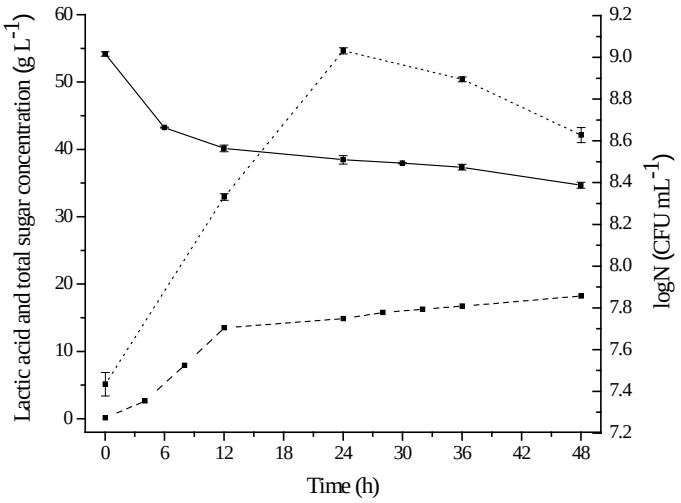

Fig 2. Kinetics of lactic acid production in batch fermentation on stillage/molasses substrate by L. rhamnosus ATCC 7469. Symbols: dashed line — lactic acid concentration, solid line — total sugar concentration, dotted line — number of viable cells

In the study of *Lu et al. (2010)* LA concentration of 50.20 g L^{-1} and productivity of 1.26 g L^{-1} h^{-1} were achieved on fermentation media which consisted of wheat bran hydrolysate, persimmon juice and glucose. The residual sugar concentration of 34.37 g L^{-1} in our study implies that the conversion of sucrose to LA by *L. rhamnosus* is not the preferable metabolic pathway. Inefficient conversion of sucrose from different raw materials had also been reported in several studies (*Senedese et al., 2015; Chan-Blanco et al., 2003; Nancib et al., 2005*). *Senedese et al. (2015)* studied LA production on molasses and yeast extract by *L. rhamnosus* ATCC 10863. The maximal achieved concentration of LA in that study was 16.5 g L^{-1} with corresponding yield of 1.17 g L^{-1} h^{-1} which is comparable with the results obtained in present study. *Nancib et al., (2005)* noticed that sucrose was not metabolized in fermentation of data juice by *L. casei*. subsp. *rhamnosus*, while glucose and fructose were simultaneously utilized. These findings are also reported by *Chan-Blanco et al., (2003)* in LA fermentation on waste banana by *L. casei*. The maximal achieved productivity on this waste substrate was only 0.13 g L^{-1} h^{-1} *(Chan-Blanco et al., 2003).* Higher productivity of 1.49 g L^{-1} h^{-1} was obtained in fermentation on waste banana enriched with yeast extract and mineral salts *(Chan-Blanco et al., 2003)*. Although, the productivity on waste banana was somewhat higher than the results obtained in our study on stillage/molasses substrate, the addition of rather expensive nitrogen source (yeast extract) and minerals was performed there. Results of the present study indicate that potato stillage is a sufficient source of nitrogen for efficient conversion of glucose to LA, but after depletion of glucose from media, remained sucrose as a carbon source was not satisfactory for further LA production by *L. rhamnosus*.

The highest number of viable *L. rhamnosus* ATCC 7469 cells (1.1×10^9 CFU mL^{-1}) was obtained after 24 h of fermentation and at the end of fermentation it amounted 4×10^8 CFU mL^{-1}. Probiotic characteristics of the strain used in this study have been extensively studied and documented *(Tuomola et al., 1998; Ampatzoglou et al., 2010)*. The recommended concentration for most probiotics is approximately 10^9 CFU kg^{-1} of feed *(Simon et al., 2005)*. Also, in digestive microflora of animals, the quantity of *Lactobacillus* probiotic species amounts 10^7-10^8 CFU g^{-1} (*Anadón et al., 2006*). Based on this data, the stillage remained after LA extraction with *L. rhamnosus* ATCC 7469 biomass has potential for using as a biomass-enriched animal feed. Presence of betaine could be another benefit of application of the residual fermented stillage in animal nutrition, since betaine has potential nutritional and physiological functions in ruminants and monogastric animals and commercially is available as feed additive (*Eklund et al., 2005*).

A significant grow of LAB biomass on this waste substrate represents great value of this process, however for more effective LA production by *L. rhamnosus* ATCC 7469 the enrichment of potato stillage with another carbon source should be examined. Alternatively, the achievement of higher LA productivity on this waste substrate could be examined by selecting the microorganism which could also effectively utilize the sucrose from the molasses.

Table 1. The values of significant parameters of lactic acid fermentation on stillage/molassess substrate

Initial sugar concentration	g L^{-1}	53.93
LA concentration	g L^{-1}	18.08
LA yield	g g^{-1}	0.34
LA yield coefficient	g g^{-1}	0.92
Volumetric LA productivity	g L^{-1} h^{-1}	0.38
Maximal volumetric LA productivity	g L^{-1} h^{-1}	1.11
Number of viable cells	CFU mL^{-1}	4×10^8

CONCLUSION

Results obtained in this study indicate that potato stillage could be used as an adequate substitution for expensive commonly used nutrient sources in LA fermentation, such as yeast extract or peptone. Waste substrate based on potato stillage and sugar beet molasses could be a suitable media for high quality animal feed production rich in probiotic LAB and betaine. However, for improvement of the process and more effective LA production a combination of potato stillage with other carbon source is suggested. In addition, this way of processing agro-industrial waste is environmentally favorable and could provide an additional value to the process.

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REFERENCES

Abdel-Rahman, A., Tashiro, Y., Sonomoto, K. (2011). Lactic acid production from lignocellulose-derived sugars using lactic acid bacteria: overview and limits. Journal of biotechnology, 156 (4), 286-301.

Ampatzoglou, A., Schurr, B., Deepika, G., Baipong, S., Charalampopoulos, D. (2010). Influence of fermentation on the acid tolerance and freeze drying survival of *Lactobacillus rhamnosus* GG. Biochemical Engineering Journal, 52 (1), 65-70.

Anadón, A., Martínez-Larrañaga, M. R., Martínez, M. A. (2006). Probiotics for animal nutrition in the European Union. Regulation and safety assessment. Regulatory Toxicology and Pharmacology, 45 (1), 91-95.

AOAC. (2000). *Official Methods of Analysis of AOAC International*, 17th edn. AOAC International, Gaithersburg, MD, USA.

Chan-Blanco, Y., Bonilla-Leiva, A. R., Velazquez, A. C. (2003). Using banana to generate lactic acid through batch process fermentation. Applied microbiology and biotechnology, 63 (2), 147-152.

Choi, S., Song, C. W., Shin, J. H., Lee, S. Y. (2015). Biorefineries for the production of top building block chemicals and their derivatives. Metabolic engineering, 31 (28), 223-239.

Cui, F., Li, Y., Wan, C. (2011). Lactic acid production from corn stover using mixed cultures of *Lactobacillus rhamnosus* and *Lactobacillus brevis*. Bioresource technology, 102 (2), 1831-1836.

Djukić-Vuković, Aleksandra, Mladenović, Dragana, Radosavljević, M., Kocić-Tanackov, Sunčica, Pejin, Jelena, Mojović, Ljiljana. (2016). Wastes from bioethanol and beer productions as substrates for l (+) lactic acid production–A comparative study. Waste Management, 29 (48), 478-482.

Djukić-Vuković, Aleksandra, Mojović, Ljiljana, Nikolić, Svetlana, Pejin, Jelena, Kocić-Tanackov, Sunčica, Mihajlovski, Katarina (2013). Distillery stillage as a new substrate for lactic acid production in batch and fed-batch fermentation. Chemical Engineering Transactions, 34, 97-102.

Eklund, M., Bauer, E., Wamatu, J., Mosenthin, R. (2005). Potential nutritional and physiological functions of betaine in livestock. Nutrition Research Reviews, 18 (01), 31-48.

Jevtić-Mučibabić, Rada, Grbić, Jasna, Mišljenović, Nevena, Koprivica, Gordana, Kuljanin, Tatjana, Radivojević, S. (2011). Nitrogen compounds in the molasses. Journal on Processing and Energy in Agriculture, 15 (3), 169-172.

Kotzamanidis, Ch., Roukas, T., Skaracis, G. (2002). Optimization of lactic acid production from beet molasses by *Lactobacillus delbrueckii* NCIMB 8130. World Journal of Microbiology and Biotechnology, 18 (5), 441-448.

Krulj, Jelena, Jevtić-Mučibabić, Rada, Grbić, Jasna, Brkljača, Jovana, Milovanović, I., Filipčev, Bojana, Bodroža-Solarov, Marija. (2014). Determination of betaine in sugar beet molasses. Journal on Processing and Energy in Agriculture, 18 (4), 179-181.

Li, Z., Han, L., Ji, Y., Wang, X., Tan, T. (2010). Fermentative production of L-lactic acid from hydrolysate of wheat bran by *Lactobacillus rhamnosus*. Biochemical Engineering Journal, 49 (1), 138-142.

Lu, Z., He, F., Shi, Y., Lu, M., Yu, L. (2010). Fermentative production of L (+)-lactic acid using hydrolyzed acorn starch, persimmon juice and wheat bran hydrolysate as nutrients. Bioresource technology, 101 (10), 3642-3648.

Martinez, F.A, Balciunas, E.M., Salgado, J.M., González, J.M., Converti, A., de Souza Oliveira, R.P. (2013). Lactic acid properties, applications and production: a review. Trends in food science & technology, 30 (1), 70-83.

Miller, G. L. (1959). Use of dinitrosalicylic acid for determining reducing sugars. Analytical Chemistry, 31 (3), 426–428.

Mojović, Ljiljana, Pejin, Dušanka, Rakin, Marica, Pejin, Jelena, Nikolić, Svetlana, Djukić-Vuković, Aleksandra. (2012). How to improve the economy of bioethanol production in Serbia. Renewable and Sustainable Energy Reviews, 16 (8), 6040-6047.

Nancib, A., Nancib, N., Meziane-Cherif, D., Boubendir, A., Fick, M., Boudrant, J. (2005). Joint effect of nitrogen sources and B vitamin supplementation of date juice on lactic acid production by *Lactobacillus casei subsp. rhamnosus*. Bioresource Technology, 96 (1), 63-67.

Senedese, A.L.C., Maciel Filho, R. and Maciel, M.R.W. (2015). L-Lactic Acid Production by *Lactobacillus rhamnosus* ATCC 10863. *The Scientific World Journal*, http://dx.doi.org/10.1155/2015/501029.

Simon, O., Vahjen, W., Scharek, L. (2005). Micro-organisms as feed additives-probiotics. Advances in pork Production, (16) 161-167.

Tuomola, E.M., Salminen, S.J. (1998). Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures, International journal of food microbiology, 41 (1), 45-51.

Wilkie, A.C., Riedesel, K.J., Owens, J.M. (2000). Stillage characterization and anaerobic treatment of ethanol stillage from conventional and cellulosic feedstocks. Biomass and Bioenergy, 19 (2), 63-102.

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