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DISTILLERY WASTES TO LACTIC ACID: BIOREFINERY APPROACH

DESTILERIJSKI OTPAD ZA PROIZVODNJU MLEČNE KISELINE I STOČNE HRANE: BIORAFINERIJSKI PRISTUP

Aleksandra ĐUKIĆ-VUKOVIĆ[*], Ljiljana MOJOVIĆ[*], Jelena PEJIN[**], Sunčica KOCIĆ-TANACKOV[**]
[*]University of Belgrade, Faculty of Technology and Metallurgy, 11000 Belgrade, Karnegijeva 4, Serbia
[**]University of Novi Sad, Faculty of Technology, 21000 Novi Sad, Bulevar cara Lazara 1, Serbia
e-mail: adjukic@tmf.bg.ac.rs

ABSTRACT

The cost of distillery wastewater treatment is an important issue in overall economy of bioethanol production. Besides traditional utilization of distillery wastewater or stillage in animal nutrition, its chemical complexity is offering other possibilities.

Complete distillery wastewater and liquid stillage from bioethanol production were studied as substrates for batch, fed-batch and repeated batch lactic acid fermentation by Lactobacillus rhamnosus ATCC 7469 for parallel production of lactic acid, probiotics and feed.

Both of the substrates, liquid stillage and complete stillage enabled effective production of lactic acid with the process productivity of approximately 1.80 g $h^{-1}L^{-1}$, however utilization of the remains was different. The first strategy enabled conversion of liquid distillery wastewater into lactic acid and high value feed additive with zeolite immobilized probiotic biomass. The second strategy enabled effective lactic acid production on the complete stillage with additional production of high quality fermented feed for monogastric animals.

***Key words**: lactic acid fermentation, stillage, animal feed, probiotics, biorefinery.*

REZIME

Troškovi tretmana destilerijske otpadne vode značajno utiču na ukupnu cenu proizvodnje bioetanola. Pored tradicionalne upotrebe destilerijske otpadne vode ili džibre u ishrani stoke, složeni hemijski sastav džibre pruža i alternativne mogućnosti iskorišćenja.

Kompletna destilerijska džibra iz proizvodnje bioetanola na otpadnom hlebu je korišćena kao supstrat za mlečno-kiselinsku fermentaciju pomoću Lactobacillus rhamnosus ATCC 7469 u cilju proizvodnje mlečne kiseline, probiotika i stočne hrane. Koncentracija mlečne kiseline, broj živih ćelija i koncentracija redukujućih šećera su praćeni u toku fermentacije.

Mlečno-kiselinska fermentacija tečnog dela destilerijske džibre je izvođena šaržno sa slobodnim i imobilisanim biokatalizatorom i takođe šaržno sa recikulacijom pomoću imobilisane biomase na zeolitu. U mlečno-kiselinskoj fermentaciji cele džibre postignuta je visoka koncentracija mlečne kiseline dok je fermentisani čvrst ostatak džibre pokazao adekvatna svojstva za upotrebu u ishrani monogastričnih životinja. Obe vrste supstrata, tečni deo džibre i kompletna džibra omogućavaju efikasnu proizvodnju mlečne kiseline sa produktivnošću od oko 1,80 g L^{-1} h^{-1}, ali nastaju različiti sporedni proizvodi. Prva strategija omogućava korišćenje tečnog dela džibre za proizvodnju mlečne kiseline i vrednog probiotskog dodatka ishrani životinja (sa brojem živih ćelija od preko 10^{10} CFU ml^{-1}) uz mogućnost iskorišćenja zaostalog nefermentisanog dela džibre za proizvodnju stočne hrane. Druga strategija podrazumeva fermentaciju cele džibre u kojoj paralelno nastaju mlečna kiselina i fermentisani čvrsti ostatak kao visoko vredna hrana za monogastrične životinje. U oba procesa nije bilo potrebno obogaćivati džibru ni sa mineralima ni sa skupim izvorima azota.

***Kljucne reći**: mlečno-kiselinska fermentacija, džibra, stočna hrana, probiotici, biorafinerijski pristup.*

INTRODUCTION

Increased production of wastes of agro-food industry represents significant environmental problem as well as an opportunity for biorefinery processes. Chemical complexity of these substrates, low price and their wide availability encourage their utilization in biotechnology. Variety of renewable substrates has shown applicability in different biorefinery processes: carbohydrate substrates like lignocelluloses, potato, corn or wheat have been utilized in production of biofuels, propanol, 1.3-propandiol, butanol, lactic acid, succinic acid and other platform chemicals (*Mennon and Rao, 2012; Zhu and Zhuang, 2012*). However, the need of these cultures for arable land and competition with food stimulate intense research on application of wastes and by-products as alternative substrates for production of biochemicals (*Star-COLIBRI, 2015*).

Potential of distillery stillage as a substrate for lactic acid production and different production approaches were studied in this paper. Lactic acid has a wide application range in pharmaceutical and food industry due to increased production of polylactides (PLA), biocompatible and biodegradable polymers. Polylactides are eco-friendly alternative to petrol based plastics and could be used both for production of food packaging or for biomedical applications (*Jiménez et al., 2015*). The important issue in the substitution of oil-based polymers by polylactides is their high price (*Okano et al., 2010*), so the establishment of efficient, sustainable and economically feasible biorefinery processes for the production of lactic acid as a precursor for polylactides is challenging and important task. The lactic acid productivities obtained in different fermentations on renewable substrates were within range of 0.73 g L^{-1} h^{-1} on recycled paper sludge (*Marques et al., 2008*) to 2.83 g L^{-1} h^{-1} on Jerusalem artichoke hydrolysate (*Shi et al., 2012*). Significant improvements were achieved by optimisation of the fermentation conditions, bioprocess intensification (using different immobilization strategies, recirculation of biomass, optimised feeding of media) and engineering of the producing microorganisms which led to higher productivities (*Abdel-Rahman et al., 2013*).

In this study both complete stillage and just its liquid part were evaluated as substrates for lactic acid fermentation.

MATERIAL AND METHOD

Stillage preparation for batch and fed-batch fermentations

The stillage remained after bioethanol production on wasted bread was obtained from Reahem Ethanol Plant (Reahem, Srbobran, Serbia). The pH of the stillage was adjusted to 6.5 with 30 % solution of NaOH (Sigma- Aldrich, USA). After that it was sterilized at 120 °C for 15 min and used as a fermentation medium. In the first set of experiments, batch lactic acid fermentation was performed with optimal initial sugar concentration of 55 g L^{-1} adjusted by addition of 70 % sterile glucose solution. In the fed-batch fermentation experiments, same fermentation media as in batch process was used for initiation of the fermentation and feeding solution was supplied when sugar concentration dropped below 20 g L^{-1} to maintain the sugar concentration at around 50 g L^{-1}. Feeding solution consisted of the sterile stillage with glucose concentration of 140 g L^{-1}. The feeding was performed until the volume of fermentation flask was filled up to 70 % of complete fermentation volume (1000 mL).

Liquid stillage preparation for batch and recycled batch fermentation

The stillage remained after bioethanol production on wasted bread obtained from Reahem Ethanol Plant (Reahem, Srbobran, Serbia) has been centrifuged (4500 rpm, 20 min, centrifuge: Sigma® model 2–16, Shropshire, UK). Solid stillage was separated from a liquid part and pH of the supernatant (liquid stillage) was adjusted to 6.5 with 30 % NaOH. After adjustment, the liquid stillage was sterilized at 121 °C for 15 min. The concentration of reducing sugars in the sterile liquid stillage (originally 12 g L^{-1}) was set at approximately 50 g L^{-1} with addition of a sterile 70 % glucose solution and used as a fermentation medium.

Microorganism

L. 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 using Anaerocult® C bags (Merck KGaA, Darmstadt, Germany) at 37 °C for 18 h in MRS broth before inoculation to fermentation medium. In batch fermentations with recirculation of immobilized *L. rhamnosus* ATCC 7469 into fresh liquid stillage media, inoculums were prepared by immobilization of *L. rhamnosus* ATCC 7469 onto the zeolite as a carrier.

Preparation of zeolite molecular sieves as a carrier

Zeolite molecular sieves (type 13X, beads, 8–12 mesh, 1 Na_2O: 1 Al_2O_3: 2.8 ± 0.2 SiO_2: xH_2O) (Technical bulletin - Sigma-Aldrich Molecular sieves, 2012) were purchased from Sigma Aldrich,

Darmstadt, Germany. Before utilization it was powdered and washed twice with demineralised water. Average particle size was 4–7 μm (90 %) with normal particle size distribution. Powdered zeolite was dried at 105 °C for 3 h and activated at 250 °C for 3 h. In this way prepared carrier was used for immobilization of *L. rhamnosus* ATCC 7469 as a lactic acid producing microorganism.

Immobilization of *l. rhamnosus* atcc 7469 onto zeolite

The culture of *L. rhamnosus* ATCC 7469 was propagated at 37 °C in 200 mL of Man Rogosa Sharpe broth (MRS) with inoculum concentration of 10 % (v/v) under anaerobic static conditions using Anaerocult ®C bags (Merck KGaA, Darmstadt, Germany). After 16 h, the culture was centrifuged (10,000 rpm, 5 min, centrifuge: Sigma® model 2–16, Shropshire, UK), twice washed with sterile 0.8 % (w/v) NaCl solution and the biomass was suspended in 200 mL of fresh MRS broth with addition of 2 % (w/v) powdered Na-zeolite. The culture prepared in this way was incubated at 41 °C, with shaking (90 rpm, KS 4000i control, IKA®, Werke GmbH & Co. KG, Staufen, Germany). After 12 h, the culture was centrifuged (1000 rpm, 5 min), supernatant with free cells was thrown, and the sediment of *L. rhamnosus* ATCC 7469 cells adsorbed onto zeolite was twice washed with sterile 0.8 % (w/v) NaCl solution and used as an inoculum for fermentation. The preparation of the free *L. rhamnosus* ATCC 7469 cells for fermentation was similar, but without addition of the powdered zeolite.

Lactic acid fermentation

All lactic acid fermentations were performed with shaking (100 rpm, KS 4000i control, IKA®, Werke GmbH and Co. KG, Staufen, Germany) at temperature of 41 °C. The fermentation was initiated by addition of 5 % (v/v) of inoculum. During the fermentations, pH was adjusted to 6.5 by addition of 30 % NaOH solution in 4 h intervals. The batch and fed-batch fermentations on the complete stillage were preformed in 1000 ml flasks with initially 400 ml of the fermentation media while batch and recycled batch fermentations with liquid stillage were preformed with 200 ml of fermentation media in 500 ml flasks. In the repeated batch fermentation, recirculation of media was applied after depletion of sugar below 10 g L^{-1}. At this point fermentation media was centrifuged (1000 rpm, 5 min), washed with sterile physiological solution and residual immobilized biomass was inoculated into the fresh fermentation media. In the fermentations with free *L. rhamnosus* cells, one fermentation cycle was preformed until complete utilization of sugars in media occurred. Microaerophylic conditions were provided by gas pack system with Anaerocult® C bags. During the fermentation: pH, sugar consumption, lactic acid concentration and a number of living cells were analysed.

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*). Calibration curve was set at 505 nm using standard glucose solutions. Lactic acid concentration was determined by enzymatic method (L-/D-Lactic acid assay, Megazyme®, Wicklow, Ireland) after deproteinization of the samples according to procedure prescribed in assay. Number of free viable *L. rhamnosus* ATCC 7469 cells was estimated using pour plate technique on MRS agar after incubation for 48 h at 37 °C. Number of viable *L. rhamnosus* ATCC 7469 cells was estimated using pour plate technique on MRS agar after detachment of cells from zeolite carrier by methodology reported

by *Đukić-Vuković et al. (2013)*. All chemicals used in experiments were analytical grade.

Statistical analysis

The experiments were done in triplicates. All values are expressed as mean ± SD. Mean values of treatments were compared by the analysis of variance (one-way ANOVA) followed by Tukey test for mean differences testing. Differences were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

The results of batch lactic acid fermentations on complete stillage and just liquid part of stillage are presented in Figure 1. Lactic acid fermentation on complete stillage was faster and utilization of sugar present in media was more efficient in complete stillage (Fig. 1). Also, it could be seen from Figure 1. that residual sugar was higher in the samples with liquid stillage as main component of the media. These findings could be explained by differences in chemical composition of complete stillage and just its liquid part. Complete stillage is higher in proteins (*Đukić-Vuković et al., 2013*) then liquid part of stillage (*Đukić-Vuković et al., 2012*) which are very important for the growth of lactic acid bacteria and the production of lactic acid. Also, in the samples with complete stillage drops in pH value during the process were lower than in liqid stillage fermentations (data not shown). This is probably an additional consequence of the presence of proteins in complete wasted bread stillage and their buffering capacity. However, around 36 g L^{-1} of lactic acid was produced on just liquid part of stillage which could be considered as a significant concentration taking into a count possibility for valorisation of remaining solids of stillage for production of DDG (dry distillers' grains) as a feed (*Đukić-Vuković et al., 2013*). Also, concentration of lactic acid obtained on triticale stillage was almost three times lower and amounted around 13 g L^{-1} (*Marković et al., 2014*), while the maximal obtained lactic acid concentration on corn liquid stillage was 18.4 g L^{-1} (*Mojović et al., 2011*).

Lactic acid yield of 0.92 g g^{-1} and corresponding volumetric productivity of 1.49 g L^{-1} h^{-1} were obtained in batch fermentation on complete stillage. The maximal productivity of the process on liquid stillage under the same conditions was around 0.66 g L^{-1} h^{-1}. In order to investigate possibilities for further improvement of the process for conversion of complete stillage into the lactic acid, fed-batch fermentation strategy was implemented. The kinetics of fed-batch lactic acid fermentation on whole stillage is presented in Figure 2. Under the proposed conditions, very high lactic acid productivity of 1.80 g L^{-1} h^{-1} was achieved and the number of viable cells exceeded 10^9 CFU ml^{-1}. Also, the lactic acid concentration at the end of fed-batch fermentation was 97.0 g L^{-1} (Fig. 2). The high lactic acid concentration is very important for cost effective extraction of product from media (*Pal et al., 2009*). Based on all these findings the fed-batch fermentation could be recommended as effective fermentation route for lactic acid production on complete stillage. Remaining fermented solids, after separation of liquid part of fermented media, were evaluated as a valuable feed with probiotic biomass of *L. rhamnosus* ATCC 7469 (*Đukić-Vuković et al., 2014*). The chemical composition of fermented and unfermented remains of the stillage qualifies it as a good feed for monogastric animals because of low content of fibers and high parameters of digestible and metabolisable energies (*Đukić-Vuković et al., 2013*).

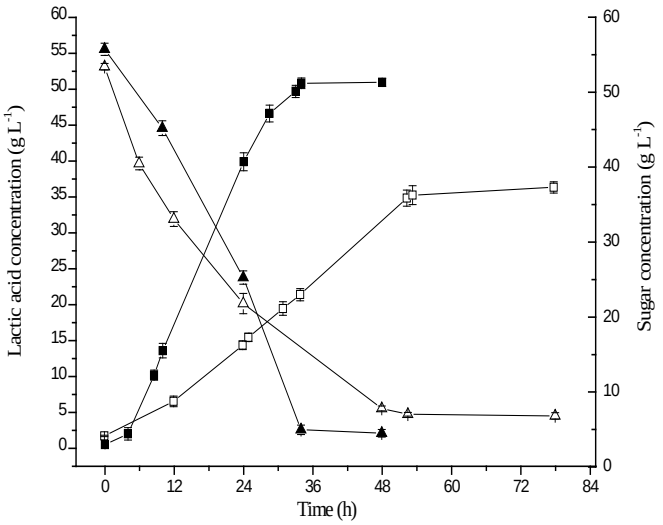

Fig. 1. Kinetics of batch lactic acid fermentation on complete stillage and liquid stillage. Symbols: square - lactic acid concentration (g L^{-1}), triangle - sugar concentration (g L^{-1}), solid symbols - whole stillage, open symbols - liquid stillage

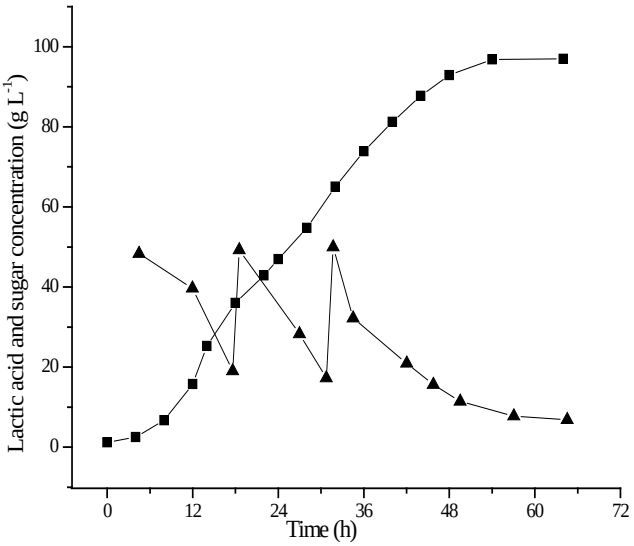

Fig. 2. Fed-batch lactic acid fermentation on complete stillage. Symbols: square - lactic acid concentration (g L^{-1}), triangle - sugar concentration (g L^{-1})

The kinetics of repeated batch lactic acid fermentation of liquid stillage is presented in Figure 3. As it could be seen in Figure 3, conversion of reducing sugars from stillage to lactic acid was faster and more efficient in repeated batch fermentation in comparison to the batch fermentation mode with the same substrate. In the repeated batch lactic acid fermentation, final overall lactic acid productivity of 1.41 g L^{-1} h^{-1} has been achieved with the high number of viable *L. rhamnosus* ATCC 7469 cells of 10^{10} CFU per g of zeolite carrier. Lignocellulose wastes are studied as substrates for lactic acid fermentation and in the study of *Adsul et al. (2007)* and maximal lactic acid productivity of 0.93 g L^{-1} h^{-1} and yield of 0.83 g g^{-1} were achieved by *L. delbrueckii* mutant Uc-3 in the fermentation of bagasse. Yield and productivity of repeated batch fermentation on liquid stillage are both higher than the results reported on fermentation of baggasse *(Adsul et al., 2007)*. Besides lactic acid as an outcome of this process, residual probiotic biomass immobilized onto the zeolite and a fraction of solid stillage from bioethanol production could be utilized in animal husbandry.

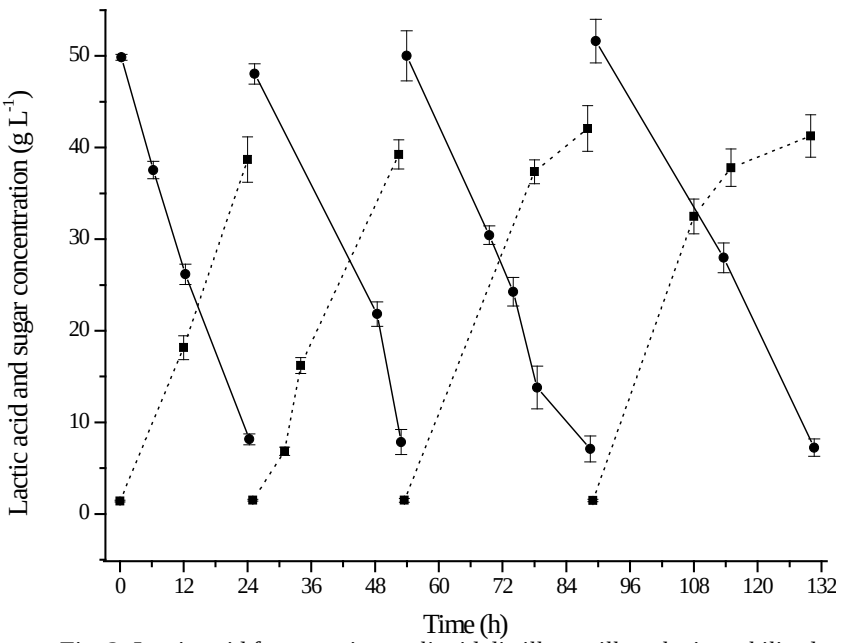

Fig. 3. Lactic acid fermentation on liquid distillery stillage by immobilized cells of L. rhamnosus ATCC 7469. Symbols: dot lines - lactic acid concentration (g L^{-1}), solid lines – sugar concentration (g L^{-1})

CONCLUSION

Based on the reported results, stillage could be recommended as a suitable substrate for growth of lactic acid bacteria and production of lactic acid without supplementation with expensive nitrogen sources. Under determined conditions lactic acid could be produced on the complete stillage or its liquid part with overall productivity of above 1.4 g L^{-1} h^{-1}. The highest lactic acid concentration of 97 g L^{-1} was achieved in fed-batch fermentation process on the complete stillage which could be recommended as the most suitable for lactic acid fermentation. Remains of the fed-batch lactic acid fermentation of complete stillage could be valorised through production of probiotic feed or feed additives with a number of viable cells above 10^9 CFU ml^{-1} while in repeated batch fermentation of liquide stillage higher cell number of above 10^{10} CFU g^{-1} was attained.

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