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ULTRASOUND AS A PHYSICAL TREATMENT OF STILLAGE FOR LACTIC ACID FERMENTATION

ULTRAZVUK KAO FIZIČKI TRETMAN ZA PRIPREMU DŽIBRE ZA MLEČNO-KISELINSKU FERMENTACIJU

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ABSTRACT

In this study, the stillage from the bioethanol production on wasted bread has been used as a substrate for fermentation by lactic acid bacteria. Ultrasound has been studied as a physical treatment of wasted bread stillage before fermentation by Lactobacillus rhamnosus ATCC 7469. In the ultrasound treated sample a 15 % higher lactic acid concentration compared to control sample and lactic acid yield of 0.87 g g^{-1} were obtained. Ultrasound could significantly increase LA productivity without effect on cell growth. Moreover, the number of viable L. rhamnosus ATCC 7469 cells was found higher in ultrasound treated samples. The study indicated that the ultrasound pretreatment could be effectively applied and used as an alternative to sterilization in lactic acid fermentation of distillery stillage.

Key words: *lactic acid, ultrasound, lactic acid fermentation, distillery stillage, Lactobacillus rhamnosus ATCC 7469.*

REZIME

Mlečna kiselina se danas dominantno proizvodi fermentacijom. Teži se zameni tradicionalnih skrobnih i sintetskih supstrata u mlečno-kiselinskim fermentacijama (MKF) jeftinijim otpadnim i sporednim proizvodima drugih industrija. Fizički postupci za tretman supstrata se intenzivno ispituju u cilju povećanja ekonomske isplativnosti i ekološke povoljnosti procesa MKF na industrijskim otpadnim proizvodima. U ovom radu je primenjen ultrazvuk kao metoda fizičkog tretmana otpadne hlebne džibre pre zasejavanja bakterijama mlečne kiseline i fermentacije u cilju proizvodnje mlečne kiseline. Ispitivan je uticaj primenjenog ultrazvuka na važnije parametre procesa MKF.

Nakon tretmana ultrazvukom, džibra je inokulisana vrstom Lactobacillus rhamnosus ATCC 7469 i u toku 48 h časova su praćeni najvažniji parametri MKF koja je izvođena na 41°C, uz mešanje, pri mikroaerofilnim uslovima. Uporedno je, pod istim uslovima, postavljen netretirani uzorak kao kontrola. U uzorku tretiranom ultrazvukom je postignuta 15% viša koncentracija mlečne kiseline uz maksimalni prinos mlečne kiseline od 0,87 g g^{-1}. Može se zaključiti da ultrazvuk može predstavljati alternativni tretman energetski zahtevnoj sterilizaciji i da obezbedi adekvatnu podlogu za rast L. rhamnosus ATCC 7469, a uz značajan porast koncentracije mlečne kiseline u medijumu i samim tim efikasniju MKF.

Ključne reči: *mlečna kiselina, ultrazvuk, mlečno-kiselinska fermentacija, džibra, Lactobacillus rhamnosus ATCC 7469.*

INTRODUCTION

Waste disposal, as a main strategy in past, has been shifted towards waste utilization during the last decade. Methanogenic anaerobic digestion, biological hydrogen production, microbial fuel cell production and various fermentations for production of valuable products were proposed as alternative biorefinery strategies for waste valorization (*Angenent et al., 2004*). Wastes have been utilized for fermentative production of bioethanol, biodiesel, biohydrogen, 1.4-butanediol, 2.3-butanediol, humic acid, succinic acid, lactic acid etc. (*Yang et al., 2015a*).

Lactic acid (LA) is an important substance widely used in chemical, food and pharmaceutical industry as an acidulant, flavour or a component for production of polymers for food packaging, grafts and other applications. Development of processes for conversion of agro-food wastes to lactic acid is mainly driven by high demand for biodegradable and biocompatible poly-lactic acid polymers (*Maharana et al., 2015*). Date waste (*Nancib et al., 2015*), mixed restaurant food and bakery wastes (*Pleissner et al., 2015; Yang et al., 2015b*), wastes from bioethanol (*Djukić-Vuković et al., 2016*) and beer production (*Pejin et al., 2014*) were studied as substrates for lactic acid production. Complexity and variability of these substrates as well as their susceptibility to contamination have been emphasized as main problems for their utilization in lactic acid fermentations. The sterilization of these complex media is energy consuming and significant amount of sugars and proteins could be lost during the thermal sterilization as a result of Maillard reaction. Also, the heterogeneity of waste substrates could reduce efficiency of the fermentation processes and physical treatments like milling were utilized, especially for lignocellulosic waste (*Ravindran and Jaiswal, 2016*) or food waste (*Jiang et al., 2014*). Based on these problems, alternative non-thermal treatments like ultrasound were proposed.

Ultrasound treatament was studied mostly for extraction purposes (*Jiang et al., 2014*) and microbial inactivation by the ultrasound has been proven but mostly for the bacterial suspensions in a simple water media (*Gao et al., 2014*). Stillage is a complex by-product of bioethanol production on different feedstocks with a high organic load and it is very susceptible to bacterial contamination. Stillage was often used as a media for fermentations (*Wilkie et al., 2000*) but it was mostly sterilized prior to inoculation or used in open-fermentations (*Islam et al., 2015*).

In this study, wasted bread stillage was treated by ultrasound and further used as a substrate for lactic acid fermentation by

Lactobacillus rhamnosus ATCC 7469. It is expected that ultrasound treatment could increase homogeneity of the media, availability of sugars and proteins and decrease number of spontaneous bacteria present in stillage. In this study, we have addressed effects of ultrasound treatment on both lactic acid and biomass production.

MATERIAL AND METHOD

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 microaerophilic conditions at 37 °C for 18 h in MRS broth prior to inoculation of fermentation medium.

Fermentation media preparation

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 the pH correction, 60 ml of stillage were treated by ultrasound (Sonopuls HD 2200, Bandelin, Germany) with sonotrode TT 13 for 10 min at actual value of amplitude 75 % and frequency of 20 kHz. Untreated control sample was sterilized at 120 °C for 15 min and used for fermentation. Ultrasound treated sample was subjected to fermentation without sterilization and the sterility was checked. The samples of ultrasound treated media were inoculated immediately after treatment to nutrient agar, malt agar and MRS agar. There were no growth observed on any of plates. In both samples, ultrasound treated and untreated sterilized media initial sugar concentration was adjusted to approximately 35 g L^{-1} by addition of 70 % sterile glucose solution. This way prepared sample were inoculated with *L. rhamnosus* and subjected to fermentation.

Lactic acid fermentation

Batch 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 *L. rhamnosus* ATCC 7469 inoculum. During the fermentations, pH was adjusted to 6.5 by addition of 30 % NaOH solution in 4 h intervals. The control samples were subjected to LA fermentation under the same conditions.

Analytical methods

The concentration of reducing sugars, calculated as glucose, was estimated by 3.5-dinitrosalicylic acid method using a 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.

Scanning electron microscopy

The morphology of the samples was studied by field emission scanning electron microscopy (FESEM) TESCAN Mira 3 XMU at 20 kV.

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

Time course of lactic acid fermentation in ultrasound treated and untreated sterilized sample is presented in Figure 1. The data of reducing sugar concentration during the LA fermentations in both samples are presented in Figure 2.

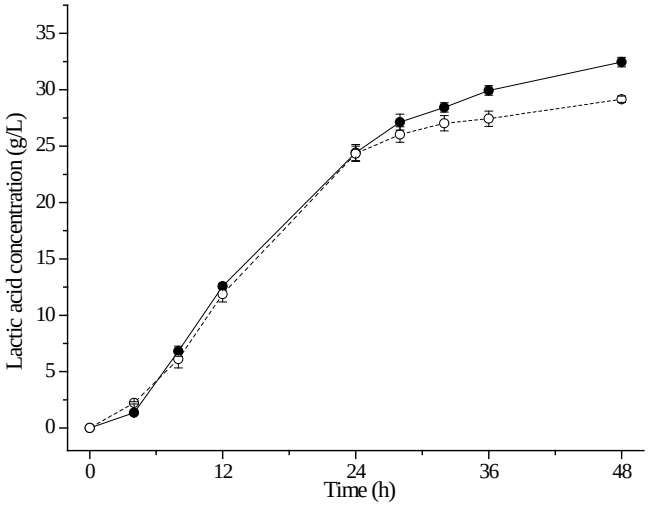

Fig. 1. Lactic acid concentration in ultrasound treated and untreated sterilized substrate during the lactic acid fermentation by Lactobacillus rhamnosus. Symbols: solid line - ultrasound treated medium, dashed line - untreated sterilized medium

It could be noticed that final LA concentration in ultrasound treated sample was higher (10 %) than in control sample and amounted 32.46 g/L. During the first 24 h of fermentation a difference between LA concentrations in ultrasound treated and sterilized samples was not significant. There was a significant difference in sugar concentrations at the end of fermentation (48 h) and the residual sugar concentration was lower in ultrasound treated sample (Fig. 2). It could be concluded that conversion of sugar to LA was more effective in the ultrasound treated sample. *Jiang et al. (2014)* observed similar effect in the anaerobic digestion of food wastes for production of volatile fatty acids, where ultrasound treatment resulted in higher chemical oxygen demand and reducing sugar concentration in substrate which increased productivity of volatile fatty acids. Also, the maximal productivity of 1.02 g L^{-1} h^{-1} was achieved in that study after 24 h of fermentation and this productivity was higher or similar to the most reported productivities on lignocellulosic waste substrates (*Zhang et al., 2015*).

In order to examine the effect of ultrasound on the growth of *L. rhamnosus* ATCC 7469 in distillery stillage, a number of viable cells during the lactic acid fermentation have been determined and it is presented in Figure 3. The number of viable *L. rhamnosus* ATCC 7469 cells was higher in ultrasound treated samples. In comparison to sterilized sample, the growth of *L. rhamnosus* was even better, so the positive effect of ultrasound was beyond reduction of spontaneous microbiome of stillage. It provided better conditions for the growth of *L. rhamnosus*.

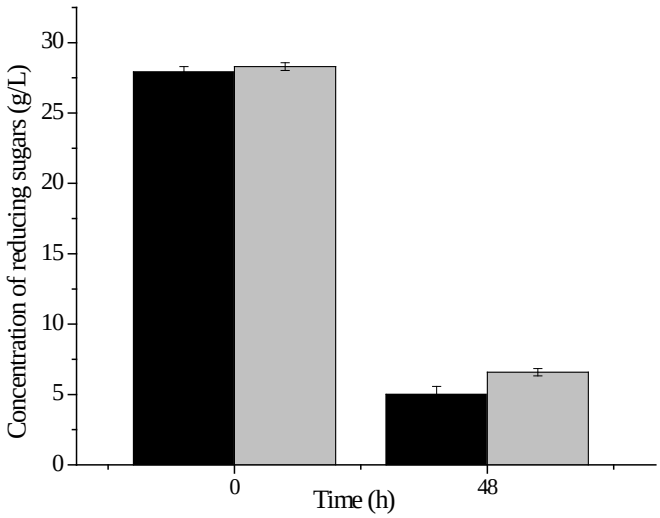

Fig. 2. Concentration of reducing sugars during the LA fermentation of ultrasound treated and untreated sterilized stillage as a substrate. Symbols: black bars - ultrasound treated medium, grey bars – untreated sterilized medium

In Figure 4, the spontaneous attachment of *L. rhamnosus* cells onto the solid particles present in stillage media is presented.

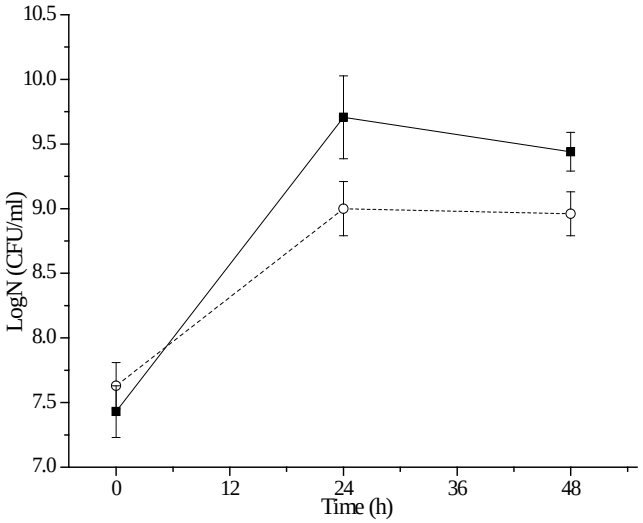

Fig. 3. The growth of Lactobacillus rhamnosus ATCC 7469 during the lactic acid fermentation on ultrasound treated and untreated sterilized distillery stillage medium. Symbols: solid line - ultrasound treated medium, dashed line – untreated sterilized medium

A high number of *L. rhamnosus* cells was attached to the solid fraction of medium in ultrasound treated samples (Fig. 4), so the conversion of sugars by bacteria in biofilm could be more effective, as already reported by *Hall-Stoodley et al. (2004)*. This indicates that ultrasound treatment could be utilized as an alternative for energy consuming sterilization process in the case of integrated LA and biomass production by *L. rhamnosus* ATCC 7469 on stillage since it causes significant reduction in number of microorganisms in media after the ultrasound treatment. Although the sterility of media could not be guaranteed and further studies have to be performed in order to check it, higher LA concentration in ultrasound treated non-sterilized samples indicates that ultrasound treatment could be a good alternative to sterilization in lactic acid production on waste substrates.

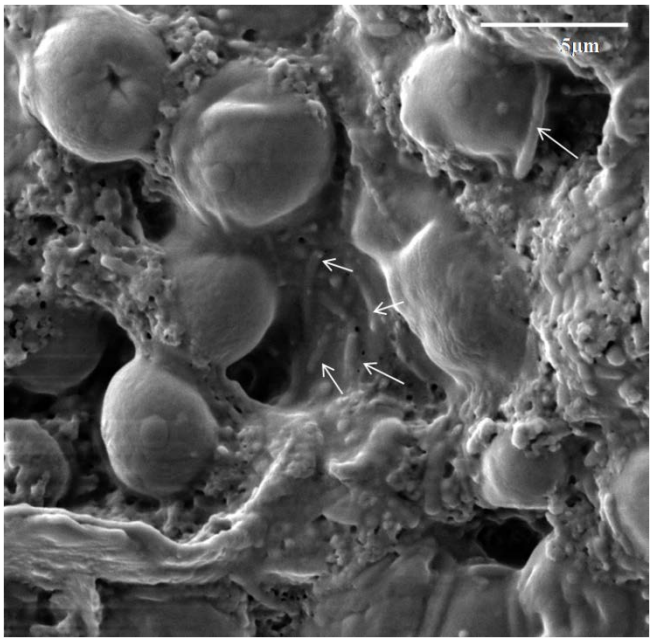

Fig. 4. Scanning electron micrography of Lactobacillus rhamnosus ATCC 7469 attached onto the surface of solid fraction of ultrasound treated stillage media after 48 h of LA fermentation (yeast cells remained from previous bioethanol fermenatation are also visible and the arrows are pointing L. rhamnosus cells)

The efficacy of conversion of sugar to LA in the ultrasound treated samples was higher also due to the higher number of viable bacteria. *L. rhamnosus* ATCC 7469 strain used in this study has shown probiotic characteristics (*Djukić-Vuković et al., 2015*) and the final number of cells of around 5×10^9 CFU ml^{-1} obtained in fermentation media was in accordance with the recommended values of 10^6-10^9 CFU ml^{-1} for probiotics in feed (*Anadón et al., 2006*). In this sense, the solid residues of fermentation media could be used in animal nutrition. The ultrasound treatment of distillery stillage enabled effective parallel lactic acid and biomass production which was accomplished in the fermentation without prior substrate sterilization.

CONCLUSION

This study indicated that the ultrasound treatment could be effectively applied in lactic acid fermentation of distillery stillage and could even replace the commonly used substrate sterilization process prior fermentation. By ultrasound treatment of distillery stillage a higher lactic acid concentration and yield as well as higher number of viable *L. rhamnosus* ATCC 7469 cells was achieved.

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REFERENCES

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, 4 (1), 91-95.

Angenent, L. T., Karim, K., Al-Dahhan, M. H., Wrenn, B. A., Domíguez-Espinosa, R. (2004). Production of bioenergy and biochemicals from industrial and agricultural wastewater. TRENDS in Biotechnology, 22 (9), 477-485.

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, 48, 478-482.

Djukić-Vuković, Aleksandra, Mojović, Ljiljana, Semenčenko, Valentina, Radosavljević, Milica, Pejin, Jelena, Kocić-Tanackov, Sunčica. (2015). Effective valorisation of distillery stillage by integrated production of lactic acid and high quality feed. Food Research International, 73, 75-80.

Gao, S., Lewis, G. D., Ashokkumar, M., Hemar, Y. (2014). Inactivation of microorganisms by low-frequency high-power ultrasound: 1. Effect of growth phase and capsule properties of the bacteria. Ultrasonics sonochemistry, 21 (1), 446-453.

Hall-Stoodley, L., Costerton, J. W., Stoodley, P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. Nature Reviews Microbiology, 2 (2), 95-108.

Islam, R., Panditharatne, C., Schellenberg, J., Sparling, R., Cicek, N., Levin, D. B. (2015). Potential of thin stillage as a low-cost nutrient source for direct cellulose fermentation by *Clostridium thermocellum*. http://www.aimspress.com/fileOther/PDF/energy/201504711.pdf (Last time accessed: February 2016)

Jiang, J., Gong, C., Wang, J., Tian, S., Zhang, Y. (2014). Effects of ultrasound pre-treatment on the amount of dissolved organic matter extracted from food waste. Bioresource Technology, 155, 266-271.

Maharana, T., Pattanaik, S., Routaray, A., Nath, N., Sutar, A. K. (2015). Synthesis and characterization of poly (lactic acid) based graft copolymers. Reactive and Functional Polymers, 93, 47-67.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry, 31, 426-428.

Nancib, A., Nancib, N., Boubendir, A., Boudrant, J. (2015). The use of date waste for lactic acid production by a fed-batch culture using *Lactobacillus casei* subsp. *rhamnosus*. Brazilian Journal of Microbiology, (AHEAD), 00-00.

Pejin, Jelena, Mojović, Ljiljana, Kocić-Tanackov, Sunčica, Radosavljević, M., Đukić-Vuković, Aleksandra, Nikolić, Svetlana. (2014). Lactic acid production on brewers' spent grain hydrolysate by *Lactobacillus rhamnosus* and *Lactobacillus fermentum*. Journal on Processing and Energy in Agriculture, 18 (4), 182-186.

Pleissner, D., Lau, K. Y., Schneider, R., Venus, J., Lin, C. S. K. (2015). Fatty acid feedstock preparation and lactic acid production as integrated processes in mixed restaurant food and bakery wastes treatment. Food Research International, 73, 52-61.

Ravindran, R., Jaiswal, A. K. (2016). A comprehensive review on pre-treatment strategy for lignocellulosic food industry waste: Challenges and opportunities. Bioresource technology, 199, 92-102.

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.

Yang, X., Choi, H. S., Park, C., Kim, S. W. (2015a). Current states and prospects of organic waste utilization for biorefineries. Renewable and Sustainable Energy Reviews, 49, 335-349.

Yang, X., Zhu, M., Huang, X., Lin, C. S. K., Wang, J., Li, S. (2015b). Valorisation of mixed bakery waste in non-sterilized fermentation for l-lactic acid production by an evolved *Thermoanaerobacterium* sp. strain. Bioresource technology, 198, 47-54.

Zhang, L., Li, X., Yong, Q., Yang, S. T., Ouyang, J., Yu, S. (2015). Simultaneous saccharification and fermentation of xylo-oligosaccharides manufacturing waste residue for l-lactic acid production by *Rhizopus oryzae*. Biochemical Engineering Journal, 94, 92-99.

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