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PRODUCTION OF LACTIC ACID AND MICROBIAL BIOMASS ON DISTILLERY STILLAGE BY USING IMMOBILIZED BACTERIA

PROIZVODNJA MLEČNE KISELINE I BAKTERIJSKE BIOMASE NA DESTILERIJSKOJ DŽIBRI POMOĆU IMOBILISANIH BAKTERIJA

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ABSTRACT

Lactic acid is versatile chemical with a wide range of applications in chemical, food, pharmaceutical, cosmetic and polymer industries. Currently, lactic acid world consumption is continually increasing mostly due to expansion of the application range of poly-lactides. Utilization of industrial distillery stillage, waste water from bioethanol production as a cheap and abundant substrate for integrated lactic acid and biomass production for animal feed could be a sustainable and environmentally friendly approach. In this study, integrated lactic acid and biomass production by fermentation with immobilized lactic acid bacteria Lactobacillus rhamnosus ATCC 7469 on an industrial waste stillage obtained from the Serbian bioethanol production plant "Reahem" was studied. The immobilization was performed onto zeolite, a microporous aluminosilicate mineral. Optimal conditions for bacterial immobilization where determined which allowed easy cell separation from the fermentation media and their reuse in repeated batch cycles. A number of viable cells of over 10^{10} CFU g^{-1} of zeolite was achieved at the end of the fourth fermentation cycle. A maximal process productivity of 1.69 g L^{-1}, maximal lactic acid concentration of 42.19 g L^{-1} and an average yield coefficient of 0.96 g g^{-1} were achieved in repeated batch fermentation with immobilized cells on the liquid stillage without mineral or nitrogen supplementation.

***Key words**: Lactic acid, Lactobacillus rhamnosus ATCC 7469, distillery stillage, zeolite.*

REZIME

Mlečna kiselina predstavlja važan proizvod koji se koristi u prehrambenoj, hemijskoj i farmaceutskoj industriji. Poslednjih godina svetska potražnja mlečne kiseline se konstantno povećava po prosečnoj godišnjoj stopi od 7% i to prvenstveno zbog veće potražnje mlečne kiseline za proizvodnju biodegradabilnih polimera. Nove tehnologije za održivu fermentacionu proizvodnju mlečne kiseline se baziraju na korišćenju sporednih i otpadnih proizvoda kao supstrata. U radu je ispitivana mogućnost korišćenja jeftinog otpadnog materijala - tečne destilerijske džibre iz proizvodnje bioetanola iz industrijskog pogona "Reahem" iz Srbobrana za integralnu proizvodnju mlečne kiseline i mikrobne biomase pomoću probiotski aktivne bakterije Lactobacillus rhamnosus ATCC 7469 imobilisane na mikroporozni aluminosilikatni mineral zeolit (13X, molekulska sita) adsorpcijom. Imobilizacijom ove bakterije na zeolit ostvarena je prilično jaka veza koja se pripisuje jakim elektrostatičkim silama izmedju bakterija i nosača kao i sposobnošću bakterija da stvaraju egzopolisaharide koji takođe utiču na jačanje veza. Na taj način je minimalizovana desorpcija i ispiranje bakterije u toku fermentacije. Značajni parametri ostvareni u fermentaciji džibre sa bakterijom L. rhamnosus ATCC 7469 imobilisanom na zeolit su upoređeni sa parametrime ostvarenim u šaržnoj fermentaciji sa slobodnom bakterijom. Korišćenjem imobilisanih bakterija bilo je moguće ostvariti jednostavnu separaciju ćelija nakon fermentacije kao i njihovo ponovno korišćenje u više uzastopnih šaržnih ciklusa. Na taj način je nakon četiri ponovljena šaržna ciklusa fermentacije u laboratorijskim uslovima ostvarena visoka produktivnost proizvodnje mlečne kiseline od 1,69 g/L·h što je bilo značajno više nego u fermentaciji sa slobodnim ćelijama. Takodje je postignuta visoka koncentracija mlečne kiseline od 42,19 g/L, visok koeficijent prinosa na supstratu od 0,96 g/g, kao i visoka koncentracija biomase ćelija od preko 10^{10} CFU/g zeolita. Značajno je i to što su ovi rezultati ostvareni na tečnoj džibri bez dodatka minerala ili izvora azota.

***Ključne reči**: Mlečna kiselina, Lactobacillus rhamnosus ATCC 7469, destilerijska džibra, zeolit.*

INTRODUCTION

In last two decades, the production of lactic acid as an important chemical has become an attractive research field. The research interest has been mainly driven by the expansion of application range of lactic acid and its derivatives. Lactic acid is commonly used as a flavour and preservative in food, cosmetic and pharmaceutical industries due to its antimicrobial characteristics. Also, it is used for production of poly-lactides, polymers with convenient characteristics (biodegradability, thermo stability, elasticity, biocompatibility, favourable controlled release profile, etc.) for pharmaceutical and plastic composite industries *(Gupta et al., 2007)*. It is predicted that global demand for lactic acid will continue to grow with an average 7% per year rate in the future *(Malveda et al., 2009)*. Fermentation is a dominant route for lactic acid production in industrial facilities *(Vink et al., 2003)* and implementation of the processes on renewable and cheap substrates is a base for cost-effective production. There are a number of studies of lactic acid production on agro-industrial starch substrates *(Anuradha et al., 1999; Rojan et al., 2005)*, lignocellulosic substrates *(Moldes et al., 2000)* and by-products of dairy industry *(Büyükkileci and Harsa, 2004)*. Processes for lactic acid production which engage agro-industrial by-products and wastes as substrates are a trend for profitable, eco-friendly and sustainable biotechnological production. The stillage remaining after bioethanol production is an abundant

waste material which, due to high organic pollution, causes serious environmental problems if not treated adequately. Recently, *Mojović et al., (2010, 2011)* and *Đukić-Vuković et al.. (2011, 2012)* studied possibilities of utilization of various types of stillage as a substrate for lactic acid fermentation.

In order to enhance the productivity of lactic acid production various production strategies could be utilized. Our previous research has shown that a fed-batch process is more productive than a batch process *(Đukić-Vuković et al., 2013)*. Immobilization of the lactic acid bacteria could enable easy cell separation from the fermentation media and their reuse in repeated batch cycles, and may possibly further increase the productivity of the process. Also, an integrated production of lactic acid and biomass offers better substrate utilization since the biomass which may posses a probiotic activity together with the stillage remaining after lactic acid fermentation could be used as a high quality feed *(Đukić-Vuković et al., 2013a)*.

The aim of this study was to investigate the possibilities of lactic acid and biomass production on a liquid stillage from bioethanol plant with Lactobacillus rhamnosus ATCC 7469 strain immobilized on powdered zeolite.

MATERIAL AND METHOD

Liquid stillage preparation

The stillage remained after bioethanol production on wasted bread was obtained from Reahem Ethanol Plant (Reahem, Srbobran, Serbia). After centrifugation (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 (Sigma-Aldrich, USA). After adjustment, the liquid stillage was sterilized at 121 ^{o}C for 20 minutes. 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. The liquid stillage consisted of proteins (43.75% of dry matter), reducing sugars (24.30% dm), lipids (11.42% dm) and ash (14.49% dm).

Preparation of zeolite for the immobilization of lactic acid bacteria

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) 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 ^{o}C for 3 hours and activated at 250 ^{o}C for 3 h. In this way, prepared carrier was used for immobilization of *Lactobacillus rhamnosus* ATCC 7469 as a lactic acid producing microorganism.

Immobilization of *L. rhamnosus* ATCC 7469 onto zeolite

L. rhamnosus ATCC 7469, a homofermentative L (+) lactic acid strain, was obtained from American Type Culture Collection (ATCC, Rockville, USA). Stock cultures of LAB were stored as lyophilized. The culture 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 (10000 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 as batch cultures, with shaking (90 rpm, KS 4000i control, IKA®, Werke GmbH & Co. KG, Staufen, Germany) at 41°C. The fermentations were preformed in 500 ml flasks with 200 ml of the liquid stillage under anaerobic conditions in the gas pack system. After depletion of sugar below concentration of 10 g L^{-1}, 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 samples with free *L. rhamnosus* cells, initially 5% of inoculum was added and one fermentation cycle was preformed until complete utilization of sugars in media occurred. During the fermentation, samples were aseptically withdrawn and pH, substrate consumption, lactic acid concentration and a number of living cells were analyzed.

Experimental analysis

The concentration of reducing sugars, calculated as glucose, was estimated by 3, 5-dinitrosalicylic acid method using spectrophotometer *(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 sample. 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 *Hrenović et al., (2009)*, with some modification. Principally, the number of viable immobilized cells (I) was determined as a difference between the total number of viable cells (T) present in fermentation media (immobilized and non immobilized cells) and the number of non immobilized cells (N). Resulting number (I) was expressed as CFU g-1 of carrier. In brief, 1 ml of fermentation media was aseptically transferred into 9 ml of sterile 0.8% (w/v) NaCl solution and mixed for 10 minutes in a tube shaker (at 50Hz) for cell detachment. This time was rather long because of strong bonds of the cells with zeolite surface. After that, serial dilutions from suspension were made and inoculated on MRS agar plates, as previously described. The colonies were counted and the remaining carrier has been dried and weighed. The total number of viable cells (T) and the number of viable non immobilized cells (N) were expressed as CFU ml-1 of fermentation media. The number of non immobilized cells (N) was determined by similar procedure. One ml of fermentation media was aseptically withdrawn for determination of non immobilized cells and transferred into 9 ml of sterile 0.8% (w/v) NaCl solution. The resulting solution was shortly mixed (10 seconds) and

serial dilutions from suspension were made and inoculated on MRS agar plates. Finally, the number of viable immobilized cells (I) was calculated by subtraction of the number of viable non immobilized (N) cells from the total number of viable cells (I=T-N).

Statistical analysis

The experiments were done in triplicates. All values are expressed as means ± standard deviation. Mean values of treatments were compared by the analysis of variance. Differences were considered significant at $p < 0.05$.

RESULTS AND DISSCUSION

Immobilization of *L. rhamnosus* ATCC 7469

Scanning electron micrograph of the surface of zeolite without cells and with *L. rhamnosus* cells attached to the surface of zeolite are presented in Figure 1.

In our study, L. rhamnosus cells were directly attached to the zeolite surface and tightly fixed with cell surface to zeolite support. The strong bond of L. rhamnosus cells with zeolite surface could be illustrated with the time required for desorption of cells from the carrier. In the study of *Hrenović et al., (2009)* detachment of cells was complete after 3 min of a vigorous shaking at 40Hz. For the detachment of immobilized L. rhamnosus cells from zeolite particles utilized in this study 10 minutes of shaking was necessary. The strong bond between L. rhamnosus and zeolite surface could be a result of electrostatic interaction between negatively charged cell surface of L. rhamnosus ATCC 7469 strain and positively charged zeolite surface. It is documented that the cell surface of L. rhamnosus ATCC 7469 is negatively charged in the wide range of pH values and it acts as a Lewis base *(Pelletier et al., 1997)*. The zeolites are well known as Lewis acids *(Miura et al., 2009)* which could enhance binding of L. rhamnosus cells with zeolite surface. In addition, L. rhamnosus ATCC 7469 strain produces exopolysaccharides (EPS) which form dense, sticky layer on the surface of bacterial cells and could also contribute to strong attachment of the cells to zeolite carrier. It is previously shown that zeolite surface could adsorb biopolymers like proteins and nucleic acids *(Kubota et al., 2008)*. Physiological role of bacterial EPS is still unknown, although due to their variability in composition and physical characteristics different functions were proposed. EPS could have protective role against harsh environmental conditions, act as adhesives for interactions with other surfaces or substrates, as substances for bacterial aggregation and stabilizers in biofilm formation *(Badel et al., 2011)*.

It is important to point out that zeolite addition in feed premixes has positive effect on animal health as an absorber and due to its ion exchange capacity it could provide high amount of important ions for bacteria in animal intestine (Mg2+, Na+, K+) *(Galindo et al., 1986)*. In addition, due to its buffering ability it can elevate low pH values attained in the lactic acid fermentation and can improve bacterial survival during the passage through the upper stomach. This is a significant issue when the immobilized biomass with remains after the lactic acid fermentation is considered for animal feed.

From the aspect of lactic acid fermentation, immobilization has several advantages such as higher cell density, better cell stability and it enables easy separation and recirculation of bacterial biomass *(Aljundi et al., 2005)*.

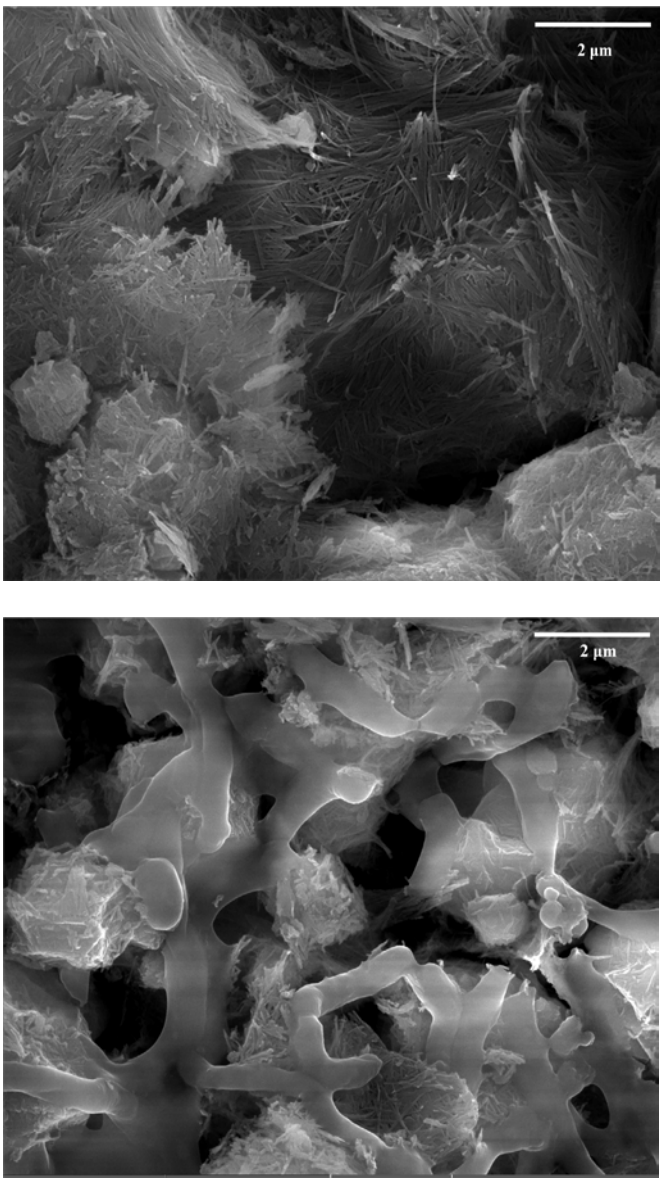

Fig. 1. First: surface of powdered zeolite molecular sieve (13X). Second: surface of powdered zeolite molecular sieve (13X) with immobilised L. rhamnosus ATCC 7469 cells

Lactic acid production with free andim mobilized *L. rhamnosus* ATCC 7469 on liquid stillage

The production of lactic acid and sugar consumption in the single batch fermentation of liquid stillage from bioethanol production by free *L. rhamnosus* ATCC 7469 cells are presented in Figure 2, while Figure 3 presents repeated batch lactic acid fermentation with zeolite immobilized bacteria. In the single batch process, a complete utilization of accessible sugars from the fermentation media was achieved after 53 h of fermentation and maximal lactic acid concentration of 34.69 g L^{-1} was achieved. Four subsequent cycles were preformed with immobilized cells until the productivity decreased to approximately 1 g L^{-1} h^{-1}. Important parameters of the single batch fermentation with free cells and the repeated batch fermentations with immobilized *L. rhamnosus* cells are presented in Table 1. A time course of viable cell numbers for free and immobilized system is presented in Figures 4 and 5.

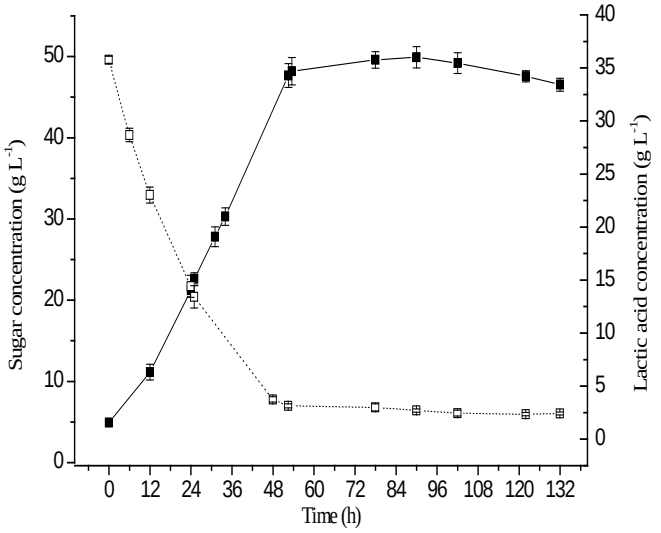

Fig. 2. Lactic acid production and sugar consumption in batch fermentation of liquid stillage by free L. rhamnosus ATCC 7469 cells. Symbols: solid line- lactic acid concentration (g L^{-1}), dot line – sugar concentration (g L^{-1})

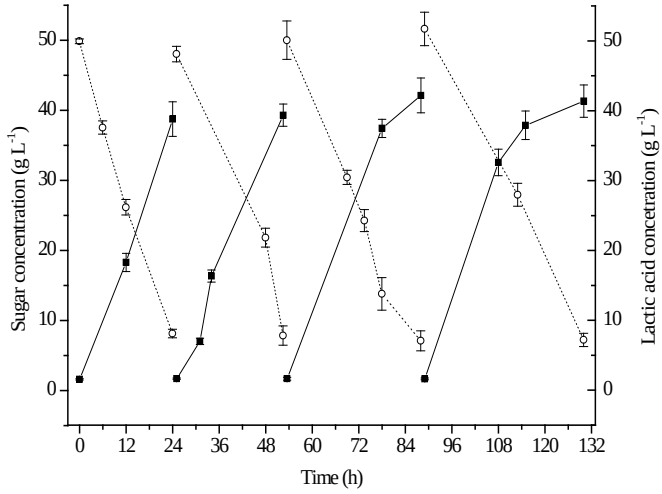

Fig. 3. Lactic acid production and sugar consumption in repeated *batch fermentation on liquid stillage by L. rhamnosus ATCC 7469 cells immobilized onto zeolite. Symbols: solid line- lactic acid concentration (g L-1), dot line – sugar concentration (g L-1)*

It can be seen from Figure 3 that both the sugar consumption and lactic acid production were faster in immobilized system comparing to the batch fermentation by free bacteria.

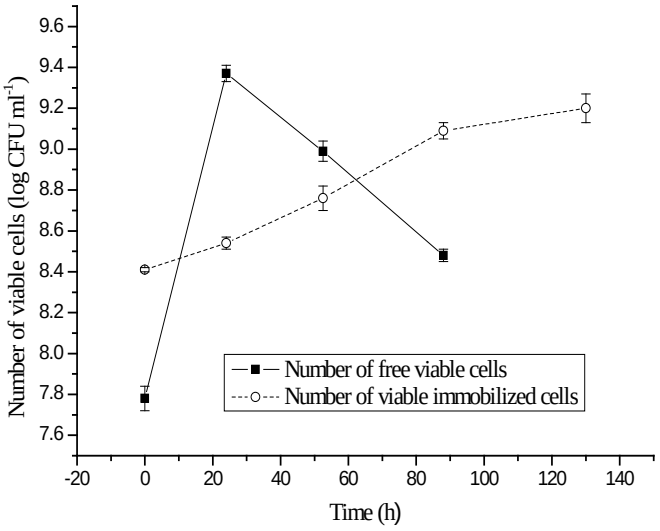

Fig. 4. Number of viable L. rhamnosus ATCC 7469 cells in free and immobilized system during the fermentation time (expressed as log CFU ml-1 of fermentation media)

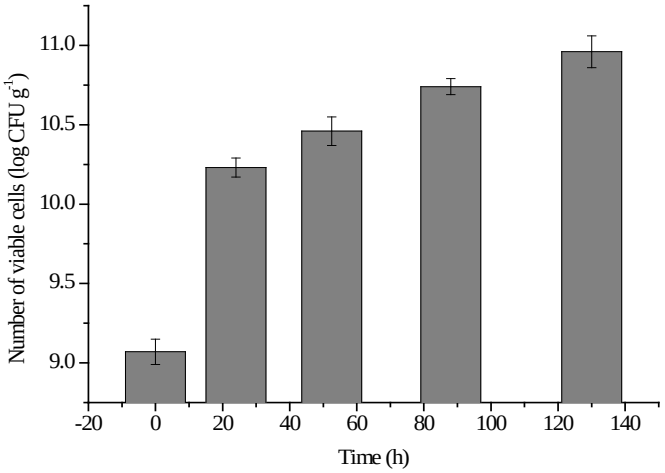

Fig. 5. Number of viable L. rhamnosus ATCC 7469 cells immobilized onto zeolite (expressed as log CFU g-1 of zeolite carrier)

Table 1. Parameters of lactic acid fermentation by free and immobilized L. rhamnosus ATCC 7469 on liquid distillery stillage

Fermentation mode		Lactic acid concentration (g L^{-1})	Lactic acid yield (g g^{-1})[a]	Yield coefficient (g g^{-1})[b]	Volumetric productivity (g L^{-1} h^{-1})
Free *L. rhamnosus* ATCC 7469[c]	Maximal values[c]	34.69±1.29	0.69±0.03	0.81±0.03	0.66±0.02
L. rhamnosus ATCC 7469 immobilized onto zeolite	1st cycle	38.80±2.48	0.78±0.04	0.93±0.05	1.62±0.10
	2nd cycle	39.36±1.59	0.82±0.01	0.98±0.03	1.43±0.06
	3th cycle	42.19±2.48	0.84±0.01	0.99±0.04	1.22±0.07
	4th cycle	41.37±2.31	0.80±0.01	0.93±0.05	1.01±0.05

[a] Lactic acid yield was expressed as g of lactic acid produced per every g of sugar present in media

[b] Yield coefficient was expressed as g of lactic acid produced per g of sugar consumed

[c] Maximal values were achieved after 52 h of fermentation

The productivity achieved in the first fermentation cycle with immobilized cells (1.62 g L^{-1} h^{-1}) was almost threefold higher than the productivity obtained in free cell system (0.66 g L^{-1} h^{-1}).

The number of viable cells initially present in immobilized system was significantly higher than in the free cell system. However, after 24 h, the viable cell number in the free cell system overcame the number of immobilized cells, but lactic acid concentration, yield, yield coefficient and productivity were still significantly higher in immobilized system (Figure 4, Table 1). This implies increased productivity of the cells immobilized onto zeolite, even with lower total number of viable cells at the end of the first cycle of repeated batch fermentation. The number of viable cells per g of carrier increased with each subsequent recirculation cycle which suggested further colonization of the zeolite surface during the fermentation (Figure 5). The continuous colonization could be explained by previously mentioned electrostatic interactions between bacterial cells and zeolite surface

and a large surface area of zeolite which was available for adsorption of bacterial cells. The lowest pH value determined in fermentation with immobilized system was 5.0 while in free cell system the lowest pH value was 3.8. Because of the basic structure of X type zeolites, a drop in pH value during the fermentation in immobilized system was lower than in the free cell system. Consequently, smaller amounts of NaOH solution were needed to maintain the constant pH value in immobilized system. *Miura et al. (2009)* reported that a number of protons are loosely bounded to the surface of different clay minerals resulting in the pH of water suspensions of zeolite of approximately 6.5. In previous study of lactic acid production on liquid stillage, a positive effect of pH control on viable cell number was noticed *(Đukić-Vuković et al., 2012)*.

The higher lactic acid productivity and better survival of bacterial cells in immobilized system is most probably a result of combined effect of zeolite buffering capacity and increased productivity of the bacteria in a biofilm on zeolite particles. Because of the advantages that could be achieved, a significant number of papers are dealing with immobilization of LAB as a good strategy for improvement of the process productivity *(Nguyen et al., 2012; Panesar et al., 2007)*. Average productivity of the process with immobilized cells was 1.32 g L^{-1} h^{-1} which is significantly higher than the productivity of 0.66 g L^{-1} h^{-1} achieved in batch fermentation with free cells (Table 1). Recently published study of *Nguyen et al. (2012)* on lactic acid production from microalgae reported a process productivity of 1.06 g L^{-1} h^{-1} which was also lower than the productivity obtained in this study.

In this study, growth of *L. rhamnosus* was intense during investigated four cycles indicating a good system stability (Figures 4 and 5) which is important for reuse of the immobilized biomass. During the fourth cycle of fermentation the sugar consumption rate as well as lactic acid production rate decreased (Table 1). However, the colonization of zeolite was still increasing during this cycle. Therefore, it can be concluded that desorption of bacteria from the carrier was not the reason for the decline in process productivity. On the contrary, high number of viable cells produced lactic acid faster at the beginning of the third and fourth fermentation cycle. The reasons for the drop of lactic acid productivity could be a product inhibition, culture aging, metabolic changes, alterations of the immobilized system, etc.

A cell number per gram of carrier is an important parameter for utilization of the remained biomass in animal nutrition. Viable cell number was more than 10^{10} CFU g^{-1} of zeolite and more than 10^{9} CFU ml^{-1} of fermentation media at the end of fourth cycle of fermentation (Figure 5). According to European Regulation *(European Union, 2008)*, a minimum content of viable bacterial cells (CFU kg^{-1}) is an important criterion for evaluation of the quality of animal feed enriched with probiotics. This number should be about 10^{9} CFU kg^{-1} in a complete feed *(Anadón et al., 2006)*. The capability of the zeolite to elevate pH value can improve the survival of *L. rhamnosus* during the passage through the upper stomach. Our future research interest is to further evaluate in detail the potential of *L. rhamnosus* ATCC 7469 immobilized onto zeolite as an animal feed supplement.

CONCLUSION

Free and zeolite immobilized cells of *L. rhamnosus* ATCC 7469 were studied for lactic acid and biomass production on the distillery stillage as a cheap waste substrate. It is shown that the zeolite immobilized system is more effective since it enables cell reuse and thus significantly higher productivity. A high number of viable cells of over 10^{10} CFU g^{-1} of zeolite were achieved at the end of fourth fermentation cycle. The results indicated that the zeolite could be used as an efficient carrier for immobilization of *L. rhamnosus* ATCC 7469 in the lactic acid fermentation of liquid stillage.

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