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# SUGAR BEET PULP AS A CARRIER FOR *LACTOBACILLUS PARACASEI* IN LACTIC ACID FERMENTATION OF AGRO-INDUSTRIAL WASTE

# REPIN REZANAC KAO NOSAČ ZA *LACTOBACILLUS PARACASEI* U MLEČNO-KISELINSKOJ FERMENTACIJI AGRO-INDUSTRIJSKOG OTPADA

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## *ABSTRACT*

*Lactic acid (LA) has gained considerable importance in the global market due to a wide range of applications. Because of the growing demand for food and feed, the use of lignocellulosic residues, by-products and waste streams is highly needed for commercial LA production. This study investigated the possibility of using potato stillage and sugar beet molasses for LA and biomass production by Lactobacillus paracasei NRRL B-4564 immobilized on sugar beet pulp.*

*Adsorption of L. paracasei enabled easy separation of bacterial biomass from the fermentation media and its efficient reuse in three successive batch cycles. Total LA concentration of 146 g $L^{-1}$ and average productivity of 1.03 g $L^{-1}$ $h^{-1}$ were achieved on waste substrate using bacterial cells immobilized on a natural carrier without mineral and nitrogen supplementation. The solid part of fermentation media remaining after LA fermentation is a valuable co-product, which could be used as animal feed rich in probiotic biomass.*

***Key words:*** *lactic acid, microbial biomass, immobilization, Lactobacillus paracasei NRRL B-4564, agro-industrial waste.*

## *REZIME*

*Mlečna kiselina (MK) zauzima značajno mesto na svetskom tržištu zbog širokih mogućnosti primene u prehrambenoj, kozmetičkoj, farmaceutskoj i hemijskoj industriji. Poslednjih godina potražnja za MK je znatno povećana zbog njene uloge u proizvodnji biodegradabilnih i biokompatibilnih polimera – polilaktida (PLA). Trenutno se na komercijalnoj skali MK proizvodi fermentacijom jestivih useva, kao što su šećerna repa, kukuruz i kasava. Zbog stalnog porasta globalne potražnje za hranom, ali i u cilju definisanja ekonomski održivog tehnološkog procesa, teži se proizvodnji MK iz alternativnih sirovina, kao što su lignocelulozni materijali, sporedni i otpadni proizvodi različitih industrija. U radu je ispitivana mogućnost korišćenja alternativnog supstrata – tečne krompirove džibre i melase šećerne repe za proizvodnju MK i mikrobne biomase pomoću soja Lactobacillus paracasei NRRL B-4564 imobilisanog na repin rezanac. Parametri ostvareni u imobilisanom sistemu su upoređeni sa parametrima postignutim u šaržnoj fermentaciji sa slobodnim ćelijama.*

*Adsorpcija L. paracasei ostvarena na površinu repinog rezanca je jednostavna i brza metoda imobilizacije koja je omogućila laku separaciju bakterijske biomase iz fermentacionog medijuma i njeno efikasno ponovno korišćenje u tri uzastopna šaržna ciklusa. Ukupna koncentracija MK od 146 g $L^{-1}$ i prosečna produktivnost od 1,03 g $L^{-1}$ $h^{-1}$ dobijena fermentacijom otpadnog supstrata na bazi krompirove džibre i melase šećerne repe sa ćelijama imobilisanim na prirodni lignocelulozni nosač je postignuta bez obogaćivanja supstrata mineralnim materijama i izvorima azota. Repin rezanac koji zaostaje nakon fermentacije zajedno sa imobilisanom mikrobnom biomasom se može koristiti kao visokokvalitetno hranivo za životinje bogato probiotskom biomasom.*

***Ključne reči****: mlečna kiselina, mikrobna biomasa, imobilizacija, Lactobacillus paracasei NRRL B-4564, agro-industrijski otpad.*

## INTRODUCTION

Lactic acid (LA) was selected as one of the most promising biochemical building blocks by the US Department of Energy in 2010 *(Bozell and Petersen, 2010)*. Besides the long history of its applications in food, pharmaceutical and chemical industry, LA has been increasingly attractive for production of polylactide (PLA) polymers. Because of its biodegradable and environmentally friendly characteristics, PLA is suitable for food packaging, rigid and soft children's toys, drug delivery systems, etc. *(Sin et al., 2012)*. On a commercial scale, LA is currently produced by LA fermentation of sugar- and starch-rich edible crops such as sugar beet, sugar cane, corn and cassava *(Harmsen et al., 2014)*. However, in order to avoid any collision in production of biobased products with food and feed production, the use of alternative feedstocks, such as kitchen garbage *(Ačanski et al., 2014)*, brewer's spent grain *(Pejin et al., 2015)*, corncob molasses *(Wang et al., 2010)* and other by-products and waste streams has been comprehensively assessed in recent years. The previous study has shown that a waste substrate based on potato stillage and sugar beet molasses could provide valuable nutrients for growth of fastidious lactic acid bacteria (LAB) in batch LA fermentation *(Mladenović et al., 2016)*. Cell immobilization has been recognized as an attractive approach for improving productivity of different fermentation processes due to higher cell concentrations, better cell stability, easier cells recovery and possibility of their reuse in repeated batch cycles *(Mojović et al., 2014)*. Cell entrapment in Ca-alginate gel is the most commonly used immobilization method for LA production. However, Ca-alginates are chemically unstable support that can easily be disrupted by lactate causing the cell release from the beads *(Chronopoulos et al., 2002)*. On the other hand, cell immobilization by adsorption on a variety of organic and inorganic supports is a simple and fast immobilization method, while utilization of low-cost and abundant agro-industrial residues as support materials could assist to create sustainable and effective biorefinery processes. Sugar beet pulp (SBP) is a

fibrous by-product of sugar beet processing. On a dry matter basis, SBP contains about 10 % proteins, 22-24 % cellulose, 30 % hemicellulose and 25 % pectin *(Vučurović and Razmovski, 2012; Šćiban et al., 2013)*. Traditionally, it is dried, pelletized and used as animal feed. For more effective utilization of SBP and consequently improved economic viability of sugar manufacturing, production of propylene glycol and bioethanol from SBP hydrolysates has been proposed *(Šćiban et al., 2013; Binczarski et al., 2015)*. The objective of this study was to investigate the possibility of using SBP as a low-cost and environmentally friendly carrier for *Lactobacillus paracasei* NRRL B-4564 in LA fermentation of combined agro-industrial waste substrate based on potato stillage and sugar beet molasses.

## MATERIAL AND METHOD

**Substrate preparation**

The stillage remaining after bioethanol production from waste potato was obtained from Reahem ethanol plant (Reahem d.o.o., Srbobran, Serbia), while sugar beet molasses was obtained from Alpis-SLC ethanol plant (Swan lake d.o.o., Belgrade, Serbia). After centrifugation of the whole stillage (4500 rpm, 20 min, centrifuge: Sigma® model 2–16, Shropshire, UK) supernatant (liquid stillage) was separated and further used for substrate preparation. The concentration of reducing sugars in the liquid stillage was set at approximately 55 g $L^{-1}$ with addition of sugar beet molasses. After adjustment of pH in the substrate to 6.5 with 30 % NaOH solution, medium prepared in this way was sterilized at 121 °C for 20 minutes and used for LA fermentation.

**Preparation of the carrier and immobilization of *Lactobacillus paracasei* NRRL B-4564**

SBP was obtained from Jedinstvo sugar plant (Sunoko d.o.o., Novi Sad, Serbia). Dry pulp pellets were chopped using food processor and the particle size fractions of 500 μm were sterilized at 121 °C for 20 minutes and used as a carrier for cell immobilization.

*L. paracasei* NRRL B-4564, a homofermentative L (+) lactic acid strain, used in these experiments was obtained from Northern Regional Research Laboratory (NRRL, Peoria, USA). The overnight culture of *L. paracasei* was propagated under anaerobic conditions at 37 °C in 200 mL of Man Rogosa Sharpe (MRS) broth with inoculum concentration of 5 % (v/v). After 18 h, the culture was centrifuged (6000 rpm, 10 min), washed with sterile 0.85 % (w/v) NaCl solution and the biomass was resuspended in 200 mL of fresh MRS broth with addition of 2 g of SBP. The culture prepared in this way was incubated at 41 °C, with shaking (100 rpm, KS 4000i control, IKA®, Staufen, Germany) for 18 h to allow cell immobilization by adsorption. After 18 h, the culture was centrifuged (1000 rpm, 5 min), supernatant with free cells was thrown, and the biomass of *L. paracasei* cells immobilized onto SBP was washed with sterile 0.85 % (w/v) NaCl solution and used as an inoculum for LA fermentation. The preparation of free *L. paracasei* cells was performed using the same procedure, but without addition of SBP.

**Scanning electron microscopy (SEM)**

Washed samples of immobilized *L. paracasei* cells on SBP were dried at 37 °C for 2 h. Dried samples were coated with Au-Pd alloy using a spatter coater. The morphology of the samples was studied by field emission scanning electron microscopy (FESEM) TESCAN Mira3 XMU at 20 kV.

**Lactic acid fermentation**

All LA fermentations were carried out as batch cultures with shaking (100 rpm, KS 4000i control, IKA®, Staufen, Germany) at the temperature of 41 °C. The fermentations were performed in 500 mL flasks with 200 mL of the fermentation media under microaerophilic conditions. After the depletion of sugar concentration below 10 g $L^{-1}$, fermentation media was centrifuged (1000 rpm, 5 min), residual immobilized biomass was washed with sterile 0.85 % (w/v) NaCl solution and inoculated into the same volume of fresh fermentation media. LA fermentation with free *L. paracasei* cells was carried out until utilization of sugars in media was completed. During the fermentation, pH was adjusted to 6.5 by adding 30 % NaOH solution in four-hour intervals. The samples were aseptically withdrawn and the substrate consumption, LA concentration and the number of living cells were further analyzed.

**Analytical methods**

In order to determine the 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. The number of viable *L. paracasei* cells was estimated using pour plate technique on MRS agar after incubation for 48 h at 37 °C. The number of viable immobilized cells was determined after detachment of cells from SBP similarly as reported by *Mojović et al., (2014)*.

**Statistical analysis**

The experiments were done in triplicates. All values are expressed as means ± standard deviation. Mean values of the treatments were compared by the analysis of variance. One-way ANOVA followed by Tukey test was applied to evaluate the effect of investigated parameters. Differences were considered significant at $p<0.05$.

## RESULTS AND DISCUSSION

**Immobilization of *Lactobacillus paracasei* NRRL B-4564 onto sugar beet pulp**

Scanning electron micrograph of the surface of SBP with immobilized *L. paracasei* cells is presented in Figure 1. As shown in Fig. 1, the surface of SBP is porous and irregular with a large number of pores and narrow channels providing high attachment area for bacterial cells. Cell immobilization on a solid surface occurs by formation of weak bonds (van der Waals forces), electrostatic and Lewis acid-base interactions *(Pelletier et al., 1997)*. Surface of SBP is heterogeneous with positively and negatively charged binding sites *(Vučurović and Razmovski, 2012)*. It was reported that *L. paracasei* strains have negatively charged cell surface in pH range 3.8-8.0 *(Pelletier et al., 1997)*, which allowed nonhomogeneous form of cell immobilization and specific cells binding on more favored sites during LA fermentation (Fig. 1). Also, cell surface hydrophobicity is important factor affecting the bacterial adhesion to a variety of surfaces. It was documented that *L. paracasei* strains have hydrophilic cell surface properties *(Pelletier et al., 1997)* and consequently a strong adhesion of *L. paracasei* cells to SBP surface can be expected.

**Lactic acid fermentation of agro-industrial waste substrate using free and immobilized cells**

LA fermentations of agro-industrial waste substrate based on potato stillage and sugar beet molasses were performed with free *L. paracasei* cells and with cells immobilized on SBP and the results are presented in Fig. 2 and Fig. 3. The main parameters of batch fermentation performed with free cells and repeated batch fermentation performed with immobilized cells are presented in Table 1.

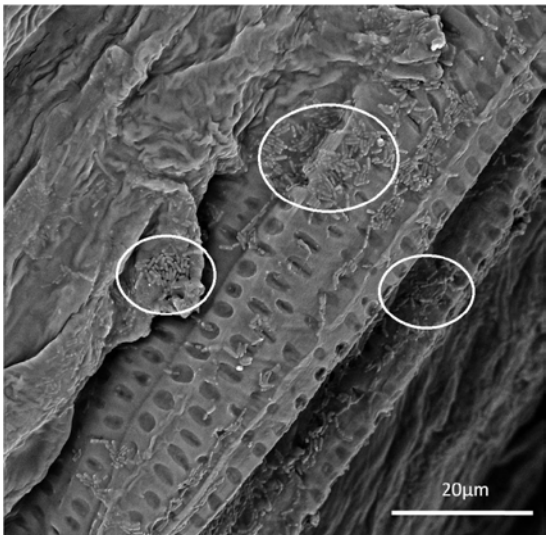


*Fig. 1. Scanning electron micrograph of Lactobacillus paracasei NRRL B-4564 immobilized onto SBP*

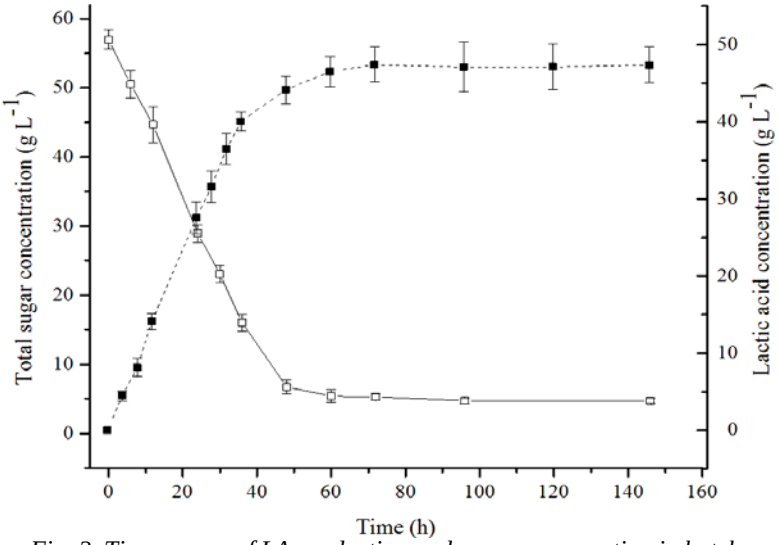


*Fig. 2. Time course of LA production and sugar consumption in batch fermentation of agro-industrial waste substrate by free L. paracasei NRRL-B 4564. Symbols: solid line – total sugar concentration, dashed line – LA concentration*

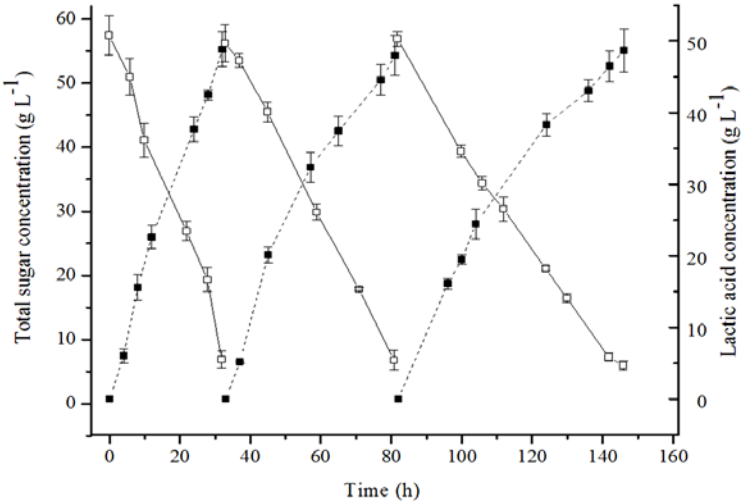


*Fig. 3. Time course of LA production and sugar consumption in repeated batch fermentation of agro-industrial waste substrate by L. paracasei NRRL-B 4564 immobilized onto SBP. Symbols: solid line – total sugar concentration, dashed line – LA concentration*

In the batch fermentation with free *L. paracasei* cells maximal LA concentration of 46.75 g $L^{-1}$ and complete sugar consumption were achieved at 72 h of fermentation. A capability of *L. paracasei* to form biofilm on the surface of SBP enabled efficient reuse of bacterial biomass in three successive batch cycles. The sugar consumption and LA production in the fermentation with immobilized cells were significantly faster compared to the same parameters of fermentation performed with free bacterial cells. The LA productivity obtained in the first batch cycle (1.53 g $L^{-1}$) was more than twofold higher in comparison to the productivity achieved in the fermentation with free cells (0.67 g $L^{-1}$). A gradual reduction of LA productivity was observed after each subsequent recirculation cycle, however after the third cycle it was still higher than the productivity obtained in the batch fermentation with free cells. In repeated batch fermentation by *L. paracasei* cells immobilized on SBP, a total LA concentration of 146 g $L^{-1}$ and average productivity of 1.03 g $L^{-1}$ $h^{-1}$ were achieved, which is significantly higher than the productivity of 0.67 g $L^{-1}$ obtained in the fermentation with free cells (Table 1).

The number of viable *L. paracasei* cells in batch and repeated batch fermentation are presented in Fig. 4. Although the initial viable cell number in batch fermentation with free cells was significantly lower than in immobilized cell system, a notable increase of free viable cell number was observed during first 24 h, and afterwards it became higher than in immobilized cell system. While the viable cell number observed in 24 h and 48 h was comparable in both fermentation modes, all significant parameters of LA fermentation achieved in the immobilized cells system were superior to those of batch fermentation with free cells. These findings are in agreement with many other studies *(Chronopoulos et al., 2002; Chantawongvuti et al., 2010; Genisheva et al., 2011; Kumar et al., 2014)* indicating that immobilized cells improved conversion efficiency and consequently resulted in superior fermentation performances. It was previously shown that natural materials used for cell immobilization could provide valuable nutrients to the medium, thus contributing to better performance of the producing microorganisms *(Genisheva et al., 2011).* As shown in Fig. 4, the maximal number of viable cells per gram of a carrier was achieved at 72 h of fermentation, followed by a slight decrease in the cell number (Fig. 4), which was probably caused by detachment of cells that were weakly bound onto SBP surface. Since LA production is dependent on cell growth, lower rates of sugar consumption and LA production during the third cycle of fermentation were observed. Although a slight decrease in the cell number after the second recirculation cycle was noticed, the high number of viable cells per gram of carrier obtained at the end of fermentation ($8\times10^{10}$ CFU $g^{-1}$) implies a satisfactory cell adsorption on SBP surface. The immobilized cell system investigated in our study has shown superior operation stability when compared to many similar studies for LA production, including bacterial

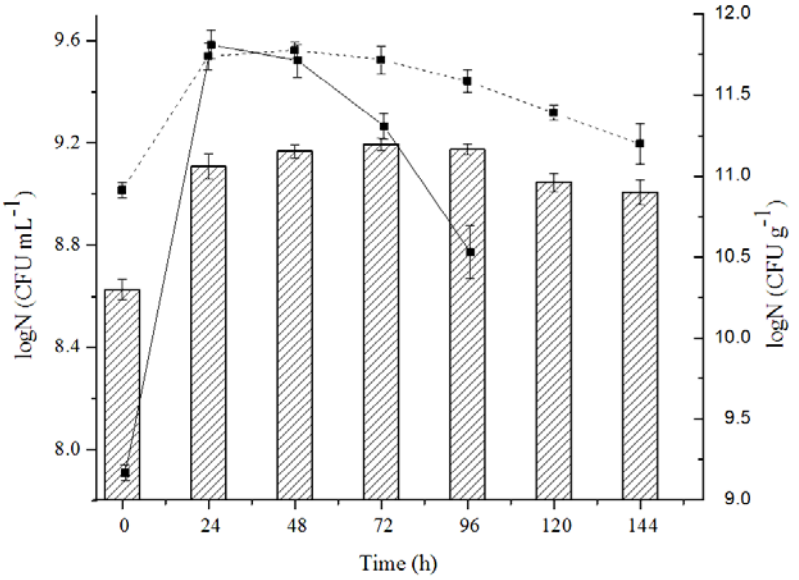


*Fig. 4. Number of viable cells in batch and repeated batch fermentation. Symbols: solid line – number of free viable cells (expressed as log CFU $mL^{-1}$ of media), dashed line – number of viable cells immobilized onto SBP (expressed as log CFU $mL^{-1}$ of media), bars – number of viable cells immobilized onto SBP (expressed as log CFU $g^{-1}$ of carrier)*

immobilization on gluten pellets *(Chronopoulos et al., 2002)*, plastic-composite supports *(Velázquez et al., 2001)*, and cellulosic materials (*Kumar et al., 2014)*. A rather strong cell adsorption and constantly high number of viable cells attached to the support surface could be explained by cellular secretion of exopolysaccharides that stabilize the cell attachment and enhance the cell retention on the surface of supporting materials *(Hsu et al., 2004)*.

*Table 1. The values of significant parameters of LA fermentation of agro-industrial waste substrate by free and immobilized L. paracasei NRRL-B 4564 cells.*

| Fermentation mode | Fermentation time (h) | | LA concentration (g $L^{-1}$) | LA yield (g $g^{-1}$) | LA yield coefficient (g $g^{-1}$) | LA productivity (g $L^{-1}$ $h^{-1}$) |
|---|---|---|---|---|---|---|
| Free cells | Maximal values | 72 | 46.75±2.07 | 0.80±0.01 | 0.85±0.01 | 0.67±0.02 |
| Immobilized cells | 1st cycle | 32 | 48.86±2.43 | 0.91±0.04 | 0.99±0.03 | 1.53±0.02 |
| | 2nd cycle | 48 | 48.38±2.80 | 0.91±0.03 | 0.98±0.02 | 1.02±0.05 |
| | 3th cycle | 62 | 48.66±2.96 | 0.90±0.01 | 0.96±0.02 | 0.78±0.03 |

The literature data on LA fermentations on waste substrate using natural and cheap materials as carriers for cell immobilization are very limited. *Kumar et al., (2014)* investigated LA fermentation of cheese whey and lactose synthetic media by bacterial cells immobilized on various cellulosic materials. After one batch cycle performed in the study of *Kumar et al., (2014)*, maximal LA concentrations of 23.8 g $L^{-1}$ and 30.2 g $L^{-1}$ and LA productivities of 0.25 g $L^{-1}$ $h^{-1}$ and 0.31 g $L^{-1}$ $h^{-1}$ were obtained on cheese whey and lactose synthetic media, respectively. These results are significantly lower than the results achieved in our present study on the combined potato stillage and sugar beet molasses media. *Chantawongvuti et al., (2010)* evaluated LA production on MRS broth by *L. salivarius* ATCC 11741 immobilized on loofa sponge coated with chitosan. In this study, LA concentration and productivity in five repeated batch fermentations were reported in the range of 25-30 g $L^{-1}$ and 0.90-1.20 g $L^{-1}$ $h^{-1}$, respectively (*Chantawongvuti et al., 2010)*. Although, the total LA concentration and productivity obtained by cells immobilized on loofa sponge coated with chitosan were comparable with the results obtained in the present study, *Chantawongvuti et al., (2010)* used synthetic MRS broth, which is expensive substrate in comparison to agro-industrial waste substrate used in our study.

Besides the high LA concentration achieved in the fermentation of agro-industrial waste substrate in this study, the high number of viable *L. paracasei* cells per gram of SBP at the end of fermentation was also achieved (Fig. 4). The LAB have the status of being generally recognized as safe (GRAS), and for decades they have been used as preserving agents during the ensiling of feed crops. Today, LAB are extensively applied in animal nutrition as probiotic feed supplement. Although drying of SBP is a common method for its long-term storage, it is usually too expensive for a small sugar factory. On the other hand, ensiling is a low-cost and more attractive storage technology for SBP and other animal feed or lignocellulosic biomass. The growth of *L. paracasei* was very intense in the combined stillage-molasses-SBP media. Taking into account the well-documented beneficial effects of LAB on the gut microbiota such as reduction of diarrhea incidence, protection against a variety of pathogen, etc. *(Gaggìa et al., 2010)*, as well as a traditional usage of SBP, stillage and molasses in animal nutrition, the solid part of fermentation media with immobilized *L. paracasei* biomass remaining after LA fermentation could be considered as high-value animal feed.

## CONCLUSIONS

The study investigated LA fermentation of agro-industrial waste substrate by free and immobilized *L. paracasei* NRRL-B 4564 cells on SBP. The studied bacterial immobilization could be applied as an attractive and effective strategy for improvement of LA production. It enabled efficient reuse of LAB biomass in three successive batch cycles. Utilization of SBP as a carrier for *L. paracasei* NRRL-B 4564 could assist to create a biorefinery process for LA and high-quality animal feed production. The valorization of distillery stillage, sugar beet molasses and SBP proposed here improves economic viability of sugar and ethanol industry and simultaneously allows for production of added value products.

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