IMMOBILIZATION OF *LACTIPLANTBACILLUS PLANTARUM* CELLS ON DISTILLERY SPENT GRAINS FOR LACTIC ACID PRODUCTION

Bilyana I. Ilieva[1], Svetla T. Danova[2], Dragomir S. Yankov[1]

[1]*Institute of Chemical Engineering, Bulgarian Academy of Sciences*
103 Acad. G. Bontchev str., 1113 Sofia, Bulgaria
[2]*The Stephan Angeloff Institute of Microbiology*
Bulgarian Academy of Sciences, 26 Acad. G. Bontchev str.
1113 Sofia, Bulgaria
Email: yanpe@bas.bg

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ABSTRACT

Many industrial fermentation processes use immobilized biocatalysts because of their undoubted advantages. From an economical point of view, the application of cheap and abundant support for immobilization is preferable. In this paper, the possibilities for using distillery spent grains as support for Lactiplantibacillus plantarum cells' immobilization for lactic acid production were investigated. The influence of different parameters (particle size, pH, temperature, support modification) on lactic acid production was studied. Best results were obtained with a 0.63 - 0.4 mm fraction of spent grains. The immobilized cells did not show differences in optimal pH and temperature compared to free cells. The immobilized L. plantarum cells kept about 75 % of the production of lactic acid, in comparison with the free ones, for more than two months and in ten consecutive runs. Distillery spent grains are very promising support for cells' immobilization with great potential.

Keywords: Lactiplantibacillus plantarum, immobilization, distiller's spent grains, lactic acid production, optimal conditions.

INTRODUCTION

Lactic (2 - hydroxypropanoic) acid is a useful chemical with both traditional (as an acidulant, neutralizer, preservative) and newer (for environmentally friendly solvents or as a precursor of biodegradable polymers) applications. The lactic acid can be manufactured by chemical synthesis (mainly by hydrolysis of lactonitrile synthesized from HCN and acetaldehyde) or by fermentation of different carbohydrate materials (sugars, whey, starchy or cellulosic hydrolysates) by various lactic acid bacteria (LAB), mainly lactobacilli. In recent years, the fermentative production of lactic acid (LA) plays a predominant role despite the high cost of substrates and medium components [1]. *Lactobacillus plantarum* (reclassify recently into a new genus - *Lactiplantibacillus)* is one of the widest spread species used in lactic acid production and/or as probiotics. Its facultative heterofermentative metabolism depends on oxygen and substrate levels in the broth [2]. In the last years, the immobilization of whole *Lactobacillus* cells attracts the attention of researchers because of the undoubted advantages in lactic acid production. Some of them are the possibility of continuous operation; ease of separation of the product; reuse of the biocatalyst; high cell density and volumetric productivity as well as protection of the cells against contamination or other chemical and physical factors, and improved process control. Various methods like adsorption [3 - 6] and entrapment [7, 8] were used for the immobilization of Lactobacillus cells. Encapsulation in different gels [9 - 11], however, is the most commonly used method for immobilization of lactic acid producing cells, with Ca-alginate being the most popular [12 - 21]. Different process parameters (polymer concentration, the growth phase of the microorganisms, effect of the bivalent ions, bead diameter, cells loading, etc.) were investigated and the better performance of Ca - alginate gels over other gels was proven by many

researchers [22 - 25]. In recent years, immobilization of different lactobacilli in Na-alginate - PVA gels was applied, with very good yields and productivity [26 - 28].

Despite numerous works devoted to the cells' immobilization, the search for new supports is still in progress. Usually, the cost of the support and the immobilization process is the major obstacle to the implementation of a given system in industrial scale production. Assuming this, "ideal" support must meet the following requirements - low price, availability, high mechanical strength, stability, reusability, high cells' loading capacity, low mass transfer limitations, nontoxicity, and biocompatibility.

Spent grains originating from breweries and distilleries are the most abundant agro-industrial waste material and respond to most of the above-listed requirements. Currently, spent grains are used mainly as animal feed. Attempts were made for using spent grains for the extraction of metals and dyes. Greater attention was paid to the hydrolysis of the grains for producing fermentable sugars, used as substrates in various bioprocesses (biomass and enzyme production, as well as the production of bioethanol and other valuable chemicals like lactic acid). A promising alternative for spent grains application is its use as carriers for enzymes and cells' immobilization. Brewer's spent grains (BSG) were used for *Saccharomyces cerevisiae* immobilization for beer or alcohol production [29 - 31] and in winemaking [32, 33]. The BSG was used without treatment or after delignification. Mussatto et al. immobilized *Aspergillus japonicus* cells on BSG for fructooligosaccharides and β - fructofuranosidase production [34]. Rocha et al. [35] and Almaida et al. [36] also used BSG as the support of cells for enzyme production. *Lactobacillus casei* was immobilized on brewery spent grains for use in sourdough wheat bread making [37]. Radosavljević et al. [38] and Mladenovich et al. [39] used brewers spent grains and other agro-industrial waste material as supports for the immobilization of lactobacilli.

Adhesion of the microorganisms on various surfaces and biofilm formation is a rather complex process, strain-specific, and depends on different factors. Some important factors affecting the adhesion are the surface properties of the cells and the matrix (surface layer proteins, electrostatic forces, surface charge, hydrophobicity, etc.) as well as the environmental factors (pH, temperature, substrate concentration) [40]. Significant differences in adhesion properties of four lactobacilli strains on eight different types of fibers were reported, resulting in different percentages of cells attached to different surfaces [41]. The cereal fibers were most suitable.

Although at first glance brewers and distillers spent grains are quite similar, there are significant differences in their chemical composition, especially in fiber, fat, lignin, and starch content. We believe that these differences will affect the adhesion of lactobacilli cells. To the best of our knowledge, there are no studies on using distiller's spent grains as support for lactobacilli immobilization for lactic acid production.

The purpose of the present work is to investigate the applicability of distiller's spent grains for cells' immobilization, especially for the production of lactic acid.

EXPERIMENTAL

Materials and Methods

Strain and growth media

Lactobacillus plantarum - AC11S, from the laboratory collection of the Institute of Microbiology - BAS, was isolated from a homemade white brined cheese [42]. The strain was identified as *Lactobacillus plantarum* by classical phenotypic methods and multiplex PCR, targeting the rec A gene. Due to the new classification of the genus *Lactobacillus* [43] today it was re-classified in the genus *Lactiplantobacillus*, but kept the species name *plantarum*. It was cultured in de Man, Rogosa, and Sharpe broth (MRS Difco, USA) and stored at –20°C in MRS broth supplemented with glycerol 20 % v/v until use in the experiments. The cells growth medium (LA broth) contained (g L^{-1}): lactose monohydrate: 11, yeast extract: 5.5, peptone from casein: 12.5, sodium acetate: 10, KH_2PO_4: 0.25, K_2HPO_4: 0.25, $MgSO_4.7H_2O$: 0.1, $MnSO_4.7H2O$: 0.05, $Fe_2(SO_4)_3$: 0.05 (all chemicals were from Fluka, p. a. grade). The inoculum was prepared from glycerol LAB stocks, pre-cultured twice in MRS broth, (Merck, Germany), 100 ml growth medium in 300 mL Erlenmeyer flasks at 30°C with an initial pH of 6.5. All experiments were carried out in flasks using a WiseCube® WIS30 shaking incubator (Witeg Labortechnik GmbH, Germany).

Carrier

The support used was spent grains from distillery Almagest AG, Bulgaria. Almagest AG produces high-

quality ethanol for the food and beverage industry, using wheat or corn as the main raw material. Dried distillers grains with soluble (DDGS), containing over 33 % protein and less than 5 % of initial starch, are separated as a by-product. In this study, DDGS from corn were used. Some characteristics of DDGS (analyzed according Standard Biomass Analytical Methods provided by the National Renewable Energy Laboratory, USA) are given below: moisture - 5.4 %; total solids - 94.6 %; acid-insoluble lignin - 13.20 %; acid-soluble lignin - 26.2 %; ash - 4.7 %; extractives - 20.3 %; other (cellulose and hemicellulose) - 35. 6 %.

Immobilization procedures

(A) Initially the *L. plantarum* AC11S was cultivated in LA growth media, described above (LA broth) for 24 h at 30°C. After cells' concentration determination, 10 g sterile spent grains were added and adsorptive immobilization was carried out at 30°C for another 24 h at gentle stirring. In the end, the concentration of the free cells was determined, and the support was separated from the broth, washed with sterile saline, and kept at 4°C in saline before further use.

(B) The immobilization was also carried out by simultaneous growth and adhesion, adding spent grains in the beginning, before growth medium seeding.

(C) To ameliorate the stability of immobilized preparation, it was treated with 100 mL 5 % glutaraldehyde, 100 mL 2 % polyethyleneimine (PEI) with pH 7.0 (H_2SO_4). After thoroughly washing with saline, the final immobilized preparation was used for stability tests.

Analytical procedures

The biomass was calculated from optical density data at 620 nm (UV-VIS spectrophotometer Milton Roy 401, Rochester, USA) using a previously prepared calibration curve. All measurements were made in duplicate. The concentrations of lactic acid were measured using an HPLC system composed of a Knauer Smartline-100 pump, Pekin-Elmer LC-25RI refractometric detector, and data processing software Eurochrome (Knauer). The column used was Aminex HPX-87H (Bio-Rad). A 0.005 M solution of H_2SO_4, was used as the mobile phase at a flow rate of 0.6 mL/min. Pure (98 % mass, Sigma), crystalline L- (+)-lactic acid was used to prepare standard solutions.

Fermentation

All experiments for lactic acid production with immobilized cells were carried out in LA broth (described above) in flasks with a volume of 300 mL at 30°C and initial pH of the medium 6.5 (except those for optimum pH and temperature determination).

RESULTS AND DISCUSSION

The attachment of cells by adsorption on natural or synthetic inert supports is the simplest method of immobilization. This technique is based on the physical interaction between cells and the support surface. Different weak forces (van der Waals, electrostatic, hydrophobic or ionic interactions, hydrogen bonds) are responsible for cells' attachment. Because of the direct contact with the nutrient medium and lack of diffusional restrictions, the cells' immobilization on properly chosen support, usually is favorable for the activity and metabolism of the attached microorganisms.

In three parallel experiments, *L. plantarum* AC11S cells were immobilized on spent grains, according to the first described procedure (A). The concentration of free biomass after 24 h of contact time (at the conditions optimal for the free cells - pH = 6.5 and T = 30°C), was measured and compared with free biomass of the control, cultivated under the same conditions. The number of cells adsorbed on spent grains varied between 4.1 x 10^3 and 5.2 x 10^3 g/g (e.g. about 20 - 30 % of the biomass was adsorbed on the grains).

Influence of pH and temperature

The pH value of the medium and the temperature of fermentation are important factors affecting the cells' growth and productivity of the immobilized cells. Usually, immobilization by adsorption does not lead to changes in the optimal pH of the immobilized cells.

In order to determine optimum conditions for immobilized *L. plantarum* AC11S cells, two series of experiments were performed. In the first one, the pH of the medium was varied from 3.0 to 8.0. Ten grams of immobilized growing cells on spent grains, (according the procedure B) were added to 100 mL LA broth, and fermentation was carried out at 30°C and under static conditions. The temperature was changed from 10 to 50°C in the second set of experiments, conducted at pH 6.5. Again, 10 grams of spent grains with immobilized

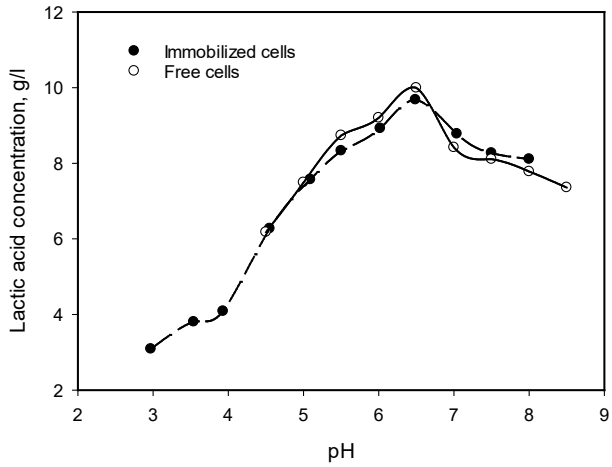

Fig. 1. Influence of the pH value on lactic acid production with immobilized cells.

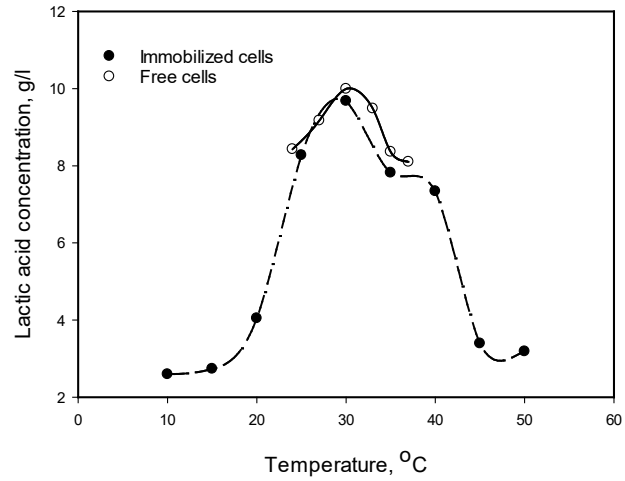

Fig. 2. Influence of the temperature on lactic acid production with immobilized cells.

growing LAB cells were added to the LA growth medium and the fermentation was carried out at static conditions, pH 6.5, and desired temperature. The obtained results are presented in Figs. 1 and 2, respectively.

It is visible from Fig. 1 and Fig. 2 that the optimum conditions were the same as for the free culture: pH 6.5 and T = 30°C. All further experiments were carried out under these conditions. It is worth to mention that immobilized cells retain relatively high activity (about 80 % of the optimal one) in a wide range of pH (5.0 to 8.5) and temperature (25 - 35°C)

Some researchers conducted fermentation with immobilized cells at the same temperature as the optimal for the growth of free cells without optimization [28, 38]. Wang et al. investigated the influence of the temperature on the lactic acid production with *Lactiplantibacillus pentosus* cells immobilized in alginate/PVA gel in the range 31 - 39°C and determined 35°C as optimal for LA production which was higher than this for free cells [44]. In the case of Ca-alginate immobilized *Lacticaseibacillus casei* cells, Gao et al. pointed out 30°C as the optimal temperature for the production of lactic acid [21]. Thakur et al. reported reasonably good LA production in the range 33 - 42°C with an optimum at 37°C [27]. Idris and Suzana also found optimal production at 37°C in the case of Ca-alginate entrapped *Lactobacillus delbrueckii* cells [16]. Mladenović et al. harvested free cells of *Lacticaseibacillus paracasei* cells cultured at 37°C but made the immobilization and fermentation at 41°C [39].

Most of the lactobacilli can grow and ferment sugars in a broad range of pH (generally between 4.0 and 8.0), however, the growth and production rates are quite different. Usually starting pH values for lactic acid production is 6.5 - 7.0 and for maximum production, the pH was controlled in this range by adding various bases. A drop in the pH of the medium due to the produced lactic acid leads to a decrease in the production rate. At a pH value close to pK_a of the lactic acid (3.86) the growth and production are fully inhibited by undissociated acid molecules, which are greater inhibitors than lactate ions. Thakur et al. reported efficient production in the pH range of 5.5 to 7.5 with an optimum around pH of 7.0 [27]. Wang et al. obtained different pH optimums for LA yield (pH 6.0) and productivity (pH 5.5) [44]. Mladenović et al. [39] performed fermentation by immobilized cells maintaining a pH of 6.5, while Radosavljević et al. [38] - at a pH of 6.2. Idris and Suzana investigated the effect of initial pH (from 4.5 to 8.5) on LA production with Ca-alginate immobilized *L. delbrueckii* cells and observed a shorter lag-phase at pH 6.5 which was optimal for the production [16].

Influence of the particle size on the immobilization efficiency

The immobilization efficiency depends on support surface properties (structure, specific area, pore size, etc.).

In the present study, the spent grains were divided into four fractions: over 1.0 mm; 1.0 - 0.63 mm; 0.63 - 0.4 mm; and 0.4 - 0.25 mm, using a sieve machine. Ten

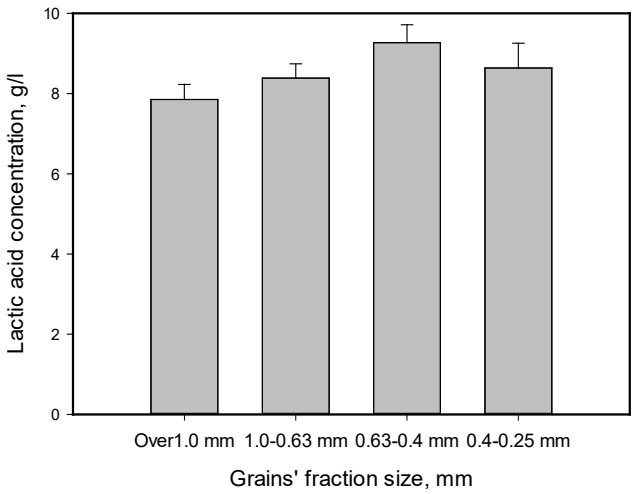

Fig. 3. Influence of grains' fraction size on lactic acid production.

grams of each fraction were used for immobilization, according to the first described procedure (A). As a control, fermentation with free cells from an exponential culture of *L. plantarum* AC11S was performed. All fermentations were carried out without pH control. The results for lactic acid production from three parallel runs are presented in Fig. 3. The best results (9.3 g L^{-1}) were obtained with a fraction of 0.63-0.4 mm.

There is no significant difference in lactic acid production during the runs. The immobilized cells produced between 70 and 90 % in comparison with free cells. The following experiments were made with spent grains of 0.63 - 0.4 mm size. Usually, there is an optimal size of support material assuring the best conditions for cells' adhesion and making diffusional restrictions negligible.

To the present, there are no published data, for optimization of particles' size in case of brewers spent grains or other agro-industrial wastes, used as supports for immobilization. Mladenovic et al. used particles with a diameter of approximately 500 μm [39]. Idris & Sizana reported maximum lactic acid production with *Lactobacillus delbrueckii* cells immobilized in Ca - alginate bead with a diameter of 1 mm [16]. The best production with 1 mm beads, was also found in the case of *Lactobacillus casei* [21]. However, the authors decided to work with 2 mm beads, because of the negligible difference in obtained lactic acid concentration. Thakur et al. proposed 2.5 mm beads diameter to be optimal for the production of lactic acid with *L. casei* cells immobilized in double-layered (with chitosan and alginate) Ca-alginate beads [27]. In the case of *Lactobacilli* cells immobilized in Ca-alginate/PVA gels, Wang et al. [26] and Radosavljević et al. [28] used particles with a diameter of 2.5 ± 0.5 mm as matrices for immobilization. Nevertheless, it is difficult to compare the influence of support size (the optimal bead's diameter) on the efficiency of production when different types of immobilization are used and different diffusional limitations are presented.

Stability of immobilized preparation

The stability of the immobilized preparation is very important for the semi-continuous or continuous processes realization, and it is related to good adhesion of the cells, as well as with cells' growth and colonization of the surface.

In order to study the stability of immobilized *L. plantarum* AC11S preparation, we carried out a new immobilization using 0.4 - 0.63 mm spent grain fraction. A series of six runs was carried out. The first three runs were executed on three consecutive days, then the immobilized preparation was stored in saline for two weeks, two more fermentations were carried out, and the last one was made 5 weeks later. The results are summarized in Fig. 4.

For the entire period of about two months, the immobilized cells reserved nearly constant activity - about 8 g L^{-1} lactic acid was produced (75 % from the free cells' control). The free biomass concentration in the broth decreased after each run and reached a constant value of about 0.1 g L^{-1} after the third run. Radosavljević et al. reported a slight decrease in lactic acid productivity after 6 consecutive runs with *Lacticaseibacillus rhamnosus* cells immobilized on brewers spent grains [38]. Maximal productivity was obtained on the 4th cycle and lactic acid yield was about 3.4 % higher than in the case of free cells.

Similar results were obtained in the case of *Lacticaseibacillus paracasei* cells immobilized on sunflower seed hull (SSH), brewers' spent grains (BSG), and sugar beet pulp (SBP). There were no significant differences between the three immobilized preparations after the first cycle, and the produced lactic acid was the same as with the free cells but significant differences were observed thereafter. Produced lactic acid decreased respectively to 96.7, 87.1, and 78.2 % at the end of the

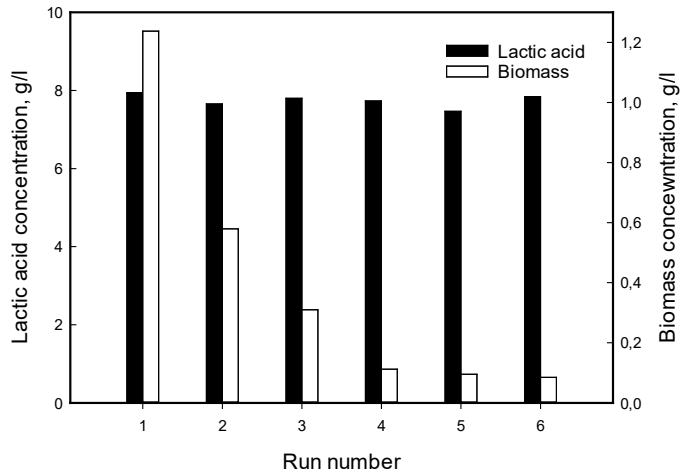

Fig. 4. Stability of immobilized *Lactiplantibacillus plantarum* AC11S preparation.

fifth cycle for SBP, BSG, and SHH.

In the case of *Lacticaseibacillus casei* cells entrapped into Ca-alginate gel, Cao et al. executed 11 cycles and found out that the concentration of produced LA passed through a maximum at the 8th cycle [21]. The conversion during the cycles varied from 70 to 77 %. Thakur et al. [27] studied the stability of *L. casei* cells immobilized by entrapment in Ca-alginate (A), Ca-alginate, coated with chitosan (AC), and Ca - alginate double-coated by chitosan and alginate (ACA) gel beads. Lactic acid production was nearly constant for 4 (A), 7 (AC), and 9 (ACA) cycles and decreased by 52, 25, and 7 % after the 9th cycle. Regarding the stability of immobilized preparations, cells entrapped in Ca - alginate/PVA gels seem to be more effective. Radosavljević et al. [28] reported constant lactic acid yields during 7, while Wang *et al*. [44] during 15 consecutive runs. Lactic acid production by *L. casei* cells immobilized in PVA cryogel decreased by 13 % at the end of the 10th cycle [45].

Influence of immobilization mode

In view to study the influence of the bacterial growth phase on the efficiency of immobilization, another procedure was used to study the influence of the bacterial growth phase on the immobilization process. The immobilized preparation was obtained after simultaneous growth and immobilization (see Materials and Methods, Immobilization procedure B). For this purpose, ten grams of the sterile spent grains were added to 100 mL of the medium and it was seeded with 10 % of 24 h inoculum. After 24 h of incubation and immobilization, the grains were separated from the broth and washed three times with sterile saline. With new immobilized preparation, three consecutive runs were executed. In Fig. 5 a comparison between two modes of immobilization (with growing and resting cells) is given. There was no difference in lactic acid production, while the free biomass concentration decreased more rapidly in comparison with the immobilized resting cells.

Subsequently, the number of consecutive runs was increased to ten and the results obtained are shown in Fig. 6. The lactic acid production remains relatively high - about 70 - 75 % from the control while free biomass concentration shows a slightly increasing tendency after the 5th run. Most probably free cells in the broth could not adhere back to the grain's surface or dead cells detach from the grains' surface. No tests were made to compare the number of living and dead free cells.

Support or immobilized preparation treatment

For ameliorating the lactic acid production efficiency, the support was treated with glutaraldehyde (GA), and polyethylene imine (PEI) - separately, or with both, consecutively. Then the modified supports were used for cell immobilization. This treatment aimed to achieve better attachment of the cells and a lower number of detached cells. Three consecutive runs were executed with each of the differently treated supports and the results were compared with those for untreated ones (Fig. 7). No significant difference in lactic acid production, but it is

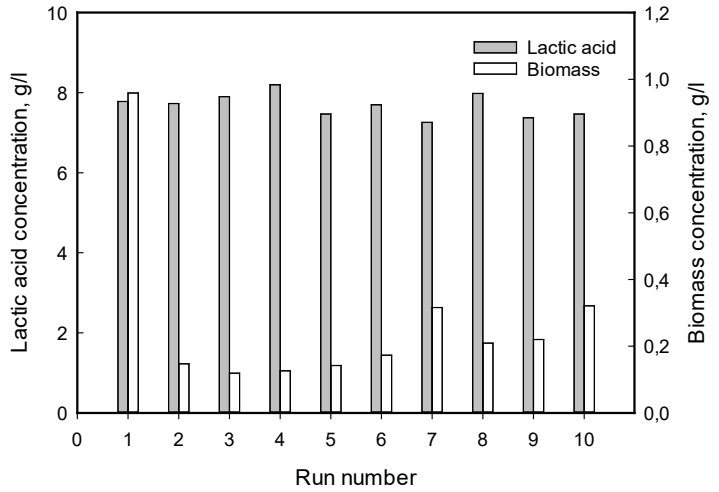

Fig. 5. Influence of mode of immobilization (growing or resting cells) on free biomass accumulation and lactic acid production.

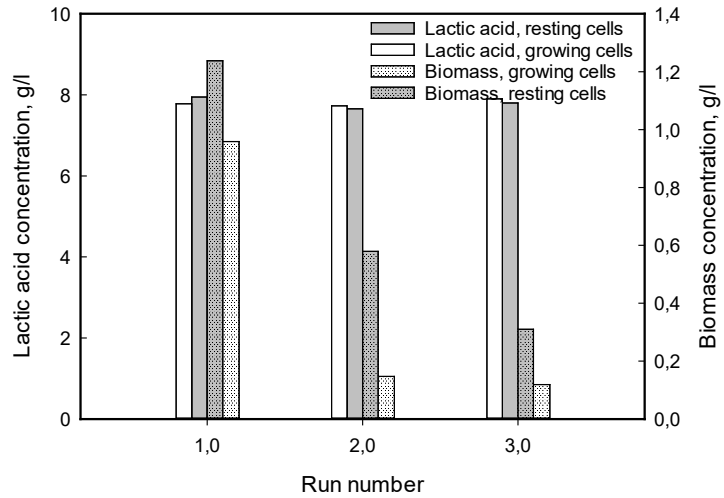

Fig. 6**.** Stability of immobilized growing LAB cells.

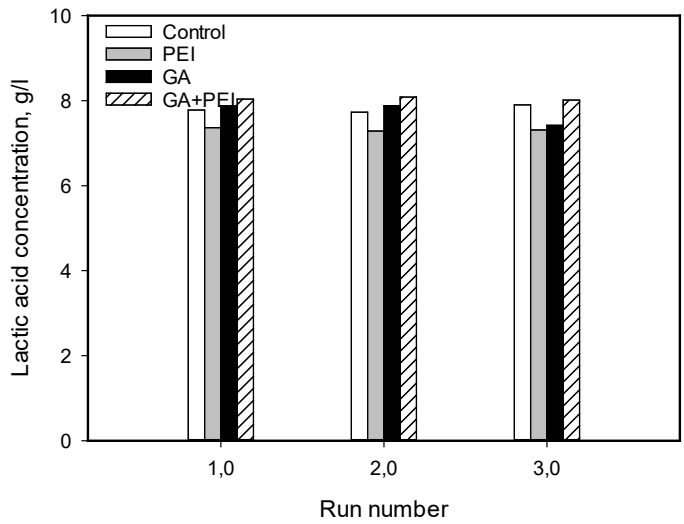

Fig. 7. Influence of additional treatment of immobilized cells (PEI, GA) on lactic acid production.

worth to mention that while PEI-treated support showed slightly lower lactic acid production, the one treated with PEI and GA showed slightly better conversion than control (untreated grans). In all cases, it is expected that PEI coated and GA cross-linked support will show better stability. The attempt to treat immobilized cells after immobilization was unsuccessful - the cells were stripped from the support and formed pellets in the solution.

CONCLUSIONS

In the present study, the possibility of distillery spent grains application for *Lactobacillus* cells immobilization, and lactic acid production was assessed. The immobilization efficiency is influenced by different parameters, such as the growth phase of LAB cells, the size of the spent grains, etc. The immobilized cells of *Lactiplantibacillus plantarum* AC11S strain showed high lactic acid productivity (about 70 - 80 % from free cells' productivity, 7.3 - 8.2 g L^{-1} lactic acid) and very good stability in at least two months of repeated batch fermentation without pH control. Moreover, no difference in optimal pH value (6.5) and temperature (30°C) for free and immobilized cells was shown. The results obtained represent a good base for studying continuous lactic acid production by immobilized on spent distillery grains *L. plantarum* or other LAB strains.

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