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The Influence of Biomass Preparation on Physiological Activity of Bacterium *Lactobacillus plantarum* Strain L4 During Storage at Different Freezing Temperatures

Utjecaj pripreme biomase na fiziološku aktivnost bakterije *Lactobacillus plantarum* soj L4 tijekom čuvanja pri različitim temperaturama zamrzavanja

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Summary

Today the lactic acid bacteria (LAB) are routinely used as starter cultures in fermented food production; species ***Lactobacillus plantarum*** *is used mostly for vegetables and olives conservation, ensiling and malolactic fermentation of wines. In this work the influence of* ***Lb. plantarum*** *L4 biomass preparation for long-term preservation by freezing at different temperatures on physiological activity (i.e., viability and metabolic activity) was investigated. After growth in optimal conditions the bacterial biomass was prepared for freezing in the following ways: a) biomass sediment obtained after centrifugation was resuspended in the whole volume of neutralized spent growth medium (BNNM); b) biomass sediment was resuspended in 1/5 th of spent growth medium volume (CB), and c) after centrifugation the bacterial biomass was rinsed and then resuspended in the physiological saline until reaching cell concentration as in a) (WB). During 100 days of storage at –20, –30 and –70 °C the viability of cells by plate count method and metabolic activity via lactic acid production were followed. At all investigated freezing temperatures through the storage period the survival of* ***Lb. plantarum*** *L4 in BNNM was complete. Meanwhile, in washed biomass (WB) the survival of cells was considerably poorer, i.e., after 100 days of storage at –20, –70 and –30 °C there was only 42, 48 and 5 % of living cells, respectively. The survival of* ***Lb. plantarum*** *L4 in CB at investigated temperatures was among 71 and 81 %. In all experimental conditions at the end of storage period the metabolic activity of frozen/ /thawed cells expressed as lactic acid production ratio of these cells and nonfrozen control was between 86 and 90 %.*

Sažetak

Bakterije mliječne kiseline danas se rutinski koriste kao starter kulture u proizvodnji fermentirane hrane; vrsta ***Lactobacillus plantarum*** *uglavnom se primjenjuje za konzerviranje povrća i maslina, siliranje, te jabučno-mliječnu fermentaciju vina. U ovom je radu istražen utjecaj načina pripreme biomase* ***Lb. plantarum*** *L4 na fiziološku aktivnost (tj. preživljavanje i metaboličku aktivnost) za dugotrajno čuvanje zamrzavanjem pri različitim temperaturama. Nakon rasta u optimalnim uvjetima bakterijska je biomasa bila pripremljena za zamrzavanje na ove načine: a) talog biomase dobiven nakon centrifugiranja pomiješan je sa cijelim volumenom neutralizirane istrošene podloge za rast (BNNM); b) talog biomase pomiješan je s 1/5 istrošene podloge za rast (CB) i c) biomasa je nakon centrifugiranja oprana, a zatim pomiješana s fiziološkom otopinom do koncentracije stanica kao u a) (WB). Tijekom 100 dana čuvanja pri –20, –30 i –70 °C praćeno je preživljavanje stanica metodom nacjepljivanja na čvrste podloge i metabolička aktivnost praćenjem proizvodnje mliječne kiseline. Pri svim temperaturama zamrzavanja tijekom cijelog razdoblja čuvanja bilo je potpuno preživljavanje bakterije* ***Lb. plantarum*** *L4 u BNNM. Međutim, u opranoj je biomasi (WB) preživljavanje stanica bilo značajno slabije, tj. nakon 100 dana čuvanja na –20, –70 i –30 °C bilo je samo 42, 48 odnosno 5 % živih stanica. Preživljavanje* ***Lb. plantarum*** *L4 u CB pri ispitivanim temperaturama bilo je između 71 i 81 %. U svim je eksperimentalnim uvjetima na kraju razdoblja čuvanja metabolička aktivnost, izražena kao odnos zamrznutih/odmrznutih stanica i nezamrzavanih stanica, iznosila između 86 i 90 %.*

Introduction

Lactic acid bacteria (LAB) are used throughout the world for the manufacture of a wide variety of fermented food products. Starter cultures of LAB are of fundamental importance in the production of fermented dairy products, meat, fish, vegetables, olives, bread and wine and also of silage for animal feed. The strains of

Lactobacillus plantarum species are included, either as pure or mixed culture, in most of the fermentation processes mentioned above *(1)*. Commercial starter cultures are mainly distributed as dried or frozen, because the cultures that have been preserved in such ways are held in a state of suspended animation *(2)*. The bacterial cells are likely to lose their viability during freezing or drying (usually freeze-drying) and subsequent storage period. The major factors that cause injuries are reviewed by Calcott *(2)* and Heckly *(3)*.

The aim of this work was to investigate the effect of biomass preparation method on viability and metabolic activity of *Lactobacillus plantarum* strain L4 during long--term storage period at different (–20, –30 and –70 °C) freezing temperatures.

Experimental

Lactobacillus plantarum strain L4 from the Culture Collection of the Faculty of Food Technology and Biotechnology, University of Zagreb was kept at 5 °C on MRS (»Merck«, Darmstadt) agar slants. Stock culture was transferred in MRS broth (pH = 6.5) and incubated at 37 °C for 12 hours before being propagated in the fermentors. One liter multistation batch fermentors (»Churchill«, L.H. Engineering Company Ltd., England) were used for the bacterial growth. Cultivation was performed in MRS broth with inoculation size of 10 mL/L, at 37 °C and pH = 6.5 for 16 hours (i.e., until early stationary phase). After cultivation the cells were concentrated by centrifugation at 8000 rpm for 15 min at 0 °C by »Beckman«, J-21B centrifuge. For the freezing experiments the bacterial biomass obtained by centrifugation was prepared in three ways: a) biomass was resuspended in previously neutralized (by 50 % NaOH) spent growth medium collected during centrifugation (BNNM); b) biomass was resuspended in 1/5 th of neutralized spent growth medium collected during centrifugation (CB), and c) biomass was three times rinsed and than resuspended in the volume of sterile physiological saline equal to supernatant of spent medium (WB). The obtained cell suspensions were asseptically distributed (by 5 mL) into 30 mL capacity glass vials with rubber stopper and mixed with equal volume of phosphate buffer (pH = 6.8). One set of samples (prepared in duplicate) was analyzed immediately and the others were frozen in mechanical freezers at –20, –30 and –70 °C, with freezing rate between 1.5 and 2.4 °C/min. Dynamics of viable cells count and metabolic activity determination during 100 days of storage at investigated freezing temperatures was: 5, 10, 15, 30, 40, 50, 60 and 100 days. All samples were thawed in the same way i.e., 1 hour in the thermostat at 37 °C. The viable cells of *Lb. plantarum* L4 were determined by plate count method placing the 10 µL of sample decimal dilutions (min. two) in quadruplicate on MRS agar plate and incubating at 37 °C for 48 hours. The results were expressed as the concentration of cells i.e., the number of cells corresponding to the number of colony forming units (CFU) per milliliter of culture (N), and represent the data calculated by statistical means (average number, standard deviation and coefficient of variation). The results with smallest dispersion (bellow 15 %) were used for survival calculations as it was described earlier *(4)*. The metabolic activity was determined by measuring the MRS broth acidification rate (i.e., the production of lactic acid) after 12 and 24 hours of stationary growth of *Lb. plantarum* L4 at 37 °C by titrating of sample aliquot with 0.1 M NaOH solution with phenolphthalein as an indicator. The results represent the ratio of lactic acid production (g/L) between thawed (P_t) and nonfrozen (P_o) cells:

$$\text{metabolic activity} = \frac{P_t}{P_o}$$

Results

The starting cells concentration (No) of *Lb. plantarum* L4 i.e., before freezing were 1.4×10^9, 1.6×10^9 and 6.5×10^9 mL^{-1} for BNNM, WB and CB, respectively. The viabilities of bacterial cells in BNNM, WB and CB during 100 days of storage at –20, –30 and –70 °C are shown in Figs. 1A, 2A and 3A, respectively. A very good viability (between 80 and 100 %) of frozen cells was obtained when the bacterial biomass was suspended in neutralized spent nutrient medium (i.e., in BNNM and CB) at all investigated temperatures (Figs. 1A, 2A and 3A). In washed cells frozen in physiological saline the viability decreased with the time of storage at –20 and –30 °C, reaching viability of 42 and 5 %, respectively after 100 days of storage. However, survival of cells stored at –70 °C was better;

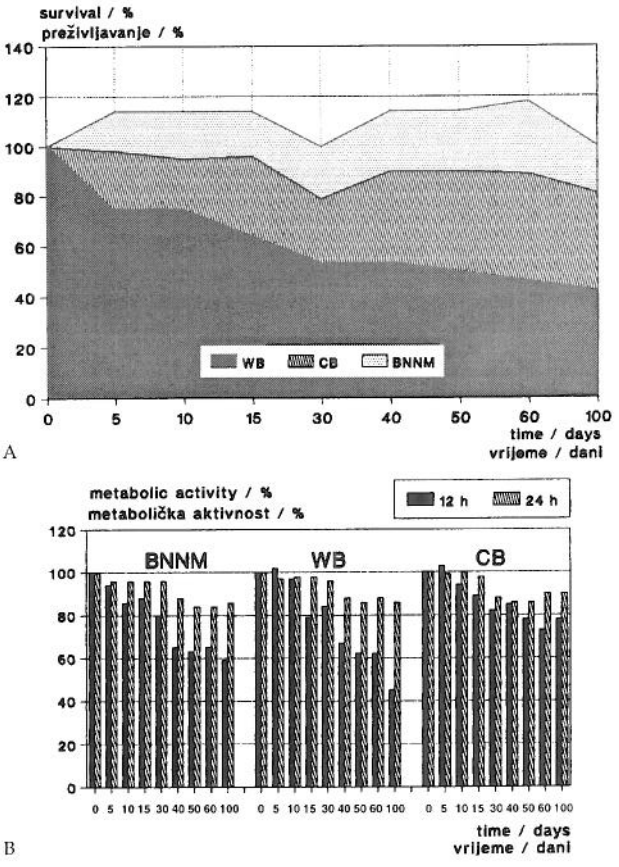

Fig. 1. Effect of biomass preparation on survival (A) and metabolic activity (B) of frozen/thawed *Lb. plantarum* L4 cells at –20 °C

Slika 1. Utjecaj pripreme biomase na preživljavanje (A) i metaboličku aktivnost (B) zamrznutih/odmrznutih stanica *Lb. plantarum* L4 pri –20 °C

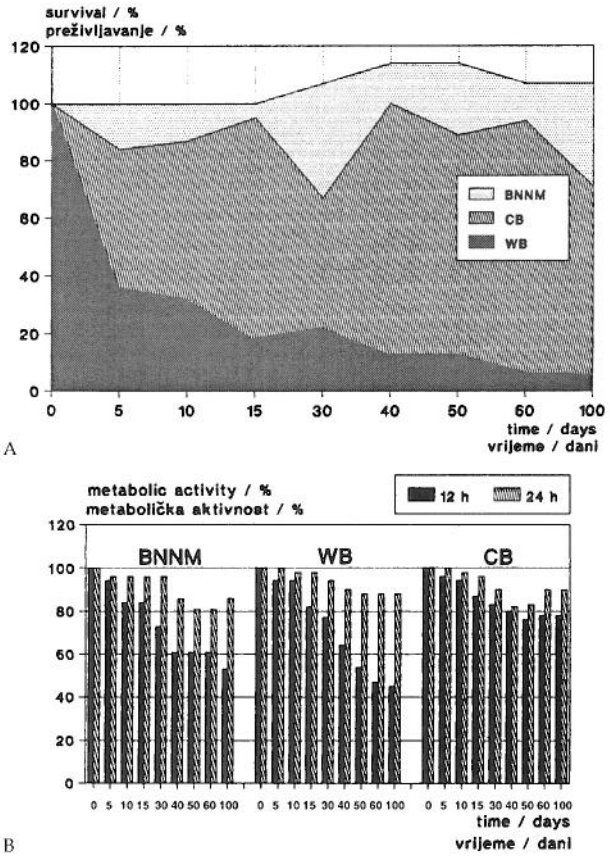

Fig. 2. Effect of biomass preparation on survival (A) and metabolic activity (B) of frozen/thawed *Lb. plantarum* L4 cells at –30 °C

Slika 2. Utjecaj pripreme biomase na preživljavanje (A) i metaboličku aktivnost (B) zamrznutih/odmrznutih stanica *Lb. plantarum* L4 pri –30 °C

there was still 84 % of living cells after 50 days of storage, and 48 % at the end of storage period. Lactic acid production of nonfrozen cells after 12 and 24 hours of growth was between 18.2–22.1 g/L and 21.6–22.1 g/L, respectively. Metabolic activity of thawed cells of *Lb. plantarum* L4, i.e., production of lactic acid during 12 and 24 hours of growth in optimal conditions is shown in Figs. 1B, 2B and 3B. Prolonged storage at freezing temperatures has an influence on metabolic activity of this bacterium. Even in BNNM where the cell viability was over 100 % the production of lactic acid after 24 hours was lower (for ca 16 %) than that of unfrozen cells. The reduction of lactic acid production was significant in exponential phase (i.e., the first 12 hours) of bacterial growth in optimal conditions (Figs. 1B, 2B and 3B). The best preserved metabolic activity was in cells frozen in CB at all freezing temperatures.

Discussion

In concentrated bacterial starter cultures production, either frozen or freeze-dryed, it is essential to ensure the conditions that can provide not only a good cells viability, but also maximal protection of wanted metabolic activities during long-term storage. It is well known that both, freezing and drying are detrimental to bacterial cells causing different types of injuries *(2,5-7)* which are not always easy to detect. Studies of the freezing effect on microbial cells, for example, are limited by the fact that cells have to be thawed before any measurements can be made. Thawing process by itself can cause additional damages of the cells *(8,9)*, or on the contrary, cells may recover *(2)* and even multiply during this process. What will really happen depends on many factors including the type and extent of injuries in the freezing process as well as the rate and temperature of thawing. The viability of *Lb. plantarum* L4 over 100 % obtained in BNNM (Figs. 1A, 2A and 3A) may be explained either by possibility of growth during thawing or by disruption of cell clumps *(9)* in the freezing/thawing process. The higher survival rates obtained in BNNM and CB in comparison with WB at all investigated temperatures (Figs. 1A, 2A and 3A) support Heckly's *(10)* findings about protective contribution (still undefined) of the spent growth medium on the viability of microorganisms during freeze-drying. This protective effect is less observable in CB, presumably, because high culture density prevents the majority of cells from being in appropriate contact with spent medium. The freezing temperature did not significantly influence the survival of *Lb. plantarum* L4 frozen in spent MRS medium (i.e., in BNNM and CB), while the survival of cells frozen in physiological saline

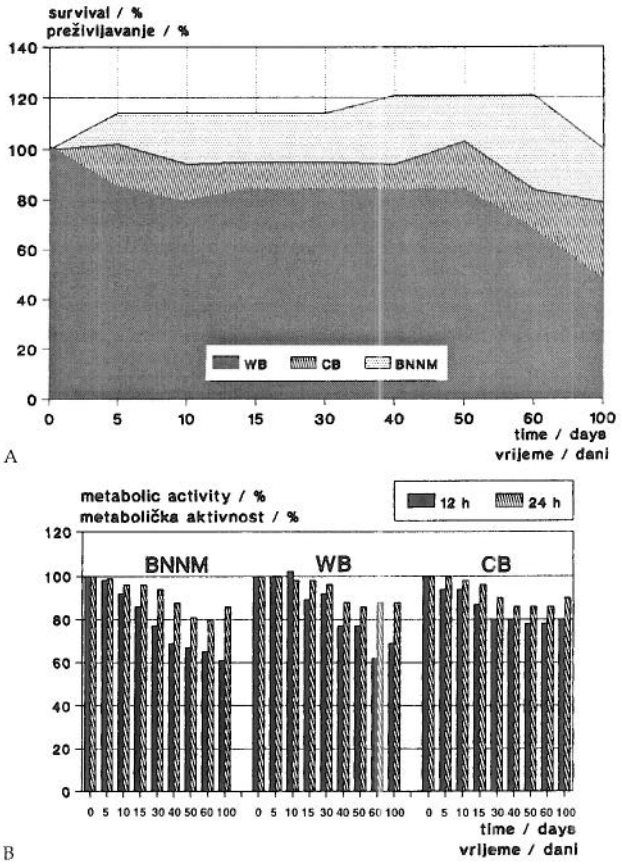

Fig. 3. Effect of biomass preparation on survival (A) and metabolic activity (B) of frozen/thawed *Lb. plantarum* L4 cells at –70 °C

Slika 3. Utjecaj pripreme biomase na preživljavanje (A) i metaboličku aktivnost (B) zamrznutih/odmrznutih stanica *Lb. plantarum* L4 pri –70 °C

(WB) at –30 °C was the most unsatisfactory (Fig. 2A). This could be explained by the number, shape and crystallization rate of ice crystals *(11,12)* in unprotected cells due to the different temperatures and rates of freezing. The ability of bacterial cells to reproduce is a criterion which provides much, but not enough information about the quality of the preservation technique *(3)*. When bacterial starter cultures are used directly for bulk medium inoculation the metabolic activity, immediately after recovery from storage, is an appropriate additional criterion for evaluating preservation. Effectiveness of the preservation method for the lactic acid bacteria used in the cheese industry has been evaluated by stimulating process (in laboratory conditions) used otherwise in the cheese *(14,15)* or cultured buttermilk *(10)* making. Direct measurement of produced lactic acid has also been used *(8,10,15)* as a criterion for preservation techniques. Determination of lactic acid production by thawed cells of *Lb. plantarum* L4 indicated that survival of cells and metabolic activity are not correlated. For example, the same metabolic activity after 24 hours of growth was achieved with both the cells frozen in WB at –30 °C (low survival rate), and cells of the highest survival rates i.e., BNNM (Fig. 2A and B). Prolonged storage at investigated freezing temperatures had greater influence on metabolic activity than freezing itself. In the first ten days of storage production of lactic acid after 12 and 24 hours of cells growth was almost equal in all samples and freezing temperatures (Figs. 1B, 2B and 3B), but after that lactic acid production during first 12 hours decreased singificantly, especially in BNNM and WB.

References

1. A. Aguilar, Community Activities in Biotechnology – The bridge »T« Project on the Biotechnology of Lactic Acid Bacteria. In: *»Lactic Acid Bacteria, Research and Industrial Applications in the Agro-Food Industries«* Adria Normandie, Cean (1992) pp. 333.
2. P. H. Calcott, *Crit. Rev. Biotechnol. 4* (1986) 279.
3. R. J. Heckly, *Adv. Appl. Microbiol. 24* (1978) 1.
4. V. Runjić-Perić, V. Bobić, I. Pavušek, *Periodicum Biologorum, 94* (1992) 179.
5. V. Runjić-Perić, *Prehrambeno-tehnol. biotehnol. rev. 26* (1988) 157.
6. M. J. Ashwood-Smith, E. Grat, *Cryobiology, 13* (1976) 206.
7. H. Fairhead, B. Setlow, W. M. Waites, P. Setlow, *Appl. Environ. Microbiol. 60* (1994) 2647.
8. E. Johannsen, *J. Appl. Bacteriol. 35* (1972) 415.
9. E. L. Ruban, *Prikladnaja biohimija i mikrobiologija 25* (1989) 291.
10. E. D. Lamprech, M. Foster, *J. Appl. Bacteriol. 25* (1963) 359.
11. R. J. Heckly, *Adv. Appl. Microbiol.* 3 (1961) 1.
12. J. Halett, Nucleation and Growth of Ice Crystals in Water and Biological Systems. In: *»Low Temperature Biology of Foodstuffs«*, J. Hawthorn, E. J. Rolfe (Eds.) (1968) pp. 23.
13. B. J. Luyet, The Formation of Ice out the Physical Behaviour of the Ice Phase in Aqueous Solution and in Biological Systems. In: *»Low Temperature Biology of Foodstuffs«*, J. Hawthorn, E. J. Rolfe (Eds.) (1968) pp. 53.
14. B. P. Keogh, *Appl. Microbiol. 19* (1970) 928.
15. G. T. Lloyd, E. G. Pont, *J. Dairy Res. 40* (1973) 157.