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ENCAPSULATION OF LACTIC ACID BACTERIA IN CALCIUM ALGINATE BEADS FOR BACTERIOCIN PRODUCTION

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Summary

Investigations on encapsulation of lactic acid bacteria in alginate beads were carried out. For this purpose a bacteriocin producing strain Enterococcus faecium A 2000 was used. For the production of capsules with a small diameter (0.8-1 mm) an electrostatic generator was applied. To enhance of the capsule stability, the fermentation was performed in modified MRS medium without phospates and with addition of $CaCl_2$. As a result of the immobilization the bacteriocin production was increased with approximately 50 %, compared with the batch fermentation with free cells. The immobilized cells could be reuses up to three times after filtration and resuspension in new medium.

Introduction

In the recent years the food industry faced the need of increasing the possibilities for better conservation and of the food products. Today, the conservation is commonly performed by sterilization or by adding sugar, salt, organic acids or by smoking. However, some of these compounds change the taste quality and the appliance of others is not healthy. For improving the quality of the products the approach by which chemicals are added must be ceased and the sterilization must be avoided as far as possible. A new protection is required, which is healthy and natural. The bacteriocins, especially these produced by food grade Starter Bacterial Cultures, could be applied as conservants.

The bacteriocins are peptides or proteins, which have an antimicrobial activity against closely related microorganisms [14]. Many of the bacteriocins produced by lactic acid bacteria are proved to inhibit or eliminate the growth of food borne pathogens such as *Listeria monocytogenes, Clostridium perfringens, Bacillus cereus* and *Staphylococcus aureus* [3, 5, 10, 12]. The GRAS (Generally Re-

cognized as a Safe) status of lactic acid bacteria and in question the bacteriocins produced by them allows their potential appliance in the food industry as non-toxic additives in the foodstuffs [13].

One of the main problems concerning the batch process is the low yield of bacteriocins. Therefore the recent research is focused on new approaches for increasing the cell concentration and bacteriocin production, respectively. One of the methods applied for maintaining high cell concentration and higher bacteriocin production is immobilization [16]. Moreover, the cell immobilization offers improvement of the plasmid stability [15]. In the case of plasmid coded bacteriocin production this fact is very important.

Among the various developed methods for cell immobilizing the encapsulation in Ca-alginate beads was used in this study. The encapsulation of the cell in capsules offers space for the cell growth and good diffusion properties. Both external mass transfer in the liquid film surrounding the particle and the internal mass transfer in the gel influence on the rate of substrate and product transport in the spherical particle. In order for better mass transfer and higher bacteriocin production techniques for immobilization, which yield small capsules, should be used. The encapsulation methods allow production of capsules with small diameter [4, 8, 11].

The aim of the recent study was increasing of bacteriocin production by means of encapsulated cells of *Enterococcus faecium* A 2000. The performed investigations included a selection of appropriate techniques, which allow the production of beads with small diameter in order for optimization of the fermentation process and to enhance the capsule stability.

Materials and Methods

Bacterial strains. *E. faecium* A 2000, which produces bacteriocin-enterococin was used in the investigation. This strain was isolated from Bulgarian homemade yellow cheese. The identification was done in the Department of Microbiology, Sofia University "Kliment Ohridski". *Listeria innocua* and *Escherichia coli* were used as indicator strains for the enterococin assay.

Media. Initial cell suspension of *E. faecium* A 2000 was obtained from normal MRS broth (Merck). Modified MRS broth (mMRS) was used for studying bacteriocin production with encapsulated cells. It is based on components of MRS medium but without phosphates and Tween, and with addition of $CaCl_2$ – 1 g/L. Elliker and BHI media were used for screening of bacteriocin activity against *L. innocua* and *E. coli*.

Encapsulation. Cells of *E. faecium* A 2000 were obtained from culture grown in 250 ml of inoculation medium at 30°C for 24 h harvested by centrifugation at 10 000g for 10 min and washed thoroughly with 0.85 % saline solution. The cells were re-suspended in 3.5 ml peptone water (1 g/L).

The cells were encapsulated by mixing of one part culture concentrate with one part Na-alginate solution (4 % SATIALGINE SG 300). This mixture was introduced in an electrostatic droplet generator. The droplets were collected in $CaCl_2$ or $BaCl_2$ solution (100 mM). An electric potential was applied between the polymer solution and the collecting solution during dropwise extrusion from the needle (Fig. 1).

The choice of this technique was made in accordance with studies performed by different authors [4, 11], who reported for production of capsules with a small diameter. The capsule diameter was detected microscopically using a graduate binnacle.

Batch fermentations with immobilized cells were performed in Erlenmeyer flasks containing 250 ml mMRS with pH 6.2, without shaking at 30°C for 96 h. Two ml from the

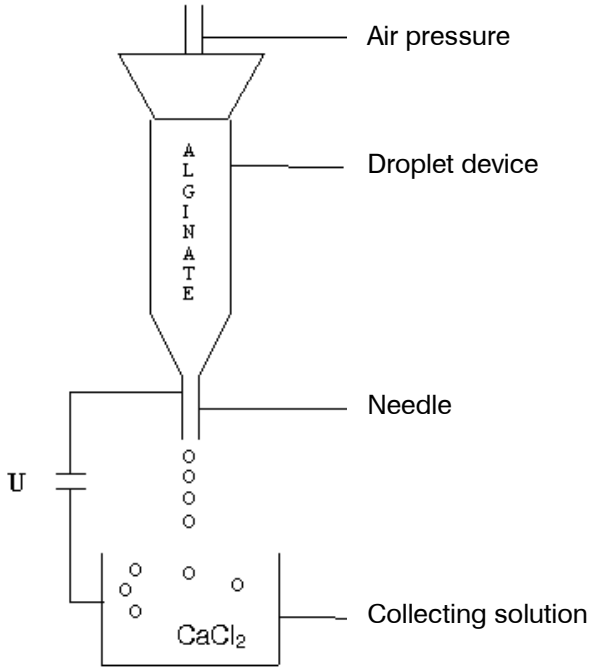

Fig. 1. Electrostatic droplet generator.

cultures were taken in every 24 h for determination of cell viability, pH and bacteriocin activity. After 96 h beads were filtered under sterile conditions, washed with saline solution and replaced in fresh media for second and third cycle of fermentation. The fermentation with free-cells was performed under the same conditions as the immobilized cells. The results obtained from this fermentation were used as a control.

Determination of the concentration of free cells in the media. The viability of cells was counted by dilution plating on MRS agar and after incubation for 48 h at 30^{o}C. The data were expressed as CFU/ml (colony forming unites per ml).

Determination of cell concentration in the capsules. One ml of alginate capsules (measured by removing of 1 ml water) was suspended in phosphate solution (concentration 0.1 M) followed by gentle shaking for 30 min for destruction of the capsules. The number of obtained cells was determined by plate counting method using MRS agar.

Determination of bacteriocin activity. Bacteriocin activity in the fermentation broth was determined by applying of the critical dilution method [2]. The activity was calculated as Arbitrary unites (AU). One AU was defined as the reciprocal of the highest dilution of the culture supernatant fluid, which gave a distinct zone of inhibition of the indicator strains.

Results

Production of beads with a small diameter and improving of the capsule stability

A significant decrease in the droplet diameter was obtained by using an Electrostatic Droplet Generator. The results showed that when the electricity potential was increased from 3000 V to 6000 V, the bead size decreased from 2.4 mm to 0.81 mm (Fig. 2).

The preliminary experiments with Ca-alginate immobilized cells showed that the beads were disintegrated in MRS medium. We supposed that the phosphates in the medium weaken the cross-links in alginate capsules causing disruption of the beads. It

was found that the capsules remained stable during the fermentation with medium without any phosphates. That is why the resulting modified medium was used for the next experiments.

Another modification for improving the capsule stability was to change the gelling agent $CaCl_2$ by $BaCl_2$. This new gelling agent provided more stable beads, which remained intact up to the end of the fermentation.

The results (not presented here) showed that the modified medium mMRS and Ba ions had no adverse effect on cell viability and bacteriocin production.

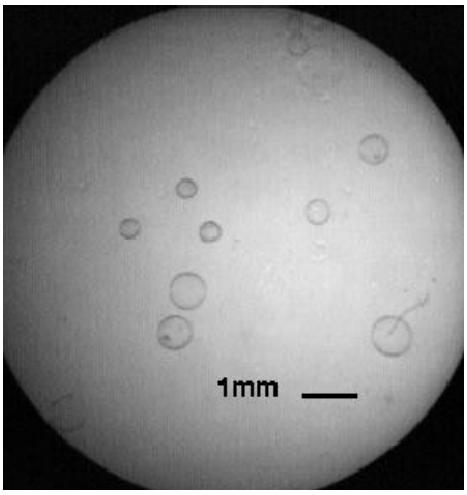

Fig. 2. Size of the alginate beads

Production of bacteriocin in batch process with free cells

The obtained results from control fermentation, performed with free cells (initial concentration about 2×10^9 CFU/ml) in mMRS showed that the maximal bacteriocin concentration of 1600 AU/ml was obtained during 48 h of fermentation (Fig. 3). After reaching a peak, the enterococin activity decreased significantly in the culture medium. Such results were also reported for other bacteriocins [6, 7]. This decreasing could be explained due to the bacteriocin digestion by proteolytic enzyme systems of the cells after the cell death.

Production of bacteriocin by immobilized bacteria in batch fermentation

The results showed that the concentration of enterococin during the fermentation with immobilized cells increased rapidly and reached the maximal level (6400 AU/ml) after 72 h and the bacteriocin was active till the end of the fermentation - 96 h (Fig. 3). It could be supposed that the alginate beads had a protective role separating the bacteriocin in the medium from the proteolytic enzymes in the beads.

After 96 h of the batch fermentation the same beads were filtrated under sterile conditions and replaced in fresh mMRS for the second cycle of batch fermentation. The concentration of enterococin produced during the second and third cycles was similar to those in the first cycle (6400 AU/ml).

Survival of *E. faecium* A2000

The CFU in the control medium significantly decreased after 72 h of fermentation while the viability of immobilized cells remained almost stable until the end of the fermentation. The cell concentration outside of the beads was also measured and the results showed that there was a decreasing of the cell viability after 72 h of batch fermentation (Fig. 4). This could be explained by the limited immunity of the free cells to their own bacteriocin in the medium. For several bacteriocin producers, it has already bean improved that the genes for bacteriocin production and immunity are regulated and transcribed simultaneously [1, 9]. Probably, the cells, which have already stopped the bacteriocin production, are sensitive to their own bacteriocin. The space separation between cells and bacteriocin, during the fermentation with immobilized cells, gave the possibilities to keep the viability of encapsulated cells.

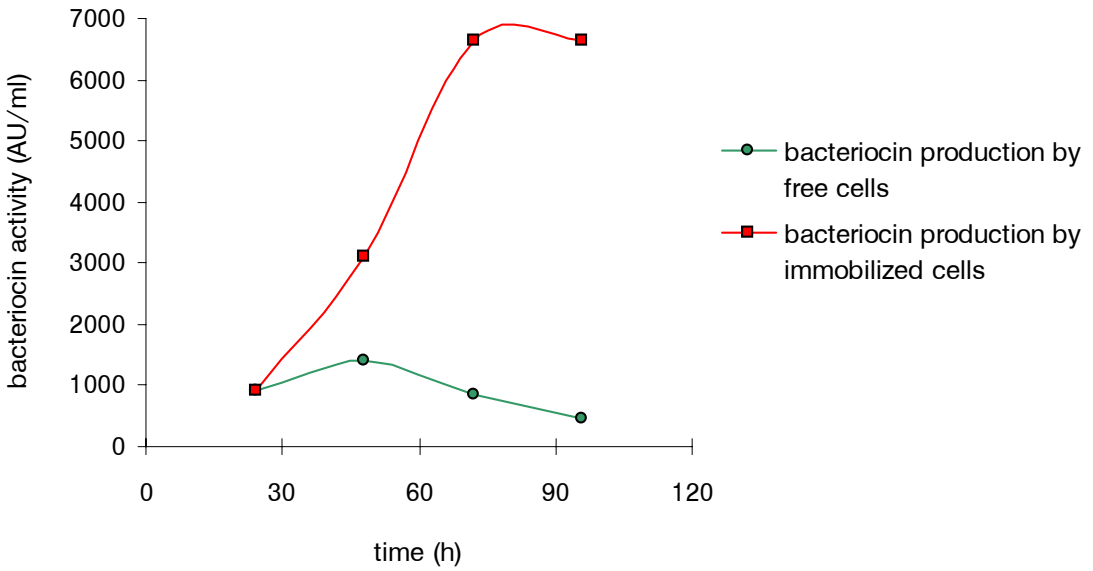

Fig. 3. Bacteriocin activity during fermentation by free and immobilized cells.

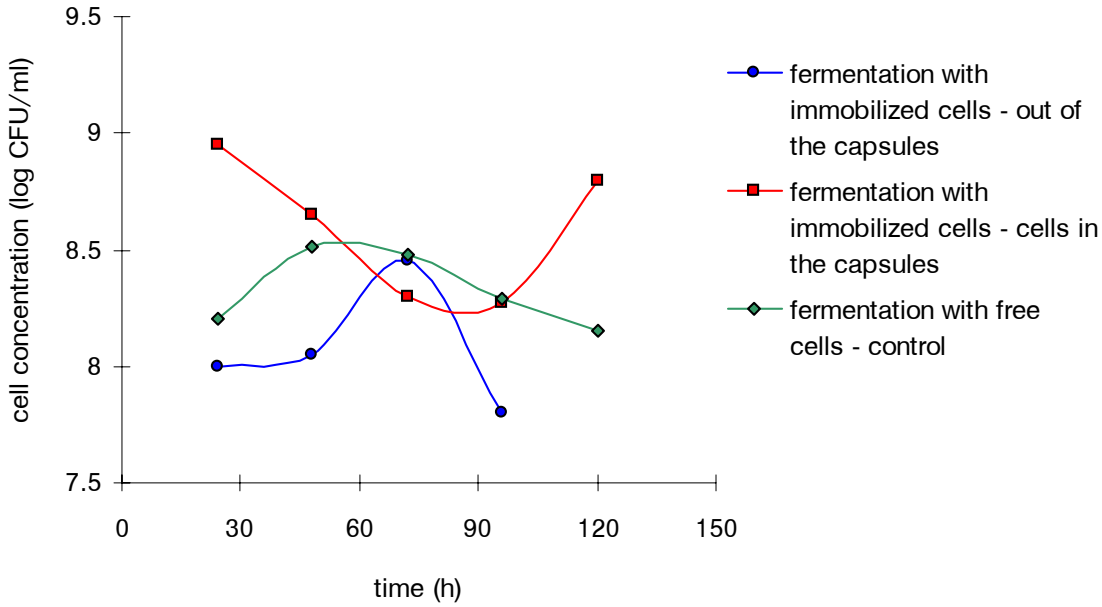

Fig. 4. Growth curves of *E. faecium* A2000 at different fermentation conditions.

Conclusion

The immobilization of the lactic acid bacteria *E. faecium* A2000 in Ca-alginate beads made possible the increasing of enterococin production as a result of the increasing of the biomass. The Electrostatic generator was used for production of capsules with small diameter (1-0.8mm). To enhance of the capsule stability, the fermentation was performed in mMRS without phosphates and with addition of $CaCl_2$. As a result of the immobilization the bacteriocin con-centration was increased by 50 %, compared with the fermentation with free cells. The bacteriocin activity and viability of cells were saved to the end of the fermentation. This allowed the same capsules to be used in the following tree cycle of batch fermentations. Moreover, the quantity of the synthesized bacteriocin was the same during this tree cycles of fermentations.

References

1. Abbe, T., 1995. *FEMS Microbiol. Lett.*, **129**,1-9.
2. Barefoot, S. F., T. R. Klaenhammer, 1983. *Appl. Envir. Microbiol.*, **45**, 1808-1815.
3. Bhunia, A. K., M. C. Johonson, B. Ray, 1988. *J. Appl. Bacteriol.*, **65**, 261-268.
4. Bugarski, B., M. F. A. Goosen, D. Poncelet, R. J. Neufeld, G.Vankak, 1994. *AIChE J.*, **40**, 1026-1031.
5. Daeschel, M. A., T. R. Klaenhammer, 1985. *Appl. Environ. Microbiol.*, **50**, 1538-1541.
6. De Vuyst, L., 1994. *Biotechnol. Let.*, **16**, 287-292.
7. De Vuyst, L., R. Callewaert, K. Crabbé, 1996. *Microbiology*, **142**, 817- 827.
8. Gyosheva, B., S. Saeva, L. Grozeva, B. Chorbanov, 1995. BG Patent.
9. Nes, I. F., D. B. Diep, L. S. Havarstein, M. B. Brurbetg, V. Eijsink, H. Holo, 1996. *Antonie van Leeuwenhoek Int. Gen. Mol. Microbiol.*, **70**, 113-128
10. Okereke, A., T. J. Montville, 1991. *Appl. Environ. Microbiol.*, **57**, 3423-3428.
11. Poncelet, D., B. Bugarski, B. G. Amsdem, J. Zhu, R. J. Neufeld, M. F. A. Goosen, 1994. *Appl. Microbiol. Biotechnol.*, **42**, 251-255.
12. Schillinger, U., M. Kaya, F. K. Lucke, 1991. *J. Appl. Bacteriol.*, **70**, 473-478.
13. Schillinger, U., R. Geisen, W. H. Holzapfel, 1996. *Trend Food Sci. Technol.*, **7**, 158-164.
14. Tagg, J. R., A. S. Dajani, L.. W. Wannamaker, 1976. *Bacteriol. Rev.*, **40**, 722-756.
15. Walls, E. L., J. L. Gainer, 1989. *Biotechnol. Bioeng.*, **34**, 717.
16. Wan, J., M. W. Hickey, M. J. J Coventry, 1995. *Appl. Bacteriol.*, **79**, 671–676.

ЕНКАПСУЛАЦИЯ НА МЛЕЧНОКИСЕЛИ БАКТЕРИИ В АЛГИНАТНИ ГЕЛОВЕ ЗА ПРОДУКЦИЯ НА БАКТЕРИОЦИНИ

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Резюме

Проведени са изследвания върху енкапсулация на млечнокисели бактерии в алгинантни гелове. За тази цел е използван щам Enterococcus faecium A 2000, продуциращ бактериоцин. Приложен е електростатичен генератор за получаване на капсули с малък диаметър (0.8-1 мм). За да се увеличи стабилността на капсулите, ферментацията е проведена в модифицирана среда MRS без фосфати и с добавяне на $CaCl_2$. В резултат на имобилизацията производството на бактериоцин е увеличено приблизително с 50 %, в сравнение с периодичното култивиране на свободни клетки. Имобилизираните клетки могат да бъдат използвани отново до три пъти след филтрация и ресуспендиране в нова среда.