Batch Propionic Acid Fermentation Using *Propionibacterium* sp. Immobilized in Different Supports

Katarzyna Czaczyk
Krystyna Trojanowska
Department of Biotechnology and Food Microbiology
University of Agriculture
Poznań

1. Introduction

Nowadays, cell immobilization technology is often used in fermentation processes (1-4). It has been studied for its potential to improve fermentation productivity. One of the most important and difficult problems encountered in the case of immobilized cells is selection of the best technique and best support for cell entrapment. A carrier is selected based on its chemical composition, reactivity, resistance to microbiological contamination, porosity and stability in environmental conditions such as temperature, pH and presence of organic solvents (5). One of the methods used now is the inclusion of microbial cells inside polymer matrices and their entrapment in gels. Entrapment of cells in polysaccharides such as alginate and carrageenan gels and inclusion inside polymer matrices are the most popular techniques used for immobilization of propionic acid bacteria (6-10).

Propionibacteria are mainly used in cheese production (11). Their other applications include the production of propionic acid and vitamin B_{12} (12,13) and biomass (14). Propionic acid is known as an important mold inhibitor. Its calcium, potassium and sodium salts are often used as food and feed preservatives. It is also used in the production of cellulose plastics, herbicides and perfumes (15). Presently, commercial production of propionic acid uses predominantly petrochemical routes, as the conventional propionic acid production is not economically competitive. Vitamin B_{12}, produced by certain strains of *Propionibacterium* sp., has several important applications in medicine and human nutrition (13).

Recently, the use of immobilized cells of *Propionibacterium* sp. in the production of propionic acid and vitamin B_{12} has been given a lot of attention (6-8, 16-18). Some authors described production of these metabolites

using cells immobilized in various support materials. Out of several carriers currently used for immobilization, the following are particularly important: polymer matrix (10), alginate, pectin, carrageenan or chitosan gels (6, 9, 16) and ceramic supports (19).

The aim of this work was to compare various supports: alginate, carrageenan, carrageenan/LBG gels and ceramic supports, as materials for immobilization of *Propionibacterium freudenreichii* subsp. shermanii.

2. Material and methods

2.1. Microorganisms

Propionibacterium freudenreichii subsp. shermanii 1 and 4, originated from our own collection. The strains were maintained on a medium containing sodium lactate supplemented with 10% glycerol. Stock cultures for inoculation were stored in refrigerator at — 70°C. For experiments, inoculum of propionic acid bacteria cultivated in a liquid casein medium was used.

2.2. Immobilization of cells in alginate gel

Sodium alginate (high viscosity, from Sigma) in 2, 3 and 4% aqueous solutions was used as support material. The solutions were sterilized at 121°C for 15 min. After cooling to 40-45°C, they were mixed with the cell suspension, containing 10^{11}CFU/ml, in a ratio of 1:1.

The mixture was introduced drop by drop into sterile 4% $CaCl_2$ solution at room temperature under continuous stirring. The beads, 3-4 mm in diameter, were hardened in $CaCl_2$ solution for 1 h. The particles were washed with sterile physiological saline to remove excess calcium ions and unentrapped cells.

2.3. Immobilization of cells in carrageenan gel

2, 4 and 6% carrageenan (type I, Sigma) aqueous solutions were used. Following the same procedures as in the case of alginate gel (sterilization, cooling, mixing with inoculum in a ratio of 1:1), the gel was cut into sections of suitable size (5 x 5 x 5 mm). The sections were hardened in 0.3M KCl solution for 1 h, then washed and used in fermentation.

2.4. Immobilization of cells in carrageenan/locust bean gum gel

2, 4 and 6% carrageenan /locust bean gum (LBG, Sigma) aqueous solutions were used in these experiments. Carrageenan and LBG were mixed in a ratio of 2:1. The procedure of immobilization was the same as in the case of carrageenan.

2.5. Immobilization of cells in the ceramic support

The ceramic carriers were designed in the Department of Chemical Physics, Maria Curie-Skłodowska University in Lublin, Poland (20). Ten grams of porous glass rings were washed with deionized water and dried at 120°C. Next, they were mixed with inoculum, incubated for 24 h at 30°C and washed with the sterile physiological saline. After adding 180 ml liquid casein medium they were used for further studies.

2.6. Cultivation methods

Fermentation was carried out in 250 ml Erlenmeyer's flasks containing 180 ml liquid casein medium (21). Twenty grams of immobilized cells were added as an inoculum. The strains were incubated statically under relatively anaerobic conditions at 30°C. During fermentation the pH of medium was adjusted to 6.8 with 25% ammonia solution and every day glucose solution (0.5 g/l) was added. The same cells were adjusted two or three times, depending on the stability of the support materials.

2.7. Analytical determination

The glucose concentration was determined by HPLC using Hewlett Packard chromatograph, model 1050, with RI detector, on Carbohydrate Analysis column (3,9 x 300 mm, Waters), with precolumn (Adsorbosphere C18, 5 m, Alltech). Operation conditions for HPLC analysis were as follows: ambient temperature, eluant — acetonitryle/water 65:35, flow rate 2 ml/min. Standard glucose (Sigma) was used to identify peaks in chromatograms, and peak areas were used to determine the sugar concentration.

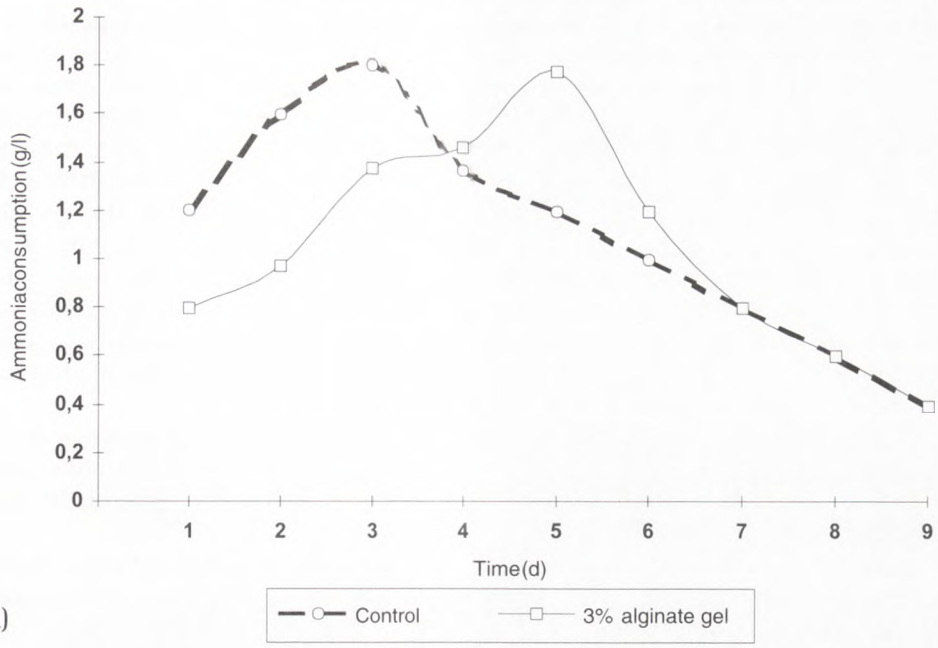
Propionic and acetic acids were determined by GC using Hewlett-Packard model GC 5890 II series, with capillary column HP-FFAP (Cross-Linked FFAP 10 m x 0,53 mm x 1 m) and FID detection. Operating conditions were: oven temperature — 140°C (isothermal), injector temperature — 18°C, FID temperature — 220°C, carrier gas — helium (flow rate 6 mL/min), quantity injected 0,4 µl. Propionic and acetic acids were quantified by computer integration operated in the mode of external standard.

Vitamin B_{12} was determined microbiologically by the plate method using *Escherichia coli* strain 113-3 (22,23).

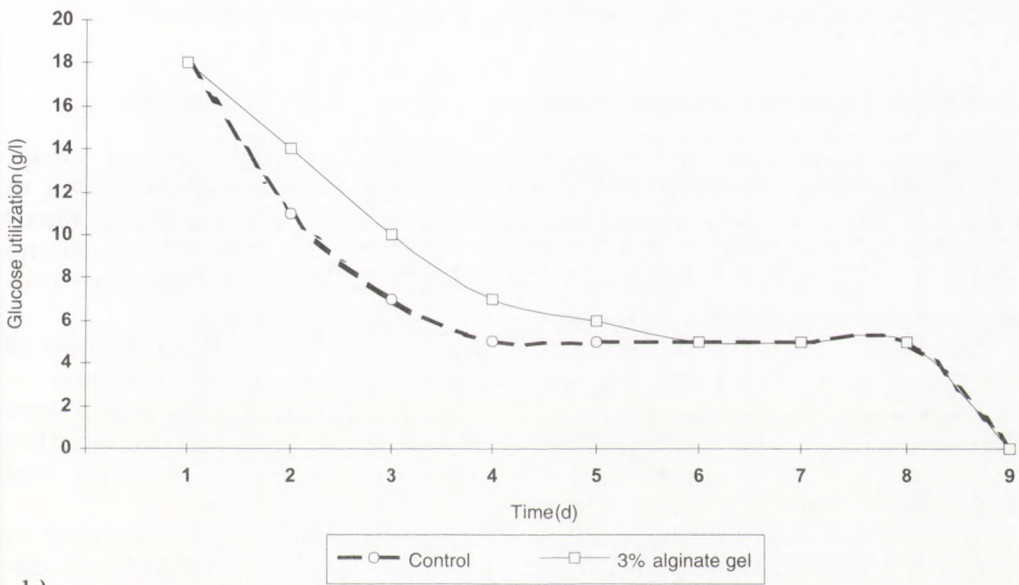
3. Results and discussion

3.1. Ammonia and glucose utilization

Propionic acid bacteria grow best at pH 6,8-7,0. The consumption of NH_4OH in the particular fermentation processes was (in g/l): 10-10,7 in the control, 9-11 in alginate gel and ceramic supports, 7,8-9,8 in carrageenan



a)



b)

Fig. 1. Ammonia consumption (a) and glucose utilization (b) by free and immobilized cells of *Propionibacterium freudenreichii* ssp. shermanii A. 1.

and carrageenan/LBG gels. The level of consumption was not dependent of the concentration of polysaccharide gels which were used in these experiments. Figure 1a presents the consumption of ammonia by free cells and cells immobilized in 3% alginate gel (first application of immobilized cells). At the beginning of this process, the free cells used more NH_4OH than the immobilized ones, but the equalization of the score was observed during the second part of fermentation and at the end, independently of the number of applications of entrapped cells. It was probably connected with some diffusion limitations between gel beads and media (24,25). The same situation was observed in the case of the cells entrapped in carrageenan and carrageenan/LBG gels for both examined species (data not presented). There were no differences in ammonia consumption between free cells of propionic acid bacteria and cells immobilized on ceramic support.

Glucose utilization was more than 99% at the end of the process for all fermentations, independently of the kind of support material, its concentration or the number of applications of immobilized cells. The differences were not statistically significant.

Figure 1b presents differences between free and immobilized cells during the fermentation process. The same situation as in the case of ammonia consumption was observed here. Free cells of propionic acid bacteria used more glucose at the beginning of the fermentation; the consumption level remained the same at the end of the process. This regularity was also observed for carrageenan and carrageenan/LBG gels.

3.2. Production of propionic acid

Production of propionic acid by *Propionibacterium freudenreichii* subsp. shermanii 1 and 4 immobilized in different support materials is shown in Figure 2. The presented values are means from three fermentations. Maximum productivity of this metabolite was observed after the 8^{th} day of the process. Generally, immobilized *Propionibacterium* sp. produced more propionic acid than the free cells.

The best results were obtained in the case of the third application of entrapped cells immobilized in 4% alginate for both examined strains — up to 9,5 g/l. 2% alginate gel was used only twice, because the beads were dissolved during their third application. It was probably due to the fact that in low concentrations polysaccharides may be damaged by propionic and acetic acids (26).

A significant increase of propionic acid production was also observed in the case of cells immobilized in carrageenan gels (Fig. 2). No significant differences were found between 2, 4 and 6% concentrations of carrageenan used as support material. Addition of LBG to carrageenan decreased productivity of this metabolite when compared with pure carrageenan. Carrageenan and LBG are known as synergistic gels (having better mechanical stability), when mixed together (27,28), but there are no data in literature indicating that their application in the fermentation process affects productivity. The

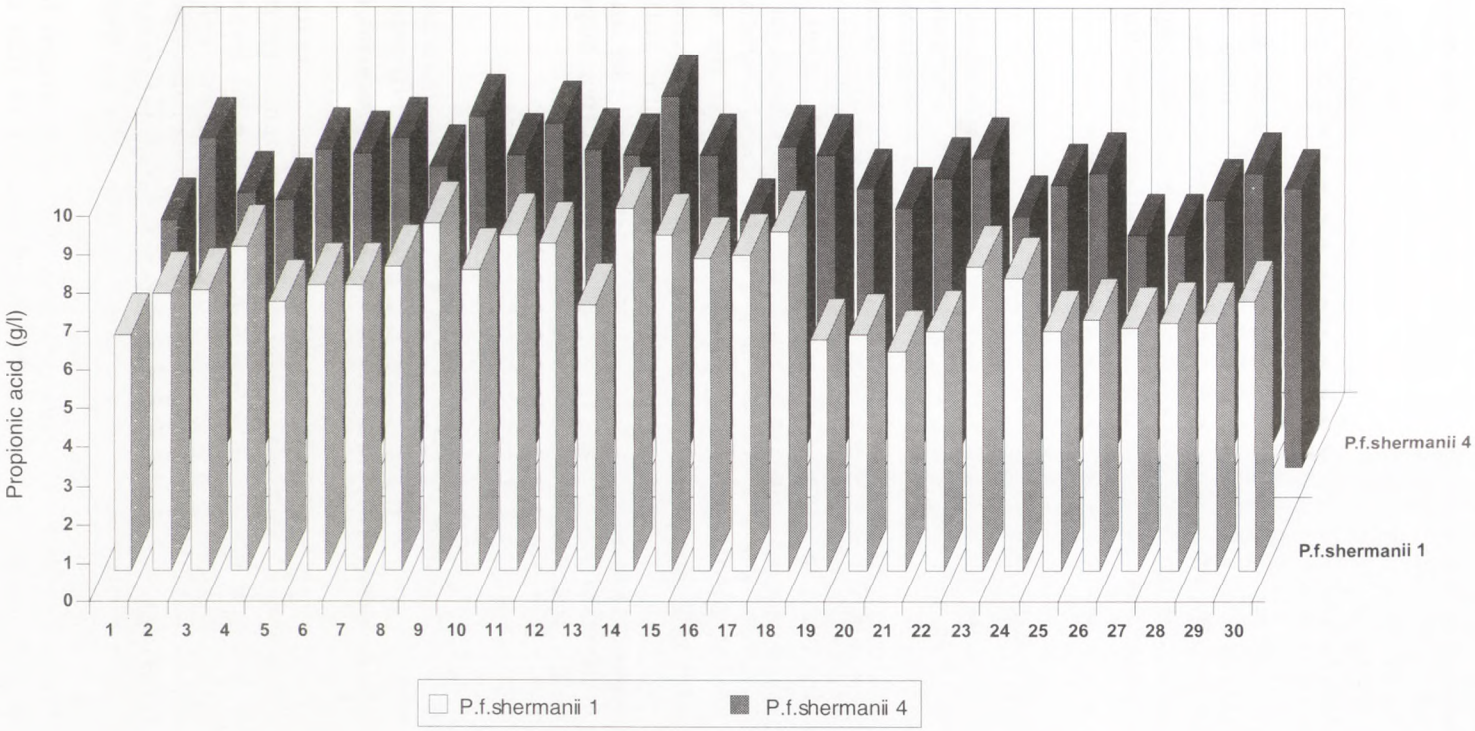


Fig. 2. Propionic acid production by propionic acid bacteria immobilized in various support materials. 1 — control; 2 — 2% alginate I; 3 — 2% alginate II; 4 — 3% alginate I; 5 — 3% alginate II; 6 — 3% alginate III; 7 — 4% alginate I; 8 — 4% alginate II; 9 — 4% alginate III; 10 — 2% carrageenan I; 11 — 2% carrageenan II, 12 — 2% carrageenan III; 13 — 4% carragennan I; 14 — 4% carrageenan II; 15 — 4% carrageenan III; 16 — 6% carrageenan I; 17 — 6% carrageenan II; 18 — 6% carrageenan III; 19 — 2% carrageenan/LBG I; 20 — 2% carrageenan/LBG II; 21 — 2% carrageenan/LBG III; 22 — 4% carrageenan/LBG I; 23 — 4% carrageenan/LBG II; 24 — 4% carrageenan/LBG III; 25 — 6% carrageenan/LBG I; 26 — 6% carrageenan/LBG II; 27 — 6% carrageenan/LBG III; 28 — ceramic I; 29 — ceramic II; 30 — ceramic III. I, II, III — application of immobilized cells.

level of biosynthesis of propionic acid by cells immobilized on ceramic support was only a little higher in comparison with free cells (Fig. 2.).

Some authors (8,17) also reported increase of production of propionic acid by immobilized *Propionibacterium* sp. In both cases, the productivity of immobilized-cell systems was ~ 4 times higher than in the case of conventional fermentation.

There were no significant differences in the production of acetic acid by free and immobilized cells of *Propionibacterium freudenreichii* subsp. shermanii (data not presented). We observed a significant increase in the propionic-to-acetic acid ratio: from 2,5:1 in the control to 3,4:1 with 4% alginate used for the third time and 3,8:1 with 2% carrageenan gel used for the third time.

3.3. Biosynthesis of vitamin B_{12}

Biosynthesis of vitamin B_{12} by immobilized and free cells is shown in Figure 3. Free cells of *Propionibacterium freudenreichii* subsp. shermanii 1 and 4 produced 4,7 mg/l of this metabolite. The production significantly decreased (less than 1 mg/l) in carrageenan, carrageenan/LBG and ceramic support, independent of the concentration of these support materials and the number of applications of immobilized cells. A similar situation was observed for the alginate gel with the exception of 2% alginate used for the second time and 4% alginate used the third time. In these cases biosynthesis of vitamin B_{12} by immobilized cells of *Propionibacterium* sp. was the same as in the control. The very low production of vitamin B_{12} by immobilized cells is due to the intercellular character of this metabolite. Some authors (9,10) reported a certain increase of production of this metabolite by immobilized cells using surfactants, such as Tween 80, or some other support materials (e.g. urethane prepolymers).

3.4. Cell release rate

Cell release rate for the selected concentration of support materials is shown as the number of free cells in cultivation media after fermentation (Fig. 4). Not statistically significant differences were observed between examined strains.

One of the important aspects of immobilized cells technique is inclusion of bacteria inside polymer matrices (alginate, carrageenan, carrageenan/LBG) or their binding on the surface of support material (ceramic support). There are not sufficient data in literature concerning the cell release rate using cell immobilization techniques. Our experiments showed that the highest cell release rate was observed using ceramic supports. In this case propionic acid fermentation was carried by free cells as well as cells entrapped on the surface of support material.

In 2% alginate gel, cell release rate was relatively high and ranged from 10^4 to 10^6 CFU/ml. It was due to worse mechanical stability of this con-

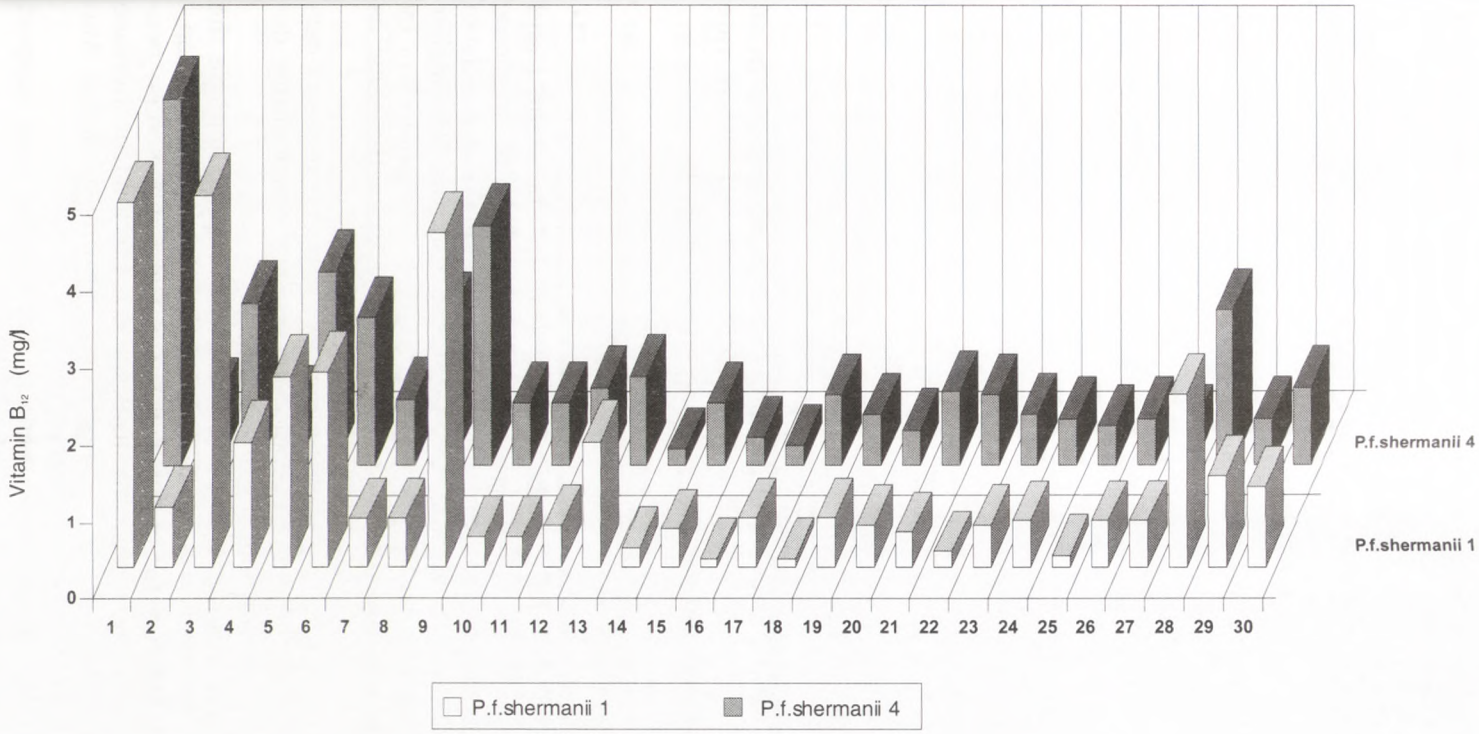


Fig. 3. Biosynthesis of vitamin B_{12} by propionic acid bacteria immobilized in various support materials. 1 — control; 2 — 2% alginate I; 3 — 2% alginate II; 4 — 3% alginate I; 5 — 3% alginate II; 6 — 3% alginate III; 7 — 4% alginate I; 8 — 4% alginate II; 9 — 4% alginate III; 10 — 2% carrageenan I; 11 — 2% carrageenan II, 12 — 2% carrageenan III; 13 — 4% carragennan I; 14 — 4% carrageenan II; 15 — 4% carrageenan III; 16 — 6% carrageenan I; 17 — 6% carrageenan II; 18 — 6% carrageenan III; 19 — 2% carrageenan/LBG I; 20 — 2% carrageenan/LBG II; 21 — 2% carrageenan/LBG III; 22 — 4% carrageenan/LBG I; 23 — 4% carrageenan/LBG II; 24 — 4% carrageenan/LBG III; 25 — 6% carrageenan/LBG I; 26 — 6% carrageenan/LBG II; 27 — 6% carrageenan/LBG III; 28 — ceramic I; 29 — ceramic II; 30 — ceramic III. I, II, III — application of immobilized cells.

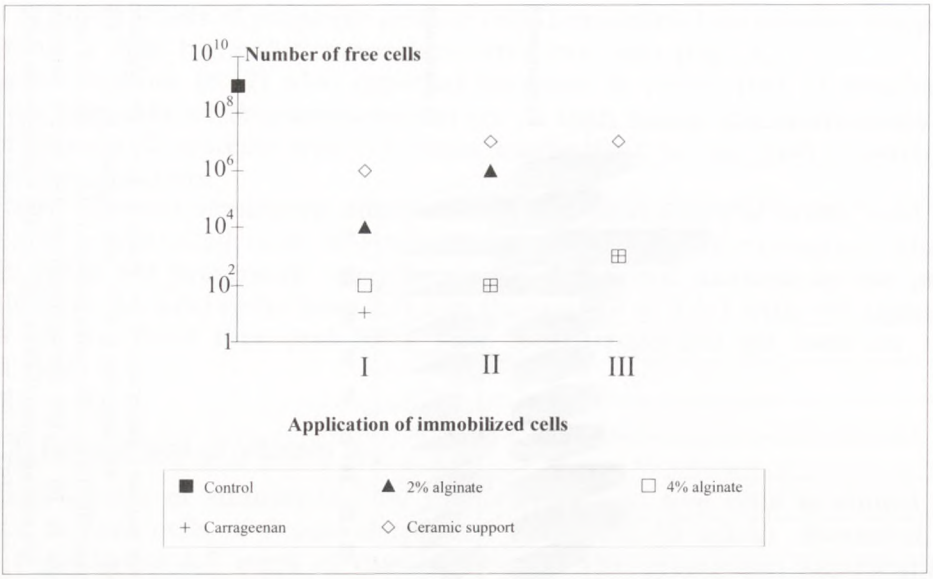


Fig. 4. Cell release rate for chosen support materials.

centration of alginate gel. In other concentrations of alginate gels and in all concentrations of carrageenan and carrageenan/LBG gels cell release rate was comparatively low and did not exceed 10^3 CFU/ml (Fig. 4).

4. Conclusion

Immobilization of *Propionibacterium freudenreichii* subsp. shermanii 1 and 4 in 2, 3 and 4% alginate gel influenced growth production of propionic acid especially in the case of the third application of immobilized cells in 4% alginate gel. In this case, biosynthesis of propionic acid was 50% higher than in the case of conventional fermentation. Production of vitamin B_{12} by propionic acid bacteria entrapped in alginate gel was lower or the same as in the control.

Immobilization of the examined species in different concentrations of carrageenan gels increased production of propionic acid, but considerably decreased biosynthesis of vitamin B_{12}.

The addition of locust bean gum to carrageenan gel did not improve the productivity of these two main metabolites of propionic acid fermentation.

The use of ceramic support for immobilization of *Propionibacterium freudenreichii* subsp. shermanii gave negative results and led to a slight increase of propionic acid production and decrease of vitamin B_{12} biosynthesis. Moreover, a high cell release rate was observed.

These investigations showed that 4% alginate gel was the best support

material for immobilization of *Propionibacterium freudenreichii* subsp. shermanii.

In general, the results showed that immobilization of propionic acid bacteria is a good method to increase propionic acid production and it has negative influence on biosynthesis of vitamin B_{12}.

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Summary

Propionibacterium freudenreichii subsp. shermanii 1 and 4 were immobilized in the living state in 2, 3 and 4% alginate gels, in 2, 4 and 6% carrageenan gels, in 2, 4 and 6% carrageenan/locust bean gum gels and on ceramic support. Ammonia consumption, glucose utilization, production of propionic and acetic acids, biosynthesis of vitamin B_{12} and cell release rate were examined. A significant increase of productivity of propionic acid and decrease of biosynthesis of vitamin B_{12} using immobilized cells were observed. The best results were obtained in the fermentation with strains immobilized in 4% alginate gel, when applied for the third time. In this case, production of propionic acid was 50% higher in comparison with free cells and biosynthesis of vitamin B_{12} was lower or the same as in the control.

Key words:
immobilization, propionic acid fermentation, alginate, carrageenan, carrageenan/locust bean gum, ceramic support.

Adres do korespondencji:
Katarzyna Czaczyk, Katedra Biotechnologii i Mikrobiologii Żywności, Akademia Rolnicza, ul. Mazowiecka 48, 60-623 Poznań.