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The continuous bioconversion of glycerol to 1,3-PD by using encapsulated *C. freundii* cells

Urszula Kowalska, Małgorzata Mizielińska*, Marcin Soból

Center of Bioimmobilisation and Innovative Packaging Materials, Faculty of Food Sciences and Fisheries, West Pomeranian University of Technology in Szczecin,
35 Janickiego Str., 71-270 Szczecin, Poland

*E-mail address: Malgorzata.Mizielinska@zut.edu.pl

ABSTRACT

The aim of the work was to decrease the costs of bioconversion process by using chip and simple chromatographic columns containing immobilized *C. freundii* cells during fermentation. The goal was also modification of medium to control of pH of process in these columns. The purpose of the study was also to ascertain the new systems of biopolymers used for encapsulation of bacterial cells. The stability of obtained membranes in different mediums has been examined. The waste glycerol conversion to 1,3-PD over continuous process has been analyzed as well. The results of the study showed that the double crosslinked alginate beads are better carriers than alginate capsules coated by chitosan during conversion of waste glycerol to 1,3-PD. Unfortunatrly these two kinds of capsules should not be used for continuous process and repeated bioconversions because of low mechanical properties of the beads. PDADMAC beads and modified (e.g. by additions) PDADMAC capsules should not be used for continuous and repeated bioconversions as well.

***Keywords*:** bioimmobilization, encapsulation, PDADMAC beads, alginate capsules

1. INTRODUCTION

Crude glycerol is the main by-product of transestrification of fats with short chain alcohol in the biodiesel production industry. This substance is therefore a chip feedstock for

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the production of chemicals and also an interesting substrate for tailored biotechnological productions (1). The main biotechnological use of the residual glycerol has been its biotransformation into 1,3-propanediol (1,3-PD) that is used as a monomer for the synthesis of the polyesters and polyurethanes as a component in the plastic polytrimethyleneterephtalate synthesis, and in production of polyesters resins for the paint industry (2). This method is easy and does not generate toxic by-products (3). Bioconversion of glycerol to 1,3-PD seems to be an attractive waste treatment practice in which both pollution control and energy recovery can be achieved (4). A number of microorganisms are able to bioconverse the glycerol to 1,3-PD. In *Klebsiella*, *Citrobacter* and *Enterobacter* species glycerol is metabolized both oxidatively and reductively (5). It is a thesis of many works that 1,3 PD synhesis by *C. freundii* has been performed under freely suspended cells batch conditions. Many would regard fed-batch bioreactors as improvements in bioconversion productivities. Unfortunately, fed-batch conditions frequently have the disadvantageous. It is why it seems reasonable to suppose that higher biomass content should cause an increase of process productivity, what could be reached in continuous (1). It is widely acknowledged that there is a tendency for culture to degenerate and for process to come instable. Culture degeneration, characterized by a progressive loss of solvent production capability, has been observed in continuous processes frequently. A condition that appears to have influenced on the stability of continuous cultures of microorganisms is to use phosphate limitation (6).

There is evidence to suggest that immobilization that protects bacterial cells by creating "microenvironment" for them could solve the problem of cells degradation, but what is also important the carriers used for immobilization of bacteria should be stable in medium containing phosphates. The immobilization has also another significant advantage. It protects the microorganisms against the negative growth conditions as well as avoids the washing- out of the population of cells upon using high dilution rates in the continuous operation mode. The immobilized cell technology appears to have become method that causes higher cell densities per unit bioreactor volume, the reuse of the same biocatalysts for prolonged periods, and smaller bioreactor volumes that could decrease the total costs of bioconversion (7). In general, immobilization of enzymes and microorganisms takes place by two main ways: adsorption by physical and chemical bonds, or physical entrapment of enzymes and cells within the carriers. The most common methods for immobilization on these matrices include adsorption, cross-linking, covalent bonding, entrapment, and encapsulation (8). Encapsulation is the method considerably similar to entrapment. In this process, microorganisms, living cells as well as for multi-enzyme systems are restricted by the membrane walls, but free-floating within the core space. The membrane itself is semi-permeable, allowing for free flow of substrates and nutrients. The frequently examined factor is the proper pore size of the membrane, attuned to the size of core material. It is also important that carrier and immobilized cells or enzymes incorporated into the capsule have no negative influence on each other. However, as every technology, microencapsulation has some disadvantages (9). One of them is that there is a tendency for microcapsules to deteriorate in mediums containing a high concentration of phoshates (6). The biopolymers useful for encapsulation are: alginic acid (alginate), chitosan, maltodextrin, cellulose and cellulose derivatives. However, double-layer microcapsules built of two different polymers are also very popular. The most common are coated with chitosan, poly-L lysine (PLL), polyvinyl acetate (PVA), gelatin, boric acid and κ-carrageenan (10).

Immobilized microorganisms were constantly used in continuous and semi-continuous production processes, allowing for significant cost decrease, as the microorganisms do not need to be refilled (9). The effect of hydraulic retention time (HRT) on the production of 1,3-PD in immobilized bioreactors was also investigated. It was revealed that HRT is an important factor and a HRT of 2 h is the best one in terms of volumetric production rate (7,11).

The aim of the work was to decrease the costs of bioconversion process by using chip and simple chromatographic columns containing immobilized *C. freundii* cells during fermentation. The goal was also modification of medium to control of pH of process in these columns. The purpose of the study was also to ascertain the new systems of biopolymers used for encapsulation of bacterial cells. The stability of obtained membranes in different mediums has been examined. The waste glycerol conversion to 1,3-PD over continuous process has been analyzed as well.

2. MATERIAL AND METHODS

The following chemicals were used for encapsulation and fermentation processes:

1-propanol (Chempur, Poland), 95% sulfuric acid VI (Chempur, Poland), cellulose (Sigma-Aldrich, Poland), NaOH (Chempur, Poland), solutions of polydiallyldimethylammonium chloride (4%) (PDADMAC) molecular weight of 8 500 g/mol (Polysciences, USA), <100 000 g/mol (Sigma Aldrich, Poland), 100 000 – 200 000 g/mol (Sigma Aldrich, Poland) oraz 200 000 – 350000 g/mol (Sigma Aldrich, Poland), KCl, $CaCl_2$ (Chempur, Poland), sodium alginate solution (4%) (Sigma-Aldrich, Poland) polycations: poliethylenimine (PEI) (Polysciences, Poland), polydiallyldimethylammonium chloride (M_w<100 g/mol), poli(chloride 2-hydroksypropyl dimetylammonium) (PHPDMA) (GreatAp Chemicals Co., Chiny), poli[dichloride oksyethyl (dimethylimino) ethyl (dimethylimino) ethylene] (GA 126) (GreatAp Chemicals Co., China) oligochitosan (M_w=10 000 g/mol) (GreatAp Chemicals Co., China), potasium hydrogenphosphate (Chempur, Poland) 48 g/L, potassium di-hydrogensulphate (Chempur, Poland) 12 g/L, ammonium sulphate (Chempur, Poland) 2 g/L, magnesium heptahydrate sulphate (Chempur, Poland) 0,4 g/L, calcium dihydrate chloride (Chempur, Poland) 0,1 g/L, cobalt heksahydrate chloride (Chempur, Poland), Syloid AL-1 FP silica (Grace Davison, USA), coloidal Ludox silica (Grace Davison, USA) 0,004 g/L, yeast extract 2 g/L (Biocorp, Poland), enzymatic hydrolizate of aminoacids 2,5 g/L (Biocorp, Poland), meat extract 1,5 g/L (Biocorp, Poland), MacConkey agar (Merck), waste glycerol (Trzebinia).

Microorganism and mediums:

C. freundii strain used in this study was obtained from the collection of Department of Biotechnology and Food Microbiology of Poznan University of Life Sciences Poznan (Poland). The following mediums were used during column fermentations (amounts per liter of distilled water):

- The culture media "M1" that has been used for the studies consisted of (g/L): 50 waste glycerol, $2.4K_2HPO_4$, $0.6KH_2PO_4$, $2(NH_4)_2SO_4$, $0.4MgSO_4 \cdot 7H_2O$, $0.1CaCl_2 \cdot 2H_2O$, $0.004CoCl_2 \cdot H_2O$, 2 yeast extract, 2.5 bactopeptone, 1.5 meat extract.

- The culture media "M2" that has been used for the studies consisted of (g/L): 50 waste glycerol, $48K_2HPO_4$, $12KH_2PO_4$, $2(NH_4)_2SO_4$, $0.4MgSO_4 \cdot 7H_2O$, $0.1CaCl_2 \cdot 2H_2O$, $0{,}004CoCl_2 \cdot H_2O$, 2 yeast extract, 2.5 bactopeptone, 1.5 meat extract.
- The culture media "M3" that has been used for the studies consisted of (g/L) 50 waste glycerol, $48K_2HPO_4$, $12\ KH_2PO_4$, $2(NH_4)_2SO_4$, $0.4\ MgSO_4 \cdot 7H_2O$, $10CaCl_2 2H_2O$, $0.004CoCl_2 \cdot H_2O$, 2 yeast extract, 2.5 bactopeptone, 1.5 meat extract.

All of the mediums were prepared according to Barbitaro et.al. composition (12,13). The amount of substrates was modified because of pH that should not change during process (increase of amount of phosphate in M2 and M3) what was important to obtain high yield of bioconversion. The amount of $CaCl_2$ was also increased (M3), bacause there was important for capsules to be stable during process. In the case of alginate capsules the icrease of $CaCl_2$ amount in medium could improve mechanical properties and the stability of these capsules.

There were: Syringe pump (New Era Pump NE-1000, USA with speed of 100 mL/h, and the diameter of the needle 0.7 mm);

Column set for continuous production:

Two simple and chip chromatographic columns (to decrease bioconversion costs) with thermo jacket XK26/20 (GE Healthcare Life Sciences, USA), thermostat (Julabo, Poland); peristaltic pump (MRC, Israel); magnetic stirrer with heating (DragonLab, China); BagMixer (Interscience, France) and Zwick/Roell Z 2.5 tensile machine used during the tests.

Immobilization of *C. freundii* strain according to Wong method (14)

In the first step of tests the beads containing *C. freundii* cells were prepared according to modified method of Wong et. al. (14). The cells were harvested from the culture medium by centrifugation at 5 °C and 9000 rpm for 10 min and were rinsed twice with 0.9 wt% NaCl solution to remove residual medium and contaminants. The bacterial cells were introduced into 4% sodium alginate. The mixture was subsequently dropped using a syringe pump into an aqueous solution containing 0.155M $CaCl_2$. After 30 minutes of crosslinking the capsules were washed with sterile water and again introduced into 0.155M $CaCl_2$ for two hours. The next step was to wash the beads with water. The immobilized cell beads were were introdeced in "M1, M2, and M3" mediums. The samples were incubated for 168 hours at room temperature.

The next step of an experiments was to ascertain the new systems of biopolymers used for immobilization of bacterial cells to obtain a capsules that would be stable in the medium with higher amount of a phosphates. It was assumed that a stable capsules would be used for continuous production of 1,3-propanediol, or for repeated fed-batch processes in which pH level is not controled.

Alginate/polycations capsules

4% sodium alginate containing polycations such as poliethylenimine (PEI), polyDADMAC (Mw > 100 g/mol), PHPDMA, Poli[dichloride oksyethyl (dimethylimino) ethyl (dimethylimino) ethylene] (GA 126) (Mw = 10 000 g/mol) were used for encapsulation. The capsules were obtained by extrusion according to two schemes (Figure 1,2). The obtained capsules were introdeced into "M2, and M3" mediums. The M1 medium were not used

because of to low amount of phosphates. The samples were incubated for 168 hours at room temperature.

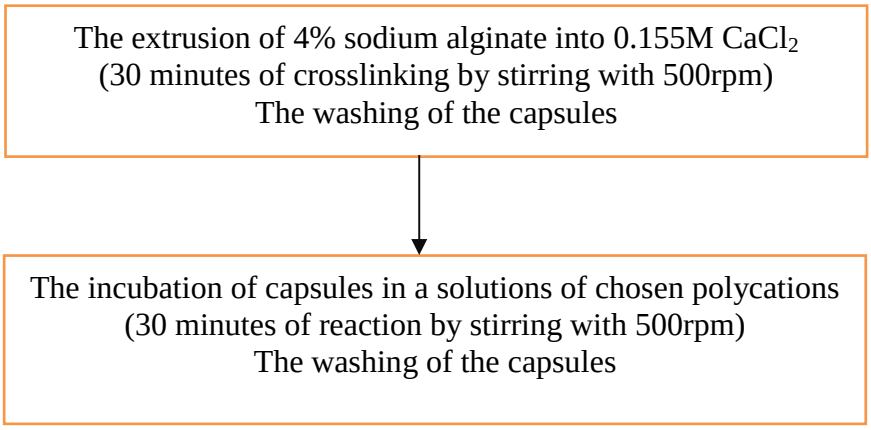


Figure 1. The capsules with polycationic coatings (Method I)

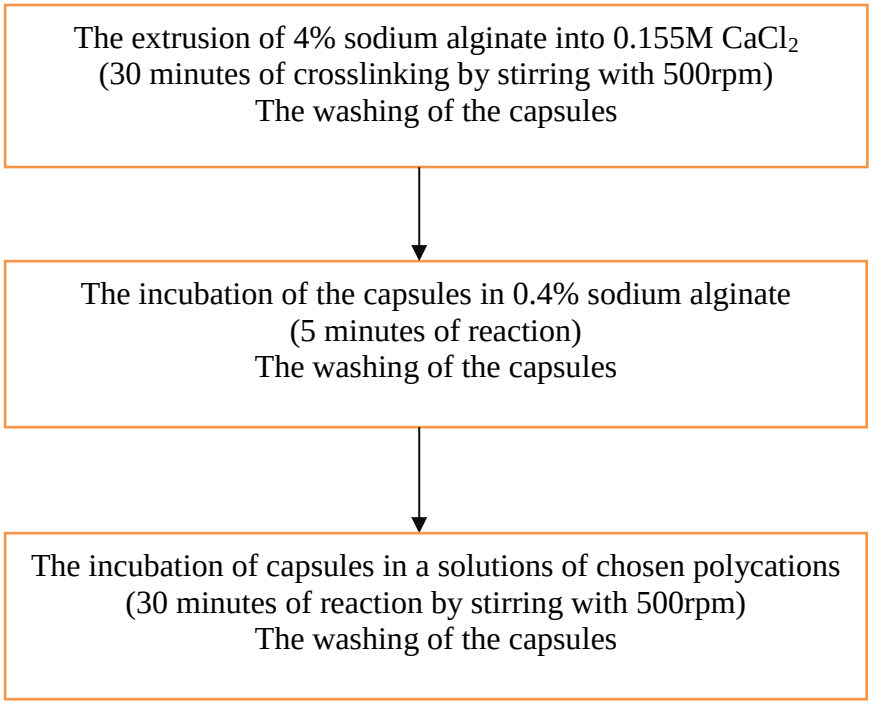


Figure 2. The capsules with polycationic coatings after incubation in dilluted sodium alginate (Method II)

Alginate capsules with oligochitosan coating

The 4% sodium alginate was extruded into 0.155M $CaCl_2$. After 30 minutes of crosslinking the capsules were washed with water. In the next step the capsules were introduced into oligochitosans solutions. The oligochitosans that were used during tests were

characterized by molecular weight of: 2500 g/mol, 7200 g/mol, and 14000 g/mol. The obtained capsules were covered by oligochitosan (30 minutes of incubation of capsules in a solutions of chosen oligochitosan by stirring with 500rpm) and introdeced into "M2, and M3" mediums. The samples were incubated for 168 hours at room temperature.

Cellulose sulfate/ PDADMAC capsules

Cellulose sulfate was used to obtain these capsules. This chemical was obtained by a modified method described by Bohlmann et al (15). Cellulose sulfate solutions were adjusted to pH = 7 using 30% NaOH and 1M NaOH. The capsules were obtaind by extrusion, using a syringe pump. The amount of 2.5 mL of sulfate was extruded into 25 mL of cellulose PDADMAC solution.

As a next step of the tests a 1.5% of kappa, lambda or iota carrageenans were introduced into the cellulose sulfate to strengthen the complex. The 0.15M salts were added into PDADMAC solution. The solutions containing kappa carrageenans were extruded into a PDADMAC solution containing 0.15 M KCl. The solutions containing iota carrageenan were extruded into a solutions containing 0.155M $CaCl_2$. Lambda carrageenan as a non-gelling fraction was injected into a solution of pure PDADMAC. The reaction time ranged from 1 to 8 hours. The obtained capsules were introdeced into "M2, and M3" mediums. The samples were incubated for 168 hours at room temperature.

Alginate capsules with silica

The 10% Syloid AL1F and 20% Ludox solultions in 4% sodium alginate were prepared. The obtained solutions were extruded into 0.155M $CaCl_2$. In the next step (after 30 minutes of crosslinking by stirring with 500rpm) the capsules were washed with water and introduced into "M2, and M3" mediums. The samples were incubated for 168 hours at room temperature.

The stability of the beads after 24 hours of incubation in mediums was determined for the capsules which were not destroyed. The tests were done after 24 hours of storage because it was important to compare the capsules from all three mediums and the beads from M2 were desintegrated after 24 hours. The bursting force was determined for each set of beads (10 beads of each complex) using Zwick/Roell Z 2.5 tensile machine. As reference (base value) the same set of measurements was conducted for new prepared capsules (without contact with M3 medium). The avarage of 10 values was counted

Encapsulation of *C. freundii* cells in alginate beads covered by oligochitosan

The cells were harvested from the culture medium by centrifugation at 5 °C and 9000 rpm for 10 min and were rinsed twice with 0.9 wt% NaCl solution to remove residual medium and contaminants. The bacterial cells were introduced into 4% sodium alginate. The mixture was subsequently dropped using a syringe pump into an aqueous solution containing 0.155M $CaCl_2$. After 30 minutes of crosslinking the capsules were washed with sterile water. In the next step the obtained capsules were covered by oligochitosan.

Bioconversion process

After immobilisation, the double crosslinked alginate beads and the alginate beads with chitosan coating were rinsed with sterile 0.9% NaCl solution and transferred to sterile column set (30mL of capsules) filled with sterile "M3" broth. The temperature in the column was kept

at 30 °C using thermo jacket connected to thermostat (Julabo, Poland). The whole continuous process including two columns with encapsulated bacterial cells under varying hydraulic retention times (ranging between 1 and 5 h) lasted for 144 hours.

Chromatography analysis

Total 1,3-PD and glycerol content was determined by HPLC (Knauer, Germany) using an Aminex HPX-87H organic acid analysis column and RI detector. The injection volume of the sample was 10 µL. The column, maintained at 25 °C, was eluted with 5 mM H_2SO_4 at a flow rate of 0.6 mL/min, samples ran for 30 minutes. Samples for chromatography analysis were taken every day after 24h. Production of 1,3-PD and consumption of glycerol were obtained by dividing the final concentration (g/L) by fermentation process time (h).

Microorganisms viability

The amount of bacterial cells in capsules were determined before and after bioconversion process. The samples from the effluent were taken and analyzed for viability of *C. freundii* cells every day. 100 µL of each effluent was put into 900 µL of medium "M3", and 1mL of beads was put into 9 mL of "M3" bullion. The samples of diluted effluent were mixed using vortex. The samples of capsules were homogenized using BagMixer. From each suspension serial dilutions were made. Cell concentration was expressed as CFU per mL and determined by making serial decimal dilutions and plating on MacConkey agar.

3. RESULTS AND DISCUSSION

The aim of the work was to decrease the costs of conversion of waste glycerol to 1,3-PD by using chip and simple chromatographic columns containing encapsulated *C. freundii* cells. One of the most important bioconversion parameter is pH that should be strictly defined to obtain high yield of process. It is known that there is not pH control system in the chromatographic columns. On the other hand it is possible to control pH level using mediums containing higher amount of phosphate salts. Unfortunately biopolymers such as calcium alginate used for encapsulation of bacteria are not stable in broth containing high amount of phosphates. It is why one of the goals of this study was to modify the medium and also to ascertain the new systems of biopolymers that could be used for encapsulation of bacterial cells. The stability of obtained membranes in different mediums has been examined. The waste glycerol conversion to 1,3-PD over continuous process using chosen beads has been analyzed as well.

The results of the study showed that double crosslinked alginate beads were stable in M1 medium after 168 hours of storage. These results were proved by Wong et.al. (14). The medium prepared by Wong and his team for 1,3-PD production contained the higher amount of pfosphates than M1 medium did, and lower amounts of these salts than M2 broth did. Unfortunately the capsules were not stable in M2 broth what was caused by to high amount of phosphates. The membranes were desintegrated after 24 hours.

The medium M2 was modified by the addition of $CaCl_2$ (M3). The results of experiments showed that the capsules were stable in M3 broth after the 168 hours of storage.

It was also noted that after 144 hours of storage the diameter of the capsules double sized. The addition of calcium chloride increased a stability of beads in M3 broth to 144 hours. The comparison of mechanical properties of the beads after 24 hours of storage in M1 and in M3 mediums showed that an avarage value of F_{max} decreased from 3.2 [N] to 1.79 [N] in the case of M1 broth. The analysis of bursting force has also showed that an avarage value of F_{max} did not change in the case of M3 medium what proved that addition of $CaCl_2$ improved stability of beads in presence of higher amount of phosphate salts.

The alginate/polycations capsules have been prepared by using Method I and Method II. After 24 hours of storage in M2 and M3 mediums the capsules have been explored. The results of the experiments showed that all of the beads which were incubated in M2 medium disintegrated. The bursting force of the capsules which had been incubated in M3 broth has been examined as well. It was shown that PEI/alginate capsules obtained by using Method I and II have been disintegrated after 24 hours of storage in M2 and in M3 mediums.

It was also demonstrated that the avarage of F_{max} of the poliDADMAC beads obtained using Method I (6.27 N) was similar to the avarage of F_{max} of the capsules obtained using Method II (6.20 N). After 24 hours of storage in M3 medium F_{max} of the poliDADMAC beads obtained using Method I decreased to 0,03 N. Unfortunately the poliDADMAC capsules obtained using method II were disintegrated. The bursting force of GA 126 beads obtained by Method I was higher (4.01 N) than the bursting force of GA 126 beads obtained by Methode II (2.12 N).

After 24 hours of storage in M3 broth F_{max} of the GA 126 capsules decreased significantly (Table 1). The results of the study has also showed that it was not possible to obtain globular PHPDMA beads using Method I. The same results have been obtained for oligochitosan capsules (Table 1).

It is also important that after 24 hours of storage in medium M3 F_{max} of both kinds of the beads obtained using Methode II decreased significantly (Table 1). The analysis of mechanical properties of alginate/polycations beads demontrated that these capsules are not sufficient to use them for waste glycerol bioconversion to 1,3-PD. As was emphasised by Zhao (10) the NaCS/PDMDAAC capsules were stable in medium containing the similar amount of phosphate salts as M1 medium had. It has been reported by this author that the NaCS/PDMDAAC microcapsules were very suitable for repeated fermentations in these conditions.

In repeated fermentations of their study, biomass of inner capsule had been enriched and cells compatible with microcapsules due to the cultivation of the first batch. In the following batches, these microcapsules with high cell density inside were directly employed. As has been demonstrated by Wong (14), The alginate beads were also suitable for repeat fermentations in broth similar to M1 conditions. They were stable enough to be used even 6 times for bioconversion process.

The experiments of this study had focused on bioplymer capsules that have been introduced into M2 and M3 medium. This study has been especially concerned with the influence of phosphates salts on the stability of these capsules. It was proved that poliDADMAC and alginate/polycation beads were not stable in M2 and M3 broths adequate to use them for repeatrd fermentations.

Table 1. The avarage values of F_{max} for alginate/polycation beads

Sample	F_{max} [N]	SD*
poliDADMAC (Methode I) before incubation	6.27	0.61
poliDADMAC (Methode I) after incubation	0.03	0.01
GA 126 (Methode I) before incubation	4.01	0.78
GA 126 (Methode I) after incubation	0.02	0.01
poliDADMAC (Methode II) before incubation	6.20	0.91
GA 126 (Methode II) before incubation	2.12	0.92
GA 126 (Methode II) after incubation	0.01	0.00
PHPDMA Oligochitosan (Methode II) before incubation	4.19	1.82
Oligochitosan (Methode II) before incubation	4.26	1.46
Oligochitosan (Methode II) after incubation	0.04	0.02

SD* - standard deviation

The next step of the experiments concerned with the stability of the beads by coating the alginate capsules using oligichitosan. Three oligochitosans with the molecular weight of 2500 g/mol, 7200 g/mol, and 14000 g/mol have been compared. The results of the experiments showed that the best was the oligochitosan with the molecular weight of 14000 g/mol. In comparison to the beads coated by oligochitosan with Mn = 2500 g/mol, and 7200 g/mol the capsules coated by oligochitosan with Mn = 14000 g/mol were stable after 24 hours of storage in medium M2.

Although the bursting force of the beads after storage decreased from 4.03 [N] to 0.1 [N] they did not desintegrated. Unfortunately the problem of these capsules was that they have been clumped after storage in medium M2 (Figure 3). To solve this problem the coating process was modified. The increase of stirring rate (stirring rate > 500 rpm) during coating prevented to clump (Figure 4). As has been demonstrated by Sarma and the team, alginate capsules which were coated with chitosan had better mechanical properties than aliginate beads devoid of coatings (16). This author proved that the capsules containing chitosan coating were durable adequate to be used during petroleum hydrocarbons biodegradation.

The modified capsules were storaged only in M3 medium because F_{max} of the beads which were incubated in M2 decreased significantly. The results of the study showed that coated capsules were stable in M3 after 168 hours of storage. Unfortunately after 194 hours

the beads were disintegrated. It was also important that after 120 hours of incubation a diameter of the capsules double-sized. The analysis of mechanical properties of these beads after 24 hours of storage in M3 broth showed that an avarage value of F_{max} decreased from 6.44 [N] to 4.13 [N] what proved that addition of $CaCl_2$ improved the stability of beads in the presence of higher amount of phosphate salts and that the capsules coated by an oligochitosan can be used in waste glycerol bioconversion process. In order to decrease the costs of bioconversion process by using chip and simple chromatographic columns it was necessary to immobilize active biomass of *C. freundii*. Two columns were used during the bioconversion processes.

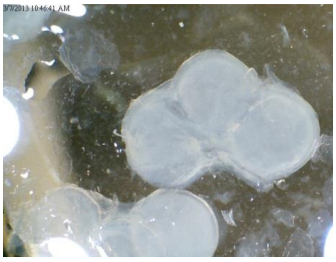
oligochitosan	The capsules before storage	The capsules after 24 hours of storage in M2
Mn = 14 000 g/mol		

Figure 3. The beads coated by oligochitosan with the molecular weight of 14000 g/mol

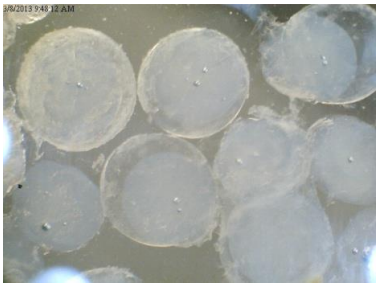
oligochitosan	The capsules before storage	The capsules after 24 hours of storage in M2
Mn = 14 000 g/mol		

Figure 4. The modified beads coated by oligochitosan with the molecular weight of 14000 g/mol

The modification of medium (M3) was necessary to pH control to keep the process stable. The purpose of the study was also to ascertain the new systems of biopolymers used for encapsulation of bacterial cells that had to be stable in M3 broth. The *C. freundii* cells

were encapsulated in alginate double crosslinked capsules and in alginate capsules coated by oligochitosan. The beads were introduced to the columns each kind separately. Therefore, a complete growth medium was supplied to the columns and temperature was maintained in an optimal range (30 °C).

In the supernatant from the columns viable cells were detected throughout all experiments indicating that the immobilisation was complete. The results of experiments showed that there were 5.3×10^6 [CFU/mL] of *C. freundii* cells in the double – crosslinked beads and the same amount of these cells in coated capsules before the process run. During the first 48 hours of bioconversion there were no bacterial cells in effluents from both of columns (Table 2). After 72 hours of conversion of glycerol to 1,3-propanediole, 3.37×10^6 [CFU/mL] of *C. freundii* in effluents from the column containing double crosslinked capsules was observed.

The protrusion of bacterial cells from the beads to an effluent was not observed in the case of coated capsules what proved that immobilization was complete. After 144 hours of bioconversion there were 2.87×10^7 [CFU/mL] of bacteria in effluent from column containing double – crosslinked beads. The increase of number of bacteria in effluent from this column was observed. The bacterial cells in effluent from column containing coated capsules were not observed even after 144 hours of process. There was 6.17×10^4 [CFU/mL] of *C. freundii* in double – crosslinked capsules after 144 hours of process.

The higher amount of bacteria in effluent than in the beads proved that immobilization was not complete or that the membranes of the capsules were not strong ample to kip the bacteria inside them. The results have also showed that there was only 8.43×10^2 [CFU/mL] of *C. freundii* in coated capsules after 144 hours of bioconversion. It was also proved that oligochitosan coating is active against immobilized cells. The significant decrease of number of bacteria (from 5.3×10^6 [CFU/mL] to 8.43×10^2 [CFU/mL] Table 2) caused low productivity of bioconversion process.

As has been argued by Gungormusler (17), the bacterial growth was seen to be exponential between 8 and 16 h of glycerol bioconversion process. In general, almost all the microorganisms studied, had relatively long lag phase lasting up to 6 h. According to the results obtained by this author, most of bacterial species had decreasing biomass concentrations at the end of fermentation period. The biomass concentration decreased even 30% in weight at the end of 48 h. According to Gungormusler it was tempting to suggest that the decrease of biomass concentration was caused by a lysis of microorganism.

The continuous bioconversion of glycerol to 1,3-PD was examined. The two columns containing immobilized *C. freundii* cells were used during processes. The M3 broth was used to synthesize 1,3-PD. The results of the study showed the average 1,3-PD concentrations obtained from each column as functions of HRT. The first of the columns was filled with double crosslinked calcium alginate beads. The second column was filled with alginate capsules covered by chitosan coatings. Because of double sizing of diameter of the capsules it was assumed that process should run 144 h.

The results of the study showed that the capsules coated by chitosan have been disintegrated after 72 hours of continuous bioconversion. From the other hand the double crosslinked beads have been stable after 144 hours of 1,3-PD synthesis despite the fact that their diameter double sized after this time (Figure 5).

The analysis of bioconversion demonstrated that the presence of bacterial cells in effluent was not observed during two first days of process. It indicated that both kind of the

beads were stable. *C. freundii* cells were observed in effluent from the column containing double crosslinked capsules after 72 hours. In the case of these capsules the higher amount of bacterial cells in the enffluent than in the capsules was observed after 144 hours of bioconverison (Table 2).

Table 2. The viability of *C. freundii* cells during bioconversion process.

The capsules	**Time [hours]**							
	24	**48**	**72**	**96**	**120**	**144**	**0 K1**	**144 K2**
	The number of bacterial cells in effluent [CFU/mL]						**The number of bacterial cells in capsules [CFU/mL]**	
Double-crosslinked	-	-	3.37×10^{-6}	4.5×10^{-7}	1.01×10^{-7}	2.87×10^{-7}	5.3×10^{-6}	6.17×10^{-4}
SD	-	-	1.53×10^{-5}	9.17×10^{-6}	2.83×10^{-5}	4.04×10^{-6}	8.9×10^{-4}	2.08×10^{-3}
Containing oligochitosan coating	-	-	-	-	-	-	5.3×10^{-6}	8.43×10^{-2}
SD	-	-	-	-	-	-	8.9×10^{-4}	3.5×10^{-1}

0K1 - The number of bacterial cells before the bioconversion
144K2 - The number of bacterial cells after the bioconversion

Time [h]	The coated beads	The double crosslinked beads
0		
48		
144		

Figure 5. The capsules with immobilized cells during bioconversion process

It was the prove that immobilisation was not complete. The lack of microorganisms cells in the effluent from the column containing coated beads after 72 hours was observed. It was caused by desintegration of the capsules. It would seem that the antimicrobial properties of chitosan in low pH could eliminate the cells. The obtained results proved that although M3 medium contained $CaCl_2$ it was not good adequate to cause the stability of the beads.

Comparing the amount of 1,3-PD in effluent from the column containing double crosslinked beads, it was observed that *C. freundii* cells have synthesized 4.31 g/L, 2.11 g/L and 2.51 g/L of this substance during the first four days of process (Table 3). After 120 hours a decrease of the amount of 1,3-PD in effluent was observed. In the case of coated capsules 2.55 g/L and 1.79 g/L of 1,3-PD were obtained during first three days of process. (Table 3). The concentrations of 1,3-PD obtained in this study were low (4.31 g/L). From the other hand the results of Wong (14) and his team proved that *Klebsiella* sp. cells encapsulated in double crossedlinked alginate beads could produce 8.75g/L of 1,3-PD. Similarly the results of the tests of these authors showed that use of waste glycerol by microorganisms was higher (42.46 g) in comparison to the results obtained in this work (7.18 g). It should be also said that these authors used the alginate beads 6 times. In contrast to their results the capsules used in this study were stable only 144 hours. After desintegration of the capsules the cells stoped to produce 1,3-PD. As seen in Tables 4 and 5, the yield and productivity of bioconversion process have also been highest during first four days in the case of double crosslinked capsules and during first three days in the case of coated beads. The results of this study showed that decomposition of waste glycerol has also been low in the case of both kinds of capsules.

The concentrations of 1,3-PD obtained from each column have been also analyzed as functions of HRT. As seen in Table 6 there was an optimum value for HRT parameter that is 1 h for both of columns. It was observed that too long (e.g., 5h) HRT resulted in lower 1,3-PD productions in both cases. The column containing bacteria encapsulated in double crosslinked alginate membranes, performed much better in terms of 1,3-PD concentrations than the column containing coated alginate capsules. Similarly HRT of 1 h was the best one for Gungormusler (7). Different results have obtained M. Gungormusler and his team (11). These authors have also studied continuous bioconversion of glycerol to 1,3-PD by using immobilized *Clostridium beijerinckii* NRRL B-593 strain. Their results showed an optimum value for HRT parameter that was 12 h. It was observed by them that too short (e.g., 2h) HRT resulted in lower 1,3-PD productions. The highest 1,3-PD concentration obtained by these authors was found to be 31 g/L. It could be said that differences between concentrations of 1,3-PD obtained in this work and by Gungormusler could be caused by different carriers used for immobilisation. Gungormusler and his team (7) confirmed these considerations. They concluded that differences between conversion rates are dependent on immobilisation material.

Summing the productivity of bioconversion of crude glycerol to 1,3-PD can be concluded that the double crossed alginate beads are better carriers than alginate capsules coated by chitosan. The amount of glycerol converted to 1,3-PD (grams glycerol per grams 1,3-PD) was described as 1,3-PD yield. As seen in Table 4, 1,3-PD yields ranged between 0.2 and 1.3 (g 1,3-PD/g glycerol) that was compatible with the theoretical expected ranges (18). The results of this study are tempting to suggest that double crosslinked capsules and the beads coated by chitosan are not suitable for use them in continuous processes in chip and simple columns devoid of pH control systems, even using modified medium. As has been shown by Wong et.al. (14), calcium alginate immobilized cells were used to increase the stability of repeated 1,3-PD production by bacterial cells. Similarly, it has been reported by Zhao (10), that the NaCS/PDMDAAC microcapsules were employed for repeated fermentations glycerol to 1,3-PD. It shoud be said that in both cases the capsules were introduced into medium which contatined low amount of phosphate as M1 had. A moment's

reflection shows that it would be better for both of the membranes analyzed in this study, to use pH control system with medium containing lower concentrations of phosphate salts (e.g. M1) than broth with higher amount of phosphates. It would appear that these chip columns could be used when solid carriers as polyurethane foams were used for immobilisation. PUFs are stable in environment containing high amount of phosphates that is very important to control pH of a medium. These considerations are confirmed by the others. As has been argued by two teams (1,7) a higher concentration of phosphates in broth had a positive influence on pH that was important to obtain a high concentration of 1,3-PD. As can been exemplified by those authors the carriers used for immobilisation should be stable in high amount of phosphates. Only if the carriers are stable they will be used for repeated bioconversions. Similarly, Jun and his team used PUF foams as immobilization material for conversion glycerol to 1,3-PD. To improve the productivity of 1,3-PD, the *K. pneumoniae* cells were immobilized using hydrophobic polyurethane media in the FBR (19). The results of these authors proved that production of 1,3-PD increased with the cycle number (1.06 gL−1h−1 versus 1.61 gL−1h−1 at the first and fourth cycle, respectively) due to successful cell immobilization. During 46 cycles of fed-batch fermentation taking place over 1.460 h, a stable and reproducible 1,3-PD production performance was observed. Based on their results, repeated fed batch with immobilized cells is an efficient fermentor configuration, and waste glycerol can be utilized to produce 1,3-PD. Kaur and hid team have mentioned (20), that the cells of *C. freundii* were immobilized on modified polyurethane foam for production of 1,3-PD as well. The latter could obtain a 2-fold increase in productivity (8.1 g/L/h) and 16.3 g/L 1,3-PD using this method.

Table 3. The amount of glycerol and 1,3-PD during bioconversion

Time [h]	The membranes	Glycerol (mL)	1,3-PD (mL)
24	Calcium alginate	53.32	0.02
	Calcium alginate with chitosan coating	54.99	0.3
48	Calcium alginate	46.44	4.31
	Calcium alginate with chitosan coating	47.92	2.55
72	Calcium alginate	50.72	2.11
	Calcium alginate with chitosan coating	30.7	1.79
96	Calcium alginate	48.47	2.51
	Calcium alginate with chitosan coating	-	-
120	Calcium alginate	52.6	1.3
	Calcium alginate with chitosan coating	-	-
144	Calcium alginate	48.86	1.37
	Calcium alginate with chitosan coating	-	-

Table 4. The yield of bioconversion.

Time [h]	**24**	**48**	**72**	**96**	**120**	**144**
Yield [mol/mol]						
Ca/ alginate beads	0	1.0	0.4	0.3	0.4	0.2
Ca/ alginate beads with chitosan coating	0	0.95	0.07	-	-	-

Table 5. The productivity of bioconversion

Time [h]	**Productivity [g/L]**					
	24	48	72	96	120	144
Ca/ alginate beads	0	1.3	0.6	0,8	0.2	0.2
Ca/ alginate beads with chitosan coating	0	0.76	0.54	-	-	-

Table 6. The yield as function of HRT

The carrier	**HRT [h]**	
	5	1
	The yield [mol/mol]	
Calcium alginate beads	0.2	0.9
Calcium alginate beads with chitosan coating	0.25	0.53

The purpose of the study has been also to obtain the capsules (not calcium alginate) that would be stable in M2 or M3 mediums. It is why cellulose sulfates with different substitution degrees (0.3 – 1.2) has been prepared (Table 8). These tests (using cellulose sulfates) have focussed on positive results obtained by two research groups (21). In the first step, 4 % PDADMAC was prepared according to (10,21,22). As seen in a work of Dautzenberg (22), the use of PDADMAC with a wide range of polydispersity in the molecular weight would have a positive impact on the formation of capsules. Therefore, a mixture of PDADMAC with molecular weights of: 8 500 g / mol (1%), <100 000 g / mol (1 %) 100 000

– 200 000 g / mol (1%), 200 000 - 300 000 g / mol (1%) was used to prepare the beads. The cellulose sulfate was extruded into PolyDADMAC solutions.

Table 7. The influence of molecular weight of 4% PDADMAC on shape and stability of the capsules

Cellulose sulphate	Molecular weight of PDADMAC				
	8 500 g/mol	**< 100 000 g/mol**	**100 000-200 000 g/mol**	**200 000-300 000 g/mol**	**Mixture with high polydispersity**
4%	irregular shape	unstable with irregular shape	precipitated	unstable with irregular shape	precipitated
6%	unstable	spherical shape	spherical shape	unstable with irregular shape	precipitated
10%	precipitated	precipitated	spherical shape	globular shape	spherical shape

Table 8. The influence of the reaction time on substitution degree of cellulose sulphate

The reaction time [minutes]	Substitution degree
60	0.3
120	0.6
240	1
480	1.2

The results of these tests were not satisfactory. In most cases, it was very difficult to obtain spherical shape of the capsules. Additionally, some of polyDADMAC solutions have precipitated after extrusion (Table 8). The results showed that the spherical beads were not stable in mediums M2 and M3. In contrast to these results, the experiments of many teams (10,15,21) demonstrated that mechanical properties of the capsules based on cellulose sulfate and PDADMAC and their stability in many solutions were very good. The negative results caused the necessity of the next tests. These experiments were focused on the addition of three different carrageenans into cellulose sulphate solutions before an extrusion.

Table 9. The influence of the addition of different carrageenan into 4% PDADMAC on shape and stability of the capsules

4% cellulose sulphate + 1,5% carrageenan	The reaction time [h]	Molecular weight of PDADMAC			
		8 500 g/mol	< 100 000 g/mol	< 100 000 g/mol + 0.3 M KCl	100 000 g/mol + $CaCl_2$ 0.3 M
Kappa	1		unstable with irregular shape	unstable with irregular shape	-
Kappa	2	unstable with irregular shape	unstable with irregular shape		
Kappa	4	unstable with irregular shape	unstable with irregular shape	precipitated	
Kappa	8		precipitated	precipitated	
Iota	1		unstable with irregular shape	-	unstable with irregular shape
Iota	2	unstable with irregular shape	unstable with irregular shape		
Iota	4	unstable with irregular shape	unstable with irregular shape		The precipitation
Iota	8		-		The precipitation
Lambda	1		unstable with irregular shape	-	
Lambda	2	unstable with spherical shape	unstable with irregular shape		
Lambda	4	unstable with irregular shape	unstable with irregular shape		
Lambda	8		unstable with spherical shape		

The results of the experiments demonstrated that the addition of carrageenan have not improved the shape and the stability of the capsules (Table 9). It was found that the capsules which had been obtained on the basis of cellulose sulfate with the addition of carrageenan lambda (with the reaction time of 2 hours) have been the best. These capsules had a regular and spherical shape. Although, the reasults of Bang et.al. (23) proved that there is very difficult to obtain the spherical lambda carrageenan capsules. As can be exemplified by these authors, the most likely shape of the capsules obtained from this biopolymer is the shape of an ellipse. The spherical shape made the capsules possible to use them during bioconverios proces. Unfortunately the beads were not stable in M2 and M3 mediums what proved that the capsules shoud not be used for 1,3-PD synthesis (Table 9).

The influence of the addition of cellulose sulphate to sodium alginate (before extrusion) has been also analyzed. It was not possible to obtain the beads when the solution of cellulose sulphate and sodium alginate was extruded into PDADMAC, the immediate precipitation has been observed. The addition of $CaCl_2$ had a positive impact on the extrusion. The spherical shape capsules has been obtained (Table 10). Unfortunately the results has also demonstrated that thes capsules had been not stable in M2 and M3 mediums.

Table 10. The influence of addition of sodium aglinate into 4% cellulose sulphate (before extrusion) on stability of analized capsules

The solution	**The reaction time [h]**	**4 % PDADMAC < 100kDa**	**4 % PDADMAC < 100kDa + 0,155 M $CaCl_2$**
4 % cellulose sulphate + 4% sodium alginate	8	precipitated	spherical capsules
4 % cellulose sulphate + 4% sodium alginate	4	precipitated	spherical capsules
4 % cellulose sulphate + 4% sodium alginate	2	precipitated	spherical capsules
4% alginian	0	precipitated	spherical capsules

The calcium alginate beads containing Syloid AL-1 FP silica and Ludox® coloidal silica have been prepared (Fig 6). Their shape was regular and spherical. As Mizielińska and Bartkowiak has mentioned (24-26), the alginate/silica beads have very good mechanical properties, and it is possible to storage these capsules in water with salinity of 11 or 35‰. It is also possible to storage them in engine oil, diesel oil, and even crude oil. There was good reason to storage the beads in M2 and M3 mediums. The results of the experiments showed that after 24 hours of incubation in bulions the capsules were almost desintegrated (Table 11). It has proved that they can not be used during bioconversion.

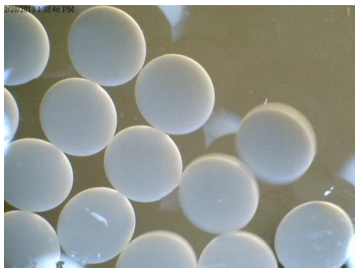
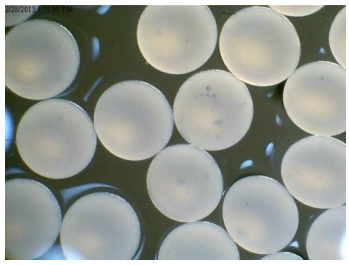
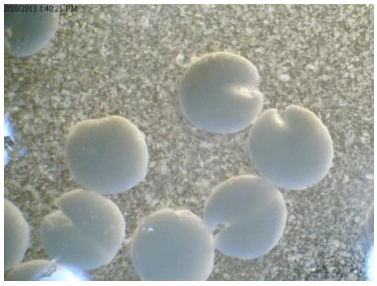
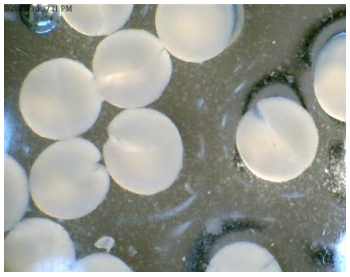
Użyta krzemionka/ Próba	4 %sodium alginate + 10 % Syloid	4 %sodium alginate + 20 % Ludox
Before incubation		
After 24 hours of incubation		

Figure 6. The alginate/silica beads

4. CONCLUSIONS

- The double crosslinked alginate beads are better carriers than alginate capsules coated by chitosan
- The double crosslinked alginate beads and the alginate capsules coated by chitosan should not be used for continous process and repeated bioconversions
- PDADMAC brads and modified (e.g. by additions) PDADMAC should not be used for repeated bioconversions.

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