WHEY PERMEATE FERMENTATION BY *Propionibacterium freudenreichii*

Fermentação de permeado de soro por *Propionibacterium freudenreichii*

Maria Inês Teixeira[1*], Juan Carlos Rossi Alva[2] e Maria Helena M. Rocha-Leão[3]

[1*] Instituto Federal de Educação, Ciência e Tecnologia do Rio de Janeiro, *campus* Rio de Janeiro – IFRJ, Rua Senador Furtado, nº 121/125, Maracanã, Rio de Janeiro, RJ, Brasil. CEP:20270-021.

[2]Coordenador do Curso de Graduação em Ciências Biológicas, Universidade Católica do Salvador (UCSAL). Av. Prof. Pinto de Aguiar 2589, Centro de Ensino I – LEMA, Patamares, Salvador, BA, Brasil. CEP: 41740-090.

[3]Programa de Pós Graduação em Ciência de Alimentos, IQ/UFRJ, Ilha do Fundão, Rio de Janeiro, RJ. Brasil. CEP: 21.910-900.

*Autor para correspondência: maria.teixeira@ifrj.edu.br

RESUMO

Os ácidos orgânicos produzidos por bactérias propiônicas podem ser usados como aditivos aromatizantes e inibidores de fungos. O objetivo deste trabalho foi avaliar os processos fermentativos para produção de ácido propiônico. A *Propionibacterium freudenreichii* foi cultivada em permeado de soro de queijo a 30ºC, sem suplementação nutricional e ajuste de pH durante os processos. Foram comparadas as produções de ácido propiônico por células livres e por células imobilizadas. O permeado de soro de queijo foi obtido da ultrafiltração do soro utilizando membranas poliméricas com massa molar de corte de 20 KDa. As concentrações de lactose e ácidos orgânicos foram determinadas por HPLC. As concentrações de ácido propiônico, após 113 horas de fermentação, obtidas de células imobilizadas e células livres foram 2,17 g/L e 2,29 g/L, respectivamente. Os resultados mostram a aplicabilidade do sistema imobilizado, já que as esferas de alginato de cálcio contendo células imobilizadas podem ser reutilizadas em várias bateladas consecutivas.

Palavras-chave: *Propionibacterium*, imobilização de células, ácido propiônico, permeado de soro, aprisionamento de células.

ABSTRACT

Volatile organic acids produced by propionibacteria can be used as mold inhibitors and aromatic additives. The aim of this work was to evaluate propionic acid production by fermentation processes. *Propionibacterium freudenreichii* was cultivated in cheese whey permeate at 30ºC, without nutrient supplementation or pH adjustment during the processes. Propionic acid production by free cells was compared to the production by immobilized cells. Whey permeate was obtained from ultrafiltration of whey with 20 KDa molecular weight cut-off membranes. Organic acids and lactose concentrations were determined by HPLC. The propionic acid concentrations after 113 hours of fermentation by immobilized and free cells were, respectively, 2.17 g/L and 2.29 g/L. The results shows the applicability of immobilized systems, since beads containing propionibacteria could be reused for several consecutive batch fermentations.

Keywords: *Propionibacterium*; cell immobilization; propionic acid; whey permeate; cell entrapment.

INTRODUCTION

The process of ultrafiltration is used to concentrate a liquid or to fractionate it into two liquids with different compositions. Ultrafiltration involves the use of membranes with a molecular weight cut-off in the range of 1-200 KDa. The application of this process for cheese production is now diversified, including manufacture of fresh, soft, hard and semi-hard cheese varieties from different milks (ROSENBERG, 1995).

Whey is a by-product of cheese and casein manufacture, largely treated by ultrafiltration for the recovery of proteins (COLOMBAM *et al.*, 1993). The ultrafiltration of whey results in retentates containing proteins, fat and colloidal minerals, and in permeate consisting of water, soluble minerals, lactose, non-protein nitrogen compounds and water-soluble vitamins (ROSENBERG, 1995). The primary use of ultrafiltration was to produce a whey protein concentrate (WPC). Separation of whey into various fractions, such as WPC, has increased the whey product values. When WPC is a desired product, the whey permeate and lactose remain as a low value by-product. The disposal of these permeate with high biological oxygen demand (BOD) is a problem in the dairy industry and is prohibited by environmental regulations (YANG and SILVA, 1995). However, they can be used as a base for biological transformations (COLOMBAM *et al.*, 1993; BARILEA *et al.*, 2009). Whey permeate can be used as a low cost carbon source for the production of propionic acid by propionibacteria (JAIN *et al.*, 1991).

Propionibacteria play important roles in the development of the characteristic flavor and eye production in Swiss-type cheese, but they also have potential usage for the production of propionic acid, vitamin B_{12}, folic acid, trehalose, biomass, aromas and antimicrobial compounds such as bacteriocins (BÉGIN *et al.*, 1992; CARDOSO, *et al.,* 2004; CHAMPAGNE *et al.* 1989; DEVLIEGHERE *et al.* 2007; HUGENHOLTZ *et al.*, 2002; LEWIS and YANG, 1992). Jan *et al*. (2002) suggests that propionibacteria could constitute probiotics efficient in digestive cancer prophylaxis via their ability to produce apoptosis-inducing short-chain fatty acids.

Immobilized cells may offer several advantages over free cell cultures (MOHAMMADI *et al.,* 2008). Since immobilization allows the concentration of active biomass, higher substrate concentrations may be used and smaller reactors may be required. Most immobilized systems are reusable and can offer long-term stability (RICKERT GLATZ *et al.*, 1998). Cell immobilization, which allows the concentration of active biomass, has been used to reduce the fermentation time in several processes (JAIN *et al*., 1991).

Propionic acid has many and varied uses as an antifungal agent in foods and feeds and as ingredient in thermoplastics, antiarthritic drugs, perfumes, flavors and solvents, and is also an important chemical used in the production of cellulose plastics and herbicides (BÉGIN *et al.*, 1992; LEWIS and YANG, 1992; LIND, JONSSON and SCHNÜRER, 2005; PAIK and GLATZ, 1994). It is commonly added to bread to increase shelf life (BÉGIN *et al.*, 1992; JAIN, *loc. cit.*). The fermented propionic acid as well as the commercial propionic acid from chemical processes were able to inhibit the growth of the fungi to the same degree (CHOOJUN and YOONPRAYONG, 2012).

The aim of this work was to evaluate fermentation processes to produce propionic acid by *Propionibacterium freudenreichii*. Both free and immobilized cell systems were compared in propionic acid production and the combined effect of culture medium on inoculum and bacterial population was also investigated.

MATERIAL AND METHODS

Bacterial culture

Propionibacterium freudenreichii PS-1 was obtained as freeze-dried culture from Chr. Hansen Ind. and Com. Ltda.

Whey Ultrafiltration

Cheese whey with 11ºD obtained from mozzarella cheese manufacturing was ultrafiltrated in a pilot-plant unit at Centro de Tecnologia do Instituto de Laticínios Cândido Tostes. Ultrafiltration was carried out in polysulfone membranes with 20 KDa molecular weight cut off. After ultrafiltration process, whey permeate was held in a freezer until used.

Culture Media, Growth Conditions and Culture Purity

Bacterial culture was activated in sodium lactate broth which was composed of 1% (w/v) yeast extract (Vetec), 1% meat peptone (Micro Med), 0,025% K_2HPO_4 (Vetec) and 1% sodium lactate syrup (Sínteses Purasal S/SP 60). Sodium lactate agar, used for maintaining bacterial stock culture, was prepared by adding 2% agar-agar (Isofar) to lactate broth and was autoclaved during 20 minutes at 120ºC, while the experimental culture medium, whey permeate, was autoclaved at 111ºC for 20 minutes. The pH of all media was set 7.00 ± 0.01 with NaOH 2N before sterilization.

Propionibacteria were cultivated in sodium lactate agar at 30ºC during 5 days. In order to prepare inocula for experiments, a single colony grown in sodium lactate agar was inoculated into 10mL sodium lactate broth and incubated aerobically at 30ºC for 3 days. Two successive 1% (v/v) transfers were made into fresh sodium lactate broth before the inoculum was used.

Tubes containing inocula were centrifuged at 939.40g for 20 minutes. Broth was discarded, the pelleted cells were washed twice with 0.85% (w/v) sterile saline solution and then the pellets were resuspended in sterile saline solution. Cell suspensions were used either in free cell and immobilized cell systems.

The culture was regularly checked for purity by microscopical examination of Gram stained preparations, colony pigmentation, cell morphological characteristics and catalase tests.

Cell Immobilization in Calcium Alginate

Cells were immobilized in a process similar to that used by Paik and Glatz (1994). Solutions of 2% (w/v) sodium alginate, 2% sodium citrate and 0.1M $CaCL_2$ were previously prepared and autoclaved at 120ºC for 20 minutes. The cell suspension after centrifugation was mixed with 2% sodium alginate (at a volumetric ratio of 1:4) and the mixture was maintained under agitation. Low agitation intensity was provided by a magnetic stirrer. The mixture was extruded through the tip of a 5mL pipette into sterile 0.1M $CaCL_2$. Alginate drops solidified in contact with $CaCL_2$, forming beads and thus entrapping bacterial cells. The beads were allowed to harden for 40 minutes. The diameter of the beads was approximately 3.0mm. Bead diameter was determined by measuring the length of 10 beads aligned along a ruler. Beads were sieved, then were put in filter-paper to dry the humidity excess and weighed. Approximately 1g (wet weight) contained 33 alginate beads.

Fermentation Conditions

In free cell system, cell suspensions after centrifugation was mixed to the medium in 500mL erlenmeyer flasks at a proportion of 25mL cell suspension into 225mL of whey permeate. The flasks were incubated statically at 30ºC during 113 hours of fermentation.

In immobilized cell system, 25g of beads were transferred to 500mL erlenmeyer flasks containing 225mL of whey permeate. The flasks were incubated under the same conditions of free cell system. Batch fermentations were carried out in duplicate and results are averages of these trials.

Analytical Methods

Whey permeate samples were analyzed for total solids (w/v) and ashes by gravimetric method; total nitrogen by Kjeldahl method and conversion factor of 6.38 for protein content (AOAC, 1997); total lipid by Bligh-Dyer method (BLIGH and DYER, 1959; PAGNO *et al*., 2009); density was measured using a lacto-densimeter instrument with thermometer; pH was measured by pH meter (HARRIGAN and MC CANCE, 1976). Lactose and organic acids were determined by HPLC (Waters mod. 510), with rheodyne injector refractive index detector mod. 410 and an integrator 747 were used. A cation-exchange column 8 X 300mm (SHODEX Rspak KC 811) was operated at 40ºC with 0.1% of phosphoric acid (1 mL/min) as eluent. Samples were filtered through Millipore 0.45µm filters prior to analysis. Standards were used to identify peaks in HPLC chromatograms and peak height was used to determine the sample concentration. Samples in free cell and immobilized cell systems were taken from the medium after addition to the flasks and every 24 hours to estimate pH, lactose and organic acids.

Viable cells were enumerated by pour plate counting method by plating serial dilutions in duplicate sodium lactate agar plates. Plates were incubated at 30ºC aerobically. Whey permeate samples from free and immobilized cells systems were counted at the beginning and at the end of the fermentation time. Were counted all colony forming units (CFU), including those of pinpoint size. Free cell system viable cell counts were expressed as CFU/mL. In immobilized system, viable cell counts were expressed as CFU per bead wet weight (CFU/g); beads were first dissolved by adding 25g of alginate beads to 225mL of 2% sodium citrate with gentle agitation. Samples from the surrounding medium also were taken to estimate cells release from the beads (CFU/mL).

All analyses of organic acids, lactose or cell concentrations were carried out at least on duplicate.

Results and Discussion

Composition of Whey Permeate Medium and its Effect on propionibacteria Growth

Small batch fermentations of free and immobilized cells of propionibacteria were incubated in whey permeate medium at an initial pH of 6.20 (after sterilization) for 113 hours without nutrient supplements, medium agitation or pH control. As it was expected, the analysis results (table 1) showed that whey permeate had low protein content (0.06% w/v). The samples from the permeate were analyzed by HPLC for organic acids determination and the results showed that propionic acid was not found, very few acetic acid (0.048g/L) and some lactic acid (0.111g/L) were found before permeate fermentation. It was investigated the combined effect of the medium on inoculum and bacterial concentrations in both systems of free and immobilized cells of propionibacteria. As it is well known, propionibacteria requires a complex nutritional medium.

Table 1. Whey permeate medium composition [a].

Total solids (% w/v)	5.08
Soluble solids (°Brix)	5
Ash (% w/v)	0.25
Protein (% w/v)	0.06
Fat (% w/v)	0.40
Lactose (% w/v)	4.02
Density (w/v)	1.021
pH	6.20
Acidity (°Dornic)	11
Lactic acid	0.111
Acetic acid (g/L)	0.048
Propionic acid (g/L)	0.000

[a] Before autoclaving and pH adjustement

Certain vitamins and minerals are required for their growth and metabolism (HETTINGA and REINBOLD, 1972). It has been shown (CHAMPAGNE *et al*., 1989; GARDNER and CHAMPAGNE, 2005; LEWIS and YANG, 1992; PAIK and GLATZ, 1994) that immobilized cells reached higher concentrations and productivities of organic acids because of higher concentration of cells in the reactor. Although permeate is a nutrient deficient medium and in the present work the permeate was not supplemented, the carbon sources – lactose and lactic acid present in the whey permeate - in addition to the high inoculum densities, provided cell growth in both free and immobilized cell systems (table 2). Our results suggest that whey permeate can be used as fermentation medium for propionibacteria, even without supplementation, when associated to high cell densities. The cell increments were the same on both systems. It was also investigated the amount of cells released from calcium alginate beads (table 2). The figure 1 shows the Scanning Electron Microscopy (SEM) image of *Propionibacterium* colonies immobilized within the matrix of calcium alginate bead. SEM image (figure 2) of entrapped cells colony shows apparently release of certain cells from bead surface. This release of cells may or may not be desirable depending on the applications (KLINKENBERG *et al.*, 2001). According to Morin *et al.* (1992) and Cavin *et al*. (1985) the immobilized-cell technology was used for the continuous production of starter cultures or prefermented milks, since cells are released from alginate beads during fermentation. On the other hand, various methods have been proposed to reduce release from alginate beads when the release of cells is undesirable (KLINKENBERG *et al.*, *loc. cit.*).

According to Mohammadi *et al*. (2008) the use of immobilized cells in industrial processes has attracted attention due to advantages such as an increase in yield and cellular stability and a decrease of process expenses due to the ease for cell recovery and reutilization over traditional processes.

Substrate Consumption and Acid Production

Table 2. *P. freudenreichii* viable cell counts at the beginning and at the end of fermentation time in whey permeate.

Culture	Viable cell counts		
	Initial time	Final time [b]	Increment cell values
Free cell system	1.23×10^{10}	1.20×10^{13}	1.20×10^{13}
	CFU/mL		
Immobilized cell system	1.60×10^{11}	1.22×10^{13}	1.20×10^{13}
	CFU/g (wet weight)		
Released cells from beads	6.20×10^{9}	3.00×10^{11}	2.94×10^{11}
	CFU/mL		

b After 113 hours of fermentation

The propionic and acetic acids production in the free cell system were just a few higher than those found for immobilized cells. The rates of 2.29g/L for propionic acid and 0.98g/L for acetic acid (table 3) in free cell system were, respectively, 5.24% and 3.06% higher than the rates in the immobilized cells. However, the volumetric productivity ($g/L.h^{-1}$) was, approximately, the same in both systems ($0.02g/L.h^{-1}$). The volumetric productivity was calculated from the final propionic acid concentration (g/L) divided by the fermentation time (h) (YANG *et al.*, 1994). Probably those few higher acid rates in free cell were due to higher cell counts in free cell system in the total amount of the medium. The ratios of propionic to acetic acids (P/A) were 2.3:1.0 in both systems of free and immobilized cells. The acid yields (table 3) obtained from the free cell system were 0.40g propionic acid/g lactose and 0.15g acetic acid/g lactose. The product yields obtained from the immobilized cell system were 0.11g propionic acid/g lactose and 0.04g acetic acid/g lactose.

The lactose amount in both systems was not exhausted (table 3). The lactose consumption (figure 3) after 113 hours of whey permeate fermentation by free and immobilized cells were 15.42% and 50.90%, respectively. Differences in acid production pattern between both systems can be observed (figures 4 and 5). As it can be seen from the figure 4, at the first 24 hours of fermentation, immobilized cells produced very few acetic, propionic and lactic acids, but the lactose consumption was almost 50%. Probably part of the energy from that catabolism was used for cell growth. After 47 hours of fermentation by immobilized cells the propionic and acetic acids concentrations began to increase, indicating that these acids are produced from the common route for propionibacteria, which is from glucose catabolism to propionic and acetic acids production without involving lactic acid pathway.

At the first 24 hours of fermentation (figure 5), free cells produced very few propionic and acetic acids rates. Nevertheless, some of the lactose has been quickly converted into lactic acid, and after 47 hours of the fermentation the lactic acid concentration decreased, probably because of its conversion to propionic and acetic acids. Probably the lactic acid pathway to propionic acid also provides the energy needs for cell maintenance.

LEWIS and YANG (1992) have been showed that propionibacteria can ferment lactate to propionic acid faster than it ferments lactose. They indicated that since the propionic acid yield is higher from lactate than from lactose, the specific propionic acid production rate with lactate would be even higher than that with lactose. It has been shown that even if propionic and acetic acids are

the two major end-products of lactose fermentation by propionic acid bacteria, other acids could be produced in the medium: lactic acid, succinic, pyruvic, malic and fumaric acids and iso-valeric and formic acids (BOYAVAL and CORRE, 1995; COLOMBAM *et al.*, 1993; CROW, 1988; THIERRY *et al.*, 2004). The fermentation study of *Propionibacterium acidipropionici* by Choi and Mathews (1994) showed that the bacteria, at controlled pH, consumed all of the glucose during the initial stage of fermentation and produced large amounts of lactic acid.

The results indicated that the immobilized cells consumed more lactose, thus reducing residual pollutant load. Besides that, the organic acids production obtained from fermentation of whey permeate by immobilized *Propionibacterium freudenreichii*, when concentrated inocula were used, achieved levels comparable to the levels observed in the free cell system. Choojun and Yoonprayong (2012) suggest that immobilized cells enhanced more propionic acid production and productivity than free cells. Compared to free-cell fermentation, propionic acid productivity increased 20% and fermentation time reduced 11% in fermentor with 40 g/L initial total sugar from whey (CHOOJUN and YOONPRAYONG, 2012).

Table 3. Lactose, organic acids contents and product yields (Y), g propionic acid/g lactose (Yp/l) and g acetic acid/g lactose (Ya/l) in free and immobilized cell systems.

System	Lactose (g/L)	Propionic acid (g/L)	Acetic acid (g/L)	Lactic acid (g/L)	Yields (Y)	
					Y p/l	Ya/l
Free cells	34.00	2.29	0.98	0.05	0.369	0.152
Immobilized cells	19.74	2.17	0.95	0.22	0.106	0.044

Effect of pH on the Growth and on the Acids Production

After autoclave sterilization the medium pH value was 6.20. The figure 6 indicates that pH decreases followed the production of organic acids. Since in the free cell system the organic acids were first produced (figure 5), mainly lactic acid at 50 hours of fermentation, the pH decreases faster followed that acids production. The pH decreases slowly in the immobilized cell system and from 95 hours of fermentation stabilized at pH 4.2, the same pH value remained steady in free cell system. Since the cell counting, by colony-forming units (CFU/mL or g), at the final fermentation time of 113 hours indicated increments, the low pH values in both systems do not seemed to interfere in the cell growth. However, at pH 4.2 the lactose consumption stopped in free and immobilized cell systems, indicating that this pH value is critical to lactose consumption by propionibacteria and, possibly, to organic acids production. Lewis and Yang (1992) showed that the pH variations strongly affect the final acid production by influencing bacterial metabolism. Their study indicated that the bacterial metabolism and fermentation pathway are altered at pH values outside the optimum range, *i.e.*, between 5.5 and 6.5 for *Propionibacterium acidipropionici*.

The results indicated (figures 3 to 6) that even when lactose consumption stopped and the low pH values were steady at 95 hours of fermentation, the production of propionic and acetic acids remained increasing until 113 hours of fermentation of the whey permeate by *Propionibacterium freudenreichii* in both systems of free and immobilized cells.

CONCLUSIONS

Propionibacterium freudenreichii was cultivated in cheese whey permeate at 30ºC, without nutrient supplementation or medium agitation, and without pH adjustment during the processes, which is an economy in energy, material and work force. The results indicated that the immobilized cell system, at the conditions studied in this work, is more effective in propionic acid production when compared to free cell system because the cells can be reutilized. The immobilized cells consumed more lactose, thus reducing residual pollutant load. Besides that, the organic acids production obtained from fermentation of whey permeate by immobilized *Propionibacterium freudenreichii*, when concentrated inocula were used, achieved levels comparable to the levels observed in the free cell system.

ACKNOWLEDGEMENTS

The authors are grateful to Leonardo R. Andrade for Scanning Electron Microscopy (SEM) Analysis, to Instituto de Laticínios Cândido Tostes for whey permeate provided, to Chr. Hansen Ind. Com. Ltda. for Propionibacteria provided and to CAPES; CNPq/PRONEX.

REFERENCES

AOAC.Official Methods of Analysis of the Association of Analytical Chemists, 16th Edn., **Association of Official Analytical Chemist**, Washington, DC. 1997.

BARILEA, D.; TAOB, N.; LEBRILLAB, C. B.; COISSONC, J.-D.; ARLORIOC, M.; GERMAN, J.B.. Permeate from cheese whey ultrafiltration is a source of milk oligosaccharides. **International Dairy Journal. September 1, 19**(9): 524–530, 2009.

BÉGIN, A.; BEAULIEU, Y.; GOULET, J.; CASTAIGNE, F.. Whey Fermentation by *Propionibacterium shermanii* Immobilized in Different Gels. **Milchwissenschaft**. **47**(77), 411- 416, 1992.

BLIGH, E.G.; DYER, W.J. A Rapid Method of Total Lipid Extraction and Purification. **Canadian Journal of Biochemistry and Physiology, 37**(8), 911-917, 1959.

BOYAVAL, P.; CORRE, C. Production of Propionic Acid – Review. **Lait, 75**, 453-461, 1995.

CARDOSO, F.S.; GASPAR, P.; HUGENHOLTZ, J.; RAMOS, A.; SANTOS, H. Enhancement of Trehalose Production in Dairy Propionibacteria through Manipulation of Environmental conditions. **International Journal of Food Microbiology, 91**, 195-204, 2004.

CAVIN, J.F.; SAINT, C.; DIVIES, C.. Continuous Production of Emmenttal Cheese Flavours and Propionic Acid Starters by Immobilized Cells of a Propionic Acid Bacterium. **Biotechnology Letters**. **7**(11), 821-826, 1985.

CHAMPAGNE, C.P.; BAILLARGEON-CÔTÉ, C.; GOULET, J.. Whey Fermentation by Immobilized Cells of *Propionibacterium shermanii.* **Journal of Applied Bacteriology**, **66**(3), 175-184, 1989.

CHOI, C.H.; MATHEWS, A.P. Fermentation Metabolism and Kinetics in the Production of Organic Acids by *Propionibacterium acidipropionici*. **Applied Biochemistry and Biotechnology**, **44**, 271-285, 1994.

CHOOJUN, S.;YOONPRAYONG, P.. Improvement of Propionic Acid Production for Antifungal Activity from Whey by Calcium Alginate Immobilization of *Propionibacterium acidipropionici* TISTR 442. **Journal of Agricultural Science and Technology, *A* 2,** 863-872, 2012.

COLOMBAM, A.; ROGER, L.; BOYAVAL, P. Production of Propionic Acid from Whey Permeate by Sequential Fermentation, Ultrafiltration and Cell Recycling. **Biotechnology and Bioengineering**, **42,** 1091-1093, 1993.

CROW, V. Polysaccharide Production by Propionibacteria during Lactose Fermentation. **Applied and Environmental Microbiology**, July, 1892-1895, 1988.

DEVLIEGHERE, F.; VERMEIREN, L.; DEBEVERE, J. New Preservation Technologies: Possibilities and Limitations. **International Dairy Journal, 14**, 273-285, 2007.

GARDNER, N.; CHAMPAGNE, C.P. Production of *Propionibacterium shermanii* Biomass and Vitamin B_{12} on Spent Media. **Journal of Applied Microbiology, 99**(5), 1236-1245, 2005.

HARRIGAN, W.F.; MC CANCE, M.E. **Laboratory Methods in Food and Dairy Microbiology**. Academic Press Inc., London, 1976. 456 p.

HETTINGA, D.H.; REINBOLD, G.W. The Propionic-Acid Bacteria – A Review I Growth. **Journal of Milk and Food Technology, 35**(5), 295-301, 1972a.

HUGENHOLTZ, J.; HUNIK, J.; SANTOS, H.; SMID, E. Nutraceutical Production by Propionibacteria.. **Lait**, **82**, 103-112, 2002.

JAIN, D.K. ; TYAGI, R.D.; KLUEPFEL, D.; AGBEBAVI, T.J. Production of Propionic Acid from Whey Ultrafiltrate by Immobilized Cells of *Propionibacterium shermanii* in Batch Process. **Process Biochemistry**. **26**, 217-223, 1991.

JAN, G.; BELZACQ; A.-S; HAOUZI, D.; ROUAULT; A. MÉTIVIER, D.; KROEMER, G. *Propionibacteria* Induce Apoptosis of Colorectal Carcinoma Cells Via Short-Chain Fatty Acids Acting on Mitochondria. **Nature**, **9**(2), 179-188, 2002.

KLINKENBERG, G.; LYSTAD, K.Q.; LEVINE, D.W.; DYRSET, N.. Cell Release from Alginate Immobilized *Lactococcus lactis* ssp. *lactis* in Chitosan and Alginate Coated Beads. **Journal Dairy Science, 84,** 1118-1127, 2001.

JAIN, D.K. ; TYAGI, R.D.; KLUEPFEL, D.; AGBEBAVI, T.J. Production of Propionic Acid from Whey Ultrafiltrate by Immobilized Cells of *Propionibacterium shermanii* in Batch Process. **Process Biochemistry**. **26**, 217-223, 1991.

JAN, G.; BELZACQ; A.-S; HAOUZI, D.; ROUAULT; A. MÉTIVIER, D.; KROEMER, G. *Propionibacteria* Induce Apoptosis of Colorectal Carcinoma Cells Via Short-Chain Fatty Acids Acting on Mitochondria. **Nature**, **9**(2), 179-188, 2002.

KLINKENBERG, G.; LYSTAD, K.Q.; LEVINE, D.W.; DYRSET, N.. Cell Release from Alginate Immobilized *Lactococcus lactis* ssp. *lactis* in Chitosan and Alginate Coated Beads. **Journal Dairy Science, 84,** 1118-1127, 2001.

LEWIS, V.P.;YANG, S.T.. Propionic Acid Fermentation by *Propionibacterium acidipropionici* Effect on Growth Substrate. **Applied Microbiology and Biotechnology**, **37**(4), 437-442, 1992.

LIND, H.; JONSSON, H.; SCHNÜRER, J. Antifungal Effect of Dairy Propionibacteria – Contribution of Organic Acids. **International Journal of Food Microbiology**, **98**, 157-165, 2005.

MOHAMMADI, M.; NAJAFPOUR, G.D.; ASADI, M.; RAHIMNEJAD, M.; NAJAFPOUR, S.; PAZOUKI, M. Production of Organic Acids in an Immobilized Cell Reactor Using *Propionibacterium acidi-propionici*. **African Journal of Biotechnology, 7**(18), 3332-3338, 2008.

MORIN, M.; BERNIER-CARDOU, M; CHAMPAGNE, C.P.. Production of Concentrated *Lactococcus lactis* subsp. *cremoris* Suspensions in Calcium Alginate Beads. **Applied Environmental Microbiology, 58**(2), 545-550, 1992.

PAGNO, C.H.; BALDASSO, C.; TESSARO, I.C.; FLORES, S.H.; JONG, E.V. Obtenção de Concentrados Protéicos de Soro de Leite e Caracterização de suas Propriedades Funcionais Tecnológicas. **Alimentos e Nutrição, 20**(2), 231-239, 2009.

PAIK, H.D.; GLATZ, B.A. Propionic Acid Production by Immobilized Cells of a Propionate-Tolerant Strain of *Propionibacterium acidipropionici*. **Applied Microbiology**, **42,** 22-27, 1994.

RICKERT, D.A.; GLATZ, C.E.; GLATZ, B.A. Improved Organic Acid Production by Calcium Alginate-Immobilized Propionibacteria, **Enzyme and Microbial Technology**, **22**, 409-414, 1998.

ROSENBERG, M. Current and Future Applications for Membrane in the Dairy Industry-Review. **Trends in Food Science & Technology**, **6**, 12-19, 1995.

THIERRY, A.; MAILLARD, M.B.; HERVÉ, C.; RICHOUX, R.; LORTAL, S.. Varied Volatile Compounds are Produced by *Propionibacterium freudenreichii* in Emmenthal Cheese. **Food Chemistry, 87**, 439-446, 2004.

YANG, S.T.; SILVA, M.. Novel Products and New Technologies for Use of a Similar Carbohydrate, Milk Lactose. **Journal Dairy Science, 78**, 2541-2562, 1995.

YANG, S-T.; HUANG, Y.; HONG, G. A Novel Recycle Batch Immobilized Cell Bioreactor for Propionate Production from Whey Lactose. **Biotechnology and Bioengineering, 45**, 379-386, 1994.

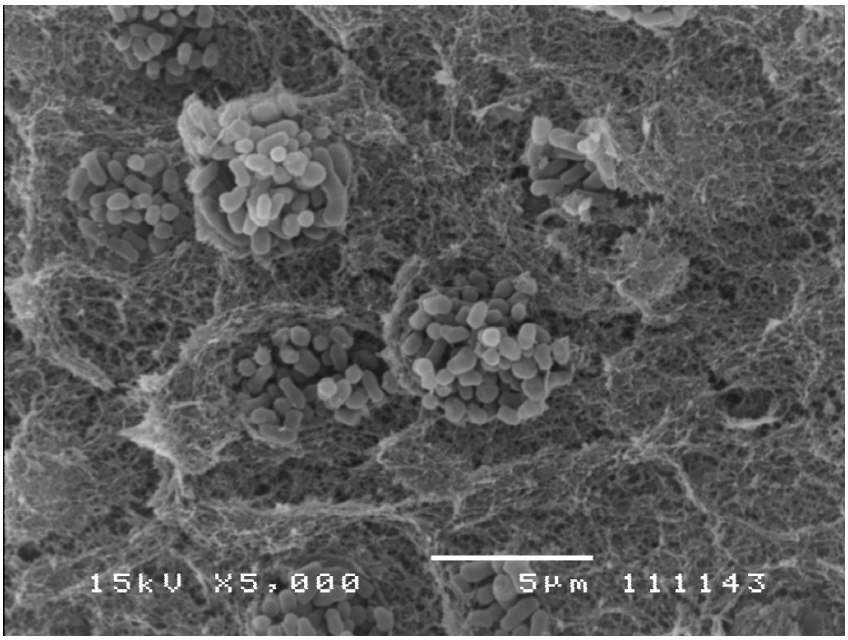

Figure 1. SEM image of *Propionibacterium* colonies immobilized within the matrix of calcium alginate bead.

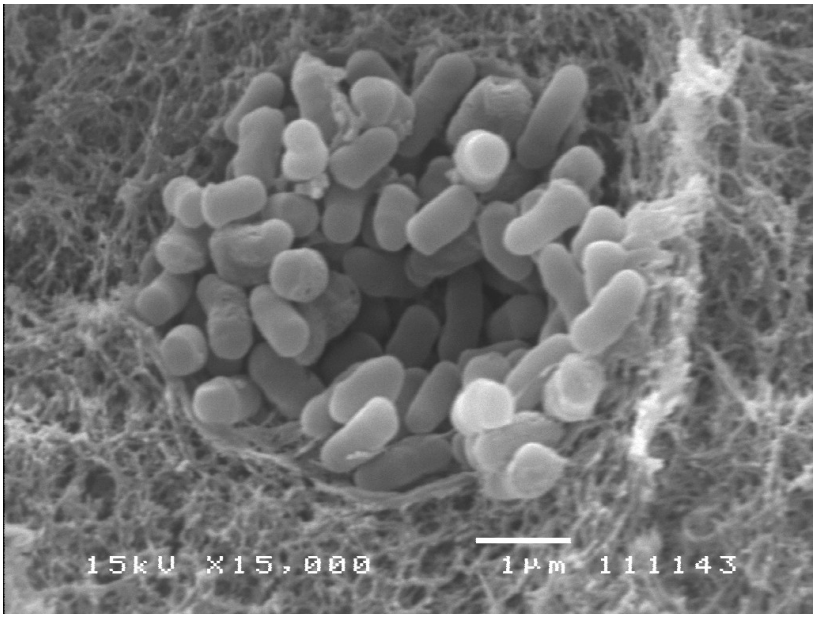

Figure 2. SEM image of entrapped cells colony shows apparently release of certain cells from bead surface.

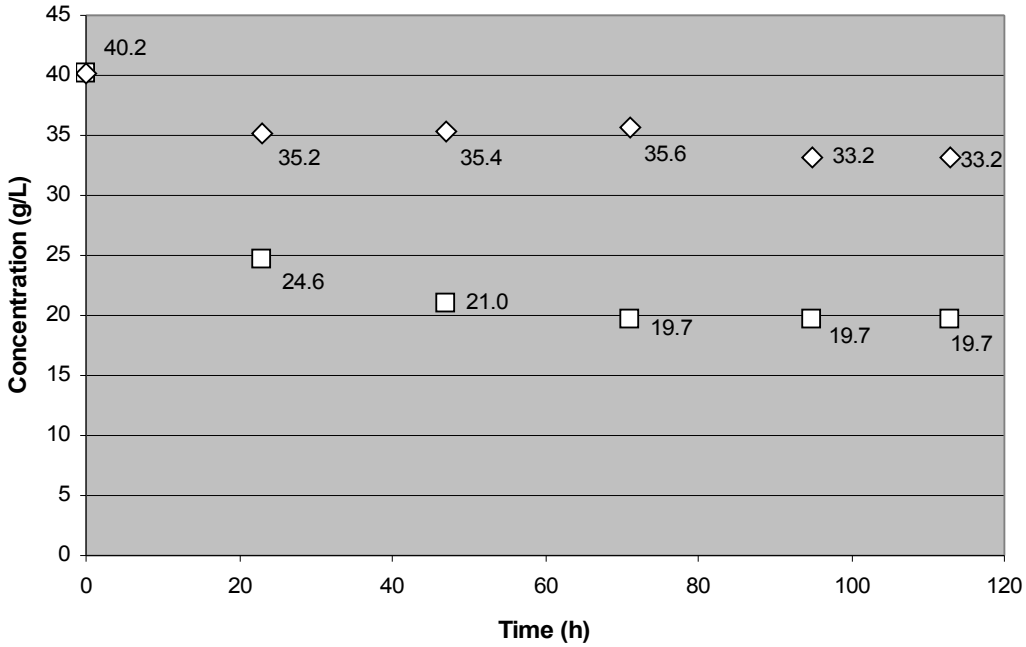

Figure 3. Valuesof the lactose consumption by free (◊) and immobilized (□) cells during 113 hours of whey permeate fermentation.

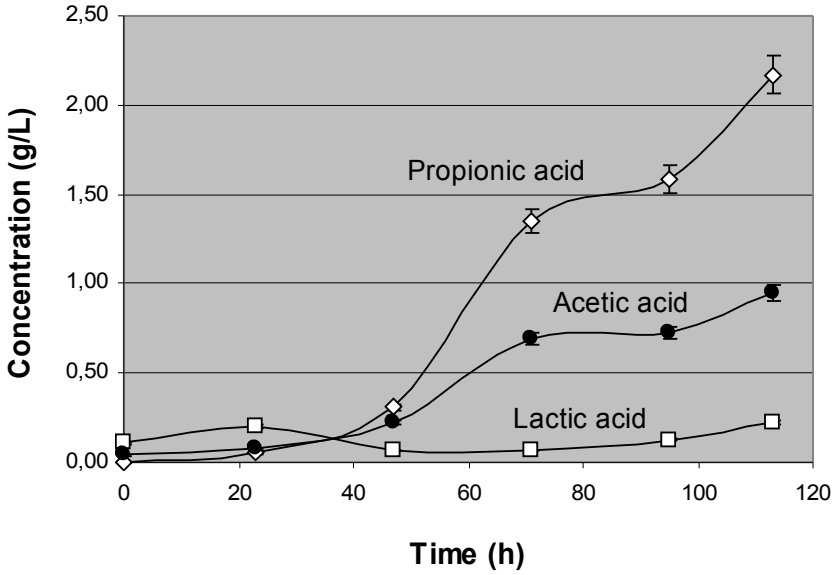

Figure 4. Organic acid productions (□, Lactic acid; ◊, propionic acid and •, acetic acid) by immobilized cell system during 113 hours of fermentation of whey permeate.

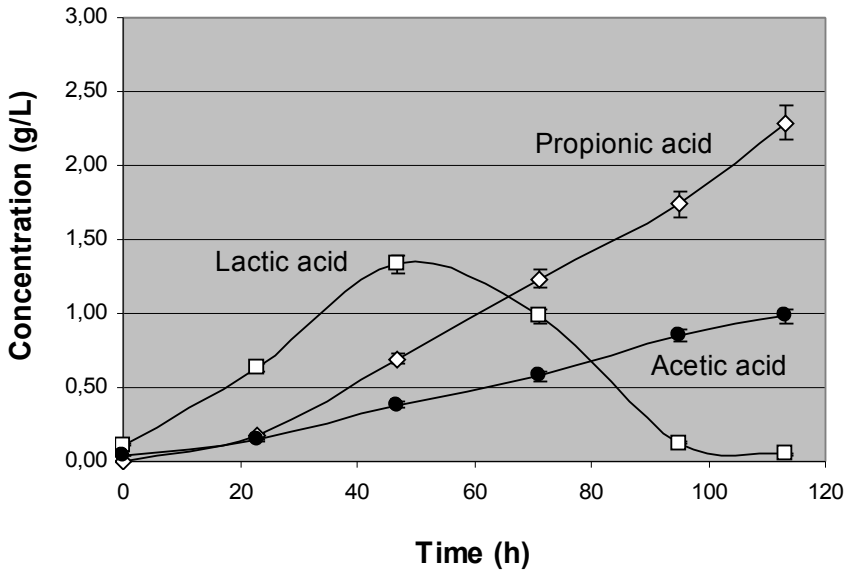

Figure 5. Organic acid productions (□, Lactic acid; ◊, propionic acid and •, acetic acid) by free cell system during 113 hours of whey permeate fermentation.

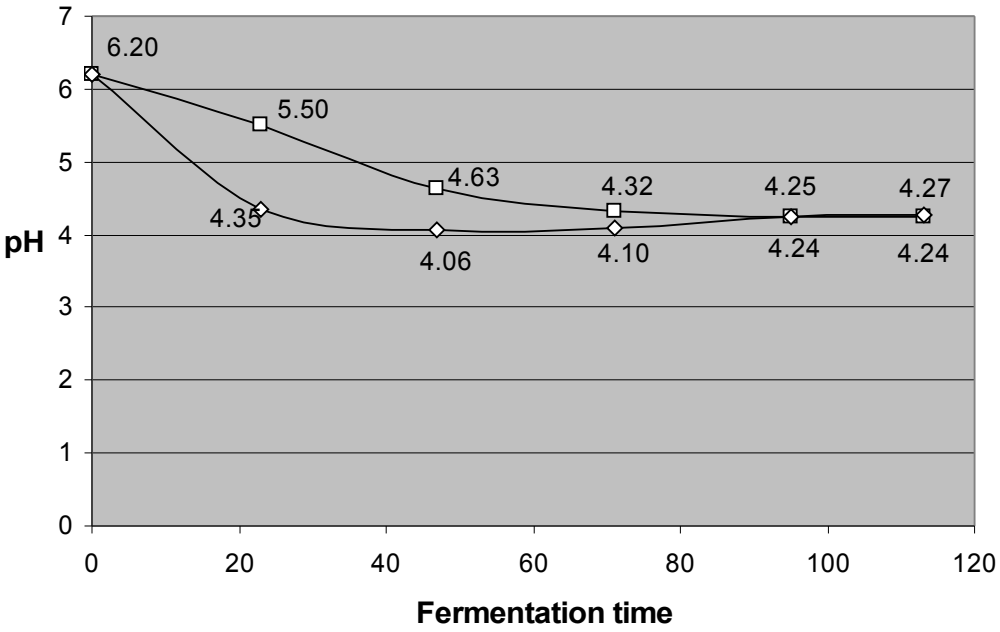

Figure 6. Decrease in pH values during 113 hours of fermentation of whey permeate by free (◊) and immobilized (□) cells.