Journal of Industrial Microbiology & Biotechnology (1998) 20, 166–170

http://www.stockton-press.co.uk/jim

Immobilization of *Bacillus licheniformis* cells, producers of thermostable α-amylase, on polymer membranes

E Dobreva[1], A Tonkova[1], V Ivanova[1], M Stefanova[1], L Kabaivanova[1] and D Spasova[2]

[1]*Department of Extremophilic Bacteria;* [2]*Department of Morphology of Microorganisms and Electron Microscopy, Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria*

Cells of *Bacillus licheniformis* 44MB82-G immobilized on different polymer membranes were used for production of thermostable α-amylase. The α-amylase yields of the membrane-immobilized cells were affected by the reactive chemical groups of the carriers and the spacer size. Formaldehyde-activated polysulphone membranes (PS-FA) were the most suitable for effective immobilization. The highest amylase yield (62% increase of the control) and operational stability (97% residual activity after 480 h repeated batch cultivation) were obtained with this system. This was confirmed by scanning electron micrographs. An additional increase of α-amylase production by PS-FA-membrane immobilized cells was achieved in a fluidized-bed reactor.

Keywords: thermostable α-amylase; cell immobilization; *Bacillus licheniformis*; polymer membranes

Introduction

Among the techniques for immobilization of living cells, entrapment in Ca-alginate or in κ-carrageenan gels is preferred for application in food technologies. This mainly due to the compatibility of the gels, the simplicity of the cell immobilization technique, and the resulting high viability and productivity of immobilized cells. However, to our knowledge there have been few reports on the use of different membranes (natural or synthetic) for bacterial cell immobilization, eg cross-linked chitosan membranes have been used for microencapsulation of lactic acid bacteria [1,3]. These biocatalysts possessed higher stability during high temperature storage and better survival to freeze-drying [1] but the productivity of the immobilized cells was similar to that of the free cells [3]. Recently Poncelet *et al* [7] reported on the formation of polyethyleneimine membranes suitable for cell encapsulation.

High levels of α-amylase production and operational stability of biocatalysts obtained from *Bacillus licheniformis* 44MB82-G cells immobilized on acrylonitrile/acrylamide membranes were reported in our previous papers [5,10]. The purpose of the present investigation was: (i) to study the potential application of other polymer membranes as carriers for immobilization of these cells; and (ii) to compare the ability of the immobilized biocatalysts for production of α-amylase in batch, repeated-batch fermentations and in a fluidized-bed bioreactor to that of free cells.

Materials and methods

Microorganism and media

Cells of *Bacillus licheniformis* strain 44MB82-G, which is resistant to catabolite repression, were used [8,9]. Nutrient broth supplemented with 1% (w/v), soluble starch and 2% (w/v) glucose was used as a seed medium. The fermentation medium contained (in %, w/v): glucose 6, beef extract 'Lab-Lemco' powder (Oxoid, UK) 1.5, peptone (Oxoid) 1.5, K_2HPO_4 1.04, cornsteep liquor 0.66, and $CaCl_2$ 0.11.

Polymer membranes, pre-treatment and activation

The following membranes were used as carriers for immobilization of *B. licheniformis* cells: (i) polysulphone membrane (PS) MF-PS with 260–290 μm thickness and pore diameter 0.43 μm and 0.16 μm; (ii) polyacrylonitrile membrane (PN) UFPN20 with 250 μm thickness and 20 kDa cut-off. Both types of membranes were products of Membrane Technologies Co, Plovdiv, Bulgaria.

The polyacrylonitrile membrane (PN) was subjected to pre-treatment for modification of the nitrile groups into amino groups as follows:

$$\underset{\text{(PN)}}{\text{P-CN}} \xrightarrow[(C_2H_5)_2O]{LiAlH_4} \underset{\text{(PA)}}{\text{P-CH}_2\text{-NH}_2}$$

The reaction was carried out for 12 h at 25°C. Additional treatment of the modified membrane (PA) with 0.2 N HCl and 0.2 N NaOH (60 min, 25°C) was carried out consecutively to eliminate Li^+ and Al^{3+}.

Activation and sterilization of PS-membranes (to PS-FA) were performed by treating with formaldehyde (Merck, 10%, 4 h, 45°C) and then abundantly washing with sterile water and phosphate buffer (0.066 M, pH 6.5).

Activation of PS- or PA-membranes (to PS-GA or PA-GA respectively) was also performed with glutaraldehyde (Fluka, 0.5%, 4 h, 25°C). The treated membranes were washed in the same manner as mentioned above and sterilized by boiling in water (30 min, three times every 24 h).

Correspondence: A Tonkova, Department of Extremophilic Bacteria, Institute of Microbiology, Bulgarian Academy of Sciences, 26 Acad G Bontchev str, 1113 Sofia, Bulgaria
Received 20 March 1997; accepted 8 January 1998

Cell immobilization and growth conditions

The activated membranes were transferred into a seed medium with growing cells and were cultivated overnight at 40°C on a rotary shaker (240 rpm). After 12–17 h the membranes were washed sequentially with sterile water, saline solution and phosphate buffer to eliminate non-immobilized cells. Fermentations were carried out in 500-ml conical flasks at 40°C on a shaker (240 rpm) for 120 h. The total membrane surface area with immobilized cells was 16 cm^2 for 50 ml production medium. As a control, free cell suspension (1%, v/v) was used and operated under the same fermentation conditions.

For repeated batch fermentations, the biocatalysts were washed with sterile water every 120 h and reintroduced into fresh medium. Parallel experiments were also run with free bacterial cells using the previous fermentation for inoculation of the next run.

The fluidized-bed reactor experiments were performed in a bubble column reactor (Figure 1) which was 350 mm tall, 25 mm internal diameter, and had a working volume of 75 ml. The gas was sterile air introduced via a rotameter and a sterile air filter into the nozzle at the bottom of the reactor. The air flow was 1.0 L min^{-1}. The whole reactor with fermentation medium was sterilized at 110°C for 30 min. The membrane surface area with immobilized cells was 7.5 cm^2. The working temperature was maintained at 40°C. Parallel batch experiments were run with immobilized cells in flasks as described above. Batch fermentations with free cells in the bioreactor were operated at the same conditions (inoculum concentration 1%, v/v).

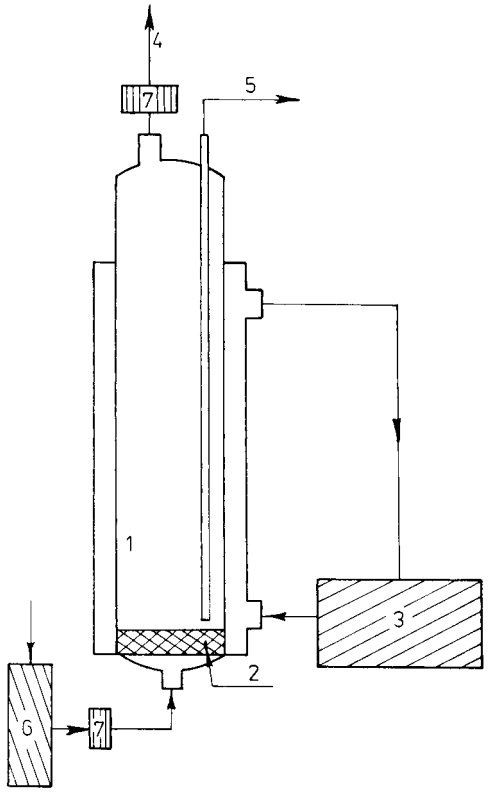

Figure 1 Experimental set-up used for α-amylase production by free and immobilized *Bacillus licheniformis* 44MB82-G cells. 1, Bubble column reactor. 2, Glass filter. 3, Thermostate. 4, Gas outlet. 5, Sample port. 6, Rotameter. 7, Airfilter.

Analytical methods

α-Amylase activity was assayed according to the method of Pantschev *et al* [6]. One unit of enzyme activity was defined as the amount of enzyme that catalysed the hydrolysis of 0.162 mg soluble starch to dextrins per min at 30°C, pH 6.5.

The concentration of free or released cells was determined by the absorbance at 650 nm. The weight of dry cells in the samples was calculated from a standard curve of cell weight *vs* A_{650}. The amount of dry biomass on membranes was measured by determining the total nitrogen of the membranes before and after the immobilization step by the method of Kjeldahl. The dry cell biomass was calculated towards the total nitrogen quantity in 1 g dry cells.

Scanning electron microscopy (SEM)

The SEM of the specimens was performed as previously described [10] on a scanning device attached to a Zeiss electron microscope (model 10C) at 20 kV accelerating voltage with a 5- to 6-nm electron beam.

Results

α-Amylase production by immobilized Bacillus licheniformis 44MB82-G cells on different polymer membranes

α-Amylase yields obtained by immobilized bacterial cells on PS-0.43 μm or PS-0.16 μm membranes treated with formaldehyde are presented in Table 1.

Both PS membranes used proved to be suitable carriers for immobilization of bacterial cells and provided an increase in the thermostable α-amylase yield of 45% (PS-0.16 μm) or 62% (PS-0.43 μm) in comparison to the control.

In order to establish the binding capacity of the PS-membranes, the quantity of immobilized dry biomass per cm^2 of carrier was determined. This parameter, as well as the released cell concentration, was dependent on the membrane pore size. A comparison of both biocatalysts showed that there was a 1.77-fold higher quantity of immobilized cells and 1.3-fold more cells released when membranes with larger pore size were used (PS-0.43 μm). On the other

Table 1 α-Amylase production by free and immobilized cells of *B. licheniformis* 44MB82-G after 120 h batch cultivation

Batch cultivation with	α-Amylase yield (U ml^{-1})	Immobilized cells (mg dry biomass per cm^2 carrier)	Released cells (mg ml^{-1})
Cells immobilized on PS-0.43 μm membranes	2750	2.44	32
Cells immobilized on PS-0.16 μm membranes	2460	1.38	24
Free cells (control)	1700	–	42[a]

[a] Free cells in the culture medium of the control.

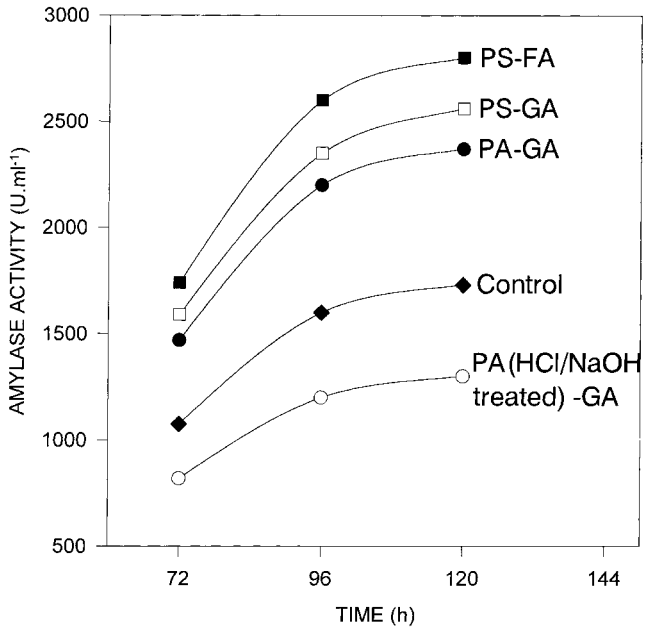

Figure 2 α-Amylase production by batch cultivation of free (control) or *B. licheniformis* 44MB82-G cells immobilized on different membranes. (■) PS-FA-membrane immobilized cells. (□) PS-GA-membrane immobilized cells. (●) PA-GA-membrane immobilized cells. (○) PA(HCl/NaOH treated)-GA-membrane immobilized cells. (◆) Free cells.

hand, the quantity of free cells (control) was 1.3- and 1.75-fold higher compared to the cells released from PS-0.43 μm and PS-0.16 μm biocatalysts, respectively. In further experiments only PS-0.43 μm membranes were used.

Preliminary chemical treatment of the carriers was performed in order to increase their chemical reactivity (as described in Materials & Methods). From the results obtained (Figure 2, Table 2), it was evident that the immobilized bacterial cells on PS-FA, PS-GA and PA-GA-membranes showed an increase of the thermostable α-amylase yields in comparison to control (free cells). This enhancement was from 37% to 62% after 120 h of cultivation. A decrease (with 24%) of the α-amylase production was found only in the case with cells immobilized on PA-membranes preliminarily treated with HCl/NaOH. The highest α-amylase production was achieved by using PS-FA-membranes (162%), moreover the amount of the released cells was the least (32 mg ml^{-1}).

The tendency established with regard to α-amylase production after 120 h of cultivation was confirmed by repeated batch fermentations of the biocatalysts (Table 2). Because of low results, PA-membranes treated with HCl/NaOH were not used for semicontinuous cultivation. The data obtained indicated an especially good perspective for application of PS-FA-membranes for immobilization of *Bacillus licheniformis* 44MB82-G cells. After the 4th cycle of repeated batch cultivation, the biocatalysts of this type showed a 58% increase of α-amylase production compared to the control and a least amount of released cells (14 mg ml^{-1}). It was obvious that the high results obtained were due to the good cell immobilization.

This fact was confirmed by SE micrographs of the immobilized bacterial cells on PS-FA-membranes (Figure 3 a, b, c). In this case immobilized cells were observed on both surfaces of the membranes, in contrast to PS-GA-membranes where the bacterial cells were bound only on the one membrane surface (Figure 3 d, e).

α-Amylase production by immobilized Bacillus licheniformis 44MB82-G cells in a fluidized-bed bioreactor

After 120 h of fermentation in a fluidized-bed reactor (Figure 1), the α-amylase yield of PS-FA-membrane immobilized cells reached 133% of the activity obtained by free cells under the same conditions (Table 3). This activity (4760 U ml^{-1}) was significantly higher than that obtained with immobilized cells in flasks (2800 U ml^{-1}).

Discussion

The kinetic behaviour of immobilized cells compared to free cells may be modified by both the immobilization step and the physico-chemical characteristics of the microen-

Table 2 α-Amylase synthesis by membrane-immobilized cells of *B. licheniformis* 44MB82-G

Batch cultivation with	Pretreatment	After 120 h of cultivation		After 4th cycle of batch cultivation	
		Enzyme activity (% of control)	Released cells (mg ml^{-1})	Enzyme activity (% of control)	Released cells (mg ml^{-1})
Cells immobilized on PS- 0.43 μm membranes	Formaldehyde	162	32	158	14
Cells immobilized on PS-0.43 μm membranes	Glutaraldehyde	148	42	133	16
Cells immobilized on PA-membranes	Glutaraldehnyde	137	65	127	18
Cells immobilized on PA-membranes	Consecutively with HCl/NaOH and glutaraldehyde	76	64	–	–
Free cells (control)	–	100	42[a]	100	22[a]

[a] Free cells in the culture medium of the control.

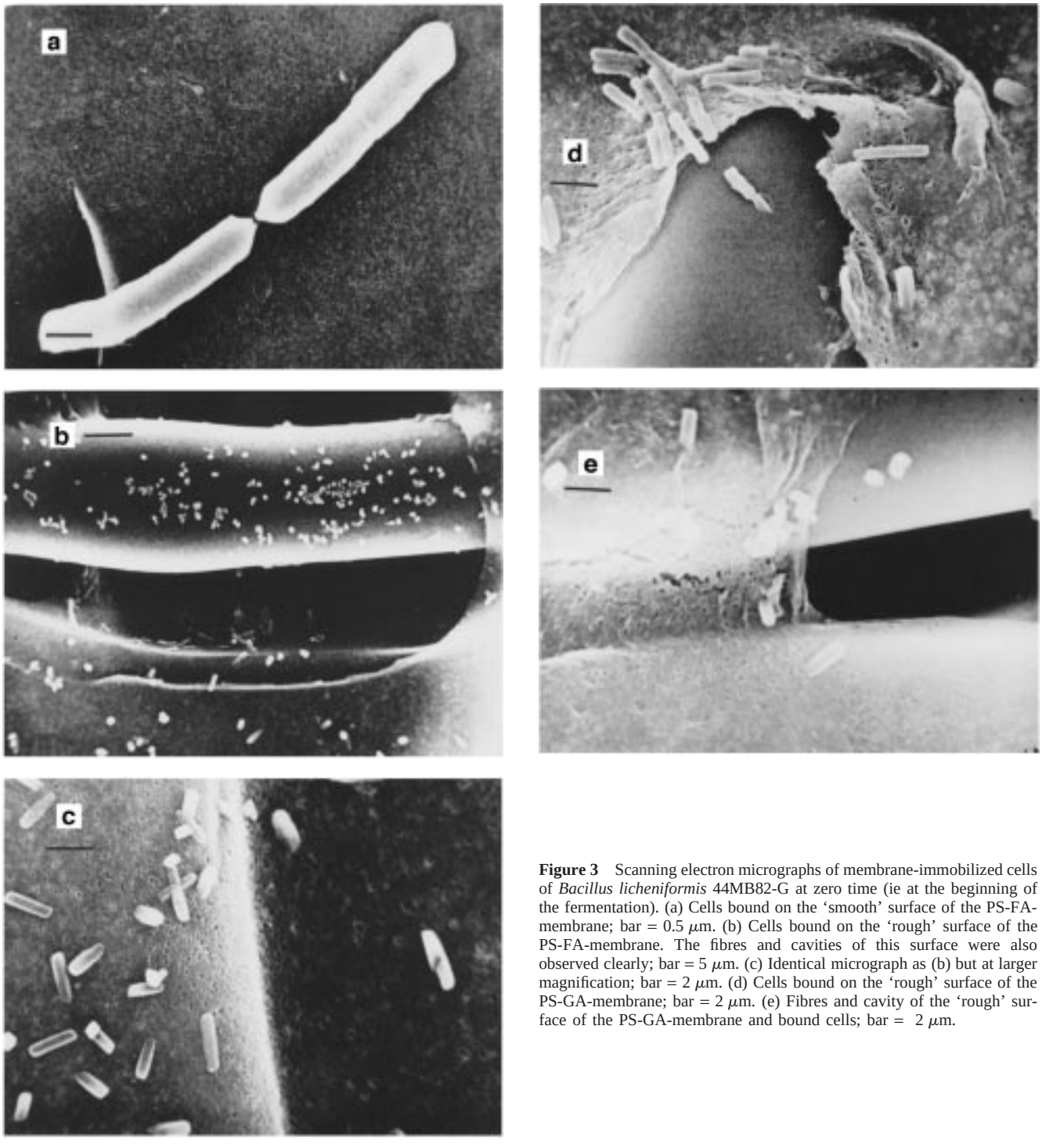

Figure 3 Scanning electron micrographs of membrane-immobilized cells of *Bacillus licheniformis* 44MB82-G at zero time (ie at the beginning of the fermentation). (a) Cells bound on the 'smooth' surface of the PS-FA-membrane; bar = 0.5 μm. (b) Cells bound on the 'rough' surface of the PS-FA-membrane. The fibres and cavities of this surface were also observed clearly; bar = 5 μm. (c) Identical micrograph as (b) but at larger magnification; bar = 2 μm. (d) Cells bound on the 'rough' surface of the PS-GA-membrane; bar = 2 μm. (e) Fibres and cavity of the 'rough' surface of the PS-GA-membrane and bound cells; bar = 2 μm.

vironment of the cells [2]. The results obtained in the present investigation indicated that α-amylase production by membrane-immobilized cells was significantly affected by the type of reactive chemical groups used for immobilization and the size of the spacer molecule. The biocatalysts studied (PS-FA, PS-GA, PA-GA) produced higher amounts of thermostable α-amylase than the free cells (37–62% higher yields after 120 h cultivation). They retained up to about 90–97% of their initial activity after 480 h at repeated batch fermentation. The formaldehyde activation of the polysulphone membranes proved to be the most suitable for effective immobilization and with this biocatalyst (PS-FA) the highest amylase yields and operational stability were obtained. This was confirmed by SEMs which showed that the cells were immobilized on both membrane surfaces. The larger space between the membrane surface and the

Table 3 Production of α-amylase by immobilized *B. licheniformis* 44MB82-G cells in fluidized-bed reactor

Fermentation	α-Amylase activity (U ml^{-1})		
	72 h	96 h	120 h
Immobilized cells in bioreactor	2850	4290	4750
Free cells in bioreactor (control I)	2040	3000	3570
Immobilized cells in flasks (control II)	1740	2600	2800

cells decreased α-amylase yield and operational stability. These results were probably due to the weak cell attachment and were confirmed also by the higher amounts of released cells (Table 2).

The aeration and stirring conditions in the bioreactor are important parameters for cell growth and bioproduction [1]. In the present study the high air flow rate (1.0 L min^{-1}) provided significantly higher amylase production by membrane-immobilized cells in comparison with the activities of free cells (under the same conditions) and those of immobilized cells in flasks. These results were similar to those of Guo *et al* [4] on α-amylase production by carrageenan entrapped *B. subtilis* cells, cultivated in a batch airlift bioreactor.

In conclusion, the high level of amylase production in a fluidized-bed reactor by PS-FA-membrane immobilized cells offers great possibility for large-scale enzyme production.

Acknowledgements

This work was supported by Grant K24 of the National Scientific Foundation, Bulgarian Ministry of Science and Education. Thanks are due to Professor A Dobrev for preparing the PA membranes and to Dr S Popandova for Kjeldahl analysis.

References

1 Champagne CP, C Lacriox and I Sodini-Gallot. 1994. Immobilized cell technologies for the dairy industry. Crit Rev Biotechnol 14: 109–134.
2 Dervakos GA and C Webb. 1991. On the merits of viable cell immobilization. Biotechnol Adv 9: 559–612.
3 Groboillot AF, CP Champagne, GD Darling, D Poncelet and RJ Neufeld. 1993. Membrane formation by interfacial crosslinking of chitosan for microencapsulation of *Lactococcus lactis*. Biotechnol Bioeng 42: 1157–1163.
4 Guo Y, F Lou, Z-Y Peng and Z-Y Yuan. 1990. Kinetics of growth and α-amylase production of immobilized *Bacillus subtilis* in an airlift bioreactor. Biotechnol Bioeng 35: 99–102.
5 Ivanova V, M Stefanova, A Tonkova, E Dobreva and D Spasova. 1995. Screening of a growing cell immobilization procedure for the biosynthesis of thermostable α-amylases. Appl Biochem Biotechnol 50: 305–316.
6 Pantchev C, G Klenz and B Hafner. 1981. Vergleichende Charakterisierung von Alpha-Amylasepraparaten. Lebensmittelindustrie 28: 71–74.
7 Poncelet D, T Alexakis, B Poncelet De Smet and RJ Neufeld. 1994. Microencapsulation within crosslinked polyethyleneimine membranes. J Microencapsulation 11: 31–40.
8 Tonkova A. 1991. Effect of glucose and citrate on α-amylase production in *Bacillus licheniformis*. J Basic Microbiol 31: 217–222.
9 Tonkova A and E Emanuilova. 1989. Method for obtaining a thermostable α-amylase. Bulgarian Patent No. 48513A.
10 Tonkova A, V Ivanova, E Dobreva, M Stefanova and D Spasova. 1994. Thermostable α-amylase production by immobilized *Bacillus licheniformis* cells in agar gel and on acrylonitrile/acrylamide membranes. Appl Microbiol Biotechnol 41: 517–522.