Immobilization of cells by attachment to alginate gels

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Modified Ca-alginate gels (containing gel 0.2 CaO SiO_2, $CaSO_4$, hydroxyethylcellulose) with immobilized yeast *S. cerevisiae* show higher biocatalytical activity in the production of alcohol by about 50 %, as well as better mechanical strength (by 15 %), when used in a reactor with static layer of catalyst. The gel 0.2 CaO · SiO_2 is formed by ionotropic gelation of the colloidal SiO_2 (a component of the alginate dispersion) by calcium ions in the $CaCl_2$ solution. The 0.2 CaO · SiO_2 + $CaSO_4$ gel represents an internal Ca source of the biocatalyst, enhancing thus its stability.

Модифицированные Ca-альгинатовые гели (содержащие гель 0,2 CaO SiO_2, $CaSO_4$, гидроксиэтилцеллюлозу) с иммобилизированными дрожжами *S. cerevisiae* проявляют на 50 % повышенную биокаталитическую активность по продукции спирта, а также обладают лучшей механической прочностью (около 15 %), при применении в реакторе со статическим слоем катализатора. Гель 0,2 CaO · SiO_2 образуется посредством ионотропной желатинизации коллоидного SiO_2 (составной части альгинатной дисперсии) с помощью ионов кальция в растворе $CaCl_2$. Гель 0,2 CaO · SiO_2 + $CaSO_4$ представляет собой внутренний источник Ca для биокатализатора, что приводит к повышению его устойчивости.

Immobilization of cells in alginate gels has become a routinely used research method [1—6]. In spite of that, much is still to be gained by generalization of the trends in the data on the use of gels in biotechnologies. We consider contributions of *Flink* and *Johansen* [3, 4, 7], concerning the influence of more

than 10 parameters on the production of ethanol to be of great significance. These authors examined the role of alginate composition (G/M — guluronic *vs.* mannuronic units), molecular mass, alginate concentration, various gel geometries, enhancement of rigidity, surface/volume ratios, presence of $CaCl_2$ in the substrate, and others.

In the absence of calcium ions in the substrate, calcium alginate is not stable enough. Consequently $CaCl_2$ is routinely added to the fermentation substrates [7, 8].

In this paper we describe an alternative method of gel stabilization by Ca^{2+} cations, built-in in the gel matrix. For that purpose insoluble calcium sulfate and calcium silicate gel were used. The latter was produced directly within the alginate gel by ionotropic gelation in the calcium chloride solution. As a matter of fact, the whole process involved both internal and external gelation of the sodium alginate, combined with the addition of further modifiers.

Experimental

Materials and methods

$CaSO_4$, $CaCl_2$ $2H_2O$, D-glucose (all of anal. grade), Biolatest (all from Lachema, Brno); SiO_2 (colloidal solution of silicon oxide with 20 nm particles, containing 28.0 mass % of SiO_2 and 0.3 mass % of Na_2O) — technical formulation Tosil (Tonaso Neštěmice, CSSR); Na-alginate (Lamitex L-10), a product of Protan A/S, Norway; hydroxyethylcellulose (HEC), type 250 HHR (Hercules BV Holland).

Dry active yeasts Blastosel kappa (Chimici per domini Spa I-Verona, strain *S. cerevisiae* "killer"), as well as *S. cerevisiae* PZ 43 CHTF were used.

D-Glucose concentration was determined by enzyme test set-Biolatest.

Concentration of ethanol was determined by GLC (chromatograph Chrom 4), equipped with a flame ionization detector and 1500 mm × 3 mm column, filled with 5 % of Carbowax 400 on silanized Chromosorb W. Peak integrator Cl 100 was used for quantitative analysis.

Gel beads were formed from droplets of Na-alginate dispersion after introduction into 0.2 M solution of $CaCl_2$. The progress of the process was monitored by chelatometric determination of Ca^{2+} ions in the solution [9].

Viscosity of alginate dispersion was measured with a standard flow viscosimeter [10].

With the instrument, depicted in Fig. 1, gel strength was measured as the force, required to compress a reference set of beads to the half of their original diameter. The diameter was measured by standard optical microscope for a random set of 21 beads.

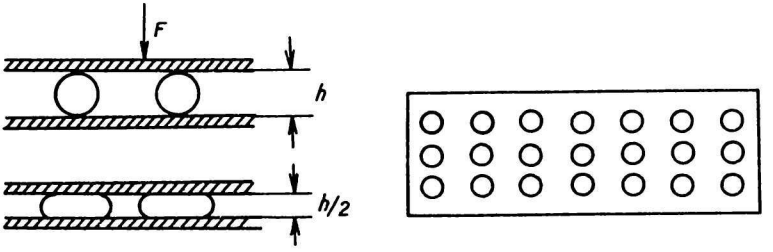

Fig. 1. Experimental arrangement, designed for the measurement of the force F, needed to compress 21 beads to half-height.

Cell immobilization

In all samples the alginate content was kept at either of the three levels — 1.5, 2.0, and 2.5 mass %. Chemical and mechanical stability of the gel was increased by Ca^{2+} ions contained in additives — calcium sulfate, hydroxyethylcellulose, and colloidal silicon oxide (Table 1). Hydroxyethylcellulose, mixed with the colloidal silicon oxide and Ca-alginate, forms a gel. In the $CaCl_2$ solution colloidal SiO_2 particles bind calcium and form a calcium silicate gel — another source of calcium, necessary for the stabilization of the alginate gel. Relative content of additives from Table 1 was kept constant, while their total amount was adjusted to three levels — a basic A level, double B and triple C level (Table 2).

Table 1

Additives serving as internal Ca^{2+} sources in alginate gels

	w(component)/%			
	Tosil	SiO_2	HEC	$CaSO_4$
Dispersion	98.48	—	0.77	0.75
Dried beads	—	94.0	2.67	2.55

Table 2

Distribution of additives content

Level	Total content	w(component)/%		
	mass %	SiO_2	HEC	$CaSO_4$
A	1.4	1.33	3.74×10^{-2}	3.57×10^{-2}
B	2.8	2.66	7.48×10^{-2}	7.14×10^{-2}
C	4.2	3.99	1.12×10^{-1}	1.07×10^{-1}

The added components were too reactive to be mixed together in the dispersion and were therefore applied as two dispersions, to be mixed immediately before gelation. The first dispersion thus contains Na-alginate, microorganism cells, and HEC. This is then admixed to the colloidal solution of SiO_2 and $CaSO_4$ and the whole mixture is blown off of the tip of a capillary with 0.3 mm bore at the rate of 500 $cm^3 h^{-1}$. Since the dispersion droplets are generated at the tip of a capillary, their size is not sensitive to viscosity ($\eta = 1$ —47 mPa s). Mean value of the diameter of beads, with the immobilized yeast *S. cerevisiae* "killer" was 3.56 ± 0.23 mm, that of beads with immobilized *S. cerevisiae* PZ 43 CHTF was 3.50 ± 0.38 mm.

Fermentation in batch and flow reactor

Fermentation in a batch reactor was used to test the activity of both immobilized and free cells. For all samples identical conditions were maintained during the fermentation (28 °C, 250 cm^3 of 10 mass % D-glucose solution, pH = 4.5, cell count 2.1×10^9, slow shaking). After 28 h fermentation ethanol content was determined. Measured by such yardstick, the activity of immobilized cells almost reached that of the free yeast cells (88.1 to 94.7 % of the free cell activity).

The flow reactor consisted of a vertically oriented 28 cm long glass tube with 3.5 cm bore, in the axis of which there was another (1.5 cm bore) brass tube, serving as a CO_2 vent. Such construction was found optimal for preventing clogging in the upper part of the reactor.

The substrate (10 mass % D-glucose solution, pH = 4.5) was continually delivered (86 $cm^3 h^{-1}$) by a micropump to the bottom of the reactor, filled to 15 cm height. At regular intervals concentration of ethanol was determined at the output of the reactor. Productivity of the bioreactor was in all experiments calculated from the formula

$$P_r = \frac{X \; S_0 \; \dot{V}}{V_R \; m}$$

where $X = (S_0 - S)/S_0$ is the substrate conversion, S_0 the starting mass concentration of the substrate (kg m^{-3}), $\dot{V}$ volume flow rate ($m^3 h^{-1}$), V_R active reactor volume (m^3), and m mass of immobilized yeasts in grams.

On the average, the system needed 15 h to reach the steady state and was further operated for another 100 h, maintaining the constant productivity. Steady-state production values, given in Table 3 were calculated from the formula

$$P_r = \frac{p}{p_0} \cdot 100$$

where p_0 represents the productivity of the reference experiment, *i.e.* 53.7 kg of ethanol from a cubic meter of reactor space in an hour, produced by a gram of yeast cells.

Table 3

Productivity of the flow reactor as a function of the gel composition

Sample	w(Alginate)/%	Additives	Relative productivity/%	
			I[a]	II[a]
26; *25*	2.5	—	100.0[b]	87.3
28; *27*	2.0	—	127.2	107.4
30; *29*	1.5	—	129.3	119.5
32; *31*	1.5	A	200.2	180.3
35; *33*	1.5	B	164.2	160.2
— *34*	1.5	C	—	125.3

a) I — data for the strain *S. cerevisiae* PZ 43 CHTF; II — data for the strain *S. cerevisiae* "killer" (Blastosel kappa).

b) Productivity of the reference experiment.

Results and discussion

Microporous beads of Ca-alginate gels were produced from the dispersion of Na-alginate, containing yeasts and additives, by dropping the mixture to the stirred aqueous solution of $CaCl_2$. The progress of the reaction was monitored by measuring the decrease in Ca^{2+} content. It has been found that the Ca^{2+} content decreased both on account of the above reaction with the Na-alginate and with the colloidal SiO_2.

Results given in Fig. 2 and Table 4 indicate that the reaction of alginate dispersion with calcium ions was very fast, in fact the reaction was practically over during the very creation of beads in $CaCl_2$ solution. Thus the content of Ca^{2+} ions in the solution, containing gel beads, remains constant during the whole monitoring. After the beads with 3.5 mm diameter were dissected, one could see no thin gel film at the surface. Independent experiments have shown that colloidal SiO_2 always reacted with Ca^{2+} ions in a fast reaction, taking place at the surface of gel beads [11] and governed by constant stoichiometry. Hence the ratio of Ca^{2+} (in the form of CaO) uptake by gels with various SiO_2 content and of SiO_2 content itself remained constant (the last column in Table 4). The reaction of Na-alginate dispersion containing SiO_2 with the stirred $CaCl_2$ solution results in the formation of a homogeneous Ca-alginate matrix, in which the gel of calcium silicate plays the role of internal Ca source. This greatly contributes to the stability of Ca-alginate gels.

Properties of alginate dispersions and gel beads are summarized in Tables 5 and 6. Viscosity and strength of gel beads increase somewhat with the increasing alginate content. Additives (SiO_2 particles, HEC, $CaSO_4$) on the other hand, had a marked influence on their viscosity, so much that already the addition of 4.2 mass % of additives (Table 5) drove the viscosity of gel beads to 47.1 mPa s, the upper limit of processability on our equipment.

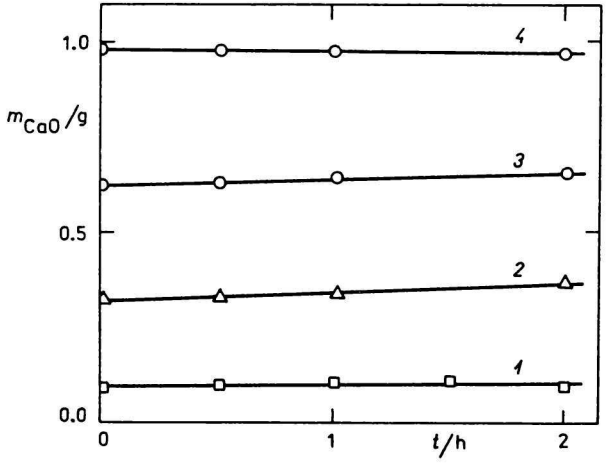

Fig. 2. The amount of calcium, represented as CaO, consumed in the reaction with alginate (*1*), or silica alginate gel (*2—4*), plotted against time for 100 g of gel (Table 3) in 200 g of the 0.2 M-$CaCl_2$ solution.

1. Without SiO_2; *2.* 1.33 mass % of SiO_2; *3.* 2.66 mass % of SiO_2; *4.* 3.99 mass % of SiO_2.

All produced gel beads had a diameter of approximately 3.5 mm, irrespective of the properties of the dispersion. By contrast, properties of the starting dispersions did significantly influence the mechanical properties of beads. This is important, since gel beads, in order to be filled in flow reactors, must have

Table 4

Dependence of the amount of chemically bound calcium (ΔCaO), expressed as mass of CaO per 100 g of silica gel, on the content of SiO_2 in gel. The gel contained 9 mass % of yeast *S. cerevisiae* "killer" (Blastosel kappa)

$\frac{m(SiO_2)}{g}$	$\frac{m(CaO)}{g}$	$\frac{m(\Delta CaO)}{g}$	$\frac{SiO_2}{\Delta CaO}$	$xCaO \cdot SiO_2$
—	0.1	—	—	—
1.33	0.36	0.26	4.77	0.21 CaO · SiO_2
2.66	0.65	0.55	4.51	0.22 CaO SiO_2
3.99	0.97	0.87	4.28	0.23 CaO · SiO_2

Table 5

Properties of gel beads, containing immobilized yeast cells of *S. cerevisiae* "killer" (Blastosel kappa, 9 mass %, 3.53×10^9 cells per 1 g of gel). Viscosity of the dispersion measured immediately after mixing the components

Sample	w(Alginate) / %	Additives	Viscosity / mPa s	Diameter / mm	Mechanical strength F/N
24	1.2	—	1.1	3.36 ± 0.19	1.30
29	1.5		1.6	3.37 ± 0.24	2.07
27	2.0	—	2.4	3.45 ± 0.16	2.11
25	2.5		4.6	3.76 ± 0.25	2.21
31	1.5	A	5.4	3.77 ± 0.17	2.28
33	1.5	B	8.5	3.60 ± 0.38	2.59
34	1.5	C	47.1	3.60 ± 0.24	2.90

Table 6

Properties of gel beads with immobilized yeast cells *S. cerevisiae* PZ 43 CHTF (9 mass %, 3.53×10^9 cells per 1 g of gel). Viscosity of the dispersion measured immediately after mixing the components

Sample	w(Alginate) / %	Additives	Viscosity / mPa s	Diameter / mm	Mechanical strength F/N
30	1.5	—	1.2	3.55 ± 0.16	2.00
28	2.0	—	1.5	3.48 ± 0.22	2.11
26	2.5	—	3.5	3.36 ± 0.75	2.21
32	1.5	A	3.1	3.75 ± 0.23	2.30
35	1.5	B	5.8	3.35 ± 0.54	2.55

certain minimal mechanical strength. Thus in reactors with static filling beads with mechanical strength lower than 1.3 N (as determined by our method) are impractical to use, since they impede the flow greatly, sometimes even clogg the reactor entirely.

Productivity of the reactor with gels without additives was inversely proportional to the alginate content in the gel, in accord with the report from the literature [7]. If an additive (colloidal SiO_2, $CaSO_4$, and hydroxyethylcellulose (Table 3)) was present, the productivity increased dramatically. Maximal productivity has been achieved with 1.5 mass % of alginate, containing 1.4 mass % of the additive. The sample No. *32* has the productivity by 54.8 % higher than the sample *30*, *i.e.* a sample without the additive. Still higher contents of additives lowered the reactor productivity.

Conclusion

Two yeast strains *S. cerevisiae* were immobilized on alginate gels, which contained calcium silicate gel (0.2 CaO SiO_2), hydroxyethylcellulose, and calcium sulfate. The calcium silicate gel was formed by external gelation of alginate beads in $CaCl_2$ solution as a consequence of the interaction of colloid SiO_2 particles with Ca^{2+} ions. Calcium sulfate acted thus as an internal gelation agent and in lieu with the calcium silicate gel also as an internal, stabilizing source of calcium. The reaction of sodium alginate as well as that of colloid particles SiO_2 (3.5 mm diameter) with Ca^{2+} ions (in 0.2 M-$CaCl_2$ solution) was very fast — it was practically over within a minute.

In conclusion we may state that the use of additives in gels brings double benefit, making the biocatalyst both mechanically stronger (by about 15 %) and more than by 50 % more productive.

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