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THE PRODUCTION OF ETHANOL BY SACCHAROMYCES CEREVISIAE IMMOBILIZED IN POLYCATION-STABILIZED CALCIUM ALGINATE GELS

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SUMMARY

Polycation treatment of preformed calcium alginate beads produced a matrix with higher resistance to phosphate ions. The treatment of immobilized *Saccharomyces cerevisiae* in the calcium alginate beads inhibited respiration of the entrapped cells but did not reduce ethanol production.

INTRODUCTION

Yeasts have been immobilized in various ways — by absorption, entrapment and coupling to supports (Kolot, 1980). Among the methods used for entrapment gels formed from calcium alginate have been demonstrated to be easily prepared and to leave the cells in an active state (Cheetham *et al.*, 1979; Kiersten and Bucke, 1977; Larsson and Mosbach, 1979; Takata *et al.*, 1977; White and Portno, 1978). These gels, however, suffer from instability in phosphate-containing media. Recently we observed that treatment of preformed calcium alginate gels (beaded-form) with higher molecular weight cationic polymers led to a surface-coated material possessing improved stability in the presence of phosphate, as one of the essential components of many biological systems. In this communication the results obtained with surface-coated calcium alginate beads with and without immobilized yeast cells will be described.

METHODS AND MATERIALS

Polyethyleneimine (PEI) was either the Dow product PEI-600 or the Cordova Chemical (North Muskegeon, Michigan) product, Corcat P-600.

Polypropyleneimine hydrobromide was prepared by condensation polymerization of 1,3 propanediamine and

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1,3 dibromopropane in refluxing methanol solution. The product was isolated by cooling and evaporating the solvent, followed by washing with methanol and drying the residue.

Polyvinylamine hydrochloride (MW -10^5) was obtained from Dynapol Corp (Palo Alto, California) (Dawson *et al.*, 1976).

All solutions of the cationic polymers were prepared by dissolving the polymer in water and adjusting the pH with either HCl or NaOH.

Sodium alginate was obtained either from Sigma Chemical (St. Louis, Mo.) or from Kelco (Kelcogel HV) (Rahway, N.J.). Calcium alginate beads were prepared by dropping solutions of sodium alginate (1-4% w/v) into $CaCl_2$ 50 mM, pH 6-8). The beads were cured 2 hours in the 50 mM $CaCl_2$ solution and washed with water followed by 20 mM $CaCl_2$ (pH 6-8) before use. All beads used had diameters of 3.0 ± 0.2 mm (average of 10 beads).

Polymer-treated beads were prepared by stirring preformed beads in aqueous solutions (polymer concentration 0.3% w/v, pH 6-8) for a given period of time and subsequently rinsed with water and 20 mM $CaCl_2$ (pH 6-8) to remove excess polymer.

Yeast (Saccharomyces cerevisiae NRCC #202036) grown on YM broth (Difco Laboratories, Detroit, Michigan, USA) was harvested by centrifugation after 18 hours at 37°C. After harvesting, 25 gm wet weight was resuspended in sterile tap water and mixed with 50 ml of 4% w/v sodium alginate (Sigma). The resulting suspension was passed through a narrow tube and dropped into $CaCl_2$ solution (50 mM, pH 6-8). Beads of 2.8-3.0 mm diameter were thus obtained. After formation the beads were cured as noted above.

Ethanol concentrations were measured using gas chromatography (Hewlett-Packard Model 5730A, column conditions: Chromosorb 102, 80-100 mesh, 6 ft × $\frac{1}{4}$ in stainless steel, 150°C, carrier gas: N_2, flow 60 ml/min, FID detector. Ethanol production yields were measured in solutions containing 10% w/v glucose. Respiration rates were tested with a YSI Oxygen meter equipped with an O_2 probe (Clark electrode) and coupled to a chart recorder. All respiration measurements were made in 1% w/v glucose solution (4 ml) using 5 beads. The number of beads was set by the activity of the yeast preparation and the meter requirements. No significant variation in the measured respiration rates when three or more sets of 5 beads were compared was noted.

RESULTS AND DISCUSSION

Prolonged treatment of preformed calcium alginate beads with cationic polymers, e.g. amine-containing polymers such as polyethyleneimine (PEI), leads to the formation of a surface-coated bead and a stabilization of the matrix when in the presence of phosphate ions (Table 1). Of the various cationic polymers tested previously (i.e. polyethyleneimine (PEI), polypropyleneimine (PPI), DEAE-dextran, polyvinylamine (PVAmine), cationic starches) (Kielland and Williams, 1980), the most effective ones were those containing a high charge density primarily composed of secondary and tertiary bases,

Table 1

Stability of PEI-Treated and Untreated Calcium Alginate Beads in Phosphate[a]

Treatment Procedure	Test Condition[b]
untreated	completely disrupted in 90 min
treated with 0.3% w/v PEI, 24 hours at room temperature	stable for >24 hours

[a] Preformed beads were prepared by dropping Na alginate (1% solution Kelcogel HVF) into 0.1 M $CaCl_2$ (pH 6-8) and curing 30 min. Beads were water-rinsed before use or treatment.

[b] Beads (10) were placed in test solution (10 ml) and incubated with occasional shaking. Test solution: 0.1 M Na_2HPO_4, pH 8, room temperature (approx. 20°C).

i.e. PEI and PPI. As indicated by combustion analysis of the dried beads the treatment caused an increase in the nitrogen content of the beads (0.0 ± 0.2% → 20.8 ± 1.0%) and a concomitent loss of calcium (10.0 ± 0.2% → 5.0 ± 0.5%). Increasing the concentration of the cationic polymer in solution caused a shrinkage of the bead (Fig. 1) and further loss of calcium.

As a test of the effectiveness of this process for stabilizing calcium alginate beads containing biological systems cells of Saccharomyces cerevisiae were incorporated into the calcium alginate gel as previously described (Kiersten and Bucke, 1977; Larsson and Mosbach, 1979). To circumvent damage to the biological system by prolonged treatment with the polycationic materials a series of short treatment times were carried out with PEI (0.3% w/v, pH 7) and their immediate effect on the respiration rates of the immobilized yeast was measured. These experiments indicated that treatment times longer than 10 min led to a reduction in the respiration rates of the entrapped yeast (Veliky and Breitkreutz, 1980).

Various polycationic materials were next tested for their effect on respiration, ethanol production and stabilization of the matrix against phosphate damage. Polyethyleneimine (PEI) polypropyleneimine (PPI) and polyvinylamine (PVAmine)- treated beads were incubated in 10% glucose solution and respiration activity and ethanol production was measured. The respiration activity of PEI- and PPI-treated beads decreased after 68 hours by 46.6% and 37.5% respectively when compared with the initial activity taken at 3 hours (adaptation time). The respiratory activity of the control

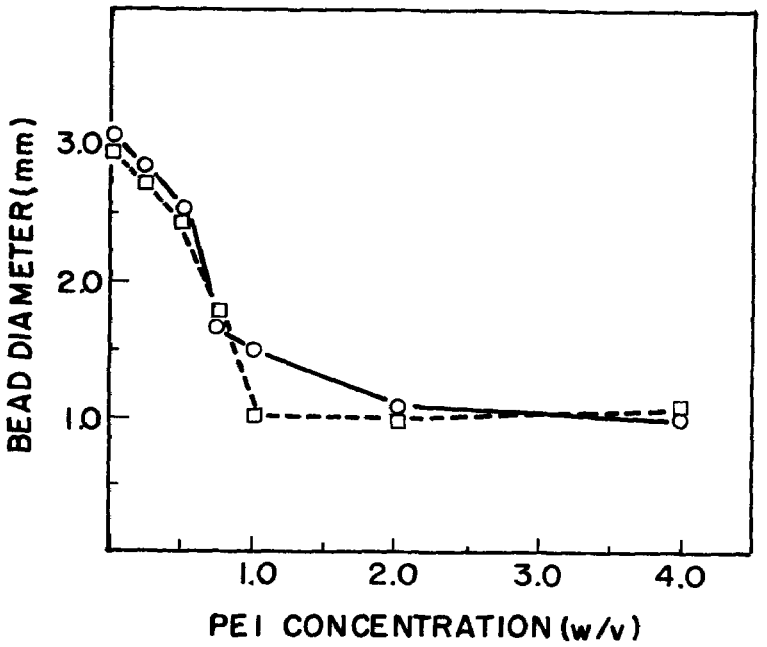

Fig. 1. Bead Shrinkage caused by Increasing Concentrations of Polyethyleneimine (PEI). Preformed Ca-alginate beads (10) were incubated with various aqueous solutions of PEI (Dow PEI-600) pH 8 (10 ml). Bead diameters were measured and averaged after 120 min (O-O) and 24 hours (□ - □) at room temperature. Averaged diameters (10 beads) have an error of 0.2 mm.

Table 2

Effect of Polycation Treatment on the Ethanol Production Rates of Immobilized Saccharomyces cerevisiae[a]

Treatment Procedure[b]	Ethanol Yield[c] 27 hrs	44 hrs	68 hrs
untreated	36.6	60.3	77.3
PEI	37.6	55.5	72.7
PPI	40.7	59.6	77.5
PVAmine	42.5	63.7	79.7

[a]Saccharomyces cerevisiae NRCC #202036 was immobilized in Ca-alginate gel. Bead incubation done in 10% glucose medium. Ethanol production detected by gas chromatography.

[b]Treatment procedure; polycation concentration: 0.3% w/v, pH 6, 10 min exposure, sterile H_2O wash to remove excess polycation.

[c]Percent of theoretical yield.

(untreated) beads and PVAmine-treated beads fell by only 12.9% during the same period. There was no indication that the 10 min treatment had any significant effect on the breakdown of glucose to ethanol since the yield of ethanol (% of theoretical) remained almost unchanged after polycation treatment (Table 2). The inhibition of respiration caused by longer PEI treatments was also detected on ethanol production. PEI treatment (0.3% w/v) for a period of 30 minutes inhibited initial respiration by 15%. After 145 hours the respiration rate of the treated beads had decreased by 60% when compared with the untreated beads. However the time course of ethanol production during the same period indicated inhibition in the PEI treated beads but at much lower degree than that detected by respiration (Fig. 2).

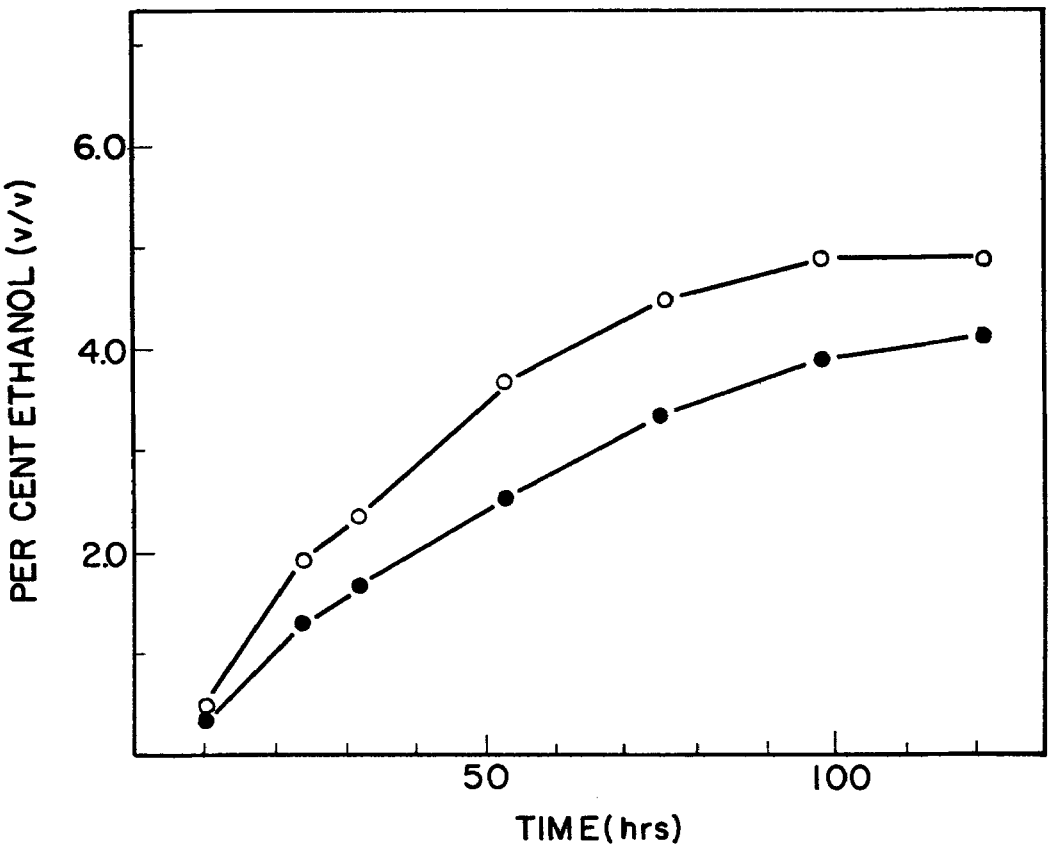

Fig. 2. Time course of ethanol concentration (v/v) during fermentation. Untreated beads (o——o) and PEI treated (0.3% w/v; 30 min.) beads (●——●). Fermentation Medium: 10% w/v glucose, $CaCl_2 \cdot 2H_2O$ 2.49 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, trace elements.

Lowered ethanol yields were also noted between PEI treated (0.3% w/v, 10 min) and untreated beads when Saccharomyces cerevisiae-containing beads were incubated in concentrations of glucose greater than 15% w/v (eg. 20% w/v glucose, 120 hrs, ethanol production 70.0% of theoretical for untreated beads vs 63.9% for treated beads).

Finally, the 10 min polycation treatment also improved the phosphate ion stability of the calcium alginate beads containing the yeast. Treatment of the beads with PEI or PPI led to beads which were stable to 0.1 M K_2HPO_4/KH_2PO_4 (pH 7.5) buffer for approximately one hour (magnetic stirrer) while the PVAmine-treated beads and the control beads were completely disrupted within 15 min.

Further experiments on polycation stabilization of alginate matrix are in progress.

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