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Immobilization of Growing Cells by Polyethyleneimine-Modified Alginate

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ABSTRACT

A unique polymer matrix that is suitable for immobilizing growing cells has been developed. Alginate was chemically modified with polyethyleneimine (PEI), and the resultant polymer aggregate was evaluated as a cell carrier. Our method of immobilization depends on reversible gelation of the PEI-modified alginate. Our hypothesis is that immobilized cells grow by dissolving the surrounding gel matrix; the dissolved polymer adduct is displaced peripherally and gelled again by the influx of calcium ion from the surrounding fermentation broth, retaining both cells and carrier polymer in the gel beads. Thus, the immobilized cells gain space for growth by expanding the carrier matrix. The PEI modification offers the following advantages: (1) improved mechanical strength; (2) improved cell retention; (3) increased catalyst life; (4) ease of pelletization; and (5) an apparent bacteriostatic capability.

When immobilized yeast cells were applied to a continuous ethanol fermentation, 94% theoretical conversion of glucose to ethanol was observed, with a reactor productivity of 15–30 g/L/h in a nonsterile reactor. A 3-mo catalyst life and minimal cell washout were observed.

Index Entries: Biocatalysis; cell immobilization; ethanol fermentation; polyethyleneimine-alginate; yeast.

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INTRODUCTION

Cell immobilization can improve handling characteristics of an industrial microorganism and increase the volumetric productivity of a bioreactor.

For an early example of live-cell immobilization, Mosbach and Larrson (*1*) prepared entrapped cells of *Curvularia lunata* in polyacrylamide gels and successfully used the gel-cell granules for hydroxylation of a steroid. Slowinski and Charm (*2*) employed gel-entrapped *Corynebacterium glutamicum* for the fermentation of glutamic acid and obtained a higher productivity compared to free cells, demonstrating the possibility of using the biocatalyst in a continuous process. Chibata et al. (*3*) and Tosa et al. (*4*) immobilized *Escherichia coli* cells in polyacrylamide gels and autolyzed the immobilized cells to obtain a highly active form of aspartase. The enzyme was stable for more than 40 d in a continuous column reactor.

Although polyacrylamide gels are suitable for immobilizing nonviable cells or resting cells, they are not suitable for immobilizing growing cells because of the lack of expansibility of the gels and the toxicity of the crosslinking agent, *N,N'*-methylene bisacrylamide. More recently, marine hydrogels, especially alginate and k-carrageenan, have been used to immobilize live cells (*5,6*). When *Saccharomyces cerevisiae* cells were immobilized in these gels, a high ethanol productivity exceeding 40 g/L/h was obtained (*7,8*). Samejima and his coworkers (*9*) recently showed possible economic advantages of an immobilized cell process over the conventional processes by obtaining a higher reactor productivity and ethanol yield in a 4000-L pilot plant.

Although alginate gels showed improvements over polyacrylamide gels as a cell carrier for growing cells, we recognized that the expansibility of the alginate gels does not seem adequate to support continued growth of cells. High expansibility is necessary to stabilize the activity of a biocatalyst by allowing continued growth of the immobilized cells for a prolonged period. Also, the susceptibility of calcium alginate to dissolution by the medium components and immobilized cells themselves results in severe loss of both cells and carrier material during ethanol fermentation.

The use of a polyethyleneimine-alginate (PEI-alginate) adduct for cell immobilization by Joung et al. (*10*), eliminated the shortcomings of sodium alginate by greatly increasing the expansibility of the resultant yeast beads. When yeast beads prepared with the new carrier were applied to ethanol fermentation, the biocatalyst expanded to 1000% of its original volume, permitting a high degree of retention of cells and carrier polymer. The yeast beads were active and stable for more than 3 mo under the harsh environment of rapid fermentation. The continuous fermentation with yeast beads yielded 94% theoretical conversion of ethanol from glucose, with a reactor productivity of 15–30 g/L/h. Furthermore,

the biocatalyst did not require a sterile system; contamination problems were avoided by simply maintaining the fermenter pH at 3.5.

METHODS

Dehydrated Baker's yeast was obtained from Universal Foods Corporation, Milwaukee, Wisconsin. Polyethyleneimine (M.W. 50,000) was purchased from Aldrich Chemical Company, Milwaukee, WI. Sodium alginate (type IV), sorbitan monooleate (Span 80), purified glucose, yeast extract, and vitamins were obtained from Sigma Chemical Company, St. Louis, MO. Kerosene (F-158301) was supplied by Quimasx Inc., Lemont, IL, and a food-grade corn oil was obtained from CPC, Summit, IL. All other chemicals employed for the study were analytical grade.

Preparation of PEI-Alginate Adduct

The PEI-alginate adduct was prepared by mixing PEI with sodium alginate at room temperature: To a 5-L Waring blender containing 2 L of 2% sodium alginate solution (aqueous, pH 7.5), 4 g of 50% PEI (aqueous, pH 12) was added with vigorous stirring. After 10 min, homogeneous dispersions of the PEI-alginate adduct resulted; the product was subjected to vacuum in order to eliminate air bubbles. The polymer adduct was stored at room temperature until it was used for bead preparation.

Preparation of Yeast Beads

An oil-phase pelletization method was employed to prepare spherical beads of yeast cells in the gel carrier. Referring to the schematic diagram in Fig. 1, the yeast–polymer mixture was prepared by combining equal volumes of 15% yeast slurry and the PEI-alginate adduct (or, alternatively, 2% sodium alginate). A mixture of 400 mL corn oil, 200 mL kerosene, and 0.5 mL sorbitan monooleate was placed in a 2-L round-bottom flask, which was equipped with an overhead stirrer. A 300-g aliquot of the yeast–polymer mixture was introduced to the oil phase with gentle agitation. As soon as the yeast–polymer mixture was completely suspended to form spheres of approximately 1.5 mm, 7 g of finely crushed calcium chloride powder was added to cure the beads. Cured beads were harvested after an agitation period of more than 20 min. Different sizes of beads were obtained by altering the amount of surfactant added to the oil phase. The yeast beads were stored in 3% calcium chloride solution at 4°C overnight before the use for fermentation.

Fermentation Medium

A 12% glucose medium was prepared in 40-L quantities, as shown in Table 1. Calcium chloride was added to the medium to facilitate reversi-

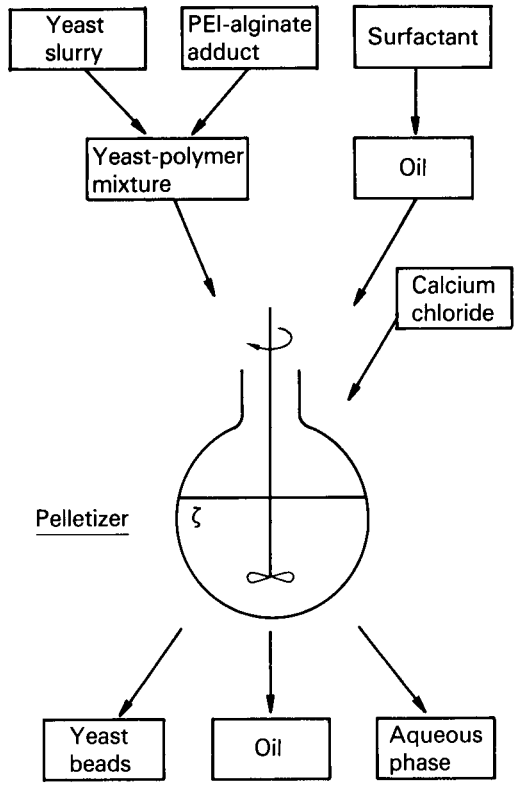

Fig. 1. A flow diagram showing preparation of yeast pellets.

ble gelation of the carrier matrix. The prepared medium was immediately autoclaved and stored at room temperature until use.

Ethanol Fermentation

The performance and stability of the yeast beads were studied by determining ethanol yield and the reactor productivity in a continuous fermenter. The effectiveness factor of the yeast beads in our reactor was also determined for various sizes of the biocatalyst (0.2–4 mm).

Figure 2 shows the lay-out of the continuous fermenter. A 7 × 40 cm glass column with a screen support served as the reactor. Approximately 400 mL of yeast beads were maintained in the reactor by frequently removing excess beads as the beads expanded during fermentation. A sterile glucose medium (12% w/v) was continuously fed to the reactor via a bubble contactor for aeration, but the reactor was open to the atmosphere. The fermented broth was removed from the reactor continuously by a sump pump. The fermentation was carried out at pH 3.5, temperature 24°C, $pO_2 < 1\%$ saturation, and the bed density of yeast beads 0.5–0.6 g/mL. The feed rate was adjusted such that the residual glucose in the effluent would remain less than 0.1%.

TABLE 1
Composition of the Fermentation Medium

Fraction	Chemical	Concentration, per L
(I) Major nutrients	Glucose	120g
	Yeast extract	1.2g
	$(NH_4)_2SO_4$	1.5g
	KH_2PO_4	0.15g
	$MgSO_4 \cdot 7H_2O$	0.5g
	$CaCl_2$	1.0g
(II) Minor nutrients	$ZnSO_4 \cdot 7H_2O$	1 mg
	$MnSO_4 \cdot 1H_2O$	1 mg
	$FeCl_3 \cdot 6H_2O$	1 mg
	$Na_2MoO_4 \cdot 2H_2O$	0.3 mg
	$CoCl_2 \cdot 6H_2O$	0.3 mg
	H_3BO_3	0.3 mg
	$CuSO_4 \cdot 5H_2O$	0.1 mg
(III) Vitamins	Biotin	0.012 mg
	Pantothenate	0.425 mg
	Folic acid	0.625 mg
	Inositol	1.0 mg
	Niacin	0.25 mg
	p-Aminobenzoic acid	0.625 mg
	Pyridoxine	0.25 mg
	Riboflavin	0.0625 mg
	Thiamine	0.25 mg

Chemical Analysis

The fermentation broth was analyzed for the content of glucose and ethanol following enzymatic methods (*11*). Samples drawn from the fermenter were immediately quenched with an equal volume of 0.4*M* perchloric acid. Glucose was analyzed by determining formation of NADPH in coupled enzymatic reactions of hexokinase and glucose-6-phosphate dehydrogenase (Sigma Glucose Assay Vials no. 15-10). Ethanol was assayed with alcohol dehydrogenase using Sigma Ethanol Assay Vials no. 330-5 and Glycine Buffer Reagent no. 332-9. The spectrophotometric measurements at 340 nm were carried out using Lambda 5 spectrophotometer (Perkin-Elmer, Norwalk, CT).

Examination of Yeast Beads

The bead size was determined by examining magnified photographs of the yeast beads. Cell content of the beads was determined after freeing

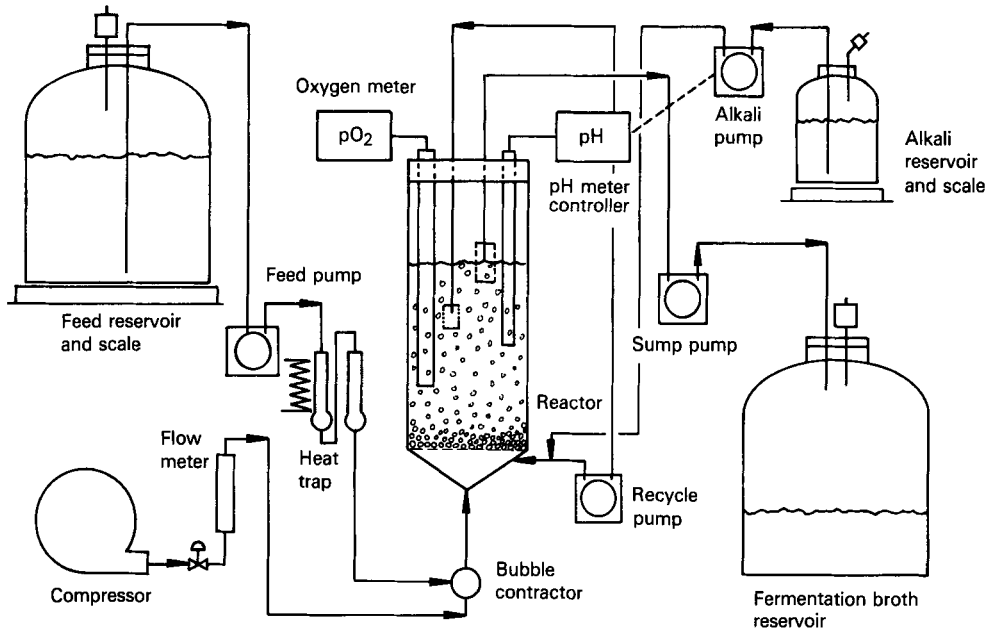

Fig. 2. Continuous fermenter with yeast beads.

the cells by dissolving the carrier gels with 2% sodium citrate at pH 6. Cells were washed with distilled water and dried at 105°C for 3 h before weighing. A portion of free cells obtained from yeast beads was stained with 3% methylene blue for microscopic determination of the cell viability. A volume-averaged bead diameter and cell viability were obtained for the calculation of the effectiveness factor and the Thiele modulus.

RESULTS AND DISCUSSION

Upon addition of the viscous PEI solution to the alginate dispersions with vigorous mixing, a stable polymer adduct was produced, presumably by strong charge attractions. The PEI-alginate adduct was clear and dispersed freely in water. The modified polymer formed resilient gels in the presence of calcium ion (tensile strength 100–200 psi). The gels readily reverted to sols when contacted with a metal chelating agent, such as citric acid, EDTA, or phosphoric acid. Furthermore, the sols reverted to resilient gels by the addition of counteracting amounts of multivalent metal ions. This reversible gelation of PEI-alginate adduct is the basis of the expandable cell carrier.

A possible molecular configuration of PEI-alginate adduct is that a molecule or molecules of PEI is (are) surrounded by many staples of linear alginate molecules neutralizing the positive charges of PEI molecule, as illustrated in Fig. 3(a). The unreacted carboxyl groups in the alginate chains can reversibly interact with metal ions, but irreversible cross-linking to other polymer adducts is prevented. Gelling occurs when mul-

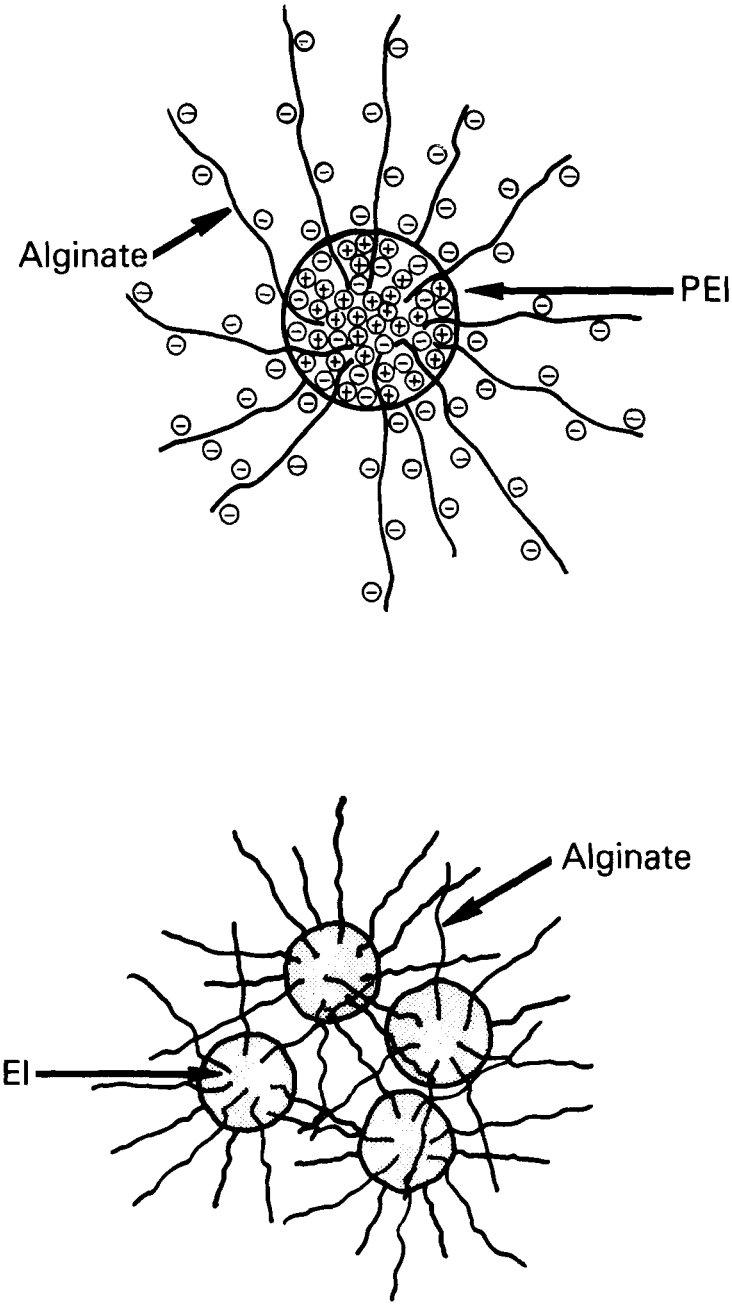

Fig. 3(a). Possible molecular configurations of the PEI-modified alginate.

tivalent metal ions are added to the polymer, as shown in Fig. 3(b), whereas dissolution of gels occurs when the metal ions are removed by a chelating agent.

The oil-phase pelletization yielded fairly uniform beads containing more than 85% cell mass on a dry-weight basis. A batch of 300 g

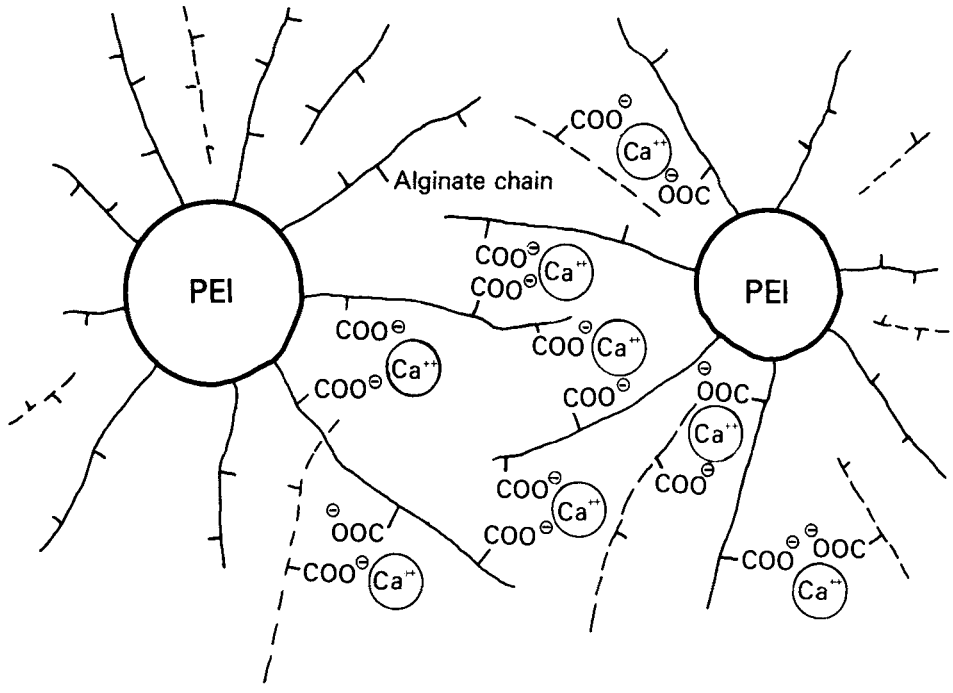

Fig. 3(b). Reversible gelation of PEI-alginate by calcium ion: Two negatively charged carboxyl groups attached on different alginate chains are bonded together in the presence of calcium ion gelling the polymer adduct, but the resultant gels can be redissolved by elimination of calcium ion with a chelating agent.

yeast–polymer mixture yielded 150 g of yeast beads, and the balancing water was removed by the curing salt. The degree of dehydration of the beads was readily adjusted by the use of different amounts of curing salt. Figure 4(a) shows a photograph of the yeast beads made of PEI-alginate adduct. The scanning electron micrograph for the cross-section of the yeast bead showed 5–10 μm cell cavities resembling honeycombs [Fig. 4(b)]. The cavities are the imprints of the yeast cells in the gel matrix. Our method of specimen preparation for the scanning electron microscopy retained the original shape and dimensions of the gel matrix, whereas the yeast cells in the bead shrank appreciably during freeze-drying. The characteristic morphologies of the gel matrix were identical for all beads prepared, regardless of the pelletization methods or the use of different polymers (oil-phase pelletization vs drip method, PEI-alginate adduct vs unmodified alginate). Selected properties of the yeast beads prepared with PEI-alginate are summarized in Table 2. The mild procedure of pelletization, as well as reacting PEI with alginate prior to mixing with yeast cells, completely eliminated cell death during pelletization. Additional benefits of using this modified polymer included the uniform size of the resultant beads and easy control of the pelletization process. Table 3 com-

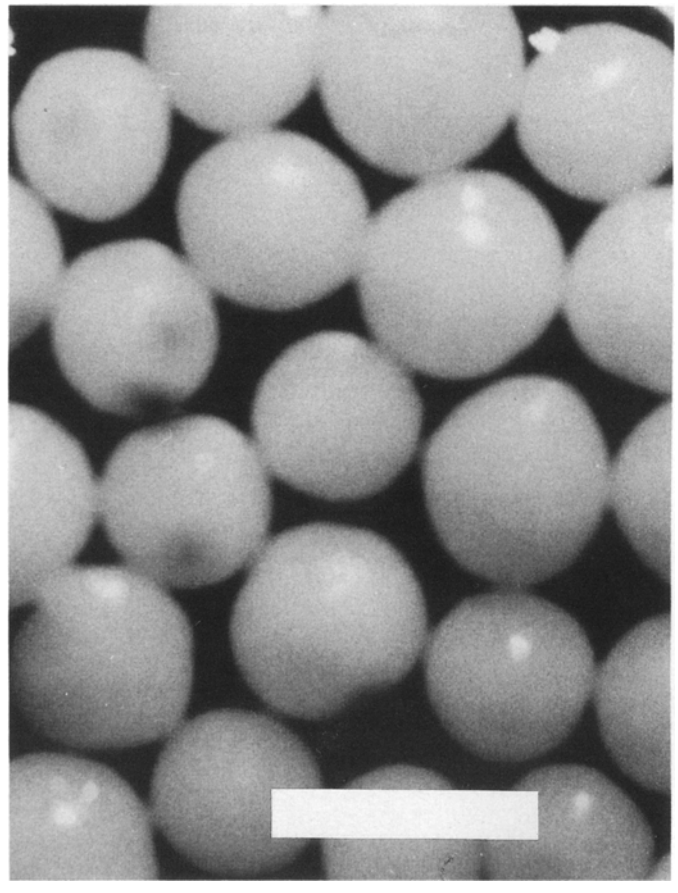

Fig. 4(a). Yeast beads prepared by the oil-phase pelletization (bar = 2 mm).

pares size distribution of beads prepared with two different materials, the polymer adduct and unmodified alginate.

The yeast beads prepared with the PEI-alginate adduct were highly active for ethanol fermentation and were physically intact even after 3 mo of continuous fermentation. The bead volume increased gradually as yeast cells grew in the beads while fermenting glucose to ethanol. The bead volume reached 1000% or more of the original volume within 10 d, after which the volume stabilized, as judged by a constant-reactor bed-volume. The yeast beads prepared with *unmodified* alginate were unstable under the harsh condition of rapid fermentation; the bed volume expanded to 400% within 2–3 d, after which it shrank gradually because of the loss of carrier alginate and cells. As yeast cells rapidly proliferated, the core of the beads were liquefied, causing bead rupture [as shown in Fig. 4(c)]. Also, the gradual disintegration of the unmodified carrier gels

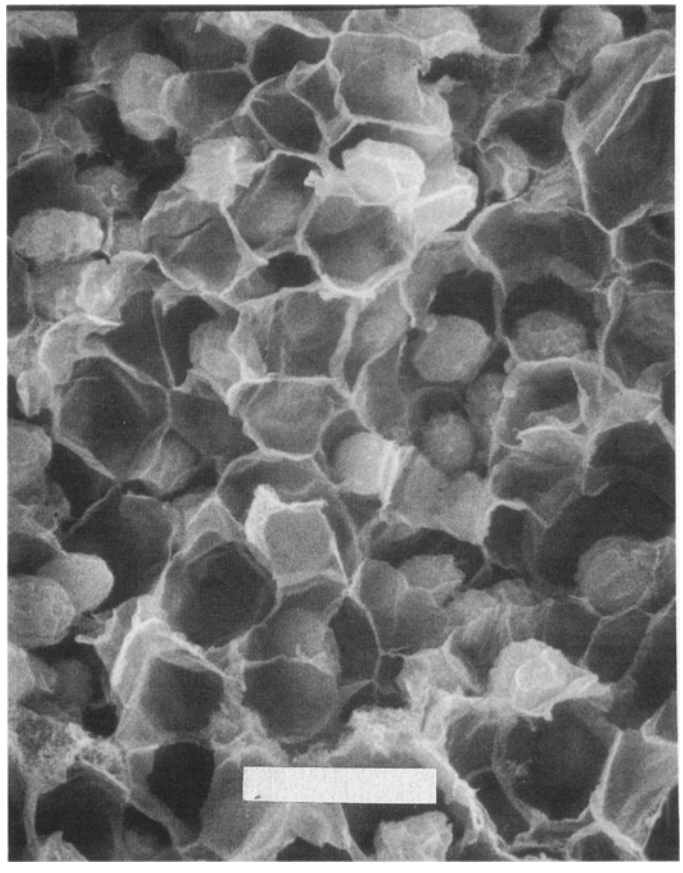

Fig. 4(b). The cross-section of the yeast bead showing imprints of the immobilized cells in the gel matrix resembling honeycombs (bar = 10 μm).

produced scum and foam, which accumulated in the reactor. The disintegration of beads was universal with unmodified alginate, regardless of the method of bead making.

Cross-sections of our yeast bead at two distinctively different stages during fermentation are compared in Fig. 5. At an early stage, yeast cells are still enclosed in the cell cavities resembling honeycombs [Fig. 5(a)]. The cell cavities were gradually expanded and interconnected as yeast cells continue to grow by dissolving the surrounding matrix. The cavities in a fully developed carrier matrix are completely open, and the space in the matrix is filled with closely packed yeast cells [Fig. 5(b)]. The bead volume, after expanding to 1000%, stabilizes as the growth rate of the yeast cells equilibrates with the rates of cell autolysis and excretion through the pores of the carrier matrix. The rate of cell washout is very small, seldom exceeding 0.3 g/kg wet bead/h. In contrast, the cell-washout rate for unmodified alginate was 3–5 g/kg wet bead/h under the fermentation conditions.

TABLE 2
Selected Properties of the Yeast Beads
Prepared with PEI-Alginate Adduct

Property	Description
Shape	Sphere to ellipsoid
Size	0.2–4 mm diameter
Specific gravity	1.07–1.08
Mechanical strength	17 psi (tensile)
Bulk density	0.7–0.73 g/cm^3
Water content	78–80%
Strength of the matrix	100–200 psi
Matrix, before activation	Closed cell structure
Matrix, after activation	Porous
Solute permeability	Permeable to all hydrophilic solutes
Stability limitations	Unstable at a high pH above 8 and to metal chelating agent

Our method of cell immobilization permitted the expansion of yeast beads, which was required for cell growth, maintaining the bead strength and geometric integrity. Yeast cells grew by dissolving the surrounding gel matrix, and the dissolved PEI-alginate adducts were displaced peripherally in the beads. The displaced polymer adducts gelled again with the aid of the influx of calcium ion from the fermentation broth, retaining the growing cells. Thus, a dynamic balance of local gel dissolution and reforming facilitated the immobilization of growing cells, providing geometric stability and mechanical strength to the yeast beads. Polyethyleneimine contributes to this balance by moderating the rate of dissolution of the carrier matrix by yeast cells, and the large molecular size of PEI-alginate minimizes loss of carrier material from the beads by retarding diffusion. Calcium chloride was required in the fermentation medium in a concentration of at least 500 mg/L in order to facilitate the reversible gelation of the carrier polymer. When the calcium chloride was

TABLE 3
Screen Analysis of Yeast Beads Prepared
With Different Carrier Polymers

US standard mesh #	Bead diameter, mm	PEI-Alginate adduct	Unmodified alginate
>12	>1.7	3	37
12–24	0.7–1.7	92	26
24–48	0.3–0.7	5	22
<48	<0.3	0	15
Total	—	100	100

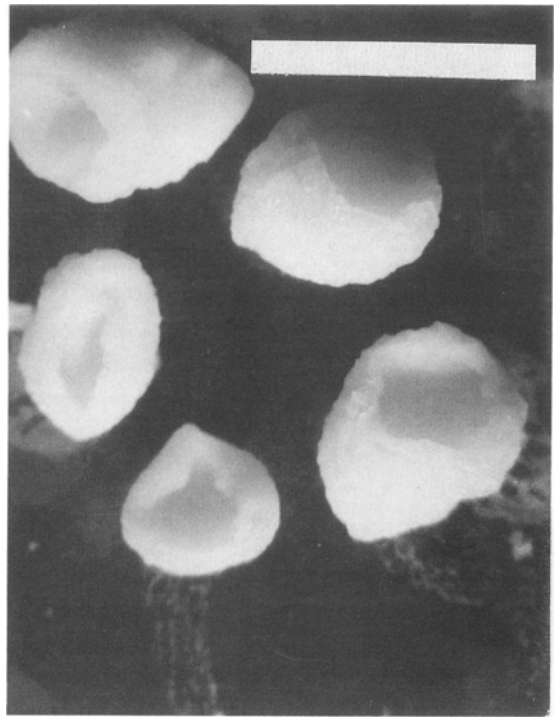

Fig. 4(c). Dissolution of the yeast beads prepared with unmodified alginate: The core of the bead was dissolved, causing rupture of the bead after 3 d continuous fermentation. Also, more gradual dissolution of the bead surfaces had started already (bar = 4 mm).

eliminated from the medium, a rapid dissolution of the carrier polymer ruined the yeast beads within a few days.

Rapid proliferation of lactic acid bacteria was another serious problem with the yeast beads prepared with unmodified alginate. A severe loss of ethanol yield, up to 20%, prevented continuous fermentation beyond 10–15 d. In contrast, yeast beads made of PEI-alginate adduct completely eliminated the infestation problem under an acidic condition of pH 3.5.

Our method of pelletization along with the PEI-alginate adduct resulted in highly active and resilient yeast beads that are suitable for ethanol fermentation. Under a higher pH condition with a luxurious medium, the ethanol productivity of the yeast beads reached as high as 150 g/kg wet beads/h (*10*). However, this high productivity could not be sustained for a prolonged period because of gradual cell death and bacterial infestation. A lean medium at an acidic pH of 3.5 (Table 1) appreciably extended the catalyst life to more than 3 mo. The catalyst activity for these conditions, however, was reduced to 30–60 g/kg/h (or the reactor productivity 15–30 g/L/h) at the ethanol concentration of 5% (w/v). The compromised productivity is still favorable when it is compared to the

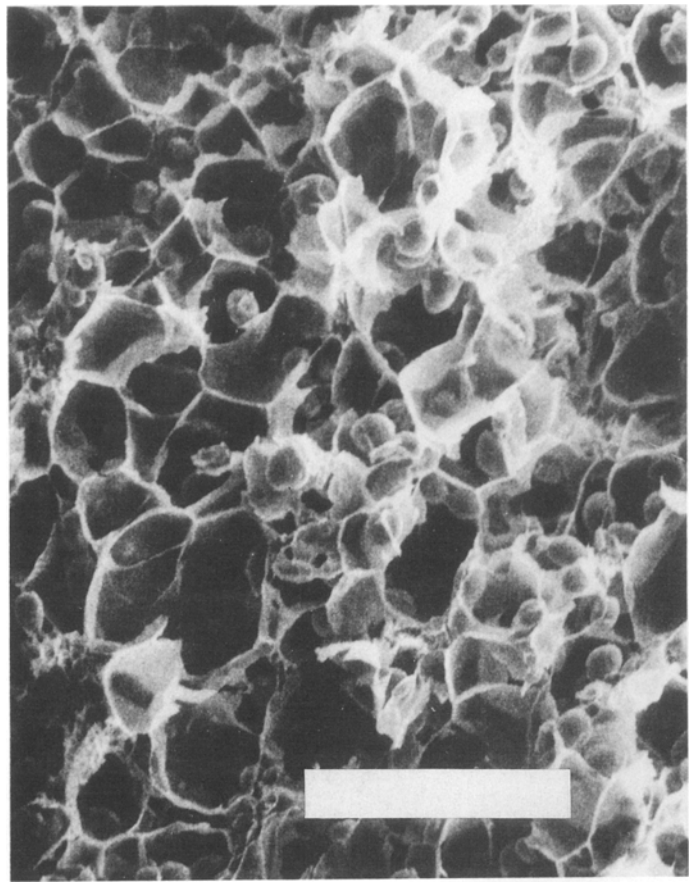

Fig. 5(a). Cross-section of the yeast bead showing enlarged or interconnected cell cavities in the carrier matrix (bar = 30 μm).

productivity of the conventional batch fermenters; 1–2 g/L/h. Bacterial infestation was no longer a problem at this low pH when the yeast beads were prepared with the PEI-alginate adduct. The average ethanol yield was also unusually high, ranging from 92 to 94% stoicheometric conversion of glucose to ethanol. Fig. 6 shows the time course of a typical run for the first 2 mo of continuous operation. The reactor productivity peaked at 26 g/L/h on the d 8 of fermentation, and a gradual decline of productivity continued until a stabilized productivity of 15 g/L/h was reached.

The declines in productivity were consistent with the declines in cell viability of the yeast beads. The ethanol yield was quite consistent, ranging from 92 to 95% after an initial surge to 92% during the period of catalyst activation.

The effectiveness factor (η) of the yeast beads ranged between 0.43 and 0.71 in our reactor, depending on the Thiele modulus (Φ) of the biocatalyst, which was varied from 1.4 to 26. Here, η is defined as the ratio

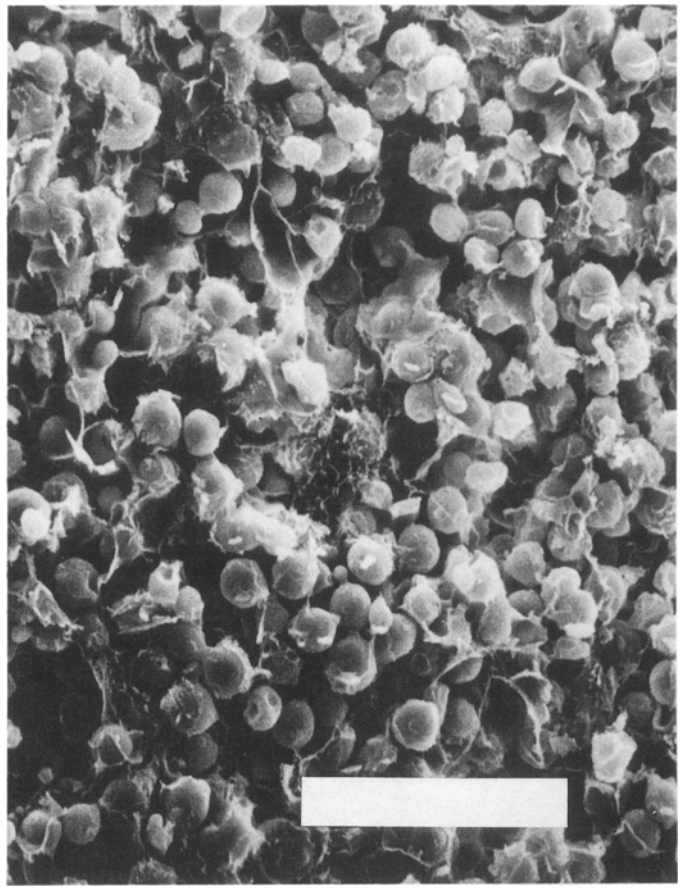

Fig. 5(b). Cross-section of the yeast bead showing luxurious cell growth and sponge-like matrix (bar = 30 μm).

of the activity of the biocatalyst to the maximum activity of free cells with saturated substrate for a given set of fermentation conditions. The Thiele modulus is defined by the following equation:

$$\Phi = R \left(\frac{V_o}{K_m D} \right)^{-1/2}$$

where R = radius of bead, V_o = the maximum activity of free cells, K_m = the substrate saturation constant for the Monod equation, and D = diffusivity of glucose. For our calculations, 1.3 g/g/h for specific activity, 6×10^{-6} cm^2/s for the diffusivity, and 0.44 g/L for the substrate saturation constant were used. Figure 7 is the η plot against Φ for our experimental results. Within the experimental range, η was best represented by the following equation:

$$\eta = 0.76\ \Phi^{-0.16}$$

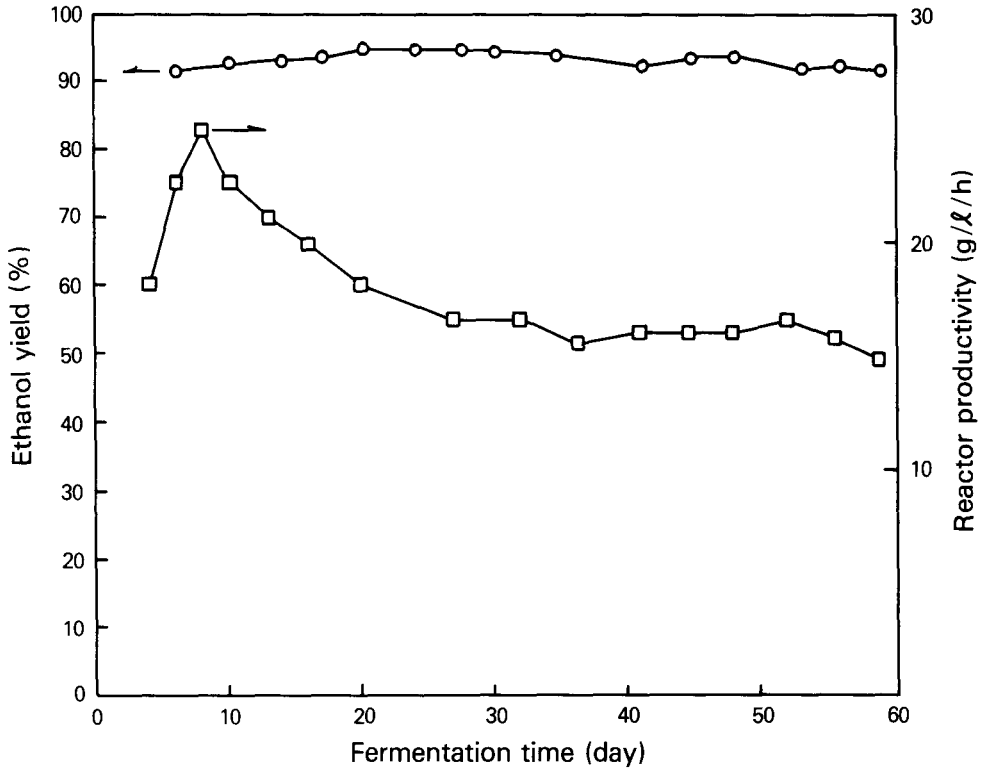

Fig. 6. The time course of a continuous fermentation with the yeast beads.

Under vigorous fermentation conditions, substrate diffusion to yeast cells affects the rate of glucose conversion appreciably. The first-order effectiveness factor shown in the graph indicates that our reactor performs well, even for a higher Thiele modulus or for larger beads. A relatively high effectiveness of our reactor in spite of the low glucose concentration in the reactor effluent indicates the existence of axial concentration gradients in the reactor. Instability of viable cell population in smaller beads was a problem even though the smaller beads were more effective for ethanol conversion. The best fermentation results were, therefore, obtained with the medium size beads (0.7–1.7 mm diameter).

In conclusion, yeast beads prepared with PEI-alginate adduct showed a high ethanol productivity and stability suitable for industrial applications.

SUMMARY

A unique polymer matrix that is suitable for immobilizing growing cells has been developed. The PEI-modified alginate matrix can expand to 1000% of its original volume as the immobilized cells grow in the matrix. Yeast beads prepared in the matrix had catalyst life for more than 3

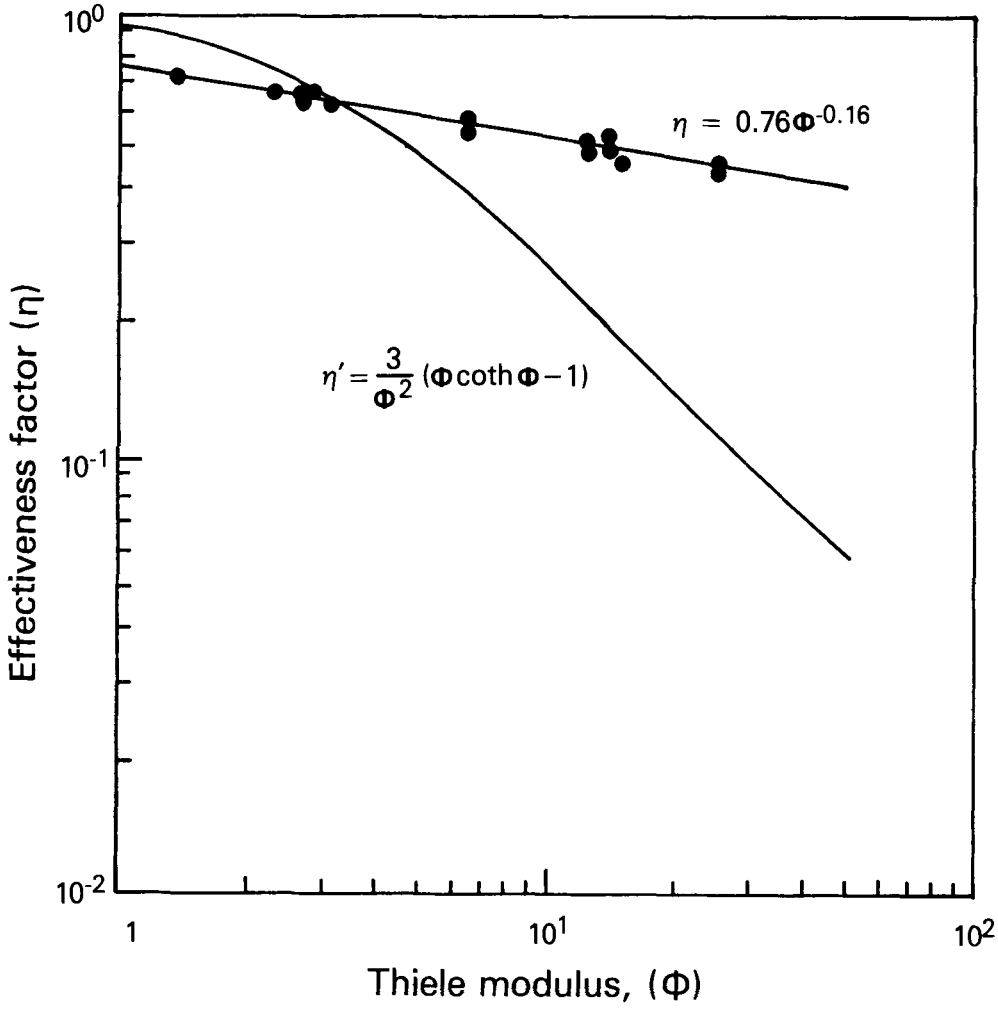

Fig. 7. The effectiveness factor (η) of the yeast catalyst in the self-stirred fermenter and the theoretical first-order effectiveness factor. (η').

mo, with high conversion efficiencies when applied to continuous fermentation of glucose to ethanol.

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