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Ethanol Production by Yeast Cells Immobilized in Open-Pore Agar

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ABSTRACT

An open-pore agar matrix has been shown to be suitable for the entrapment of microbial whole cells required for use in reactions that involve cell growth and gas evolution. Beads of porous agar with entrapped yeast cells have been used for the continuous fermentation of sugar cane molasses to ethanol, without apparent bead rupture, even after periods of 3 mo of use. The agar gel does not erode during prolonged operation, unlike porous gelatin cross-linked with glutaraldehyde.

Index Entries: Open-pore agar gel; carrier matrix, for *Saccharomyces sp.* immobilization; agar beads with entrapped yeast cells; packed bed reactor; sugarcane molasses, continuous fermentation; porous agar bead stability, in prolonged continuous fermentation.

INTRODUCTION

We had described earlier a procedure for obtaining open-pore gelatin beads as a carrier for the entrapment of yeast cells (*1*). Erosion from the interior of beads has been observed during a period of 2–3 mo when the gelatin with entrapped yeast cells had been used in packed-bed reactors for the continuous fermentation of sugar cane molasses to ethanol.

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The degradation was apparently caused by microbial utilization of the matrix. An obvious alternative carrier that is resistant to microbial degradation, in general, is agar gel, which finds extensive use as a solid support for the growth of cell cultures. Applications of agar for whole-cell immobilization have been described in the literature, and these include the entrapment of *Escherichia coli* for its β-glucosidase activity (*2,3*), yeast for its invertase activity (*4*), *Rhodospirillum rubrum* for its hydrogenase activity (*5*), and plant cells for their secondary metabolites (*6*).

A general disadvantage with agar as a carrier is that of marked diffusional limitations (*4*). A specific problem in its use in reactions involving cell growth and gas production is its brittle nature and consequent rupture of the carrier gel during such fermentation, unless strengthened by incorporation of polymers, like polyacrylamide (*7*). The present paper demonstrates that these disadvantages can also be overcome by rendering the agar support porous through the selective leaching of calcium alginate from a composite matrix containing calcium alginate and agar. This approach had been used by us earlier for obtaining open-pore gelatin beads (*1*).

MATERIALS AND METHODS

Yeast

Saccharomyces sp, designated as Y-10, was used for the fermentation of cane molasses to ethanol. The isolate, which was substrate- and ethanol-tolerant, was obtained from sugar cane juice by Mr. D. V. Gokhale of our laboratory. Cells were grown in a New Brunswick Labroferm batch fermenter of 14 L capacity under standard conditions. Cell suspensions were either used directly for immobilization or harvested on the Sharples supercentrifuge and used without storage.

Molasses and Chemicals

The sample of sugar cane molasses was obtained from a local sugar factory and analyzed by the dinitrosalicylic acid method for total reducing sugars after inversion with HCl (*8*). The molasses sample contained 58% w/w sugars, 90% of which were fermentable. Agar and sodium alginate were from LOBA-Chemie Indoaustranal Co. Ethanol was analyzed by gas chromatography, using Chromosorb 101.

Immobilization Procedures

Agar Gel with Pregrown Yeast Cells

Open-pore gel beads, with the entrapped yeast cells, were obtained by selective leaching of calcium alginate from an agar-calcium alginate

composite matrix. The procedure was essentially similar to the earlier method described for obtaining open-pore gelatin beads (*1*), except that the glutaraldehyde treatment was omitted.

A slurry of 20 g of wet, packed, yeast cells in 100 mL of an aqueous solution containing 2% agar and 2% sodium alginate at 45°C was dropped into a stirred solution of 2% calcium chloride and the beads removed immediately after the end of the addition. The diameter of the beads was about 3 mm. The calcium alginate in the composite beads was leached out by washing with 0.05*M* potassium phosphate buffer at pH 7.5 until the washing was clear. The resulting porous agar beads were left overnight at pH 4.5 in the cane molasses diluted to 16% fermentable sugars, and packed the next day into columns for continuous fermentation.

Yeast Cell Growth Within the Bead Matrix

For obtaining the open-pore agar bead system with entrapped inoculum, 20 mL of *Saccharomyces* Y-10 cell suspension in the growth medium were mixed with 80 mL of a solution containing 2% agar + 2 g sodium alginate at 45°C. The growth medium contained in 1 L: glucose, 50 g; yeast extract, 3 g; malt extract, 3 g; and peptone, 5 g. Beads were obtained as described above and the calcium alginate leached out with potassium phosphate buffer.

Calcium alginate beads with the yeast inoculum were prepared from a suspension obtained by adding 20 mL of the inoculum to 80 mL of an aqueous solution containing 4 g of sodium alginate at 30°C. The suspension was dropped into a stirred solution of 2% calcium chloride and the beads left 1 h in the calcium chloride solution.

Beads in both cases were about 3 mm in diameter, and the yeast cell density was approximately 1×10^7 cells/g bead.

Packed-Bed Reactors

Water-jacketed columns of the type described by Krouwel et al. (*9*) were used (top, 5 cm id; bottom 3 cm id; and height, 18 cm). The bead vol was about 65% of the working bioreactor vol of 200 mL. The initial immobilized cell concentration was about 38 g dry wt/L bead vol. The feed stream was cane molasses containing 16% fermentable sugars supplemented with 0.1% urea and pH adjusted to 4.5. The column was operated at 30°C. Ethanol productivity (g/L/h) was determined on total reactor volume basis.

Scanning Electron Micrographs

The immobilized yeast was fixed, as described earlier (*1*), and sections observed in a Cambridge StereoScan model 150 Electron Microscope.

RESULTS AND DISCUSSION

Scanning Electron Micrographs of Gel-Entrapped Yeast Cells

The effect of leaching the calcium alginate–agar beads with potassium phosphate buffer at pH 7.5 can be seen in Fig. 1. The open-pore structure of the leached bead in which the entrapped cells are fully exposed (Fig. 1a) is clearly distinct from the covered cells observed in the unleached composite matrix (Fig. 1b). The effect of leaching on the carrier structure was similar to that observed with gelatin matrix (*1*).

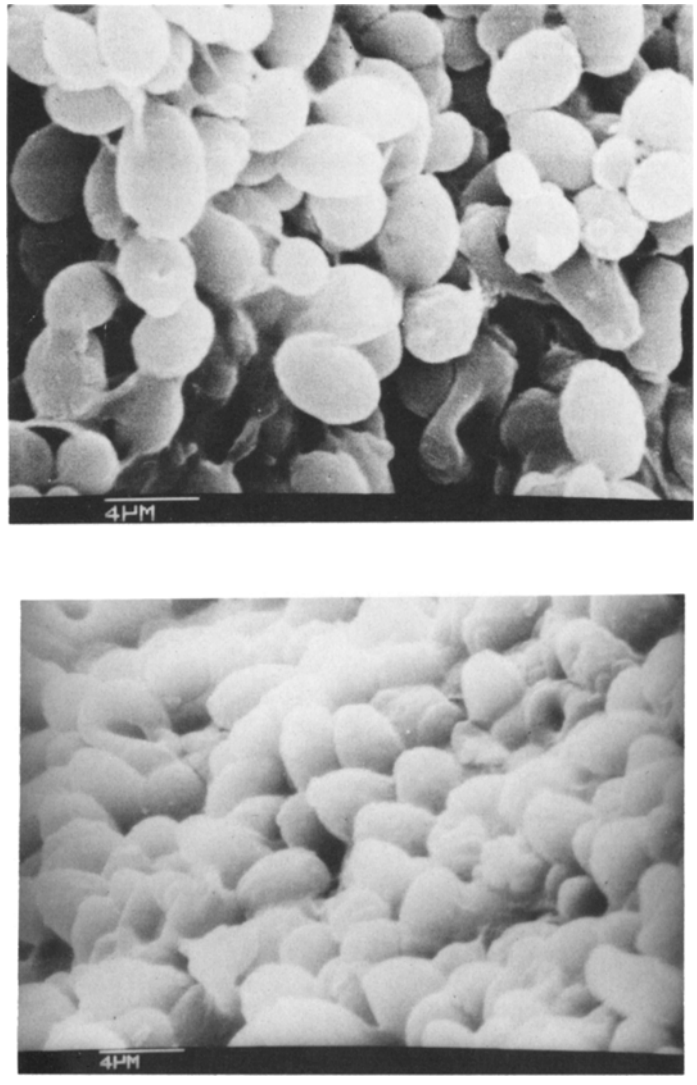

Fig. 1. Scanning electron micrographs of pregrown yeast cells entrapped in (a) porous agar bead and (b) calcium alginate–agar composite bead.

Figure 2 shows differences in cell growth from inoculum entrapped within the open-pore agar bead (Fig. 2a) and plain calcium alginate bead system (Fig. 2b). The cell density within the open-pore agar matrix is uniform and abundant throughout the bead, although the growth in the calcium alginate bead is markedly lower. The more porous nature of the former system could account for the enhanced availability within the beads of nutrients required for cell growth.

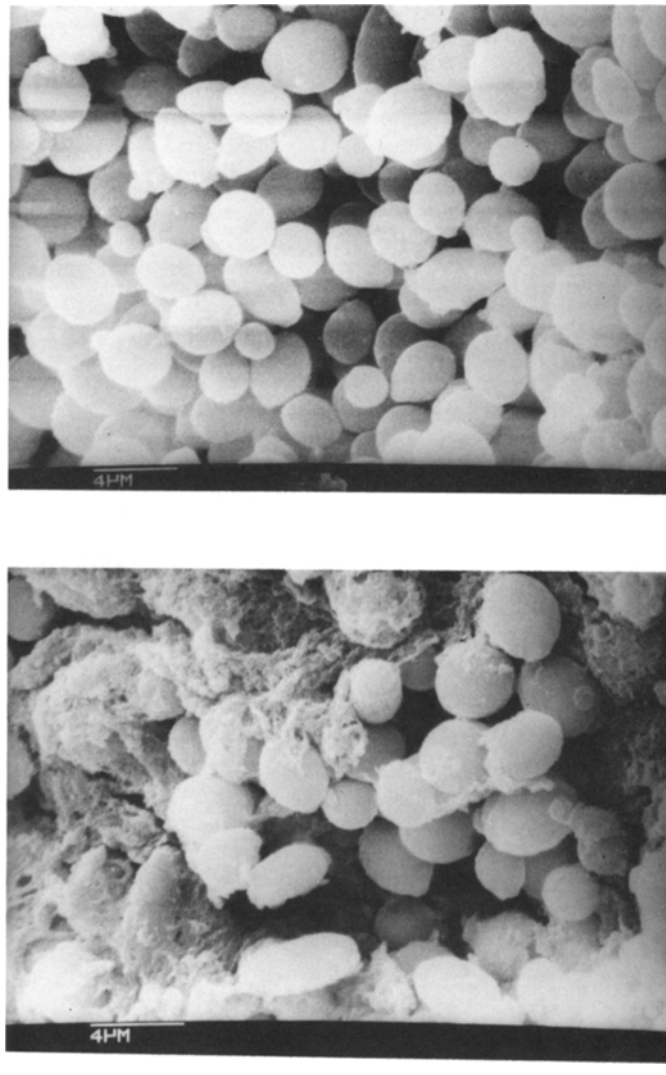

Fig. 2. Scanning electron micrographs showing growth of *Saccharomyces* Y-10 cells in (a) porous agar bead and (b) calcium alginate bead.

Continuous Fermentation of Cane Molasses to Ethanol

Preliminary experiments carried out, using the composite agar–calcium alginate beads before leaching the calcium alginate with phosphate treatment, showed that beads rupture during a period of 1–2

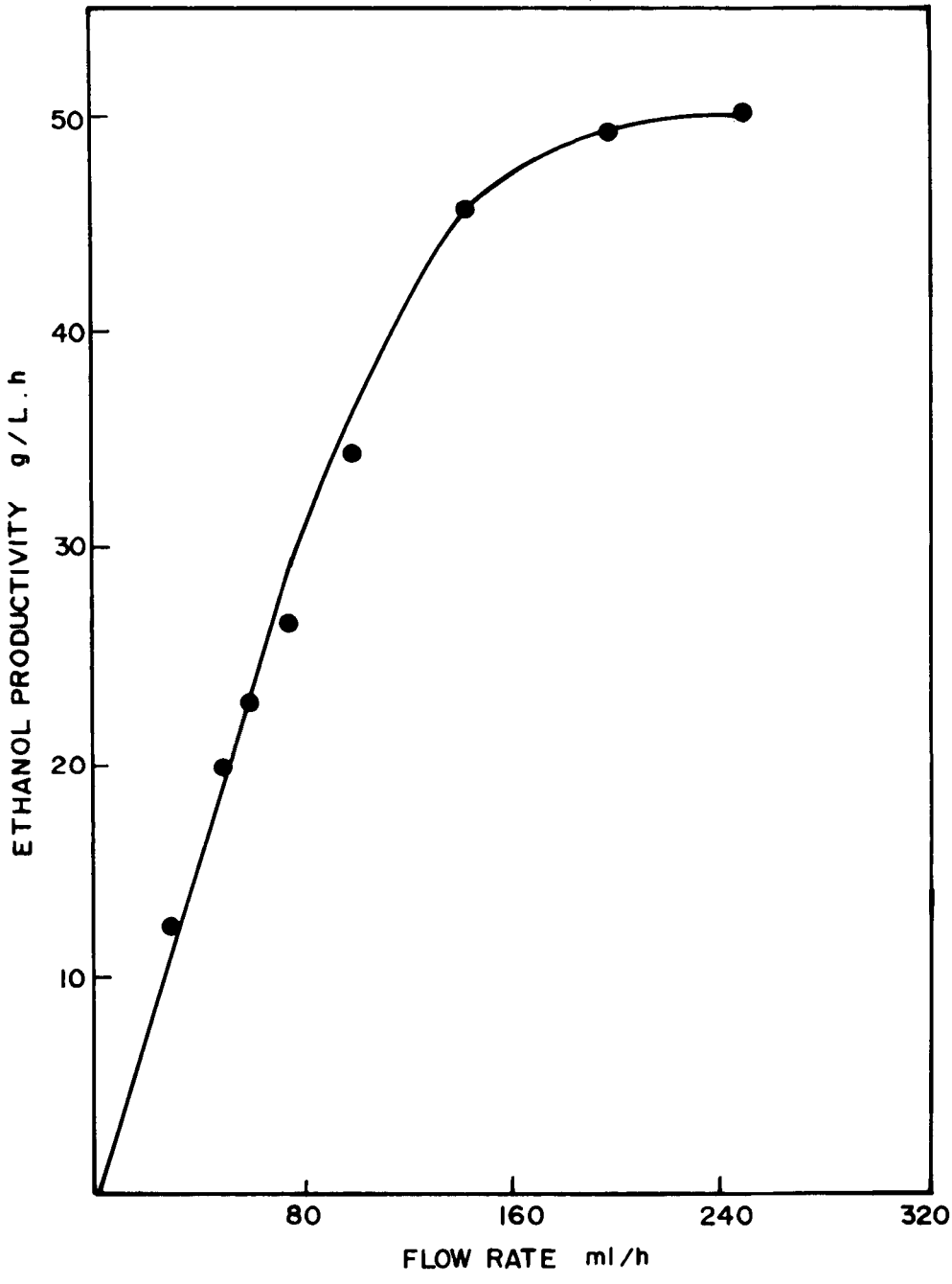

Fig. 3. Effect of flow rate on ethanol productivity of *Saccharomyces* Y-10 cells immobilized in open-pore agar beads, using cane molasses with 16% fermentable sugars.

TABLE 1
Ethanol Productivities at 30°C and Varying Flow Rates of Molasses Containing 16% Fermentable Sugars

Flow rate	% Utilization of feed sugar	Productivity, g/Lh
50	100	20
60	95	23
73	85	26
145	80	46

wk under the conditions of gas evolution and cell growth; this, however, was not observed with the porous agar beads obtained from leaching out of the calcium alginate component.

Figure 3 shows the relationship of ethanol productivity as a function of flow rates through a reactor column packed with open-pore agar beads with entrapped, pregrown *Saccharomyces* Y-10 cells. The ethanol productivities at various percentages of substrate utilization are summarized in Table 1.

The productivity of ethanol at 95% conversion efficiency and about 77 g/L product concentration is 23 g/L h which is comparable to that reported for calcium alginate gel bead immobilized system operated in fluidized bead reactors for continuous fermentation of cane molasses to ethanol (*10*).

The operational stability of the packed-bed reactor with open-pore beads used continuously for the fermentation of cane molasses to ethanol is shown in Fig. 4. No decrease in ethanol productivity was observed up to a month of operation, after which the conversion efficiency dropped

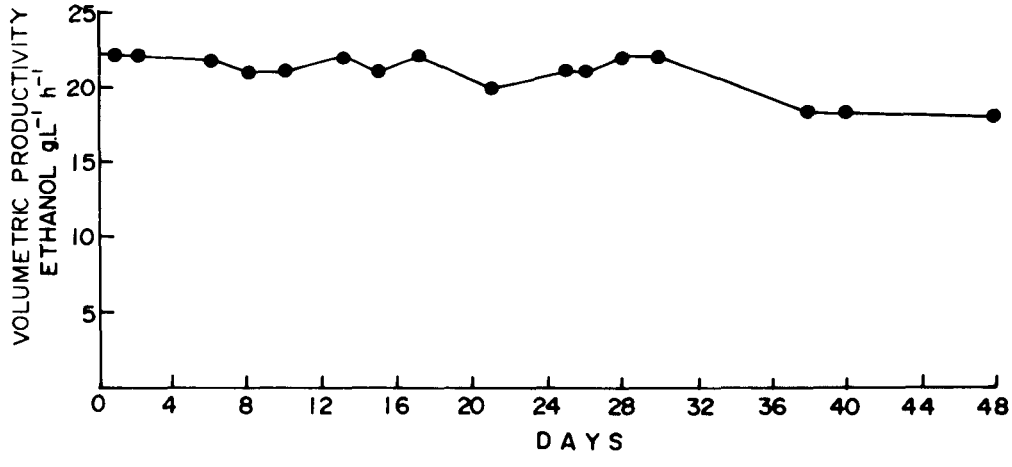

Fig. 4. Operational stability of packed-bed bioreactor in the continuous fermentation of cane molasses to ethanol, using *Sacchromyces* Y-10 immobilized in open-pore agar beads. Cane-molasses feed stream, containing 16% fermentable sugars; 30°C. Initial product concentration was 77 g/L ethanol.

and the ethanol concentration in the effluent became limiting at about 58 g/L, with a consequent decrease in the volumetric ethanol productivity to about 17–18 g/L/h. No apparent change in bead structure could be observed even after periods of about 2 mo of continuous use.

CONCLUSIONS

Open-pore agar beads can be obtained by leaching calcium alginate out of a composite matrix of agar and calcium alginate. The porous agar matrix is suitable for entrapment of yeast cells and is operationally stable even with gas evolution during the fermentation of molasses to ethanol.

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