0273-2289/92/3435—0477$02.00

A Two-Phase Method to Produce Gel Beads

Application in the Design of a Whole Cell β-Galactosidase Catalyst

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ABSTRACT

A method for producing entrapped whole cell biocatalyst is described. The procedure consists of the direct mixing of a gel suspension with an oily phase. Three biopolymers are used: gelatin, agar, and carrageenan using *K. fragilis* cells containing β-galactosidase activity as a design model. For a given gel type and concentration, the bead's particle size is a direct function of the agitation rate and the type of impeller, as well as the geometry of the system. Therefore, the particle size distribution is obtained as a function of the impeller's Reynolds number in order to define a scale-up criteria. The β-galactosidase biocatalyst is characterized considering the effect of particle size and substrate concentration on the effectiveness factor. This method does not require the usual extrusion equipment and, as shown here, is adequate for scaling up.

Index Entries: Immobilized cells; gel beads; β-galactosidase.

NOMENCLATURE

De = effective diffusivity (cm^2s^{-1})
D_i = impeller diameter (cm)
D_t = tank diameter (cm)

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dp = particle diameter (cm)
IMC = Immobilized Cell Catalyst
kE_t = maximum reaction rate (Ug^{-1})
K_m = Michaelis Menten constant (gl^{-1})
L = liquid height (cm)
N = impeller speed (rpm)
r = distance from external surface of particle (cm)
R = radius of particle (cm)
Re = impeller's Reynolds number
S = substrate concentration (gl^{-1})
v_{obs} = observed reaction rate of catalyst (Ug catalyst^{-1})
v^* = reaction rate of catalyst with no diffusional limitations (Ug catalyst^{-1})
z = dimensionless radius of particle

Greeks
β = substrate modulus
ϕ = Thiele modulus
ν = effectiveness factor
μ = viscosity of continuous phase (g $cm^{-1}s^{-1}$)
ρ = density of continuous phase (gl^{-1})

INTRODUCTION

Immobilization of whole cells for the application of a single enzyme activity is an expanding alternative in enzyme technology. This situation is illustrated in Table 1 (*1,2*), by the increasing number of industrial enzyme catalysts formulated with whole cells. The main advantage of immobilized cell catalysts (IMC) is that the enzyme extraction and purification processes are avoided resulting in lower catalyst production costs (*3*). A wide variety of materials and methods for IMC production have been proposed, gel entrapment being the most frequent of them all. However, in this type of catalyst, the internal mass transfer resistance of the immobilization matrix is important in determining the operational activity of the immobilized cells (*4*). In order to overcome the problem caused by internal diffusional resistance, two alternatives are available. Either the particle size or the enzyme activity can be reduced. In practice, however, the only viable alternative is to produce particles having a small diameter, since the particles must have the highest specific activity possible.

Several methods for IMC production by gel entrapment have been proposed. Most of them are produced by injecting a suspension of cells and polymer through a nozzle, letting individual drops separate either by gravity or by atomization. The particles are received in an aqueous phase. Oil has been used as a receiving solution in order to enhance the formation of beads. In this type of system, 2–5 mm diameter beads are typically formed (*5*). Small beads can only be obtained using small nozzle diameters

Table 1
Examples of Industrial Enzymatic Processes with Immobilized Whole Cells

Microorganisms	Enzyme	Product	Company
E. coli	Aspartase	L-aspartic acid	Tanabe Seiyaku Genex Corp.
E. coli	Penicillin acylase	6-aminopenicilanic acid	Tanabe Seiyaku Novo Industri Toyo Yozo
Actinoplanis missourienses	Glucose Isomerase	Fructose syrups	Gist Brocades
Bacillus coagulans	Glucose Isomerase	Fructose syrups	Novo Industri
E. coli	Tryptophanase	Tryptophan	Genex Corp.
Brevibacterium ammoniagenes	Fumarase	Malic acid	Tanabe Seiyaku
Erwinia rhapontici	Isomaltulose sinthase	Isomaltulose	Miles Lab.
Serratia plymuthica	Isomaltulose sinthase	Isomaltulose	South German Sugar
Mortierella vinacea	α-galactosidase hydrolysis	Raffinose	Hokkaido Sugar
Pseudomonas dacunhae	Aspartate decarboxylase	Alanine	Tanabe Seiyaku
Klebsiella aerogenes	Serine hydroxymethyl transferase	Serine	Genex Corp.
Rhodotorula rubra	Phenylalanine ammonialyase	Phenylalanine	Genex Corp.
Rhodococcus sp	Nitrilase	Acrylamide	Nitto Chem. Ind.
Achromobacter sp	L-histidine ammonia-lyase	Urocanic acid	Tanabe Seiyaku
Aspergillus niger	Fructosyl-transferase	Fructo-oligosaccharides	Meiji Seika
Protaminobacter	α-glucosyl-transferase	Palatinose	Mitsui Sugar
Pseudomonas chloroaphis	Nitrile hydratase	Acrylamide	Nitto Chem.
Streptomyces carbophilus	Hydroxylase	Pravastatin (inh. cholesterol synthesis)	Sankyo

that result in the need for extremely small liquid flow rates (*6*), increasing also the clogging frequency of the nozzle. The particle size distribution in a two-fluid annular atomizer has been found very broad for high flow rates even though small beads were formed: Narrow distributions were obtained only at flow rates lower than 90 cm^3h^{-1} (*7*).

A second alternative is to produce the catalyst by direct mixing of the oil phase with the cell and biopolymer suspension. In this case, the particle shape and size will be a function of the hydrodynamic conditions, the volume of the various phases, and the temperature at which the operation takes place.

In this article, we present the design and characterization of immobilized cell catalysts in three different biopolymers: agar, carrageenan, and gelatin. To produce the catalyst beads, a water suspension of the gel and cells is added to a vessel containing vegetable oil, and provided with mechanical agitation to form a macroemulsion. After cooling, the gelified beads are recovered by filtration. The effect of agitation on the size and distribution of the particle size is studied.

MATERIALS AND METHODS

Microorganism

Cells of *Kluyveromyces fragilis* NRRL-1109 with β-galactosidase activity were grown and permeabilized as has already been described (*8*). The activityof the cells was 1600 Ug $cell^{-1}$.

Enzymatic Activity

Lactase activity was measured following the release of glucose from a 5% lactose solution in 0.1*M* phosphate buffer (pH = 6.6), with the glucose test reagent (*9*). In order to measure the biocatalyst activity, the initial glucose production rate from lactose was measured in 20-mL vol with 400 mg of immobilized cells and with samples taken during the first 10 min. For batch reactions, the volume was increased to 50 or 100 mL. The activity is reported in terms of a unit weight of dry cell (Ug $cell^{-1}$) or dry catalyst (Ug cat^{-1}). One unit is defined as the amount of enzyme required to hydrolyze 1 μmol of lactose in 1 min. The cells produced contained 1.666 Ug $cell^{-1}$ and followed Michaelis-Menten kinetics with a $K_m = 5.33$ g l^{-1}, galactose being a competitive inhibitor (*10*).

In order to account for internal diffusion, the effectiveness factor was calculated. It is defined as:

$$\eta = (v_{obs} / v^*) = (v_{obs} / kE_t S / K_m + S) \quad (1)$$

and was experimentally determined by comparing the activity of a given gel catalyst (v_{obs}) with the activity of the same catalyst after mechanical disintegration (v^*), corresponding to the observed reaction rate in the presence of diffusional resistances and the kinetic rate, respectively.

All equations were solved numerically with the aid of simulation language ISIM® developed by Salford University Industrial Center Ltd., Salford, England. This language utilizes four different integration methods to solve differential equations.

Cell Immobilization

IMC were produced by a dispersion process in a two-phase system previously described by Nilsson et al. (*11*) and optimized for carrageenan by Audet and Lacroix (*12*). Three gels were used: gelatin (Hormel Industries, USA), carrageenan (Quimica Hercules, Mexico), and agar (Difco, USA). In all cases, 10% (w/v) of cells was mixed with 3% (w/v) of gelatin or agar and 2% (w/v) in the case of carrageenan. The cell suspension was poured into corn oil in a jacketed vessel with a $L/D_t = 1$ and $Di/D_t = 1/3$. The vessel was provided with mechanical agitation with a marine impeller. In all cases, the production temperature was 3°C higher than the gelification temperature. Each production process was characterized by the impeller's Reynolds number (Re) defined as:

$$Re = (D_i^2 N \rho / \mu) \quad (2)$$

After formation, the gel beads were cooled to 5°C, filtered, and washed with a 1% Tween 80 solution to wash off the oil. Catalysts produced with gelatin and agar were hardened for 1 h, with 1.25% glutaraldehyde and carrageenan with 0.3*M* KCl. Afterwards, all catalysts were sieved to determine the particle size distribution in a Brinkmann vibrating siever, model VS (Rexdale, Canada). Stainless-steel sieves with mean diameters of 0.59, 0.50, 0.35, and 0.23 mm were selected. Drained beads (100 g) were weighed and sieved. After 15 min, each sieve fraction was collected, dried, and weighed. Weight values were expressed as mass percentage for each sieve mean diameter. These results are plotted for a graphical description of the particle size distribution. Each bead sample was sieved in triplicate with good reproducibility.

The mean particle size of the distribution is defined as

$$\overline{dp} = (\Sigma\, xi\, dpi / \Sigma\, xi) \quad (3)$$

where xi is the mass percentage for each sieve of mean diameter dpi. A standard deviation is also calculated for each distribution.

RESULTS AND DISCUSSION

Catalyst Design

In the first part of the experiments, the maximum dispersed phase load in a given oil phase volume was determined. At dispersed phase volumes higher than 30% of the total system volume, a stable microemulsion was formed during mixing. This microemulsion made particle separa-

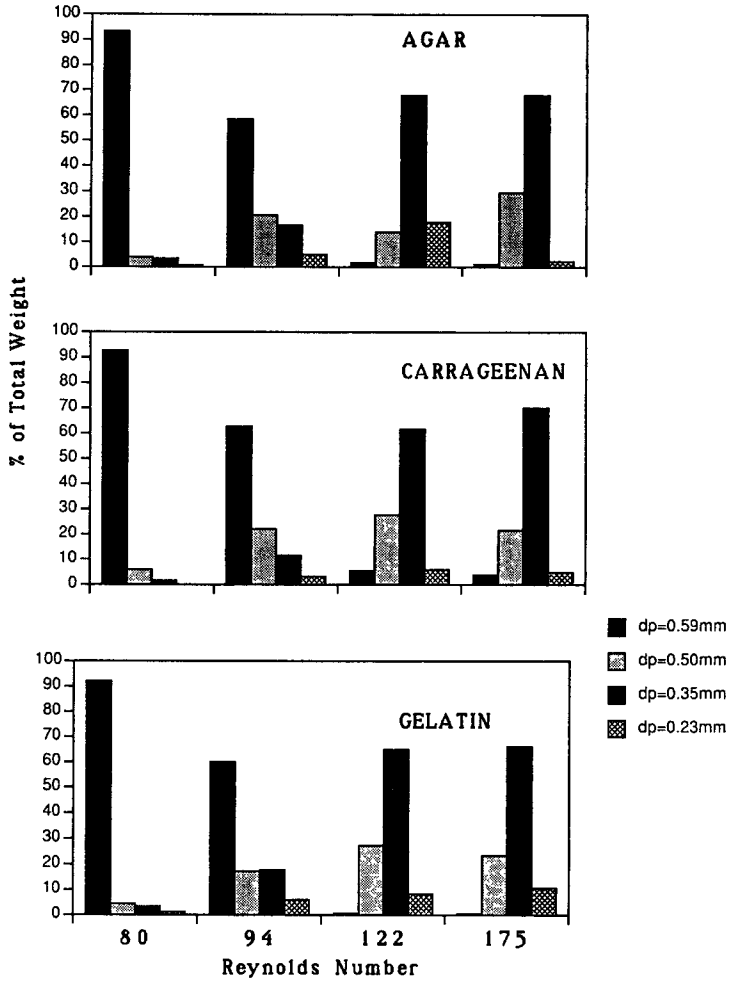

Fig. 1. Particle size distribution for three biopolymers as a function of the impeller's Reynolds number in a two-phase catalyst production system. ■ dp=0.59 mn, ▨ dp=0.50 mn, ■ dp=0.35 mn, ▨ dp=0.23 mn.

tion difficult. Therefore, a dispersed phase volume of 30% was used in the IMC production process.

IMC catalysts were produced by mixing the two immiscible phases at different agitation rates between 50–900 rpm. At agitation rates under 300 rpm, the system was poorly mixed, and at rates higher than 700 rpm, no differences in particle size were found. In Fig. 1, the particle size distribution (PSD) obtained for the three biopolymers at different impeller's Reynolds numbers (Re) are shown. It can be observed that, in all cases at Re higher than 100, the average diameter of the particles is near 0.35 mm. At low Re numbers (e.g., 94 and 80), the majority of the beads have diameters greater than 0.60 mm. In a previous report (*1*), it was established that effectiveness factors near 1 were obtained for *K. fragilis* yeast cells entrapped in gelatin with beads of 0.40 mm.

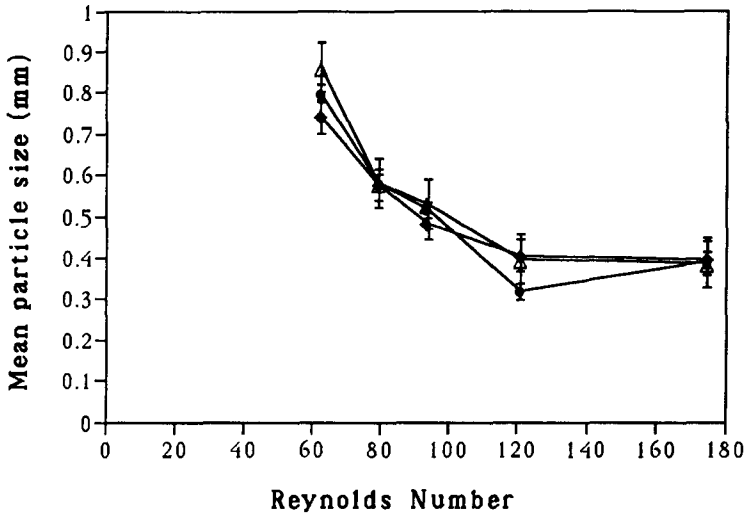

Fig. 2. Mean particle size for three biopolymers as a function of impeller's Reynolds number. —◆— Gelatin, —△— carrageenan, —●— agar.

In Fig. 2, the data presented in Fig. 1 is analyzed in terms of the mean particle size and standard deviation of the distribution. It is clear that, at high Re numbers, the mean particle size becomes independent of the Re number. Such a behavior resembles the relationship between the Power and Re numbers during mixing. This result is important, since it defines a viable criteria for scale-up of the IMC production process.

At impeller's Reynolds number of 120, the influence of agitation time was studied using gelatin. The PSD obtained after only 5 min of agitation is the same as the one obtain after 20 min, shown in Fig. 1. Therefore, only a few minutes are required in order to obtain the final catalyst particle size distribution. The distribution changes only if the Re is increased during the production process.

Catalyst Characterization

In the characterization of IMC, the diffusional limitations should be evaluated in order to establish the efficiency of the enzyme used in the process. This was carried out by direct measurement of the effectiveness factor, defined in Materials and Methods. In order to predict this parameter, the mass balance equation inside the spheroidal particle neglecting external mass transfer resistances, in this case in dimensionless form, was solved (*1*):

$$(d^2\beta / dz^2) = -(\phi^2\beta / 1 - \beta) - (2d\beta / zdz) \quad (4)$$

where:

$$\phi = (R / 2)\,[(kE_t / D_e K_m)]^{1/2} \quad (5)$$

$$\beta = S/K_m$$

$$z = r/R$$

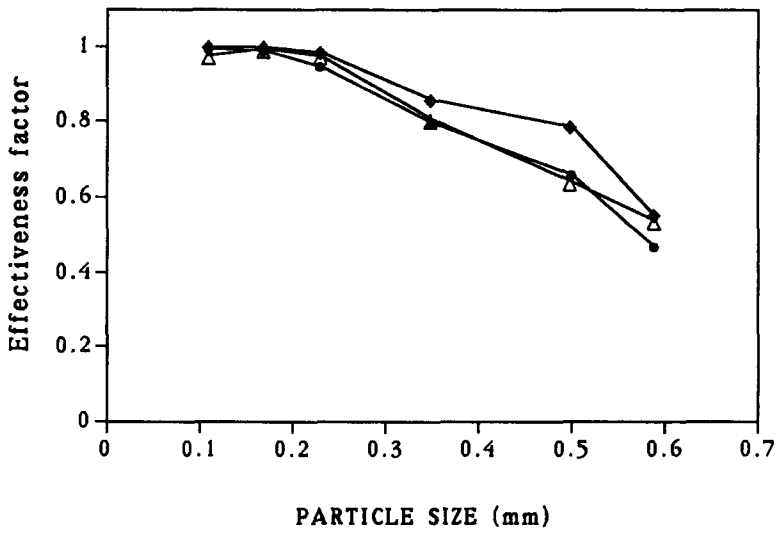

Fig. 3. Effectiveness factor as a function of particle size for a β-galactosidase catalyst. (Each point is the average of three experiments. In no case was the deviation higher than 5%). —◆— Gelatin, —△— carrageenan, —●— agar.

For a given Thiele modulus (ϕ) and an initial substrate concentration, after integration of the concentration profile obtained from Eq. 4, the effectiveness factor, can be determined. If, on the other hand, the effectiveness factor is experimentally determined and the kinetic parameters are known, then the effective diffusivity may be calculated from the calculated Thiele modulus given by Eq. 5 (*13*).

In Fig. 3, the effectiveness factor found from *K. fragilis* cells immobilized in the three biopolymers at various particle sizes is presented. It is found that particle sizes higher than 0.60 mm result in low effectiveness factors, whereas for particles lower than 0.25 mm, the efficiency is almost 1.0. In general for all three materials, the behavior is the same, which is a consequence of a similar pore structure. This is further demonstrated from the estimated effective diffusivities for lactose in the three materials. For a particle size of 0.50 mm and two different substrate concentrations, the effective diffusivity is calculated from Eqs. 4 and 5. These results are presented in Table 2, where it can be observed that there are no significant differences in the effective diffusivity values for the immobilization matrix of the three materials. The values are also compared with data from the literature.

Finally, the effect of substrate concentration on the effectiveness factor is studied for a particle size of 0.50 mm. These results are shown in Fig. 4, where it may be observed that the efficiency of the catalyst decreases with decreasing substrate concentration. This is important to consider when designing enzymatic reactors, since the effectiveness factor is usually taken as constant for a given system.

Table 2
Lactose Diffusivities in Various Systems

Lactose	De, cm^2s^{-1} Gelatine	agar	Carrageenan
50 g/L	0.211×10^{-8}	0.144×10^{-8}	0.180×10^{-8}
70 g/L	0.211×10^{-8}	0.134×10^{-8}	0.150×10^{-8}

From reference *14*: *K. Fragilis*—gelatine De (cm^2s^{-1})=4.21×10^{-8}, *E. coli*—carrageenan and locust bean gum De (cm^2s^{-1})=1.2×10^{-8}, carrageenan and locust bean gum De (cm^2s^{-1})=3.5×10^{-8}.

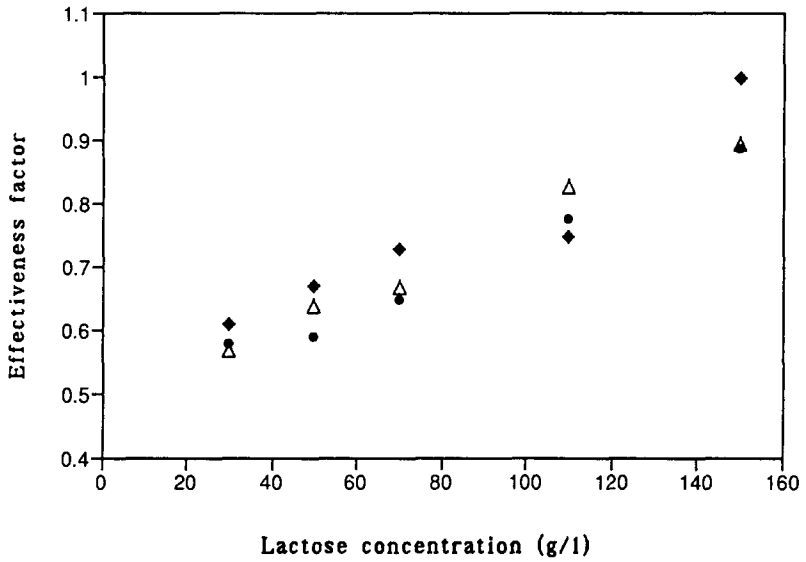

Fig. 4. Effect of substrate concentration on effectiveness factor for a β-galactosidase catalyst with a particle size of 0.50 mm. (Each point is the average of three experiments. In no case was the deviation higher than 5%). —◆— Gelatin, —△— carrageenan, —●— agar.

CONCLUSION

A two-phase dispersion process for the production of an immobilized cell catalyst with β-galactosidase activity was studied. The particle size distribution and the mean particle size were determined as a function of agitation rate, characterized by the impeller's Reynolds number. This is an adequate criteria for scaling up the catalyst production process. The IMC is also characterized in terms of the effectiveness factor defining the particle size required for kinetic control of the system. The biocatalyst production system studied here is one of the simplest methods reported in the literature, without sophisticated equipment requirements and with

low production cost to obtain immobilized cell catalysts by gel entrapment. It allows the production of small beads with high productivities and may be scaled up at any desired capacity.

ACKNOWLEDGMENTS

The authors acknowledge technical assistance from Javier Torres and style reviewing from Octavio T. Ramírez.

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