Effect of Particle Loading on GM-CSF Production by *Saccharomyces cerevisiae* in a Three-Phase Fluidized Bed Bioreactor

Chin-Hang Shu and Shang-Tian Yang*
Department of Chemical Engineering, The Ohio State University, 140 West 19th Avenue, Columbus, Ohio 43210

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Continuous production of a recombinant murine granulocyte-macrophage colony-stimulating factor (MuGM-CSF) by immobilized yeast cells, *Saccharomyces cerevisiae* strain XV2181 (a/a, Trp1) containing plasmid pαADH2, in a fluidized bed bioreactor was studied at a 0.03 h^{-1} dilution rate and various particle loading rates ranging from 5% to 33% (v/v). Cells were immobilized on porous glass beads fluidized in an air-lift draft tube bioreactor. A selective medium containing glucose was used to start up the reactor. After reaching a stable cell concentration, the reactor feed was switched to a rich, nonselective medium containing ethanol as the carbon source for GM-CSF production. GM-CSF production increased initially and then dropped gradually to a stable level. During the same period, the fraction of plasmid-carrying cells declined continuously to a lower level, depending on the particle loading. The relatively stable GM-CSF production, despite the large decline in the fraction of plasmid-carrying cells, was attributed to cell immobilization. As the particle loading rate increased, the plasmid stability also increased. Also, as the particle loading increased from 5% to 33%, total cell density in the bioreactor increased from 16 to 36 g/L, and reactor volumetric productivity increased from 0.36 to 1.31 mg/L·h. However, the specific productivity of plasmid-carrying cells decreased from 0.55 to 0.07 mg/L·g cell. The decreased specific productivity at higher particle loading rates was attributed to reduced growth efficiency caused by nutrient limitations at higher cell densities. Both the reactor productivity and specific cell productivity increased by two- to threefold or higher when the dilution rate was increased from 0.03 to 0.07 h^{-1}.
Key words: bioreactor, fluidized bed • murine granulocyte-macrophage colony stimulating factor • *Saccharomyces cerevisiae*

INTRODUCTION

The yeast, *Saccharomyces cerevisiae*, grows rapidly in a relatively simple medium to high cell densities with high levels of product secretion. *S. cerevisiae* also possesses an eukaryotic subcellular organization capable of some posttranslational modifications, which are required for most mammalian proteins. Thus, *S. cerevisiae* has been used as a host organism for production of several recombinant proteins,[12] including granulocyte-macrophage colony-stimulating factor (GM-CSF).[28] GM-CSF is one of the four specific glycoproteins that stimulate a population of committed granulocyte-macrophage progenitor cells to generate granulocytes and macrophages, two important white blood cells.[17] The carbohydrate portion of GM-CSF is not required for biological activity. GM-CSF has been cloned and expressed in bacterial, yeast, and mammalian cells and is now mass-produced for clinical uses.[18] However, information regarding the production of recombinant GM-CSF is scarce in the available literature.[3]

One major concern in recombinant cell fermentation is plasmid loss during fermentation, which results in reduced product yield and reactor productivity.[4] Immobilization of recombinant cells offers an attractive alternative to traditional fed-batch and chemostat processes. Because of reduced cell growth and selection between plasmid-containing and plasmid-free cell populations, improved plasmid stability and productivity can be obtained with cell immobilization.[8,13,23,25,29,33,36,37] Through immobilization, the productive plasmid-carrying cells can be retained in the reactor while daughter cells, revertant and unproductive, are washed out. The increased plasmid stability in immobilized cells also may result from mechanical properties of the gel bead system used, which limits cell divisions.[2]

Fluidized bed reactors (FBR) have been widely used in biological wastewater treatment. Recently, FBRs have also attracted great attention for potential applications in fermentation because of their capability to achieve high mass transfer rates, high cell densities, and high reactor productivities.[6,10,14,15,21,26,27,30,34,35,38] Compared with other types of immobilized cell bioreactors, the fluidized bed provides better solid and liquid mixing, higher oxygen transfer, easier CO_2 removal, easier cell regeneration, and a more uniform cell population in the reactor than does the membrane or packed bed bioreactor. However, cell density per unit volume is potentially lower in a fluidized bed than in a packed bed or membrane system because of packing considerations. The overall performance of the fluidized bed bioreactor

* To whom all correspondence should be addressed.

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depends on its operating conditions. A draft tube three-phase fluidized bed bioreactor is considered to give higher overall gas loading and solid loading than those from conventional three-phase fluidized beds.[9,22] Because of its simple construction, a three-phase draft tube fluidized bed bioreactor reduces the possibility of contamination.[32] Additionally, the use of gas to circulate the contents of a fermentor through a draft tube is a convenient means of achieving good mixing and aeration. Such airlift design is of simple construction and operation, has low power consumption yet improved oxygen transfer,[19] and is thus attractive for large-scale operation.[5] The main objective of this work was to study the feasibility of using a three-phase, draft-tube, fluidized bed bioreactor for continuous production of GM-CSF by recombinant yeast cells immobilized on porous glass beads. Fermentation kinetics and reactor stability were studied.

Hydrodynamic behavior of three-phase fluidized bed bioreactors is rather complicated and not well understood. Of the many hydrodynamic properties of three-phase fluidized bed bioreactors, phase holdup is one major factor affecting the mass transfer characteristics and reaction rate in the reactor. Solid loading in three-phase fluidized bed bioreactors also affects the volume of immobilized cell supports. An increased solid loading rate implies that increased cell density can be obtained for fermentation. As the solid loading rate increases, the hydrodynamic behavior of a fluidized bed bioreactor also changes. Consequently, the physiology of immobilized cells and reactor performance also could be affected by solid loading rate. The effects of solid loading rate on GM-CSF production, reactor productivity, and plasmid stability in the continuous fermentation with a nonselective rich medium were thus studied and are reported in this article.

MATERIALS AND METHODS

Culture

The *Saccharomyces cerevisiae* strain XV2181 used in this study was obtained from Immunex (Seattle, WA). This yeast cell contained the vector pαADH2, which consisted of pBR322, the yeast TRP1 gene, 2μ origin of replication, and cDNA for murine GM-CSF. Gene expression of the protein was controlled by the glucose-repressible ADH2 promoter. The selective marker for this tryptophan auxotroph is TRP1 gene. Detailed description of this vector can be found elsewhere.[28] The recombinant yeast culture was maintained on agar plates with biweekly transfer.

Media

Two different media were used. The selective medium contained 6.7 g/L yeast nitrogen base without amino acids (Difco), 5 g/L casamino acid (Difco), 20 g/L glucose, 10 mg/L adenine, and 20 mg/L uracil. The use of acid-hydrolyzed casamino acid provided a tryptophan-free amino acid source; thus the medium is selective for the plasmid-carrying cells. This selective medium was used in preparing inocula and for reactor start-up. A rich (nonselective) medium containing 10 g/L yeast extract, 20 g/L peptone, 80 mg/L adenine, 80 mg/L uracil, and 20 g/L ethanol was used in continuous fermentation study. Ethanol, instead of glucose, was used in the rich medium to ensure that the ADH2 promoter was not repressed by glucose and to attain high product expression level. The pH of these media was about 6.5. For agar plates used in colony counts, the media also contained 2% (wt/v) Bacto-agar.

Bioreactor Construction

A 0.5-L draft tube air-fluidized bioreactor with a working volume of 350 mL was used in this work. The bioreactor was constructed with a glass column (30 cm $\times$ 5 cm), an inner glass tube (20 cm $\times$ 2.6 cm) located at the center as the draft tube, and a sintered glass filter disk (3.0 cm diameter; 60 μm pore size). Porous glass beads (Siran, Cat. # 012-0.5-120A, Schott Engineering, Mainz, Germany) were used for cell immobilization. These glass beads had an average diameter of 1 mm, porosity of 60%, pore diameter of 120 μm, and density of 2.07 g/cm^3. The surface of the beads was hydrophobic, with a surface area of 0.2 m^2/g. The bioreactor was equipped with a dissolved oxygen (DO) probe and a pH electrode to monitor DO and pH. The reactor was maintained at a constant temperature by circulating constant-temperature water through the reactor water jacket. Unless otherwise noted, the bioreactor was maintained at 30°C and was aerated at a volumetric air flow rate of 1.5 std L/min.

Reactor Start-Up

The bioreactor containing the selective medium was inoculated with 5 mL of 24-hour-old culture grown in the selective medium. The cells were allowed to grow in the bioreactor for ~24 h. The bioreactor was then continuously fed with the selective medium at a dilution rate of 0.03 h^{-1} for 3 to 4 days to allow plasmid-carrying cells to grow to a high density in the reactor. During this start-up period, cells were also immobilized onto the glass beads by adsorption. After the cell density in the effluent had reached a steady value, an indication of obtaining an equilibrium immobilized cell density on glass beads, the fermentation was shifted to GM-CSF production phase by feeding the reactor with the rich medium at the same dilution rate of 0.03 h^{-1}.

Continuous Fermentation Studies

The continuous fermentation was studied for four particle loading rates: 5%, 10%, 20%, and 33% (v/v). The

reactor pH was not controlled, and the effluent pH varied from 3.1 to 5.7, depending on the conversion, which varied because of differences in cell density in the immobilized cell bioreactor. The reactor performance was studied at a low dilution rate of 0.03 h^{-1} for a period of 80 to 160 h. To evaluate the effect of dilution rate on reactor productivity, the dilution rate was then switched between 0.03 and 0.11 h^{-1} every 12 h. The reactor was operated at the average dilution rate of 0.07 h^{-1} for ~120 h. Effluent samples were taken and assayed for cell density, cell viability, substrate and product concentrations, and plasmid stability. At the end of each bioreactor study, the total cell density in the immobilized cell bioreactor was estimated. Immobilized cells in the glass beads were also analyzed for cell viability and plasmid stability, and examined by scanning electron microscopy (SEM).

Determination of Immobilized Cell Concentration

The concentration of immobilized cells on glass beads was determined by dry weight measurement. The cell dry weight was calculated by the dry weight difference before and after cell removal from the glass beads. A sample of glass beads of about 10.0 g was removed from the reactor at the end of each bioreactor study, and the sample was dried in a vacuum oven at 70°C overnight. After this sample was weighed, the immobilized cells were removed and lysed by boiling in 4.0N KOH, followed by washing with distilled water. A similar size sample of glass beads without cell immobilization was also treated in the same way to correct for possible corrosive effects on glass beads caused by the base used in removing cells. The results showed that there was ~4% weight loss of glass beads caused by treatment with the base. All measurements were duplicated and the average value is reported.

Removal of Immobilized Cells from Glass Beads

At the end of each fermentation study, a sample of ~3-g glass beads was taken as sample from the reactor. The sample was put into a test tube containing 10 mL of sterile distilled water. The immobilized cells were removed from glass beads by vortexing vigorously for 1 min. The removed cells in suspension were then separated from the glass beads. These cells removed from the first wash were considered as loosely immobilized. The glass beads were washed again with new water to remove more strongly attached cells. The wash process was repeated several times until no more cells could be removed. The viability and fraction of plasmid-carrying cell population in the suspended cells collected from each wash were assayed. Cell samples from all washes were also combined and assayed to determine the fraction of plasmid-carrying cells in the total immobilized cell population. All samples and measurements were duplicated.

Scanning Electron Microscopy

Samples of glass beads removed from the bioreactor were immersed in 2.5% glutaraldehyde solution overnight at 4°C, completely rinsed with distilled water, and then dehydrated progressively with 20% to 70% ethanol, in increments of 10%, by holding them at each concentration for 30 min. The partially dehydrated samples were left in 70% ethanol for overnight at 4°C, and then dehydrated progressively with 80% to 100% ethanol. These samples were then cryogenically dried at critical point with liquid CO_2. The completely dried samples were coated with gold/palladium before SEM photographs were taken using a Joel Model JSM-820 SEM.

Assay Methods

Cell Concentration

Cell concentration was determined by measuring the optical density at 660 nm (OD_{660}) of the cell suspension in a 1.5-mL polystyrene cuvette (with a light path of 10 mm) and comparing the measured value to a standard curve. One unit of OD was found to be equivalent to 0.64 g/L cell when OD was less than 0.45. Samples were diluted with distilled water if the OD was greater than 0.4.

Glucose and Ethanol Concentrations

Concentrations of ethanol and glucose in the sample solution were analyzed with high performance liquid chromatography (HPLC) following procedures previously described elsewhere.[40]

GM-CSF Concentration

The concentration of GM-CSF was determined using a reverse-phase HPLC.[31] Twenty-five microliters of cell-free sample solution were injected into a reverse-phase column (Vydac C4, 5 mm reverse-phase silica). A binary solvent system (Solvent A: 0.1% trifluoroacetic acid [TFA, Sigma] in water; Solvent B: 0.1% TFA in acetonitrile) was used as the eluent, at a flow rate of 1.0 mL/min, to separate the GM-CSF from other proteins in the sample. A gradient controller (Isco Model 2360 gradient programmer) was used to provide linear gradients of 0% to 100% Solvent B in the eluent at the following increasing rates: 3% per min for 10 min, 0.67% per min for 30 min, and 5% per min for 10 min. Absorbance at 280 nm was measured using a UV spectrophotometer (Waters Lambda-Max Model 481 LC). A standard containing 100 mg/L murine GM-CSF provided by Im-

munex Corp. was used to determine the concentration of GM-CSF in fermentation samples. Two to three peaks located within close distances on the chromatogram were identified as GM-CSF proteins with different glycosylations from recombinant yeast fermentation.[28] The concentration of GM-CSF in each sample was determined from the total peak area of these peaks.

Cell Viability

The viability of yeast cells present in the effluent and in the glass beads was measured using a modified staining method.[16] The staining solution used was Ringer salt solution containing 0.03% methylene blue (composition: NaCl, 0.9 g; KCl, 0.042 g; $CaCl_2$, 0.048 g; $NaHCO_3$, 0.02 g; methylene blue, 0.03 g; distilled water, to 100 mL). Cell samples were diluted with Ringer salt solution to $\sim 3.0 \times 10^8$ cells/mL. Then, 0.1 mL of the diluted sample was mixed with 0.9 mL of the staining solution. A hemocytometer was used to count the numbers of colorless cells (viable) and blue-colored cells (dead) within 10 min. The viability was determined by the number of viable cells divided by the total cell count.

Fraction of Plasmid-Carrying Cells

The fraction of plasmid-carrying cells in the total cell population was determined from the ratio of the colony counts on the selective medium agar plate and the rich (nonselective) medium agar plate. Cell samples were diluted to get the colony counts within the range of 30 to 300. All plate counts were taken from the average of at least three replicates.

RESULTS AND DISCUSSION

Reactor Start-Up

Typically, the bioreactor reached steady state, as indicated by the stable glucose, ethanol, and cell concentrations in the reactor effluent, within 70 h. During the 3-day start-up period, the reactor pH decreased to ~3.0, the effluent cell density increased to 2.6 g/L, glucose concentration stabilized at 4.6 g/L, cell viability declined to ~80%, and the fraction of plasmid-carrying cells remained at ~96%.

Reactor Performance

The time-course data of four continuous fermentations at 5%, 10%, 20%, and 33% particle loading rate are shown in Figures 1 to 4, respectively. As is seen in these figures, the fraction of plasmid-carrying cells dropped significantly as fermentation time increased. The production of GM-CSF increased initially but then decreased gradually to a stable lower level. Both cell density and ethanol concentration in the effluent were stable

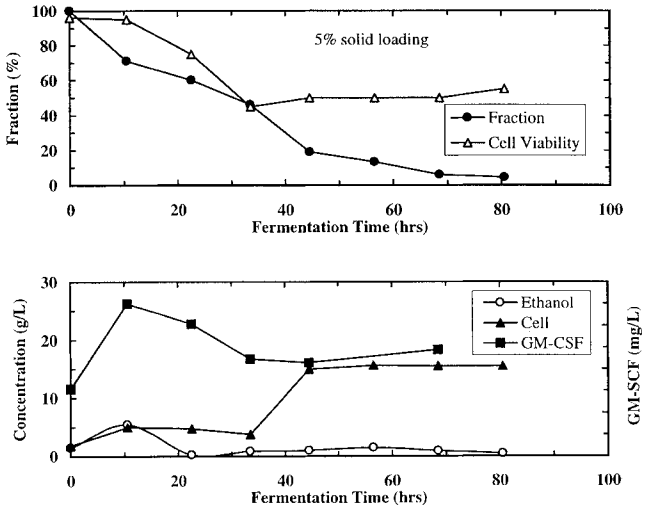

Figure 1. Kinetics of continuous fermentation in the fluidized bed with 5% (v/v) particle loading (Fraction: fraction of plasmid-carrying cells).

throughout these continuous fermentations. The effluent pH from reactors with lower solid loading (5% and 10%) remained at ~ 3.2 throughout the fermentation, but pH value from reactors with high solid loading (20% and 33%) increased with fermentation time to ~5.5. Cell viability in effluent from reactors with high solid loading was generally higher than 75%. However, with low solid loading, cell viability decreased gradually and reached a low value of ~50%.

Immobilized Cell Density

The scanning electron micrographs (Fig. 5) of glass beads removed at the end of the study showed cells were immobilized by attachment to the external as well as internal pore surfaces of the glass bead.

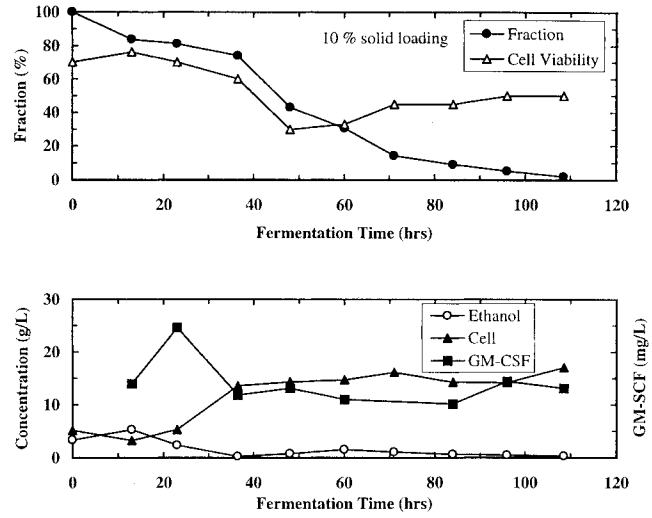

Figure 2. Kinetics of continuous fermentation in the fluidized bed with 10% (v/v) particle loading (Fraction: fraction of plasmid-carrying cells).

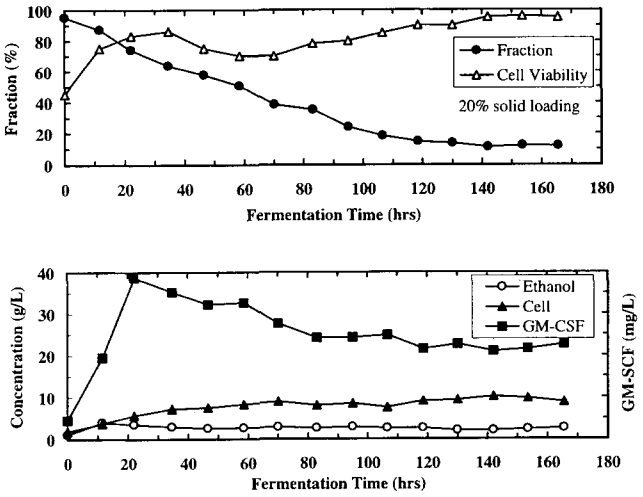

Figure 3. Kinetics of continuous fermentation in the fluidized bed with 20% (v/v) particle loading (Fraction: fraction of plasmid-carrying cells).

As shown in Table I, as particle loading increased from 5% to 33%, the density of immobilized cells increased from 4.88 to 28.68 g/L, but the concentration of freely suspended cells decreased from 11.28 to 6.63 g/L. Although the total cell density in the reactor increased linearly from 16.16 to 35.31 g/L with increasing particle loading, the immobilized cell density per gram of glass beads decreased from 0.16 to 0.098 g/g as particle loading increased (Fig. 6). The lower immobilized cell density per particle at the higher particle loading indicated that there might be nutrient limitations at high particle loading and high cell density under the conditions studied.

Cell Viability

In general, cell viability of the immobilized cells should be lower than, if not the same as, that of free cells

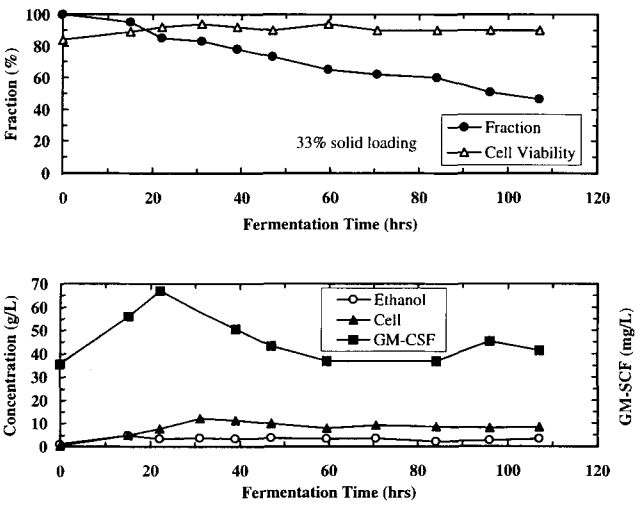

Figure 4. Kinetics of continuous fermentation in the fluidized bed with 33% (v/v) particle loading (Fraction: fraction of plasmid-carrying cells).

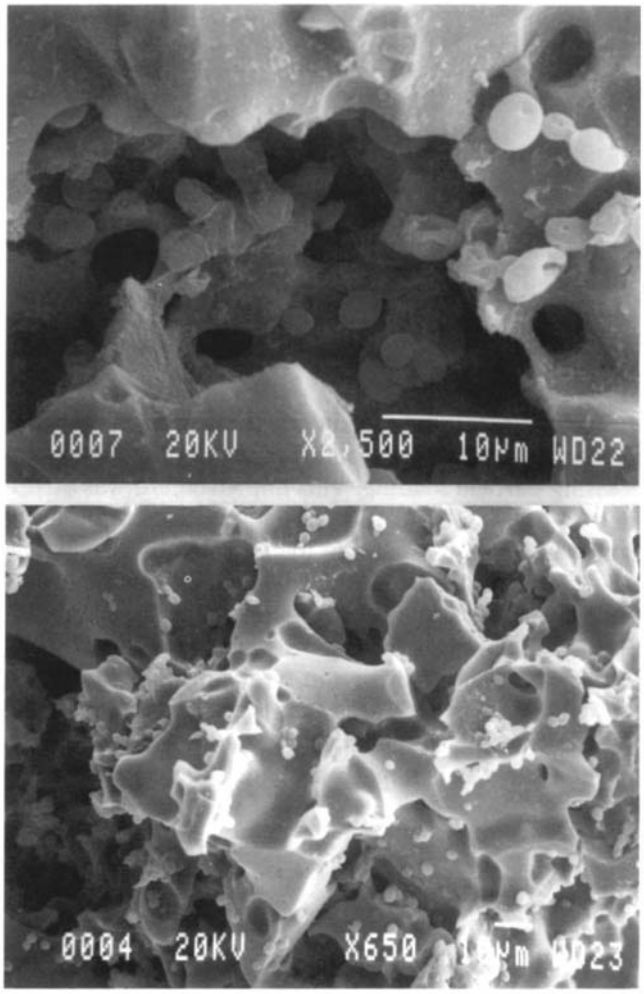

Figure 5. Scanning electron micrographs of yeast cells immobilized in the glass bead.

since immobilized cells should be older and subjected to nutrient limitations. Viability of immobilized cells was 5% and 15% lower than that of suspended cells for reactors with 20% and 33% particle loading, respectively. However, at the two lower particle loading rates (which also had a lower pH), viability of suspended cells was ~50%, but viability of immobilized cells was much higher, 75% to 85%. This indicated that immobilization might have protected cells from adverse environmental conditions. For the reactors with lower particle loading, there were more suspended cells subjected to shear and particle–particle collision damages, resulting in greater mortality and higher fraction of dead cells in the effluent cell population. When the reactor dilution rate was increased to 0.07 h^{-1}, the suspended cell concentration decreased and cell viability increased to 85% or higher (Table II).

Plasmid Stability

The fraction of plasmid-carrying cells in the reactor effluent decreased with time. As shown in Figure 7, the decay of the fraction of plasmid-carrying cells was

Table I. Cell concentrations in the fluidized bed bioreactor with various particle loading rates.

Particle loading rate (v/v)	Immobilized cells Conc. (g/L)	Immobilized cells Viability (%)	Suspended free cells Conc. (g/L)	Suspended free cells Viability (%)	Total cells conc.[a] (g/L)
5%	4.88	85	11.28	50	16.17
10%	8.12	75	11.28	50	19.40
20%	14.48	85	7.17	90	21.65
33%	28.68	75	6.63	90	35.31

[a]Total cell concentration in the bioreactor on the basis of reactor working volume.

slower for reactors with higher particle loading rates, indicating that plasmid stability was improved by increasing particle loading. This improvement was attributed to the immobilization effect, which became more profound when the particle loading was higher.

For all samples from different particle loading rates studied, the fraction of plasmid-carrying cells in immobilized cells removed from glass beads was found to be similar to that found for the freely suspended cells in the effluent. This indicated that there existed low or no selectivity of plasmid-carrying cells by immobilization on the beads. This might simply be due to the physical characteristics of the particle support used or might result from the hydrodynamics, which had high mixing and particle velocities, in the fluidized bed.

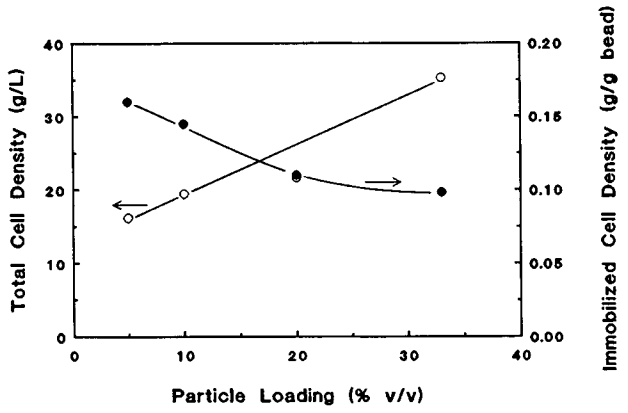

Figure 6. Effects of particle loading on total cell density in the reactor and the immobilized cell density on glass beads.

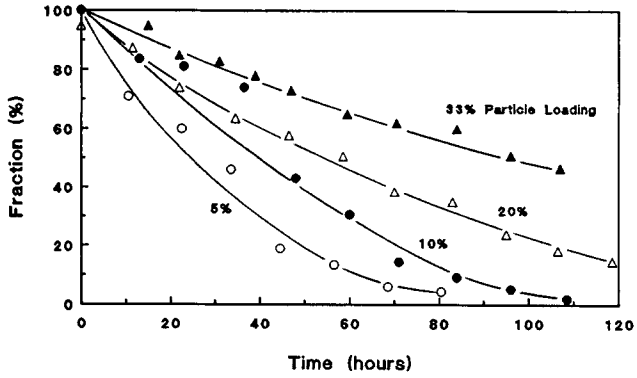

Figure 7. Effect of particle loading on plasmid stability in the immobilized cell bioreactor.

Cell Yield and Product Yield

Based on differences in ethanol, cell, and product concentrations between feed and effluent, cell yield and product yield were calculated. As particle loading increased, cell yield decreased from 0.66 to 0.41 g/g, but product yield increased from 0.7 to 2.7 g/g (Table II). The decreased cell yield at higher particle loading might

Table II. Effects of particle loading rate and dilution rate on GM-CSF fermentation.

D (h^{-1})	PLR (%)	Viab. (%)	$Y_{x/s}$ (g/g)	$Y_{p/s}$ (mg/g)	$Y_{p/x}$ (mg/g)	P (mg/L·h)	P/X^+ (mg/h·g)
0.03	5	50	0.66	0.70	1.06	0.36	0.55
	10	50	0.65	0.74	1.14	0.39	0.39
	20	90	0.47	1.42	3.02	0.68	0.22
	33	90	0.41	2.71	6.68	1.31	0.07
0.07	5	90	0.59	1.74	2.95	1.98	—
	10	85	0.55	1.47	2.67	1.71	—
	20	95	0.41	1.30	3.17	1.50	0.91
	33	93	0.38	2.67	7.03	3.25	0.79

D: dilution rate; PLR: particle loading rate; Viab.: cell viability in the effluent stream; $Y_{x/s}$: cell yield; $Y_{p/s}$: product yield; $Y_{p/x}$: specific GM-CSF production; P: productivity; P/X^+: specific productivity.

be attributed to nutrient limitation at high cell density as a result of high particle loading. The increased product yield at higher particle loading was attributed to the higher plasmid-carrying cell fraction.

Productivity

The average product concentration during the last 48 h of fermentation was used to estimate reactor volumetric productivity. As shown in Table II, productivity increased from 0.36 to 1.31 mg/L·h as particle loading increased from 5% to 33%. However, these productivities were considerably lower than those found with batch cultures, 1.06 mg/L·h at pH 4.0 and 1.77 mg/L·h at pH 6.0.[31] The low productivity obtained from these immobilized cell fermentations was attributed to the low dilution rate, 0.03 h^{-1}, used and the low fraction of plasmid-carrying cells in the reactor. Since GM-CSF production by this recombinant yeast cell was found to be growth-associated, and plasmid stability was better at higher dilution (growth) rate,[31] higher productivities should be attained when the bioreactor is operated at higher dilution (growth) rates than the one used in this study.

The specific cell productivity was calculated based on the density of viable plasmid-carrying cells in the final period of each continuous fermentation. As also shown in Table II, average specific productivity decreased from 0.55 mg/h·g cell to 0.07 mg/h·g cell as particle loading increased. The lower specific productivity at high particle loading rates was attributed to the lower growth rate caused by nutrient limitations at high cell density. Because the growth substrate, ethanol, was not completely consumed, the limiting nutrient should be either the oxygen or nitrogen source. The gas–liquid mass transfer in a fluidized bed is known to be dependent on the particle concentration. In general, the k_La value decreased with increasing particle concentration once the particle concentration was greater than 5% in a well-mixed fluidizing system.[9,11,20,24,39] It is thus important to improve oxygen transfer in the fluidized bed bioreactor at high particle concentrations.

Effects of Dilution Rate

The effects of dilution rate on cell yield, product yield, specific GM-CSF production, volumetric productivity, and specific productivity can be seen in Table II. Increasing the dilution rate from 0.03 to 0.07 h^{-1} improved product yield at the two lower particle loading rates and improved the reactor productivity for all particle loading rates studied. The productivity could be further improved if a higher dilution rate was used. Low dilution rate may result in a low growth rate of immobilized cells and adversely affect the growth-associated recombinant protein production.[41] The specific productivity was also higher at the higher dilution rate, which was also observed in other related studies.[31,41] High dilution rate also could improve plasmid stability.[31]

CONCLUSIONS

It is clear from this study that the fluidized-bed bioreactor can be used for continuous production of recombinant protein. The porous glass beads used in this work were effective for cell immobilization and protected cells from external force damage. Plasmid stability was greatly improved by cell immobilization. In the immobilized cell system, maintenance of a plasmid-carrying cell population should be easier than in a suspended free cell system. This is because segregational plasmid loss and overgrowth of plasmid-free cells are less likely to happen as cell growth is reduced.[1,36] Furthermore, the compartmental cell distribution and mass transfer limitation in the immobilized cell environment also limit the growth of plasmid-free cells.[2,7,8] Cell immobilization also offers the advantages of providing high cell density and high reactor productivity and, thus, can be a preferable alternative to conventional free-cells fermentation processes. The performance of the fluidized bed bioreactor can be further improved by optimizing the particle loading rate and the feed rate. The dilution rates used in this work were low and might have limited the reactor productivity because of nutrient limitations.

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References

1. Bailey, K., Vieth, W. R., Chotani, G. K. 1987. Analysis of bioreactor containing immobilized recombinant cells. Ann. NY Acad. Sci. **506**: 196–208.
2. Barbotin, J. N., Berry, F., Briasco, C., Huang, J., Nasri, M., Sayadi, S., Thomas, D. 1989. Immobilization effects on the stability of recombinant microorganisms. Chimicaoggi **Nov**: 49–52.
3. Caldwell, J., Locey, B., Clarke, M. F., Emerson, S. G., Palsson, B. O. 1991. Influence of medium exchange schedules on metabolic, growth, and GM-CSF secretion rates of genetically engineered NIH-3T3 cells. Biotechnol. Prog. **7**: 1–8.
4. Caunt, P., Impoolsup, A., Greenfield, P. F. 1988. Stability of recombinant plasmids in yeast. J. Biotechnol. **8**: 173–192.
5. da Fonseca, M. M., Black, G. M., Webb, C. 1986. Reactor configurations for immobilized cells, pp. 63–74. In: C. Webb, G. M. Black, and B. Atkinson (eds.), Process engineering aspects of immobilized cell systems. Institution of Chemical Engineers, Rugby, Warks, UK.
6. Davison, B. H., Scott, C. D. 1992. A proposed biparticle fluidized-bed for lactic acid fermentation and simultaneous adsorption. Biotechnol. Bioeng. **39**: 365–368.
7. De Taxis du Poet, P., Dhulster, P., Barbotin, J.-N., Thomas, D. 1986. Plasmid inheritability and biomass production: comparison between free and immobilized cell culture of *Escherichia coli* BZ18(pTG201) without selection pressure. J. Bacteriol. **165**: 871–877.

8. Dincbas, V., Hortacsu, A., Camurdan, A. 1993. Plasmid stability in immobilized mixed cultures of recombinant *Escherichia coli.* Biotechnol. Prog. **9**: 218–220.
9. Fan, L. S., Hwang, S.-J., Matsuura, A. 1984. Hydrodynamic behavior of draft tube gas-liquid-solid spouted bed. Chem. Eng. Sci. **39**: 1677.
10. Gilson, C. D., Thomas, A. 1995. Ethanol production by alginate immobilized yeast in a fluidized bed bioreactor. J. Chem. Technol. Biotechnol. **62**: 38–45.
11. Gollakota, S. V., Guin, J. A. 1984. Comparative study of gas–liquid mass transfer coefficients in stirred autoclaves, tubing bomb microreactors, and bubble columns. Ind. Eng. Chem. Res. Dev. **23**: 52.
12. Heslot, H., Gaillardin, C. 1991. Molecular biology and genetic engineering of yeasts. CRC Press, Boca Raton, FL.
13. Kanayama, H., Sode, K., Karube, I. 1988. Continuous hydrogen evolution by immobilized recombinant *E. coli* using a bioreactor. Biotechnol. Bioeng. **32**: 396–399.
14. Kratje, R. B., Wagner, R. 1992. Evaluation of production of recombinant human interleukin-2 in fluidized bed bioreactor. Biotechnol. Bioeng. **39**: 232–242.
15. Kratje, R. B., Reimann, A., Hammer, J. 1994. Cultivation of recombinant baby hamster kidney cells in a fluidized bed bioreactor system with porous borosilicate glass, Biotechnol. Prog. **10**: 410–420.
16. Lee, S. S., Robinson, F. M., Wang, H. Y. 1981. Rapid determination of yeast viability. Biotechnol. Bioeng. Symp. **11**: 641–649.
17. Metcalf, D., 1985. The granulocyte-macrophage colony-stimulating factors. Science **229**: 16–22.
18. Metcalf, D. 1991. Control of granulocyte and macrophages: molecular, cellular, and clinical aspects. Science **254**: 529–533.
19. Mercer, D. G. 1981. Flow characteristics of a pilot-scale airlift fermentor. Biotechnol. Bioeng. **23**: 2421–2431.
20. Mills, D. B., Bar, R., Kirwan, D. J. 1987. Effect of solids on oxygen transfer in agitated three-phase systems, AIChE J. **33**: 1542–1549.
21. Morikawa, Y., Karube, I., Suzuki, S. 1980. Continuous production of bacitracin by immobilized living whole cells of *Basillus* sp. Biotechnol. Bioeng. **22**: 1015–1023.
22. Muroyama, K., Mitani, Y., Yasunishi, A. 1985. Hydrodynamic characteristics and gas–liquid mass transfer in a draft tube slurry reactor. Chem. Eng. Commun. **34**: 87.
23. Nasri, M., Sayadi, S., Barbotin, J.-N., Dhulster, P., Thomas, D. 1987. Influence of immobilization on the stability of pTG201 recombinant plasmid in some strains of *Escherichia coli.* Appl. Environ. Microbiol. **53**: 740–744.
24. Nienow, A. W., Konno, M., Bujaiski, W. 1986. Studies on three-phase mixing: a review and recent results. Chem. Eng. Res. Des. **64**: 35.
25. Oriel, P. 1988. Immobilization of recombinant *Escherichia coli* in silicone polymer beads. Enzyme Microb. Technol. **10**: 519–523.
26. Paz, E. D. D., Santana, M. H. A., Eguchi, S. Y. 1993. Enhancement of the oxygen transfer in a circulating three-phase fluidized bed bioreactor. Appl. Biochem. Biotechnol. **39/40,** 455–466.
27. Petersen, J. N., Davison, B. H. 1995. Development of a predictive description of an immobilized-cell, three-phase, fluidized-bed bioreactor. Biotechnol. Bioeng. **46**: 139–146.
28. Price, V., Mochizuki, D., March, C. J., Cosman, D., Deeley, M. C., Klinke, R., Clevenger, W., Gills, S., Baker, P., Urdal, D. 1987. Expression, purification and characterization of recombinant murine granulocyte-macrophage colony-stimulating factor and bovine interleukin-2 from yeast. Gene **55**: 287–293.
29. Sayadi, S., Nasri, M., Barbotin, J. N. Thomas, D. 1989. Effect of environmental growth conditions on plasmid stability, plasmid copy number, and catechol 2,3-dioxygenase activity in free and immobilized *Escherichia coli* cells. Biotechnol. Bioeng. **33**: 801–808.
30. Shen, B., Greenfield, P., Reid, S. 1994. Calcium alginate immobilized hybridomas grown using a fluidized bed perfusion system with a protein-free medium. Cytotechnology **14**: 109–117.
31. Shu, C. H. 1992. Multiphase bioreactors for recombinant yeast fermentation, Ph.D. dissertation, Ohio State University, Columbus, OH, USA.
32. Siegel, M. H., Hallaile, M., Merchuk, J. C. 1988. Air-lift reactors: design, operation, and applications, pp. 79–124. In: Upstream processes: equipment and techniques. A. R. Liss, New York.
33. Sode, K., Morita, T., Peterhans, A., Meussdoerffer, F., Mosbach, K., Karube, I. 1988. Continuous production of α-peptide using immobilized recombinant yeast cells. J. Biotechnol. **8**: 113–122.
34. Terashima, S., Kamihira, M., Ogawa, T., Ohno, M., Iijima, S., Kobayashi, T. 1994, Continuous production of human erythropoietin by immobilized recombinant L-929 cells. J. Ferment. Bioeng. **77**: 52–56.
35. Tzeng, J.-W., Fan, L. S., Gan, Y.-R. 1991. Ethanol fermentation using immobilized cells in a multistage fluidized bed bioreactor. Biotechnol. Bioeng. **38**: 1253–1258.
36. Walls, E. L., Gainer, J. L. 1989. Retention of plasmid bearing cells by immobilization. Biotechnol. Bioeng. **34**: 717–724.
37. Walls, E. L., Gainer, J. L. 1991. Increased protein productivity from immobilized recombinant yeast. Biotechnol. Bioeng. **37**: 1029–1036.
38. Weuster, B. D. 1993. Continuous ethanol production by *Zymomonas mobilis* in a fluidized bed reactor. Part I. Kinetic studies of immobilization in macroporous glass beads. Appl. Microbiol. Biotechnol. **39**: 679–684.
39. Wiedman, J. A., Steiff, A., Weinspach, P. M. 1985. Suspension behavior of two and three-phase stirred reactors. Ger. Chem. Eng. **8**: 321.
40. Yang. S. T., Tang, I. C., Okos, M. R. 1987. Kinetics of homoacetic fermentation of lactate by *Clostridium formicoaceticum.* Appl. Environ. Microbiol. **53**: 823–827.
41. Yang, S. T., Shu, C. H. Kinetics and stability of GM-CSF production by recombinant yeast cells immobilized in a fibrous-bed bioreactor. Biotechnol. Prog. (in press).