Kinetics and Stability of GM-CSF Production by Recombinant Yeast Cells Immobilized in a Fibrous-Bed Bioreactor

Shang-Tian Yang* and Chin-Hang Shu

Department of Chemical Engineering, The Ohio State University, 140 West 19th Avenue, Columbus, Ohio 43210

The continuous production of murine granulocyte−macrophage colony-stimulating factor (GM-CSF) by recombinant yeast cells immobilized in a fibrous-bed bioreactor was studied. A high cell density of ~68 g/L and a GM-CSF productivity of ~3.5 mg/L·h were attained in the fibrous-bed bioreactor fed with a rich (nonselective, pH 6.7) medium at a dilution rate of 0.16 h^{-1}. The GM-CSF production was stable even though the fraction of plasmid-carrying cells in the reactor effluent gradually dropped below 5% over a period of 2 weeks. At the end of that period, the immobilized cells in the fibrous matrix still had a high fraction, ~26%, of plasmid-carrying cells. Similar results were obtained with reactors operated at 0.05 h^{-1} dilution rate and pH 4.0. Although the GM-CSF production was lower at pH 4, the reactor was stably operated for over 4 weeks without contamination or significant loss of productivity. The stable long-term GM-CSF production from the fibrous-bed bioreactor was attributed to the effect of cell immobilization on plasmid stability. Because GM-CSF production was growth-associated, as was found in batch fermentation with free cells, this stabilization effect cannot be attributed solely to the reduced cell growth in the immobilized cell environment. Plasmid-carrying cells were preferentially retained in the fibrous matrix, perhaps because their abilities to adhere to the fiber surface and to form cell aggregates were higher than those of plasmid-free cells.

Introduction

Granulocyte−macrophage colony-stimulating factor (GM-CSF) is one of the four specific glycoproteins that stimulate a population of committed granulocyte−macrophage progenitor cells to generate the granulocytes and macrophages, two important types of white blood cells (Metcalf, 1985). 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 (Metcalf, 1991). However, information regarding the production of recombinant GM-CSF is scarce in the available literature (Caldwell et al., 1991).

The yeast *Saccharomyces cerevisiae* grows rapidly in a relatively simple medium to high cell densities with high levels of product secretion. The yeast also possesses a eukaryotic subcellular organization capable of some posttranslational modifications, which are required for most mammalian proteins. Thus, yeast has been used as a host organism for the production of several recombinant proteins (Heslot and Gaillardin, 1991), including GM-CSF (Price et al., 1987). The recombinant *S. cerevisiae* strain XV2181 containing the vector pαADH2, which consists of pBR322, the yeast *TRP1* gene, the 2μ origin of replication, and cDNA for murine GM-CSF, was studied in this work. Gene expression of the GM-CSF protein was controlled by the glucose-repressible ADH2 promoter. The selective marker for this tryptophan auxotroph is the *TRP1* gene. A detailed description of this vector has been given by Price et al. (1987), but little is known about the fermentation kinetics of this recombinant yeast.

Plasmid instability is a major concern in industrial fermentations using recombinant microorganisms to produce heterologous protein products (Caunt et al., 1988). The synthesis of a foreign gene product is usually toxic to the host cell; therefore, poor plasmid stability and a low growth rate of recombinant cells are usually found. Plasmid loss during the course of a fermentation causes the formation of nonproductive, plasmid-free cells, which usually outgrow plasmid-carrying cells in fermentation unless selective pressure can be effectively employed. The consequence of plasmid instability is a significantly reduced production level of the desired protein product. Although the employment of selective pressure during fermentation may effectively maintain plasmid stability, it is generally cost-ineffective and undesirable for large-scale fermentations.

Cell immobilization has been used to overcome or alleviate plasmid instability problems and to enhance reactor productivity in recombinant cell fermentations (Barbotin, 1994; D'Angio et al., 1994; Dincbas et al., 1993; Joshi and Yamazaki, 1987; Kanayama et al., 1988; Nasri et al., 1987; Oriel, 1988; Sayadi et al., 1989; Sode et al., 1988; Walls and Gainer, 1991). In the immobilized cell system, the maintenance of a plasmid-carrying cell population becomes easier as segregational plasmid loss and overgrowth of plasmid-free cells are less likely to happen because of the reduced cell growth (Bailey et al., 1987; Walls and Gainer, 1989) and the compartmental cell distribution and mass-transfer limitation in the immobilized cell environment (Barbotin et al., 1989; De Taxis du Poet et al., 1986; Dincbas et al., 1993). Immobilization also offers the advantage of providing high cell density and high reactor productivity and, thus, may be a preferable alternative to conventional free cells batch or chemostat processes.

However, conventional cell immobilization methods using either covalent cross-linking with glutaraldehyde or entrapment in a gel matrix are usually complicated by problems such as alteration of cell physiology (Doran and Bailey, 1986; Kolot, 1988), decreased efficiency of

* Author to whom correspondence should be addressed: telephone, (614) 292-6611; fax, (614) 292-3769; e-mail, yangst.15@osu.edu.

S8756-7938(96)00037-9 CCC: $12.00

nutrient and product transport (Fonseca et al., 1986; Tanaka and Nakajima, 1990), and increased risk of contamination during the cell immobilization process. Thus, a simple *in situ* immobilization method, such as cell adsorption and entrapment in a fibrous matrix packed in a bioreactor, as previously described by Lewis and Yang (1992), would be desirable. The fibrous-bed bioreactor has been shown to yield high productivity with excellent long-term stability for several bacterial fermentations because of the high viable cell densities maintained in the reactor and its unique packing structure, allowing the free flow of gas, liquid, and solid in the reactor bed (Yang et al., 1994; Silva and Yang, 1995).

In this work, the feasibility of using the fibrous-bed bioreactor to achieve stable, long-term production of a recombinant GM-CSF with a rich (nonselective) medium was studied. Fermentation kinetics and reactor stability were studied at two different dilution rates. The performance of the immobilized cell bioreactor was compared with that of batch fermentations with free cells. The mechanism of cell immobilization and its effects on reactor productivity and stability are also discussed.

Materials and Methods

Culture. *Saccharomyces cerevisiae* XV2181 (pαADH2) used in this study was obtained from Immunex (Seattle, WA). A detailed description of the vector pαADH2 can be found elsewhere (Price et al., 1987). 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. This selective medium was used in preparing inocula for batch fermentations and during reactor startup for continuous fermentations. 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 the 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 a high product expression level. Without any adjustment, the pH of both of these media was about 6.7. A low-pH medium (pH 4.0) was obtained by adding HCl to the medium. For agar plates used in colony counts, the media also contained 2% (wt/vol) Bacto agar.

Batch Fermentation. Batch fermentations were carried out in 5-L fermentors (Marubishi MD-300) at 30 °C. The fermentor containing 3 L of the rich medium was inoculated with a 100-mL flask culture grown overnight in the selective medium. The fermentation was controlled at a constant pH by adding 0.5 N HCl. The agitation rate was 500 rpm and the aeration rate was 3 L/min (air at 1 atm and 20 °C). Plasmid stability and the concentrations of ethanol, biomass, and GM-CSF were monitored throughout the fermentation.

Continuous Fermentation. ***Bioreactor Construction.*** Figure 1 shows the schematic diagram of the fibrous-bed bioreactor system used in this study. The bioreactor was made of a glass column (5-cm i.d., 35-cm height) containing a spiral-wound fibrous sheet material and had a total volume of ~0.5 L. A sintered glass filter disk (3-mm diameter, 50-μm pore size), which was fitted to the bottom of the glass column, was used as an air sparger to generate fine bubbles for efficient aeration. The fibrous matrix used for cell immobilization was made of knitted cotton cloth (13 × 30 cm and ~5 mm thick) on a stainless steel wire cloth (mesh #20, Small Parts Inc.). A spiral configuration with an ~8-mm gap between two adjacent layers was formed by winding the matrix around a stick. The fibrous matrix was placed 2 cm above the sparger with the axis of the spiral parallel to the fluid motion. Both ends of the glass column were close-fitted with no. 14 rubber stoppers. The packed fibrous matrix in the bioreactor was 13 cm long and had ~95% void volume. The reactor working (liquid) volume was ~300 mL. The feed was pumped into the bioreactor by using a peristaltic pump. 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 0.25 std L/min. The reactor pH was not controlled for the single-pass continuous fermentation.

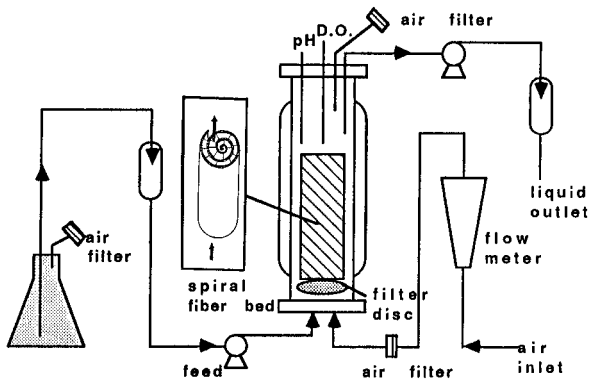

Figure 1. Schematic diagram of the fibrous-bed bioreactor system.

Reactor Startup and Operation. For reactor startup, a 5-mL, 24-h-old tube culture was introduced into the bioreactor containing 300 mL of the selective medium. The yeast cells were allowed to grow in the bioreactor for 18 h. Then the bioreactor was continuously fed with the selective medium at a dilution rate of 0.05 h^{-1} for ~7 days to allow plasmid-carrying cells to grow to a high density in the reactor. During this startup period, cells were immobilized in the fibrous matrix by adsorption and entrapment. The feed medium was then changed to the rich medium to provoke GM-CSF production in the fibrous-bed bioreactor. The dissolved oxygen (DO) level in the reactor was monitored and maintained at 40% of saturation or higher by adjusting the air flow rate.

Reactor Study. The continuous fermentation was first studied with the high-pH medium (pH 6.7) at a dilution rate of 0.16 h^{-1}. Under this condition, the reactor effluent pH was ~6.0. This study continued for ~2 weeks and was then stopped when contamination was found in the reactor effluent at the end of the 15-day operation. To minimize the risk of contamination during long-term continuous operation, the low-pH medium (pH 4.0) was then used in the second continuous bioreactor study at a dilution rate of 0.05 h^{-1}. No contamination was found during the 4-week period studied. A third bioreactor study was also carried out with the low-pH medium (pH 4.0), but the reactor dilution rate was switched between 0.05 and 0.16 h^{-1} every 12 h. Effluent samples were taken and assayed for cell density, cell viability, substrate and product concentrations, and plasmid stability.

Immobilized Cells in the Fibrous Matrix. At the end of each bioreactor study, the total cell density in the immobilized cell bioreactor was estimated. Immobilized cells in the fibrous matrix were also analyzed for cell viability and plasmid stability and examined with scanning electron microscopy (SEM).

Cell Concentration. All of the liquid present in the bioreactor was drained, and its volume and optical

density (OD) were measured and used to estimate the concentration of suspended cells in the bioreactor. Then the fibrous matrix was removed from the drained bioreactor. Several small pieces (1 × 1 cm) of fibrous material were cut and used for SEM and other studies. The remaining fibrous sheet was dried at 70 °C overnight in a vacuum oven. The density of immobilized cells was determined from the total weight of the dried fibrous material containing cells, subtracting the dried weight of the fibrous material prior to use for cell immobilization in the bioreactor.

Removal of Immobilized Cells from Cotton Cloth. Immobilized cells were washed off the cotton cloth sample by vortex mixing for 2 min in a test tube containing 10 mL of sterile distilled water. The washed water containing the cells was collected, and the cotton cloth was washed again with new water. This was repeated five times or until the washed water was clear. Cells loosely entrapped within the fibrous matrix came off in the first wash. Cells attached to or adsorbed on fiber surfaces came off after several washes, depending on the strength of the attachment. The viability and fraction of plasmid-carrying cell population in the suspended cells collected from each wash were assayed. Almost all cells were removed from the fibrous matrix after the fifth wash. Cell samples from all five washes were also combined and assayed to determine the overall cell viability and the fraction of plasmid-carrying cells in the total immobilized cell population. All sample analyses were duplicated, and average values are reported.

Scanning Electron Microscopy. Samples of cotton cloth removed from the fibrous-bed bioreactor were immersed in a 2.5% glutaraldehyde solution overnight at 4 °C, completely rinsed with distilled water, and then progressively dehydrated with 20−70% ethanol, in increments of 10%, by holding them at each concentration for 30 min. The partially dehydrated samples were left in 70% ethanol overnight at 4 °C and then progressively dehydrated with 80−100% ethanol. These samples were then dried cryogenically at the critical point with liquid CO_2. The completely dried samples were coated with gold/palladium before SEM photographs were taken using a JEOL Model 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 equivalent to 0.64 g/L of cells when OD was less than 0.45. Samples were diluted with distilled water if the OD was greater than 0.45.

Glucose and Ethanol Concentrations. Concentrations of ethanol and glucose in the sample solution were determined by using high-performance liquid chromatography (HPLC) as previously described by Yang et al. (1987).

GM-CSF Concentration. The concentration of GM-CSF was determined by using reverse-phase high-performance liquid chromatography (Shu, 1992). Cell-free sample solution (25 μL) was injected into a reverse-phase column (Vydac C4, 5-mm reverse-phase silica). A binary solvent system (solvent A, 0.1% trifluoroacetic acid (TFA) 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 0−100% solvent B in the eluent at the following increasing rates: 3%/min for 10 min, 0.67%/min for 30 min, and 5%/min for 10 min. Absorbance at 280 nm was measured by using a UV spectrophotometer (Waters Lambda-Max Model 481 LC). A standard containing 100 mg/L murine GM-CSF (Mochizuki et al., 1986) provided by Immunex Corp. was used to determine the concentration of GM-CSF in fermentation samples. Two to three peaks located within a narrow range of retention times on the chromatogram were identified as GM-CSF proteins with different glycosylations from recombinant yeast fermentation (Price et al., 1987). The concentration of GM-CSF in each sample was determined from the total peak area of these peaks.

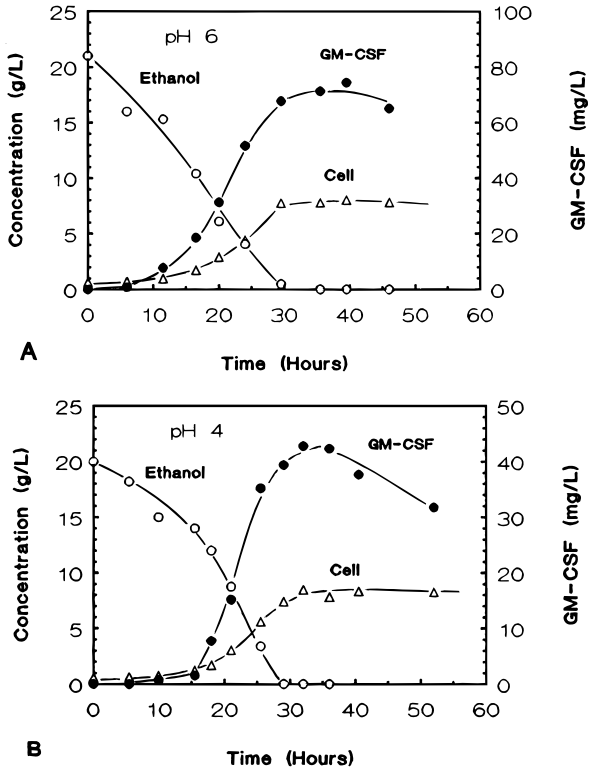

Figure 2. Kinetics of batch fermentations: (A) pH 6.0; (B) pH 4.0.

Cell Viability. The viability of yeast cells present in the effluent and in the fibrous matrix was measured by using a modified staining method (Lee et al., 1981). The staining solution used was a 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 the Ringer salt solution to ~3.0×10^8 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 on the rich (nonselective) medium agar plate. Cell samples were diluted to get the colony counts within the range of 30−300. All plate counts were determined from the average of at least three replicates.

Results and Discussion

Batch Fermentation. The kinetics of GM-CSF production from yeast grown on ethanol as the substrate at pH 6 and 4 are shown in Figure 2. As is seen in the figure, the production of GM-CSF was growth-associated; the GM-CSF concentration increased during the expo-

Table 1. Batch Fermentation Results

pH	μ (h^{-1})	$Y_{x/s}$ (g/g)	$Y_{p/s}$ (mg/g)	$Y_{p/x}$ (mg/g)	P (mg/L·h)	$P/[X^+]$ (mg/h·g)
6.0	0.130	0.44	4.3	9.8	1.77	0.28
4.0	0.126	0.44	2.8	6.4	1.06	0.18

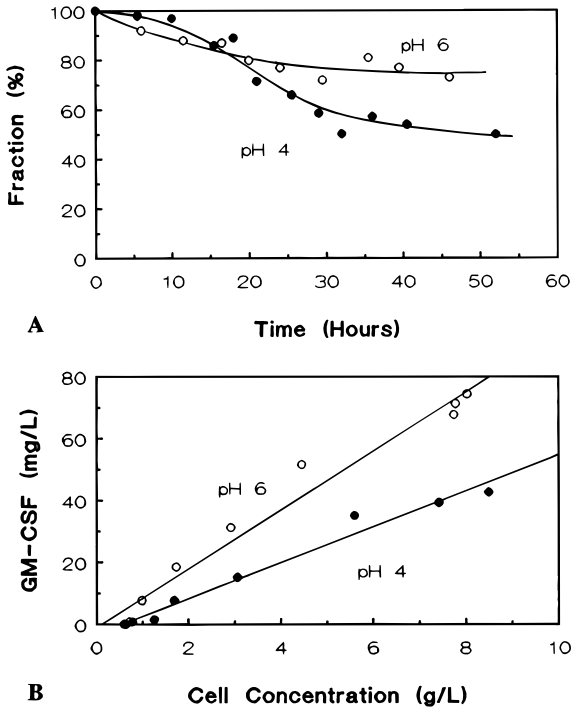

Figure 3. Effects of pH on (A) plasmid stability and (B) specific GM-CSF production. (Fraction: the fraction of plasmid-carrying cells in the total cell population.)

nential growth phase but remained nearly unchanged in the stationary phase. It took about 40 h for these batch fermentations to reach a maximum GM-CSF concentration of 70.8 mg/L at pH 6 and 42.5 mg/L at pH 4.

Cell yield, $Y_{x/s}$, and product yield, $Y_{p/s}$, were determined from the linear plots of cell concentration vs ethanol concentration and of GM-CSF concentration vs ethanol concentration, respectively. The specific growth rate, μ, was determined from the slope of the semilogarithmic plot of cell concentration vs time. The pH did not have significant effects on cell growth; both μ and $Y_{x/s}$ at pH 4 and 6 were about the same (Table 1). However, the medium pH had profound effects on plasmid stability and GM-CSF production. The effects of pH on plasmid stability and specific GM-CSF production, $Y_{p/x}$, which was indicative of the product expression level in the cells, are shown in Figure 3. The lower pH resulted in a lower GM-CSF production and poorer plasmid stability (Figure 3A). The specific GM-CSF production was 9.8 mg/g cell at pH 6 and 6.4 mg/g cell at pH 4, as was determined from the slope of the plot shown in Figure 3B. The reactor productivity, P, as determined from the GM-CSF concentration produced divided by the batch fermentation time, was 1.77 mg/L·h at pH 6 and 1.06 mg/L·h at pH 4. The specific cell productivity, $P/[X^+]$, as estimated from the productivity divided by the plasmid-carrying cell concentration, $[X^+]$, was approximately 0.28 mg/h·g of cells at pH 6 and 0.18 mg/h·g of cells at pH 4. These results are also summarized in Table 1.

Continuous Fermentation. ***Reactor Startup.*** Typical reactor startup performance with pH 6.7 glucose selective medium is shown in Figure 4. After the continuous feed started at 18 h, the effluent pH dropped quickly to ~3.0. The effluent glucose concentration also decreased quickly and stabilized at ~0.9 g/L. Both

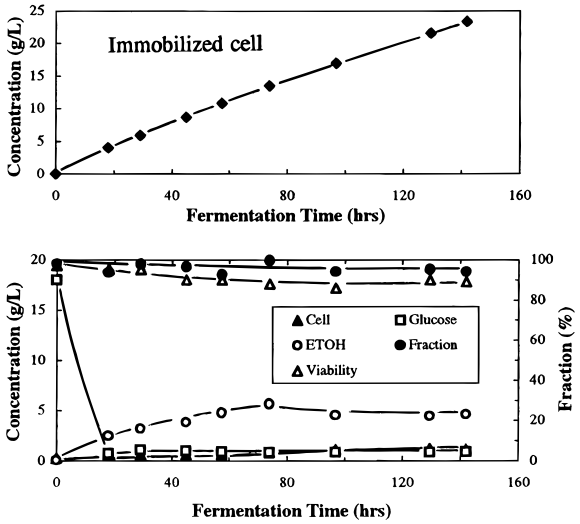

Figure 4. Reactor startup performance (ETOH: ethanol).

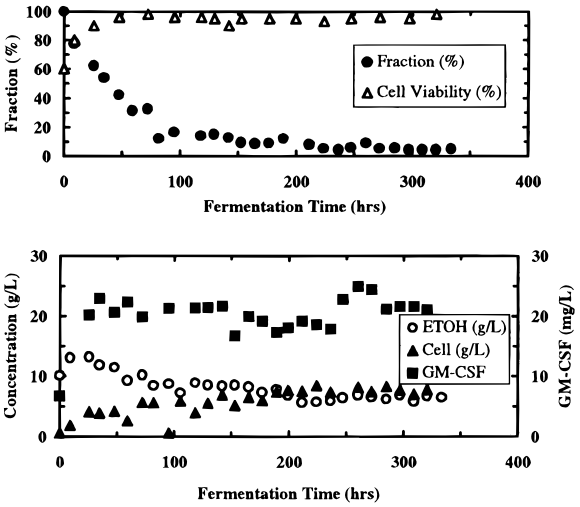

Figure 5. Continuous fermentation with the fibrous-bed bioreactor at 0.16 h^{-1} dilution rate (D) and pH 6.2 (reactor 1).

ethanol and cell concentrations in the effluent increased and stabilized at ~4.5 and ~1.1 g/L, respectively. During the entire 1-week startup period, cell viability was higher than 90% and the fraction of plasmid-carrying cells was higher than 95%, indicating high plasmid stability with the selective medium. The cell yield for growth on glucose in the selective medium was previously found to be 0.2 g/g glucose (Shu, 1992). On the basis of the total material balance, the immobilized cell density in the reactor reached ~24 g/L reactor volume at 142 h (Figure 4). The reactor feed was then switched to the rich medium to study GM-CSF production.

Reactor Performance. Figure 5 shows the kinetics of continuous fermentation at the dilution rate of 0.16 h^{-1}. As shown, after changing to the rich medium (pH 6.7), the GM-CSF production immediately increased to ~21 mg/L and then stayed at about the same level with some fluctuations for the following 330-h period. During the same period, effluent cell concentration increased to a steady level of ~7.8 g/L, ethanol decreased to ~6.4 g/L, effluent pH remained almost constant at ~6.2, cell viability remained at ~95%, and the fraction of plasmid-carrying cells in the effluent dropped to ~5%. The reactor productivity remained almost unaffected by the apparent loss in the plasmid-carrying cell population in the effluent. The observed stable long-term GM-CSF production was attributed to the cell immobilization effect discussed later in this paper. This reactor study was stopped at 340 h because of a contamination problem.

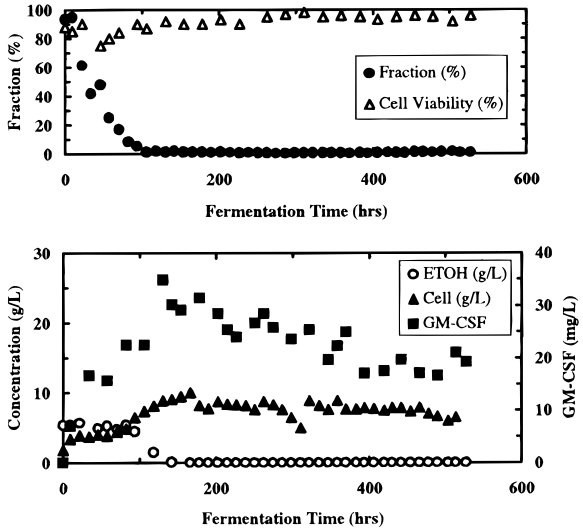

Figure 6. Continuous fermentation with the fibrous-bed bioreactor at 0.05 h^{-1} dilution rate (D) and pH 4.0 (reactor 2).

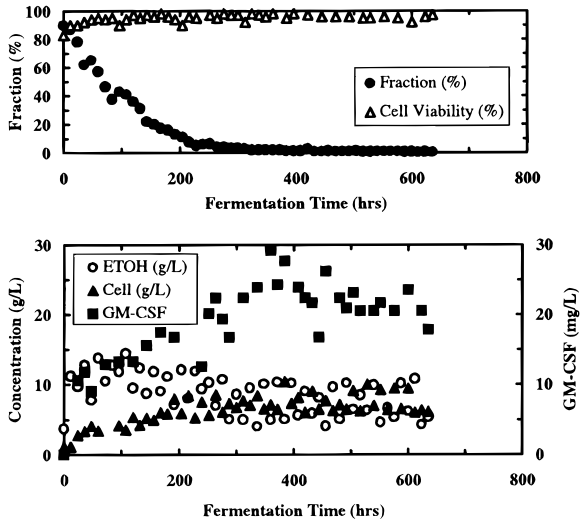

Figure 7. Continuous fermentation with the fibrous-bed bioreactor at a dilution rate oscillating between 0.05 and 0.16 h^{-1} every 12 h and pH 4.0 (reactor 3).

The long-term performance of the second reactor at 0.05 h^{-1} dilution rate with pH 4.0 medium is shown in Figure 6. At the low pH and dilution rate, the reactor gave poorer GM-CSF production than was observed in the first reactor study. GM-CSF production slowly increased to a maximum level of 35 mg/L at 130 h, even though the plasmid-carrying cell fraction in the effluent quickly dropped to ~2% in the same period. GM-CSF concentration then gradually decreased and settled at a steady level of ~19.5 mg/L for a period of 400 h despite the apparently low plasmid-carrying cell fraction in the reactor effluent, which was only ~0.8%. There was no contamination problem with this reactor for the entire 4-week period studied. The lower pH helped to prevent contamination at the expense of lower productivity. Ethanol was depleted in the reactor effluent, indicating that the reactor might also have been limited by the growth substrate at this low dilution rate.

Figure 7 shows the results from the third reactor study, which was fed with the pH 4 medium but was operated with cyclic oscillation in the dilution rate between 0.05 and 0.16 h^{-1} with an oscillation period of 12 h. It has been previously shown by other researchers that oscillation of the reactor dilution rate may improve the plasmid stability and reactor productivity of a recombinant cell fermentation (Impoolsup et al., 1989; Stephens et al., 1992; Weber and San, 1988). However, no work has been reported for the periodic operation of an immobilized recombinant cell system. As shown in Figure 7, GM-CSF production was maintained at ~21 mg/L, which was slightly higher than that obtained from the second reactor, for a period of ~400 h.

Table 2. Continuous Fermentation Results

reactor	pH	D (h^{-1})	$Y_{x/s}$ (g/g)	$Y_{p/s}$ (mg/g)	$Y_{p/x}$ (mg/g)	P (mg/L·h)	$P/[X^+]$ (mg/h·g)
1	6.2	0.16	0.56	1.61	2.9	3.50	0.26
2	4.0	0.05	0.38	0.98	2.6	0.98	0.15
3	4.0	0.05/0.16	0.58	1.70	2.9	2.31	0.27

Plasmid Stability. The fraction of plasmid-carrying cell population in the reactor effluent may be used as an indication of the plasmid stability of the immobilized cells in the fibrous-bed bioreactor. The rate of decrease in the fraction of plasmid-carrying cells in the effluent at the higher dilution rate was slower than that at the lower dilution rate. This indicated that the plasmid stability was better at the higher dilution rate. This is consistent with the finding of chemostat culture (Shu, 1992). The difference in plasmid stability also could be partially attributed to the pH effect as found in the batch fermentation study. The plasmid stability in the third reactor with oscillating dilution rate (av 0.105 h^{-1}) was better than at the lower dilution rate (0.05 h^{-1}), but about the same as at the higher dilution rate (0.16 h^{-1}). The effect of periodic operation in improving plasmid stability was thus apparent.

Cell Yield and Product Yield. The cell yield, product yield, and specific GM-CSF production were calculated on the basis of the pseudo-steady-state concentrations of ethanol, biomass, and GM-CSF in the effluent during the last ~100-h period. The results are listed in Table 2. In general, the cell yield was slightly higher than or about the same as that found in the free cell batch fermentation, but the product yield and specific GM-CSF production were much lower for the immobilized cell system. The lower product yield and specific GM-CSF production were attributed to the lower fraction of plasmid-carrying cells as a result of plasmid loss over the long operation period. Except for reactor 2, which might have suffered from severe nutrient limitations due to operation at a low dilution rate, the immobilized cell bioreactor apparently had an higher cell yield than that from batch fermentation. This could be attributed to the large amount of plasmid-free cells present in the bioreactor after a long operation period. Plasmid-free cells usually grow faster and have higher cell yields than plasmid-carrying cells.

Volumetric Productivity. In general, a higher reactor productivity can be obtained from the immobilized cell bioreactor than from the free cell batch system because of increased cell concentration. The productivity from the first bioreactor was approximately 3.5 mg/L·h, which was about 2 times that obtained in batch fermentation at pH 6. However, a relatively low productivity of 0.98 mg/L·h was obtained from the second reactor operated at pH 4 and a dilution rate of 0.05 h^{-1}. Since GM-CSF production was growth-associated, the low productivity could be attributed to the low dilution rate (low specific growth rate) used. With an increased average dilution rate, reactor 3 also had a higher productivity than that obtained in batch fermentation at pH 4.

Immobilized Cells in the Fibrous Matrix. ***Scanning Electron Micrograph.*** At the end of each reactor study, the cells immobilized in the fibrous matrix were examined with SEM and analyzed for their total concentration, viability, and fraction of plasmid-carrying cell

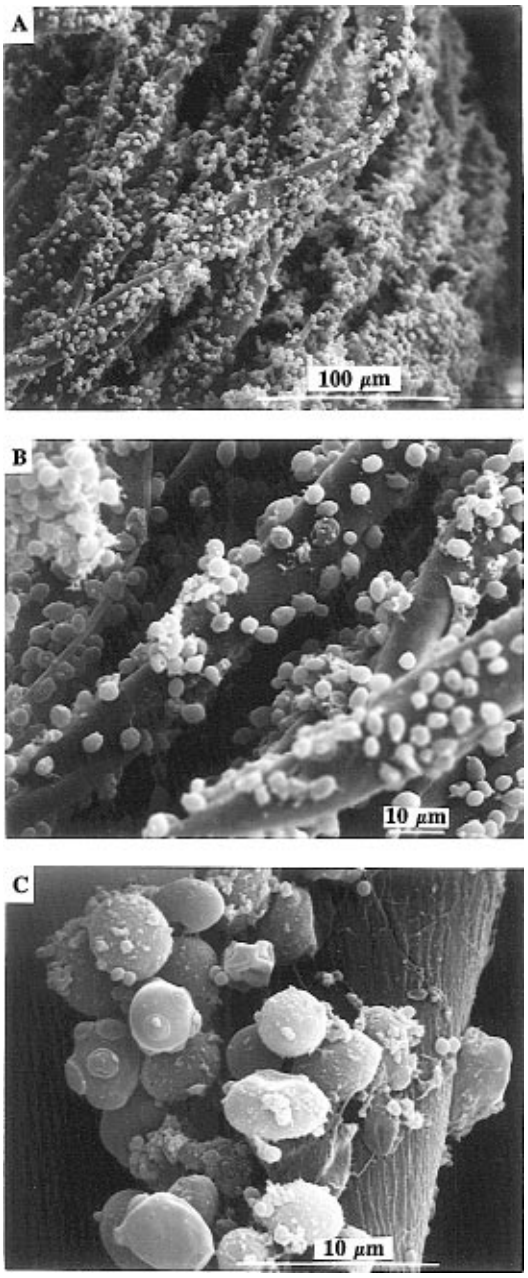

Figure 8. Scanning electron micrographs of yeast cells immobilized in the fibrous matrix.

population. As is seen in the SEM pictures shown in Figure 8, the fibrous matrix was full of yeast cells adsorbed on the fiber surface and entrapped in the interstitial spaces within the fibrous matrix (Figure 8A). Many yeast cells were strongly attached to the fiber (Figure 8B). The budding yeast cells seen in Figure 8C indicate that most of the immobilized cells in the bioreactor were actively growing cells.

Cell Concentration. The concentrations of immobilized cells and free cells in fibrous-bed bioreactors at the end of each reactor study are listed in Table 3. The first bioreactor had 18.63 g of cells immobilized in 27 g of cotton cloth and 1.8 g of free cells suspended in 230 mL of liquid medium. The total amount of cells in this bioreactor was 20.4 g, and the cell density was ~68 g/L reactor volume. The second bioreactor had 12.24 g of cells immobilized in 18 g of cotton cloth and 2.3 g of free cells suspended in 310 mL of liquid medium. The total amount of cells in this bioreactor was 14.6 g or equivalent to a cell density of ~47 g/L reactor volume. Similarly, the cell density in the third bioreactor was found to be ~55 g/L reactor volume. The cell density in the fibrous matrix was ~0.69 g/g fiber for all three reactors, indicating that the maximum immobilized cell density in the fibrous matrix was ~0.69 g/g. The immobilized cell density in the fibrous-bed bioreactor thus is dependent on the packing density of the cotton cloth in the reactor.

Table 3. Cell Concentrations in Fibrous-Bed Bioreactors

	immobilized cells			suspended free cells			total cells	
reactor	density (g/g of fiber)	total fiber (g)	total (g)	concn (g/L)	liq volume (mL)	total (g)	total amt (g)	concn, $[X_{tot}]^a$ (g/L)
1	0.69	27	18.63	7.8	230	1.8	20.4	68.0
2	0.68	18	12.24	7.5	310	2.3	14.6	47.1
3	0.69	21	14.5	7.5	280	2.1	16.6	55.3

[a] $[X_{tot}]$, total cell concentration in the bioreactor based on a reactor working volume of ~300 mL.

Table 4. Viability and Fraction of Plasmid-Carrying Cells in Fibrous-Bed Bioreactors

	reactor 1		reactor 2		reactor 3	
cell population	α	F	α	F	α	F
free cells in effluent	97%	5%	96%	0.9%	98%	0.5%
immobilized cells						
1st wash	80%	25%	80%	15%	87%	16%
3rd wash	78%	34%	75%	28%	82%	30%
5th wash	76%	75%	65%	50%	75%	42%
overall	80%	26.5%	75%	22%	82%	21%

Cell Retention Time and Turnover Rate. On the basis of the total cell concentration ($[X_{tot}]$) in the bioreactor, the effluent cell concentration ([X]) at pseudo-steady state, and the dilution rate (D), the average cell retention times ($RT = [X_{tot}]/[X]D$) in the fibrous-bed bioreactor were estimated to be 55, 125, and 69 h for reactors 1−3, respectively. Thus, during the entire operation period (t), the numbers of cell turnover (t/RT) in the bioreactor were 6, 4, and 9, respectively. The numbers of cell turnover can also be estimated from the total amount of cells produced over the entire operation period. On the basis of the total amount of ethanol consumed and the calculated cell yield at pseudo-steady state, about 90 g of cells (equivalent to 300 g/L reactor volume) were produced in reactor 1. With a total cell density of ~60 g/L, the cell turnover number thus was about 5. Similarly, about 66 g of cells (220 g/L reactor volume) were produced in reactor 2 and the cell turnover number was about 4.7.

In the same operating period, ~78 g of cells (equivalent to 260 g/L reactor volume) and ~57 g of cells (190 g/L reactor volume) left reactors 1 and 2, respectively. Therefore, there was a net increase in cell concentration in the reactor during the operation with ethanol feed. This increase occurred mainly during the initial transient period, but not during the pseudo-steady-state period. There were already ~24 g/L of cells present in the reactor from reactor startup with the glucose medium. Thus, the total cell density in the reactor estimated from the total material balance is consistent with the measured total cell density at the end of the experiment. No material balance was done for reactor 3 because of the complexity generated from feed oscillation.

Cell Viability. As shown in Table 4, the viability (α) of the immobilized cells was 75−82%, which was significantly lower than that of the free cells (over 95%). Also, the viability of immobilized cells decreased from 80−87% for the loosely entrapped cells removed at the first wash to 65−76% for the strongly attached cells removed at the fifth wash. This was expected because the more strongly immobilized cells were likely to be the oldest cells in the reactor. They were also closer to or at the surface of the fiber and were covered with layers of other cells and, thus, had a limited nutrient supply.

Fraction of Plasmid-Carrying Cells. Immobilized cells in the fibrous matrix had a much higher fraction of plasmid-carrying cell population than did the freely suspended cells in the reactor, at over 20% and less than 5%, respectively (Table 4). As also shown, the fraction (F) was higher for the cells from later washes or more strongly immobilized cells. It is clear that cell immobilization in the cotton cloth resulted in the retention of a significant amount of plasmid-carrying cells, even after long period of operation with a nonselective medium feed. This explains the stable GM-CSF production attained with the fibrous-bed bioreactor.

Specific Productivity. The specific productivity, $P/[X^+]$, was calculated on the basis of total viable plasmid-carrying cells ($[X^+] = [X_{tot}]F\alpha$) present in the fibrous-bed bioreactor at the end of the period studied. As also listed in Table 2, $P/[X^+]$ was 0.26 mg/h·g of cells for reactor 1 and 0.15 mg/h·g of cells for reactor 2. These specific productivities were almost the same as those found in batch fermentations at similar pH conditions (see Table 1). Compared to batch fermentation with free cells at the same pH, the slightly lower specific productivity from immobilized cell bioreactors (reactors 1 and 2) indicated that not all immobilized plasmid-carrying cells were as productive as free cells, possibly because of mass-transfer limitations.

The lower specific productivity at pH 4 (reactor 2) than at pH 6 (reactor 1) was consistent with the pH effect observed in batch fermentation. However, a lower dilution rate also may result in a lower growth rate of immobilized cells and may adversely affect the growth-associated recombinant protein production. A higher specific productivity at a higher dilution rate has been reported for several other recombinant cell fermentations (Lee et al., 1988; Seo and Bailey, 1986). With oscillation in the dilution rate, reactor 3 had a significantly higher specific productivity than did reactor 2 or batch fermentation at the same pH. Thus, oscillation in the dilution rate (specific growth rate) improved the specific productivity.

Effects of Cell Immobilization. The improved plasmid stability in an immobilized cell system has been attributed to reduced cell growth, which makes the maintenance of plasmid-carrying cell population easier as segregational plasmid loss and overgrowth of plasmid-free cells are less likely to happen (Bailey et al., 1987; Walls and Gainer, 1989), and to compartmental cell distribution and mass-transfer limitation in the space-limited immobilized cell environment, preventing plasmid-free cells from outgrowing plasmid-carrying cells (Barbotin et al., 1989; De Taxis du Poet et al., 1986; Dincbas et al., 1993). A physiological change in immobilized cells and the increased plasmid copy number at reduced growth rate may also improve segregational stability (De Taxis du Poet et al., 1987; Sayadi et al., 1989). The mechanism of maintaining the high fraction of plasmid-carrying cells in the fibrous-bed bioreactor is not clear, however. Since GM-CSF production was growth-associated and the cell turnover rate in the fibrous-bed bioreactor was significant, this stabilization effect cannot be solely attributed to the reduced cell growth in the immobilized cell environment.

The plasmid-carrying cell population might have been maintained in the fibrous matrix through the following two possible mechanisms: (1) most available adsorption sites on the fiber surface had been preoccupied by plasmid-carrying cells during reactor startup, and thus not all cells from new growth could be effectively immobilized and were washed out; (2) there were sufficient adsorption sites available, but plasmid-carrying cells from new growth were favorably retained in the fibrous matrix and most plasmid-free cells were freely suspended in the liquid medium and thus were washed out.

The specific GM-CSF production, $Y_{p/x}$, was 2.9 for the first bioreactor, which was ~30% of the value found in batch fermentation at pH 6. For the second reactor, $Y_{p/x}$ was ~40% of that found in batch culture at pH 4. Since GM-CSF production is growth-associated, these percentages should approximate the fraction of cell growth from plasmid-carrying cells. The first mechanism thus was unlikely to be responsible for the observed immobilization effect since the fraction of plasmid-carrying cells in the reactor effluent (5% or lower) was far lower than these percentages. It is apparent that cells from new growth were disproportionately discharged from the reactor; most plasmid-carrying cells stayed in the reactor while almost all plasmid-free cells left the reactor.

The effect of selective immobilization or retention of plasmid-carrying cells in the fibrous-bed bioreactor is similar to that obtained from selective flocculation and recycling of plasmid-carrying cells (Henry et al., 1990). In general, plasmid-carrying cells grow slower than plasmid-free cells and have a higher probability of remaining attached to the cotton fiber surface or to each other to form cell aggregates. Thus, they can be better retained by adsorption and by entrapment in the fibrous matrix than can plasmid-free cells. On the other hand, plasmid-free cells grow faster and have a higher tendency to become freely suspended in the liquid medium. They thus can be washed out from the reactor more easily than plasmid-carrying cells. In a related study with cells immobilized in porous glass beads in a three-phase fluidized-bed bioreactor, no significant difference in the fraction of plasmid-carrying cells was found between the immobilized cells and the free cells (Shu and Yang, 1996). The different results from these two different bioreactor studies could be attributed to the different immobilization media used, though one cannot rule out the possible effects from different hydrodynamic and mass-transfer environments.

It is also noted that the observed stabilization effect on GM-CSF production in the fibrous-bed bioreactor also could be partially attributed to increased plasmid copy number for cells grown at low growth (dilution) rate (De Taxis du Poet et al., 1987). The recombinant yeast cell used in this work had a high plasmid copy number of ~30 (Price et al., 1987). The immobilized plasmid-carrying cells in the fibrous-bed bioreactor could have a higher plasmid copy number due to the limited growth nutrients and slower growth rate. In a related chemostat culture study using an air-lift bioreactor, the specific productivity from the plasmid-carrying cell population was found to increase significantly as time proceeded, indicating an increase in plasmid copy number or in the fraction of high-copy-number cell population (Shu and Yang, 1996).

Conclusions

Compared to free cell batch fermentation, the production of recombinant GM-CSF was improved in reactor volumetric productivity and long-term stability by using the fibrous-bed bioreactor because of the high density of viable plasmid-carrying cells initially immobilized in the bioreactor and the preferential retention of plasmid-carrying cells in the fibrous matrix. However, possibly because of diffusion limitations, the immobilized cell system had a lower cell growth rate that resulted in a lower specific cell productivity because the production of GM-CSF was growth-associated. A higher dilution rate thus should be used to improve GM-CSF production. Higher dilution rates would result in higher specific growth rates. Higher flow rates may also wash out

plasmid-free cells and, hence, may help in the maintenance of a high fraction of plasmid-carrying cells in the reactor, resulting in improved productivities and yields. Also, oscillation in the dilution rate improved both plasmid stability and specific productivity.

Acknowledgment

We thank Immunex for providing the yeast culture and GM-CSF proteins used in this study. Technical assistance provided by Dr. Price of Immunex during the course of this study is also acknowledged.

Literature Cited

Bailey, K.; Vieth, W. R.; Chotani, G. K. Analysis of bioreactors containing immobilized recombinant cells. *Ann. N.Y. Acad. Sci.* **1987**, *506*, 196−208.

Barbotin, J. N. Immobilization of recombinant bacteria: a strategy to improve plasmid stability. *Ann. N.Y. Acad. Sci.* **1994**, *721*, 303−309.

Barbotin, J. N.; Berry, F.; Briasco, C.; Huang, J.; Nasri, M.; Sayadi, S.; Thomas, D. Immobilization effects on the stability of recombinant microorganisms. *Chimicaoggi* **1989** (Nov), 49−52.

Caldwell, J.; Locey, B.; Clarke, M. F.; Emerson, S. G.; Palsson, B. O. Influence of medium exchange schedules on metabolic, growth, and GM-CSF secretion rates of genetically engineered NIH-3T3 cells. *Biotechnol. Prog.* **1991**, *7*, 1−8.

Caunt, P.; Impoolsup, A.; Greenfield, P. F. Stability of recombinant plasmids in yeast. *J. Biotechnol.* **1988**, *8*, 173−192.

D'Angio, C.; Beal, C.; Boquien, C. Y.; Corrieu, G. Influence of dilution rate and cell immobilization on plasmid stability during continuous cultures of recombinant strains of *Lactococcus lactis* subsp. *lactis*. *J. Biotechnol.* **1994**, *34*, 87−95.

De Taxis du Poet, P.; Dhulster, P.; Barbotin, J.-N.; Thomas, D. Plasmid inheritability and biomass production: Comparison between free and immobilized cell culture of *Escherichia coli* BZ18(pTG201) without selection pressure. *J. Bacteriol.* **1986**, *165*, 871−877.

De Taxis du Poet, P.; Arcand, Y.; Bernier, R., Jr.; Barbotin, J.-N.; Thomas, D. Plasmid stability in immobilized and free recombinant *Escherichia coli* JM105(pKK223-200): Importance of oxygen diffusion, growth rate, and plasmid copy number. *Appl. Environ. Microbiol.* **1987**, *53*, 1548−1555.

Dincbas, V.; Hortacsu, A.; Camurdan, A. Plasmid stability in immobilized mixed cultures of recombinant *Escherichia coli*. *Biotechnol. Prog.* **1993**, *9*, 218−220.

Doran, P. D.; Bailey, J. E. Effects of immobilization on growth, fermentation properties, and macromolecular composition of *Saccharomyces cerevisiae* attached to gelatin. *Biotechnol. Bioeng.* **1986**, *28*, 73−87.

Fonseca, M. M. da; Black, G. M.; Webb, C. Reactor configurations for immobilized cells. In *Process Engineering Aspects of Immobilized Cell Systems*; Webb, C., Black, G. M., Atkinson, B., Eds.; Pergamon Press: New York, 1986; pp 63−74.

Henry, K. L.; Davis, R. H.; Taylor, A. L. Continuous recombinant bacterial fermentations utilizing selective flocculation and recycle. *Biotechnol. Prog.* **1990**, *6*, 7−12.

Heslot, H.; Gaillardin, C. *Molecular Biology and Genetic Engineering of Yeasts*; CRC Press: Boca Raton, FL, 1991.

Impoolsup, A.; Caunt, P.; Greenfield, P. F. Stabilization of a recombinant yeast plasmid in nonselective medium by cyclic growth rate changes. *Biotechnol. Lett.* **1989**, *9*, 605−608.

Joshi, S.; Yamazaki, H. Stability of plasmid pbr322 in *Escherichia coli* immobilized on cotton cloth during use as resident inoculum. *Biotechnol. Lett.* **1987**, *9*, 825−830.

Kanayama, H.; Sode, K.; Karube, I. Continuous hydrogen evolution by immobilized recombinant *E. coli* using a bioreactor. *Biotechnol. Bioeng.* **1988**, *32*, 396−399.

Kolot, F. B. Changes in cell properties after immobilization. In *Immobilized Microbial Systems: principles, techniques and industrial applications*; R. E. Krieger: Florida, 1988; pp 13−18.

Lee, S. S.; Robinson, F. M.; Wang, H. Y. Rapid determination of yeast viability. *Biotechnol. Bioeng. Symp.* **1981**, *11*, 641−649.

Lee, S. B.; Ryu, D. D. Y.; Siegel, R.; Park, S. H. Performance of recombinant fermentation and evaluation of gene expression efficiency for gene product in two-stage continuous culture system. *Biotechnol. Bioeng.* **1988**, *33*, 805−820.

Lewis, V. P.; Yang, S. T. Continuous propionic acid fermentation by immobilized *Propionibacterium acidipropionici* in a novel packed-bed bioreactor. *Biotechnol. Bioeng.* **1992**, *40*, 465−474.

Metcalf, D. The granulocyte-macrophage colony-stimulating factors. *Science* **1985**, *229*, 16−22.

Metcalf, D. Control of granulocyte and macrophages: molecular, cellular, and clinical aspects. *Science* **1991**, *254*, 529−533.

Mochizuki, D. Y.; Eisenman, J. R.; Conlon, P. L.; Park, L. S.; Urdal, D. L. Development and characterization of antiserum to murine granulocyte-macrophage colony-stimulating factor. *J. Immunol.* **1986**, *136*, 3706−3709.

Nasri, M.; Sayadi, S.; Barbotin, J.-N.; Dhulster, P.; Thomas, D. Influence of immobilization on the stability of pTG201 recombinant plasmid in some strains of *Escherichia coli*. *Appl. Environ. Microbiol.* **1987**, *53*, 740−744.

Oriel, P. Immobilization of recombinant *Escherichia coli* in silicone polymer beads. *Enzyme Microb. Technol.* **1988**, *10*, 519−523.

Price, V.; Mochizuki, D.; March, C. J.; Cosman, D.; Deeley, M. C.; Klinke, R.; Clevenger, W.; Gills, S.; Baker, P.; Urdal, D. Expression, purification and characterization of recombinant murine granulocyte-macrophage colony-stimulating factor and bovine interleukin-2 from yeast. *Gene* **1987**, *55*, 287−293.

Sayadi, S.; Nasri, M.; Barbotin, J. N; Thomas, D. 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.* **1989**, *33*, 801−808.

Seo, J. H.; Bailey, J. E. Continuous cultivation of recombinant *Escherichia coli*: existence of an optimum dilution rate for maximum plasmid and gene product concentration. *Biotechnol. Bioeng.* **1986**, *28*, 1590−1594.

Shu, C. H. Multiphase Bioreactors for Recombinant Yeast Fermentation. Ph.D. Dissertation, Ohio State University, Columbus, OH, 1992.

Shu, C. H.; Yang, S. T. Production of Recombinant GM-CSF by *Saccharomyces cerevisiae* in a Three-Phase Fluidized Bed Bioreactor. *Biotechnol. Bioeng.* **1996**, in press.

Shu, C. H.; Yang, S. T. Kinetics of Continuous GM-CSF Production by Recombinant *Saccharomyces cerevisiae* in an Air-Lift Bioreactor. *J. Biotechnol.* **1996**, in press.

Silva, E. M.; Yang, S. T. Continuous Production of Lactic Acid from Acid Whey by *Lactobacillus helveticus* in a Fibrous-Bed Bioreactor. *J. Biotechnol.* **1995**, *41*, 59−70.

Sode, K.; Morita, T.; Peterhans, A.; Meussdoerffer, F.; Mosbach, K.; Karube, I. Continuous production of α-peptide using immobilized recombinant yeast cells. *J. Biotechnol.* **1988**, *8*, 113−122.

Stephens, M. L.; Christensen, C.; Lyberatos, G. Plasmid stabilization of an *Escherichia coli* culture through cycling. *Biotechnol. Prog.* **1992**, *8*, 1−4.

Tanaka, A.; Nakajima, H. Application of immobilized growing cells. *Adv. Biochem. Eng./Biotechnol.* **1990**, *42*, 97−131.

Walls, E. L.; Gainer, J. L. Retention of plasmid bearing cells by immobilization. *Biotechnol. Bioeng.* **1989**, *34*, 717−724.

Walls, E. L.; Gainer, J. L. Increased protein productivity from immobilized recombinant yeast. *Biotechnol. Bioeng.* **1991**, *37*, 1029−1036.

Weber, A. E.; San, K. Y. Enhanced plasmid maintenance in a CSTR upon square-wave oscillations in the dilution rate. *Biotechnol. Lett.* **1988**, *8*, 531−536.

Yang, S. T.; Tang, I. C.; Okos, M. R. Kinetics of homoacetic fermentation of lactate by *Clostridium formicoaceticum*. *Appl. Environ. Microbiol.* **1987**, *53*, 823−827.

Yang, S. T.; Zhu, H.; Li, Y.; Hong, G. Continuous Propionate Production from Whey Permeate Using a Novel Fibrous Bed Bioreactor. *Biotechnol. Bioeng.* **1994**, *43*, 1124−1130.

Accepted May 14, 1996.[⊗]

BP960037G

[⊗] Abstract published in *Advance ACS Abstracts,* July 1, 1996.