Journal of Biotechnology 143 (2009) 60–68

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Airlift-driven fibrous-bed bioreactor for continuous production of glucoamylase using immobilized recombinant yeast cells

Peter Kilonzo, Argyrios Margaritis*, Maurice Bergougnou

Department of Chemical and Biochemical Engineering, University of Western Ontario, London, Ontario N6A 5B9, Canada

ARTICLE INFO

Article history:
Received 5 March 2009
Received in revised form 3 May 2009
Accepted 5 June 2009

Keywords:
Recombinant *Saccharomyces cerevisiae*
Glucoamylase
Fibrous bed
Immobilization
Airlift
Bioreactor

ABSTRACT

Continuous production of a fungal glucoamylase by immobilized recombinant *Saccharomyces cerevisiae* strain C468 containing plasmid pGAC9. Yeast cells were immobilized on hydrophilic cotton cloth in an inverse internal loop airlift-driven bioreactor. Free-cell culture in the airlift and stirred tank bioreactors confirmed the plasmid instability of the recombinant yeast. Enhanced glucoamylase productivity and plasmid stability were observed both in the free and immobilized cell cultures in the airlift bioreactor system. The glucoamylase level of the free-cell culture in the airlift bioreactor was ~20% higher than that in the in stirred tank bioreactor due to high cell density (cell dry weight/volume of bioreactor) and fraction of the plasmid-carrying cells. A potentially high glucoamylase activity of 161 U/L and a corresponding volumetric productivity of 3.5 U/L h were achieved when a cell density of ~85 g/L (or 12.3 g/g fiber) was attained in the fibrous-bed immobilized cell bioreactor system. The stable glucoamylase production was achieved after five generations, at which time a fraction of ~62% of the plasmid-carrying cells was realized in the immobilized cell system. Plasmid stability was increased for the immobilized cells during continuous culture at the operating dilution rate. The volumetric and specific productivities and fraction of plasmid-carrying cells in the immobilized cell system were higher than in the free-cell counterpart, however. This was in part due to the high viability (~80%) in the immobilized cell system and the selective immobilization of the plasmid-carrying cells in the fibrous bed, and perhaps increased plasmid copy number.

1. Introduction

In recent years, there has been a keen interest in the production of heterologous proteins by utilizing recombinant DNA technology. Some of the heterologous proteins have already been produced at the industrial level. In general, however, the recombinant proteins produced accumulate in the microorganisms. With regard to further downstream process and the stability of the protein produced, the secretion of these proteins in the external medium would be desirable. The application of yeast cells to the production of foreign proteins offers several advantages. For example, many foreign proteins are produced in soluble form in yeast cells, and post-translational modification could be expected in yeast cells. Furthermore, by utilizing yeast proteins secretion systems, foreign peptides can be secreted to the external medium. In this respect, by utilizing yeast secretion systems, such as α-factor signal peptide, attempts were made to produce several recombinant proteins (Kilonzo et al., 2008; Gorgens et al., 2001; Bannister and Wittrup, 2000; Shiba et al., 1994; Vainio et al., 1994; Chau et al., 2000; Chen et al., 2006; Ekino et al., 2002).

Plasmid instability is a major concern in industrial production of heterologous protein products using recombinant cells (Kilonzo et al., 2008). This is related to the tendency of the recombinant cells to loss their engineered characteristics. This problem is especially important in recombinant yeast strains. The yeast multiplies by non-uniform budding, which leads to an uneven partitioning of the plasmids from mother to daughter cells. The formation of multiple buds compounds this problem thereby leading to a high degree of segregational instability (Gupter and Mukherjee, 2001). The loss of plasmids frequently results in a significant loss of productivity of the desired gene products and impairs industrial exploitation of the recombinant DNA technology.

The problem of plasmid loss can be at least partly overcome by process intensification through immobilization of the recombinant microorganisms on fibrous support beds combined with the use of airlift bioreactors. An airlift-packed bed bioreactor is a potential candidate for bioconversion processes (Kilonzo et al., 2007b, 2006; Lo and Hwang, 2003). The internal loop airlift (ILA) bioreactor features with effective mixing, pneumatic agitation with no mechanical devices, effective tuning of liquid circulation, moderate shear stress and good interface mass transfer. Moreover, under

* Corresponding author. Tel.: +1 519 661 2146; fax: +1 519 661 3498.
E-mail address: amarg@uwo.ca (A. Margaritis).
URL: http://www.eng.uwo.ca/people/amargaritis/ (A. Margaritis).

0168-1656/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2009.06.007

Nomenclature

E	ethanol (or EtOH) concentration, g/L
F^+	fraction of plasmid-carrying cells in the liquid phase, %
F_i^+	fraction of plasmid-carrying cells in the immobilization phase, %
GA	glucoamylase activity, U/L
P	product concentration, U/L
R	regression coefficient, –
S	substrate concentration, g/L
S_0	initial substrate concentration, g/L
X	total cell concentration, g/L
X^+	plasmid-carrying cell concentration, g/L
X^-	plasmid-free-cell concentration, g/L

Greek letters

ϕ_p	porosity (void fraction) of the packing, –

continuous operation the ILA bioreactor is characterized by a well-stirred behavior that may positively affect substrate-inhibition bioconversions.

The application of the immobilization technique to bioprocess is widely accepted to have many advantages compared to conventional chemostat processes (Kilonzo et al., 2007a; Yang and Shu, 1996) and has been employed to minimize plasmid instability and to improve bioreactor productivity (Chau et al., 2000; Chen et al., 2006; Huang et al., 1996; Shu and Yang, 1996a; Yang and Shu, 1996; Barbotin, 1994; D'Angio et al., 1994). In the immobilized cell system, the maintenance of a plasmid-bearing cell population becomes easier as segregational instability and overgrowth of plasmid-free cells are less likely to happen because of reduced cell growth rate and selection between plasmid-carrying and plasmid-free-cell populations (Chau et al., 2000; Yang and Shu, 1996; Zhang et al., 1997a) and the compartmental cell distribution and mass transfer limitations in the immobilized cell environment (Yang and Shu, 1996).

Traditional cell immobilization methods are associated with problems such as alteration of cell physiology, decreased efficiency of nutrient and product transport, and increased risk of contamination during the cell immobilization process (Yang and Shu, 1996). Recently, fibrous matrices (some soft and highly porous materials such as fibers cotton, polyester, glass, nylon, ryon, polymer foam and sponge) have been developed as alternative support for cell immobilization because of their high surface-to-volume ratios, constant surface-to-volume ratio leading to both smaller pressure drops and lower mass transfer resistance as compared to microcarrier beads, high void volume, low cost, high mechanical strength, and high permeability (Hsu et al., 2004; Ma et al., 1999). Also, their availability, maximum loading, low diffusion problems, nontoxicity, biodegradability, and durability (Silva and Yang, 1995; Huang and Yang, 1998; Melo and D'Souza, 1999) make fibrous matrices more attractive as cell immobilization supports than other materials (Yang et al., 1994; Silva and Yang, 1995; Yang et al., 1995). The fibrous-bed bioreactor has been successfully used for several organic acid fermentations (Huang and Yang, 1998; Silva and Yang, 1995; Yang et al., 1994, 1995) and only few attempts have been made with recombinant cell processes (Zhang et al., 1997a; Yang and Shu, 1996).

In this work, the recombinant yeast *S. cerevisiae* C468/pGAC9 expressing glucoamylase gene was used as the model organism to investigate effect of immobilization on the stability of continuous production of glucoamylase. To avoid the problems encountered with cell entrapment in hydro-gels, a surface adsorption immobilization method was employed using a fibrous matrix to develop a novel airlift-driven fibrous-bed immobilized cell bioreactor for the recombinant *S. cerevisae* fermentation.

The *Aspergillus awamori* glucoamylase enzyme [GA; (1 → 4) (1 → 6)-α-D-glucan glucohydrolase; EC3.2.1.3] is a glycosylated protein that hydrolyzes starch by attacking both α-1,4 and α-1,6 glucosidic linkages from the non-reducing end of the molecule. It is one of the enzymes used industrially for the conversion of liquefied corn starch to glucose syrup (Withers et al., 1998; Gomes et al., 2005). It is used in glucose production and quantitative determination of glycogen and starch (Kilonzo et al., 2008). One of its major applications is that of saccharification during bioethanol production from starchy materials (Kilonzo et al., 2007a). Moreover, glucoamylase is used in medicine in a coupled assay with α-D-glucosidase in the diagnosis of pancreatic disease (Kilonzo et al., 2008).

2. Materials and methods

2.1. Host strain and plasmid

The recombinant *S. cerevisiae* strain C468/pGAC9, which secretes glucoamylase into the extracellular medium (Kilonzo et al., 2008; Zhang et al., 1997a,b), was employed in this study. The *S. cerevisiae* strain C468/pGAC9 (ATCC 20690) contains the hybrid plasmid vector pGAC9. The plasmid pGAC9 contains a portion of the yeast 2μ plasmid (2μ micron circle), a DNA fragment which encodes the *LEU* gene product (leucine), and a section of a glucoamylase gene from *A. awamori*, under control of the yeast enolase I promoter and terminator. The *S. cerevisae* host strain C468 (α *leu2-3 leu2-112 his311 his3-15 mal*$^-$) (ATCC 62995) is haploid, with auxotrophic markers for leucine and histidine and carries mutation (*mal*$^-$) blocking the utilization of maltose as carbon source. Therefore, the host cell is complementary to the leucine prototrophy by inserting the selectable marker (*LEU* 2) into the expression plasmid and the presence of the glucoamylase gene on the plasmid allows the host cell to grow on maltose (Kilonzo et al., 2008; Zhang et al., 1997a,b).

2.2. Growth media

Two different media were utilized in this study. The selective YNBG medium containing 6.7 g/L yeast nitrogen base (YNB) without amino acids (Sigma), 0.04 g/L L-histidine (Sigma), and 20 g L D-glucose. The stock slant culture was grown in the YNBG and maintained on YNBM containing 2% (w/v) maltose. The selective medium was used in preparing inocula for batch cultures and during bioreactor startup for continuous cultures. A complex non-selective YEPG medium containing 5 g/L yeast extract (Becton Dickinson), 10 g/L peptone (Becton Dickinson), and 20 g/L D-glucose was used in chemostat culture. Without adjustment, the pH of these media was 5.0. For agar plates, the media also contained 2% (w/v) Fermtech-agar. The medium components other than D-glucose and maltose in YNBG, YEPG and YNBM, respectively were sterilized by filtration (0.2 μm filter). D-Glucose and maltose were sterilized separately in an autoclave for 40 min at 121 °C and 20 psi pressure. A selection procedure (Zhang et al., 1997a,b) was applied to minimize any structural instability of the plasmid constructs during the preparation of the pre-culture.

2.3. Cell growth and development

The selective YNBG medium was used in the inoculum and cell development stages as described above. Inoculum (A) was grown in 250-mL Erlenmeyer flasks up to a concentration $A_{570\,nm} = 0.33$, CDW = 0.25 g/L (i.e. 1.5×10^{10} cells/mL) and then used to inoculate inoculum (B) flasks. A cell suspension at cell concentration $OD_{600\,nm} = 0.52$, CDW = 0.33 g/L (i.e. 1.4×10^{11} cells/mL) was then

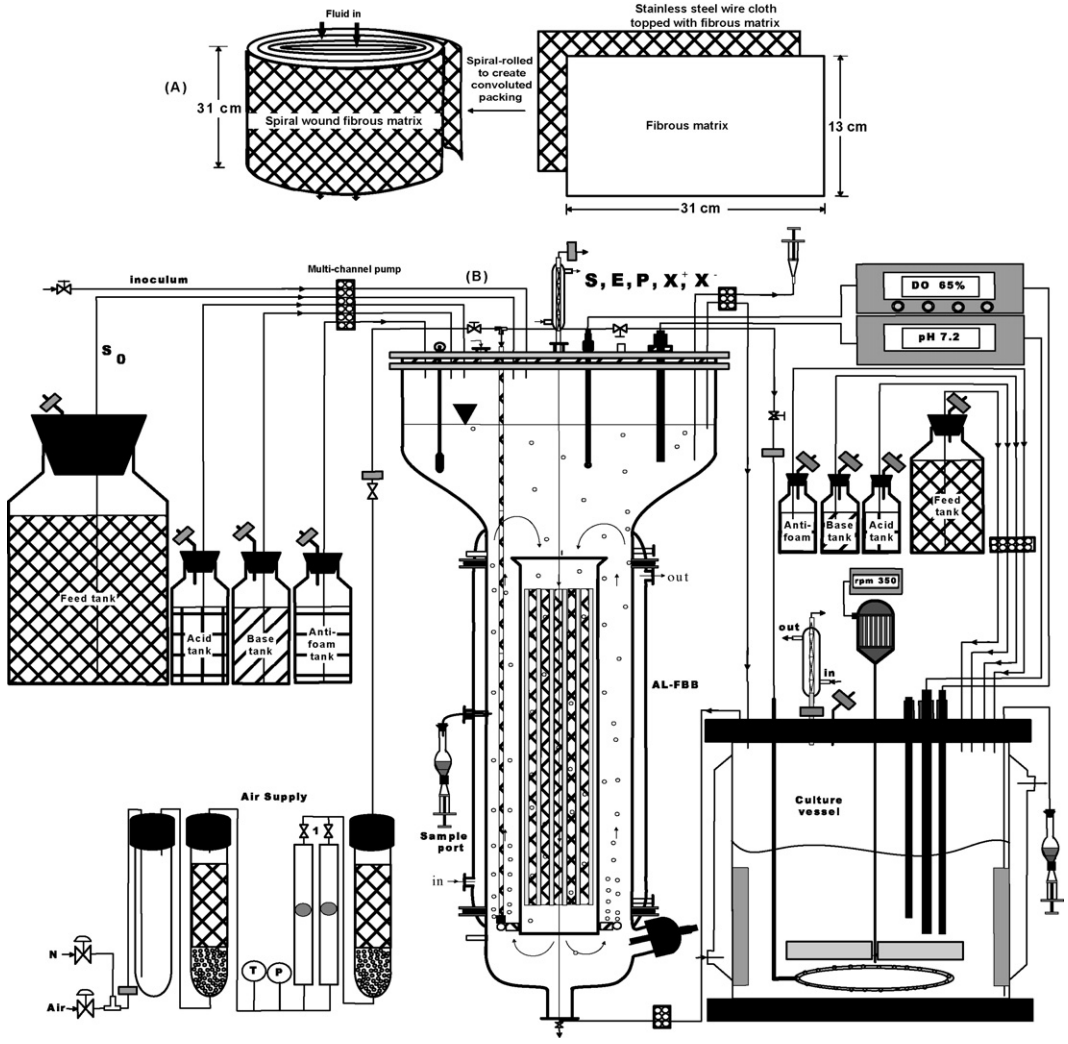

Fig. 1. Schematic representation of the inverse internal loop, airlift-driven fibrous-bed bioreactor. (A) Construction of the fibrous matrix, (B) setup for immobilized recombinant yeast fermentation.

used to inoculate the cell development stage. The cells intended for cell immobilization studies were developed in stirred tank bioreactor (STBR) (see Fig. 1). At the end of 20 h cultivation time, the cell density reached $OD_{600\ nm}$ = 2.65, CDW = 8.2 g/L (i.e. 7.7×10^9 cells/mL) prior immobilization.

2.4. Selection of fibrous materials

Seven different fabric sheet materials were used to test the attachment condition of recombinant *S. cerevisiae* cell. They were: (1) 100% heavy bleached cotton (woven), (2) 100% poly-ester (PE), (3) 65% poly-ester (PE)/35% cotton (woven), (4) 100% nylon (woven), (5) 100% polyurethane foam (PUF), and (6) cellulose re-enforced polyurethane. Small twenty pieces (1 cm × 1 cm) of each of the fibrous material #1 (0.054 g), material #2 (0.029 g), material #3 (0.018 g), material #4 (0.038 g), material #5 (0.024 g), and material #6 (0.120 g) were placed inside a 250-mL Erlenmeyer flask and treated twice with 95% ethanol for 2 h each time, once with 0.5 M HCl for 10 min, and once in 0.5 M NaOH for 10 min at room temperature, and were washed with double distilled water and phosphate buffer solution (0.1 M PBS at pH 7.4) (0.079 M Na_2HPO_4 + 0.027 M NaH_2PO_4) after each treatment. After treatment, the flasks with the fibrous materials were filled with 100 mL of PBS solution and autoclaved at 121 °C for 30 min, after which time the PBS solution was poured out and replaced with 100 mL of complex non-selective YEPG medium. The pH of the contents in the flasks was adjusted to 5.0 using 2 M HCl and/or 2 M NaOH and then autoclaved again under the same conditions. Each flask was inoculated with 5% incolum medium containing a final cell concentration of 3.1×10^8 cells/L (i.e., OD at 600 nm = 0.291, 0.071 CDW/L) and incubated in a shaker at 30 °C for 7 days to allow attachment.

2.5. Fibrous-bed bioreactor construction

Experiments were conducted in an inverse (annulus sparged) internal loop airlift fibrous-bed bioreactor (I-IL-AL-FBB). The schematic diagram of the experimental setup is shown in Fig. 1. The internal loop airlift-driven bioreactor containing a spiral-wound fibrous sheet material had a cylindrical column (I.D.: 0.106 m) containing an inner concentric draft tube (downcomer) of 0.35 m in height and a diameter of 0.06 m. The bioreactor, with a total working volume of 9 L, was made of Plexiglas (poly-methyl methacrylate) and surrounded by a water jacket for temperature control. The bottom clearance h_b between the draft tube bottom and the base plate was kept constant at 0.059 m. The riser-downcomer combination gave an A_r/A_d of 1.84 and a bioreactor volume of 9.0×10^{-3} m^3. The aspect ratio (L_d/D_c) of the bioreactor was 3.43 (Kilonzo et al., 2007a,b, 2006; Li et al., 1995).

The fibrous matrix used for cell immobilization was a heavy bleached cotton material [(0.13 m × 0.31 m × ~0.0005 m and porosity ϕ_P = 90–96% (Yang et al., 1994) 96%, kindly donated by Mountain Weavers Ltd., Dorset, VT, 05251, U.S.A)] on stainless steel wire cloth (SUS 304, porosity: 0.972: mesh #20, Small parts Inc., USA) (Guo et al., 2001). A spirally wound configuration with c_p = 0.002–0.004 m gaps between two adjacent layers of the fibrous sheet matrix which allows medium fluid to flow or pass through the bioreactor unhindered was formed by winding the matrix around a stick. The fibrous sheet material and the stainless steel mesh also created high specific surface area and porosity (~0.935). These properties can allow larger amounts of biomass to attach on the fibrous bed (Guo et al., 2001). The fibrous matrix was loosely packed in the downcomer (draft tube) section of the I-IL-ALB. The top end h_{pt} of the packing was kept constant at 0.114 m below the medium level and the bottom end h_{pb} at 0.125 m above the bioreactor bottom.

2.6. Cell Immobilization

In order to attach biomass on to the fibrous bed, the I-IL-AL-FBB unit was connected to the STB. The cell suspension containing recombinant *S. cerevisiae* C468/pGAC9 was introduced at the bottom of the I-IL-AL-FBB and returned from the top outlet to the culture vessel for re-aeration at a flow rate of ~25 L/min to allow cells to attach and become immobilized onto the fibrous matrix. After about 36–48 h of continuous circulation, most of the cells were immobilized and no change in the cell density in the medium could be identified. The medium circulation rate was then increased to ~100 mL/min, and the bioreactor was operated in a repeated batch mode to increase the cell density in the fibrous bed to a stable, high level (>50 g/L).

2.7. Bioreactor start-up

The yeast cells, with negatively charged cell wall surface were able immobilize on to the fibrous matrix in a passive manner. After 54 h, the circulation was discontinued and then spend medium in the immobilized cell-I-IL-AL-FBB bioreactor was drained. The bioreactor was rinsed with sterile YNBG medium for 2 h to remove suspended and loosely attached cells.

The bioreactor was maintained at 30 °C and aerated at a volumetric air flow rate of 9 L/min (i.e.0.028 m/s). YNBG medium was re-introduced to the bioreactor and then pumped out after 24 h to remove any suspended cell that may have been detached by hydrodynamic forces. The bioreactor was then continuously fed with the YNBG selective medium at a dilution of 0.05 h^{-1} for 3–4 days to allow plasmid-bearing cells to grow to a high density in the bioreactor. Cells got immobilized onto the fibrous bed by adsorption. After the effluent cell density reached steady state, the fermentation was shifted to glucoamylase production phase by feeding the bioreactor with the rich non-selective YEPG medium at the same dilution rate of 0.05 h^{-1}.

2.8. Continuous fermentation studies

The continuous fermentation was studied at 0.10 h^{-1}dilution rate. Effluent samples were taken and assayed for cell density, glucose and ethanol concentrations, glucoamyalse activity, and the fraction of plasmid-carrying cells in the total cell population. At the end of each dilution rate study, total cell density and the fraction of plasmid-carrying cells in the total cell population immobilized on the fibrous bed were analyzed. In the case of free-cell cultures, the fibrous bed was removed from the bioreactor. In this case, the total liquid volume in the bioreactor was 9 L.

2.9. Immobilized 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 pieces of 1 cm × 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 vaccum 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.

2.10. Removal of immobilized cells from fibrous matrix

Immobilized cells were washed off the fibrous matrix sample by vortex mixing for 2 min in a test tube containing 10 mL of sterile distilled water. 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.

2.11. Scanning electron micrograph

One piece (1 cm × 1 cm) of the fibrous material was removed from each flask and cut aseptically into 0.5 cm × 0.5 cm samples. The samples were immersed in a 25 mL of 2.5% glutaraldehyde solution for 48 h at 4 °C and washed three times with 0.1 M PBS for 30 min, three times with 0.9% saline (9 g NaCl + 1000 mL d-H_2O) solution for 30 min, and completely rinsed with sterile distilled water. The washed samples were then progressively dehydrated with 20–70% [i.e., (60 mL EtOH + 240 mL d-H_2O) – (210 mL EtOH + 90 mL d-H_2O)], in increments of 10%, by holding them at each concentration for 30 min. The partially dehydrated samples were left in 70% ethanol (i.e. 210 mL EtOH + 90 mL d-H_2O) over night at 4 °C and then progressively dehydrated with 80–100% ethanol [i.e., (240 mL EtOH + 60 mL d-H_2O – 300 mL-EtOH + 0 mL d-H_2O)]. These samples were then dried cryogenically using liquid CO_2. The dried samples were then coated with gold/palladium and pictures were taken using SEM using UWO Crossbeam Model 820 SEM.

2.12. Analytical methods

2.12.1. Biomass analysis

Free-cell concentration was measured by colorimetric and dry weight and optical density methods. The correlation between dry cell weight (X) and optical density (OD) was determined as X = 1.6835 OD (R = 0.9986, R^2 = 0.9971).

2.12.2. Glucose and ethanol determination

Glucose concentrations in the fermentation broth were determined using a glucose assay kit GAGO20-kiT (Sigma No. 027K8600). Samples from starch solutions were analyzed according to the method described by Kilonzo et al. (2007a). Ethanol concentration was determined by alcohol dehydrogenase enzyme Kit according to the procedure described by procedure described recently by Kilonzo et al. (2007a,b).

2.12.3. Glucoamylase activity assay

Glucoamylase activity was determined according to a modification of the assay described by Zhang et al. (1997a,b). To 0.3 mL of enzyme solution, 0.5 mL of 1.5% soluble starch solution and 0.7 mL of 0.2 M acetate buffer (pH 5.0) was added and incubated at 37 °C for

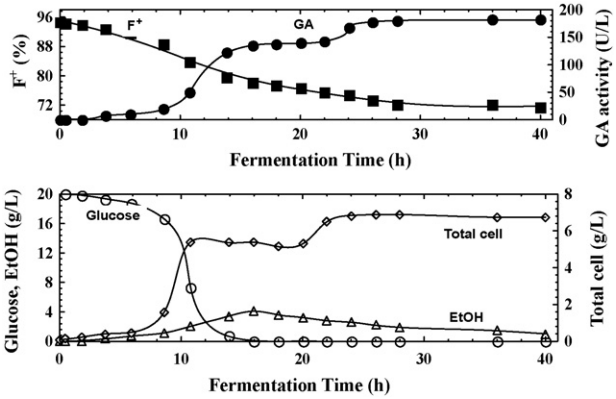

Fig. 2. Typical time course of GA (glucoamylase) production of in a stirred-tank bioreactor batch culture using YEPG non-selective medium containing glucose as the carbon source. F^+, the fraction of plasmid-carrying cells in the total population; EtOH, ethanol concentration.

30 min. The reaction was stopped by adding 2.0 mL of 12 N H_2SO_4 into the reaction tube and the solution was assayed for reducing sugars (glucose) produced using glucose kit (unless otherwise stated). One unit of glucoamylase activity is defined as the amount of enzyme in 1 mL to produce 1 μmol of equivalent glucose in per minute from soluble starch in 0.2 M citrate buffer at pH 5.0 and 37 °C (Kilonzo et al., 2008; Zhang et al., 1997a,b).

2.12.4. Fraction of plasmid-bearing cells determination

The fraction of plasmid-harboring cells determined by spreading about 100 μL of diluted (1×10^{-5} to 1×10^{-6}) sample onto both YNBG selective and YEPG non-selective agar plates (each five plates) (Kilonzo et al., 2008; Zhang et al., 1997a,b), and then incubating at 30 °C for 2 days. All viable cells will multiply on YEPG non-selective agar plates, but only the plasmid–carrying cell can grow on YNB-D selective agar plates. The fraction of plasmid-carrying cells was found by counting the colony-forming units (CFU) on both types of plates (100–300 colonies per plate). All plate counts were taken from the average of at least five replicates.

3. Results and discussion

3.1. Batch production with free-cell culture

The time course of batch production of glucoamylase using free-cell culture of recombinant *S. cerevisiae* C468/pGAC9 grown in the YEPG non-selective medium is shown in Fig. 2. Glucose was first consumed for predominantly for ethanol production due to Crabtree effect. Cell growth and glucoamylase production was minimal at this phase. After about ~14 h glucose was completely exhausted and ethanol reached its maximum, then the yeast cells began to utilize ethanol (EtOH) as the carbon source for continued cell growth in a second exponential phase, and a large amount of glucoamylase was produced in this period. The cell yield resulting from growth on glucose was 0.19 ± 0.002 g/g, but was 0.46 ± 0.004 g/g based on the total carbon consumption during the entire batch culture period. Glucoamylase production in this batch culture increased with increasing cell concentration, reaching its maximum value of 181 U/L after 28 h when the cell concentration approached the stationary phase. This suggests that GA production was growth associated (Kilonzo et al., 2008; Yang and Shu, 1996; Zhang et al., 1997a). As shown in Fig. 2, the fraction of plasmid-carrying cells dropped to about 72% at the end of the exponential phases but then remained almost at the same level even when the glucose was depleted. This might be a result of the difference in the slow death rate between plasmid- carrying cells and plasmid-free cells.

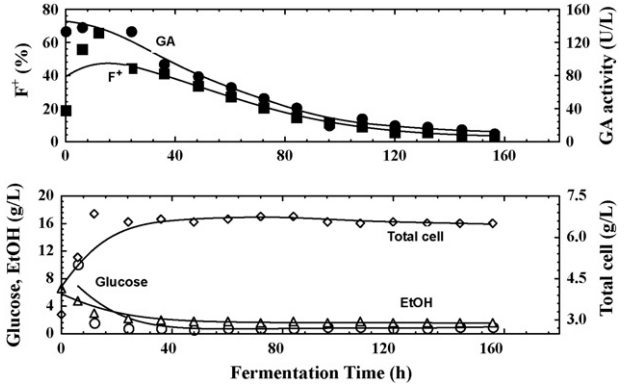

Fig. 3. GA (glucoamylase) production by a continuous free-cell culture of recombinant *S. cerevisiae* strain C468/pGAC9 in the stirred tank bioreactor at 0.10 h^{-1} dilution rate; EtOH, ethanol concentration.

3.2. Continuous production with free-cell culture

The corresponding continuous free-cell fermentations were also carried in the stirred tank bioreactor using the YEPG nonselective medium. After 12 h of batch cultivation period, continuous feeding of the medium was initiated to give a dilution rate of 0.10 h^{-1}. The time-course data of continuous culture of the recombinant *S. cerevisiae* strain C468/pGAC9 carried out in the stirred tank and airlift bioreactors at 0.10 h^{-1} dilution rate is shown in Figs. 3 and 4, respectively. As shown in these figures, the cell and ethanol concentrations, reached relatively stable levels within 40 h. The steady-state glucose concentration was <1.0 g/L. It can be seen from Figs. 3 and 4 that after 120 h, the fraction of plasmid-bearing cells in the stirred tank and airlift bioreactors dropped rapidly to ~10% and 40%, respectively. In response to the change from selective medium to non-selective medium, the glucoamylase (GA) production initially increased with cell concentration and reached a maximum of 65.8 U/L in the STB and ~200 U/L in the ALB after 12 h, then decreased continuously during the rest of the fermentation period. After 156 h (~26 generations), glucoamylase activity dropped to about 10% of its maximum value.

3.3. Continuous culture immobilized in an airlift-driven fibrous-bed bioreactor

Fig. 5 shows the results for a continuous fermentation in the airlift-driven fibrous-bed immobilized cell bioreactor using the original recombinant *S. cerevisiae* strain C468 at 0.10 h^{-1} dilution

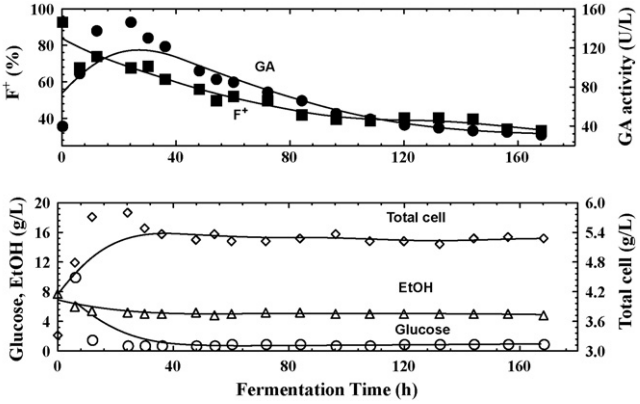

Fig. 4. GA (glucoamylase) production by a continuous free-cell culture of recombinant *S. cerevisiae* strain C468/pGAC9 in an airlift-driven bioreactor at 0.10 h^{-1} dilution rate; EtOH, ethanol concentration.

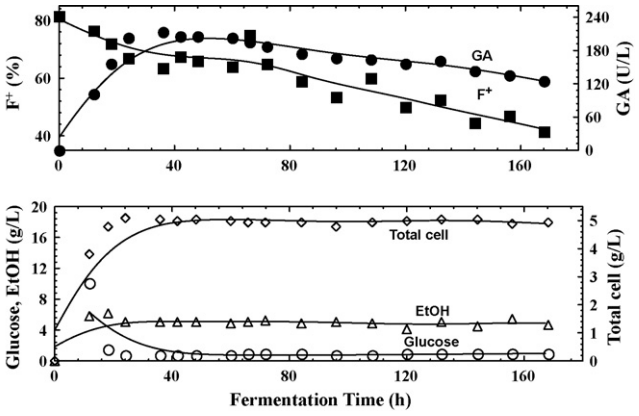

Fig. 5. Continuous production of GA (glucoamylase) in an airlift-driven fibrous-bed immobilized cell bioreactor using recombinant *S. cerevisiae* strain (C468/pGAC9) at 0.10 h^{-1} dilution rate; EtOH, ethanol concentration.

rate. The glucose concentration in the bioreactor decreased rapidly and reached steady-state at a concentration less than 1.0 g/L. The ethanol (EtOH) concentration reached maximum shortly after 12 h (~2 generation), and after 36 h it reached a steady state concentration of 5.38 g/L. The total cell concentration increased to a maximum, reaching a steady-state concentration of 5.55 g/L only after 36 h.

The steady-state immobilized cell concentration was ~85 g/L (i.e., 1089 or 820 mg/cm^2), which is 2 times higher than that obtained by Yang and Shu (1996). As shown in Fig. 5, GA production initially increased to a maximum value of about 212 U/L, and then decreased gradually to ~136 U/L. The maximum GA activity in the immobilized cell bioreactor was about 1.5 times higher than that in the corresponding free-cell system.

Cell viability increased to ~70% (data not shown), and the fraction of plasmid-carrying cells in the effluent dropped to about ~55%. However, the fraction of plasmid-carrying cells was decreasing at a faster rate than the GA activity.

3.4. Comparison between free- and immobilized-cell systems

Fig. 6 shows that the fraction of the plasmid-carrying cells in the effluent of the immobilized cell bioreactor system decreased more slowly than that in the free-cell system. Moreover, the protein production in the immobilized system was higher than the corresponding free-cell culture system. Fig. 5 also indicates that the immobilized cell system maintained a higher percentage of plasmid-carrying cells. This is supported by the low declining rate of the GA activity in the immobilized cell system.

Table 1
Glucoamylase levels, volumetric productivity, and specific productivity for continuous suspension and immobilized cultures.

Bioreactor	P (U/L)	P (U/L h)	P (U/g N^+ h)	Generation number
STB (free)	48.4 ± 10.0	2.8 ± 0.39	0.8 ± 0.34	2
ALB (free)	71.3 ± 9.07	1.4 ± 0.54	0.8 ± 0.28	3
ALFBB (imm)	160.8 ± 10.22	3.5 ± 0.49	1.1 ± 0.14	5

Table 1 compares these values for free versus immobilized runs. As can be seen, similar specific productivity (average) was observed for the free-cell culture in both stirred tank bioreactor (STB) and airlift bioreactor (ALB). However, the volumetric productivity in the STB was twice that in the ALB. The amount of glucoamylase increased from 71 U/L of the free-cell culture to 160 U/L of immobilized cell-culture. Similar improvement in volumetric and specific productivities was also observed. It is also evident from Table 1 that the averaged glucoamylase productivity was enhanced from 1.4 U/L h of free-cell culture (after 3 generations) to 3.5 U/L h of immobilized cultivation (after five generations). These findings are consistent with other studies (Chau et al., 2000; Zhang et al., 1997b; Yang and Shu, 1996).

3.5. Immobilized cells in the fibrous support

Fibrous samples from various parts of the fibrous bed were examined with scanning electron microscope (SEM) and analyzed for their total cell density and distribution, viability, and fraction of plasmid-carrying cell population at the end of each bioreactor study. Fig. 7 shows the attachment of recombinant *S. cerevisiae* cells on the fiber surface. As shown in Fig. 7, the fibrous bed was colonized by well-distributed yeast cells adsorbed on the fiber surface and entrapped in the interstitial spaces (Fig. 7A and B) within the fibrous support. Initially, the yeast cells were adsorbed on the fiber surface without forming large cell clumps or aggregates (cf. Fig. 7A). Until later, majority of the yeast cells were strongly attached to the fiber surface in large cell aggregates (cf. Fig. 7B and C). The budding yeast cells shown in Fig. 7D indicate that most of the immobilized cells in the fibrous-bed bioreactor were actively growing.

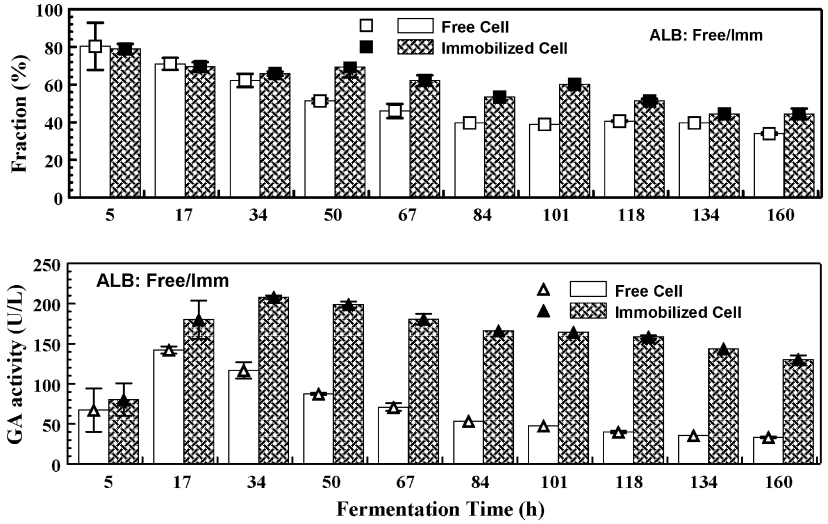

Fig. 6. Comparison of plasmid stability and GA production in continuous free-and immobilized selected strain (C468/pGAC9) of recombinant *S.cerevisiae* cells in an airlift-driven fibrous-bed immobilized cell bioreactor at 0.10 h^{-1} dilution rate.

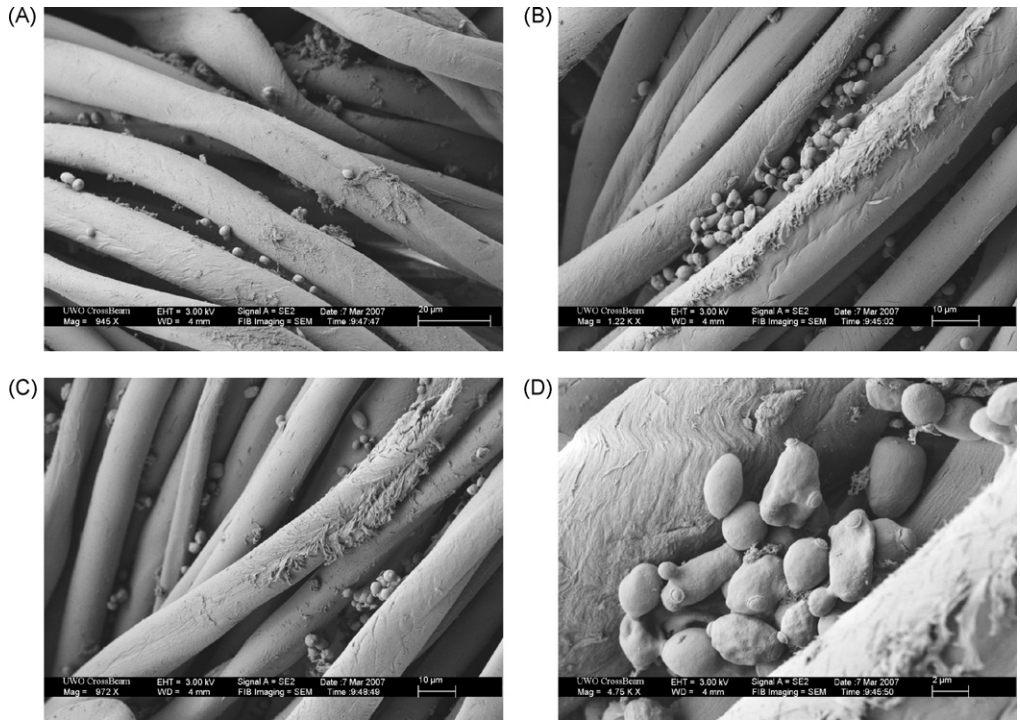

Fig. 7. Scanning electron micrographs (SEM) of recombinant yeast cells immobilized in the fibrous matrix.

3.6. Immobilized cell concentration

At the end of each bioreactor study, the concentration of immobilized and free cells in the fibrous-bed bioreactor was determined and as shown in Fig. 8. As shown, immobilized cell bioreactor 1 had 767.0 g of cells immobilized in 61.1 g of cotton fiber (i.e., 12.5 g cells/g fiber) and 74.2 g of free cells suspended in 8.3 L of liquid medium. The total amount of cells in this bioreactor was 841.2 g, and the cell density was 101.3 g/L bioreactor volume. The second and third fibrous-bed bioreactors had 671.0 and 658.8 g of cells immobilized in 55.2 g (in 2nd) and 54.4 g (in 3rd) of cotton fiber used in the cotton fibrous beds (i.e., 12.2 g cells/g fiber for 2nd, and 12.1 g cells/g fiber for the 3rd one), and 125.5 g and 95.4 g of free cells in the liquid phase, respectively. Similarly, the bioreactor was found to be 717.0 g/L (i.e., 12.3 g cells/g fiber). The average cell density cell density of cells immobilized in 58.3 g of the cotton fiber used in the fourth fibrous bed suspended in the liquid medium in each of the four fibrous-bed bioreactors was ~12.0 g/L of the bioreactor volume. Similarly, the averaged cell density for cells in the immobilizing fibrous matrix was ~85 g/L (~1089 or 819 mg/cm^2 fiber), or ~12.3 g/g fiber for all four bioreactors, indicating that the maximum immobilized cell density in fibrous matrix was 12.3 g/g. This shows that the immobilized cell density was depended on the packing density of the cotton fiber in the bioreactor.

3.7. Cell viability

Figs. 9–11 show the viability and fraction of plasmid-carrying cells in the fibrous-bed bioreactor. As shown in Fig. 9, the viability of recombinant cells in the effluent (suspension) of the fibrous-bed bioreactor was very high in the neighborhood of 94–100%, although the fraction of plasmid-carrying cells was very low, <10%. Figs. 10 and 11 show viability and fraction of plasmid-carrying cells after the 1st and 7th wash of the fibrous matrix. It is evident from these figures that viability decreases while fraction of plasmid-carrying cells increases with the level of washing the fibrous matrix.

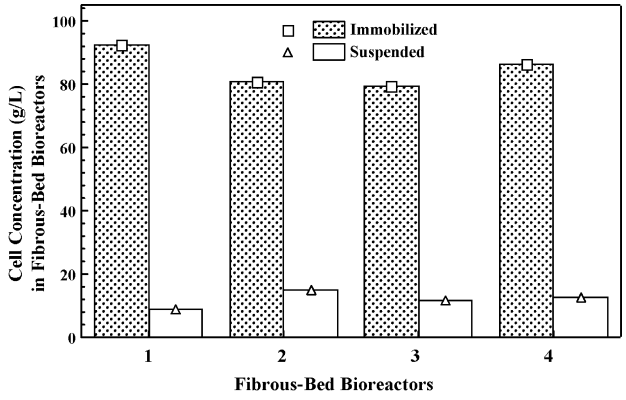

Fig. 8. Cell concentrations in fibrous-bed bioreactors (Note: The total cell concentration in the bioreactor based on a bioreactor working volume of 9 L, and the bioreactor void volume (V_p/V_L) was ~0.08).

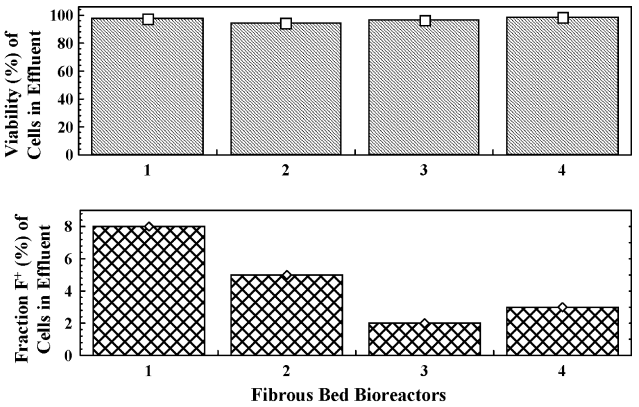

Fig. 9. Viability and fraction of plasmid-carrying cells in the effluent of the fibrous-bed bioreactor.

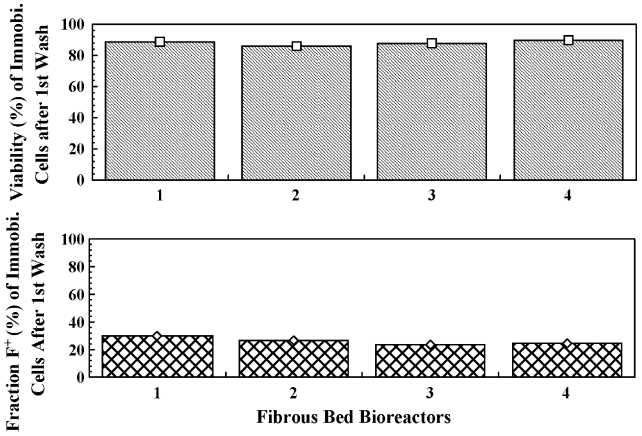

Fig. 10. Viability and fraction of plasmid-carrying cells in the immobilized phase of the fibrous-bed bioreactor after the 1st wash.

After the 7th wash, the recombinant yeast cell viability decreases from 89% to 68% whiled the fraction of plasmid-carrying cells increased from 25% to 88%. As shown in Figs. 9–11, the viability of the immobilized cells was 78–83% lower than that of the free cells (>94%). Also, the viability of immobilized cells decreased from 86% to 90% for the loosely entrapped cells removed at the first was to 60–90% for the strongly attached cells removed at the seventh wash. This was anticipated because the more strongly immobilized cells were likely to be the oldest cells in the bioreactor. They were also closer to the surface of the fiber and were covered with layers of other cells and, thus, had limited nutrient supply. Also, it was found that increasing the level of washing reduced cell viability by more than 30%, although the fraction of the plasmid-carrying cells increased by more than 95%. Thus, F_i^+ was higher for the cells from later washes or more strongly immobilized cells. It is evident from these results that the cell immobilization in the cotton fibrous matrix led to the retention of a large amount of the plasmid-carrying cells during the operation period with YEPG nonselective medium. This was the reason for the stable GA production in the airlift-driven fibrous-bed bioreactor.

3.8. Effect of immobilizing recombinant yeast cells

The increased stability and hence the activity of the targeted protein in the immobilized recombinant cell system has been associated with: (1) reduced specific growth rate, which increases the chance of the plasmid-carrying cells being retained in the immobilized cell bioreactor and reduces the likely hood of segregational and competitive instability to occur (Walls and Gainer, 1991), (2) compartmental cell distribution and mass transfer limitation in the immobilized cell environment, thus limiting the overgrowth of the plasmid-free-cell population (Dincbas et al., 1993), (3) physiological changes in the immobilized cells and increased plasmid copy number at reduced growth rate, and (4) selective immobilization or preferential retention of plasmid-carrying cells in the fibrous bed (Zhang et al., 1997a,b; Yang and Shu, 1996).

In principle, plasmid-carrying cells grow at a slow phase compared to their plasmid-free counterparts and have a higher probability of remaining attached to the fibrous-matrix surface or to each other to form cell aggregates. Thus, they can be better retained by adsorption and entrapment in the fibrous matrix than can plasmid-free cells. In contrast, plasmid-free cells grow at a faster rate than plasmid-carrying cells and have an increased tendency to become freely suspended in the liquid medium. They thus can be washed out from the bioreactor more easily than plasmid-carrying cells.

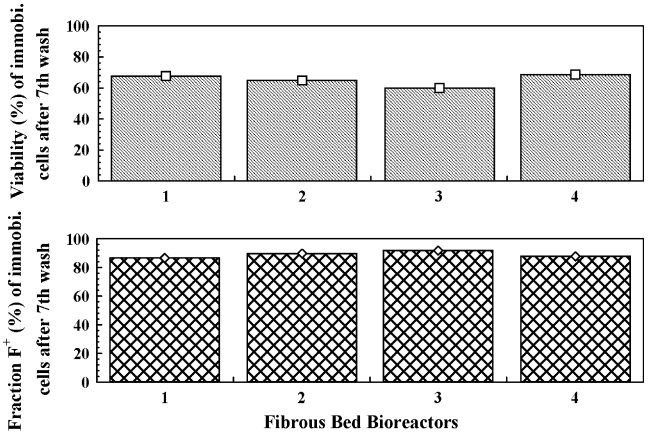

Fig. 11. Viability and fraction of plasmid-carrying cells in the immobilized phase of the fibrous-bed bioreactor after the 7th wash.

4. Conclusions

The amount of glucoamylase expressed by recombinant *S. cerevisiae* C686/pGAC9 cells was increased by cell immobilization during continuous cultures. In contrast, continuous free-cell cultures were unstable and rapidly lost the plasmid. In the immobilized cell system, the increase in productivity appeared to have resulted from both increased plasmid stability and increased specific productivity during continuous culture due to reduction in the probability of plasmid loss as a result of reduced specific growth rate, and perhaps a preferential retention of the plasmid-carrying cells in the fibrous-bed immobilized cell system. Similar increases in both volumetric and specific productivity have been demonstrated for protein production in immobilized *E. coli* JM101 cells (Chen et al., 2006).

Acknowledgement

The authors acknowledge financial support of this work by the Natural Science and Engineering Research Council of Canada (NSERC) through individual research grants awarded to Professor Argyrios Margaritis and Professor Maurice Bergougnou.

References

Bannister, S.J., Wittrup, K.D., 2000. Glutathione expression in response to heterologous protein secretion in *Saccharomyces cerevisiae*. Biotechnol. Bioeng. 68 (4), 809–879.

Barbotin, J.N., 1994. Immobilization of recombinant bacteria: a strategy to improve plasmid stability. Ann. N. Y. Acad. Sci. 506, 196–208.

Chau, T.L., Guillan, A., Roca, E., Nunez, M.J., Lema, J.M., 2000. Enhancement of plasmid stability and enzymatic expression by immobilizing recombinant *Saccharomyces cerevisiae*. Biotechnol. Lett. 22, 1247–1250.

Chen, X.-A., Xu, Z.-N., Cen, P.-L., Wong, W.K.R., 2006. Enhanced plasmid stability and production of hEGF by immobilized recombinant *E. coli* JM101. Biochem. Eng. J. 28, 215–219.

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

Dincbas, V., Hortascu, A., Camurdan, A., 1993. Plasmid stability in immobilized mixed cultures of recombinant *E. coli*. Biotchnol. Bioeng. 9, 218–220.

Ekino, K., Hayashi, H., Moriyama, M., Mtasuda, M., Goto, M., Yoshino, S., Furukawa, K., 2002. Engineering of polyploid *Saccharomyces cerevisiae* for secretion of large amounts of fungal glucoamylase. Appl. Environ. Microbiol. 68, 5693–5697.

Gomes, E., de Souza, S.R., Grandi, R.P., Da Silva, R., 2005. Production of thermostable glucoamylase by newly isolated *Aspergillus flavus* A 1.1 and *Thermomyces lanuginosus* A13. 37. Braz. J. Microbiol. 36, 75–82.

Gorgens, J.F., van Zyl, W.H., Knoetze, J.H., 2001. The metabolic burden of the *PGK1* and *ADH2* promotor system for heterologous xylanase production by *Saccharomyces cerevisiae* in defined medium. Biotechnol. Bioeng. 73 (5), 238–245.

Gupter, J.C., Mukherjee, K.J., 2001. Stable maintenance of plasmid in continuous culture of yeast under non-selective conditions. J. Biosci. Bioeng. 28, 89–99.

Guo, G.-L., Tseng, D.-H., Huang, S.-L., 2001. Co-metabolic degradation of trichloroethylene by *Pseudomonas putida* ina fibrous bed bioreactor. Bioetchnol. Lett. 23, 1653–1657.

Hsu, C.H., Chu, Y.E., Argin-Soyal, S., Hahm, T.S., Lo, Y.M., 2004. Effects of surface characteristics and xanthan polymers on the immobilization of *xanthomonas Campestris* to fibrous matrices. J. Food Sci. 69 (9), E441–E448.

Huang, Y.L., Shu, C.H., Yang, S.T., 1996. Kinetics and modeling of GM-CSF production by immobilized recombinant yeast in a three-phase fluidized bed bioreactor. Biotechnol. Bioeng. 53, 470–477.

Huang, Y., Yang, S.-T., 1998. Acetate production from whey lactose using co-immobilized cells of homolactic and homoacetic bacteria in a fibrous-bed bioreactor. Biotechnol. Bioeng. 60, 499–507.

Kilonzo, P.M., Margaritis, A., Bergougnou, M.A., 2008. Effects of medium composition on glucoamylase production during batch fermentation of recombinant *Saccharomyces cerevisiaae*. J. Inst. Brew. 114 (2), 83–95.

Kilonzo, P., Margaritis, A., Yu, J.T., Ye, Q., 2007a. Bioethanol production from starchy biomass by direct fermentation using *Saccharomyces diastaticus* in batch free and immobilized cell systems. Int. J. Green Energy 4, 1–14.

Kilonzo, P.M., Margaritis, A., Bergougnou, M.A., Yu, J.T., Qin, Y., 2007b. Effect of geometrical design on hydrodynamic and mass transfer characteristics of a rectangular-column airlift bioreactor. Biochem. Eng. J. 34, 279–288.

Kilonzo, P.M., Margaritis, A., Bergougnou, M.A., Yu, J.T., Qin, Y., 2006. Influence of the baffle clearance design on hydrodynamics of a two riser rectangular airlift reactor with inverse internal loop and expanded gas-liquid separator. Chem. Eng. J. 121, 17–26.

Li, G.-Q., Yang, S.-Z., Chen, J.-Y., 1995. Mass transfer and gas-liquid circulation in an airlift bioreactor with viscous non-Newtonian fluids. Chem. Eng. J. 56, B101–B107.

Lo, C.-S., Hwang, S.-J., 2003. Local hydrodynamic properties of gas phase in an internal-loop airlift reactor. Chem. Eng. J. 91, 3–22.

Ma, T., Yang, S.T., Kniss, D.A., 1999. Development of an invitro human placenta model by cultivation of human trophoblasts in a fiber-based bioreactor system. Tissue Eng. 5, 91–102.

Melo, J.S., D'Souza, S.F., 1999. Simultaneous filtration and immobilization of cells from a flowing suspension using a bioreactor containing polyethylenimine coated cotton threads: application in the continuous inversion of concentrated sucrose syrups. World J. Microbiol. Biotechnol. 15 (1), 23–27.

Shiba, S., Nishida, Y., Park, Y.S., Iijima, S., Kobayashi, T., 1994. Improvement of cloned α-amylase gene expression in Fed-batch cultures of recombinant *Saccharomyces cerevisiae* by regulating both glucose and ethanol concentration using a fuzzy controller. Biotechnol. Bioeng. 44, 1055–1063.

Shu, C.H., Yang, S.T., 1996a. Effect of particle loading on GM-SCF production *Saccharomyces cerevisiae* in a three-phase fluidized bed bioreactor. Biotechnol. Bioeng. 51, 229–236.

Silva, E.M., Yang, S.T., 1995. Kinetic and stability of a fibrous-bed bioreactor for continuous production of lactic from unsupplemented acid whey. J. Biotechnol. 41 (1), 59–70.

Vainio, A.E.I., Lantto, R., Parkkinen, E.E.M., Helena, T., Torkkeli, H.T., 1994. Production of *hormoconis resinae* glucoamylase by a stable industrial strain of *Saccharomyces cerevisiae*. Appl. Microbiol. Botechnol. 41, 53–57.

Yang, S.-T., Huang, Y., Hong, G., 1995. A novel recycle batch immobilized cell bioreactor for propionate production from whey lactose. Biotechnol. Bioeng. 45, 379–386.

Yang, S.T., Shu, C.H., 1996. Kinetics and stability of GM-CSF production by recombinant yeast cells immobilized in a fibrous-bed bioreactor. Biotechnol. Prog. 12, 449–456.

Yang, S.T., Zhu, H., Li, Y., Hong, G., 1994. Continuous propionate production from whey permeate using a novel fibrous bed bioreactor. Biotechnol. Bioeng. 41 (11), 1124–1130.

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

Withers, J.M., Swift, R.J., Wiebe, M.J., Robinson, G.D., Punt, P.J., van den Hondel, C.A., 1998. Optimization and stability of glucoamylase production by recombinant strains of *Aspergillus niger* in chemostat culture. Biotechnol. Bioeng. 59, 407–418.

Zhang, Z., Scharer, J.M., Moo-Young, M., 1997a. Plasmid instability kinetics in continuous culture of a recombinant *Saccharomyces cerevisiae* in airlift bioreactor. J. Biotechnol. 55, 31–41.

Zhang, Z., Scharer, J.M., Moo-Young, M., 1997b. Mathematical model for aerobic culture of a recombinant yeast. Bioproc. Bioeng. 17, 235–240.