J Ind Microbiol Biotechnol (2010) 37:773–783
DOI 10.1007/s10295-010-0719-4

ORIGINAL PAPER

# Repeated-batch production of glucoamylase using recombinant *Saccharomyces cerevisiae* immobilized in a fibrous bed bioreactor

**Peter M. Kilonzo · Argyrios Margaritis · Maurice A. Bergougnou**

Received: 28 December 2009 / Accepted: 26 March 2010 / Published online: 21 April 2010
© Society for Industrial Microbiology 2010

**Abstract** The recombinant *Saccharomyces cerevisiae* strain C468/pGAC9 has an unstable hybrid plasmid pGAC9, which directs production of glucoamylase. A fibrous cotton material with a good adsorption capability for recombinant *S. cerevisiae* cells was used as the immobilization matrix in an internal loop airlift-driven fibrous bed bioreactor (ILALFBB) system. With batch cultures in the ILALFBB, the fraction of plasmid-carrying cells was 72% after more than 2 days cultivation, which was two times higher than that in the conventional free-cell culture. Correspondingly, a high activity of glucoamylase (GA; 113 U/l) was achieved with a high productivity of 43 U/l/h. The ILALFBB system also maintained a high fraction of viable plasmid-carrying of 74% for glucoamylase production during repeated-batch cultures, achieving a high glucoamylase activity of 140 U/l with a productivity of 19–130 U/l/h in all 14 batches studied during 19.8 days. The stable and long-term glucoamylase production from the ILALFBB was attributed to the effect of cell immobilization on plasmid stability. Plasmid-carrying cells were preferentially retained in the fibrous matrix because of their ability to adhere to the fiber surface and to form cell aggregates higher than those of plasmid-free cells. The repeated batch using immobilized cell of recombinant *S. cerevisiae* in the ALALFBB system thus provides a feasible method for stable, long-term and high-level production of glucoamylase.

P. M. Kilonzo · A. Margaritis (✉) · M. A. Bergougnou
Department of Chemical and Biochemical Engineering, University of Western Ontario, London, ON N6A 5B9, Canada
e-mail: amarg@uwo.ca
URL: http:/www.eng.uwo.ca/people/amargaritis/

**Keywords** Recombinant *Saccharomyces cerevisiae* · Fibrous bed bioreactor · Immobilization · Glucoamylase

**List of symbols**

| | |
|---|---|
| $A$ | Cross-sectional area ($m^2$) |
| $c_p$ | Spacing between adjacent fiber surfaces (m) |
| $F^+$ | Fraction of plasmid-carrying cells (%) |
| GA | Glucoamylase activity (U/l) |
| $h$ | Clearance (m) |
| $N$ | Cell density of total cell population (g/l) |
| $N^+$ | Cell density of plasmid-carrying cells (g/l) |
| $P$ | Productivity concentration (U/l) |
| $Q$ | Volumetric productivity (U/l/h) |
| $S$ | Substrate concentration (g/l) |
| $Y$ | Yield |

**Subscript**

| | |
|---|---|
| b | Bottom |
| d | downcomer |
| i | Immobilized |
| p | packed bed |
| r | Riser |
| t | Top |

## Introduction

The fungal glucoamylase enzyme [GALase; (1 → 4) (1 → 6)-α-D-glucan glucohydrolase; EC3.2.1.3] is a glycoprotein 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 to saccharify starchy feedstocks in commercial processes for glucose syrup [8, 27] and bioethanol [17] productions. In addition, glucoamylase is used in medicine in a coupled assay with α-D-glucosidase in the diagnosis of pancreatic

disease. Furthermore, glucoamylase is also used in quantitative determination of glycogen and starch [18].

The production of glucoamylase by recombinant strains of fungi are much lower as compared to those of homologous proteins, and usually only reach levels that do not exceed a few tens of milligrams per liter of culture medium [9]. Even with efficient secretion of heterologous proteins in filamentous fungi, a major problem is the degradation of heterologous proteins by extracellular proteases, which hinder the development of filamentous fungal strains as hosts for recombinant strains [9, 10, 26]. Therefore, a suitable host organism for glucoamylase gene expression and secretion is needed to improve the yield.

Recombinant yeast expression systems such as *Saccharomyces cerevisiae* often play an important role in the production of value-added proteins with high fidelity to their native forms [19, 29]. As a simple eukaryote, *S. cerevisiae* can accomplish many post-translational modifications such as oligomeric assembly, disulfide bond formation, glycosylation and proteolytic cleavage [6, 7, 24]. In addition, proteins can be secreted which would simplify and reduce the cost of downstream processing [16]. Due to these inherent superior characteristics, a gene having a sequence complementary to that of the glucoamylase polypeptide mRNA from *Aspergillus awamori* was cloned into a mutant *S. cerevisiae* C468 (α *leu2-3 leu2-112 his311 his3-15 mal*$^-$) (ATCC 62995) as a host strain. Several recombinant *S. cerevisiae* strains including C468/pAC1, C468/pGC21, 468/YEpMAC101, C468/YEpGAC9, C468/YEpPM16, C468/YEpPM18 and C48/pGAC9 have been constructed using the host strain [18]. The *A. awamori* glucoamylase expressed in *S. cerevisiae* C468/pGAC9 was found to be stable in YEPG (containing 2% D-glucose) batch fermentation, exhibiting plasmid stability as high as 92% [18]. But, the expression levels in continuous culture were relatively low. Especially the productivity of glucoamylase in *S. cerevisiae* strain C468/pGAC9 was greatly limited by the low cell density (0.3–1.3 $\times$ $10^{10}$ cells $l^{-1}$) of plasmid-carrying cells as well as low plasmid stability (14–34%) during continuous fermentation [16]. Therefore, it is necessary to increase cell density and plasmid stability during the cultivation process in order to improve the production of recombinant proteins in *S. cerevisiae* C468/pGAC9.

Cell immobilization is now a widely accepted technique to minimize plasmid instability and to improve bioreactor productivity [1, 4]. Over-production of a recombinant protein may unbalance metabolic processes and ultimately result in cell death because of the over-expression of a cloned gene or to the expression of a heterologous protein inside a bioreactor [1]. As a result, conventional immobilized cell bioreactor systems usually suffer from productivity loss over an extended operation period because of accumulation of the dead cells and poor mass transfer under high cell density conditions.

Recently, a fibrous-bed bioreactor (FBB) with cells immobilized in the fibrous matrix packed in the reactor has been successfully used for several organic acid fermentations [14, 28] with significantly improved cell immobilization efficiency, productivity, final productivity yield and concentration [33]. It has been shown that the FBB could continuously renew the cell population and adapt cells to the bioreactor environment [2, 12]. Fibrous sheet materials, such as commonly used cotton clothes, which have rough surfaces and provide more surface area per unit weight than smooth surfaces can, were used in the FBB. Furthermore, it was suggested that the rough surface of cotton fibers could induce turbulence, hence increasing the contact frequency of cells with immobilizing carriers and shielding the adsorbed cells from being washed off the immobilization carriers.

Repeated-batch cultivation is a well-known method for enhancing the productivity of microbial cultures because it skips the turnaround time and the lag phase, thus increasing the process productivity [13, 25]. This method has been applied to increase the yield of several fermentation processes such as the production of gibberellic acid [21], hyaluronic acid [13], mycophenolic acid [27], butyric acid [14], ethanol [3, 5, 15, 23], biohydrogen [20] and lipase [30]. Chen et al. [2] observed a long-term production of soluble human Fas ligand (hFasL) obtained by immobilizing *Dictyostelium discoideum* in a FBB using a cotton towel. With batch cultures in the FBB, a highly immobilized cell density of 1.37 $\times$ $10^8$ cell/ml and a hFasL productivity of 23 μg/l/h were achieved after 110-h cultivation. The FBB also maintained a high density of viable cells for production during repeated batch cultures achieving a productivity of 9–10 μg/l/h in three batches. A similar operation may be promising to improve productivity of a recombinant yeast bioprocess.

In this work, the recombinant yeast *S. cerevisiae* C468/pGAC9 expressing the *A. awamori* glucoamylase gene was used as the model organism to investigate the effect of immobilization on the stability of repeated batch production of glucoamylase. To avoid the problems encountered in conventional immobilized cell systems, a surface adsorption immobilization method was employed using a fibrous matrix to develop a novel fibrous bed bioreactor for the recombinant yeast fermentation.

## Materials and methods

### Recombinant yeast strain and plasmid

The recombinant *S. cerevisiae* strain C468/pGAC9, used earlier in other studies [16, 18, 32], was also employed in

this study. This strain contains the hybrid plasmid vector pGAC9 shown in Fig. 1. The plasmid contains a portion of the yeast 2-$\mu$ plasmid (2-μm circle), a DNA fragment that 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 [22].

## Culture media

The recombinant yeast was grown in selective YNBG (YNB-yeast yeast nitrogen base without amino acids, G-glucose) 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, and maintained on YNBM (YNB-yeast yeast nitrogen base without amino acids, M-maltose) containing 2% (w/v) maltose. A complex non-selective YEPG (YE-yeast extract, P-peptone, G-glucose) medium containing 5 g/l yeast extract (Becton-Dickinson), 10 g/l peptone (Becton-Dickinson) and 20 g/l D-glucose was used for the yeast cultivation. 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.

## Adsorption of recombinant *S. cerevisiae* on fibrous matrix

The fibrous sample of heavy white woven cotton material (0.5 cm thick) was tested for cell adsorption capacity by placing five pieces of cotton, each 0.87 g (or of size 4 cm × 4 cm × 0.4 cm) in a 500-ml shake flask containing 200 ml precultured recombinant *S. cerevisiae* C468/pGAC9 broth for 3–4 days with a cell density of $4.4 \times 1.0^{12}$ cells/l (corresponding to $OD_{600} \cong 2.8$, CDW = 10 g/l). The flask was incubated and shaken at 200 rpm, 30°C, and monitored in the following 300 min, after which time the cell numbers in suspension and in the fibrous matrices were counted to estimate the rate of cell immobilization.

## Construction of the fibrous-bed (FB) matrix

The fibrous materials used in this study were the heavy bleached woven cotton fabric shown in Fig. 1a and b.

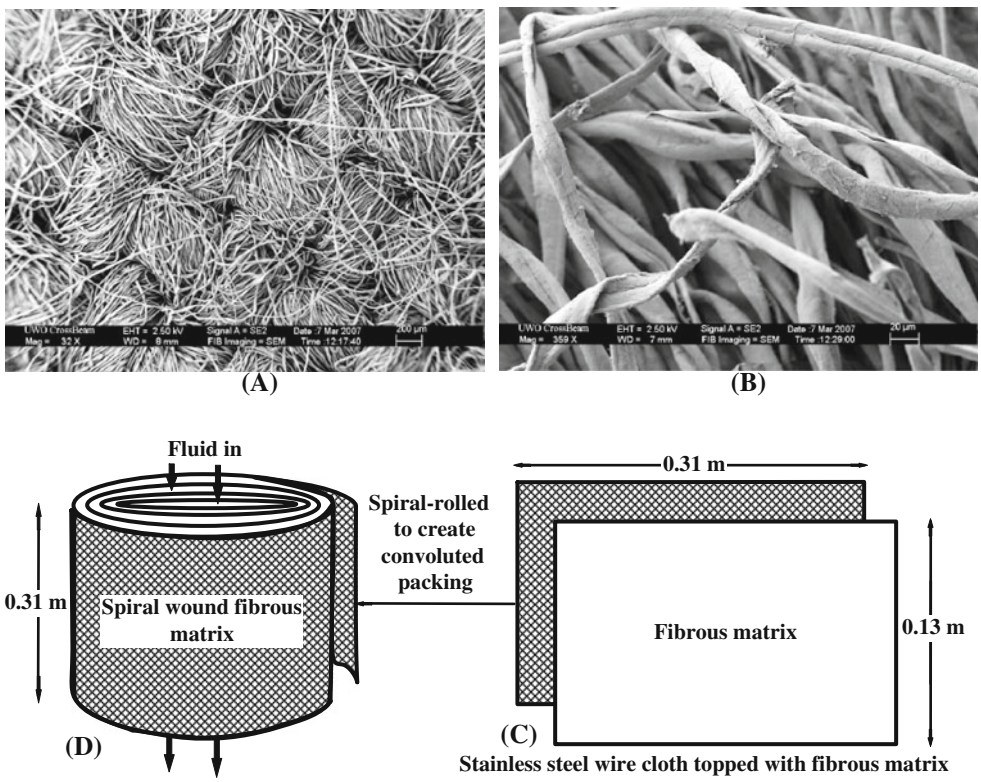


**Fig. 1** Schematic diagram of the construction of the proposed spiral-wound fibrous bed bioreactor: **a** and **b** cotton, **c** stainless steel wire cloth topped with fibrous matrix, **d** spiral-wound fibrous matrix

The cotton matrix (0.61-l) [13 cm × 94 cm × 0.05 cm and porosity of 96%] was kindly donated by Mountain Weavers Ltd., Dorset, VT. A stainless steel wire cloth (SUS 304, porosity: 0.972: mesh no. 20, Small Parts Inc., Logansport, IN) topped with the fibrous cotton sheets (Fig. 1c) was wound into a spiral configuration along the vertical axis as shown in Fig. 1d. The spiral-wound configuration had $c_p = 0.2$–$0.6$ cm gaps between two adjacent layers of the fibrous sheet matrix to allow fluid flow through the bioreactor unhindered. The fibrous sheet material and the stainless steel mesh also created a highly specific surface area and porosity ($\sim 0.935$). These properties can allow larger amounts of biomass to attach on the fibrous bed.

## Construction of the internal loop airlift fibrous-bed bioreactor (ILALFBB)

The fibrous-bed matrix was loosely incorporated into the downcomer (draft tube) section of an inverse (annulus sparged) internal-loop airlift reactor (88 cm height) that had a cylindrical column (i.d.: 10.6 cm) containing an inner concentric draft tube (downcomer) of 35 cm in height and 6 cm diameter. The schematic diagram of the experimental setup is shown in Fig. 2. The bioreactor, with a total working volume of 9 l, was made of Plexiglas (polymethyl 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

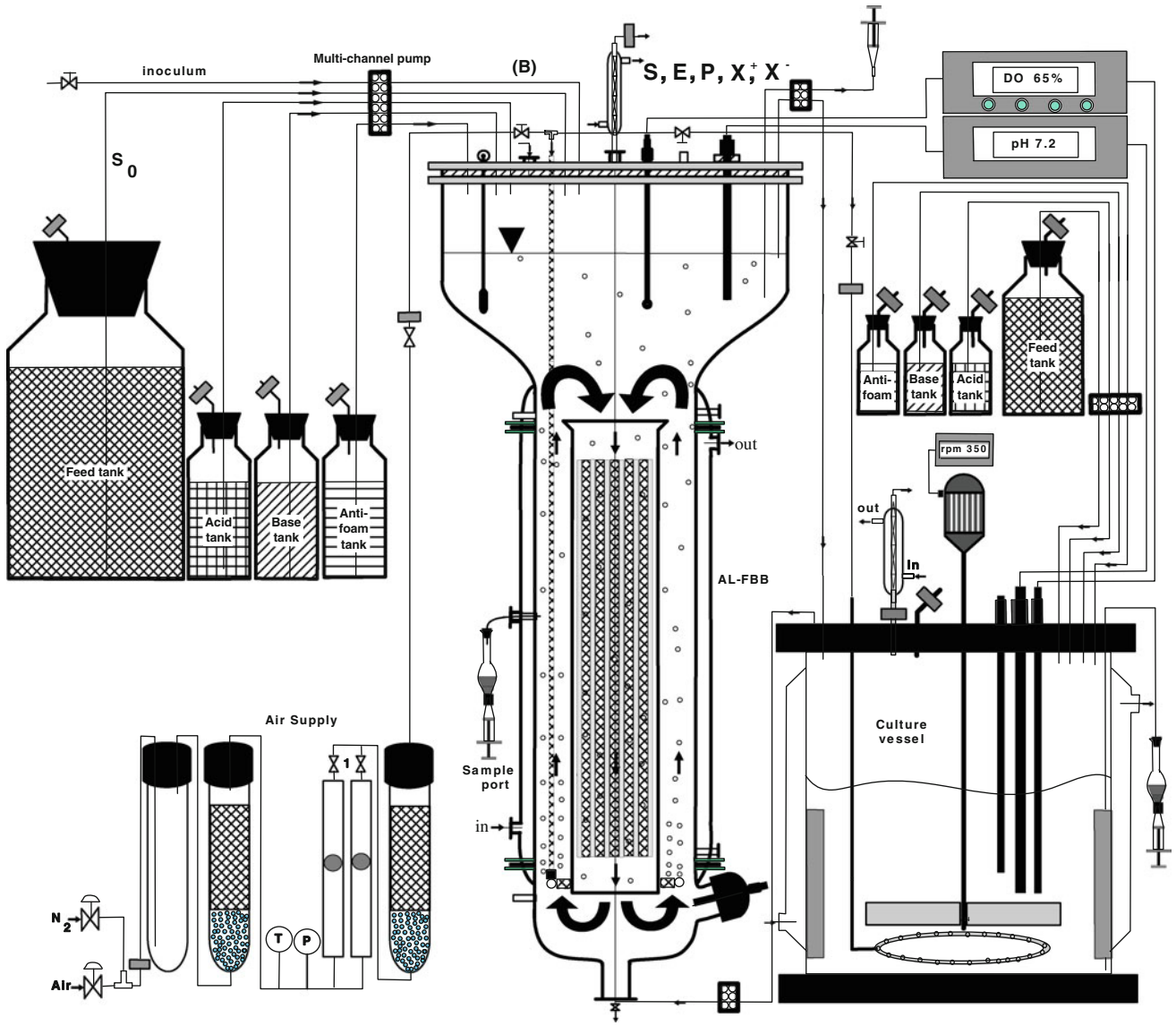


**Fig. 2** Schematic description of a fibrous bed airlift bioreactor (ILALFBB). The ILALFBB consist of an airlift bioreactor column, a culture vessel (standard stirred tank bioreactor), air supply, acid, base, antifoam and feed tanks, pH and DO meters

5.9 cm. The riser-downcomer combination gave an area ration $A_r/A_d$ of 1.84. The top end $h_{pt}$ of the fibrous bed was kept constant at 11.4 cm below the medium level and the bottom end $h_{pb}$ at 12.5 cm above the bioreactor bottom.

### Cell immobilization and fermentation in the ILALFBB

The cells intended for cell immobilization studies were developed in a stirred tank bioreactor (STBR) (see Fig. 2) and allowed to attain a cell density of $OD_{600\ nm} = 2.65$, CDW $\cong$ 8.2 g/l (i.e., $7.7 \times 1.0^{12}$ cells/l) prior to immobilization. Cell suspension containing the recombinant *S. cerevisiae* C468/pGAC9 was introduced at the bottom of the ILALFBB and returned from the top outlet to the culture vessel for re-aeration at a broth flow rate of 25 ml/min to allow a natural attachment of the cells.

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 from 25 to 100 ml/min, and the bioreactor was operated in a continuous and repeated batch mode to increase the cell density in the fibrous bed to a highly (>50 g/l) stable level. The yeast cells, with negatively charged cell wall surfaces, were passively immobilized onto the fibrous matrix surface.

After 54 h, the circulation was discontinued, and the spend medium was replaced with a fresh one, which was continuously fed at a dilution of 0.05/h to allow plasmid-bearing cells 3–4 days to grow to a high density in the bioreactor. The bioreactor was maintained at 30°C and aerated at a volumetric airflow rate of 9 l/min (i.e., 0.028 m/s). After the effluent cell density reached steady state, the spent fermentation broth was pumped out and then replaced with 8.1 l of freshly prepared YEPG nonselective medium to initiate glucoamylase production. The fermentation proceeded in the batch mode for 24 h. The culture fluid was pumped out from the bioreactor, and about 900 ml was retained as the inoculum for the next batch fermentation. The next batch fermentation was initiated by pumping the 8.1 l of fresh YEPG non-selective medium into the bioreactor. This procedure was repeated every 24 h. The repeated-batch fermentation was performed for 2.8 weeks. During this period, samples were taken at regular time intervals to monitor the total cell concentration, D-glucose, starch, ethanol and glucoamylase concentrations, and plasmid stability.

### 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 the 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 were reported.

### 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 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 + 1,000 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-$H_2O$) overnight 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.

## Analytical methods

### Free cell concentration

Free cell concentration was measured by colorimetric and dry weight and optical density methods. The correlation between dry cell weight ($N$) and optical density (OD) was determined as $N = 1.6835\ \mathrm{OD}$ ($R = 0.9986, R^2 = 0.9971$).

### Immobilized cell concentration

All of the liquid present in the bioreactor was drained, and its volume and 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 1 × 1-cm pieces 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.

### 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 Zanone et al. [31]. Ethanol concentration was determined by alcohol dehydrogenase enzyme kit according to the procedure described recently by Kilonzo et al. [16, 18].

### Glucoamylase activity assay

Glucoamylase activity was determined according to a modification of the assay described by Zhang et al. [32]. 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) were added and incubated at 37°C for 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 per minute from soluble starch in 0.2 M citrate buffer at pH 5.0 and 37°C [22].

### Fraction of plasmid-bearing cells determination

The fraction of plasmid-harboring cells determined by spreading about 100 μl of diluted ($10^{-5}$–$10^{-6}$) sample onto both YNBG selective and YEPG non-selective agar plates (each five plates) [32], 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.

## Results and discussion

### Adsorption of recombinant *S. cerevisiae* cells on fibrous matrix

The fibrous matrix was initially examined for the adsorption of recombinant *S. cerevisae* cells prior to its use as the immobilization matrix. The immobilization rate of the recombinant *S. cerevisae* cells is shown in Fig. 3. Because of the short period in which the cells were subjected to the fibrous matrix structure, the effect of cell growth was negligible. Figure 3 shows that the fibrous matrix had a fast cell immobilization rate and adsorbed >83% of the

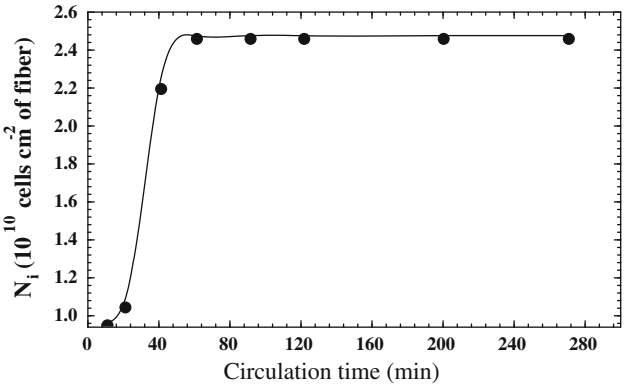


**Fig. 3** Immobilization from a flowing suspension; variation of the amount immobilized per unit area as a function of the duration of circulation. Flow velocity is 0.10 m $s^{-1}$. $N_i$-immobilized cell concentration

suspended cells within the first 60 min (1 h). After 3–4 days of immobilized cultivation of the recombinant yeast, pieces of the fibrous matrix were examined under scanning electron microscope.

As shown in Fig. 4, the recombinant yeast cells colonized the fiber surface (cf. Fig. 4b, c), and some of them were actively attached to the fiber surface in network form (cf. Fig. 4d), resulting in the formation of large cell aggregates, and some others had started to bud (cf. Fig. 4).

### Batch fermentation

Prior to the repeated-batch operation, characteristics of glucoamylase (GA) production from YEPG non-selective medium were investigated by batch fermentation. As shown in Fig. 5, glucose was first consumed predominantly for ethanol production because of the crabtree effect [16, 18, 32]. The ethanol concentration reached a maximum of about 7 g/l after 18 h, when the residual glucose concentration in the fermentation broth was about 11 g/l. At the end of this period, the cells were simultaneously consuming the glucose and ethanol. Cell growth and GA production were minimal at this phase.

Glucose was depleted after 28 h, and ethanol consumption was continued for 15 h more for further cell growth in a second exponential phase. The recombinant *S. cerevisiae* cells were grown to a cell density of about 25 g/l after 51 h of cultivation; then, feeding was commenced at a rate of 2 ml/min and a corresponding dilution rate of 0.1/h. The cell yield resulting from growth on glucose was 0.74 ± 0.11 g/g. The concentration of free cells in suspension reached a maximum at 5 g/l and then continued to decline to a lower level. As seen from this figure, the production of GA continued after the employment of the ILALFBB and reached a high concentration of ~125 U/l. The fraction of the plasmid-carrying cells was

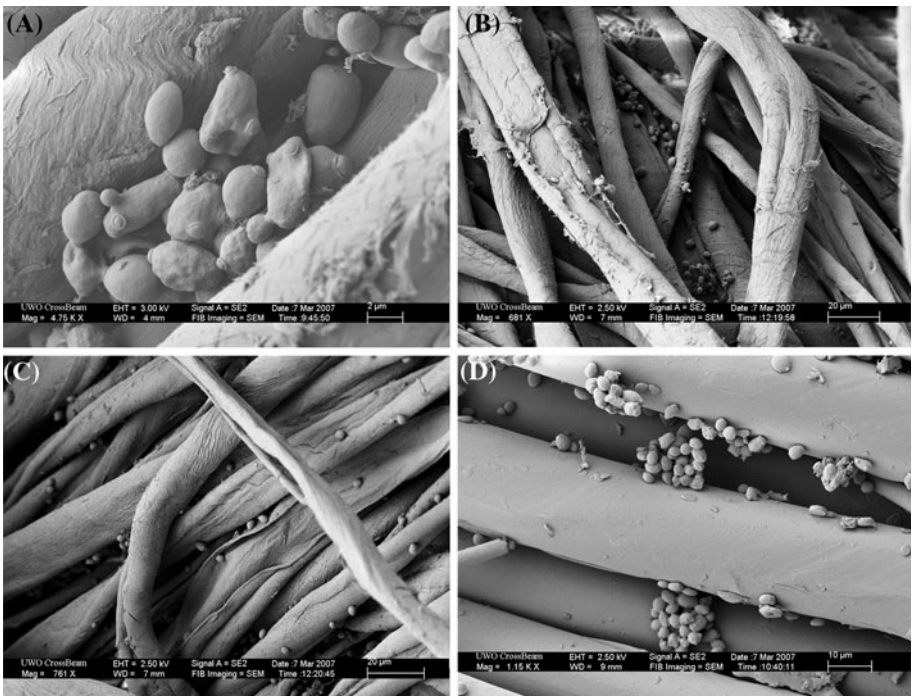


**Fig. 4** Scanning electron micrograph of fibrous cotton support surface with immobilized recombinant *S. cerevisiae* C468/pGAC9 cells after 3–4 days

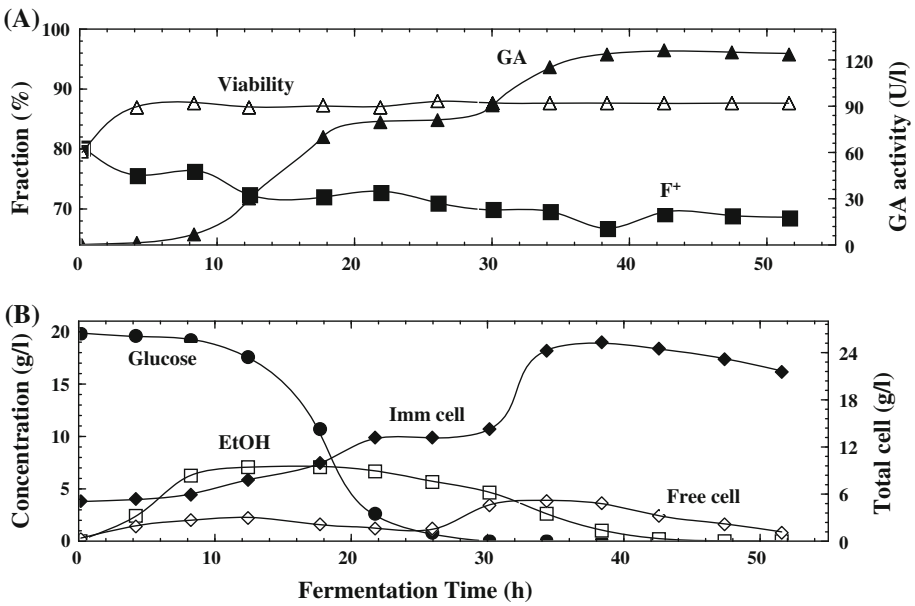


**Fig. 5** Batch production of glucoamylase (GA) with immobilized cells of recombinant *S. cerevisiae* C468/pGAC9 in the ILALFBB. **a** Fraction of plasmid-carrying cells, viability and glucoamylase, **b** glucose, ethanol, immobilized and free cells. *Arrow*: Timing of starting feeding for repeated batch fermentation

also high, about 72%. The productivity of the GA in this ILALFBB system was very high, 52 U/l/h based on the packed volume of the fibrous bed. The GA production can be continued with the extension of the fermentation time by utilizing the immobilized cells repeatedly.

## Repeated batch fermentation

To evaluate the long-term performance of the ILALFBB system with YEPG nonselective medium, repeated-batch fermentation was studied at pH 5.0. Figure 6 shows the ILALFBB performance at various fermentation times. The similar results from both of the bioreactors studied demonstrate the reproducibility of the airlift-driven fibrous bed bioreactor performance.

As shown in Fig. 6a and b, there was no lag phase in all 14 batches, which represents an adaptation period necessary for the cells to live in a new environment. In contrast, the cells in the fermentation broth grew rapidly during the following 24 h after replacing with fresh medium, indicating most cells immobilized in the fibrous bed have a high vitality. This is consistent with the fact that most

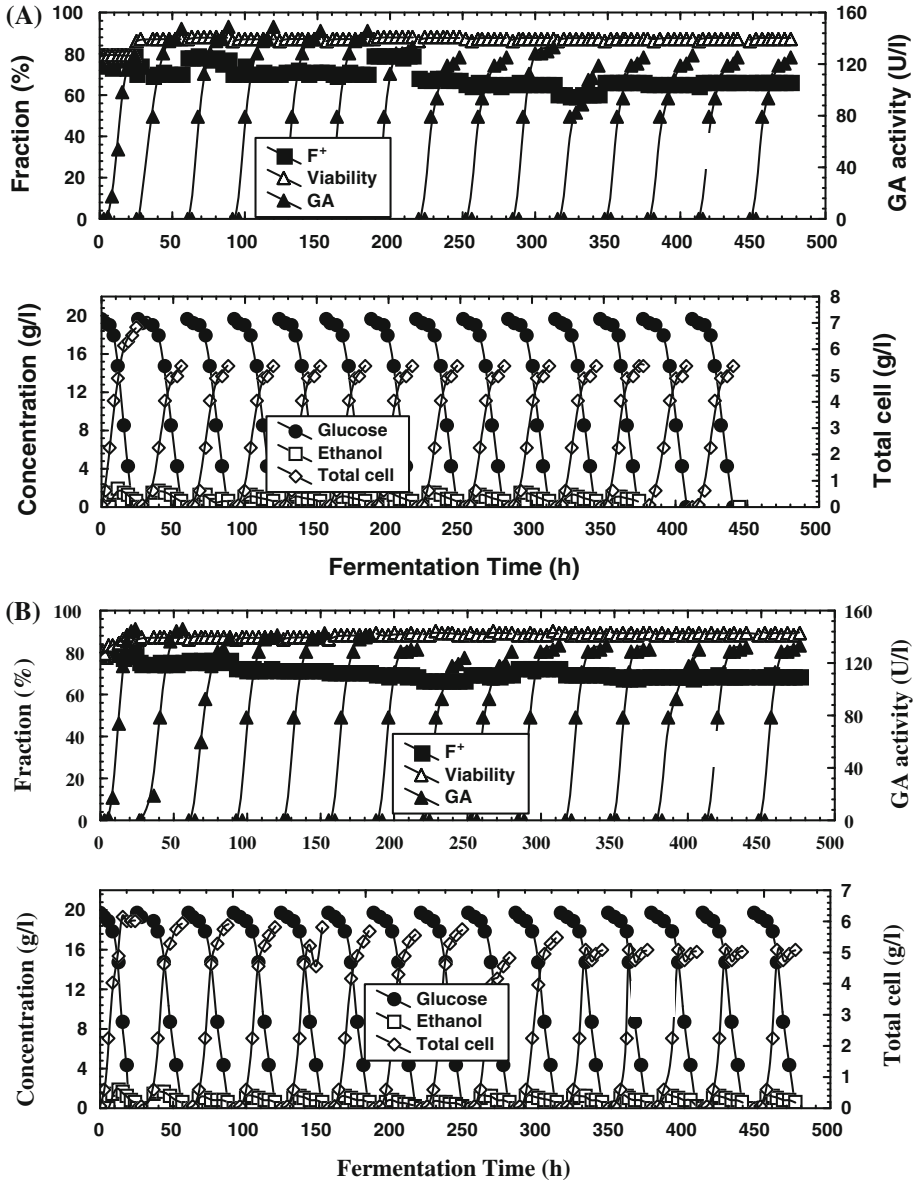


**Fig. 6** GA production by a repeated batch fermentation of recombinant *S. cervisiae* immobilized in ILALFBB at 30°C, pH 5.0: **a** reactor A, **b** reactor B

reactions occurred in the fibrous bed bioreactor instead of in the fermentor. The cells consumed all the D-glucose (cf. Fig. 6) within 28 h. The recombinant yeast also underwent a diauxic growth, first consuming D-glucose as the carbon source for glucoamylase, cell, $CO_2$ and ethanol production. Secondly, when glucose was depleted in the medium, the ethanol produced during the first growth phase was utilized as a carbon source for more glucoamylase, cells and $CO_2$.

The glucoamylase production was also associated with growth (cf. Fig. 6). As shown in Fig. 6, the average immobilized cell density was kept steady at 4.93 ± 0.12 g/l for 18.8 days after medium exchange was started. It is known that recombinant *S. cerevisiae* C468/pGAC9 cells excreted the glucoamylase, which accumulated to a maximum of 148.6 U/l after the fourth batch. The corresponding fraction of the plasmid-carrying cells was 71%. After 14 medium exchanges (19 days), the average glucoamylase activity was kept at 140 ± 2.44 U/l and fraction of plasmid-carrying cells at 74 ± 1.47%. The ethanol concentration increased to a highest value of 1.9 g/l, but then dropped slightly and stayed at a relatively low and stable level till the end of batch fermentation. These findings agree well with results reported earlier by Kilonzo et al. [16, 18] and Zanone et al. [31] for glucoamylase production by recombinant yeast strains.

As shown in Fig. 7, the cell yield ($Y_{N/S}$) in all batch fermentations studied varied from 0.20 to 0.30 g/g, with an average of 0.25 ± 0.01 g/g. The glucoamylase yield ($Y_{P/S}$) varied from 6.0 to 8.0 U/g of substrate, with an average of 6.65 ± 0.12 U/g. The volumetric productivity ranged from 19 to 130 U/l/h, with an average of 31.1 ± 8.34 U/l/h, whereas specific productivity varied from 20 to 42 U/g/h

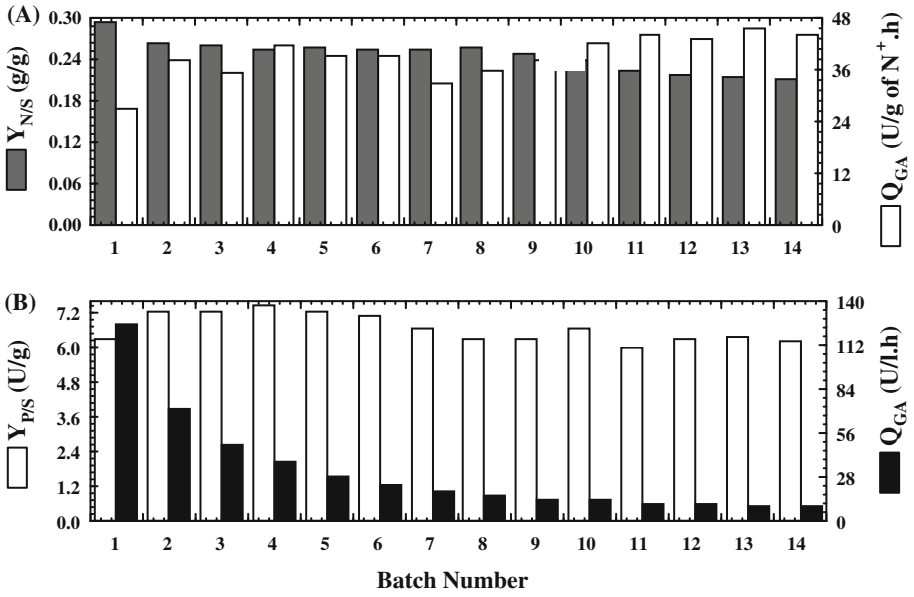


**Fig. 7** Repeated batch fermentations for GA production with immobilized recombinant *S. cerevisiae* in ILALFBB at pH 5.0. **a** Cell yield, specific productivity and **b** GA yield, volumetric productivity

(based on plasmid-carrying cells), with an average of 39.1 ± 1.32 U/g/h, for all seven batches during the fermentation period of 180 h. The variations in yield and productivity were related to the initial glucose concentration at each batch.

Table 1 compares the results of cell density, fraction of plasmid-carrying cells, glucoamylase (GA) activity and productivity under different culturing conditions. The highest fraction of plasmid-carrying cells (i.e., stability), GA activity, and productivity were 74 ± 0.6%, 140 ± 2.5 U/l and 50.4 ± 8.3 U/l/h, respectively, when the cells were immobilized in the fibrous matrix. Because of the high fraction of the plasmid-carrying (productive) cells in the fibrous bed bioreactor, the glucoamylase concentration and productivity in the ILALFBB culture were much higher than those in the suspension cultures [11]. The fraction of plasmid-carrying cells in the in the ILALFBB was over 70%, which was about two times higher than those in the conventional suspension cultures (cf. Table 1). The glucoamylase productivity was also more than 30-fold higher in the ILALFBB than those from suspension cultures. This is because compartmental cell distribution in porous space and mass transfer limitation in the immobilized cell environment limit the growth of plasmid-free cell population. These phenomena decreased the probability of plasmid loss and the overgrowth of the plasmid-free cells.

The performance of the repeated-batch cultures in the ILALFBB was stable in the 19.8-day cultivation period studied. However, the glucoamylase volumetric productivity and the fraction of plasmid-carrying cells during the repeated batches in the ILALFBB were lower than those in the batch culture. This could be an indication of nutrient limitation in the fibrous matrix, which might have restrained the immobilized cell growth and glucoamylase production. With the retention of high density of productive cells in the fibrous matrix, it was obvious that the dissolved oxygen level inside the fibrous matrix was low since the oxygen transfer in the fibrous bed was controlled by diffusion. A low $DO_2$ level could severely limit cell growth and their capability to express the glucoamylase gene.

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

| Culture system | Operation mode | Immob. matrix | $F^+$ (%) | GA (U/l) | $Q_{GA}$ (U/l/h) | $N$ (g/l) | Cultivation time (day) | Reference |
|---|---|---|---|---|---|---|---|---|
| Flask | Batch | - | 82 ± 0.6 | 181 ± 6.6 | 6.6 ± 0.3 | 3.3 ± 0.2 | 1.7 | [4] |
| ILALB | Continuous | - | 41 ± 1.1 | 50 ± 2.2 | 0.4 ± 0.2 | 5.4 ± 0.8 | 1.5 | [28] |
| ILALB | Continuous | - | 60 ± 4.3 | 111 ± 14.8 | 3.4 ± 1.3 | 6.8 ± 0.3 | 8.0 | [12] |
| ILALCFB | Continuous | Cotton | 60 ± 1.5 | 100 ± 2.1 | 0.8 ± 0.3 | 7.7 ± 0.0 | 5.4 | [28] |
| ILALFBB | Batch | Cotton | 72 ± 0.3 | 113 ± 3.5 | 42.6 ± 5.5 | 25.2 ± 1.5 | 2.1 | This work |
| ILALFBB | Repeated batch | Cotton | 74 ± 0.6 | 140 ± 2.5 | 50.4 ± 8.3 | 84.8 ± 1.5 | 19.8 | This work |

*ILALCFB* internal loop airlift cell-film bioreactor, *ILALFBB* internal loop airlift-driven fibrous bed bioreactor, *GA* glucoamylase, *N* total cell, $Q_{GA}$ volumetric GA production

## Conclusion

The novel, fibrous-bed airlift bioreactor system proposed in this work demonstrated that the production of glucoamylase in recombinant *S. cerevisiae* could be enhanced and maintained for an extended cultivation period by adopting an internal ILALFBB system coupled with an immobilization strategy. The expression of glucoamylase was attained effectively with batch and repeated cultures of recombinant *S. cerevisiae* cells immobilized in the fibrous bed airlift bioreactor. The strong adsorption of recombinant *S. cerevisiae* cells on the fibrous matrix provided the possibility of maintaining an extended production of glucoamylase in the FBB system. As the concentration of free cells in suspension was kept at a minimum level of 5/l, the production of glucoamylase was attributed to the immobilized cells in the ILALFBB. It was also observed that the fraction of plasmid-carrying cells was kept at 70% and cell viability at 88%, suggesting that the fibrous matrix preferentially adsorbed and protected a high proportion of viable (productive) plasmid-carrying cells having a high plasmid copy number and less segregational instability, and the nonviable (non-productive) plasmid-free and dead cells were continuously washed off the fibrous matrix, therefore providing an enhanced genetic stability. This selective immobilization feature allowed the ILALFBB to maintain a high productivity of glucoamylase for extended repeated batch cultivation of the recombinant *S. cerevisiae* cells.

It should be noted that the production of glucoamylase by immobilized recombinant *S. cerevisiae* cultures in the ILALFBB was stable throughout the fermentation period studied because of the continuously renewed viable (productive) cell population in the fibrous matrix. Therefore, it can be concluded that immobilization of recombinant *S. cerevisiae* cells in the fibrous bed bioreactor is feasible for long-term production of glucoamylase.

**Acknowledgments** The authors acknowledge financial support for this work from the Natural Science and Engineering Research Council of Canada (NSERC) through individual research grants awarded to Profs. Argyrios Margaritis and Maurice A. Bergougnou.

## References

1. Chau TL, Guillan A, Roca E, Nunez MJ, Lema JM (2000) Enhancement of plasmid stability and enzymatic expression by recombinant *Saccharomyces cerevisiae*. Biotechnol Lett 22:1247–1250
2. Chen J, Chen H, Zhu X, Lu Y, Yang S-T, Xu Z, Cen P (2009) Long-term production of soluble human Fas ligand through immobilization of *Dictyosteliu discoideum* in a fibrous bed bioreactor. Appl Microbiol Biotechnol 82:241–248
3. Chen J-P, Wu K-W, Fukuda H (2008) Bioethanol production from uncooked raw starch by immobilized surface-engineered yeast cells. Appl Biochem Biotechnol 145:59–67
4. Chen X-A, Xu Z-N, Cen P-L, Wong WKR (2006) Enhanced plasmid stability and production of hEGF by immobilized recombinant *E. coli* JM101. Biochem Eng J 28:215–219
5. Choi G-W, Kang H-W, Moon S-K (2009) Repeated-batch fermentation using flocculent hybrid, *Saccharomyces cerevisiae* CHFY0321 for efficient production if bioethanol. Appl Microbiol Biotechnol 84:261–269
6. Cho KM, Yoo YJ (1999) Novel SSF process for ethanol production from microcrystalline cellulose using the $\delta$-integrated recombinant yeast, *Saccharomyces cerevisiae* L2612$\delta$GC. J Microbiol Biotechnol 9:340–345
7. Chung BH, Kim WK, Rao KJ, Kim CH, Rhee SK (1999) Downstream processing of recombinant hirudin produced in *Saccharomyces cerevisiae*. J Microbiol Biotechnol 9:179–183
8. Gomes E, de Souza SR, Grandi RP, Da Silva R (2005) Production of thermostable glucoamylase by newly isolated *Aspergillus flavus* A 1.1 and *Thermomyces lanuginosus* A 13.37. Braz J Microbiol 36:75–82
9. Gouka RJ, Punt PJ, van den Hondel CAMJJ (1997) Efficient production of secreted proteins by *Aspergillus niger*. Appl Microbiol Biotechnol 47:1–11
10. Gouka RJ, Punt PJ, van den Hondel CAMJJ (1996) Analysis of heterologous proteins in defined recombinant *A. awamori*. Appl Environ Microbiol 62:1951–1957
11. Gupter JC, Pandey G, Mukherjee KJ (2001) Two-stage cultivation of recombinant *Saccharomyces cerevisiae* to enhance plasmid stability under non-selective conditions: experimental study and modeling. Enzyme Microbiol Technol 28:89–99
12. Guo G-L, Tseng D-H, Huang S-L (2001) Co-metabolic degradation of trichloroethylene by *Pseudomonas putida* in a fibrous bed bioreactor. Biotechnol Lett 23:1653–1657
13. Huang W-C, Chen S-J, Chen T-L (2008) Production of hyaluronic acid by repeated batch fermentation. Biochem Eng J 40:460–464
14. Jiang L, Wang J, Liang S, Wang X, Cen P, Xu P (2009) Butyric acid fermentation in a fibrous-bed bioreactor with immobilized *Clostridium tyrobutyricum* from cane molasses. Bioresour Technol 100:3403–3409
15. Kajiwara S, Aritomi T, Suga K, Ohtaguchi K, Kobayashi O (2000) Overexpression of the OLE1 gene enhances ethanol fermentation by *Saccharomyce cerevisiae*. Appl Microbiol Biotechnol 53:568–574
16. Kilonzo P, Margaritis A, Bergougnou MA (2009) Plasmid stability and kinetics of continuous production of glucoamylase by recombinant *Saccharomyces cerevisiae* in an airlift bioreactor. J Ind Microbiol Biotechnol 36(9):1157–1169
17. Kilonzo P, Margaritis A, Yu JT, Ye Q (2007) Bioethanol production from starchy biomass by direct fermentation using *Saccharomyces diastaticus* in batch free and immobilized cell systems. Intern J Green Energy 4:1–14
18. Kilonzo PM, Margaritis A, Bergougnou MA (2008) Effects of medium composition on glucoamylase production during batch fermentation of recombinant *Saccharomyces Cerevisiaae*. J Inst Brew 114(2):83–95
19. Kim M-D, Han K-C, Kang H-A, Rhee S-K, Seo J-H (2003) Coexpression of BIP increased antithrombotic hirudin production in recombinant *Saccharomyces cerevisiae*. J Biotechnol 101:81–87
20. Liu DW, Zeng RJ, Angelidaki I (2008) Enrichment and adaptation of extreme-thermophilic (70°C) hydrogen producing bacteria to organic household solid waste by repeated batch cultivation. Int J Hydrogen Energy 33:6397–6492
21. Meleigy SA, Khalaf MA (2009) Biosynthesis of gibberellic acid from milk permeate in repeated batch operation by a mutant Fusarium moliniforme cells immobilized on loofa sponge. Bioresour Technol 100:374–379

22. Nunberg J, Flatgaard JE, Innis MA (1988) Glucoamylase c-DNA. US Patent 4,794,175, Cetus Corporation, Emeryville, CA, USA, pp 1–32
23. Ogbonna JC, Mashima H, Tanaka H (2001) Scale-up of fuel ethanol production from sugar beet juice using loofah sponge immobilized bioreactor. Bioresour Technol 76:1–8
24. Park JN, Shin DJ, Kim HO, Kim DH, Lee HB, Chun SB, Bai S (1999) Expression of *Schwanniomyces occidentalis* α-amylase gene *Saccharomyces cerevisiae var. diastaticus*. J Microbiol Biotechnol 9:668–671
25. Radmann EM, Reinehr CO, Costa JAV (2007) Optimization of the repeated batch cultivation of microalga *Spirulina platensis* in open raceway ponds. Aquaculture 265:118–126
26. Wieber MG, Karandikar A, Robinson GD, Trinci AP, Candia JL, Trappe S, Wallis G, Rinas U, Derkx PM, Madrid SM, Sisniega H, Faus I, Montijn R, van den Hodel CAMJJ, Punt PJ (2001) Production of tissue plasminogen activator (t-PA) in *Aspergillus niger*. Biotechnol Bioeng 76(2):164–167
27. Withers JM, Swift RJ, Wiebe MJ, Robinson GD, Punt PJ, van den Hondel CAMJI (1998) Optimization and stability of glucoamylase production by recombinant strains of *Aspergillus niger* in chemostat culture. Biotechnol Bioeng 59:407–418
28. Xu Z-N, Yang S-T (2007) Production of mycophenolic acid by *Penicillium brevicompactum* immobilized in a rotating fibrous-bed bioreactor. Enzyme Microb Technol 40:623–628
29. Yang ST, Shu CH (1996) Kinetics and stability of GM-CSF production by recombinant yeast cells immobilized in a fibrous-bed bioreactor. Biotechnol Prog 12:449–456
30. Yang X, Wang B, Cui F, Tan T (2005) Production of lipase by repeated batch fermentation immobilized *Rhizopus arrhizus*. Proc Biochem 40:2095–2103
31. Zanone JP, Peres MFS, Gattas EAL (2007) Colorimetric assay of ethanol using alcohol dehydrogenase from dry Baker's yeast. Enzyme Microb Technol 40:466–470
32. Zhang Z, Scharer JM, Moo-Young M (1997) Plasmid instability kinetics in continuous culture of a recombinant *Saccharomyces cerevisiae* in airlift bioreactor. J Biotechnol 55:31–41
33. Zhu H, Yang S-T (2004) Long-term continuous production of monoclonal antibody by hybridoma cells immobilized in a fibrous-bed bioreactor. Cytotechnol 44:1–14