Surface Modifications for Controlled and Optimized Cell Immobilization by Adsorption: Applications in Fibrous Bed Bioreactors Containing Recombinant Cells

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Abstract

The effects of surface properties of *S. cerevisiae* strains 468/pGAC9 and 468 on adhesion to polyethylenimine (PEI) and/glutaraldehyde (GA) pretreated cotton (CT), polyester (PE), polyester + cotton (PECT), nylon (NL), polyurethane foam (PUF), and cellulose re-enforced polyurethane (CPU) fibers were investigated. Process parameters (circulation velocity, pH, ionic strength, media composition and surfactants) were also examined. 80, 90, and 35% of the cells were adsorbed onto unmodified CT, PUF and PE, respectively. PEI-GA pre-treated CT and alkali treated PE yielded 25% and 60% cell adhesion, respectively. Adsorption rate (K_a) ranged from 0.06 to 0.17 for CT and 0.06 to 0.16 for PE at varied pH. Adhesion increased by 15% in the presence of ethanol, low pH and ionic strength, and decreased by 23% in the presence of yeast extract and glucose. Shear flow and 1% Triton X-100 detached 62 and 36% nonviable cells from PE and CT, respectively, suggesting that cell immobilization in fibrous-bed bioreactors can be controlled to optimize cell density for long-term stability.

Keywords: Fibrous-matrices; Fibrous bed bioreactor; Immobilization; Adsorption; Surface modification; Recombinant cells

Nomenclature: CEPUF: cellulose re-enforced polyurethane; CT: cotton (fiber) ; E: concentration of active adsorption sites on the support; EtOH: ethanol (g l^{-1}); FBB: fibrous-bed bioreactor; G: glucose(g l^{-1}); GA: glutaraldehyde; K_a: adsorption rate (h^{-1}); K_d: desorption rate (g l^{-1} h^{-1}); K_s : saturation constant (U g^{-1}) ; M: maltose(g l^{-1});MMSS: modified Morita Salts Solution; N: cell concentration (g l^{-1}); N_0: initial cell concentration (g l^{-1});NL: nylon (fiber); P: peptone (g l^{-1}); P_c: critical pressure (psi); PE: polyester (fiber); PECT: polyester-cotton (fiber); PEI: polyethyleneimine; PEI-GA: polyethyleneimine-glutaraldehyde; PUF: polyurethane foam (fiber); YE: yeast extract (g l^{-1}); YEPG: yeast extract-peptone-glucose; YNB: yeast nitrogen base (g l^{-1}); YNBG: yeast nitrogen base supplemented with glucose; YNBM:yeast nitrogen base supplemented with maltose; t: time (h); T_c: critical temperature (˚C)

Introduction

The immobilization of viable cells has been defined by several investigators as the physical confinement or localization of viable microbial cells to a certain defined region of space in such a way as to limit their free migration and exhibit hydrodynamic characteristic which differ from those of the surrounding environment while retaining their catalytic activities for repeated and continuous use [1].

Cell immobilization is often used to improve the performance of traditional continuous fermentation process by increasing the amount of cells per bioreactor volume, and cell deposition on supports or inclusion in gel matrices has also been applied to promote plasmid stability of recombinant cells [1-3]. Lebrun et al. [4], attempted immobilizing cells in microporous beads or gels by entrapment. However, in both systems the anticipated benefits such as increased cell density, stability, productivity, permeability, and enhanced resistance to environmental perturbations, and expedited cofactor regeneration [5,6], were not realized due to accumulation of dead cells and poor mass transfer under high cell density conditions.

Different from the inherent problems associated with cell entrapment [7], cell immobilization through adsorption provides a direct contact between nutrients and the immobilized cells, thus eliminating such concerns. This cell immobilization technique involves the transport of the cells from the bulk phase to the surface of support, followed by the adhesion of cells, and subsequent colonization of the support surface. Both electrostatic and hydrophobic interactions govern the cell-support adhesion, which is the key step in controlling the cell immobilization on the support.

The adsorption capacity and strength of binding are the two major factors that affect the selection of a suitable support material [8]. In contrast to ceramics, wood chips and straw, fibrous matrices provide adequate supporting surfaces for cell adsorption [9,10], due to their high specific surface area, void volume, mechanical and permeability, low pressure drop, diffusion problems and toxicity, maximum loading, biodegradability and durability and low cost and high availability [11-13]. Their natural configuration also allows them to trap more cells than other materials [12,14,15]. Recently, we succeeded in immobilizing recombinant *Saccharomyces cerevisiae* C468/pGAC9 cells by adsorption in an airlift-driven fibrous-bed bioreactor (FBB) [2], for continuous production of *Aspergillus awamori* glucomylase.

Nevertheless, the strength of the cell adsorption depended greatly on the yeast cell wall composition as well as on fibrous matrix surface properties, which can be easily controlled for effective cell immobilization by treatment with polyethylenimine and/or glutaraldehyde. Surface pre-treatment prevents unwanted non-specific adsorption of non-productive cells, allows immobilization of

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most viable cells, and increases stability and long-term performance immobilized cell bioreactors [9,16,17]. It also means that an additional surface modification strategy is required to facilitate cellular adhesion [16]. The use of glutaraldehyde and polyethylenimine in the immobilization of microbial cells in alginate beads has been investigated [18-21], to overcome cell leakage from the matrix. Kawaguti et al. [17], observed increased stability and a longer period of conversion when polyethylenimine (PEI) and glutaraldehyde (GA) were used as cross-linking agents in the immobilization of *Erwinia* sp. SD2 cells in calcium alginate for isomaltulose production. Only limited information on the application of PEI and GA in the immobilization of cells in fibrous matrices exist in the current literature [8]. Based on PEI's ability to interact with negatively charged species, D'Souza and Kamath [22], used PEI-coated cotton cloth and observed improved stability of immobilized yeast cells and high enzyme activity. In contrast, Hsu et al. [8], observed increased immobilization efficiency and xanthan production with untreated cotton, than with PEI-coated cotton. Most recently, Chu et al. [9], observed stable immobilization of bioluminescent *E. coli* cells for viscose fiber treated with 0.667% PEI, which resulted in a long sensing period, a quick response time, and better signal reproducibility.

Besides, full exploitation of the potential advantages of fibrous matrices will strongly depend on the wise selection of a set of processing parameters allowing for high productivity combined with extended operational stability. These include growth conditions of cells, media components, pH, inorganic salts and surfactant concentrations, and bioreactor hydrodynamics [23,24]. For example, medium composition affects operational stability by its influence on cell growth and de novo protein synthesis as well as changing the environmental conditions for prolonged product synthesis. It also affects cell matrix interactions via swelling of gels or weakening of cell-adsorbent interaction [24]. D'Souza and Kamath [22], observed that the presence of 1 M KCl salt concentration in the cell suspension did not alter the binding capacity of yeast cells. Understanding of the effect of these parameters on operational stability of immobilized cells will allow rational systematic design and evaluation of novel fibrous-bed bioreactor systems. To date, no information exists on the effect of process parameters in the immobilization of recombinant cells in fibrous matrices.

In the present study, immobilization of recombinant *S. cerevsiae* C468/pGAC9 strain (ATCC # 20690) and non-recombinant *S. cerevisiae* C468 strain (ATCC # 26599) immobilized by adhesion on fibrous matrices treated with polyethylenimine and glutaraldehyde was examined. Various operating parameters affecting cell adhesion were studied including shear rate, culture age, and different components in the media. The effect of pH and ionic strength on cell adhesion was examined to study the role of electrostatic interaction. The effect of hydrophobic interaction was investigated by determining the effects of different surfactants (including a concentration series of Triton X-100) on cell desorption.

Kinetics of cell adsorption and desorption

Cell adhesion to the support (fiber) can be modeled by a reversible first-oder surface reaction as follows:

$$N + E \underset{Kd}{\overset{Ka}{\rightleftharpoons}} N - E \tag{1}$$

where N is the cell concentration, E is the concentration of active adsorption sites on the support, N-E is the cell-support complex, and K_a and K_d are the adsorption and desorption rate constants, respectively. When there is no diffusion limitation, the rate of formation of the cell-

support complex or cell adsorption rate can be expressed as:

$$-\frac{dN}{dt} = k_a[N][E] - k_d[N-E] \tag{2}$$

where t is the reaction time. Initially, adsorption of cells on the support predominates as [N-E] is zero or negligibly small. Therefore, the adsorption rate is much larger than the desorption rate, and desorption term in equation (2) can be neglected. Also, when [E] >> [N], [E] can be considered as a constant and [E] [E_0]. Equation (2) is thus reduced to equation (3):

$$-\frac{dN}{dt} = K_a[N][E] \approx K_a[N][E_0] = K_a[N] \tag{3}$$

where $K_a = k_a E_0$. Furthermore, equation (3) can be integrated to the following form:

$$\ln\left(\frac{N}{N_0}\right) = -K_a t \tag{4}$$

where N_0 is the initial cell concentration and E_0 is the initial concentration of active adsorption sites on the support. If cell adsorption follows the first-order reaction kinetics, a semi-logarithmic plot of N/N_0 vs. time t should yield a straight line with a slope equal to $-K_a$, negative value of the adsorption rate constant.

The kinetics of desorption can be simplified in a similar way. In the beginning of desorption using a desorbing agent, the concentration of cells in the suspension is negligible. Therefore, the adsorption term $k_a[N][E]$ in equation (2) can be neglected comparing to the desorption term $k_d[N-E]$. Also, [N-E] can be considered as a constant when compared to the concentration of cells in suspension, as [N-E] >> [N]. As a result, $k_d[N-E] = k_d$ and equation (2) can be simplified to a pseudo-zero-order reaction as:

$$-\frac{dN}{dt} = K_d \tag{5}$$

The desorption rate constant K_d can be calculated from the initial slope of the plot of cell concentration *vs.* time t during the desorption period.

Materials and Methods

Chemicals

Unless otherwise stated chemicals and laboratory components were obtained from either, Sigma Aldrich chemicals (Oaksville, Mississauga, ON, Canada), VWR Internal or Spectrum Labs Inc. and they were all of analytical grade.

Yeast culture

The recombinant *S. cerevisiae* strain C468/pGAC9 (ATCC # 20690) and the host *S. cerevisiae* strain C468 (ATCC # 62995) used in this study were obtained from American type culture collection (ATCC).

Growth media

S. cerevisiae strain 468/pGAC9 (ATCC 20690) was stored on selective YNBG agar slants containing 6.7 g L^{-1} yeast nitrogen base (YNB) without amino acids (Sigma), 0.04 g L^{-1} L-histidine (Sigma), and 20 g L^{-1} D-glucose and cultivated in YNBM supplemented with 2% (w v^{-1}) maltose and Fermtech-agar [2]. Nonselective YEPG containing YE-yeast extract, 5 g L^{-1}; P-peptone, 10 g L^{-1}; and D-glucose, 20 g L^{-1} was used to store *S. cerevisiae* strain C468 (ATCC 62995).

The strain was grown on YEP supplemented with 2% (w v^{-1}) glucose and Fermtech-agar. It was stored in a fridge at 4°C. Selective YNBG and nonselective YEPG media were used to prepare inoculums for *S. cerevisiae* 468/pGAC9 (ATCC 20690) and *S. cerevisiae* C468 (ATCC 62995), respectively. The yeasts were grown in 500-mL shake flasks containing 300 mL of sterile YEP-G containing 5 g L^{-1} yeast extract (Becton Dickinson), 10 g L^{-1} peptone (Becton Dickinson), and 20 g L^{-1} D-glucose. Without adjustment, the pH of these media was 5.0. For agar plates, the media also contained 2% (w v^{-1}) 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.

Cell growth and development

The yeast strains were grown in 250-mL cotton plugged Erlenmeyer flasks containing 50 mL of YNBG or YEPG medium as pre-culture A. The medium pH was adjusted to 5.0 using 2 M HCl and/or 2M NaOH before autoclaving for 20 min at 121°C, 1.1 atm (i.e. 111.4 kPa). Inoculum was obtained by transferring 2-3 loops of slant stock culture into the flasks. Following incubation at 30°C in a reciprocating shaker at 200 rpm for 24 h, the cells were transferred to the second stage to increase cell numbers further (pre-culture B). A 10% cell suspension volume of pre-culture A at a cell concentration corresponding to an OD$_{570\,nm}$ of 0.33 (equivalent to CDW = 0.25 g L^{-1} or 1.5 x 10^{10} cells mL^{-1}) was transferred into 4 x 500-mL Erlenmeyer flasks each containing 325 mL sterile YEPG nonselective medium containing double the amount of D-glucose and 12 pieces of the fibrous material (cotton, polyester, 65% polyester + 35% cotton, and nylon). After incubation under similar conditions as in stage 1 (pre-culture A), the cells reached the stationary phase after 32 h. After 48 h, the cells were separated from fermentation broth by centrifugation at 4°C, 5000 rpm (6,289 x g) for 10 min. A cell suspension with an OD$_{600\,nm}$ of 0.52 (equivalent to CDW = 0.33 g L^{-1}, or 1.4 x 10^{11} cells mL^{-1}) was used to inoculate the cell development stage. Cell cultivation temperature was maintained by circulating water at 30°C temperature. No pH adjustments were made. Cells intended for cell immobilization were also grown in 500-mL Erlenmeyer flasks containing 325 mL sterile YEPG nonselective medium. After 20 h cultivation, the OD$_{600\,nm}$ reached 2.65 (equivalent to CDW = 8.2 g L^{-1}, or 7.7 x 10^9 cells mL^{-1}).

Fibrous materials

Heavy bleached cotton (CT) (woven) (Mountain Weavers Ltd., Dorset, VT, U.S.A), polyester (PE) (Lincon Fabric Ltd., St. Catherines, ON, Canada), 65% polyester (PE) + 35% cotton (PECT) (woven) (Local textile Stores), nylon (NL) (woven) (Lincon Fabric Ltd., St. Catherines, ON, Canada), polyurethane foam (PUF) (Spondex Inc., US), and cellulose re-enforced polyurethane (CEPUF) (Spondex Inc. US) were used to study effects of surface treatment and process parameters on immobilization of recombinant *S. cerevisiae* cell. The fabric materials were cut into small pieces (1 x 1 cm). Before use, twenty pieces of CT (0.054 g), PE (0.029 g), PECT (0.018 g), NL (0.038 g), PUF (0.024 g), and CEPUF (0.120 g) were placed inside 250-mL Erlenmeyer flasks.

Cleaning and treatment of fibers

The fibrous materials were soaked twice in 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 rinsed with double distilled water and phosphate buffer solution (0.1 M PBS at pH 7.4) (0.079 M Na$_2$HPO$_4$ + 0.027 M NaH$_2$PO$_4$) after each treatment. The flasks with the fibrous materials were filled with 100 mL of PBS solution and autoclaved at 121°C for 30 min. The PBS solution was poured out and the flasks were filled 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 at the same conditions. Each flask was inoculated with 5% incolum medium containing a final cell concentration of 3.1 x 10^8 cells L^{-1} (i.e., OD at 600 nm = 0.291, 0.071 CDW L^{-1}) and incubated in a rotary shaker operated at 250 rpm and 30°C. The medium in the flasks was changed after three and six days, and cell attachment to these fibrous materials was examined using a scanning electron microscope (SEM) after seven days.

Treatment by polyethylenimine (PEI) coating: The fibrous materials were soaked for 2 hours in 0.2% aqueous solution of PEI adjusted to pH 7.0 with HCl. The PEI soaked materials were then extensively rinsed with distilled water and allowed to dry at room temperature.

Treatment by glutaraldehyde (GA) cross-linking: The PEI-treated fibrous materials were placed in the solution of 1% (w v^{-1}) GA in 0.05 M pH 7.0 PBS for 2 h, rinsed with distilled water and air-dried. All the modified fibrous matrices were stored at 4°C until use.

Scanning electron microscopy (SEM)

One piece (1 cm x 1 cm) of the fibrous material was removed from each flask and cut aseptically into 0.5 cm x 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$_2$O) 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$_2$O) – (210 mL EtOH + 90 mL d-H$_2$O)], 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$_2$O) over night at 4°C and then progressively dehydrated with 80%-100% ethanol [i.e., (240 mL EtOH + 60 mL d-H$_2$O – 300 ml-EtOH + 0 mL d-H$_2$O)]. These samples were dried cryogenically in a critical point drier (Model: EMS-850, Electronic Microscope Sciences, Hatfield, PA, USA) at the critical point (critical temperature T$_c$ = 31.1^{o}C, critical pressure P$_c$ = 1072 psi) with liquid CO$_2$, mounted on circular stainless steel moulds and coated with gold/palladium before SEM photographs were taken using UWO Crossbeam Model 820 SEM.

Static adsorption and desorption

The static experiments were conducted to study the adsorption of yeast onto cotton (CT), polyester (PE), polyurethane foam (PUF) and cellulose re-enforced polyurethane (CEPUF).

Cells in the stationary phase were harvested by centrifugation (10 min at 6,289 x g), washed and re-suspended in 0.2 M phosphate buffer (pH 6.0) solution to obtain an initial OD$_{600\,nm}$ of 1.0-1.5. Dry CT, PE, PUF and CEPU pieces (4 cm x 4 cm each) were soaked in 100 ml of cell suspensions in 250-mL flasks with gentle shaking on a rotary shaker (150 rpm) at room temperature. The experiments were conducted for approximately 4 to 5 hours until the cell concentration in the suspension did not change. The effect of liquid circulation velocity on cell adsorption was studied at airflow rate ranging from 0 to 14 L min^{-1}.

At the end of adsorption experiments, the non-ionic surfactant Triton X-100 and anionic surfactant Tween 80 at a concentration of 0.1% (w v^{-1}) in phosphate buffer (0.2 M, pH 6.0) were used to detach adsorbed cells from the support.

Samples were taken at 2-h interval to monitor changes in the cell concentration in the suspension. The unbound cells on the fibers were removed by washing the fibers repeatedly with distilled water. Total cell loading on the fibers was then estimated from the mass balance.

In the pH effect experiment, cells were suspended in 0.2 M Na_2HPO_4-$Na_3C_6H_5O_7$ buffer at different pHs from 3.0 to 8.0. Ionic strength experiments were carried out in 0.2 M phosphate buffer, pH 6.0 with NaCl concentrations ranging from 0.01 to 0.5 M. Six different media (d-H_2O with 15 g L^{-1} of ethanol; d-H_2O with 10 g L^{-1} yeast extract; d-H_2O with 20 g L^{-1} D-glucose; filtered final fermentation broth with no residual glucose, all adjusted to pH 6 with 2 M NaOH) were used to study the effect of media composition

Dynamic adsorption and desorption

Cells in the stationary phase were harvested by centrifugation (10 min at 6,289 x g), washed and re-suspended in 150 mL modified Morita Salts Solution (MMSS) to obtain an initial $OD_{600\ nm}$ of 3.0. A re-circulating-type reactor system used earlier [2] was applied to study cell adsorption/desorption under dynamic flow conditions. The glass reactor (dimensions: 0.10 m i.d. X 1.19 m height) with a volume of 18000 mL was packed with pre-wetted fibrous material colaminated with a steel wire mesh support in a spiral would configuration. The well mixed cell suspension in the recirculation vessel was pumped (peristaltic pump, Masterflex, Cole Parmer, Canada, Mississauga, ON) through the packed fibrous bed at a flow rate (0 to 14 l min^{-1}) to maintain the well-mixed condition in the system. Each adsorption experiment was continued for 2 h to allow most cells initially in the suspension to be adsorbed to the fibrous matrix.

For the desorption experiment, the medium in the system was replaced with the phosphate buffer (0.2 M, pH 6.0) containing non-ionic surfactant Triton X-100 at a concentration ranging from 0 to 1%. All experiments were conducted at room temperature. Samples were taken at intervals of 2 h regularly to monitor changes in the cell concentration in the suspension.

Cell immobilized by adsorption

At the end of each study, the liquid present in the bioreactor was drained, and its volume and optical density (OD) were measured to estimate the concentration of suspended cells in the bioreactor. The fibrous matrix was removed from the drained bioreactor, and several pieces of 1 x 1 cm of fibrous material were cut and used for SEM and other immobilized cell 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.

Analytical methods

Biomass concentration: Free cell concentration was measured using dry weight and optical density methods. The dry weight was determined by centrifuging 20 mL of sample in a 25 mL centrifuge tube at 6,289 x g to separate the cells from the spent fermentation broth. The clear supernatant was stored for use in the analytical stage. The cells were washed with deionized water twice and then dried overnight at 90 - 108°C. The optical density (OD) of the fermentation broth was measured at 600 nm (OD_{600}) in a 4-mL quartz cuvette using a UV/VIS spectrophotometer (752S, Micro Photonics Inc., Allentown PA). Samples were diluted if the OD_{600} was above 0.3. The correlation

between dry cell weight (N) and optical density (OD) was determined as N = 1.6835 x OD (R = 0.9986, R^2 = 0.9971).

Cell viability assay: After dynamic adsorption and desorption experiments using 1% Triton X-100 immobilized cells were washed off from the fibrous matrix sample by vortexing the fibrous matrix gently in a test tube containing 10 mL sterile pH 6.0 phosphate buffer. The washed buffer containing the cells was collected. The viability of yeast cells present in the effluent and in the fibrous matrix was measured by using a modified staining method [2,14]. The staining solution 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) [12]. Cell samples were diluted with Ringer salt solution to a concentration of 3.0 x 10^8 cells mL^{-1} and 0.1 mL of the diluted sample was mixed with 0.9 mL of the staining solution. A heamocytometer was used to count the number of colorless cells (viable) and blue-colored cells (dead) within 10 min. The number of viable cells divided by the total cell count determined the viability. The analysis was duplicated and average values are reported.

Results and Discussions

Passive adsorption of cells to untreated fibers

The efficiency of initial cell adsorption without surface treatment was evaluated using actively growing recombinant *S. cerevisiae* 468/pGAC9 cell suspension. The yeast cells, with negatively charged cell wall surface [25], were immobilized in a passive manner by circulating the suspension through fibrous bed packing.

Figure 1a shows that the amount of immobilized cells increased to a maximum and then remained constant at 5750 mg/g fiber, 4600 mg/g fiber, 3700 mg/g fiber, and 2950 mg/g fiber for cotton (CT), polyester (PE), nylon (NL), polyurethane foam (PUF), respectively, for up to (Figure 1)170 min in contact with the fibers at a constant circulation velocity of 0.1 cm s^{-1}. The limitation to about one-half the maximum density value may be attributed either to an electrostatic repulsion of the cells already attached, which have a slightly negative surface charge, or to the fact that the cells already immobilized prevented, for hydrodynamic reasons, a contact between the cells in suspension and the area of the fibrous support, which remained uncovered. It was observed that initial cell concentration did not affect the adsorption rate; however, cell loading, which indicates the maximum amount of cells adsorbed, was dependent on the duration of cell contact with the support (Figure 1a). The result in the present study is in agreement with those reported elsewhere [25], in which the cell layer resisted the largest flow velocity, ca. 50 cm s^{-1}. Therefore, for comparison purpose, all experiments were carried out at a similar level of initial cell concentration. Among the fibers investigated, untreated CT showed the highest efficiency of cell adsorption throughout the period studied,
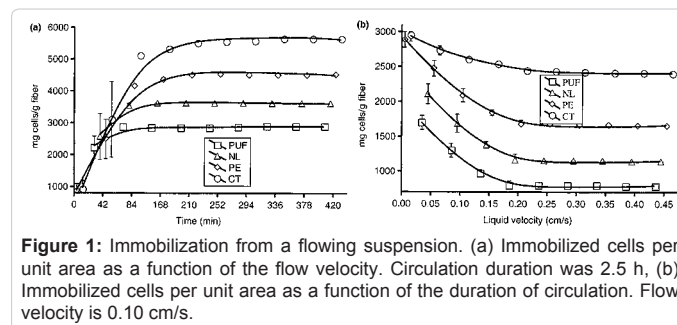

Figure 1: Immobilization from a flowing suspension. (a) Immobilized cells per unit area as a function of the flow velocity. Circulation duration was 2.5 h, (b) Immobilized cells per unit area as a function of the duration of circulation. Flow velocity is 0.10 cm/s.

as indicated by the highest cell dry weight (mg cells/g fiber) of cells retained (adsorbed) on the fiber. On untreated polyurethane (PUF), on the contrary, the adsorbed cells were only about 50% of the amount on the cotton fiber. According to Yang and Lo [12], with untreated cotton showing higher surface roughness than untreated Viscose Rayon towel, it seemed plausible to attribute the poor adsorption efficiency of polyurethane foam to the inferior roughness and may be hairless on the polymer surface.

Busscher and Weerkamp [26], categorized cell adsorption in the absence of cross-linking agents into several stages. When the distance between the cells and the contact surface is > 50 nm, the first operative interaction for adhesion is van der Waals force, a nonspecific force only relevant to particle size and separating distance. At a reduced distance (around 10 to 20 nm), the cells starts to experience both van der Waals force and repulsive electrostatic interactions. Because most natural and artificial surfaces are negatively charged as reported by Hsu et al. [8], and Chu et al. [9], for cotton (except positive charge for nylon), the repulsive interactions exist because yeast cell surface also carry a negative charge under common physiological conditions [22].

Surface treatment of fibers

Due to the smooth surface of the polyester fabric, it was first treated with NaOH in order to increase its hydrophilicity by exposing more hydroxyl groups and surface roughness to provide cells with shelter from the fluid shear. Then surface treatment by PEI and GA was carried out on cotton, polyurethane, nylon and alkali-treated polyester as shown in Figure 2.

PEI is a highly branched polymer, possessing primary, secondary, and tertiary amine groups in a ratio of approximately 1:2:1. Its high cationic density has been used to modify surface charge of cells or support in cell immobilization [8,9,17]. GA is usually used for intermolecular cross- linking in enzyme immobilization. In protein chemistry, GA reacts mainly with lysine in the protein structure. Therefore, GA may react readily with the protein in the lipid bilayer of cell membrane [27,28]. Its aldehyde groups can also cross-link the amino groups on PEI-coated materials and the surfaces to promote cell-support interaction by chemical bonding [28,29].

At the end of each study, the cells adsorbed in various fibrous matrices were examined with SEM to visualize the attachment of cells on the fiber surface and to assess cell morphology (see supplied data). The increases of immobilized cells on PUF and CPUF were more significant than those on other fibers due to the interstitial spaces within the (Figure 2) matrix, which still showed an observable level of increase in cells attached to its surface as well. Cotton and polyester were partially colonized by cells, while the yeast cells poorly colonized nylon. Most cells appeared strongly attached to the fiber surface (see supplied data). Whereas some cells formed clumps at locations where no fiber surface was available for attachment, the cells on cotton, polyester and nylon fibers appeared to be unevenly distributed and to form multi-layer.

PEI, a highly branched cationic polymer, has been widely used to coat cellulose surfaces to enhance biocatalyst and cell immobilization [22,30]. The structure difference between cotton and other fibers may lead to different hydrophilic capacities, which consequently may result in different levels of immobilization although both surfaces are preferable for to PEI to impregnate. Since the amino pendant groups of PEI function as enzyme carriers, it is reasonable to suggest formation of peptide bonds between the abundant urea groups of polyurethane
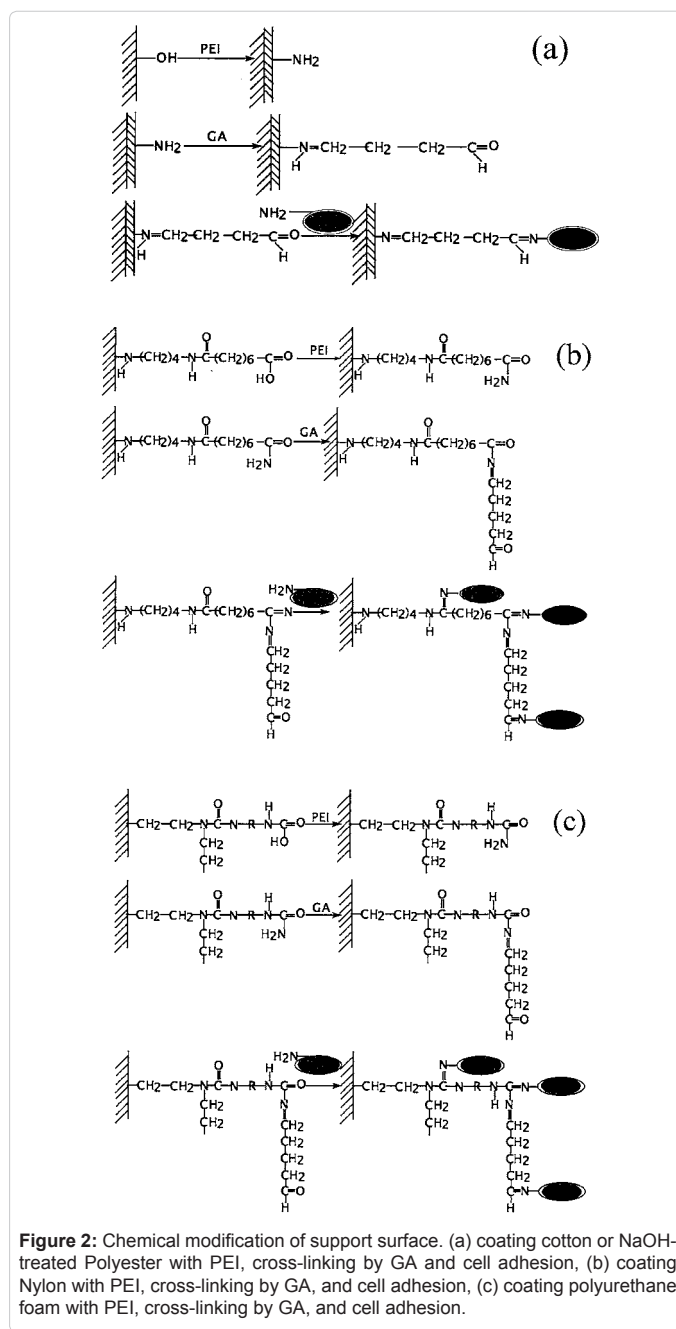

Figure 2: Chemical modification of support surface. (a) coating cotton or NaOH-treated Polyester with PEI, cross-linking by GA and cell adhesion, (b) coating Nylon with PEI, cross-linking by GA, and cell adhesion, (c) coating polyurethane foam with PEI, cross-linking by GA, and cell adhesion.

foam surface and the amine groups of PEI, resulting in increased cell immobilization. However, further research is needed to elucidate the PEI-cell interaction mechanisms.

Static adsorption of cells to different fibers

The static adsorption results for cells harvested from different growth phases are shown in Figure 3a. In terms of cell loading, the original *S. cerevisiae* C468 and the selected *S. cerevisiae* C468/pGAC9 exhibited almost similar level of adhesion. Results from the present study showed that the surfaces of yeast cells are hydrophobic. (Figure 3)The average size of the yeast cells was 2.8 to 4 (m, and the greater adhesion is probably due to the large contact surface between cells and the hydrophobic, as indicated by a large contact angle of 35°, measured

from the SEMs with the aid of a protractor. Thus, hydrophobic interaction might be another factor responsible for enhanced cell adhesion. Based on the weight of the fiber, nylon fiber had a lower cell loading due to its higher density, and perhaps, due to its smooth and hydrophobic surface nature. Cotton fiber usually had a lower cell loading than polyester due to its higher density than that of polyester. However, for the both yeast cells studied, more cells were adsorbed on cotton than polyester with the same size. Up to 80-90% of yeast cells were adsorbed on cotton or modified cotton, compared to only 30-40% of yeast cells on polyester. Because cotton is more hydrophilic than polyester, other mechanisms might be present for better cell adhesion under experimental conditions. It has been proposed that surface roughness of cotton facilitates cell adhesion and prevents cell from being sheared off [8,12]. A rough surface also has a greater surface area and the depression in the roughened surface can provide more favorable sites for colonization [31]. This reasoning can be applied to the polyurethane foam fiber, which outperformed all the other fibers in terms of cell loading. More than 90% of the yeast cells had been adsorbed on to the polyurethane foam fiber. In addition to the above-mentioned reasoning, the increased active sites for cell adsorption also contributed to cell adhesion.

As shown in Figure 3a, with PEI-GA treatment, cell loading increased by 10-40% for cotton and up to 60% for alkali-treated polyester. The cell loading for PEI-GA treated nylon and polyurethane increased by about 10-13%. Cells adhering to PEI-GA modified supports had strong adhesion and less likely to be removed by hydrodynamic shear. Chemical bonding provides a stronger adhesion forces for the cells. Treatment with PEI alone was not sufficient to increase cell adhesion, as seen from the data for cell adhesion on PEI-only modified fibers, indicating that increasing the positive charge between cells and support could not effectively enhance cell adhesion under the experimental conditions.

Alkali-treatment increased cell loading on polyester fiber by about 40% as shown in Figure 3a. However, for yeast cells from mid-log
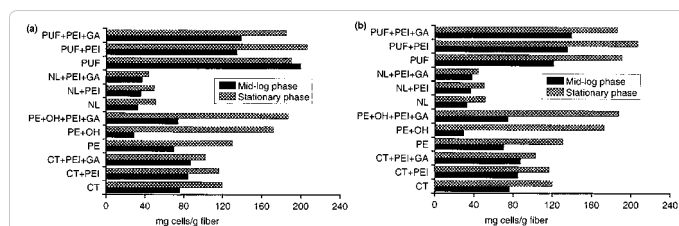
Figure 3: Static adsorption of cells to different fibers in pH 6.0 phosphate buffer. Symbols: CT-cotton, PE-polyester, NL-nylon, PUF-polyurethane foam, PEI-polyethylenimine, GA-glutaraldehyde, OH-NaOH. The initial cell concentration was 0.7 (10 g l⁻¹). **(a)** *S. cerevisiae* C468/pGAC9, **(b)** *S. cerevisiae* C468.

Fiber	Adsorption rate K_a (h⁻¹)	Adsorbed cells (% of total cells)	Cell loading (mg cells g⁻¹ fiber)
Cotton	0.041±0.007	85.8	63.78
Modified cotton	0.0040.008	97.3	69.26
Polyester	0.1720.005	73.9	142.10
Modified polyester	0.069±0.002	76.9	148.10
Nylon	0.451±0.016	36.9	27.43
Modified nylon	0.0790.016	41.8	29.78
Polyurethane foam (PUF)	1.718±0.061	90.7	104.60
Modified PUF	2.9930.062	99.6	113.59

Note: Cells were harvested from the stationary phase and suspended in PBS buffer. The initial cell concentration was ~5 g l⁻¹.

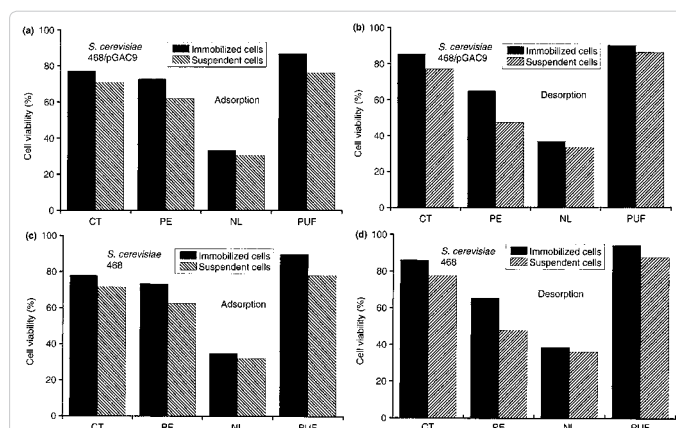**Table 1:** Dynamic adsorption of recombinant *S. cerevisiae* cells to different fibers.

Figure 4: Comparison of viability between immobilized cells and cells in suspension after dynamic adsorption and desorption using 1% Triton X-100. The materials used were PEI and GA modified cotton, polyester, nylon and polyurethane. Symbols: CT = cotton, PE = polyester, NL = nylon, PUF = polyurethane foam. **(a)** adsorption and **(b)** desorption of *S. cerevisiae* 468/pGAC; **(c)** adsorption and **(d)** desorption of *S. cerevisiae* 468.

phase cell loading decreased by more than 80% after NaOH treatment. Additionally, the adhesion strength between adsorbed cells and NaOH-treated polyester was not strong enough to resist fluid shear. Similar or smaller amounts of cells were retained on NaOH-treated polyester after washing the fiber as compared to untreated polyester. By contrast, at least 2-fold higher amount of cells was retained on PEI-GA modified polyester.

Having established that PEI-GA modification increased cell adsorption, in the following experiments, it was used throughout in the preceding studies to modify the fibers and those fibers after modification were represented as "modified cotton", "modified polyester", "modified nylon", or "modified polyurethane." In the dynamic adsorption experiments, the rates of adsorption of cells on different matrices were determined from the slopes of the change in cell concentration (N/N$_0$) with time (Eq. 4). The calculated K_a, the maximum amount of adsorbed cells, and cells loading are listed in Table 1. PEI-GA modification promoted greatly the adhesion of yeast cells on the fibrous matrices as indicated by the higher K_a, higher percentage of adsorbed cells, and higher cell loading. It was noticed that, at the end of 2-hour run, almost all of the yeast cells had been adsorbed onto the PEI-GA modified cotton, polyester and polyurethane foam matrices. The viability of cells in suspension and cells immobilized onto the modified matrices were also measured and compared (Table 1). As shown in Figure 4a-d, immobilized cells on PEI-GA modified fibers had higher viability than those did in the suspension. For unmodified fibers, the viability of immobilized cells was only slightly higher than that of suspended cells (2% and 6%) (Figure 4) more viable for immobilized yeast on cotton and polyester, respectively. These results indicated that viable yeast cells were more likely to be immobilized on to the PEI-GA modified fiber than dead cells due to the cross-linking by GA. This result is also consistent with the finding of decreased adhesion.

Thus, it can be concluded that PEI-GA modification can be an affective method to improve cell immobilization on fibrous matrices [28,29]. PEI is non-toxic and has been approved by the FDA for use in potable water [22]. GA is well known for its wide application in biological cross-linking. Therefore, PEI-GA modified fibrous matrices should have a good potential for large-scale use in recombinant cell immobilization [28,29].

Factors affecting initial cell adsorption

Effects of liquid circulation velocity: In order to investigate the velocity dependence of the adhesion process, a set of experiments was performed using two durations of circulation (1 and 2.5 h) and selecting various flow velocities in the range 0.10-0.40 cm s^{-1}. The results presented in Figure 1b show that the amount immobilized is small when the flow is higher than 0.20 cm s^{-1}; the immobilized amount obtained with a velocity of 0.10 cm s^{-1} is about one-half the maximum value. Visual and microscopic observation studies of the separation front between a cell suspension and clear water at various flow velocities showed that the rate of immobilization of the cells was of the order of 0.15 cm s^{-1}. The best result of immobilization obtained at a flow velocity of 0.10 cm s^{-1} may be attributed to the fact that the net velocity of the cells was very low during the phase where the liquid was flowing upwards. It may be concluded, and it appears indeed logical, that the best conditions to achieve adhesion on a surface parallel to the direction of flow is to use a flow velocity balancing the rate of immobilization, so that the cell particles are practically stationary with respect to the surface. This is more crucial for yeast cell flocs than for smaller particles, since the critical parameter should be the ratio of the mass, governing the kinetic energy, to the area of contact, governing the energy of adhesion.

Effect of Ph: Electrostatic interactions play a very crucial role in the adsorption behavior of microorganisms. To evaluate the significance of electrostatic interactions, the effects of pH and ionic strength of the media on cell attachment were tested. Na$_2$HPO$_4$-Na$_3$C$_6$H$_5$O$_7$ buffer at different pHs was used for the pH effect study. Figure 5a-b depicts the pH-dependent adsorption behavior of yeast cells adsorbed on unmodified fibrous matrices. Clearly, cell loading onto fibers was pH dependent and increasing with decreasing the pH. A similar (Figure 5.) pH effect was observed on the adsorption rate constant as shown in (Table 2) Most yeast cells have negative charge, as do most solid surfaces. Thus electrostatic repulsion between surfaces of like charge will tend to prevent their close approach. Controlling the pH conditions for cell attachment can reduce electrostatic repulsion between cells and support surface. At low pH values, the surfaces of the cells were less negatively charged. Therefore, the electrostatic repulsion is reduced, further promoting the adsorption of cells on fibrous matrices (Table 2).
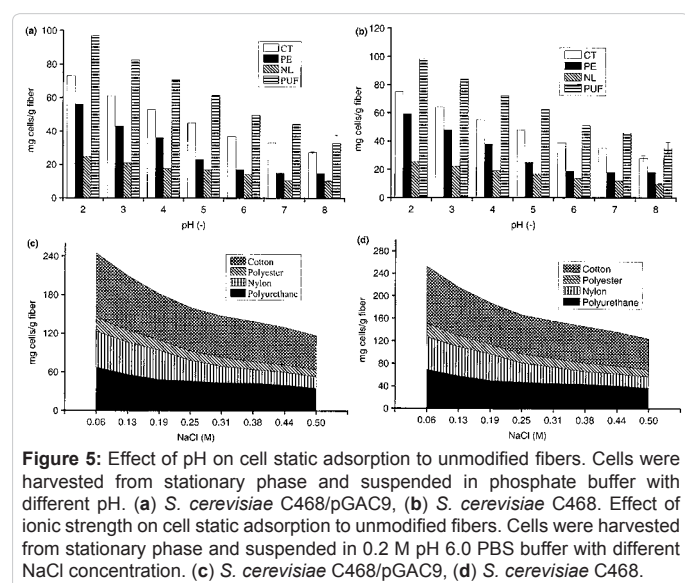

Figure 5: Effect of pH on cell static adsorption to unmodified fibers. Cells were harvested from stationary phase and suspended in phosphate buffer with different pH. (**a**) *S. cerevisiae* C468/pGAC9, (**b**) *S. cerevisiae* C468. Effect of ionic strength on cell static adsorption to unmodified fibers. Cells were harvested from stationary phase and suspended in 0.2 M pH 6.0 PBS buffer with different NaCl concentration. (**c**) *S. cerevisiae* C468/pGAC9, (**d**) *S. cerevisiae* C468.

Fiber	Ph	Adsorption rate constant (h^{-1})	
		S. cerevisiae C468/pGAC	*S. cerevisiae* C468
Cotton	2.0	0.1685	0.2006
	3.0	0.1541	0.1835
	4.0	0.1398	0.1664
	5.0	0.1102	0.1312
	6.0	0.0890	0.1059
	7.0	0.0730	0.0869
	8.0	0.0685	0.0816
	9.0	0.0641	0.0763
Polyester	2.0	0.1635	0.1947
	3.0	0.1525	0.1816
	4.0	0.1415	0.1685
	5.0	0.1068	0.1271
	6.0	0.0776	0.0924
	7.0	0.0735	0.0875
	8.0	0.0678	0.0807
	9.0	0.0621	0.0739

Table 2: Effect of pH on the adsorption rate constant in static adsorption on unmodified fibers.

Effect of Ionic Strength: The effect of ionic strength on adsorption is shown in Figure 5c-d. Low ionic strength promoted cell adhesion and high salt concentration inhibited adhesion. This result is contradictory to some of the results reported in previous studies. The adhesion of *S. cerevisiae* to glass plate was increased by increasing the aluminum ions on the surface [25], because of reduced electrostatic repulsion and increased ion-bridging at higher electrolyte concentrations. Other studies [32], have found no apparent relationship between electrolyte concentration and adhesion. Yee et al. [32], also reported that the extent of *Bacillus subtilis* adsorption onto corundum increased with decreasing ionic strength. It is however, suggested that, under the experimental conditions, a high ionic strength might destabilize immobilized cells and prevents the interaction between cells and supports surfaces, and therefore, reduced cell adhesion.

Effect of media composition: The media composition has a significant effect on cell adsorption on PEI-GA modified cotton. As shown in (Figure 6.) the adsorption rate was maximal in the medium containing 20 g L^{-1} glucose, although cell loading in this medium was not the highest. This may be due to reduced affinity of glucose molecules for cells wall and support [33]. The presence of 15 g L^{-1} ethanol, increased cell adhesion by 15% compared to distilled water. Enhanced hydrophobic interactions between cells and support may be able to explain this effect. The addition of yeast extract lowered cell adsorption due to the presence of amino acids and peptides. However, cell loading in this medium was still higher than in distilled water. Coating the support by these soluble macromolecules also facilitated the subsequent yeast cell adhesion. A further decrease in cell adhesion was observed in the fermentation broth. In this case, glucose was absent while the presence of some yeast extract and other unknown components at the end of fermentation exhibited inhibitory effect on cell adhesion (Figure 6).

Effects of surfactants: Hydrophobic interactions regular cell adhesion to solid surfaces. Evidence for hydrophobic interactions was provided by experiments with surfactants measuring the detachment of cells. As summarized by Neu [34], surfactants can alter cell adhesion by adsorbing to the cell surface. If the hydrophobic ends of the surfactant molecules adsorb to cell surface, then the hydrophilicity ends remain in the aqueous phase. This adsorbed layer should make the surface

more hydrophilic. This surface modification should decrease the hydrophobic interaction between the cells and support and reduced adhesion [35].

Triton X-100 and Tween 80 at a concentration of 0.1% increased cell detachment from the unmodified fibrous matrices under static conditions (Figure 7a-b). Moreover, the removal of cells was more pronounced with polyester, the more hydrophobic of the two surfaces. Tween 80 was less effective than Triton X-100 at releasing cells from the support under this concentration. Based on the results of cell static desorption by surfactants, we carried out cell dynamic desorption experiments using Triton X-100. Specifically, we were interested in the effect of different concentrations of Triton X-100. (Figure 7c-d) shows cell desorption from various matrices as indicated by increased cell concentration in the suspension. Desorption of cells from unmodified polyester fiber had the highest desorption rate, and it increased with increasing the concentration of Triton X-100 from 0 to 1% w v^{-1}. Yeast cells have a more hydrophobic surface (Figure 7) [36-39], than polyester fiber, which is more hydrophobic than cotton fiber [40]. It is expected that hydrophobic interaction might play more important role in adhesion between the surfaces of yeast and polyester. In other systems, the relationship between changes in the desorption rate and followed desorption kinetic model very well.

The desorption rate constants were estimated from the data and are shown in (Table 3) Surfactant concentrations were similar, but the extent of increase was not as marked, demonstrating that hydrophobic interaction cannot be the primary basis for all adhesive interactions of these microorganisms. Probably electrostatic interactions were making
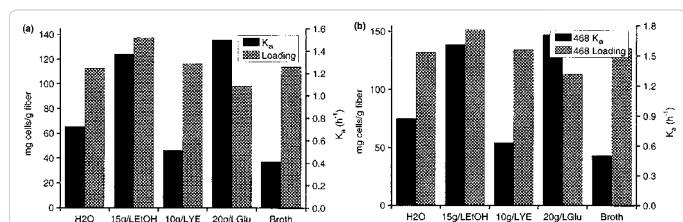

Figure 6: Effect of media composition on adsorption rate and adsorption capacity of static cell adhesion using PEI-GA modified cotton at pH 6.0. (**a**) *S. cerevisiae* C468/pGAC9, (**b**) *S. cerevisiae* C468.
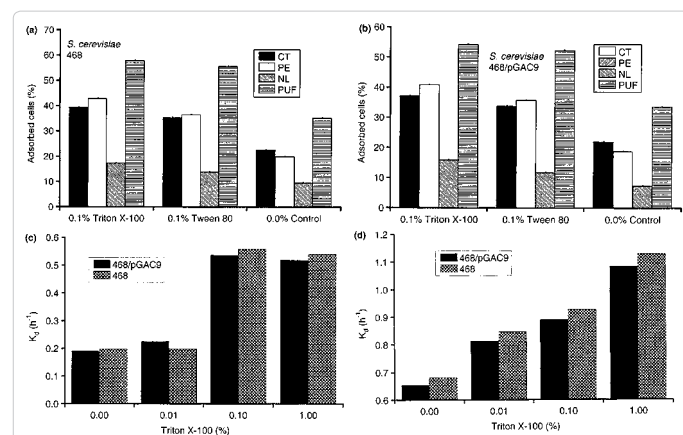

Figure 7: (a and b) Cell static desorption by surfactant. The experiments were carried out in pH 6.0 phosphate buffer after static adsorption experiment. (**a**) *S. cerevisiae* C468, (**b**) *S. cerevisiae* 468/pGAC9. Dynamic desorption of *S. cerevisiae* C468 and *S. cerevisiae* 468/pGAC9 with Triton X-100, (**c**) cotton, (**d**) polyester.

Support	Triton X-100 Concentration (% w/v)	Desorption rate constant K_d (h^{-1})	
		S. cerevisiae C469/pGAC9	*S. cerevisiae* C468
Cotton	Control	0.190±0.001	0.198±0.004
	0.01	0.2250.013	0.198±0.004
	0.1	0.536±0.011	0.558±0.013
	1	0.518±0.022	0.540±0.027

Table 3: Effect of Triton X-100 concentration on recombinant yeast cell dynamic desorption.

a significant contribution to the adhesive interaction as well (Table 3) According to these experimental results, 1% w v^{-1} of Triton X-100 was used as desorbing agent to dislodge attached cells from fibrous matrices under dynamic conditions. Each was lasted 30 min and then cell suspension was replaced by fresh desorbing solution. It was noticed after the 3rd wash that no more cells. Totally, about 62.1% of cells were removed from polyester, and 36.1% of cells were released from cotton.

The viability of desorbed cells in the suspension and cells remained on the fibrous matrices was also examined (see Figure 4). In general, cells attached to the fiber were more viable than those being washed off by surfactant. Therefore, Triton X-100 can selectively desorb cells from the fiber. When pH 6.0 phosphate buffer was used as the desorbing agent, no difference on cell viability was found between immobilized and suspended cells. This result show that Triton X-100 can selectively interact with nonviable cells by hydrophobic interaction and disrupts cell adhesion on the support. It is probably due to the fact that changes in cell surface properties due to cell death led to stronger interaction between nonviable cells and the surfactants, and the physical adsorption between cells and support was not strong to prevent cells from being washed off.

It was found that, in a separate experiment, the viability of cells in the pH 6.0 phosphate buffer remained almost unchanged after the addition of the surfactant. Therefore, the surfactant was not toxic to the cells at this concentration and toxicity is not the mode of action of the surfactant, but rather disruption of hydrophobic interaction between the cell surface and fibrous matrices. The finding could be very useful for controlling cell density in cell immobilization. Conventional immobilized cell bioreactors usually suffer from productivity loss after long-term running due to accumulation of dead cells and culture degeneration. This problem can be overcome by the removal of non-productive cells using the surfactant.

Conclusion

Immobilization of recombinant and non-recombinant *S. cerevisiae* on untreated fibrous materials had poor adsorption efficiency (5750-mg cells/ g fiber for cotton and 4600-mg cells/ g fiber for polyester). Treatment of the fibrous matrices with PEI and GA resulted in 85% yeast cells on cotton and 35% on polyester suggesting increased adsorption sites on the fiber surfaces. PEI-GA combined treatment also increased cell loading by 35% on cotton and 60% on alkali treated polyester suggesting enhanced yeast cell adhesion. Studies examining the influence of media composition suggested that low pH and ionic strength promoted cell adhesion by 29%, while yeast extract and/or glucose decreased adhesion by about 15%. Presence of shear flow and 1% Triton X-100 selectively detached 62% and 36% of nonviable cells from polyester and cotton, respectively, suggesting that cell immobilization in fibrous-bed bioreactors can be controlled to optimize cell density for long-term stability.

Acknowledgements

References

1. Margaritis A, Kilonzo P (2005) Production of Ethanol Using Immobilized Cell Bioreactor Systems. Applications of Cell Immobilization Technology Vol: 8B 375-405.

2. Kilonzo P, Margaritis A, Bergougnou MA (2009) Airlift-driven fibrous bed bioreactor for continuous production of glucoamylase using immobilized recombinant yeast cells. J Biotechnol 143: 60-68.

3. Gupta JC, Mukherjee KJ (2001) Stable maintenance of plasmid in continuous culture of yeast under non-selective conditions. J Biosci Bioeng 92: 317-323.

4. Lebrun L, Junter GA, Jouenne T, Mignot L (1994) Exopolysaccharide production by free and immobilized microbial cultures. Enzyme Microb Technol 16: 1048-1054.

5. Hemachander C, Bose N, Puvanakrishnan R (2001) Whole cell immobilization of *Ralstonia pickettii* for lipase production. Process Biochem 36: 629-633.

6. West TP, Strohfuls B (1998) Polysaccharide production by *Aureobasidium pullulans* cells immobilized by entrapment. Microbiol Res 153: 253-256.

7. Braschler T, Johnn R, Heul M, Metref L, Renaud P (2005) Gentle cell trapping and release on a microfluidic chip by in situ alginate hydrogel formation. Lab Chip 5: 553-559.

8. Hsu CH, Chu YF, Argin-Soysal S, Hahm TS, Lo YM (2004) Effect of surface characteristics and xanthan polymers on the immobilization of *Xanthomonas campestris* to fibrous matrices. J Food Sci 69: E441-E448.

9. Chu YF, Hsu CH, Pavan KS Lo YM (2009) Immobilization of bioluminescent *Escherichia coli* cells using natural and artificial fibers treated with polyethylenimine. Biores Technol 100: 3167-3174.

10. Talabardon M, Scwitzguelbel JP, Peringer P, Yang ST (2000) Acetic acid production from lactose by anaerobic thermophilic coculture immobilized in a fibrous-bed bioreactor. Biotechnol Prog 16: 1008-1017.

11. Huang Y, Yang ST (1998) Acetate production from whey lactose using co-immobilized cells of homolactic and homoacetic bacteria in a fibrous-bed bioreactor. Biotechnol Bioeng 60: 498-507.

12. Yang ST, Lo YM (1998) Production of Cell-free Xanthan gum fermentation broth by cell adsorption on fibers. Biotechnol Prog 14: 259-264.

13. Melo JS, D'Souza SF (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: 17-21.

14. 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.

15. Dicosmo F, Facchini PJ, Neuman AW (1998) Does the spontaneous adhesion of cultured plant cells to polymer surfaces have potential as an immobilization technique. Trends Biotechnol 6: 137-140.

16. Kim DN, Park J, Koh WG (2009) Control of cell adhesion on poly(ethylene glycol) hydrogel surface using photochemical modification and micropatterning technique. J Journal of Industrial and Engineering Chemistry 15: 124-128.

17. Kawaguti HY, Buzzano MF, Orsi DC, Suzuki GT, Sato HH (2006) Effect of the additive polyethylenimine and glutaraldehyde on the immobilization of *Erwinia* sp. D12 cells in calcium alginate for isomaltulose production. Process Biochem 41: 2035-2040.

18. Barranco-Florido E, Garcia-Garibay M, Gomez-Ruiz L, Azaola A (2001) Immobilization system of *Klyuveromyces marxianus* cells in barium alginate for inulin hydrolysis. Process Biochem 37: 513-519.

19. Elibo M, Moreira AR (2003) Production of extracellular alkaline protease by immobilization of the marine bacterium *Teredinobacter turnirae*. Process Biochem 38: 1445-1450.

20. Nighojkar S, Phanse Y, Sinha D, Nighojkar A, Kumar A (2006) Production of polygalacturonase by immobilized cells of *Aspergillus niger* using orange peel as inducer. Process Biochem 41: 1136-1140.

21. Hann EC, Sigmund AM, Hennessey SM, Gavagan JE, Short DR, et al. (2002) Optimization of an immobilizedcell biocatalyst for production of 4-cyanopentanoic acid. Org Process Res Dev 6: 492-496.

22. D'Souza SF, Kamath N (1988) Cloth Bioreactor Containing Yeast Cells Immobilized on Cotton Cloth Using Polyethylenimine. Appl Microb Biotechnol 29: 136-140.

23. Scott JA, O'Reilly AM (1996) Co-immobilization of selected yeast and bacteria for controlled flavour development in an alcoholic cider beverage. Process Biochem 31: 111-117.

24. Freeman A, Lilly MD (1998) Effect of processing parameters on the feasibility and operational stability of immobilized cells. Enzyme Microb Technol 23: 335-345.

25. Van Haecht JL, Bolipombo M, Rouxhet PG (1985) Immobilization of *Saccharomyces cerevisiae* by Adhesion: Treatment of the Cells by Al Ions. Biotechnol Bioeng 27: 217-224.

26. Busscher HJ, Weerkamp AH (1987) Specific and nonspecific interactions in bacterial adhesion to solid substrata. FEMS Microbiol Rev 46: 165-173.

27. Alonso N, López-Gallego F, Betanco L, Hidalgo A, Mateo C, et al. (2005) Immobilization and stabilization of glutaryl acylase on aminated sepabeads supports by the glutaraldehyde crosslinking method. J Mol Catal B: Enzym 35: 57-61.

28. Lopez-Gallego F, Betanco L, Mateo C, Hidalgo A, Alonso-Morales N, et al. (2005) Enzyme stabilization by glutaraldehyde crosslinking of adsorbed proteins on aminated supports. J Biotechnol 119: 70-75.

29. Vallejo-Becerra V, Vasquez-Bahen JM, Santiago-Hernandez JA, Hidalgo-Lara ME (2008) Immobilization of recombinant invertase INVB from Zymomonas mobilis on Nylon-6. J Ind Microb Biotechnol 35: 1289-1295.

30. Albayrak N, Yang ST (2002) Immobilization of (-galactosidase on fibrous matrix by polyethyleneimine for production of galacto-ologosaccharides from lactose. Biotechnol Prog 18: 240-251.

31. Merritt K, An YM (2000) Factors influencing bacterial adhesion. In: An YH, Friedman RJ (Eds.), Handbook of bacterial adhesion: Principles, methods, and applications. The Humana Press Inc., Totowa, NJ, pp. 53-72.

32. Yee N, Fein JB, Daughney CJ (2000) Experimental study of the pH, ionic strength, and reversibility behavior of bacterial-mineral adsorption. Geochim Cosmochim Acta 64: 609-617.

33. Bar R, Gainer JL, Kirwan DJ (1987) Ethanol fermentation by ionically adsorbed *Zymomonas mobilis*: Environmental effects on cell immobilization. Biotechnol Bioeng 29: 1045-1049.

34. Neu TR (1996) Significance of bacterial surface-active compounds in interaction of bacterial with interface. Crit Rev Microbiol 18: 151-166.

35. Stelmack PL, Gray MR, Pickard MA (1999) Bacterial Adhesion to Soil Contaminants in the Presence of Surfactants. Appl Environ Microbiol 65: 163-168.

36. Azeredo J, Ramos I, Rodriguks L, Oliveira R, Teixeira J (1997) Yeast flocculation: A new method for characterizing cell surface interactions. J Inst Brew 103: 359-361.

37. Silva TMJ, Glee PM, Hazen KC (1995) Influence of cell surface hydrophobicity on attachment of *Candida albicans* to extracellular matrix proteins. J Medic Veterin Mycol 33: 117-122.

38. Suzzi G, Romano P, Vannini L (1994) Cell surface hydrophobicity and flocculence in *Saccharomyces cerevisiae* wine yeasts. Collo Surfa B: Biointerfa 2: 505-510

39. Vazquez-Juarez R, Andlid T, Gustafsson L (1994) Cell surface hydrophobicity and its relation to adhesion of yeasts isolated from fish gut. Collo Surfa B: Biointerfa 2: 199-208.

40. Gao Q, Zhu Q, Guo Y (2009) Formation of Highly Hydrophobic Surfaces on Cotton and Polyester Fabrics Using Silica Sol Nanoparticles and Nonfluorinated Alkylsilane. Ind Eng Chem Res 48: 9797-9803.

41. White JS, Walker GM (2011) Influence of cell surface characteristics on adhesion of *Saccharomyces cerevisiae* to the biomaterial Hydroxylapatite. Antonie van Leeuwenhoek 99: 201-209.