Appl Biochem Biotechnol (2011) 165:98–108
DOI 10.1007/s12010-011-9236-9

Control and Optimization of *Clostridium tyrobutyricum* ATCC 25755 Adhesion into Fibrous Matrix in a Fibrous Bed Bioreactor

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Received: 16 December 2010 / Accepted: 28 March 2011 /
Published online: 12 April 2011

Abstract The great performance of a fibrous bed bioreactor (FBB) is mainly dependent on the cell adhesion and immobilization into the fibrous matrix. Therefore, understanding the mechanism and factors controling cell adhesion in the fibrous matrix is necessary to optimize the FBB setup and further improve the fermentability. The adhesion behavior of a strain of *Clostridium tyrobutyricum* isolated from an FBB was studied, which was proven to be affected by the different environmental conditions, such as growth phase of cells, pH, ionic strength, ionic species, and composition of media. Our results also suggested that electrostatic interactions played an important role on bacteria adhesion into the fibrous matrix. This study demonstrated that the compositions of fermentation broth would have a significant effect on cell adhesion. Consequently, a two-stage glucose supply control strategy was developed to improve the performance of FBB with higher viable cell density in the operation of the FBB setup.

Keywords *Clostridium tyrobutyricum* · Fibrous matrix · Adhesion · Electrostatic interaction · Fibrous bed bioreactor

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Introduction

A fibrous bed bioreactor (FBB), high cell density immobilized in a fibrous matrix packed in the reactor, has been studied in the laboratory during the last 15 years as a novel cell immobilization process involving both adsorption and entrapment [1–3]. A fibrous matrix has been developed as the support for cell immobilization because of its high specific surface area, high void volume, high mechanical strength, high permeability, low cost, and low pressure drop [4]. By circulating cell suspension through the FBB, a simple in situ immobilization of the cells can be carried out. The FBB has been successfully used for the bioproduction of a series of chemicals, including organic acids [5–7], enzymes [8, 9], xanthan gum [10], and pharmaceuticals, such as, developmental endothelial locus-1 [11], embryonic stem cells [12], GM-CSF [13], hepatocytes [14], monoclonal antibody [15], and soluble human Fas ligand [16].

Clostridium tyrobutyricum ATCC 25755 has been chosen for butyric acid production preferably due to its high butyric acid concentration and selectivity in the fermentation broth, and high productivity of the bioreactor, especially in the FBB system [17–19]. Our previous study has also proven that a significantly improved final butyric acid yield with the high cell density in the fibrous matrix (>40 g cells per liter) was achieved when using crude cane molasses as the available carbon source [20]. However, the fundamental mechanism and environmental factors involved in bacterial adhesion in the FBB are poorly understood and have not been well defined. The aims of the present study were to test the effect of various environmental conditions on cell-support adhesion, including the growth phase of cells, pH, ionic strength, ion species, and composition of media, as well as to optimize the cell immobilization process in the FBB setup.

Materials and Methods

Materials

Cotton towel was selected as the fibrous matrix for adhesion, offered by Yantai Huaxing Towel Co., Ltd. China, with a thickness of 0.20 cm and a density of 0.25 g/cm^3. Before adhesion assays, the cotton towel was cut into rectangular chips (4×2 cm), cleaned by soaking in a boiling water bath for 30 min and then dried. 2,3,5-Triphenyl-2H-tetrazolium chloride (TTC) was obtained from Aldrich Chemical Co., WI, USA. Other chemicals of analytical grade were obtained from standard sources.

Strain and Cultivation Conditions

The *C. tyrobutyricum* ATCC 25755 used herein was isolated from our previous study with crude cane molasses as the available carbon source [20] and stored at 4 °C. The synthetic medium used for the microorganism culture contained (per liter of distilled water): 30 g of glucose, 5 g of yeast extract, 5 g of peptone, 3 g of $(NH_4)_2SO_4$, 0.6 g of $MgSO_4 \cdot 7H_2O$, 0.03 g of $FeSO_4 \cdot 7H_2O$. The cultivation conditions were 37 °C, pH 6.0, and anaerobiosis was kept by sparging the medium with N_2 before inoculation.

The fermentation system consisted of a 5-L stirred-tank fermentor (B. Braun, B. Braun Biotech International, Melsungen, Germany) connected to a 0.5-L fibrous bed bioreactor.

The synthetic medium mentioned above was totally added into the fermentor at the beginning of the bioreactor setup. Details about the conventional bioreactor setup have been given elsewhere [21]. When the glucose concentration in the fermentation broth decreased to zero, the cells in the fibrous matrix were washed off by vortexing the cotton towel in sterile potassium phosphate buffer (0.1 M, pH 6.0) under anaerobic conditions. The washed buffer containing the cells was collected at 4 °C for further assay.

Adsorption Experiments

The experiments were conducted to study the adsorption of *C. tyrobutyricum cells* into a cotton towel. The immobilized cells in the FBB were removed from the fibrous matrix by vortexing the matrix in sterile distilled water under anaerobic conditions. Unless otherwise stated, the microbial cells were harvested in the stationary phase from the cell fermentation broth, separated by centrifugation for 10 min at 8,400×g at 4 °C, washed twice and resuspended in a potassium phosphate buffer (0.1 M, pH 6.0) to obtain an initial OD_{600} of 0.7–0.9. A dry cotton piece was soaked in the cell suspension (volume, 100 mL) in a 500-mL serum bottle with gentle shaking (150 rpm) on a shaker at room temperature. The experiments were conducted for approximately 5 to 6 h until the cell concentration in the suspension did not change. To study the effect of the different growth phase on cell adhesion, the cells were harvested in the early log phase, mid-log phase, and stationary phase, respectively. In the pH effect experiment, the cells were suspended in 0.1 M potassium phosphate buffer over a wide range of pH (3.0 to 7.0). Ionic strength experiments were carried out in the phosphate buffer (pH 6.0) with different NaCl concentrations from 0.01 to 0.5 M. In the ionic species experiment, seven different ionic species including NaCl, KCl, $CaCl_2$, $MgCl_2$, $MnSO_4$, and $Al_2(SO_4)_3$ with the concentrations of 0.01 and 0.1 M, respectively, were added into a modified Morita salt solution (MMSS) as adhesion media for cells. MMSS contained 2.6 g L^{-1} of NaCl, 0.56 g L^{-1} of $MgCl2{\cdot}6H_2O$, 0.76 g L^{-1} of $MgSO_4{\cdot}7H_2O$, and 0.15 g L^{-1} of $CaCl_2{\cdot}2H_2O$ [22]. To study the influence of the medium composition, the five media were as follows: the MMSS solution as the control, the MMSS with 30 g L^{-1} glucose, the MMSS with 10 g L^{-1} yeast extract, the MMSS with 30 g L^{-1} butyrate, and the MMSS with 10 g L^{-1} cane molasses. All these solutions were adjusted to pH 6.0 with either H_2SO_4 or NaOH (0.1 M). The amount of attached cells were indirectly estimated by measuring the decrease of OD_{600} using a spectrophotometer (Ultrospec 3300 pro, Amersham Pharmacia Biotech, Cambridge, UK). One unit of OD_{600} was equivalent to 0.68 g L^{-1} cell dry weight for *C. tyrobutyricum*. All the adsorption experiments were conducted in an anaerobic chamber.

Cell Viability Assay

The viability of the cells was measured by the TTC method previously described by Suwannakham and Yang [2]. Briefly, TTC was added to the cell suspension to give a final concentration of 0.1% (*w*/*v*). The cell suspension was incubated at room temperature for 30 min and centrifuged (12,000×g, 10 min, 4 °C). The cell pellet was collected and then suspended in methanol to extract the pink color. The absorbance of the methanol extract at 485 nm, which is proportional to the viable cell number, was measured with a spectrophotometer. The amount of 1 mg of active cells was correspondent to an absorbance of 0.242 at 485 nm. The cell viability (percent) was expressed with cells cultured in serum bottles and harvested in the exponential phase as the control with 100% viability.

Scanning Electron Mircoscopy

After the incubation period, several small pieces of the fibrous material were taken as samples from the drained fibrous matrix and rinsed three times in 0.1 M potassium phosphate buffer to remove the unattached cells. These samples were immersed in 2.5% glutaraldehyde solution for 2 h and then rinsed completely with double-distilled water.

The samples were gradually dehydrated with 20–100% ethanol in increments of 10% by holding the samples at each concentration for 30 min. The fibers were then air-dried and coated with gold before being observed with a JSM-6390 scanning electron microscope (JEOL, Japan).

Results and Discussion

Effect of Growth Phase on Adhesion

Usually, the greater hydrophobicity of cells and support causes greater attractive forces, resulting in a higher degree of adhesion [23]. It has been found that the hydrophobicity in the different phases of *Pseudomonas fluorescens* cells was dissimilar [24], and nutrient starvation could enhance yeast immobilization onto the glass fibers [25]. Therefore, the growth phase has a large influence on cell-support adhesion. In the preliminary experiment, it was found that the initial cell concentration did not affect the adhesion rate; however, cell loading, which indicates the maximum amount of cells adsorbed, was dependent on the initial cell concentration. Consequently, for comparison, all experiments for the cells were carried out at the same initial cell concentration (OD_{600} of 0.7–0.9). In this work, *C. tyrobutyricum* cells were harvested after culturing for 24, 36, and 48 h, that is, in the period of the early log phase, mid-log phase, and stationary phase, respectively. As shown in Fig. 1, adhesion of the cells was closely related to the growth phase, as adhesion increased with cell age. Obviously, older cells in the stationary phase were more capable of adhesion into the fibrous matrix, which was 4.65- and 1.33-fold higher amount of cells than those in the early- and mid-log phase, respectively (19.31 vs. 4.15 and 14.48 mg cells per gram of

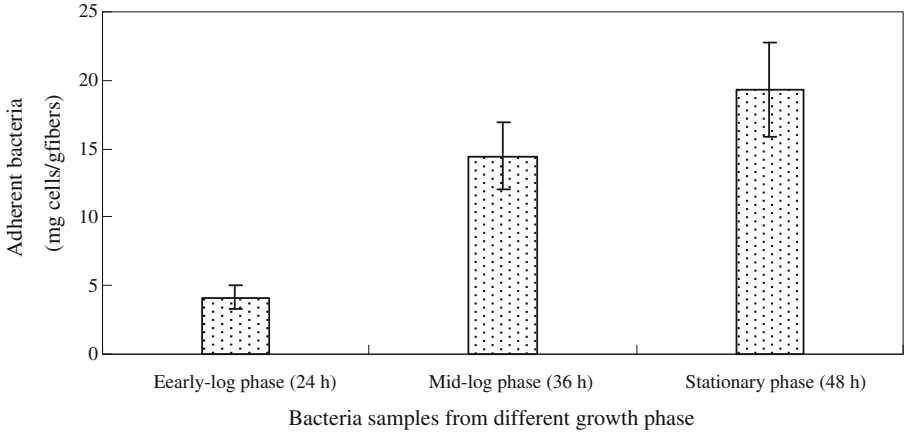

Fig. 1 Effect of growth phase on cell adhesion into fibrous matrix in a potassium phosphate buffer (0.1 M, pH 6.0)

fiber). Changes in cell surface hydrophobicity, as well as the charge and amino groups for chemical bonding might give reasons for the enhanced cell-support adhesion [26].

Effect of pH on Adhesion

Many investigations into the physicochemical forces between the bacteria and the support have also shown the involvement of electrostatic interactions [27–29]. To evaluate the significance of electrostatic interactions, the effect of pH on cell-support adhesion was tested. Potassium phosphate buffer (0.1 M) at different pHs from 3.0 to 7.0 was conducted for the pH effect study (Fig. 2). On the whole, cell loading into fibrous matrix was pH dependent, increasing with decreasing pH in the range studied. Changes in pH between 5.0 and 7.0 had little effect but below pH 5.0 there was an obvious increase in adhesion, especially more remarkable when the pH decreased from 4.0 to 3.0. The maximum adhesion amount was up to 30.50 mg cells per gram of fiber at pH 3.0. It has been reported that most bacteria have a net negative charge, as do most solid surfaces [29, 30]. The excess surface charges lead to the formation of a solvation layer surrounding the bacteria, which will enhance the stability of the cells in the liquid phase and inhibit their approach to the solid surface with the same charge [31]. This electrostatic repulsion between cells and support surface can be reduced by pH control. At low pH values, the surface of the cells was less negatively charged due to the deprotonation decrease of various chemical groups, e.g., carboxyl (COO^-), phosphate (PO_4^{2-}) and amine groups (COO^-), and proteins, etc. [32]. This should result in a decreased electrostatic repulsion and enhanced adhesion between the bacteria and the support.

Effect of Ionic Strength on Adhesion

The ionic strength of the medium is another important factor affecting the electrostatic interactions. The dependence of the adhesion on the ionic strength was illustrated in Fig. 3. The concentration of NaCl in the adhesion medium from 0.01 to 0.1 M caused a steeper slope of the bacteria adhesion, while a further increase of ionic strength from 0.1 to 0.5 M resulted in a slight increase in the adhesion of *C. tyrobutyricum* cells, and the maximum adhesion amount was found to be 23.50 mg cells per gram of fiber at 0.5 M

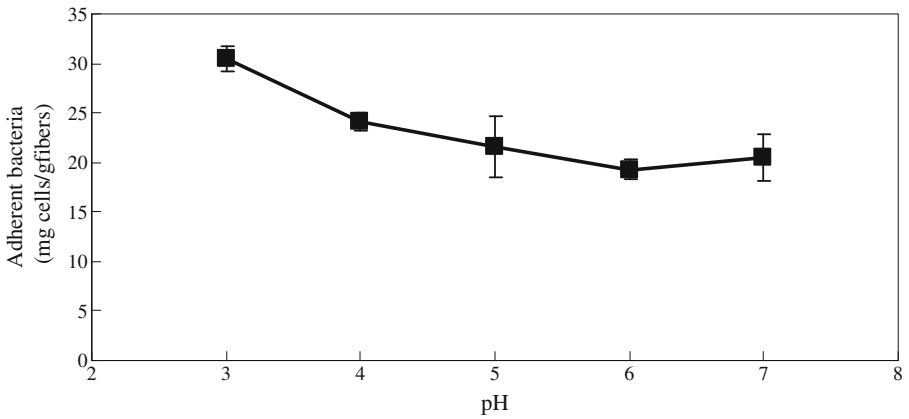

Fig. 2 Effect of pH on cell adhesion into fibrous matrix in 0.1 M potassium phosphate buffer

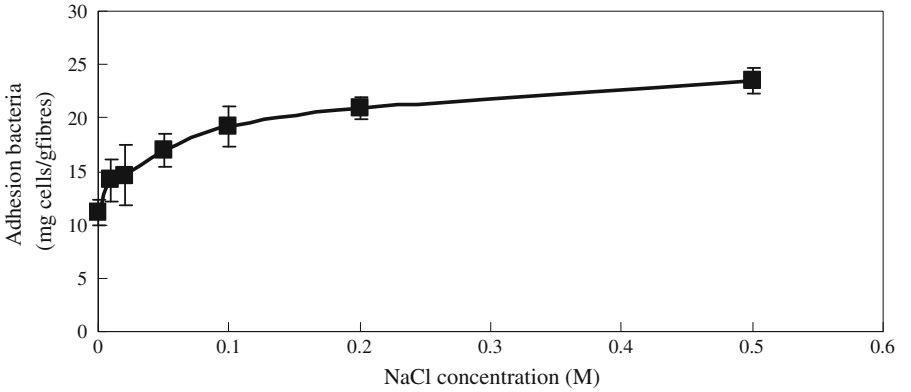

Fig. 3 Effect of ionic strength on cell adhesion into fibrous matrix in a potassium phosphate buffer (0.1 M, pH 6.0)

NaCl. A similar phenomenon was observed with the adsorption experiments conducted by previous reporters with other bacteria [33–35]. These observations might be quantitatively described by the extended Derjaguin and Landau, Verwey and Overbeek (DLVO) theory [36]. According to the extended DLVO theory, an increase in ionic strength reduces repulsive electrostatic forces, allows bacteria to approach closer to the support, and consequently facilitates adhesion through the action of attractive Lifshitz-van der Waals and Lewis acid–base forces. However, this result was contradictory to some of the results reported in other studies, which indicated that low ionic strength promoted cell adhesion and high salt concentration inhibited adhesion or there was no apparent relationship between electrolyte concentration and adhesion [30, 37, 38]. It is suggested that, under the experimental conditions, a high ionic strength might reduce repulsive electrostatic forces, allow bacteria to approach closer to the surface, and consequently facilitate adhesion through the action of attractive Lifshitz-van der Waals and Lewis acid–base forces.

Effect of Ionic Species on Adhesion

It was reported by our previous study that crude cane molasses, one of the industrial grade raw materials, which had been successfully exploited for butyric acid production, contained a specified amount of metal ions such as calcium, potassium, sodium, iron, magnesium, copper, etc. [20]. The following experiment was employed to clarify the relationship between electrolyte valency of inorganic salt and adhesion. The results presented in Table 1 showed that the behavior of *C. tyrobutyricum* adhesion was different when using various kinds of cations, but this did not seem significant. Indeed, the adhesion increased slightly with the increasing ionic strengths (from 0.01 to 0.1 M), whether the adhesion occurred in various salt solutions containing monovalent (sodium, potassium) or divalent (calcium, magnesium, manganese). The average increment came up to 10.0–30.2% in 0.01 M electrolyte and 13.2–21.9% in 0.1 M electrolyte compared to the control, respectively. However, the adhesion of *C. tyrobutyricum* was reduced in the presence of Al^{3+} cations, decreasing by 3.0% (0.01 M) and 0.7% (0.1 M), respectively. It was also reported by Marshall et al. [31] that adhesion of a marine pseudomonad to glass decreased in the presence of trivalent cations ($A1^{3+}$, La^{3+}).

Table 1 Effect of ion species on cell adsorption into fibrous matrix in a modified Morita salt solution (pH 6.0)

Suspending liquid		
Ion	Concentration (M)	Adhesion bacteria (mg cells per gram of fiber)
Control	0.01	15.23±2.00
	0.1	20.12±0.69
NaCl	0.01	18.60±0.74
	0.1	23.89±0.13
KCl	0.01	18.84±0.99
	0.1	23.69±1.72
$MgCl_2$	0.01	19.44±1.84
	0.1	24.53±2.82
$CaCl_2$	0.01	19.83±1.24
	0.1	22.78±1.70
$MnSO_4$	0.01	16.75±0.71
	0.1	24.04±0.64
$Al_2(SO_4)_3$	0.01	14.78±0.56
	0.1	18.65±0.20

Effect of the Media Composition on Adhesion

As the influence of media compositions under industrial conditions on the adhesion between cells and fibrous matrix was complex, especially with crude cane molasses as the carbon source, therefore, the main component in the medium was listed separately with the MMSS solution as the control. As can be seen in Fig. 4, the medium composition had a significant effect on the adhesion of *C. tyrobutyricum* into the fibrous matrix. The MMSS solution with 30 g L^{-1} glucose-enhanced cell adhesion rate compared with the control. Bar et al. [39] have pointed out that an affinity of glucose molecules to the cell wall might cause a screening of the electrical change, which might reduce the electrostatic repulsion between cells and fibrous matrix, but the cell

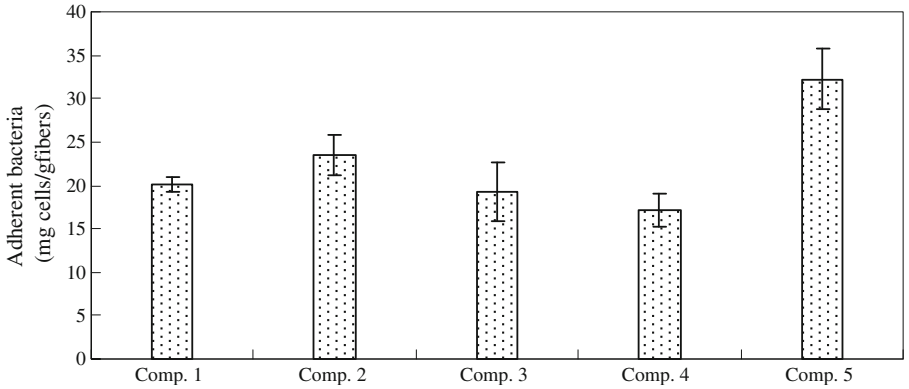

Fig. 4 Effect of media compositions on cell adhesion into fibrous matrix. The details of each media composition were as follows: the MMSS solution as the control, the MMSS with 30 g L^{-1} glucose, the MMSS with 10 g L^{-1} yeast extract, the MMSS with 30 g L^{-1} butyrate, the MMSS with 10 g L^{-1} cane molasses

loading in this medium was not the highest. The adhesion bacteria was found to be maximal in the medium containing 10 g L^{-1} crude cane molasses, which was probably due to the high level of ionic strength. However, the addition of yeast extract interfered with cell adhesion as shown by the lower cell loading. It might be a result of the competitive adhesion of amino acids and peptides, which is a composition of yeast extracts. As expected, a further decrease in cell adhesion was observed with 30 g L^{-1} of butyrate, that means that separation of butyrate from the FBB system was a good choice not only for prevention of product inhibition, but also for better cell immobilization. In this case, at the end of the fermentation broth, glucose was absent and some yeast extract and other unknown compositions were present which might have an inhibitory effect on cell adhesion.

Optimization of the Cell Immobilization Process

Based on the aforementioned results of cell adsorption, a new strategy for improving the performance of the FBB was developed with the optimization of the cell immobilization procedure in the process of bioreactor setup. The initial glucose concentration in the fermentation medium herein was reduced, from 30 to 15 g L^{-1}, yet the concentrations of the three mineral salts were all doubled (see "Materials and Methods"). After the inoculation of approximately 150 mL of cell suspension into the fermentor, the strains were allowed to grow until the cell concentration reached approximately 7.0 g L^{-1}, which indicated that the cells were in the stationary phase, and then the cell immobilization was carried out. During the 2-day immobilization procedure, another 15 g L^{-1} of glucose was continuously feeding by pump with the residual glucose levels remained above zero. Both adhesion amount and relative viability of cells immobilized into the fibrous matrix were measured comparing the optimized immobilization method with no optimization (see "Materials and Methods"). As shown in Table 2, immobilized cells in the fibrous matrix after optimization was much more than those before optimization, increasing by 43.8% (168.6±15.60 vs. 117.2±10.45 mg cells per gram of fiber), as well as the increasing of the cell viability, from 70.3% before to 77.9% after optimization. Table 2 also showed a higher butyric acid concentration (10.83± 0.31 vs. 9.05±0.23 g L^{-1}) and a lower acetic acid concentration (2.11±0.45 vs. 3.10± 0.10 g L^{-1}) produced after optimization. Furthermore, as can be seen in the scanning electron micrographs, the cells scattered on the fiber surface before the optimization of the cell immobilization procedure (Fig. 5a), after which, the cells formed a large amount of

Table 2 Comparison of cell immobilization and acid production before and after the optimization of the process of bioreactor setup

	Before optimization	After optimization
Cell immobilization		
Cell adhesion (mg cells per gram of fiber)	117.2±10.45	168.6±15.60
Cell relative viability (%)	70.3 ±4.32	77.9±5.41
Acid production		
Butyric acid concentration (g L^{-1})	9.05±0.23	10.83±0.31
Butyric acid yield (g g^{-1})	0.30±0.03	0.36±0.02
Acetic acid concentration (g L^{-1})	3.10±0.10	2.11±0.45
Acetic acid yield (g g^{-1})	0.10±0.012	0.06±0.010
B/A ratio (g g^{-1})	2.92±0.50	5.13±0.49

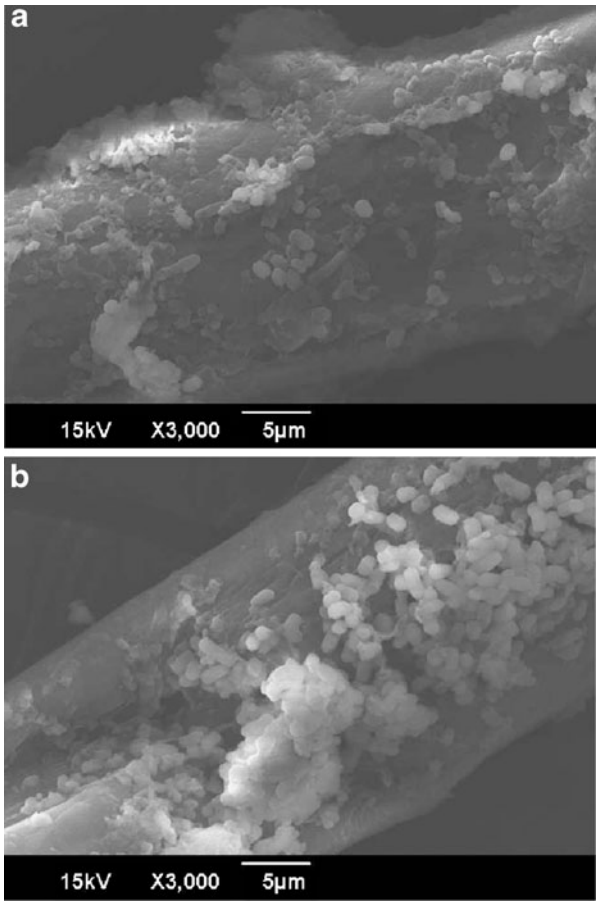

Fig. 5 Scanning electron micrographs of *C. tyrobutyricum* cells adhering to cellulose fibers before (**a**) and after (**b**) optimization

clumps and present almost full in the void space of the porous fibrous matrix (Fig. 5b). These results allowed verification that more viable cells were likely to be immobilized into the fibrous matrix when extending the culture age and increasing the ionic strength of the medium. The two-stage glucose supply control strategy could not only present the glucose during the whole immobilization process, but also postpone the accumulation of butyrate, which both benefit the cell adhesion.

Conclusions

The different characteristics of cells and environmental conditions can greatly affect cell adhesion. In general, the cells harvested at the stationary phase were more capable of adhering to the support than those from early- or mid-log phase. The relatively low pH value and high ionic strength of the medium could enhance the adsorption behavior of microorganisms because of electrostatic repulsion reduction between cells and support surface. However, there was no apparent relationship between ionic species and adhesion. Furthermore, the addition of cane molasses and glucose to the medium increased the adhesion while the presence of yeast extract and butyrate decreased the adhesion.

Consequently, these factors were taken into consideration in the optimization of bacteria immobilization in the process of FBB setup with a two-stage glucose supply control strategy and resulted in the marked increase in the amount of adsorbed cells (by 43.8%) as well as the cell viability (by 10.8%). These results suggest that cell immobilization in the FBB can be controlled to achieve an optimal viable cell density, further enhance the reactor productivity and long-term stability.

Acknowledgments This work was supported by the National High Technology Research and Development Program of China (2009AA02Z206) and the National Basic Research Program of China (2009CB724700), the Ministry of Science and Technology, China, and the Key Program of the National Natural Science Foundation of China (no. 20936002).

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