Whole-Cell Immobilization Using Cell Surface-Exposed Cellulose-Binding Domain

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Specific adhesion of *Eshcherichia coli* with surface-exposed cellulose-binding domain (CBD) to cellulosic materials was investigated. Whole-cell immobilization was very specific, forming essentially a monolayer of cells onto the different supports. Cells with surface-exposed CBD bound specifically and tightly to cellulose supports at a wide range of pH. In contrast to CBD, which shows the highest binding to cellulose at 4 °C, highest cell loading was observed at 37 °C. The extent of immobilization was dependent on the amount of surface-exposed CBD. Cells binding increased with increasing amount of CBD until binding was saturated. Even induction of very low level of CBD (0.05 mM IPTG) was sufficient to provide specific and tight binding to cellulose support. Because optimal binding can be obtained under physiological conditions such as pH 7 and 37 °C, the results demonstrate the general utility of surface-exposed CBD as an efficient means of whole-cell immobilization.

Introduction

The use of immobilized cells has found applications in a wide range of biological processes, ranging from the production of ethanol (*1*) to the degradation of phenol (*2*). Industrial methods for cell immobilization are classified under two broad categories: immobilized-"free"-cell method and "modified"-cell methods. In the free-cell method, cells are immobilized by confining them behind dialysis/filtration membranes. An example of this is the hollow fiber system (*3*). However, the cost of hollow fibers and a steady decline in the filtration rate are the two major limitations. In the modified-cell methods, cells are either bound to a support or entrapped within a matrix such as polyacrylamide, polyurethane, alginate, collagen, and *k*-carragenan. Entrapment inside a matrix has limitations of a high degree of mass transfer resistance between the cell and the surroundings (*4*). Additionally, the use of entrapment sometimes requires harsh conditions and may result in damage or loss of viability.

Cells could be attached to the surface by taking advantage of their property to naturally adhere to surfaces. Although easy to perform, mild on the cells, and potentially free of diffusion, cell immobilization by simple adsorption has serious drawbacks. Changes in pH, temperature, or ionic strength can easily release the cells from the support matrix. These limitations can be alleviated by covalently coupling the cells to the support matrix (*5*). This technique involves linkage of any reactive component, generally the amine groups, on the cell to an activated support. This technique produces a system free of diffusion limitation and provides very strong binding. However, covalent immobilization involves the use of cross-linking agents such as glutaraldehyde for the attachment of cells to the support, which results in a loss of activity and cell viability. Additionally, since covalent bonds are very strong and for most purposes irreversible, the immobilization matrix cannot be regenerated when the biological activity decreases to an undesirable level. A significant improvement, in terms of both economics and technology, could be achieved with reversible and specific adhesion to the support.

Many biological molecules can be attached non-covalently to a support by receptor-mediated specific adhesion. Advantages of immobilization through an affinity tag are specificity and the ability to reverse the binding for regeneration of the support matrix when the whole-cell activity drops to an undesirable level. Several non-covalent adhesions such as biotin−avidin (*6*) and antibody−antigen (*7*) have been reported for immobilization of proteins. However, to date, use of such tags has not been demonstrated for whole-cell immobilization. Additionally, because these supports are relatively expensive, their commercial application for cell immobilization may be economically infeasible.

Cellulose-binding domains (CBD) provide a new class of affinity tags with appealing attributes (*8*−*10*). Interactions between CBD and cellulose are very specific and strong (*11*). Cellulose is a naturally abundant, low-cost affinity matrix with inherently low, non-specific binding characteristics and is available in many different forms. Specific adhesion of whole cells to cellulosic materials with high affinity has been demonstrated by anchoring CBD from *Cellulomonas fimi* on the surface of *E. coli* (*12*). Recombinant cells with surface-expressed CBD can bind tightly and rapidly to cellulose fibers, thus providing a very simple and economical way of whole-cell immobilization.

To gain a better understanding of this new technology for whole-cell immobilization, we have investigated here several factors influencing whole-cell immobilization via the specific adhesion with surface-expressed CBD. The effects of pH, temperature, and the amount of surface-expressed CBD were investigated to define the optimal conditions for whole-cell immobilization.

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Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions. *Escherichia coli* strains XL1-Blue (*rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 (r_k^-, m_k^+), *sup*E44, *rel*A1, *lac* [F′, *pro*AB, *lac*I^{q}ZΔM15, Tn10 (Tet^r)]) and JM105 (*end*A1, *thi*, *rps*L, *sbc*B15, *hsd*R4, Δ(*lac-pro*AB), [F′, *tra*Δ36, *pro*AB, *lac*I^{q}ZΔM15]) were used in this study. Plasmids pUCBD, pKCBD and pKQCBD were used for expression of Lpp-OmpA-CBD on the cell surface. The *lpp-ompA-cbd* fragment was PCR amplifed from pTX210 (*12*) and subcloned into *Pst*I/*Hind*III digested pUC18, pK184 (*13*) and pKQV4, (*14*), to give pUCBD, pKCBD, and pKQCBD, respectively. Primers CBD1: dGGGCTGCAGCTCTAGAGGGTATTAATAATGAAAGCTCBD2 and CBD2: dGGGAAGCTTCGTCAGCCGACCGTGCAGGG were used. Expression of CBD from pKQCBD is tightly regulated due to the presence of the *lacI*^{q} gene on the same plasmid. Plasmid pET-38b(+) (Novagen), which contains the *cbd* gene from *Cellulomonas fimi* was used for the production of CBD in the cytoplasm. Cells carrying pK184 was used as a control for cellulose binding assay.

E. coli cells were cultivated in buffered LB media (10 g/L Difco trptone, 5 g/L Difco yeast extract, 10 g/L NaCl, 1 g/L KH_2PO_4, 3 g/L K_2HPO_4, pH 7.0) supplemented with either ampicillin (100 μg/mL) or kanamycin (20 μg/mL). Except for cells bearing pUCBD, full induction of Lpp-OmpA-CBD was achieved by adding IPTG to 1 mM final concentration at an OD_{600} of 0.5. Cells were harvested 48 h after induction.

Cell Fractionation. Cell fractionation experiments were conducted as described (*15*). Briefly, cells were harvested by centrifugation, resuspended in 100 mM potassium phosphate buffer, pH 7.5, and lysed by passing through a French press operated at 20,000 psi. The lysates were centrifuged for 5 min at 1,500 × *g* to pellet any unbroken cells and then ultracentrifuged for 45 min at 115,000 × *g*. The membrane pellets were resuspended in potassium phosphate buffer.

Immunofluorescence Microscopy. Cells were harvested, washed, and resuspended in PBS/3%BSA buffer (OD_{600} = 0.5) containing rabbit anti-CBD_{cex} antibody (Novagen) diluted at 1:1000, and incubated at 4 °C for 4 h. After five washes with PBS buffer, the cell−antibody complex was incubated overnight at 4 °C with goat anti-rabbit IgG conjugated with FITC at a dilution of 1:3000. Prior to microscopy, cells were washed five times with PBS buffer, and then a photograph was taken using a fluorescence microscope.

Immunoblotting. A total of 200 μL of cells (OD_{600} = 1) was combined with 40 μL of disruption buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2%(w/v) SDS, 5% 2-mercaptoethanol, 0.05%(w/v) bromophenol blue) and boiled for 10 min. Samples were then electrophoresed through 12.5% (w/v) acrylamide SDS−PAGE gels prior to immunoblotting analysis. Immunoblotting was performed using a BioRad Immun-Blot GAR-AP kit (BioRad, Hercules, CA). The antibody against CBDcex was obtained from Novagen. Prestained low range protein markers (BioRad) were utilized for estimation of protein molecular weights.

Proteinase Accessibility. To demonstrate the surface localization of the Lpp-OmpA-CBD, a proteinase-K treatment was employed. Samples of XL1-Blue(pET38b) and XL1-Blue(pKCBD) (approximately 1 OD_{600} centrifuged and resuspended in 900 μL of 15% (w/v) sucrose; 15 mM Tris-HCl, pH 7.8) were incubated with 5 μL of 20 mg/mL proteinase-K for 60 min at 30 °C. Following the incubation, samples were used for Western blot analysis.

Whole-Cell Immobilization. Cells were harvested and resuspended in the following buffers: 150 mM carbonate-bicarbonate buffer, pH 9.5 (30% (v/v) 150 mM sodium carbonate and 70% (v/v) 150 mM sodium bicarbonate) and 150 mM citrate phosphate buffers at pH 4.0, 6.0, 7.0, or 8.0 (150 mM sodium phosphate dibasic titrated with 0.5 M citric acid to the respective pH), respectively. For each immobilization experiment, a piece (16 cm × 5 cm) of cellulose fabric (Bemliese 606 from Asahi Chemical Industry Co., Ltd., Japan) or Whatman filter paper (#1) was wrapped on a plastic frame of 4.7 cm diameter and merged in 150 mL of cell suspension at an OD_{600} of 0.2 in a 250-mL glass beaker. The apparatus was put on a stir plate, and the cell suspension was mixed at 200 rpm for 24 h at the appropriate temperature and pH as indicated. Cell suspension was sampled for protein content and wet weight determination before and after the incubation. The fabric was then carefully removed and washed in 150 mL of buffer of appropriate pH at the same conditions as immobilization for 24 h. After washing, 32 mL of the washing buffer was collected for protein content and wet weight measurements. Samples of cell suspension stirred at the same condition without the fabric was used to determine the spontaneous drop in cell density because of cell lysis.

Protein content of the cells was determined by centrifuging 5 mL of cell suspension at 16,000 × *g* and resuspending in 50 μL of buffer. A total of 425 μL of 0.1 N NaOH was then added, and the sample was heated at 80 °C for 10 min followed by 425 μL of 0.1 N HCl to neutralize and 100 μL of 0.5 M citrate phosphate pH 8 buffer to give a final volume of 1 mL. Then, 200 μL of the above solution was mixed with 800 μL of concentrated dye reagent (BioRad) and incubated for 5 min. Optical density at 595 nm was recorded and converted to protein concentration following the Bradford method.

The cell wet weight was determined by measuring the weight of the cell pellet recovered from 5 mL of cell suspension centrifuged at 16,000 × *g* in a preweighed empty eppendorf tube.

Results and Discussion

Regulated Expression of Lpp-OmpA-CBD. To regulate the level of surface-expressed CBD, a low copy number vector, pK184, was used. The *lpp-ompA-cbd* fragment was PCR amplified from pTX210 and subcloned into pK184 to give pKCBD. Expression of Lpp-OmpA-CBD can be easily induced by IPTG. Production of Lpp-OmpA-CBD was verified by immunoblotting with CBD_{cex} antiserum (Figure 1). The localization of Lpp-OmpA-CBD in the membrane fraction was also verified by immunoblotting (Figure 1A). The majority of the Lpp-OmpA-CBD fusion was detected in the membrane, while unfused CBD was detected only in the soluble fraction. The surface localization of CBD was demonstrated by immunofluorescence microscopy. Cells were probed with rabbit anti-CBD serum and then fluorescently stained with FITC-labeled goat anti-rabbit IgG antibody. As shown in Figure 2A, cells harboring pKCBD were brightly fluorescent, while control cells carrying only pK184 were not stained at all (Figure 2B). In addition, protease accessibility experiments were carried out to ascertain the percentage of CBD on the cell surface. Since proteinase K cannot readily diffuse across the cell membrane, degradation should only occur with CBD exposed on the surface. When cell suspensions were incubated with proteinase K for 1 h, the 30 kDa band corresponding to Lpp-OmpA-CBD disappeared, while no effect was observed with the intracellularly expressed CBD (Figure

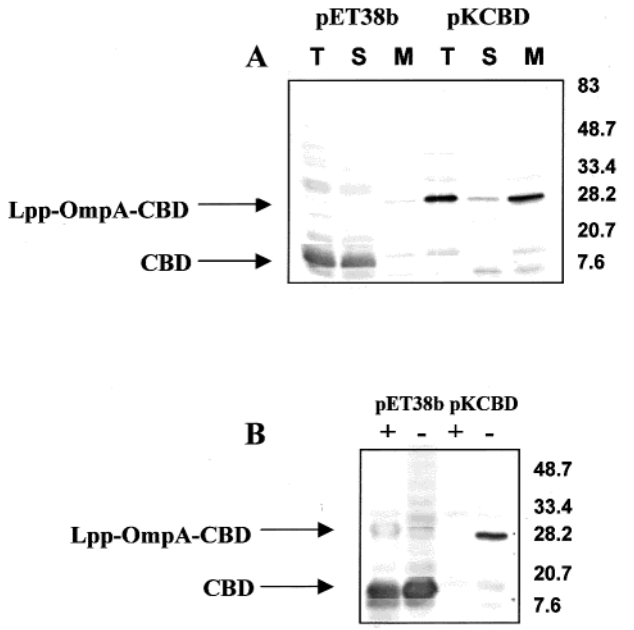

Figure 1. Western blot analysis of protein fractions from XL1-Blue(pET38b) and XL1-Blue(pKCBD). Anti-CBD antisera were used at 1:1000 dilution. (A) Total protein, soluble fraction, and membrane fraction are denoted T, S, and M, respectively. (B) Membrane fractions of samples with (+) or without (−) proteinase K treatment.

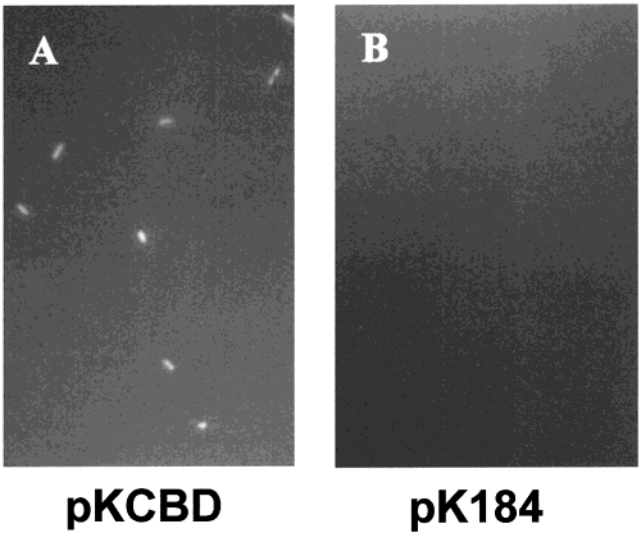

Figure 2. Immunofluorescence micrographs of *E. coli* XL1-Blue harboring (A) pKCBD and (B) pK184. Cells were probed with anti-OPH antisera and fluorescently stained with goat anti-rabbit IgG-FITC conjugate.

1B). From all these results, we concluded that the majority of CBD was indeed displayed on the cell surface using this low-copy number construct.

Whole-Cell Immobilization on Cellulose Supports. To determine the functionality of surface-exposed CBD, two different types of cellulose supports were used to demonstrate whole-cell immobilization via the surface-exposed CBD. Cell suspensions were incubated with either Whatman filter papers or cellulose fabrics at room temperature for 24 h. Subsequently, the supports were washed with citrate-phosphate buffer and whole-cell binding was observed with scanning electron microscopy (SEM). Control cells with no CBD on the surface showed virtually no binding to either support (Figure 3A and 3C),

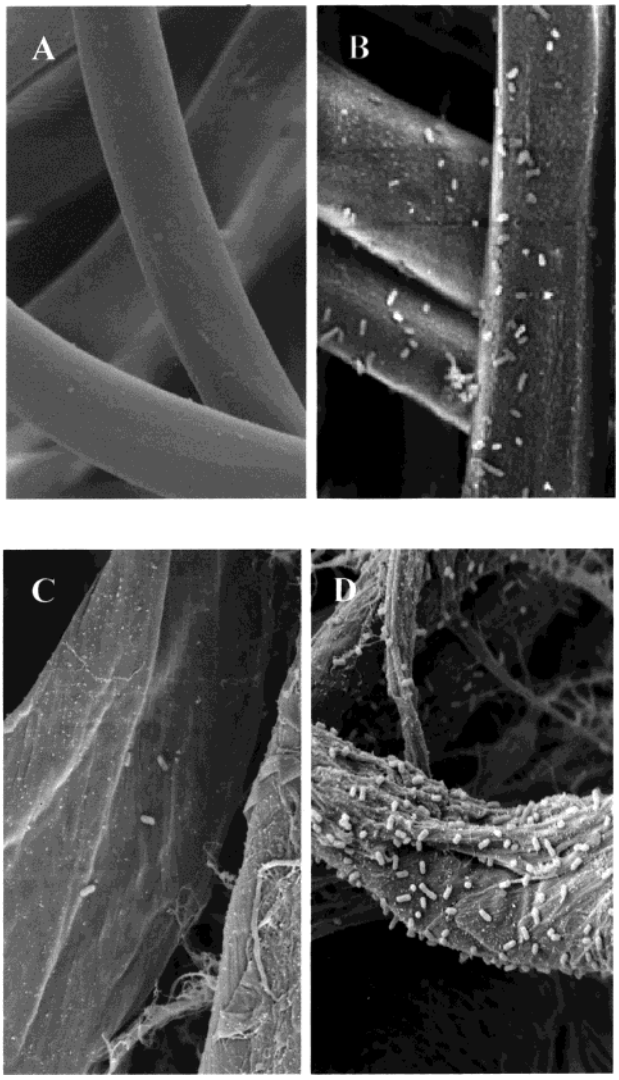

Figure 3. Scanning electron micrograph of cellulose supports from whole binding experiments. Cellulose fabric or Whatman filter paper was immobilized with (A, C) XL1-Blue(pK184) or (B, D) XL1-Blue(pKCBD).

whereas cells with surface-exposed CBD formed a monolayer on the supports (Figure 3B and 3D).

Effect of pH and Temperature on Whole-Cell Immobilization. The binding of CBD_{cex} to cellulose materials is affected by temperature (*11*), pH (*11*, *16*), etc. Previous studies employing CBD_{cex} as an affinity tag for enzyme immobilization have also shown the same influences (*17*). Although whole-cell immobilization via the action of surface-expressed CBD has been demonstrated, the precise conditions for optimal binding have not been elucidated. Our focus was to identify the optimal whole-cell immobilization conditions for future bioreactor applications.

Previous reports have shown that the binding of CBD_{cex} fusions is affected by pH, with optimal binding occurring around pH 7 (*17*). Binding decreases dramatically at higher pH. To investigate the effect of pH on cell binding, cells carrying pKCBD or pK184 were incubated with a 16 cm × 5 cm cellulose fabric at room temperature for 24 h at pH ranging from 6 to 9.5. After the support was carefully removed from the cell suspension, the amount of cells (based on total protein and wet weight measurements) bound to the support was measured. As shown in Figure 4, cells with CBD on the surface bound much stronger to the cellulose support than the control cells

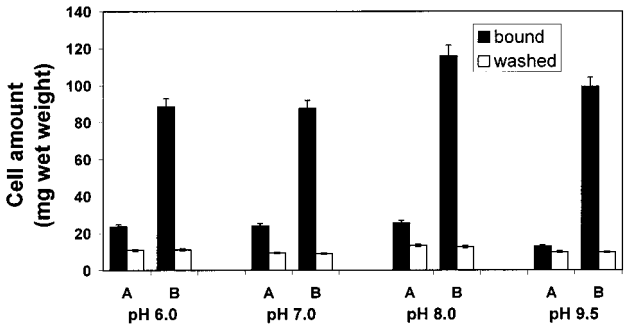

Figure 4. Whole-cell immobilization experiments at pH ranging from 6 to 9.5. The amount of cells immobilized or washed out was expressed as mg wet weight per support. Results are the average of four experiments: (A) XL1-Blue(pK184) and (B) XL1-Blue(pKCBD).

at all pH. The amount of non-specific binding decreased significantly at pH 9.5. Since most metabolic reactions have optimal pH higher than 6.0, our results suggest that surface-exposed CBD is ideal for providing specific adsorption of whole cells onto cellulose matrices under these preferred pHs. Specifically, one of the applications we are investigating with this technology is to prepare immobilized whole-cell biocatalysts for the detoxification of organophosphate pesticides. Organophosphorus hydrolase, the enzyme responsible for the detoxification has a pH optimum around 9 (*18*), a condition that strongly favors whole-cell immobilization with surface-exposed CBD.

Figure 5 shows the effect of temperature on the amount of cell bound to cellulose support through CBD-cellulose interaction. Cell binding was determined as before. For CBD_{cex} or CBD fusion enzymes, binding affinity has always been shown to be the highest at 4 °C. However, our results indicated that binding at 37 °C actually provided the highest whole-cell immobilization. This observation is unexpected, as even Francisco et al. (*12*) have reported their initial whole-cell immobilization experiments at 4 °C. Since our aim is to eventually operate the immobilized cell bioreactor at normal physiological conditions, these results suggest that 37 °C may be ideal not only for normal metabolic functions but also for whole-cell immobilization.

Stability of Immobilized Cells. Although cells are attached to the cellulose surface by the affinity interaction between CBD and cellulose, they are still susceptible to any shear or attrition that results from the relative motion of the support and fluid inside the bioreactor. To determine the stability of the immobilized cells, supports were subjected to extensive washing and the amount of cells washed out was used as an indicator of stability. As shown in Figures 3 and 4, around 60−90% of the immobilized cells harboring pK184 were removed from the support after 24 h of washing. In contrast, less than 10% of cells with surface-expressed CBD were removed. These results demonstrated that cells with surface-exposed CBD bound tightly to cellulose supports. This agrees very well with the properties of CBD_{cex}, which is known to bind cellulose very tightly and becomes dissociated only by denaturing conditions or incubation in low ionic strength buffers.

Cell Binding as a Function of Surface-Exposed CBD. Immobilized fusion enzymes through the action of CBD were found to leak from the support at a slow rate (*19*). This leakage could be overcome by using two CBDs in the fusion enzymes instead of one. Similarly, it can be expected that the number of CBD molecules anchored

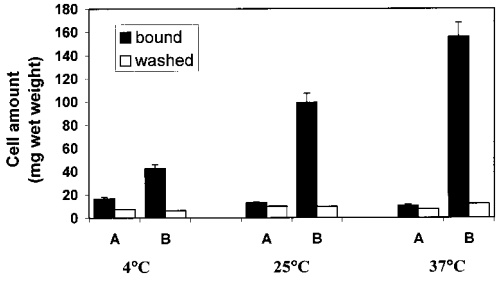

Figure 5. Whole-cell immobilization experiments at different temperatures. The amount of cells immobilized or washed out was expressed as mg wet weight per support. Results are the average of four experiments: (A) XL1-Blue(pK184) and (B) XL1-Blue(pKCBD).

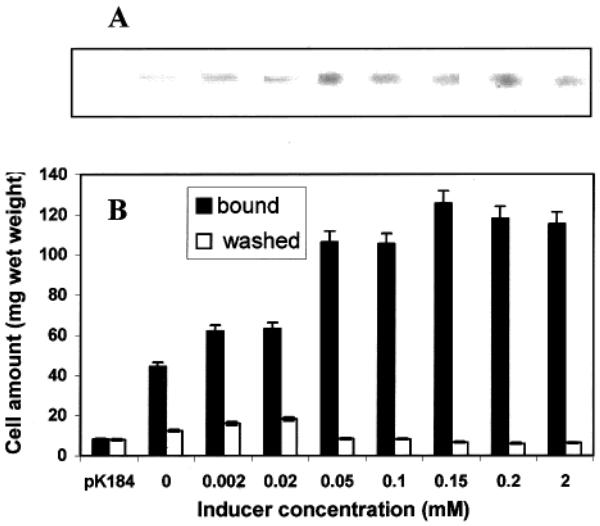

Figure 6. Effect of surface-exposed CBD on whole-cell immobilization. The level of surface-exposed CBD was varied by adding different amounts of IPTG. The amount of cells immobilized or washed out was expressed as mg wet weight per support. Results are the average of four experiments: (A) Western blot analysis for the production of Lpp-OmpA-CBD. (B) Whole-cell immobilized at different IPTG concentrations.

on the cell surface could also influence whole-cell binding to cellulose. To investigate this effect, a regulated expression system (pKQCBD) was employed to fine-tune the expression of CBD on the cell surface. As shown in Figure 6, low level expression of Lpp-OmpA-CBD was detected even with the noninduced cultures. Addition of as little as 0.002 mM of IPTG was sufficient to induce the production of a significant amount of Lpp-OmpA-CBD (Figure 6A). Clearly, the amount of CBD anchored on the surface has a direct correlation with cell immobilization. Whole-cell binding increased with higher CBD expression until binding was saturated. The stability of the immobilized cells was also affected by the amount of surface-exposed CBD. Close to 30% of the immobilized cells at low induction were removed after 24 h of washing, compared to only around 10% for cultures with higher induction. Since one of the major goals is to allow very tight cell binding without resulting in a significant metabolic burden on the cells, our result suggests that even very low level of induction may be sufficient to provide maximum cell immobilization efficiency.

Conclusions

E. coli cells with surface-exposed CBD were generated, enabling single-step immobilization onto different cellulose materials. The binding characteristics of whole

cells with surface-exposed CBD were comparable with those of the native CBD. The pH profile of whole-cell binding was similar to that of native CBD. In contrast, optimal whole-cell immobilization onto cellulose support was observed at 37 °C rather than 4 °C for the native CBD. Immobilized cells remained tightly bound even after extensive washing. In addition, other cellulose materials such as cellulose beads or sponges may also be used for whole-cell immobilization. Therefore, specific cell adhesion onto cellulose represents a very simple, mild, and inexpensive technique of immobilization for the preparation of whole-cell biocatalysts.

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