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Immobilization of Cells with Surface-Displayed Chitin-Binding Domain

Jen-You Wang and Yun-Peng Chao*

Department of Chemical Engineering, Feng Chia University, 100 Wenhwa Road, Taichung, Taiwan

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To explore chitin-binding domain (ChBD)-based cell immobilization, a tripartite gene fusion consisting of an in-frame fusion of ChBD to *lpp* and *ompA* was constructed and expressed in *Escherichia coli*. ChBD-displayed cells exhibited highly specific and stable binding to chitin within a wide range of pHs (5 to 8) and temperatures (15 to 37°C). These results illustrate the promising use of this approach for engineering applications.

Active immobilization of cells on inert supports enables facilitation of segregation of cells from the aqueous phase and recovery of cells after a bioreaction is completed. To achieve cell immobilization, entrapment of cells within porous matrices appears to be the most applicable method. The polymers commonly used in such a preparation include agar, alginate, κ-carrageenan, polyacrylamide, and chitosan (1). Alternatively, cell immobilization could be achieved by adsorption of cells on the support surface. This approach allows direct exposure of immobilized cells to substrates. However, the former method is frequently made difficult by the problem of limited transport of substrates and the mechanical fragility of materials, while the latter has difficulty in stably retaining cells in the case of environmental changes, particularly in the presence of hydrodynamic shear.

Recombinant DNA technology allows fusion of a protein with an affinity tag to endow it with the ability for specific binding to an unnatural cognate ligand. This approach has received wide acceptance on the basis of the following merits: strong and reversible binding of enzymes to the support, proper exposure of active domains of immobilized enzymes, mild adsorption conditions, and the lack of diffusion constraints (14). A broad spectrum of fusion tags have been explored (16), and the selection of an affinity tag appropriate for use remains biased. Among the peptide tags previously developed, FLAG, poly-His, c-*myc*, and glutathione *S*-transferase are the most commonly used. However, reports on the use of these peptides for cell immobilization are virtually absent, probably due to the economic infeasibility of their corresponding ligand materials. The cellulose-binding domain (CBD) appears to be an attractive alternative because of the low cost and availability of the binding matrix, cellulose. Serving as a fusion tag, CBD has found extensive application in the field of biotechnology (11). In particular, the anchorage of whole cells on cellulosic materials was first demonstrated with *Escherichia coli* cells, whose surfaces were displayed with CBD (7). It was later shown that the adherence of CBD-exposed *E. coli* cells to cellulose was very specific and stable over a wide range of pHs and temperatures (17).

* Corresponding author. Mailing address: Department of Chemical Engineering, Feng Chia University, 100 Wenhwa Road, Taichung, Taiwan. Phone: 886 2451 7250 3677. Fax: 886 4 2451 0890. E-mail: ypchao@fcu.edu.tw.

The chitin-binding domain (ChBD) of chitinase A1 from *Bacillus circulans* WL-12 is a small peptide consisting of 45 amino acids. Isolated ChBD is found to assume a tightly packed structure and exhibits notable integrity (9). ChBD binds very specifically to insoluble chitin via a hydrophobic interaction, and bound ChBD can be liberated by controlling pH (9). These remarkable features of ChBD have made it appealing for versatile applications. Apart from this, the binding matrix, chitin, is the most abundant naturally existing polysaccharide from the cell wall structure of fungi and the exoskeletons of invertebrates (21). From the perspectives of economics and technology, a useful method of cell immobilization could be explored by exploitation of ChBD-exposed cells. Therefore, an attempt was made to develop ChBD-based immobilization of cells in this study.

The usefulness of the Lpp-OmpA hybrid has been well recognized to direct heterologous polypeptides to the cell surface (6). To stably present ChBD outside of the cell, plasmid pLOA-ChBD was constructed to carry the in-frame fusion of ChBD to *lpp* and *ompA* (*lpp-ompA*-ChBD) under the control of the T7 promoter as follows. With the aid of PCR, the DNA containing *lpp-ompA* was amplified with two oligomers (ATT ACATATGAAAGCTACTAAACTGGTAC and CATGAAT TCCCATGGGTTGTCCGGACGAGTGC) by priming plasmid pSD192 (provided by G. Georgiou). The resulting PCR product was cleaved by NdeI and EcoRI and spliced into plasmid pET-20bI (19) to obtain plasmid pLOA. By PCR, ChBD was obtained from the genome of *Bacillus circulans* WL-12 with the primer set GCAAAGCTTGGCCTGACCGG TCTGAAC and TTCCTCGAGCCCGGGTTGAAGCTGCC ACCAGGCAG. After being predigested with HindIII-XhoI, the PCR-amplified ChBD and plasmid pLOA were ligated together to give plasmid pLOA-ChBD. Subsequently, plasmid pLOA-ChBD was transformed into *E. coli* strain BL21(DE3), and one transformant conferring ampicillin resistance was scored and designated BL21(DE3)/pLOA-ChBD. In a similar manner, strain BL21(DE3)/pLOA was constructed to serve as a control.

The shake-flask culture of the plasmid-bearing strains was carried out in Luria-Bertani (LB) medium (12) supplemented with ampicillin (50 μg/ml). The cell culture was maintained in an orbital shaker operated at 200 rpm and 30°C, and cell growth was monitored turbidimetrically by the optical density at 550 nm (OD_{550}). A cell density of 1 at OD_{550} was deter-

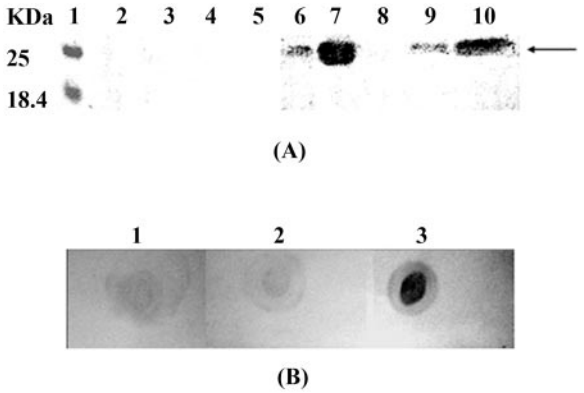

FIG. 1. (A) Western blot analysis of cellular proteins from strains BL21(DE3)/pLOA and BL21(DE3)/pLOA-ChBD. Plasmid-bearing *E. coli* strains were cultured and induced by IPTG to initiate the production of the fusion proteins Lpp-OmpA and Lpp-OmpA-ChBD, respectively. At the end of cultivation, cells were harvested by centrifugation and subsequently disrupted by sonication. After centrifugation, the supernatant was removed as the soluble fraction and the insoluble fraction was obtained from the precipitate. A further treatment of insoluble fraction as described previously (17) gave the membrane fraction. Immunoblotting was carried out by first analyzing the fractioned proteins with sodium dodecyl sulfate-polyacrylamide gel electrophoresis conducted on 12% acrylamide gels as described previously (3). After electrophoresis, protein spots were blotted onto nitrocellulose membranes using a Bio-Rad electrophoretic transfer cell and then subjected to hybridization with a rabbit ChBD-specific antibody (New England Biolabs) at a 1:1,000 dilution for 3 h. After two washes, the horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) (Sigma) was administered for 3 h. Finally, another two washes were applied, and the addition of 4-chloro-1-naphthol allowed the HRP-mediated color change to develop. Lane 1, protein marker; lane 2, soluble fraction of the IPTG-induced strain BL21(DE3)/pLOA; lane 3, soluble fraction of strain BL21(DE3)/pLOA-ChBD; lane 4, soluble fraction of the IPTG-induced strain BL21(DE3)/pLOA-ChBD; lane 5, insoluble fraction of the IPTG-induced strain BL21(DE3)/pLOA; lane 6, insoluble fraction of strain BL21(DE3)/pLOA-ChBD; lane 7, insoluble fraction of the IPTG-induced strain BL21(DE3)/pLOA-ChBD; lane 8, membrane fraction of the IPTG-induced strain BL21(DE3)/pOLA; lane 9, membrane fraction of strain BL21(DE3)/pLOA-ChBD; lane 10, membrane fraction of the IPTG-induced strain BL21(DE3)/pLOA-ChBD. The arrow indicates the position of Lpp-OmpA-ChBD. (B) Colony blotting of strains BL21(DE3)/pLOA and BL21(DE3)/pLOA-ChBD. As modified from the previous report (7), one drop of cell equilibrant (OD_{550} of 10) was layered on nitrocellulose membranes. Subsequent incubation with anti-ChBD antibodies was conducted for 1 h and followed by a gentle wash. After that, the color development mediated by HRP was carried out by mixing the anti-rabbit IgG and 4-chloro-1-naphthol. Panel 1, IPTG-induced strain BL21(DE3)/pLOA; panel 2, strain BL21(DE3)/pLOA-ChBD; panel 3, IPTG-induced strain BL21(DE3)/pLOA-ChBD.

mined to correspond to 1.16 mg of wet cell weight (WCW) per ml. To induce the production of the fusion protein within cells, 50 μM isopropyl-β-D-thio-galactopyranoside (IPTG) was added to culture broth when cell density reached 0.46 mg WCW/ml. After induction for 6 h, cells were harvested by centrifugation for further experiments. The location of ChBD was identified by immunoblotting with anti-ChBD serum, and the result revealed the presence of ChBD predominantly in the insoluble fraction and the outer membrane of the IPTG-induced strain BL21(DE3)/pLOA-ChBD (Fig. 1A). A similar result was also obtained for this strain without receiving IPTG, but very faint signals were detected. In contrast, this method gave no indication of ChBD in the induced control strain, BL21(DE3)/pLOA. These results suggest controllable expression of *lpp-ompA*-ChBD in response to IPTG and the potential for targeting ChBD to the outer membrane. A further analysis by colony blotting showed that the color was developed around the induced strain BL21(DE3)/pLOA-ChBD, whereas the color became absent for the uninduced counterpart and the induced control strain (Fig. 1B). After being pretreated with trypsin (10 μg/ml) for 1 h at 30°C, the induced strain BL21(DE3)/pLOA-ChBD, subjected to the same blotting analysis, became free of color (data not shown). Owing to the unlikely diffusion of antiserum and trypsin into the cells, this result indicates the proper assembly of Lpp-OmpA on the cell envelope to which ChBD was attached and consequently protruded from the cell surface.

To examine the functionality of ChBD-exposed cells, chitin beads and chitin flakes were chosen as a binding matrix for demonstration of cell immobilization. Chitin flakes (Ly Tone Co., Taiwan) were shaped into squares with a dimension of 0.5 by 0.5 cm and immersed in HCl (6 N) solution for 30 min. This was followed by being washed with deionized water, and then the chitin flakes were incubated in NaOH (5 N) and heated in boiled water for 30 min. Subsequently, chitin flakes were neutralized by extensive washing with deionized water. To perform whole-cell immobilization, 2 g of chitin flakes was added to a glass beaker containing 20 mg WCW of cells suspended in the adsorption buffer (20 ml). At pH 7.0 (20 mM sodium phosphate buffer) and 4°C, the cell slurry was stirred at 4°C for 24 h. After the removal of chitin, the remaining cells in the solution were then determined by measuring their WCW in a preweighed empty Eppendorf tube by recording the weight gained after centrifugation or by turbidimetric measurement. To account for cell loss due to lysis, cells treated under the same condition without chitin were determined. Alternatively, 1-ml chitin beads (New England Biolabs) were first spun down and washed with a 20-fold volume of adsorption buffer. Another centrifugation was applied to harvest chitin beads followed by soaking in 1 ml adsorption buffer. Subsequent addition of 10 ml sodium phosphate buffer (pH 7) containing 20 mg WCW of cells allowed the adsorption reaction to proceed at 4°C. The incubation time lasted for 24 h, during which the mixture was occasionally stirred. Finally, the chitin beads thus treated were gathered by centrifugation and rinsed with a 20-fold volume of the same buffer. As a result of the proceeding of an adsorption reaction, recovered chitin in either form was washed with adsorption buffer and then observed by variable vacuum scanning electron microscopy (VVSEM). This showed that a monolayer of ChBD-displayed cells attached to the surface of chitin beads, whereas the sporadic presence of cells without ChBD was visualized (Fig. 2). A similar result was obtained with chitin flakes (data not shown).

The stability of immobilized cells was further tested. After adsorption, the recovered chitin flakes were washed and subsequently placed in a glass container containing the adsorption buffer (pH 7.0). Chitin dispersed in the container was continuously stirred at room temperature, and liquid solution was carefully withdrawn at 12-h intervals to determine the WCW of desorbed cells or the amount of cells released by turbidimetric measurement. No liberated cells from chitin were detected

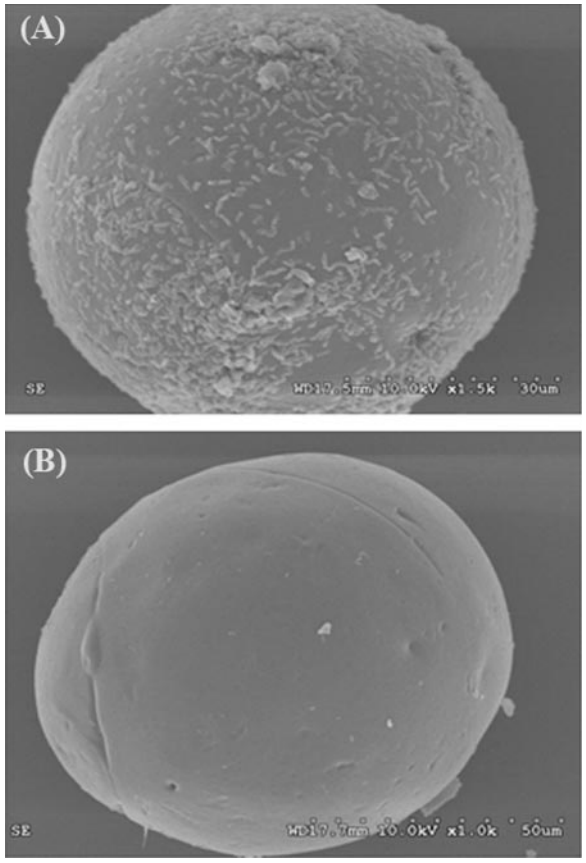

FIG. 2. Electron micrographs of chitin beads used for cell immobilization. Refer to the text for details. Strains BL21(DE3)/pLOA (control) and BL21(DE3)/pLOA-ChBD were cultured and induced for hybrid protein production. Subsequently, the induced cells and the induced strain BL21(DE3)/pLOA-ChBD, treated with trypsin, were prepared for the adsorption experiment, and the result was visualized by VVSEM. The trypsin-treated strain BL21(DE3)/pLOA-ChBD gave a cell binding result similar to that of the control strain (data not shown). (A) IPTG-induced strain BL21(DE3)/pLOA-ChBD. (B) IPTG-induced strain BL21(DE3)/pLOA.

during 72 h of incubation, indicating the strong association of ChBD-exposed cells with chitin.

As reported previously (9), the binding of ChBD to chitin resides mainly in hydrophobic interactions. Therefore, the efficiency of this cell binding appears to be affected by many environmental factors. A first step to investigate these determinants was taken to examine the effect of pH on cell immobilization. Cell fixation was carried out in a similar fashion by incubating the IPTG-induced BL21(DE3)/pLOA-ChBD cells along with chitin flakes in a buffer solution of the indicated pH. After adsorption for 24 h at 4°C, chitin flakes were removed and washed with the adsorption solution and the unadsorbed cells remaining in the solution were then determined by the WCW and turbidimetric measurement. As depicted in Fig. 3A, the ChBD-expressed cells bound most strongly at pH 7.0 while the number of adsorbed cells on chitin dropped dramatically as pH exceeded 9. In general, conditions were favorable for the adsorption of ChBD-exposed cells to chitin at neutral pH and became unfavorable at pH close to alkalinity. Meanwhile, at various concentrations of sodium phosphate buffer (pH 7.0), adsorption of cells appeared to reach saturation in buffer solution of 20 to 100 mM. As in water, the binding force of cells turned out to be relatively weak in lower concentrations of buffer (Fig. 3B).

In addition, the dependence of cell immobilization on adsorption temperatures was studied. The mixtures of cells and chitin flakes were incubated at pH 7.0 and various temperatures for 24 h. Upon the end of adsorption, the determination of unbound cells indicated a higher affinity of cells to chitin between 25 and 37°C (Fig. 4A). Moreover, it took 24 h for cells to achieve saturated attachment (Fig. 4B).

In contrast to conventional methods, the display of affinity tags on the cell surface is an easy, reversible, direct, and relatively stable way to achieve cell immobilization. This approach was pioneered by demonstrating the anchorage of the CBD-displayed cells on cellulose (7) and found a useful application for detoxification of organophosphate (18). Like the function of CBD, ChBD plays a role in assisting the specific binding of chitinase A1 to chitin, which in turn leads to the hydrolysis of chitin (20). Owing to superior integrity and controllability of ChBD, the first illustration of ChBD as an affinity tag was reported to facilitate on-column purification of proteins in conjunction with the protein splicing element intein (4). In this study, we have placed our emphasis on the development of ChBD-based cell immobilization. To direct ChBD to the cell surface, a T7 expression system-based plasmid, pLOA-ChBD, was constructed by joining ChBD to the anchor motif consisting of *lpp-ompA*. This dual gene fusion comprises the signal sequence of lipoprotein linked to a transmembrane domain from outer membrane protein A (6). As analyzed by immunoblotting and colony blotting (Fig. 1), ChBD could be efficiently directed and displayed on the outer surface of IPTG-induced cells with the aid of *lpp-ompA*. However, like a problem commonly encountered in the T7 expression system (2), the leakiness of plasmid pLOA-ChBD resulted in weak detection of ChBD in the cells without receiving induction (lanes 6 and 9 in Fig. 1A). This detection signal was entirely absent from the same strain bearing plasmid pLOA, which contained no ChBD.

Furthermore, an adsorption process was designed to permit the attachment of ChBD-exposed cells to chitin. Taken by VVSEM, a micrograph disclosed the monolayer coverage of ChBD-displayed cells on the surface of chitin beads (Fig. 2). This indicates the successful resumption of active ChBD structure outside the cells aided by *lpp-ompA*. As reported by the previous study (9), ChBD from chitinase A1 exhibits highly specific binding toward chitin in a soluble or crystalline form but shows no preference for soluble derivatives of chitin such as chitosan. According to the manufacturer (New England Biolabs), chitin beads were initially prepared from the emulsified chitosan and subsequently acetylated to chitin. Therefore, the "bare" region of chitin bead surface as visualized from Fig. 2 may be indicative of deacetylation degree (DD). With the aid of the spectrophotometric measurement (10), the DD of chitin beads was found to reach 18%. As prepared previously (8, 10), the DDs of various chitinous materials were also examined, with 8% found for colloidal chitin (PCI, Japan) and 22% found for chitin flakes. Under similar adsorption conditions (pH 7 and 4°C), ChBD-displayed cells showed high binding to chitinous substrates such as colloidal chitin, chitin beads,

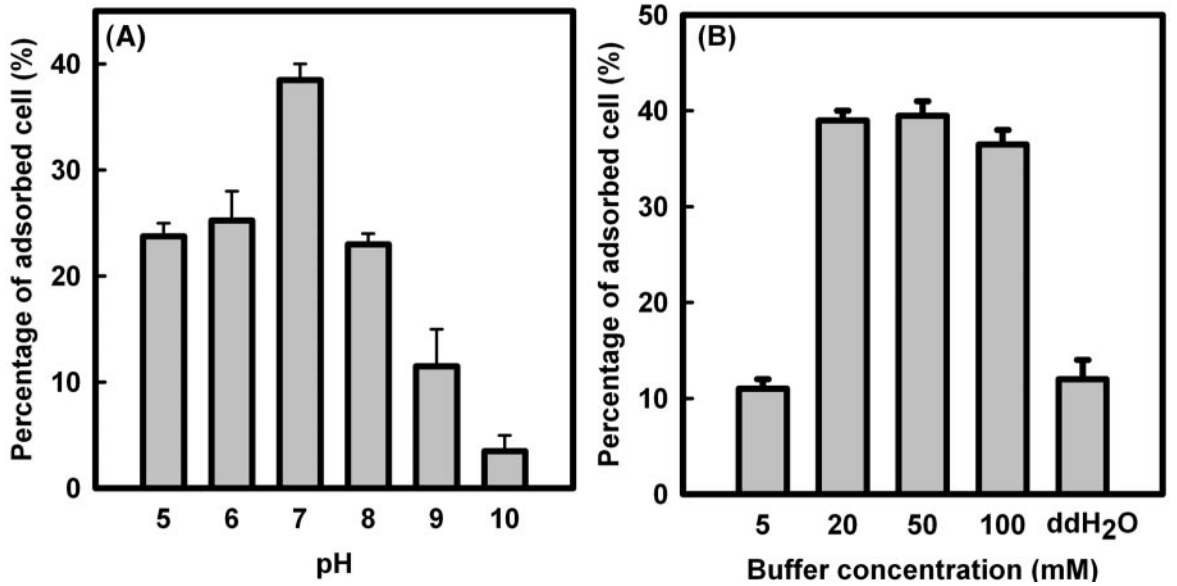

FIG. 3. (A) Dependence of cell immobilization on pH. The binding of the IPTG-induced strain BL21(DE3)/pLOA-ChBD to chitin was carried out at 4°C for 24 h in 20 mM buffer solution at the pHs indicated. After the termination of adsorption, the removed chitin received an extensive wash, and the number of cells remaining in the solution was then determined. The amount of WCW or OD thus measured relative to that before adsorption was expressed as the percentage of cells adsorbed. The adsorption solution used here included sodium citrate buffer (pH 5 and 6), sodium phosphate buffer (pH 7), Tris-HCl buffer (pH 8), and glycine-sodium hydroxide buffer (pH 9 and 10). (B) Effect of buffer concentration on cell binding. In a similar fashion, cells were adsorbed to chitin in sodium phosphate buffer (pH 7.0) at various concentrations. For parallel comparison, the pH of double-distilled water (ddH_2O) was adjusted to 7.0. The data shown represent three independent experiments.

and chitin flakes and the percentages of adsorbed cells were 92%, 61%, and 53%, respectively. Although chitin beads and chitin flakes have a similar DD, the former exhibits higher adsorption efficiency—probably due to the likely heterogeneity of the latter (see below). In addition, less than 1% of ChBD-containing cells could be adsorbed on cellulose (Chrom Tech, Taiwan) and agarose (Cambrex, Rockland, ME), the nonchitinous substrates.

To search for the optimal condition of cell adsorption, chitin flakes were used instead because they could be easily separated

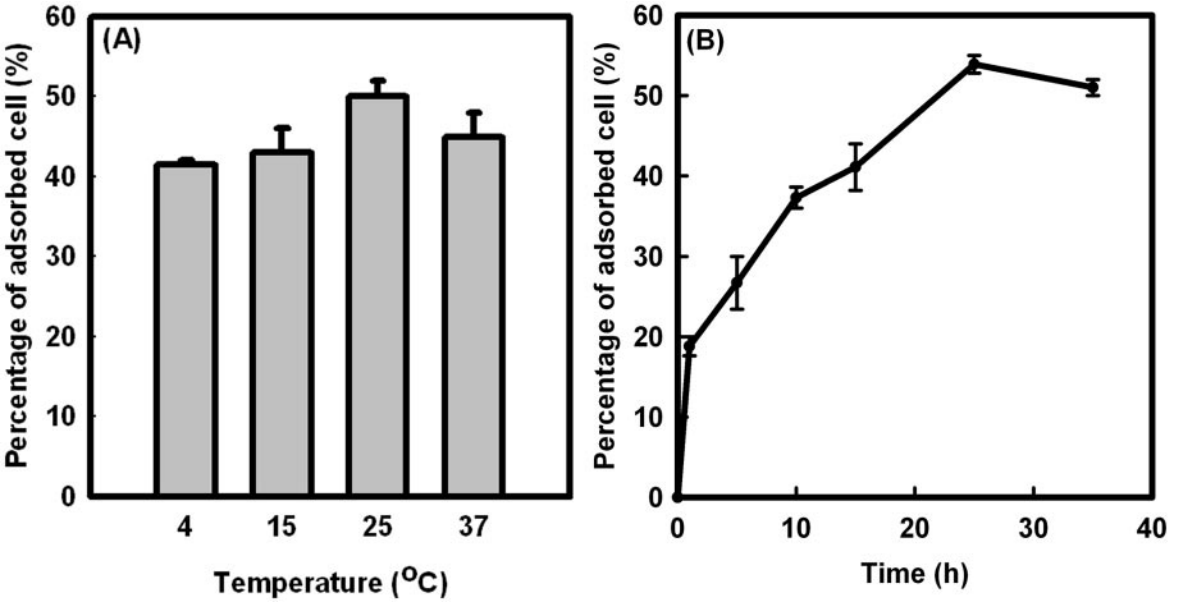

FIG. 4. (A) Dependence of cell immobilization on temperature. Similar to the adsorption conditions described in the legend to Fig. 3, cell binding was performed in 50 mM sodium phosphate buffer (pH 7.0) at various temperatures for 24 h. (B) Effect of adsorption time on cell binding. The adsorption conditions remained unchanged, although the temperature was kept at 4°C for various times. The data were adopted from three independent experiments.

from the aqueous phase by brief centrifugation. As prepared from crude extracts of shrimp shell, chitin flakes must be carefully pretreated, as described here, for the removal of contaminants to ensure their best performance. In a survey of pH-dependent cell adsorption, ChBD-displayed cells exhibited a better binding activity under the acidic condition and the maximal adsorption appeared at neutral pH (Fig. 3A). In contrast, ChBD was reported to display the highest binding at pH 9, and its binding activity could be improved at pH 6 in the presence of NaCl (9). In this case, ChBD-displayed cells were prone to lysis for unknown reasons at higher pHs. Together with this phenomenon, the potential of nonspecific charge interactions between cell surface and chitin might contribute to such a shift in pH dependence of cell binding. Indeed, we found that higher concentrations of the buffer solution were beneficial for cell adsorption (Fig. 3B). This is in agreement with a previous result and supports the expectation that hydrophobic interactions play a major role in the adsorption of ChBD (8). Moreover, cell viability was not affected by this immobilization approach. The investigation concluded that 1 g of chitin flakes after cell adsorption at various pHs was recovered and incubated in 10 ml of LB medium at 30°C. The cell density was increased at a rate characteristic of the free cell counterpart after a time lag. The lag phase could be avoided if a transfer of cells freshly immobilized on chitin to solution was conducted.

The presence of more ChBD molecules on the cell surface appears to support the binding of cells to chitin. Should the subject of optimization for cell binding be addressed, this issue obviously deserves further discussion. The question that then follows is how many displayed ChBD molecules would be sufficient (or optimal)? An unequivocal answer here seems unlikely. However, to find out the solution, a prerequisite is to have a more reliable and delicate method for the detection of individual ChBD molecules. Moreover, this requires a homogenous population of cells upon induction, and this is particularly relevant to the topic of engineering. As proposed in a related report, cell homogeneity and cell viability, in essence, are the keys to achieve optimization of preserving a diversity of surface-displayed libraries (5). However, widespread among the most commonly employed promoters such as *lac* and *araBAD* promoter (13, 15), the phenomenon known as an "autocatalytic" induction mechanism (13) describes a perceived situation in which the cell population consists of induced and uninduced cells at subsaturating concentrations of inducers. Obviously, analogous to the *lac* promoter, the expression vector developed in this study has the same phenomenological consequence. Therefore, to eliminate cell heterogeneity and achieve optimization, the application of the developed system in a *lacY*-deficient strain is necessary (13).

In conclusion, as illustrated in this study, the approach using ChBD-based cell immobilization is marked by high stability as well as simplicity and is applicable to a wide range of pHs and temperatures. This is the first example illustrating the feasibility of fixing cells with the surface display of ChBD on chitin, and it clearly opens up a new route in bioprocess engineering.

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