Applied Usage of Yeast Spores as Chitosan Beads

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In this study, we present a nonhazardous biological method of producing chitosan beads using the budding yeast *Saccharomyces cerevisiae*. Yeast cells cultured under conditions of nutritional starvation cease vegetative growth and instead form spores. The spore wall has a multilaminar structure with the chitosan layer as the second outermost layer. Thus, removal of the outermost dityrosine layer by disruption of the DIT1 gene, which is required for dityrosine synthesis, leads to exposure of the chitosan layer at the spore surface. In this way, spores can be made to resemble chitosan beads. Chitosan has adsorptive features and can be used to remove heavy metals and negatively charged molecules from solution. Consistent with this practical application, we find that spores are capable of adsorbing heavy metals such as Cu²⁺, Cr³⁺, and Cd²⁺, and removal of the dityrosine layer further improves the adsorption. Removal of the chitosan layer decreases the adsorption, indicating that chitosan works as an adsorbent in the spores. Besides heavy metals, spores can also adsorb a negatively charged cholesterol derivative, taurocholic acid. Furthermore, chitosan is amenable to chemical modifications, and, consistent with this property, dIT1Δ spores can serve as a carrier for immobilization of enzymes. Given that yeast spores are a natural product, our results demonstrate that they, and especially dIT1Δ mutants, can be used as chitosan beads and used for multiple purposes.

Chitosan is a linear polysaccharide composed of β-1,4-linked D-glucosamine. Because of the positive charges on its amino groups, chitosan can attract negatively charged molecules. Furthermore, chitosan has a chelating ability, so the polymer also can adsorb transition metal ions. Besides these adsorptive properties, the amino groups as well as some hydroxyl groups are chemically reactive, which makes chitosan amenable to chemical modification (1, 2). Based on these unique properties, chitosan has been used in a variety of fields, including water purification and the food, chemical, and medical industries (3–5). Chitosan is industrially produced by chemical deacetylation of chitin, primarily that derived from crustacean shell waste. However, this method presents several challenges, including the potentially unstable supply of raw materials and production of harmful waste. Thus, chemoenzymatic and biological strategies have been investigated as alternative methods for chitosan production (3, 6).

In the budding yeast *Saccharomyces cerevisiae*, chitosan is specifically used as a component of the spore wall and does not exist in the vegetative cell wall (7). Yeast sporulation is a developmental program which is triggered by nutrient starvation (8). Through this process, four haploid spores are created within a diploid mother cell so that the mother cell plasma membrane becomes the ascospore membrane (9). The spore wall consists of four layers (10) composed (from the inside out) of mannoprotein, β-glucan, chitosan, and dityrosine layers (7, 11). β-Glucan and mannoproteins are shared components of both the spore and vegetative cell walls, whereas the chitosan and dityrosine layers are structures unique to the spore wall (12). Compared to vegetative cells, yeast spores are more resistant to environmental challenges. The stress resistance properties of the spore depend largely on protective features of the outer two layers (13).

The spore wall is assembled *de novo* in a sequential manner such that chitosan layer formation is followed by dityrosine deposition (14). If the chitosan layer is absent or severely defective, the dityrosine layer is not assembled, though spores are viable even in the absence of the outer two layers (15, 16). In sporulation, chitosan synthesis involves two steps: first, chitin is synthesized by the chitin synthase, Chs3 (15), and then the N-acetylglucosamine residues of chitin are deacetylated by the sporulation-specific chitin deacetylases Cda1 and Cda2 to produce chitosan (17–19). Synthesis and deposition of dityrosine require three genes: DIT1, DIT2, and DTR1 (14, 20, 21). DIT1 is involved in the first step to produce dityrosine from L-tyrosine in the cytosol, and its deletion causes a lack of the dityrosine layer (22).

For practical uses of chitosan, beads are a convenient form that makes use of the polymer’s adsorptive and chemically reactive features, and thus, various chemical methods have been developed for the production of chitosan beads (23). Yeast spores, which contain chitosan, exhibit a bead-like morphology, consisting of spores ~3 μm in diameter. Although the spore’s chitosan layer is covered with the dityrosine layer, it is possible to prevent dityrosine deposition by genetic manipulations. Thus, in this study, we attempted to use yeast spores as chitosan beads.

**MATERIALS AND METHODS**

**Yeast strains and growth media.** Unless otherwise noted, standard media and genetic techniques were used (24). Yeast strains used in this study are listed in Table 1. AN120 was used as the wild-type strain and has been deposited in The Culture and Information Centre of Industrial Microorganisms of China Universities (CICIM; accession number Y0702). HW3 (dIT1Δ/dIT1Δ) was constructed as follows: a DNA fragment for *DIT1* disruption was generated by PCR using pFA6a-HIS3MX6 (25) as the template and HXO34 (ATTTTGTAAATATCCCTATTCGGTAAAGTCATTTGT

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TABLE 1. S. cerevisiae strains used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Reference and/or source</th>
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</thead>
<tbody>
<tr>
<td>AN120 (wild type, diploid)</td>
<td>MATα MATα ARG4/arg4-Nsp1 his3ΔSK/his3ΔSK hо/LYS2/hо/LYS2 leu2/leu2</td>
<td>43; CICIM Y0702</td>
</tr>
<tr>
<td>AN117–4B</td>
<td>MATα ura3 leu2 trp1 his3ΔSK arg4-Nsp1 lys2 hо/LYS2 met15::LEU2</td>
<td>43</td>
</tr>
<tr>
<td>AN117–16D</td>
<td>MATα ura3 leu2 trp1 his3ΔSK hо/LYS2</td>
<td>43</td>
</tr>
<tr>
<td>AN162</td>
<td>MATα MATα ARG4/arg4-Nsp1 his3ΔSK/his3ΔSK hо/LYS2/hо/LYS2 leu2/leu2</td>
<td>44</td>
</tr>
<tr>
<td>HW3 (dit1Δ/dit1Δ)</td>
<td>MATα MATα ARG4/arg4-Nsp1 his3ΔSK/his3ΔSK hо/LYS2/hо/LYS2 leu2/leu2</td>
<td>This study</td>
</tr>
<tr>
<td>HW143 (crr1Δ/crr1Δ)</td>
<td>MATα MATα ARG4/arg4-Nsp1 his3ΔSK/his3ΔSK hо/LYS2/hо/LYS2 leu2/leu2</td>
<td>This study</td>
</tr>
<tr>
<td>HW146 (cda1Δ cda2Δ/cda1Δ cda2Δ)</td>
<td>MATα MATα ARG4/arg4-Nsp1 his3ΔSK/his3ΔSK hо/LYS2/hо/LYS2 leu2/leu2</td>
<td>This study</td>
</tr>
</tbody>
</table>

CGAGACATTTAAGAAACGGATCCCCGGTTAATTTAA) and HX035 (TGT GTTAAGTAAAAAGGATCAGACATTTAGCGCTCCTAC TTGAGAATTCGACAGGTTGTTAAAC) as primers. The resulting PCR fragment was integrated into haploid AN117–4B and AN117–16D cells, and the resulting strains were mated to generate the diploid dit1Δ dit1Δ disruptant. Other mutants were constructed in the same way. For deletion of CDA1, CDA2, and CRR1, the primer pairs Cda1-F (CTTCTTAATTAGAATTTACCTTTCTAAATTAGAGGAACATCGGTGCTTTAAC), HX0518 (CTTTTTAAATTGTAATTAATTCATAAGAGATCAAAGATATATAGAGAAACACTTGAATTC CTCCATTTTTCTTCAATCCTGCCGAAGAAATTCGACAGGTTGTTAAAC) and Cda1-R (TGGAGGTTGAAATTTACCTTTCTAAATTAGAGGAACATCGGTGCTTTAAC) were used to generate knockout cassettes, respectively. pFA6a-TRP1, pFA6a-HIS3MX6, and pFA6a-kanMX6 (25) were used as templates to disrupt CDA1, CDA2, and CRR1, respectively. The cda1Δ cda2Δ double mutant was constructed beginning with cda1Δ haploid cells. The crr1Δ crr1Δ and cda1Δ cda2Δ/cda1Δ cda2Δ strains were designated HW143 and HW146, respectively.

Yeast sporulation and spore purification. For sporulation, yeast cells derived from a single colony were grown in 5 ml of YPAD (1% yeast extract, 2% peptone, 30 mg/liter adenine, 2% glucose) medium overnight. A 2-ml portion of the culture was then shifted to 100 ml of YPA (1% yeast extract, 2% peptone, 2% potassium acetate) and grown overnight. The cells were harvested by centrifugation, washed with water, resuspended in 2% potassium acetate medium at a concentration of 3 × 10⁸ cells/ml, and cultured for at least 24 h. Sporulation efficiency was determined under the microscope, and cultures with efficiencies greater than 90% were used for further processes.

To remove spores from asc, the asc wall was first digested with lyticase (Sigma-Aldrich, Shanghai, China). For this purpose, asc were resuspended in 2 ml of water and treated with lyticase (625 U/gram cells) at 37°C for 1 h with shaking at 250 rpm. The asc membrane was then disrupted by sonication.

Spores were purified by Percoll gradient centrifugation based on a previously described method (26). Spores were washed three times with 0.5% Triton-X. After the washing, the resulting pellet was resuspended in 1 ml of 0.5% Triton-X and layered on top of Percoll (Sigma-Aldrich, Shanghai, China) gradients (50 to 80% Percoll, 10% 2.5 M sucrose, and 0.5% Triton-X). After centrifugation at 15,000 × g at 4°C for 1 h, the top three layers, consisting of vegetative cells and debris, was removed. The remaining spore layer was washed with 0.5% Triton-X and freeze-dried. Purified spores were freeze-dried as follows. First, spores were frozen in a −20°C freezer for more than 2 h. They were then freeze-dried with an YELEA FD-1000 freeze dryer (Tokyo Rikakikai, Tokyo, Japan) at −50°C for 72 h under a pressure of 25 Pa.

Heavy metal adsorption assays. CuSO₄, Cr(NO₃)₃, Ni(NO₃)₂, Zn(NO₃)₂, Cd(NO₃)₂, and Pb(NO₃)₂ were prepared in deionized water to a concentration of 1 mM. Various amounts of wet or freeze-dried cells were incubated in 1 ml of 1 mM heavy metal solutions at 30°C for 5 h with shaking at 200 rpm. After incubation, cells were centrifuged at 21,500 × g for 10 min and the supernatants were used to determine the residual heavy metal concentrations by atomic absorption spectroscopy.

Taurocholic acid adsorption assays. Taurocholic acid (Sangon, Shanghai, China) was dissolved in deionized water. Various amounts of freeze-dried cells were incubated in 1 ml of 3 mM taurocholic acid solution at 37°C for 2 h with shaking at 200 rpm. After centrifugation at 21,500 × g for 10 min, the supernatants were used to measure the residual amounts of taurocholic acid by high-performance liquid chromatography (HPLC) (Hitachi, Tokyo, Japan) under the following conditions: a Symmetry C₁₈ reversed-phase column (4.6 mm by 250 mm; Waters) was used; the temperature was 30°C; the flow rate was 1 ml/min; mobile phase A was acetonitrile and mobile phase B was 20 mM KH₂PO₄-NAOH buffer (pH 6.8); elution conditions consisted of a linear gradient from 30:70 (A/B) to 45:55 in 20 min.

Immobilization of β-galactosidase on spores. Freeze-dried cells were incubated with 2% glutaraldehyde (Sangon, Shanghai, China) dissolved in deionized water at 30°C for 1 h with stirring at 200 rpm. β-Galactosidase (Sigma-Aldrich, Shanghai, China) was dissolved in water at 0.2 mg/ml. This concentration was determined by using a bicinchoninic acid (BCA) protein assay kit (Beyotime, Jiangsu, China). The experimentally measured specific activity of β-galactosidase in this solution was 1,048 U/g. Activated freeze-dried cells were then incubated in 200 μl of the β-galactosidase solution, and the cross-linking reaction took place in a tabletop rotary shaker at 4°C for 4 h. After cross-linking, the supernatant was used to measure the residual amount of the enzyme, and cells were washed with water and stored in 0.1 M sodium acetate buffer (pH 4.6) at 4°C for further experiments. The amount of immobilized β-galactosidase was determined by subtracting the residual amount of the enzyme from that of the original amount (40 μg). A BCA protein assay kit was used to measure protein amounts. For β-galactosidase assays, cells prepared as...
described above (5 mg) were incubated in 500 μl of Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 10 mM dithiothreitol) containing 5 mM o-nitrophenyl-o-galactopyranoside (ONPG) (Sangon, Shanghai, China) at 30°C for 30 min with shaking at 200 rpm. For the thermostability assay, the incubation was performed at various temperatures from 25°C to 80°C. The increase in the amount of o-nitrophenol production was measured at 410 nm by spectrophotometry. For repetitive assays of the immobilized enzyme, the spores were washed with Z buffer prior to each subsequent round of the assay. One unit of β-galactosidase activity was defined as the amount of enzyme required to release 1 μmol of o-nitrophenol produced per min per ml.

Quantification of chitosan in spores. Quantification of chitosan in yeast cells was performed by a modified version of the method of Pochanavanich and Suntornsuk (27) and Nitschke et al. (28). First, 0.3 g of freeze-dried cells prepared as described above was ground and suspended in 9 ml of 1 M NaOH. The cell suspensions were then sonicated at 121°C for 15 min. Cell pellets were collected by centrifugation (12,000 × g for 15 min) and washed three times with 1 ml of water. Pellets were resuspended in 6 ml of 10% (vol/vol) acetate and incubated at 100°C for 8 h to extract chitosan. The suspensions were centrifuged (12,000 × g for 15 min), and the supernatant was used for the quantification assay. For standard solutions, chitosan (Sigma-Aldrich, Shanghai, China) was dissolved in 10% acetate. The amount of chitosan detected in 10 μl of cell extracts and standard solutions was spotted onto a thin-layer chromatography (TLC) plate (Qingdao Haiyang Chemical, Qingdao, China). The spots were detected with Lugol’s solution (Sigma-Aldrich, Shanghai, China). After color formation, plates were dried and scanned using an ImageScanner III (GE Healthcare Bio-Science, Uppsala, Sweden). Optical density of the spots was analyzed with ImageQuant TL software (GE Healthcare Bio-Science, Uppsala, Sweden).

Statistics. Statistical significance was determined with Student’s t-test (one-tailed, two-sample unequal variance) using Microsoft Excel software. Differences between the analyzed samples were considered significant at a P value of <0.05.

RESULTS

Yeast spores have the ability to adsorb Cu²⁺. Because yeast spores contain chitosan as a spore wall component, we speculated that spores would adsorb heavy metals. To verify this hypothesis, we performed a copper ion (Cu²⁺) adsorption assay. Various numbers of wet yeast spores were incubated in 1 ml of 1 mM CuSO₄ solution, and after removal of the spores, the residual amount of Cu²⁺ in the solution was measured by atomic absorption spectrometry. Under our experimental conditions, sporation efficiency was greater than 90%. Spore samples were prepared by treating asci with lyticase and sonication. In this experiment, amounts of spores were adjusted by turbidity before asci were lysed. As shown in Fig. 1, even intact asci were capable of adsorbing a certain amount of Cu²⁺, spores exhibited superior adsorption capacity: 10⁴ asci removed 45.1 μg (70%) of Cu²⁺, whereas the same number of spores removed 58.2 μg (91%) of Cu²⁺ (Fig. 1).

Spores can survive hazardous conditions, such as ether treatment, because of the presence of the outermost dityrosine layer (14). However, if spores are intended for use as an adsorbent, the dityrosine layer would present an obstacle to solutes access to chitosan. Therefore, removal of the dityrosine layer may improve adsorptive properties of spores. To test this possibility, we prepared dit1Δ spores and measured their Cu²⁺ removal ability as described above. Dit1 is required for dityrosine synthesis, so the dityrosine layer is absent in dit1Δ spores (14, 22). Strikingly, we found that dit1Δ spores removed Cu²⁺ more efficiently than wild-type spores. As shown in Fig. 1, 62.1 μg (97%) of Cu²⁺ was removed by 8 × 10⁸ dit1Δ spores, whereas the same amount of wild-type spores removed 51.0 μg (80%) of Cu²⁺. To examine whether the chitosan layer is involved in Cu²⁺ adsorption, we then prepared chs3Δ mutant spores. CHS3 encodes a chitin synthase, and both the chitosan and dityrosine layers are absent in the mutant spore wall (15). As shown in Fig. 1, in chs3Δ spores, Cu²⁺ adsorption was decreased to a level lower than that of wild-type spores. These results indicate that yeast spores have the ability to adsorb Cu²⁺, and exposure of the chitosan layer on the surface increases their adsorption capacity of spores.

dit1Δ spores adsorb Cu²⁺ more efficiently than vegetative cells do. In the above-described experiments, we used wet cells, and their amount was adjusted by turbidity. However, wet cells are inconvenient for precise measurement of cell amount. Furthermore, the spore samples prepared as described above contain residual debris from the ascal and cell membranes, which could affect adsorption. For these reasons, we next prepared purified and freeze-dried spore samples. Percoll gradient centrifugation was used to purify spores, and the amount of freeze-dried cells was adjusted by weight. The amount of chitosan detected in 1 g of freeze-dried wild-type spores was 20.3 mg (see Fig. S1 in the supplemental material). We verified that vegetative cells and chs3Δ spores did not contain detectable amounts of chitosan. Figure 2A shows the result of a Cu²⁺ adsorption assay using purified and freeze-dried dit1Δ spores. As the amount of spores was increased, more Cu²⁺ was removed from the solution, indicating that the freeze-dried spores also had the ability to adsorb Cu²⁺. Consistent with the assay using wet cells (Fig. 1), dit1Δ spores adsorbed more Cu²⁺ than other spores. With 6 mg of freeze-dried wild-type, dit1Δ, and chs3Δ spores, 20.8, 24.3, and 19.2 μg of Cu²⁺ was removed, respectively (Fig. 2B). We then prepared freeze-dried vegetative cells, because previous studies have shown that vegetative cells also have the ability to remove heavy metals from solution (29). However, in our assay, freeze-dried vegetative cells were not able to adsorb Cu²⁺ as efficiently as wild-type or dit1Δ spores: 6 mg of vegetative cells removed 17.9 μg of Cu²⁺ (Fig. 2B). Thus, dit1Δ spores work better as a bioadsorbent than vegetative cells.

dit1Δ spores are capable of removing heavy metals. The above findings prompted us to examine if dit1Δ spores also can...
and Pb$_2^+$ sorptive capacity among the four cell types. Remarkably, this observation indicates that even the inner two layers of the spore wall (the β-glucan and mannoprotein layers) can adsorb more heavy metals than those in the vegetative cell wall. Heavy metal adsorption assay data are summarized in Table 2. The amounts of heavy metals adsorbed by dit1Δ spores was in the following order; Cr$_{3+}^+$ > Cu$_{2+}^+$ > Cd$_{2+}^+$ > Zn$_{2+}^+$ > Pb$_{2+}^+$ > Ni$_{2+}^+$.

The presence of chitosan and removal of the dityrosine layer are required for efficient adsorption of heavy metals by spores. To further verify that chitosan in the spore wall is required for efficient adsorption of heavy metals, we made a cda1Δ cda2Δ double mutant, CDA1 and CDA2 encode chitin deacetylases, and thus deletion of these genes causes a defect in making chitosan and a failure of dityrosine layer formation (19). cda1Δ cda2Δ spores generally adsorbed more heavy metals than chs3Δ spores (Fig. 3). This probably because chitin also has the ability to adsorb heavy metals (30). We found that dit1Δ spores can adsorb more Ni$_{2+}^+$, Cr$_{3+}^+$, Cd$_{2+}^+$, and Pb$_{2+}^+$ than cda1Δ cda2Δ spores, though statistically significant differences for Cu$_{2+}^+$ and Zn$_{2+}^+$ adsorption were not detected (Fig. 2B and 3).

CRR1 is another gene involved in proper spore wall assembly. CRR1 encodes a putative transglycosidase, and the dityrosine layer in crr1Δ spores is more permeable than that in wild-type spores (31). If the dityrosine layer obstructs heavy metal adsorption, adsorption ability of spores might be improved by CRR1 deletion. However, as shown in Fig. 2B and 3, the adsorption levels are not significantly affected by CRR1 deletion. Thus, it may be that the dityrosine layer does not work only as a barrier that prevents access of heavy metals to the chitosan layer. Collectively, our results show that both the presence of chitosan and absence of the dityrosine layer are required for efficient adsorption of heavy metals by spores.

dit1Δ spores also can adsorb taurocholic acid. In acidic solution, chitosan carries a positive charge, permitting adsorption of negatively charged molecules in addition to heavy metals. Thus, if dit1Δ spores work as chitosan beads, these spores should adsorb negatively charged molecules as well. This possibility was tested using a bile acid, taurocholic acid. Bile acids are cholesterol derivatives; previous reports have shown adsorption by chitosan (32, 33). Following incubation of freeze-dried spores with 3 mM taurocholic acid solution, the residual amount of taurocholic acid was measured by HPLC and used to calculate removal by spores. Figure 4A shows that dit1Δ spores remove taurocholic acid from solution. With 1 mg of wild-type spores, dit1Δ spores, chs3Δ spores, and vegetative cells, 0.74, 0.92, 0.60, and 0.54 μmol of taurocholic acid were removed, respectively (Fig. 4B), showing remove other heavy metals, such as chromium (Cr), nickel (Ni), zinc (Zn), cadmium (Cd), and lead (Pb). Six milligrams of freeze-dried vegetative cells (vege) or wild-type (wt), dit1Δ, chs3Δ, cda1Δ cda2Δ, or crr1Δ spores was incubated in 1 mM Cu(NO$_3$)$_3$, Ni(NO$_3$)$_2$, Zn(NO$_3$)$_2$, Cd(NO$_3$)$_2$, or Pb(NO$_3$)$_2$ solution for 5 h at 30°C. After removal of the cells, residual amounts of heavy metals were measured by atomic absorption spectrometry. Amounts of heavy metals removed from the original solution are shown. Data are the means ± SE from three independent experiments. *, P < 0.05; **, P < 0.01; NS, not significant.

FIG. 2 dit1Δ spores remove Cu$_{2+}^+$ more efficiently than vegetative cells do. (A) Various amounts of freeze-dried dit1Δ spores were incubated in 1 ml of 1 mM CuSO$_4$ solution for 5 h at 30°C. After removal of the cells, residual amounts of Cu$_{2+}^+$ in the solution were measured by atomic absorption spectrometry. The amount of Cu$_{2+}^+$ removed by the spores is shown. (B) Six milligrams of freeze-dried vegetative cells (vege) or wild-type (wt), dit1Δ, chs3Δ, cda1Δ cda2Δ, or crr1Δ spores was incubated in 1 mM CuSO$_4$ solution for 5 h at 30°C. Residual amounts of Cu$_{2+}^+$ were measured by atomic absorption spectrometry. Cu$_{2+}^+$ amounts removed from the original solution are shown. Data are the means ± SE from three independent experiments. *, P < 0.05; **, P < 0.01; NS, not significant.

FIG 3 Removal of heavy metals by yeast spores and vegetative cells. Six milligrams of freeze-dried vegetative cells (vege) or wild-type (wt), dit1Δ, chs3Δ, cda1Δ cda2Δ, or crr1Δ spores was incubated in 1 ml of 1 mM Cr(NO$_3$)$_3$, Ni(NO$_3$)$_2$, Zn(NO$_3$)$_2$, Cd(NO$_3$)$_2$, or Pb(NO$_3$)$_2$ solution for 5 h at 30°C. After removal of the cells, residual amounts of heavy metals were measured by atomic absorption spectrometry. Amounts of heavy metals removed from the original solution are shown. Data are the means ± SE from three independent experiments. *, P < 0.05; **, P < 0.01; NS, not significant.

FIG. 4 Removal of taurocholic acid by yeast spores. (A) Yeast spores remove taurocholic acid in a concentration-dependent manner. Various amounts of freeze-dried vegetative cells (vege) or wild-type (wt), dit1Δ, chs3Δ, cda1Δ cda2Δ, or crr1Δ spores were incubated in 1 ml of 1 mM Cr(NO$_3$)$_3$, Ni(NO$_3$)$_2$, Zn(NO$_3$)$_2$, Cd(NO$_3$)$_2$, or Pb(NO$_3$)$_2$ solution for 5 h at 30°C. After removal of the cells, residual amount of taurocholic acid was measured by HPLC and used to calculate removal by spores. (B) Six milligrams of freeze-dried vegetative cells, wild-type spores, or chs3Δ spores were incubated in 1 ml of 1 mM CuSO$_4$ solution for 5 h at 30°C. After removal of the cells, residual amount of taurocholic acid was measured by HPLC and used to calculate removal by spores. Data are the means ± SE from three independent experiments. *, P < 0.05; **, P < 0.01; NS, not significant.
that dit1Δ spores also adsorbed taurocholic acid more efficiently than other spores or vegetative cells. These results further support the idea that dit1Δ spores work as chitosan beads.

*dit1Δ spores can be used as carriers for enzyme immobilization.* We also attempted to use yeast spores as carriers for enzyme immobilization. A variety of enzymes have been immobilized on chemically prepared chitosan beads for practical purposes by coupling to the chemically reactive amino groups on chitosan (34). In this study, we used β-galactosidase as a test case for such enzyme immobilization. According to a previously reported strategy to immobilize β-galactosidase onto chitosan beads (35), freeze-dried spores were first treated with glutaraldehyde, and then activated spores were incubated in β-galactosidase solution. This procedure was first performed with dit1Δ spores, and we found that the spores can immobilize substantial amount of β-galactosidase (Fig. 5). Compared to dit1Δ spores, the amounts of immobilized β-galactosidase were markedly lower with vegetative cells and with wild-type, chs3Δ, cda1Δ cda2Δ, and crr1Δ spores (Fig. 5B). We found that dit1Δ spores exhibited higher activities than the other cells (Fig. 6A), though the activity in dit1Δ spores was not so high considering the amount of immobilized enzyme. The specific activity of soluble β-galactosidase used in this experiment was 1,048 U/g (see Materials and Methods). Since 5 μg of the enzyme was immobilized on dit1Δ spores (Fig. 5B), the predicted total activity on the spores was 5.2 mU. However, experimentally measured total activity on the spores was 1.6 mU (specific activity was 0.31

**TABLE 2 Amounts of heavy metals adsorbed by freeze-dried spores or vegetative cells**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Cu²⁺</th>
<th>Cr³⁺</th>
<th>Ni²⁺</th>
<th>Zn²⁺</th>
<th>Cd²⁺</th>
<th>Pb²⁺</th>
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<tr>
<td>dit1Δ spores</td>
<td>63.8 ± 1.9</td>
<td>68.6 ± 1.1</td>
<td>24.7 ± 0.5</td>
<td>33.9 ± 1.3</td>
<td>47.4 ± 0.7</td>
<td>25.8 ± 0.6</td>
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<tr>
<td>Wild-type spores</td>
<td>54.7 ± 1.7</td>
<td>43.3 ± 2.8</td>
<td>14.8 ± 0.8</td>
<td>25.5 ± 1.3</td>
<td>15.1 ± 0.4</td>
<td>22.2 ± 0.6</td>
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<tr>
<td>chs3Δ spores</td>
<td>50.4 ± 2.4</td>
<td>55.4 ± 1.1</td>
<td>13.3 ± 0.3</td>
<td>25.0 ± 3.1</td>
<td>16.3 ± 0.2</td>
<td>20.8 ± 0.6</td>
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<tr>
<td>cda1Δ cda2Δ spores</td>
<td>59.3 ± 2.9</td>
<td>61.9 ± 1.5</td>
<td>19.3 ± 0.6</td>
<td>28.5 ± 0.4</td>
<td>37.6 ± 0.5</td>
<td>23.3 ± 0.2</td>
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<tr>
<td>crr1Δ spores</td>
<td>50.4 ± 2.2</td>
<td>57.7 ± 2.2</td>
<td>13.9 ± 1.1</td>
<td>27.1 ± 0.7</td>
<td>17.7 ± 0.9</td>
<td>20.9 ± 0.5</td>
</tr>
<tr>
<td>Vegetative cells</td>
<td>47.0 ± 1.3</td>
<td>23.1 ± 1.8</td>
<td>11.1 ± 1.0</td>
<td>14.3 ± 1.2</td>
<td>12.5 ± 1.2</td>
<td>16.7 ± 0.1</td>
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</table>

*Calculated based on data obtained from the adsorption assays using 6 mg of cells. Values are means ± SE (n = 3).

FIG 4 dit1Δ spores adsorb taurocholic acid more efficiently than other cells. (A) Various amounts of freeze-dried dit1Δ spores were incubated in 1 ml of a 3 mM taurocholic acid solution for 2 h at 37°C. After removal of the spores, residual amounts of taurocholic acid in the solution were measured by HPLC. Amounts of taurocholic acid removed by the spores are shown. (B) One milligram of freeze-dried vegetative cells (vege) or wild-type (wt), dit1Δ, or chs3Δ spores were incubated in the taurocholic acid solution under the conditions described above, and residual amounts of taurocholic acid were measured by HPLC. Amounts of taurocholic acid removed from the original solution are shown. Data are the means ± SE from three independent experiments. *, P < 0.05; **, P < 0.01.

FIG 5 Immobilization of β-galactosidase on dit1Δ spores. (A) Various amounts of freeze-dried dit1Δ spores were first treated with 500 μl of 2% glutaraldehyde for 1 h and then incubated in 200 μl of a 0.2-mg/ml β-galactosidase solution for 4 h at 4°C. Amounts of β-galactosidase removed from the original solution are shown. (B) Five milligrams of freeze-dried vegetative cells (vege) or wild-type (wt), dit1Δ, chs3Δ, cda1Δ cda2Δ, or crr1Δ spores were treated as described above, and amounts of β-galactosidase removed by the cells are shown. Data are the means ± SE from three independent experiments. *, P < 0.05; **, P < 0.01.
U/g [Fig. 6A]. For \( \beta \)-galactosidase immobilized on wild-type spores, predicted and measured total activities were 0.7 and 1.1 mU, respectively (calculated from data in Fig. 5B and 6A). Thus, it may be that covalently immobilized \( \beta \)-galactosidase in the chitosan layer partially loses its activity. The immobilized \( \beta \)-galactosidase on \( \Delta \text{dit} \) spores can be used repeatedly. We repeated the activity assay and washed the spores several times. After the third wash, the activity retained in \( \Delta \text{dit} \) spores was 67% of the original activity, whereas that in wild-type spores was 30% (Fig. 6B). We further assessed whether the immobilization affects thermostability of the enzyme. As shown in Fig. 6C, thermostability of \( \beta \)-galactosidase is improved by the enzyme’s being immobilized on \( \Delta \text{dit} \) spores. These results show that \( \beta \)-galactosidase can be immobilized on \( \Delta \text{dit} \) spores.

**DISCUSSION**

In this study, we have demonstrated that yeast spores, especially \( \Delta \text{dit} \) spores, can be used as chitosan beads and used for several purposes, such as removal of hazardous heavy metals and immobilization of enzymes. Yeast cells have been studied extensively as a bioadsorbent, but those previous studies were all performed using vegetative cells (29). In \( \text{Cu}^{2+} \) adsorption assays, we found that yeast spores removed \( \text{Cu}^{2+} \) more efficiently than vegetative cells. In the yeast spore wall, the chitosan layer is covered with the dityrosine layer. Compared to wild-type spores, \( \Delta \text{dit} \) spores, in which the dityrosine layer is absent, can adsorb more \( \text{Cu}^{2+} \) than wild-type spores. Thus, the dityrosine layer obstructs \( \text{Cu}^{2+} \) adsorption. We further demonstrated that spores are capable of removing various heavy metals besides \( \text{Cu}^{2+} \). Notably, \( \Delta \text{dit} \) spores adsorbed \( \text{Cd}^{2+} \) significantly more than other cells, suggesting that the chitosan in the spore wall has higher affinity for this major hazardous heavy metal.

The presence of chitosan is required for efficient adsorption of heavy metals by spores. In \( \Delta \text{chs}3 \) spores, both the chitosan and dityrosine layers are absent (15). \( \Delta \text{dit} \) spores generally adsorb a larger fraction of each heavy metal than \( \Delta \text{chs}3 \) spores do. Furthermore, we found that \( \Delta \text{dit} \) spores can adsorb more \( \text{Ni}^{2+} \), \( \text{Cr}^{3+} \), \( \text{Cd}^{2+} \), and \( \text{Pb}^{2+} \) than \( \text{cda}1 \) \( \Delta \text{cda}2 \) spores. \( \text{CDA1} \) and \( \text{CDA2} \) encode chitin deacetylases, and the double mutant is defective in production of chitosan (17). Unlike \( \Delta \text{chs}3 \) spores, \( \text{cda}1 \) \( \Delta \text{cda}2 \) spores contain chitin, which also has the ability to adsorb heavy metals (19, 30). Probably for this reason, \( \text{cda}1 \) \( \Delta \text{cda}2 \) spores can adsorb more heavy metals than \( \Delta \text{chs}3 \) spores. \( \text{Crr1} \) is a putative transglycosidase and is considered to be required for a linkage of chitosan to the \( \beta \)-glucan layer (31). Although the dityrosine layer in \( \text{crr1} \) spores is more permeable than that in wild-type spores (31), we found that the ability of \( \text{crr1} \) spores to adsorb heavy metals was lower than that of \( \Delta \text{dit} \) \( \Delta \text{cda}1 \) \( \Delta \text{cda}2 \) spores. One possible reason for this is that the character of the chitosan layer is altered by linkage to dityrosine molecules, as mentioned below. Alternatively, cross-linking of chitosan to \( \beta \)-glucan may contribute to the increase in the adsorption capacity of spores. Despite the lack of the chitosan layer, \( \Delta \text{chs}3 \) spores are still able to adsorb certain amounts of heavy metals. This implies that other components of the spore wall are adsorbent in addition to chitosan. Previous work has shown that the biosorptive ability of vegetative cells is largely dependent on the cell wall materials, which are composed mainly of mannoproteins and \( \beta \)-glucans (36). Since the inner two layers of the spore wall are also composed of these two materials (12), it seems likely that these components adsorb heavy metals in \( \Delta \text{chs}3 \) spores. In this context, it should be noted that, for all the heavy metals that we examined, \( \Delta \text{chs}3 \) spores showed higher adsorption activity than did vegetative cells; this distinction was especially notable for \( \text{Cr}^{3+} \), of which the former adsorbed more than twice as much as the latter. This result suggests that mannoproteins and \( \beta \)-glucans in the spore wall may have more affinity for heavy metals than those in vegetative cells. Though the vegetative wall and the inner spore wall have similar constituents, the mannan and \( \beta \)-glucans are organized differently in the two structures (10, 37). Thus, these organizational differences might alter the affinity for heavy metals.
These days, chitosan is available commercially because oral administration of chitosan has been shown to decrease serum cholesterol levels (38). One possible mechanism underlying this phenomenon is that bile acids, which are synthesized from cholesterol, may be adsorbed by chitosan in the gut and then discharged to the outside of the body. In this way, the chitosan could act as a sink to lower serum cholesterol via excretion of bile acids. Heavy metals and bile acids interact with chitosan in different ways: the former bind to chitosan by chelation, whereas the latter associate with chitosan by electrostatic interactions (1, 5). As seen for heavy metals, dit1Δ spores removed taurocholic acid more efficiently than wild-type or chs3Δ spores, suggesting that the chitosan layer is also responsible for increasing the adsorption of taurocholic acid, whereas the dityrosine layer prevents bile acid adsorption. Thus, dit1Δ spores can adsorb taurocholic acid as well as heavy metals. This further supports the idea that the spores can be used as chitosan beads and used to adsorb versatile molecules.

As another application for spores, we showed that dit1Δ spores can be used to immobilize an enzyme. In this study, glutaraldehyde was used to activate amino groups, permitting cross-linking to β-galactosidase. Consistent with general features of immobilized enzymes, the β-galactosidase on the Δdit1 spores could be assayed repeatedly; the immobilized enzyme exhibited improved thermostability compared to the soluble enzyme. These findings demonstrate that dit1Δ spores are amenable to chemical modifications and can be used as a carrier to immobilize enzymes. The amount of β-galactosidase immobilized on wild-type spores is markedly lower than that on dit1Δ spores. This is consistent with the previous finding that amino groups of dityrosine molecules in the spore wall are formylated (39). Neither chs3Δ nor cda1Δ cda2Δ spores can immobilize β-galactosidase very well, showing that chitosan is required for the immobilization. The immobilized enzyme was catalytically active, although dit1Δ spores did not exhibit significantly higher activity than other cell types. One possible reason for the loss of the activity is that β-galactosidase partially loses its activity by covalent immobilization in the chitosan layer. In other cells, a large fraction of the β-galactosidase may associate with some cellular component rather than binding covalently through glutaraldehyde bridge, so that the enzyme retains full activity. Consistent with this idea, the activity in wild-type spores is washed out more efficiently than that in dit1Δ spores. In the repetitive-use assay, we found that the activity in the dit1Δ spores decreased by 0.27 mU (about 17% of the original activity) during the first wash. After the first wash of the dit1Δ spores, we measured the activity in the supernatant of the wash solution and found that total activity in the wash solution was 0.11 mU (data not shown). This suggests that loss of the activity due to enzyme leakage from the dit1Δ spores is about 7%. Thus, it seems that the loss of the activity during the wash is not only due to leakage of the enzyme. Some loss of activity may also be due to loss of spores from pellet during the washing steps.

Our results suggest that the dityrosine layer prevents access of heavy metals, taurocholic acid, and β-galactosidase to the chitosan layer. For bile acids and exogenous enzymes, these findings are consistent with the notion that the dityrosine layer works as a size-dependent diffusion barrier (40). However, this size-dependent exclusion does not explain the decrease in heavy metal adsorption by the dityrosine layer: heavy metal ions are much smaller than glucose, a molecule that can be imported into spores through the spore wall when spores initiate germination (41). It has been reported that dityrosine is covalently attached to the chitosan layer through an unknown linkage (14). Thus, this linkage might disrupt the arrangement of the NH2 groups, which is necessary for effective chelation.

Taken together, our results show that spores are apparently better than vegetative cells in terms of adsorption abilities and chemical modification. Additionally, considering the rigidity of the spore wall, spores are more attractive than vegetative cells for use as beads and application to practical purposes. A previous study showed that 1 mg of chemically produced pure chitosan flake can adsorb 9.9 µg of Cd2+, 11.7 µg of Pb2+, and 20.9 µg of Cu2+ (42). Based on the data in Table 2, 1 mg of dit1Δ spores can adsorb 5.3 µg of Cd2+, 5.3 µg of Pb2+, and 4.1 µg of Cu2+. Thus, if only adsorption capacity per unit mass is taken into consideration, it seems that the spores may not be a superior adsorbent. However, compared to chemically produced chitosan or chitosan beads, yeast spores have a great advantage in that they are totally natural products, so that no hazardous wastes are produced to obtain them. Furthermore, since yeasts are used in a variety of industries, including brewed beverages and bioethanol production, it is possible to prepare yeast cells en masse. Once yeast cells are obtained, spores are easily produced by incubation in nonfermentable carbon sources such as acetic acid. For these reasons, yeast spores may find uses in many of the procedures that call for chitosan beads.

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