Applied uses of yeast spores as chitosan beads

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Abstract

In this study, we present a non-hazardous 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 Cu$^{2+}$, Cr$^{3+}$ and Cd$^{2+}$ and removal of the dityrosine layer further improves the adsorption. Removal of 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, especially *dit1Δ* mutants, can be used as chitosan beads and applied for multiple purposes.
Introduction

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 in the food, chemical and medical industries (3-5).

Chitosan is industrially produced by chemical deacetylation of chitin primarily as derived from crustacean shell waste. However, this method presents several challenges including the potentially unstable supply of raw materials and production of harmful wastes. 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 a 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 ascal 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 the 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 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 sphere of ~3 micrometers 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 which 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 the DIT1 disruption was generated by PCR using pFA6a-HIS3MX6 (25) as template, and HX034 (AATTTGTTAATATCCTAA TCTTATTACGACATTAACAAAAACGGATCCCCGGGTTAATTAA) and HX035 (TGTCTAAATAGAAACAAAAAGGTAGACCAATGTAGCGCTCTTA CTTTAGAATTGGAGCGCTTTAAAAC) as primers. The resulting PCR fragment was integrated into haploid cells AN117-4B and AN117-16D, and the resulting strains were mated to generate the diploid dit1Δ disruptant. Other mutants were constructed in the same way. For deletion of CDA1, CDA2 and CRR1, Cda1-F (GCTTAAGAAAGCGAATAGAGAAATACAAAAATTAAGTGATAGACGG ATCCCCGGGTTAATTAA) and Cda1-R (TGTCAGCTCGATATTAAATAAGAGAAGAGTAAGTTTAAAGTATCATCAAGGGCTTTTGCACGAAATAAAAATCGGATCCCCGGG TTAATTAA) and HX0521 (CAGTCGTCTAGTTTAAAGTATCATCAAGGGCTTTTGCACGAAATAAAAATCGGATCCCCGGG TTAATTAA) and HX0520 (TAGAGGATCCGCTACGGAAGAGAGAACTTCGTAA TAGAGGATCCGCTACGGAAGAGAGAACTTCGTAA TAGAGGATCCGCTACGGAAGAGAGAACTTCGTAA).
GACATTATTGCTGTGAGAATTCGAGCTCGTTTAAAC) were used as primers 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/cda1Δ cda2Δ/cda2Δ strains were designated as 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 liquid media overnight. 2 ml of the culture was then shifted to 100 ml of YPAcetate (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 media at a concentration of $3 \times 10^7$ cells/ml, and cultured for at least 24 hours. Sporulation efficiency was determined under the microscope and cultures with efficiencies of greater than 90% were used for further processes.

To take spores out from asci, the ascal wall was first digested with lyticase (Sigma-Aldrich, Shanghai, China). For this purpose, asci were resuspended in 2 ml of water and treated with lyticase (625 U/gram cells) at 37°C for 1 hour with shaking at 250 rpm. The ascal 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 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 (50-80% Percoll, 10% 2.5 M sucrose and 0.5% Triton-X). After centrifugation at 15,000 × g at 4°C for 1 hour, the top of three layers which 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 hours. They were then freeze-dried by EYELA FD-1000 freeze dryer (Tokyo Rikakikai, Tokyo, Japan) at -50°C for 72 hours under the pressure of 25 Pa.

**Heavy metal adsorption assays**

CuSO$_4$, Cr(NO$_3$)$_3$, Ni(NO$_3$)$_2$, Zn(NO$_3$)$_2$, Cd(NO$_3$)$_2$ or Pb(NO$_3$)$_2$ were prepared in deionized water to a the 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 hours 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 hours 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 HPLC (Hitachi, Tokyo, Japan) under the following conditions: Symmetry C18 reverse
column (4.6 mm × 250 mm, Waters); temperature, 30°C; flow rate, 1 ml/min; mobile
phases, (A) acetonitrile and (B) 20 mM KH$_2$PO$_4$/NaOH buffer (pH 6.8); elution condition,
linear gradient from A/B (30:70) 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 hour with stirring at 200 rpm. β-galactosidase (Sigma-Aldrich, Shanghai, China) was dissolved in water at the concentration of 0.2 mg/ml. This concentration was determined by using BCA protein assay kit (Beyotime, Jiangsu, China). The experimentally measured specific activity of β-galactosidase in this solution was 1048 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 table top rotary shaker at 4°C for 4 hours. 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). BCA protein assay kit was used to measure protein amounts. For β-galactosidase assays, the cells prepared as above (5 mg) were incubated in 500 μl of Z-buffer (60 mM Na$_2$HPO$_4$, 40 mM NaH$_2$PO$_4$, 10 mM KCl, 1 mM MgSO$_4$, 10 mM dithiothreitol) containing 5 mM of o-nitrophenyl-o-galactopyranoside (ONPG) (Sangon, Shanghai, China) at 30°C for 30 minutes with shaking at 200 rpm. For the thermostability assay, the incubation was performed at various temperatures from 25°C
to 80°C. Increased 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 method of Pochanavanich and Suntornsuk (27) and Nitschke et al. (28). First, 0.3 g of freeze-dried cells prepared as above were ground and suspended in 9 ml of 1 M NaOH. The cell suspensions were then autoclaved at 121°C for 15 minutes. 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% (v/v) acetate and incubated at 100°C for 8 hours to extract chitosan. The suspensions were centrifuged (12,000 × g for 15 min) and supernatant was used for the quantification assay. For standard solutions, chitosan (Sigma-Aldrich, Shanghai, China) was dissolved in 10% acetate. The amount of chitosan was colorimetrically determined as follows: 2 µl of cell extracts and standard solutions were 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-tail, two-sample unequal variance) using Microsoft Excel software. Differences between the analyzed samples were considered significant at $P < 0.05$. 
**Results**

**Yeast spores have the ability to adsorb Cu\(^{2+}\)**

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 (Cu\(^{2+}\)) adsorption assay. Various numbers of wet yeast spores were incubated in 1 ml of 1 mM CuSO\(_4\) solution and, after removal of the spores, the residual amount of Cu\(^{2+}\) in the solution was measured by atomic absorption spectrometry. Under our experimental conditions, sporulation 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, although even intact asci were capable of adsorbing a certain amount of Cu\(^{2+}\), spores exhibited superior adsorption capacity: \(10^9\) asci removed 45.1 µg (70%) of Cu\(^{2+}\), whereas this number of spores removed 58.2 µg (91%) of the provided Cu\(^{2+}\) (Fig. 1).

Spores can survive through 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. If so, removal of the dityrosine layer may improve adsorptive properties of spores. To test this possibility, we prepared \(dit1\Delta\) spores and measured their Cu\(^{2+}\) removal ability as described above. \(DIT1\) is required for dityrosine synthesis, so the dityrosine layer is absent in \(dit1\Delta\) spores (14, 22). Strikingly, we found that \(dit1\Delta\) spores removed Cu\(^{2+}\) more efficiently than wild-type spores. As shown in Fig. 1, 62.1 µg (97%) of Cu\(^{2+}\) was removed by \(8 \times 10^8\) \(dit1\Delta\) spores whereas the same amount of wild-type spores...
removed 51.0 μg (80%) of Cu\textsuperscript{2+}. To examine whether the chitosan layer is involved in Cu\textsuperscript{2+} adsorption, we then prepared chs3\Delta 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 chs3\Delta spores, Cu\textsuperscript{2+} adsorption was decreased to an adsorption level lower than that of wild-type spores. These results indicate that yeast spores have the ability to adsorb Cu\textsuperscript{2+}, and exposure of the chitosan layer on the surface increases their adsorption capacity of spores.

**dit1\Delta spores adsorb Cu\textsuperscript{2+} more efficiently than vegetative cells do**

In the above 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 above contain residual debris of 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 (Fig. S1). We verified that vegetative cells and chs3\Delta spores did not contain detectable amount of chitosan. Fig. 2A shows the result of a Cu\textsuperscript{2+} adsorption assay using purified and freeze-dried dit1\Delta spores. As the amount of spores was increased more Cu\textsuperscript{2+} was removed from the solution, indicating that the freeze-dried spores also had the ability to adsorb Cu\textsuperscript{2+}. Consistent with the assay using wet cells (Fig. 1), dit1\Delta spores adsorbed more Cu\textsuperscript{2+} than other spores. With 6 mg of freeze-dried wild-type, dit1\Delta or chs3\Delta spores, 20.8, 24.3 or 19.2 μg of Cu\textsuperscript{2+} 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 Cu\(^{2+}\) as efficiently as wild-type or dit\(1\)\(\Delta\) spores: 6 mg of vegetative cells removed 17.9 µg of Cu\(^{2+}\) (Fig. 2B). Thus, dit\(1\)\(\Delta\) spores work better as a bio-adsorbent than vegetative cells.

**dit\(1\)\(\Delta\) spores are capable of removing heavy metals**

The above findings prompted us to examine if dit\(1\)\(\Delta\) spores also can remove other heavy metals such as chromium (Cr), nickel (Ni), zinc (Zn), cadmium (Cd) and lead (Pb). 6 mg of freeze-dried vegetative cells, wild-type spores, dit\(1\)\(\Delta\) spores or chs3\(\Delta\) 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\(_3\))\(_2\) solutions, and amounts of these metal ions removed were calculated. As shown in Fig. 3, for all of these heavy metals, dit\(1\)\(\Delta\) spores showed the highest adsorption capacity; by contrast, vegetative cells exhibited the lowest adsorptive capacity among the four cell types. Remarkably, dit\(1\)\(\Delta\) spores showed significantly higher adsorption of Cd\(^{2+}\), compared to the other cells. It should be noted that chs3\(\Delta\) spores adsorbed Zn\(^{2+}\), Cr\(^{3+}\) and Pb\(^{2+}\) more efficiently than vegetative cells; notably, chs3\(\Delta\) spores adsorbed more than twice as much Cr\(^{3+}\) as vegetative cells (Fig. 3). This observation indicates that, even the inner two layers of the spore wall, that is, the β-glucan and mannoprotein layers, can adsorb more heavy metals than those in the vegetative cell wall. Heavy metal adsorption assay data is summarized in Table 2. The amounts of heavy metals adsorbed by dit\(1\)\(\Delta\) spores was in the...
following order: Cr<sup>3+</sup> > Cu<sup>2+</sup> > Cd<sup>2+</sup> > Zn<sup>2+</sup> > Pb<sup>2+</sup> > Ni<sup>2+</sup>.

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 cda<sub>1</sub>Δ cda<sub>2</sub>Δ 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). cda<sub>1</sub>Δ cda<sub>2</sub>Δ double mutant spores generally adsorbed more heavy metals than chs<sub>3</sub>Δ spores (Fig. 3). This probably because chitin also has an ability to adsorb heavy metals (30). We found that dit<sub>1</sub>Δ spores can adsorb more Ni<sup>2+</sup>, Cr<sup>3+</sup>, Cd<sup>2+</sup> and Pb<sup>2+</sup> than cda<sub>1</sub>Δ cda<sub>2</sub>Δ double mutant spores, though statistically significant differences for Cu<sup>2+</sup> and Zn<sup>2+</sup> 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 crr<sub>1</sub>Δ 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 only work 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 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 acids, 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. Fig. 4A shows that dit1Δ spores remove taurocholic acid from solution. With 1 mg of wild-type, dit1Δ spores, chs3Δ spores or vegetative cells, 0.74, 0.92, 0.60 or 0.54 µmol of taurocholic acid was removed, respectively (Fig. 4B), showing 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. 5A and B). Compared to dit1Δ spores, the amounts of immobilized β-galactosidase were markedly lower with vegetative cells or wild-type, chs3Δ, cda1Δ cda2Δ or 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 1048 U/g (see Materials and Methods). Since 5 µg of the enzyme was immobilized on dit1Δ spores (Fig. 5B), 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 U/g as shown in Fig. 6A). For β-galactosidase immobilized on wild-type spores, predicted and measured total activities were 0.7 and 1.1 mU, respectively (calculated from Fig. 5B and Fig. 6A data). Thus, it may be that covalently immobilized β-galactosidase in the chitosan layer partially loses its activity. The immobilized β-galactosidase on Δdit1 spores can be used repeatedly. We repeated the activity assay and washed several times. After the third wash, the activity retained in Δdit1 spores was 67% of the original activity whereas that in wild-type spores was 30% (Fig. 6B). We further assessed whether the immobilization affects on thermostability of the enzyme. As shown in Fig. 6C, thermostability of β-galactosidase is improved by being immobilized on dit1Δ spores. These results show that β-galactosidase can be immobilized on dit1Δ spores.
Discussion

In this study we have demonstrated that yeast spores, especially dit1Δ spores, can be used as chitosan beads and applied for several purposes, such as removal of hazardous heavy metals and immobilization of enzymes. Yeast cells have been studied extensively as a bio-adsorbent, but those previous studies all were performed using vegetative cells (29).

In Cu^{2+} adsorption assays, we found that yeast spores removed 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, dit1Δ spores, in which the dityrosine layer is absent, can adsorb more Cu^{2+} than wild-type spores. Thus, the dityrosine layer obstructs Cu^{2+} adsorption. We further demonstrated that spores are capable of removing various heavy metals besides Cu^{2+}. Notably, dit1Δ spores adsorbed Cd^{2+} 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 chs3Δ spores, both the chitosan and dityrosine layers are absent (15). dit1Δ spores generally adsorb a larger fraction of each heavy metal than chs3Δ spores do. Furthermore, we found that dit1Δ spores can adsorb more Ni^{2+}, Cr^{3+}, Cd^{2+} and Pb^{2+} than cda1Δ cda2Δ spores. CDA1 and CDA2 encode chitin deacetylases and the double mutant is defective in production of chitosan (17). Different from chs3Δ spores, cda1Δ cda2Δ spores contain chitin which also has an ability to adsorb heavy metals (19, 30). Probably for this reason, cda1Δ cda2Δ spores can adsorb more heavy metals than chs3Δ spores. Crr1 is a putative transglycosidase and is considered to be required for a linkage of chitosan to the β-glucan layer (31). Although the dityrosine layer in crr1Δ spores is more permeable than
that in wild-type spores (31), we found that the adsorption ability of crr1∆ spores for heavy metals was lower than that of dit∆ and cda1∆ cda2∆ spores. One possible reason for this is that the property of the chitosan layer is altered by linkage to dityrosine molecules as mentioned below. Alternatively, cross-linking of chitosan to β-glucan may contribute to increase the adsorption capacity in spores. Despite the lack of the chitosan layer, chs3∆ 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 is mainly composed of mannoproteins and β-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 chs3∆ spores. In this context, it should be noted that, for all the heavy metals that we examined, chs3∆ spores showed higher adsorption activity than did vegetative cells; this distinction was especially notable for Cr³⁺, for which the former adsorbed more than twice as much as the latter. This result suggests that mannoproteins and β-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 β-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 applied to adsorb versatile molecules.

As another application of 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 compared to 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 that seen in 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 bind 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 unknown linkage (14). Thus, this linkage might
disrupt the arrangement of the NH$_2$ groups which is necessary for effective chelation.

Taken together, our results show that spores are apparently better than vegetative cells in the 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 has shown that 1 mg of chemically produced pure chitosan flake can adsorb 9.9 µg of Cd$^{2+}$, 11.7 µg of Pb$^{2+}$ and 20.9 µg of Cu$^{2+}$ (42). Based on Table 2 data, 1 mg of dit1Δ spores can adsorb 5.3 µg of Cd$^{2+}$, 5.3 µg of Pb$^{2+}$ and 4.1 µg of Cu$^{2+}$. Thus, if only taking adsorption capacity per unit mass 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 non-fermentable carbon sources such as acetic acid. For these reasons, yeast spores may find use in many of the procedures that call for chitosan beads.

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Figure Legends

**FIG 1 Removal of Cu$^{2+}$ by yeast spores**

Indicated numbers of asci or wild-type (wt), dit1Δ or chs3Δ wet spores were incubated in 1 ml of 1 mM CuSO$_4$ solution (total amount of Cu$^{2+}$ was 64 µg) for 5 hours at 30°C. Amounts of spores were measured before asci were lysed. After removal of the cells, residual amounts of Cu$^{2+}$ in the solution were measured by atomic absorption spectrometry and its amount removed by the cells was calculated. Data presented are the mean ± SE of three independent experiments.

**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 hours at 30°C. After removal of the cells, residual amounts of Cu$^{2+}$ in the solution were measured by atomic absorption spectrometry. Amount of Cu$^{2+}$ removed by the spores was shown. (B) 6 mg of freeze-dried vegetative cells (vege) or wild-type (wt), dit1Δ, chs3Δ, cda1Δ cda2Δ or crr1Δ spores were incubated in 1 mM CuSO$_4$ solution for 5 hours 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 presented are the mean ± SE of three independent experiments. * P < 0.05; ** P < 0.01; NS, not significant.

**FIG 3 Removal of heavy metals by yeast spores and vegetative cells**

6 mg of freeze-dried vegetative cells (vege) or wild-type (wt), dit1Δ, chs3Δ,
*cda1Δ cda2Δ or err1Δ* spores were incubated in 1ml of 1 mM Cr(NO$_3$)$_3$, Ni(NO$_3$)$_2$, Zn(NO$_3$)$_2$, Cd(NO$_3$)$_2$ or Pb(NO$_3$)$_2$ solutions for 5 hours 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 presented are the mean ± SE of three independent experiments. * $P < 0.05$; ** $P < 0.01$; NS, not significant.

**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 3 mM taurocholic acid solution for 2 hours 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) 1 mg of freeze-dried vegetative cells (vege) or wild-type (wt), dit1Δ or chs3Δ spores were incubated in the taurocholic acid solution under the above conditions and residual amounts of bile acids were measured by HPLC. Amounts of taurocholic acid removed from the original solution are shown. Data presented are the mean ± SE of 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 hour, and then incubated in 200 μl of 0.2 mg/ml β-galactosidase solution for 4 hours at 4°C. Amounts of β-galactosidase removed from the original solution are shown. (B) 5 mg of freeze-dried vegetative cells (vege) or wild-type (wt), dit1Δ, chs3Δ,
cda1Δ cda2Δ or crr1Δ spores were treated as above and amounts of β-galactosidase removed by the cells are shown. Data presented are the mean ± SE of three independent experiments. * P < 0.05; ** P < 0.01.

FIG 6 dit1Δ spores can be used to display immobilized β-galactosidase enzymatic activity

(A) Enzymatic activities of β-galactosidase immobilized on 5 mg of freeze-dried vegetative cells (vege) or wild-type (wt), dit1Δ, chs3Δ, cda1Δ cda2Δ or crr1Δ spores are shown. The cells were incubated in 500 μl of 5 mM ONPG solution at 30°C for 30 min and increased amount of o-nitrophenol production was measured by absorbance at 410 nm. 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. (B) dit1Δ or wild-type (wt) spores harboring immobilized β-galactosidase (5 mg) were subjected to repeated enzymatic assays. After the enzymatic activity was measured as above (first time), spores were washed with Z-buffer and the activity was assayed again (second time). This cycle was repeated three times. (C) Activities of soluble or immobilized β-galactosidase on 5 mg of freeze-dried dit1Δ spores were assayed at the indicated temperatures but otherwise under the same conditions as above. The activity obtained at 30°C was determined as 1.0 and relative activities are shown. Data presented are the mean ± SE of three independent experiments. * P < 0.05; ** P < 0.01.
References


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Suda Y, Rodriguez RK, Coluccio AE, Neiman AM. 2009. A screen for spore wall permeability mutants identifies a secreted protease required for proper spore wall


FIG 4
Table 1. *S. cerevisiae* strains used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>AN120</td>
<td>MATα/MATα ARG4/arg4-NspI his3ΔSK/his3ΔSK (wild-type, diploid)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ho::LYS2/ho::LYS2 leu2/leu2 lys2/lys2 RME1/rme1::LEU2 trp1::hisG/trp1::hisG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ura3/ura3</td>
<td></td>
</tr>
<tr>
<td>AN117-4B</td>
<td>MATα ura3 leu2 trp1 his3Δsk arg4-NspI lys2 ho::LYS2 rme1::LEU2</td>
<td>(43)</td>
</tr>
<tr>
<td>AN117-16D</td>
<td>MATα ura3 leu2 trp1 his3Δsk lys2 ho::LYS2</td>
<td>(43)</td>
</tr>
<tr>
<td>AN262</td>
<td>MATα/MATα ARG4/arg4-NspI his3ΔSK/his3ΔSK (ditΔ/ditΔ) ho::LYS2/ho::LYS2</td>
<td>(44)</td>
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<td>leu2/leu2 lys2/lys2 RME1/rme1::LEU2 trp1::hisG/trp1::hisG ura3/ura3</td>
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</tr>
<tr>
<td></td>
<td>chs3Δ::his5+/chs3Δ::his5+</td>
<td></td>
</tr>
<tr>
<td>HW3</td>
<td>MATα/MATα ARG4/arg4-NspI his3ΔSK/his3ΔSK (crr1Δ/crr1Δ) ho::LYS2/ho::LYS2</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>leu2/leu2 lys2/lys2 RME1/rme1::LEU2 trp1::hisG/trp1::hisG ura3/ura3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>crr1Δ::kan'/crr1Δ::kan'</td>
<td></td>
</tr>
<tr>
<td>HW143</td>
<td>MATα/MATα ARG4/arg4-NspI his3ΔSK/his3ΔSK (cda1Δ cda2Δ/ cda1Δ cda2Δ) ho::LYS2/ho::LYS2 leu2/leu2 lys2/lys2</td>
<td>This study</td>
</tr>
<tr>
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<td>RME1/rme1::LEU2 trp1::hisG/trp1::hisG ura3/ura3 cda1Δ::his5'/cda1Δ::his5'</td>
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<tr>
<td></td>
<td>cda2Δ::TRP1/cda2Δ::TRP1</td>
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Table 2. Amounts (moles) of heavy metals adsorbed by freeze-dried spores or vegetative cells\(^a\)

<table>
<thead>
<tr>
<th>Cells</th>
<th>Amounts of heavy metals (nmol / mg cells)(^b)</th>
<th>Cu(^{2+})</th>
<th>Cr(^{3+})</th>
<th>Ni(^{2+})</th>
<th>Zn(^{2+})</th>
<th>Cd(^{2+})</th>
<th>Pb(^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>dit1Δ spore</td>
<td></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>wt</td>
<td></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>
</tr>
<tr>
<td>chs3Δ spore</td>
<td></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>
</tr>
<tr>
<td>cda1Δ</td>
<td></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>
</tr>
<tr>
<td>cda2Δ spore</td>
<td></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>crr1Δ spore</td>
<td></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>
</tr>
</tbody>
</table>

Vegetative cell

\(^a\) These amounts were calculated based on data obtained from the adsorption assays using 6 mg of cells

\(^b\) Values are expressed as mean ± SE (n=3)