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BIOTECHNOLOGY METHODS

Bioconversion of D-glucose to D-psicose with immobilized D-xylose isomerase and D-psicose 3-epimerase on *Saccharomyces cerevisiae* spores

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Abstract *Saccharomyces cerevisiae* spores are dormant cells, which can tolerate various types of environmental stress. In our previous work, we successfully developed biological and chemical methods for enzyme immobilization based on the structures of *S. cerevisiae* spore wall. In this study, we employed biological and chemical approaches for the immobilization of D-xylose isomerase (XI) from *Thermus thermophilus* and D-psicose 3-epimerase (DPEase) from *Agrobacterium tumefaciens* with yeast spores, respectively. The enzymatic properties of both immobilized XI and DPEase were characterized and the immobilized enzymes exhibit higher thermostability, broader pH tolerance, and good repeatability compared with free enzymes. Furthermore, we established a two-step approach for the bioconversion of D-glucose to D-psicose using immobilized enzymes. To improve the conversion yield, a multi-pot strategy was adopted for D-psicose production by repeating the two-step process continually. As a result, the yield of D-psicose was obviously improved and the highest yield reached about 12.0 %.

Keywords *S. cerevisiae* spore · D-Xylose isomerase · D-Psicose 3-epimerase · Enzyme immobilization · D-Psicose synthesis

Introduction

Saccharomyces cerevisiae initiates a sporulation program that leads cell to enter the developmental pathway of meiosis and spore morphogenesis in response to nitrogen starvation and the presence of a nonfermentable carbon source [23, 24]. *S. cerevisiae* spore is a dormant cell, which can resist various types of environmental stress. Compared with vegetative cell wall, *S. cerevisiae* spore wall is more extensive and consists of four morphologically distinct layers. The first and second outermost layers of spore wall are composed of dityrosine and chitosan, which are responsible for the enhanced resistance of spores to many stresses [4, 7, 23, 26]. The most unique feature of the spore wall is the dityrosine layer, which is composed of neither protein nor polysaccharide [5]. Instead, the major composition of this layer is the modified, cross-linked *N*–*N*-bisformyl dityrosine [5, 6]. Our previous study showed that the dityrosine layer of spore wall can hold soluble enzymes and the activity of encapsulated enzyme can be improved in *osw2*Δ mutant due to the minor defects of the dityrosine layer [28]. It was reported that disruption of the *DIT1* gene required for the synthesis of dityrosine layer leads to the loss of this layer but an intact chitosan layer exposed to the outside [16]. Chitosan, a natural polyaminosaccharide, is recommended as an ideal support material for enzyme immobilization, especially for food, pharmaceutical, and medical applications due to its excellent properties, such as non-toxicity, biocompatibility, and biodegradability [1, 27, 31].

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D-Psicose, the C-3 epimer of D-fructose, exhibits special properties of being an ideal sucrose substitute. It is 70 % as sweet as sucrose but produces almost no calories due to its inhibition effect toward hepatic lipogenic enzymes [18, 30]. It has also been discovered that foods containing D-psicose show higher antioxidant activity [30]. The most common method of D-psicose synthesis is through the epimerization of D-fructose at C-3 position catalyzed by D-tagatose 3-epimerase (DTEase, EC 5.1.3.-) family enzymes. Until recently, eleven DTEases from *Pseudomonas* sp. ST-24, *Agrobacterium tumefaciens*, *Rhodobacter sphaeroides* SK011, *Clostridium cellulolyticum* H10, *Clostridium scindens* 35704, *Clostridium bolteae*, *Treponema primitia* ZAS-1, *Ruminococcus* sp., *Mesorhizobium loti*, *Desmospora* sp, and *Clostridium* sp. have been characterized and employed for D-psicose synthesis [9, 11, 12, 20, 21, 32, 33, 36–40]. DTEases from *A. tumefaciens*, *C. cellulolyticum* H10, *C. scindens* 35704, *C. bolteae*, *T. primitia*, *Ruminococcus* sp., *Desmospora* sp, and *Clostridium* sp. are usually renamed as D-psicose 3-epimerase (DPEase) due to their high substrate specificity toward D-psicose. As the epimerization reaction catalyzed by DTEase enzymes is an equilibrium process, thus mass production of D-psicose from D-fructose with a high yield as well as product purification still remains a challenge. To reduce the cost, D-psicose could be prepared through a dual-enzyme coupling reaction directly from D-glucose as the starting material and the conversion yield from D-glucose to D-psicose was about 10 % at equilibrium state [10]. During this process, D-xylose isomerase (XI, EC 5.3.1.5) firstly catalyzes the conversion of D-glucose into D-fructose. Then D-psicose is generated from the intermediate D-fructose catalyzed by DPEase.

In our previous work, we successfully developed two new methods of enzyme immobilization based on the structure of yeast spore wall [28, 35]. For the biological approach, the target enzyme was expressed in sporulating cells and the outmost layer dityrosine can function as a barrier to prevent the diffusion of soluble proteins [29]. Moreover, the enzyme encapsulated in yeast spores acquires resistance to environmental stresses such as proteinase digestion and high temperature. For the chemical approach, the free enzyme was immobilized on the chitosan layer of *dit1*Δ spores to function as "chitosan beads." It is worth noting that the immobilized enzyme on *dit1*Δ spores exhibited higher stability than soluble enzyme and could be utilized repeatedly without significant loss of activity. As a specific example, we employed these two approaches to immobilize XI from *Thermus thermophilus* and DPEase from *A. tumefaciens* on yeast spores, respectively, and developed a simple approach for the bioconversion of D-glucose to D-psicose with immobilized enzymes.

Materials and methods

Microorganisms and culture conditions

The strains used in this study are listed in Table 1. *Escherichia coli* strains DH5α and BL21(DE3) were grown at 37 °C in LB medium (yeast extract 5 g/L, tryptone peptone 10 g/L, NaCl 10 g/L). All *S. cerevisiae* strains used in this study are in the background of fast-sporulating SK-1 strain and were grown at 30 °C in YPAD (yeast extract 10 g/L, peptone 20 g/L, adenine 0.03 g/L, and glucose 20 g/L) or SD media (yeast nitrogen base 6.7 g/L and glucose 20 g/L) with appropriate supplemental amino acids. The sporulation process was performed as previously described [14].

Constructions of recombinant plasmids

Primers and plasmids used in this study are listed in Tables 2 and 3. The *xylA* gene encoding XI was amplified

Table 1 Strains used in this study

Strains	Genotype	Source or reference
BL21(DE3)	F$^-$ *ompT hsdSB*($r_B^- m_B^-$) *gal dcm* (DE3)	Lab stock
AN120(wild-type)	*MATα/MATa ARG4/arg4-NspI* his3ΔSK/his3ΔSK ho::LYS2/ho::LYS2 *leu2/leu2 lys2/lys2 RME1/rme1::LEU2 trp1::hisG/trp1::hisG ura3/ura3*	[24]
HW83(*osw2*Δ)	*MATα/MATa ARG4/arg4-NspI* his3ΔSK/his3ΔSK ho::LYS2/ho::LYS2 *leu2/leu2 lys2/lys2 RME1/rme1::LEU2 trp1::hisG/trp1::hisG ura3/ura3 osw2*Δ*::his5 +/osw2*Δ*::his5+*	[28]
HW3(*dit1*Δ)	*MATα/MATa ARG4/arg4-NspI his3*Δ*SK/his3*Δ*SK ho::LYS2/ho::LYS2 leu2/leu2 lys2/lys2 RME1/rme1::LEU2 trp1::hisG/trp1::hisG ura3/ura3 dit1*Δ*::his5 +/dit1*Δ*::his5+*	[35]

Table 2 Primers used in this study

Primers	Sequences
HXO153	5′-TAAAACTAGTAAGTAAATGGTTTCGTTCAG-3′ (*Spe*I)
xylA-F1	5′-TATACATATGATGTACGAGCCCAAACCGGA-3′ (*Nde*I)
xylA-F2	5′-GCGCCTGCAGATGTACGAGCCCAAACCGG-3′ (*Pst*I)
xylA-R1	5′-TCCGGTTTGGGCTCGTACATGGAAACAGGATTACAGTTTA-3′
xylA-R2	5′-TATAGAATTCTCACCCCCGCACCCCCAGGA-3′(*EcoR*I)
HA-F1	5′-ACCTCCTGGGGGTGCGGGGGGCTGGTTACCCATACGATGT-3′
HA-R1	5′-GCGCGAATTCTCAGCACTGAGCAGCGTAAT-3′ (*EcoR*I)
dpe-F1	5′-TATAGCTAGCATGAAGCACGGTATCT-3′ (*Nhe*I)
dpe-R1	5′-TATAGAATTCTTATCAACCACCTAAAACG-3′ (*EcoR*I)

Table 3 Plasmids used in this study

Plasmids	Descriptions	Source (ref.)
pET-28a	5369 bp, T7 promoter, pBR322 origin, Kan^r	Novagen
pET28a-*xylA*	pET-28a containing *xylA* gene	This study
pRS424-TEF_{pr}	pRS424 plasmid with *TEF* promoter, *TRP1*	[22]
pRS424-TEF_{pr}-*ss-xylA*	pRS424-TEF_{pr} containing *xylA* gene with *ss* sequence	This study
pRS424-TEF_{pr}-*ss-RFP*	pRS424-TEF_{pr} containing *RFP* gene with *ss* sequence	[28]
pRS424-TEF_{pr}-*ss-RFP-xylA*	pRS424-TEF_{pr}-*ss-RFP* containing *xylA* gene at C-terminal of RFP	This study
pRS424-TEF_{pr}-*ss-xylA*-3HA	pRS424-*TEFpr-ss-xylA* with 3 × HA tag at C-terminal of XI	This study
pUC57-*dpe*	pUC57 containing a synthesized *dpe* mutant gene	GenScript
pET28a-*dpe*	pET-28a containing the *dpe* mutant gene for expression of I33L S213C DPEase variant	This study

by PCR using *xylA*-F1 and *xylA*-R2 as primers with the genomic DNA of *T. thermophilus* strain HB8 as the template purchased from ATCC (Manassas, VA). The *xylA* fragment was digested with *Nde*I and *EcoR*I and ligated to pET-28a to obtain the plasmid pET28a-*xylA*. To express XI fused with the signal peptide (first 24 amino acids) from Spr1 under a constitutive *TEF* promoter, the plasmid pRS424-TEF_{pr}-*ss-xylA* was constructed as follows: firstly, the signal sequence (*ss*) fragment from *SPR1* was amplified using HXO153 and *xylA*-R1 as primers with plasmid pRS424-TEF_{pr}-*ss-RFP* as the template [28]. Then *ss-xylA* fusion gene was amplified using *ss* and *xylA*-R2 as primers with plasmid pET28a-*xylA* as the template. Finally, *ss-xylA* PCR product was digested by *Spe*I and *EcoR*I and ligated into pRS424TEF$_{Pr}$ [22] to obtain plasmid pRS424-TEF_{pr}-*ss-xylA*. To construct plasmid pRS424-TEF_{pr}-*ss-xylA-3HA* expressing XI fused with three copies of a hemagglutinin (3 × HA) tag, the 3 × HA fragment was firstly amplified using HA-F1 and HA-R1 as primers with pFA6a-3HA-His3MX6 as a template. Then *ss-xylA-3HA* fragment was amplified using HXO153 and 3HA fragment as primers with pRS424-TEF_{pr}-*ss-xylA* as a template. Next, the *ss-xylA-3HA* fragment was digested with *Spe*I and *EcoR*I, and cloned into pRS424-TEF_{pr} to obtain pRS424-TEF_{pr}-*ss-xylA-3HA*. In order to determine the localization of XI, the plasmid pRS424-TEF_{pr}-*ss-RFP-xylA* was constructed to express the labeled protein monomeric red fluorescent protein (mRFP) fused with N-terminal of XI. The constructions were as follows: firstly, the *xylA* gene was amplified using *xylA*-F2 and *xylA*-R2 as primers with the genomic DNA of *T. thermophilus* HB8 as the template. Next, the *xylA* fragment was digested with *Pst*I and *EcoR*I, and cloned into pRS424-TEF_{pr}-*ss-RFP* to obtain pRS424-TEF_{pr}-*ss-RFP-xylA*. To construct plasmid pET28a-*dpe* expressing I33L S213C double-site DPEase variant from *A. tumefaciens*, the *dpe* gene was amplified with *dpe*-F1 and *dpe*-R2 as primers with plasmid pUC57-*dpe* containing a synthesized *dpe* mutant gene encoding I33L S213C double-site DPEase as a template. The *dpe* fragment was digested with *Nhe*I and *EcoR*I, and ligated into pET-28a. All the recombinant plasmids were transformed into DH5α strain for amplifying and sequencing.

Overexpression and purification of XI and DPEase

The recombinant plasmid pET28a-*xylA* was transformed into *E. coli* BL21(DE3) to overexpress XI. 2 mL overnight culture of *E. coli* BL21(DE3) with the plasmid was inoculated to 100 mL LB medium containing 50 μg/mL kanamycin and cultivated at 37 °C, 200 rpm. When the OD_{600} reached to 0.8, the culture was incubated at 16 °C and IPTG was added at a final concentration of 0.1 mM to induce

the expression of XI for 20 h. Then cells were harvested by centrifugation at 4000×g for 15 min and resuspended into 10 mL Tris–HCl (pH 7.5) buffer (50 mM Tris–HCl, 100 mM NaCl, and 10 mM imidazole). After disrupted by sonication on ice, the cell lysate was clarified by centrifugation at 12,000×*g* for 30 min at 4 °C. The supernatant was loaded onto Ni^{2+}-NTA column equilibrated with Tris–HCl (pH 7.5) buffer (50 mM Tris–HCl, 100 mM NaCl, and 10 mM imidazole). Then the column was washed with the Tris–HCl buffer above. Finally, XI protein was eluted with elution buffer (pH 7.5; 50 mM Tris–HCl, 100 mM NaCl, and 500 mM imidazole). The purified enzyme was concentrated and desalted with Amicon Ultra (10 kDa) with 50 mM Tris–HCl buffer (pH 7.5) and stored at −20 °C. The recombinant plasmid pET28a-*dpe* was transformed into *E. coli* BL21(DE3) to overexpress DPEase, the expression and purification of DPEase were performed with similar method of XI. The purities of the target proteins were determined by 12 % (v/v) SDS-PAGE and the gels were stained by Coomassie Brilliant Blue. The protein concentrations were determined using BCA Protein Assay Kit according to the instructions.

Expression and immobilization of XI on yeast spores with biological approach

The recombinant plasmid pRS424-TEF_{pr}-*ss-xylA* was transformed into *S.cerevisiae* AN120, *osw2*Δ mutant, and *dit1*Δ mutant. The sporulation process was performed with the similar method reported [14]. Then the cells were harvested by centrifugation at 8000×*g* for 5 min and the pellets were washed and resuspended in PBS (pH 7.4) buffer (KH_2PO_4 0.27 g/L, Na_2HPO_4 1.42 g/L, NaCl 8 g/L, and KCl 0.2 g/L). In order to release spores from asci, yeast cells were firstly treated with lyticase (625 U per 1 g wet cells) for 0.5 h at 37 °C. Then cells were washed with PBS buffer and sonicated to completely disrupt the ascal membrane. The purification of spores was performed by Percoll gradient centrifugation as previously reported [2, 13] and freeze dried. In order to visualize the localization of XI by fusion of RFP, the recombinant plasmid pRS424-TEF_{pr}-*ss-RFP-xylA* was constructed and transformed into *S. cerevisiae* AN120, *osw2*Δ mutant, and *dit1*Δ mutant. The sporulation process and preparation of spores were performed as described above. Microscopy images were obtained using laser confocal microscopy system model C2 (Nikon, Tokyo, Japan).

Western blotting

Yeast cells containing pRS424-TEF_{pr}-*ss-xylA-3HA* were firstly cultured overnight in 5 mL of SD (without tryptophan) medium. Then 1 mL of the culture was transferred into 30 mL YPA medium (1 % yeast extract, 2 % peptone, and 2 % potassium acetate) and cultured for 24 h. Next, cells were transferred to 2 % potassium acetate medium for sporulation and spores were purified as previously described. After that, spores were washed with deionized water, suspended in 500 μL of 8 M urea, and disrupted by sonication for 1 h on ice. Finally, cell lysate was centrifuged at 4000×*g* for 5 min and the supernatant containing 50 μg protein was used for SDS-PAGE analysis (5 % stacking gel and 10 % separating gel) and Western blotting analysis. Mouse anti-HA antibody (Sigma-Aldrich, Shanghai, China) was used as primary antibody at a dilution of 1:3000. Goat anti-mouse IgG-horseradish peroxidase (HRP) (Life Science, Shanghai, China) was used as secondary antibody at a dilution of 1:2000. Clarity Western ECL Substrate (Bio-Rad, Shanghai, China) was used to visualize signals and ImageQuant LAS4000 (GE Healthcare Bio-Science, Uppsala, Sweden) was used to obtain images.

Immobilization of DPEase onto *dit1*Δ spores with chemical approach

Freeze-dried *dit1*Δ spores (10 mg) were added into 1.5 mL 50 mM citrate phosphate buffer containing a series of different concentrations of glutaraldehyde from 0.5 % (v/v) to 3.5 % (v/v). The mixtures were incubated into a shaker (200 rpm) at 30 °C for 30 min. Then the activated spores were harvested and washed twice with deionized water. Next, the activated spores suspended in citrate phosphate buffer were mixed with 4 μL purified DPEase enzyme (0.40 mg/mL) and incubated at 4 °C for 4 h. After that, spores immobilized with enzyme were collected by centrifugation and washed with high concentration of salt solution (0.6 M NaCl and 1 % Triton X-100). Finally, the above pellets were washed twice with deionized water to afford the immobilized DPEase on yeast spores. To determine the optimal pH value for cross-linking, the enzyme was immobilized on spores at varying pH values from 3.0 to 8.0. The buffer solutions were as follows: 50 mM sodium acetate/acetic acid buffer (pH 3.0, 4.0, 5.0, and 6.0) and 50 mM phosphate buffer (pH 7.0 and 8.0). To determine the optimal cross-linking time, the reaction was incubated at 4 °C for 1, 2, 3, and 4 h under the optimal pH. To determine the amounts of enzyme immobilized on *dit1*Δ spores, different amounts of freeze-dried spores were activated with 2 % glutaraldehyde. After cross-linking with DPEase under optimal conditions, the residual amounts of enzyme in the supernatants were measured and the amounts of DPEase immobilized on *dit1*Δ spores could be calculated by subtracting the residual amounts of the enzyme from original amounts.

Enzyme assay

The activity of XI was measured in 1.5 mL 100 mM Tris–HCl buffer (pH 7.0) containing 2 g/L D-glucose, 10 mM $MnCl_2$. 5 μL XI free enzyme (0.6 mg/mL) or 10 mg spores containing XI was added to start the reaction. The reaction was performed at 85 °C for 0.5 h and stopped in the boiling water for 10 min [34]. The activity of XI was assayed by HPLC (HITACHI chromaster) under the following conditions: Bio-Rad Aminex HPX-87H column(300 mm × 7.8 mm), 5 mM H_2SO_4 as the mobile phase with a flow rate 0.5 mL/min, column temperature 60 °C, and 5450 RI Detector. The activity of DPEase was measured in 1.5 mL 20 mM Tris–HCl buffer (pH 8.0) containing 2 g/L D-fructose, 1 mM $MnCl_2$. 5 μL DPEase free enzyme (0.6 mg/mL) or 10 mg spores containing DPEase was added to start the reaction. The reaction was performed at 60 °C for 0.5 h and stopped in the boiling water for 5 min [40]. The activity of DPEase was assayed by HPLC (HITACHI chromaster) under the following conditions: Bio-Rad Aminex HPX-87C column (250 mm × 4 mm), H_2O as the mobile phase with a flow rate 0.3 mL/min, column temperature 80 °C, and 5450 RI Detector.

Characterization of free and immobilized enzymes

The effects of temperature on free and immobilized XI were assayed from 60 °C to 95 °C and the reactions were performed at pH 7.0 for 0.5 h. The effects of pH on free and immobilized XI were determined in 100 mM buffer at varying pH values from 5.0 to 9.0 (sodium acetate, pH 5.0 and 6.0; Tris–HCl, pH 7.0, 8.0, and 9.0) under optimal temperature. The reusability of immobilized XI by D-glucose conversion was assayed under the optimal conditions in a continuous cycle. After each cycle, spores were collected by centrifugation and washed with high concentration of salt solution in the presence of detergent (0.6 M NaCl and 0.1 % Triton X-100). According to the same procedure, batched reactions were performed. The effects of temperature on free and immobilized DPEase were assayed from 40 °C to 80 °C and the reactions were performed at pH 7.0 for 0.5 h. The effects of pH on free and immobilized DPEase were determined in 20 mM buffers with varying pH values from 5.0 to 10.0 (sodium acetate, pH 5.0 and 6.0; Tris–HCl, pH 7.0, 8.0, and 9.0; glycine/NaOH, pH 10.0) under optimal temperature. The reusability of immobilized DPEase was assayed by D-fructose conversion under the optimal conditions in a continuous cycle similar with that of XI.

D-Psicose synthesis via a two-step approach with immobilized enzymes on spores

A two-step approach was established for D-psicose synthesis from D-glucose catalyzed by immobilized enzymes on yeast spores. In the first step, the conversion of D-glucose into D-fructose was performed in the first vessel at 85 °C in the presence of 60 mM Tris–HCl buffer (pH 8.0), 3 g/L D-glucose, 5 mM $MnCl_2$, and 20 mg *osw2*Δ spores containing XI. In the second step, the conversion of D-fructose into D-psicose was performed at 60 °C by transferring the supernatant in the first step into the second vessel and mixed with 10 mg *dit1*Δ spores containing DPEase. To determine the optimal reaction time, XI-catalyzed reactions were performed at 85 °C for 1 to 6 h, and then the corresponding supernatants were reacted with DPEase at 60 °C for the same time. In order to obtain the highest yield from D-glucose to D-psicose, a multi-pot method was employed by repeating the two-step method continuously. The process was described as follows: the supernatant after reacted with DPEase in the first cycle was transferred to the first vessel containing *osw2*Δ spores and reacted at 85 °C for 5 h, then the supernatant was transferred to the second vessel containing *dit1*Δ spores and reacted at 60 °C for 5 h. The two-step process was repeated for five cycles and the reactions were monitored by HPLC as previously described.

Results

Expression and immobilization of XI on yeast spores with biological approach

In order to determine which kind of spores were the most appropriate for the expression of XI from *T. thermophilus*, XI was expressed in AN120 wild-type spores, *osw2*Δ spores, and *dit1*Δ spores, respectively. Then the activities of XI on different spores were assayed. The results showed that *dit1*Δ spores exhibited the highest activity among three different yeast spores and *osw2*Δ spores had higher activity than wild-type spores which was consistent with our previous results [28] (Fig. 1a). To determine the localization of XI on different yeast spores, the labeled protein mRFP fused with N-terminal of XI was constructed and expressed in spores. Under laser confocal microscopy, clear fluorescence signals were all observed at the periphery of wild-type, *osw2*Δ, and *dit1*Δ spores (Fig. 1b), which indicated that the target proteins were all localized to yeast spore wall. To further investigate the expression levels and stabilities of XI on different spores, Western blot analysis of spores expressing 3 × HA-tagged XI was performed. As shown in Fig. 1c, XI was successfully expressed in three different yeast spores. The expression levels of XI in wild-type and *osw2*Δ spores were very similar, while the expression level in *dit1*Δ spores was much lower than wild-type and *osw2*Δ spores. Moreover, the amounts of XI retained on the spore wall of wild-type, *osw2*Δ, and *dit1*Δ spores after washing with high salt solution were also examined by

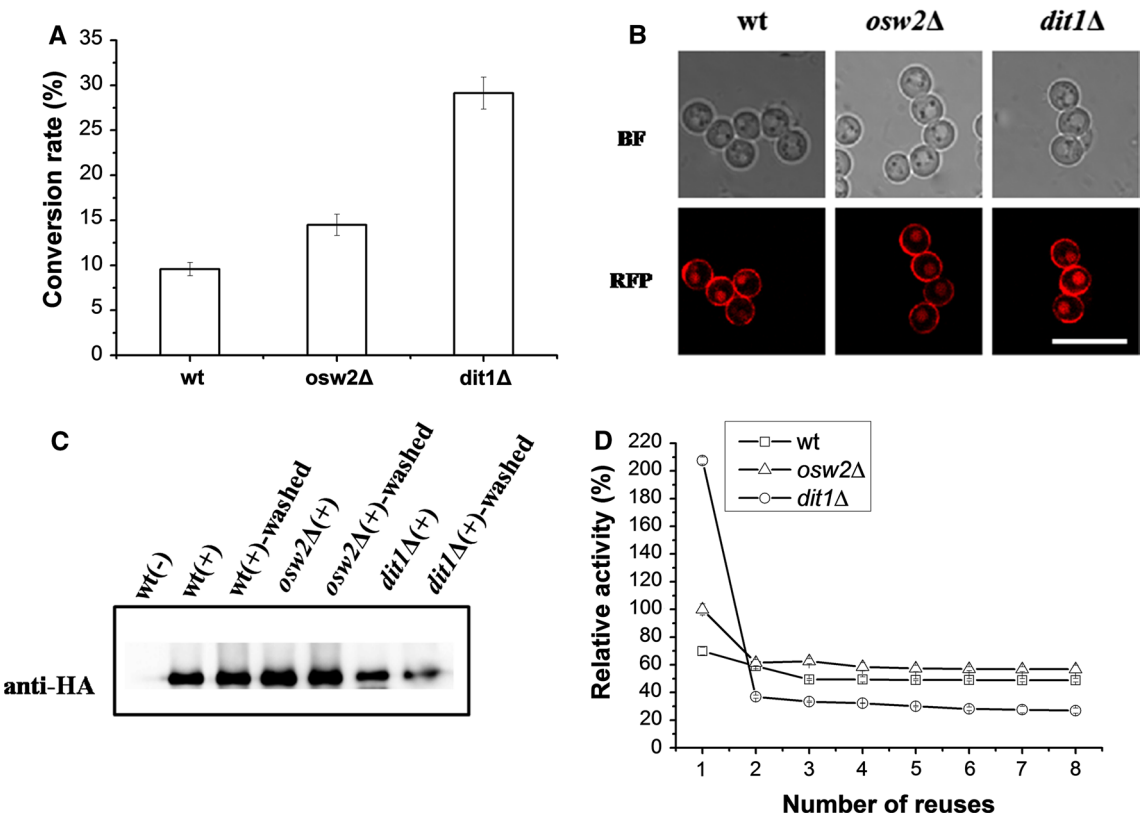


Fig. 1 **a** Comparison of the activities of XI immobilized on wild-type (wt), *osw2*Δ, and *dit1*Δ spores. Data presented are the mean ± SE of three independent experiments. **b** Localization analysis of RFP-XI fusion protein expressed in wt, *osw2*Δ, and *dit1*Δ spores. Spores were observed under bright-field (BF) or laser confocal (RFP) microscopy. *Bar* 10 μm. **c** Western blot analysis of the expression levels and stabilities of XI in wt, *osw2*Δ, and *dit1*Δ pure spores expressing XI-3HA fusion proteins before and after washing with 0.6 M NaCl solution containing 0.1 % Triton X-100. Wild-type spores containing empty vectors were used as a control. **d** Comparison of the repeatability of XI immobilized on wt, *osw2*Δ spores, and *dit1*Δ spores. The relative activity of *osw2*Δ spores for the first use was defined as 100 %. Data presented are the mean ± SE of three independent experiments

Western blot. As a result, the amounts of protein in *dit1*Δ spores were obviously decreased after washing. However, the amounts of protein in wild-type and *osw2*Δ spores were very stable and no significant decrease was observed. In order to investigate the repeatability of immobilized XI on different spores, spores were collected and washed with high salt solution after reactions and the activities were re-examined. It was observed that the repeatability of *dit1*Δ spores was the worst, while wild-type and *osw2*Δ spores showed good repeatability (Fig. 1d). As *osw2*Δ spores showed higher activity than wild-type spores (Fig. 1d), *osw2*Δ spores were chosen as the best carrier for XI expression and immobilization in the following study.

Biochemical characterization of free and immobilized XI

The effects of temperature on the activities of both free and immobilized XI (XI immobilized on *osw2*Δ spores) were firstly examined at a series of temperatures from 60 to 95 °C. It was reported that free XI exhibited the highest activity at 85 °C [34]. From Fig. 2a, we could observe that both free and immobilized XI showed the highest activity at 85 °C. It is worth noting that immobilized XI still had 60 % of the highest activity at a high temperature of 95 °C. The effects of pH on the activities of both free and immobilized XI were also investigated. Both free and immobilized XI had highest activity at pH 7.0 (Fig. 2b). Compared with the free enzyme, immobilized XI could tolerate a broader pH values and catalyze the reaction efficiently at pH between 6.0 and 9.0. Moreover, the immobilized enzyme still exhibited 70 % of the maximum activity at pH 9.0, while the free enzyme only had 20 % of the highest activity at this pH value.

The immobilization of DPEase on yeast spores by chemical approach

To immobilize DPEase on yeast spores by chemical approach, firstly DPEase from *A. tumefaciens* was

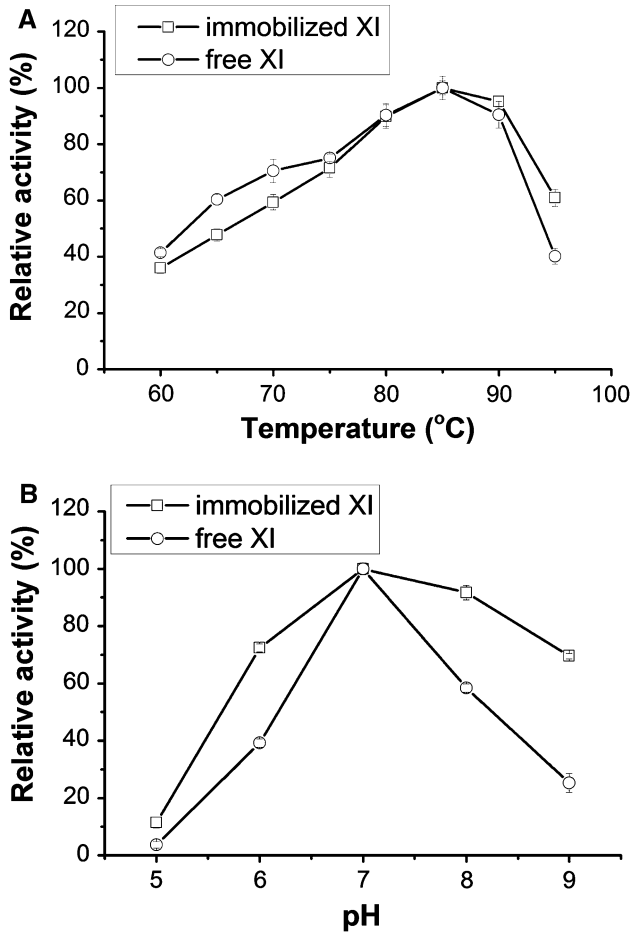


Fig. 2 The effects of temperature (**a**) and pH (**b**) on the activities of free XI expressed and purified from *E. coli* BL21(DE3) and immobilized XI on *osw2*Δ spores. Data presented are the mean ± SE of three independent experiments

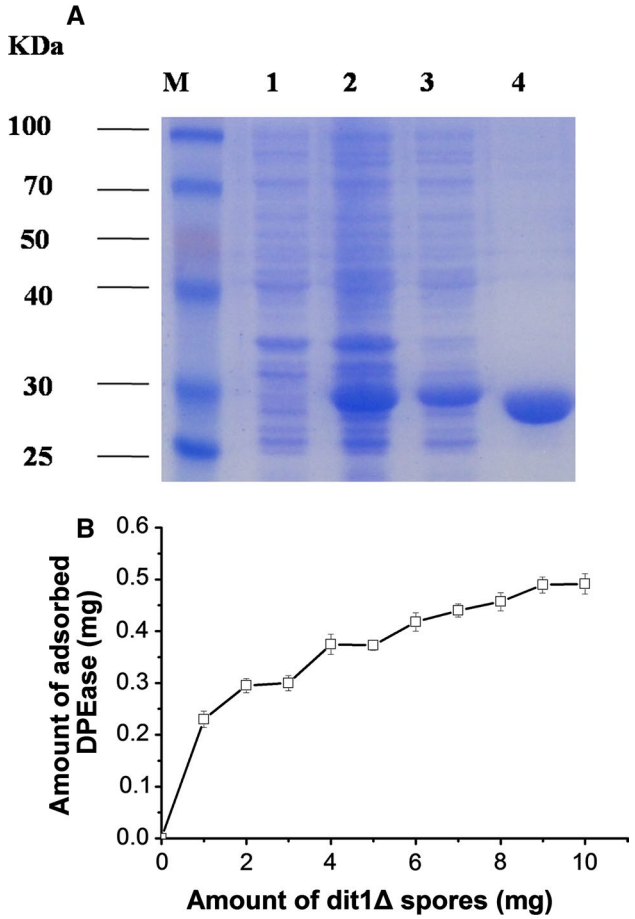


Fig. 3 **a** SDS-PAGE analysis of DPEase expression and purification. *Lane M* marker, *lane 1* total cell protein not induced, *lane 2* total cell protein after IPTG induction, *lane 3* supernatant of the cell lysate, *lane 4* purified DPEase. **b** Measurement of the amounts of enzyme immobilized onto *dit1*Δ spores. The amounts of enzyme could be calculated by subtracting the residual amounts in the solution from original amounts. Data presented are the mean ± SE of three independent experiments

heterologously expressed in *E. coli* BL21(DE3). After purification with Ni^{2+}-NTA column, the purity of the DPEase was above 90 % and the molecule weight of the enzyme was consistent with the theoretical value according to SDS-PAGE analysis (Fig. 3a). After *dit1*Δ spores were activated by glutaraldehyde, free DPEase was immobilized on the activated spores by cross-linking. To immobilize DPEase on spore wall as much as possible, it is necessary to optimize the concentration of glutaraldehyde and other immobilization conditions, such as pH value and absorption time. The results showed that the optimal concentration of GA was 2 % and the optimal pH value for immobilization was 5.0 (Data not shown), respectively. However, the effect of adsorption time on the immobilization was not obvious (Data not shown). As shown in Fig. 3b, *dit1*Δ spores can immobilize substantial amounts of DPEase under optimum conditions.

Biochemical characterization of free and immobilized DPEase

The temperature effects on the activities of both free and immobilized DPEase were firstly studied at a series of temperatures from 40 °C to 80 °C. It was observed that both enzymes demonstrated the highest activities at 60 °C (Fig. 4a). Compared with the free enzyme, the immobilized enzyme could tolerate a broader temperature range and the relative activity at 75 °C remained about 70 % of the maximum activity. However, free DPEase almost lost its activity at such high temperature. The effects of pH on the activities of both free and immobilized DPEase were also examined. The results showed that the optimal pH value for the

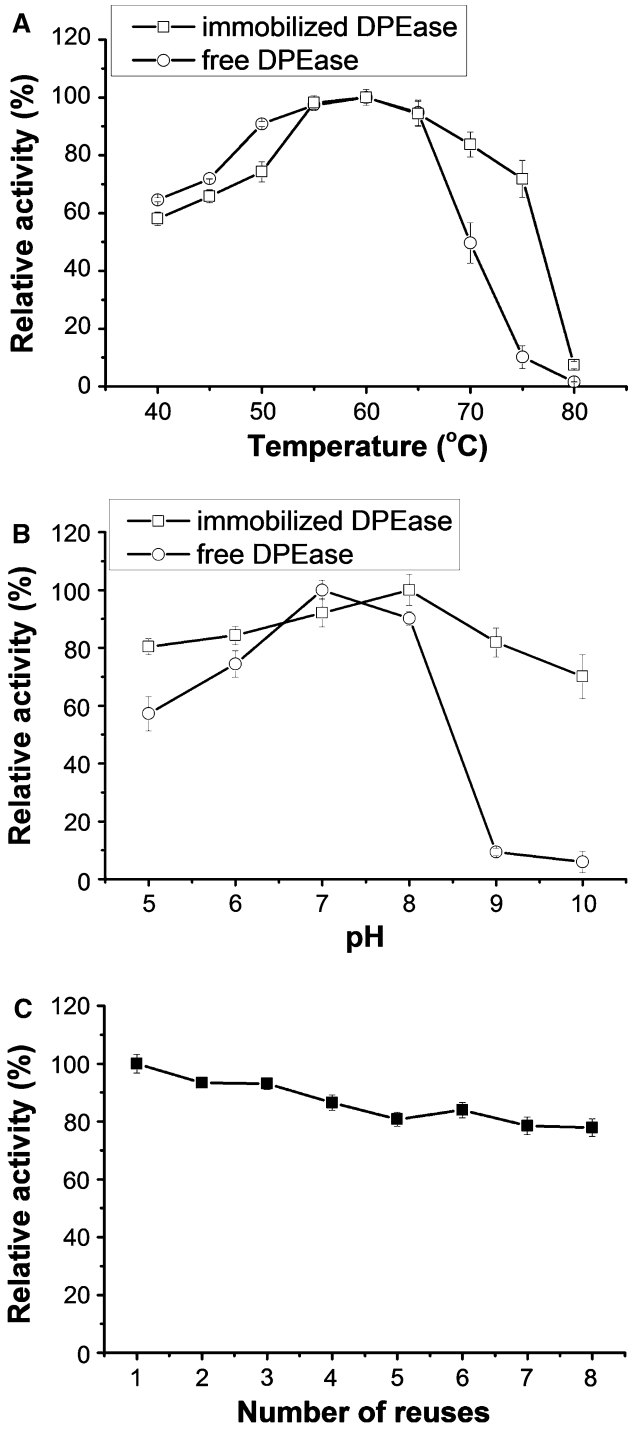
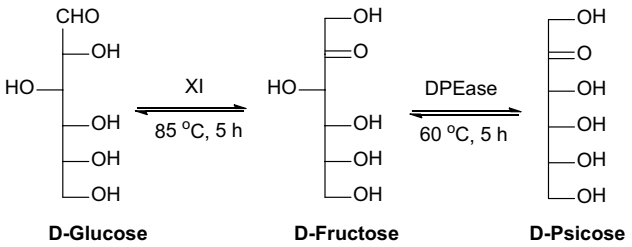


Fig. 4 The effects of temperature (**a**) and pH (**b**) on the activities of free DPEase and immobilized DPEase on *dit1*Δ spores. Data presented are the mean ± SE of three independent experiments. (**c**) Analysis of the reusability of immobilized DPEase on *dit1*Δ spores. Relative activity was defined as the relative value to the maximum enzyme activity (first assay). Data presented are the mean ± SE of three independent experiments



Scheme 1 D-psicose synthesis via a two-step approach with immobilized enzymes on spores

immobilized enzyme was around 8.0, which was slightly higher than that of free enzyme (pH 7.0) (Fig. 4b). In contrast to the free enzyme, the immobilized enzyme showed a broad pH tolerance and catalyzed the reaction efficiently at pH between 5.0 and 9.0. The immobilized enzyme still exhibited 70 % of the maximum activity at pH 10.0, while the free enzyme almost lost its activity completely at this alkaline pH value. To investigate the reusability of the immobilized DPEase, the batch reactions were repeatedly performed. The results clearly showed that the immobilized DPEase exhibited good reusability as the enzymatic activity was kept approximately 80 % even after eight cycles (Fig. 4c).

D-Psicose synthesis via a two-step approach with immobilized enzymes on spores

As described above, the optimal temperatures for immobilized XI and DPEase were 85 °C and 60 °C, respectively, which were very different from each other. Thus, one-pot two-enzyme synthesis of D-psicose from D-glucose may be inefficient. Instead, a two-step approach for D-psicose production was established in our study (Scheme 1). In the first step, the conversion of D-glucose into D-fructose was performed at 85 °C, pH 8.0 in the presence of *osw2*Δ spores containing XI. In the second step, the conversion of D-fructose to D-psicose was realized by transferring the supernatant of reaction mixture in the first step into a vessel and reacted with *dit1*Δ spores containing DPEase at 60 °C. Our results demonstrated that the highest conversion rate of D-psicose from D-glucose was about 5 % when both reaction steps were performed for 5 h, respectively (Fig. 5a). Considering the low yield of two-step method, a multi-pot strategy was employed for D-psicose production by repeating the two-step process continually. As a result, the yield of D-psicose was obviously improved and the highest conversion rate was about 12.0 % (Fig. 5b).

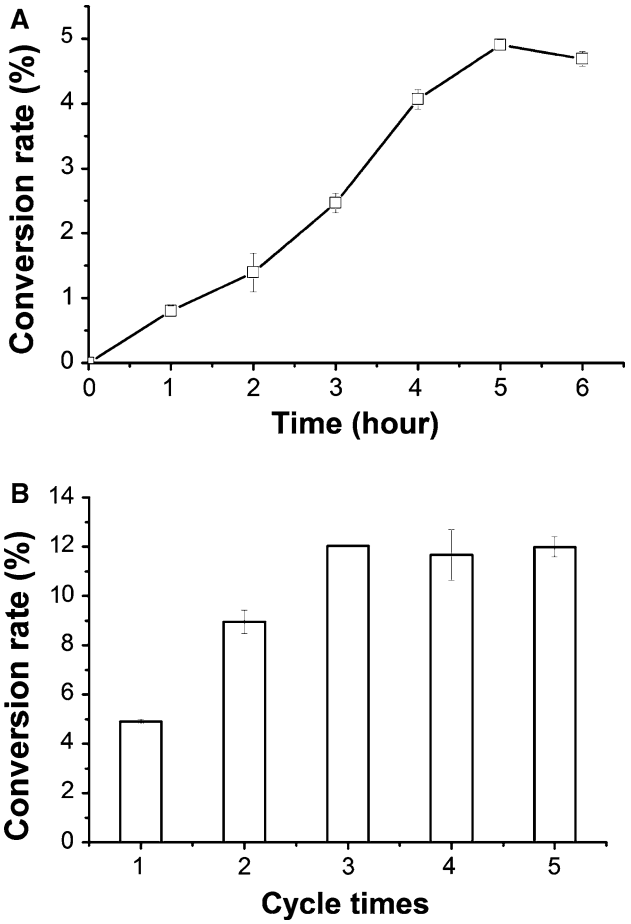


Fig. 5 **a** The time course of the conversion from D-glucose to D-psicose via a two-step approach. **b** The multi-pot strategy could improve the conversion rate of D-psicose by repeating the two-step process continually. Data presented are the mean ± SE of three independent experiments

Discussion

XI is a key enzyme in xylose metabolism which is industrially important for the conversion of glucose (xylose) into fructose (xylulose), respectively. In order to endow *S. cerevisiae* with the ability to utilize xylose, expressions of many different sources of XIs were widely investigated. However, only a few XIs could be expressed in *S. cerevisiae* with active forms [3, 15, 17, 25, 34]. Among the many different sources of XIs, XI from *T. thermophilus* was firstly expressed in *S. cerevisiae* with an active form. In this study, XI from *T. thermophilus* was successfully immobilized on yeast spores in a biological way based on the microencapsulation technology we previous established [28]. One prominent advantage for this approach is that soluble enzymes secreted from yeast cells could be biologically immobilized on spore wall and avoid the purification and cross-linking of the target enzyme. In order to evaluate which kind of spores are the best choice for XI encapsulation, a secretory form of XI was expressed and immobilized on wild-type, *osw2*Δ and *dit1*Δ spores, respectively. We found that the XI activity in *dit1*Δ spores was the highest among three different spores. It is possible that the dityrosine layer could be an obstacle for substrates to interact with the enzymes, especially for the macromolecule substrates. The high activity of XI in *dit1*Δ spores could be attributed to the speculation that the substrate accessibility to the enzyme was improved by removal of the dityrosine layer. Besides *dit1*Δ spores, we found that the activity of XI expressed in *osw2*Δ spores had higher activity than wild-type spores. The possible reason was that the loose dityrosine layer of *osw2*Δ spores facilitated the reaction between the substrate and the enzyme.

The localization of XI on different yeast spores was determined by observing the fluorescence signals of RFP-XI fusion protein under laser confocal microscopy. Unexpectedly, RFP-XI fusion protein could also be localized to the *dit1*Δ spore wall without the outmost dityrosine layer similar with wild-type and *osw2*Δ spores. The possible reason was that the chitosan layer was able to entrap RFP-XI to some extent because of some particular properties of XI, which could associate with chitosan layer by electrostatic interactions in a way similar with α-galactosidase we previously studied [28]. According to the Western blot analysis, the expression level of XI in *dit1*Δ spores was the lowest among three different structures of yeast spores and the amounts of protein were obviously decreased after washing with high salt solution, which could be explained by the speculation that the outmost dityrosine layer of spore wall could function as a diffusion barrier and *dit1*Δ spore wall without dityrosine layer leads to the leakage of protein from spore wall. In contrast, for wild-type and *os*w2 spores, the expression levels of XI were very similar and no significant differences were observed before and after washing. This suggested that *osw2*Δ spores could efficiently prevent the release of XI from spore wall, although the dityrosine layers may have some minor defects.

It seems that *dit1*Δ spores are the best carrier for XI immobilization, since the activity was the highest. However, XI immobilized on *dit1*Δ spores showed the worst repeatability, which is a very important feature for immobilized enzyme. The bad repeatability of *dit1*Δ spores could attribute to the fact that spores without dityrosine layer could not efficiently retain enough enzymes and high reaction temperature (85 °C) aggravated the release of XI from spore wall. Therefore, XI immobilized on *dit1*Δ spores was not ideal for synthetic purpose. In contrast, wild-type and *osw2*Δ spores showed good repeatability. As *osw2*Δ spores showed higher activity than wild-type spores, *osw2*Δ spores were chosen as the ideal material for XI immobilization and the enzymatic properties were determined

accordingly. Compared with the free enzyme, the pH and temperature stabilities of the immobilized enzyme were obviously improved.

It was reported that the thermostability of DPEase from *A. tumefaciens* could be obviously improved by using I33L S213C variant [8]. Thus, a *dpe* mutant gene encoding I33L S213C double-site DPEase variant was synthesized with codon optimization for improved expression in *S. cerevisiae*. We firstly attempted to express and immobilize DPEase on yeast spores with biological approach similar with that of XI. Unfortunately, we failed to express DPEase with an active form. As the post-translational nascent protein in yeast cell was matured after a series of modification in endoplasmic reticulum and Golgi apparatus, this process may result in the inactivation of DPEase. To eliminate the glycosylation effects on the activity of DPEase, two asparagines of potential N-glycosylation sites were substituted with glutamine by site-directed mutagenesis, but mutant DPEase still showed no activity. In our previous study, β-galactosidase was successfully immobilized on the chitosan layer of *dit1*Δ spores by chemical method [35]. In contrast to chitosan chemically obtained, *dit1*Δ spores could function as natural chitosan beads, which could be easily obtained with low cost. Therefore, we overexpressed and purify DPEase I33L S213C variant in *E. coli* BL21 (DE3) and adopted the chemical approach for DPEase immobilization on *dit1*Δ spores activated by a cross-linking agent glutaraldehyde. Although free and immobilized DPEase both showed the highest activity at 60 °C, the thermostability of the immobilized enzyme was obviously improved. Moreover, compared with the free enzyme, the optimal pH value of immobilized enzyme was shifted from 7.0 to 8.0 and pH tolerance was greatly improved. Importantly, immobilized DPEase showed good reusability, as the activity was not significantly decreased even after eight cycles.

As the optimal temperatures for immobilized XI and DPEase on yeast spores were not consistent, a two-step approach for D-psicose production from D-glucose was developed based on immobilized enzymes. To improve the conversion yield of D-psicose, a multi-pot strategy was employed by repeating the two-step process continually and the highest yield reached about 12.0 % improved by 2.4-fold, while the conversion rate of D-psicose from D-glucose using free enzymes was 6 %. Importantly, the XI and DPEase enzymes immobilized on yeast spores could be easily harvested and reused for five cycle times without significant loss of activity. However, the yield was still low for D-psicose production in large scale compared with that of one-step reaction catalyzed by DPEase free enzyme using D-fructose as the substrate [21, 38, 39]. In contrast, Sun and coworkers developed a one-step process for D-psicose production from D-glucose via co-expression of XI and DPEase in *E. coli* with a conversion yield of 16 % under optimal conditions [19]. It should be mentioned that, although spores germinate in the presence of glucose, the germination process was actually prevented by performing the reactions at high temperature in our study. In addition, several yeast mutants incapable of germination were isolated in our laboratory (unpublished results). Nevertheless, it would be significant to find a convenient method to handle this process for practical applications. Moreover, the cross-linking agent glutaraldehyde is a cytotoxic chemical, which is not safe for the production of D-psicose. Therefore, food-grade cross-linking agents instead of glutaraldehyde are on the way.

Conclusions

D-Xylose isomerase from *T. thermophilus* and D-psicose 3-epimerase from *A. tumefaciens* were successfully immobilized on *S. cerevisiae* spore by using biological and chemical methods based on the unique structures of *S. cerevisiae* spore wall, respectively. In contrast to free enzymes, the immobilized enzymes exhibit favored enzymatic properties, such as higher thermostability, broader pH tolerance, and good repeatability. Furthermore, we established a two-step approach for the economic production of D-psicose from D-glucose using immobilized enzymes. In addition, a multi-pot strategy was adopted to improve the conversion yield of D-psicose by repeating the two-step process continually. To further improve the yield and productivity of D-psicose, it is necessary to obtain higher activity enzyme by reforming the structure of protein molecule or screening other new enzymes. In the meantime, bioreactor technology can also be employed to achieve automatic and continuous reaction.

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Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

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