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ORIGINAL ARTICLE

Display of a sucrose isomerase on the cell surface of *Yarrowia lipolytica* for synthesis of isomaltulose from sugar cane by-products

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Abstract

Isomaltulose (α-D-glucopyranosyl-1,6-D-fructofuranose) is an important industrial and raw food material, which can be synthesised from the by-products of sugar cane processing through sucrose isomerization conversion. In this study, we constructed a surface display vector of sucrose isomerase from *Pantoeadispersa* (*p*SIase) by a glycosylphosphatidylinositol (GPI)-cell wall protein (CWP) anchor signal sequence and successfully displayed *p*SIase on the cell surface of *Yarrowia lipolytica,* thereby increasing the conversion efficiency of isomaltulose. The highest activity of the displayed *p*SIase reached 2910.3 U/g of cell dry weight. Compared with the free *p*SIase, the displayed enzyme showed good stability at a broad range of temperatures (20–45 °C). The half-life at 40 °C increased from 62 to 141 min and the deactivation constants (k_d) reached 4.91×10^{-3} min^{-1}. Using low-cost cane molasses as the substrate, the isomaltulose conversion rate remained at 85% even after 9 batches were processed, which is a highly desired outcome for industrial use.

Keywords Sugar cane · Isomaltulose · Sucrose isomerase · Cell surface display

Introduction

Sugar cane is one of the most important crops in the world and a major source of biofuels and sugar production. In Brazil, the first and second generation ethanol produced from sugar cane has a mature commercial market (Maeda et al. 2013). Recently, bioconversion of isomaltulose from cane molasses has drawn wide attention. Isomaltulose (also known as palatinose) is a well-known carbohydrate that releases glucose into blood at a slow rate, thus avoiding a sudden peak and sudden drop in glucose levels (Fleddermann et al. 2016; Hamada 2002). Isomaltulose is often used as an energy bar material for astronauts and special workers due to its more balanced and longer energy supply. More importantly, isomaltulose has accessible carbonyl groups and is preferred as a renewable raw material in the manufacture of bioproducts such as polymers and surfactants over petroleum-based materials (Cartarius et al. 2001).

Both isomaltulose (a-D-glucosylpyranosyl-1,6-D-fructofuranose) and trehalulose (a-D-glucosyl-pyranosyl-1,1-D-fructofuranose) are structural isomers of sucrose. Isomaltulose is 30% as sweet as sucrose and can be readily crystallized for use as a solid additive (Cheetham et al. 1982) whereas trehalulose is extremely water-soluble (Ooshima et al. 1991) and can only be used in highly sweetened foods such as jellies and jams which require highly concentrated sugar solutions. Currently, more than 90% isomaltulose is converted from sucrose using microbial cells and enzymes. Microbial conversion has been accomplished with either free or immobilized cells (Ahn et al. 2003; Kawaguti et al. 2007). However, two key factors limit microbial conversion: (1) biosafety of some strains is unclear. For instance, *Klebsiellapneumonia* (Aroonnual et al. 2007), *Pantoeadispersa* (Wu and Birch 2004) and *Serratiaplymuthica* (Véronèse and Perlot 1999) are considered to be potential pathogens. Therefore, isomaltulose produced by such microbes can not be used in the food industry (2) the isomaltulose conversion ratio is between 60 and 90% and it is not only difficult to

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separate isomaltulose from the other contaminating microbial metabolites, but the yields and recovery of the desired isomer is also lower (Salvucci et al. 2003; Véronèse and Perlot 1999). Recently, sucrose isomerase (SIase) has been used in industries for the production of isomaltulose. Many SIase have been purified and characterized, including the natural form of enzymes from *Erwiniarhapontici* (Li et al. 2011), *Pantoeadispersa* (Wu and Birch 2004) as well as the recombinant enzymes from *Enterobacter* sp. (Cha et al. 2009), *Protaminobacterrubrum* (Lee et al. 2008) and *Pseudomonasmesoacidophila* (Watzlawick and Mattes 2009). Enzymes have remarkable catalytic reactions and substrate specificity compared to microbial conversion as neither the substrate nor the products have to cross the cell membrane barrier. In some cases biocatalytic methods use purified enzyme which are time consuming and expensive. Moreover, the enzyme preparation can only be used once for the desired reaction and is subsequently discarded as waste.

Yeast surface display is an extension of the enzyme application technique. One of the most attractive features of surface display is that enzyme molecules are simultaneously synthesized and self-immobilized on the microbial cell surface and the living whole-cell biocatalyst is easily produced by microbial cultivation techniques (Chen et al. 2012; Hiraishi et al. 2012; Ueda and Tanaka 2000). No further work is required to either purify or immobilize the enzymes. Recently, lipase, glucosidase, amylases, cellulases, xylanases and other enzymes have been successfully immobilized on yeast cells and their potential applications have been evaluated (Adachi et al. 2013; Schüürmann et al. 2014; Yue et al. 2008; Yuzbasheva et al. 2015). Inspired by such works we constructed a surface display vector for sucrose isomerase from *P. dispersa* (*p*SIase) by glycosylphosphatidylinositol (GPI)-cell wall protein. *p*SIase is an excellent glycosylated transferase from *P.dispersa* as it shows a high activity and low Km (40 mM) toward surcose compared to other SIase (Cha et al. 2009; Lee et al. 2008; Wu and Birch 2004, 2005). After 96 h cultivation, the *p*SIase was successfully displayed on the cell surface of *Yarrowialipolytica*. This dispalyed *p*SIase shows a high thermal stability and activity compared to the free *p*SIase. Using *Y.lipolytica* harboring displayed *p*SIase as whole-cell biocatalysts, cane molasses can be efficiently converted to the isomaltulose. Our study suggests that cell surface display is a valuable method in isomaltulose systhesis.

Materials and methods

Materials

pMD19-T vector was purchased from TaKaRa (Japan). The surface display vector pINA1317-CWP110, which contains the C-terminal end of CWP110 from *Y.lipolytica*, was donated by ZM Chi. The *Y.lipolytica* yeast strain used for cell surface display was Po1h (genotype: *MatA*, *ura3*-302, *xpr2*-322, *axp1*-2; phenotype: *Ura* − , *AEP*, *AXP*, *Suc* +). In order to produce displayed protein by yeast transformants, the phosphate vitamin B (PPB) medium described by Jolivalt (2005) was used. Sucrose, isomaltulose and trehalulose were supplied by Sangon Inc (Shanghai, China). Cane molasses, the by-product of the sugar cane industry, was obtained from Media Sugar Refinery (Qingdao, China).

Display *p*SIase on the cell surface of *Y. lipolytica*

A forward primer S1: 5′-GGCCGTTCTGGCCGCCAG CCCGTTAACTAAGCCATCGA-3′ (underlined bases encode *Sfi*I restriction site) and a reverse primer P15′-GGATCCTCAGTTCAG CTTATAGATCCCGGCTTG -3′ (underlined bases encode *BamH*I restriction site) were designed to amplify the *p*SIase gene from *P.dispersa* (ATCC14589, GenBank accession number AY223549.1) using PCR. Gene amplification and expression were performed as described by Yue et al. (2008). The resulting plasmid carrying *p*SIase gene was designated as pINA1317-CWP110-*p*SIase (Fig. 1). The positive transformants carrying *p*SIase gene were grown in PPB liquid medium for 96 h. The cells of *Y.lipolytica* Po1h carrying only the yeast cassette without *P*SIase gene were used as controls.

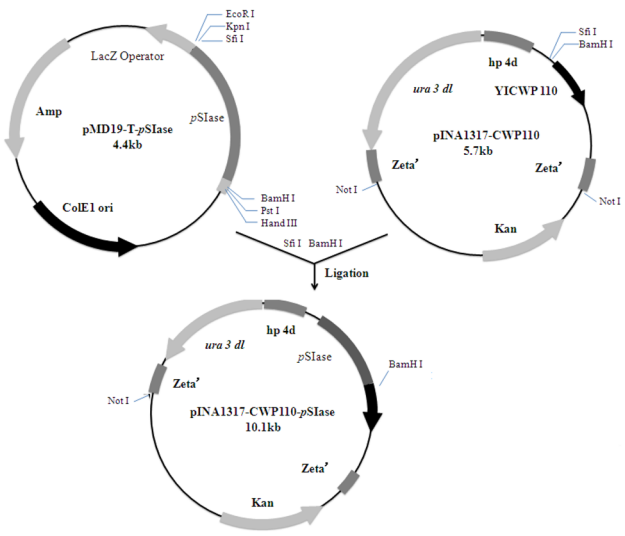

Fig. 1 Construction of the plasmid pINA1317-YlCWP110-*p*SIase for surface display on *Y. lipolytica* cells

Determination of *p*SIase activity

The fermentation broth (1000 mL) was centrifuged at 9,000 rpm for 5 min at 4 °C, and the cell pellet was rinsed with deionized water and resuspended in 100 mL 50 mM sodium acetate buffer (pH 6.0). The activity of *p*SIase was measured at 30 °C in 50 mM sodium phosphate buffer (pH 6.0) with 10% sucrose solutions as a substrate. Specifically, 100 µL wet cells solution was mixed with 900 µL substrate solution and incubated for 15 min. The reaction was stopped by boiling the mixture in a 100 °C water bath for 5 min. Resolution and quantification of sucrose, isomaltulose and trehalulose were achieved by High-Performance Liquid Chromatography (HPLC) system equipped with a Thermo Hypersil APS2 NH2 (150 × 4.6 mm) and Refractive Index (RI) detector. The mobile phase was acetonitrile–water (80:20), the flow rate was 0.8 mL/min and the column and detector temperatures were 30 °C. Sucrose, isomaltulose and trehalulose were used as standards (25 mg/L). One unit of *p*SIase activity is defined as the amount of cell dry weight that produces 1 µmol of isomaltulose per min under the conditions described above.

Thermal characterization of the displayed *p*SIase

The optimal temperature for displayed *p*SIase was determined in the temperature range between 20 and 45 °C. Temperature stability of the displayed *p*SIase was determined by pre-incubating the enzyme for 60 min at different temperatures ranging from 20–45 °C and the residual activity was measured immediately as described above. The deactivation constants (k_d) was calculated from the semi-logarithmic plot of residual activity versus time (Eq. 1), where E_t is the residual enzyme activity after heat treatment for time, E_0 is the initial enzyme activity before heat treatment. The half-lives of the free and displayed *p*SIase were calculated using Eq. 2.

$$\ln\left[E_t/E_0\right] = -k_d t, \tag{1}$$

$$t_{1/2} = \ln 2/k_d. \tag{2}$$

Pretreatment of cane molasses

The cane molasses an important by-product of the cane sugar industry contains 45.6% (w/w) sucrose, 4.9% (w/w) glucose, 6.3% (w/w) fructose, 2.5% (w/w) other carbohydrates, 4.2% (w/w) crude protein, 0.1% (w/w) crude fat, 8.3% (w/w) ash, 7.9% (w/w) metal ions (e.g., calcium, potassium, sodium, iron, magnesium and copper) and 20.3% (w/w) water. Cane molasses was diluted with distilled water to obtain a total sugar concentration of 200 g/L. In order to promote colloid, protein flocculation and precipitate of the divalent metal ions, its pH was adjusted to 3.5 with 5 M H_2S0_4. Then, 5% (w/v) activated carbon powder was added to the acidic cane molasses for decolorization, followed by incubation at 65 °C for 1 h after which the activated carbon was removed by filtration and the pH adjusted to 6.0–6.5 with 10 M NaOH.

Reusability of the displayed *p*SIase using cane molasses as substrate

A batch reaction was performed with 30 ml wet cells (approximately 8500 U SIase) mixed with 200 mL pretreated cane molasses and the necessary nutrients for *Y.lipolytica* (5 g/L yeast extract, 10 g/L tryptone) in a 500 mL flask. The reactions were incubated in a shaking bath at 30 °C until the substrate was completely catalyzed to products. Then, the products were washed with deionized water after each batch. All samples of each batch were analyzed by HPLC.

Results

Cell surface display of *p*SIase

CWP110 is a gene isolated from *Y.lipolytica*, which encodes GPI-cell wall protein and promotes the immobilization of the targeted protein on the yeast cell surface (Yue et al. 2008). In order to anchor *p*SIase to the cell surface, the gene encoding *p*SIase (1731 bp in length without the signal sequence) was fused with the vector pINA1317-CWP110. The constructed pINA1317-CWP110-*p*SIase was transfered into *Y.lipolytica*, followed by cultivation in the PPB medium for 96 h. The positive transformants would carry CWP110-*p*SIase while the control cells would carry only CWP110. The correlation of the culture time with the displayed *p*SIase activity and cell growth was investigated (Fig. 2). The displayed *p*SIase activity began to increase at 36 h and reached to a peak value at 82 h. The biomass of the cell reached 17 g dry weight/L and the *p*SIase activity reached 2910.3 U/g of cell dry weight. Among the tested transformants, the displayed *p*SIase activity varied significantly due to the differences of the copy numbers within the integrated cassette. The highest SIase activity transformants was then chosen for further cultivation.

Thermal characterization of the displayed *p*SIase

The optimal temperature and thermostability of the displayed *p*SIase were determined. The displayed *p*SIase showed optimal activity at 30 °C with the performance being the same as the free *p*SIase (Fig. 3a). However, the thermostability of the displayed *p*SIase was higher than that of the free enzyme in the range of 25–45 °C. As shown in

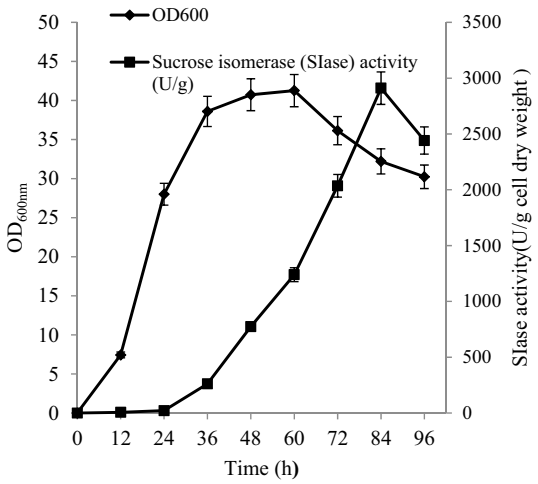

Fig. 2 The growth curve and producing enzyme curve of *Y. lipolytica* harbing *p*INA1317-CWP110-*p*SIase. Data are given as the mean ± SD, $n = 3$

Fig. 3b, the displayed *p*SIase retained approximately 52% of its enzyme activity upon incubation at 45 °C for 1 h, whereas the free *p*SIase lost 71% of its hydrolytic activity at the same condition.

The half-life of the *p*SIase activity was determined at a constant temperature. As shown in Fig. 4a, the residual enzyme activity of the free *p*SIase dropped to about 22% of the original after 120 min incubation at 40 °C. In contrast, the displayed *p*SIase retained approximately 61% of its enzyme activity under the same conditions. The deactivation constants (k_d) for the first-order thermal deactivation were calculated using Eq. (1). The k_d of the displayed *p*SIase at 40 °C was 4.91×10^{-3} min^{-1} in comparison with $k_d = 1.12 \times 10^{-2}$ min^{-1} of free *p*SIase (Fig. 4 b). The displayed *p*SIase exhibited a half-life of 141 min, which was higher than that of the free *p*SIase (62 min). This finding suggests that the *p*SIase was thermostable when immobilized on the yeast cell wall.

The effect of reaction temperatures on products

To date there is still no consistent conclusion reported on the effect of temperature on enzyme products. Wu and Birch reported that low temperatures promoted the release of isomaltulose but suppressed trehalulose formation (Wu and Birch 2005), while others observed the opposite phenomena. For example, the maximum production of SIase from *S.plymuthica* was obtained at 30 °C for isomaltulose and at 25 °C for trehalulose (Véronèse and Perlot 1999). Using the displayed *p*SIase, we determined the effect of reaction temperatures on the ratio of the products. As shown in Fig. 5, with the decrease of reaction temperatures, the ratio of isomaltulose increased from 84 to 93%. We speculated that the increased products ratio was due to the thermal isomeric effect. Sugar isomerization is a two-step reaction mechanism. First, the enzyme binds sugar to form an enzyme-sugar complex, followed by cleaved at the α-1,2 bond (fructosyl moiety) and produces a second enzyme form (enzyme-glucose complex). Then, the free fructose is isomerized at the active site and *p*SIase catalyzes the enzymatic rearrangement of the α-1,2 linkage between glucose and fructose to an α-1,6 linkage (producing isomaltulose) or α-1,4 linkage (producing trehalulose). Isomaltulose formation requires rotation of the fructose residue through 180° (Cheetham 1984). Upon changes in the reaction temperature, enzyme molecules reduced the activation energy by changing the structure rigidity and flexibility to ensure the progress of the reaction. As a result, the structural constraints of active site of the enzyme will be changed correspondingly, which leads to the disturbance of the rotation of the fructose and allows the formation of kinetically or thermodynamically preferred products, thereby forming the different sucrose isomer.

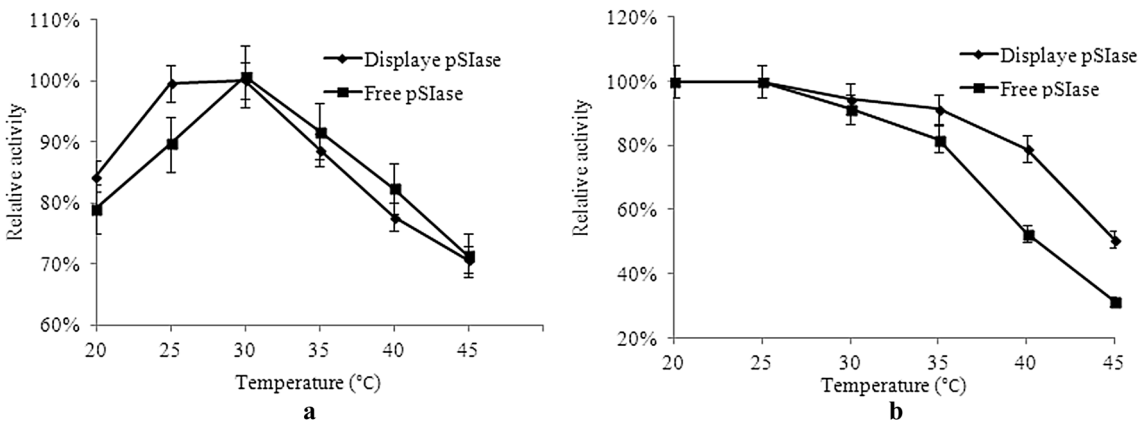

Fig. 3 Effects of different temperatures on activity **a** and stability **b** of displayed and free *p*SIase, respectively. Data are given as the mean ± SD, $n = 3$

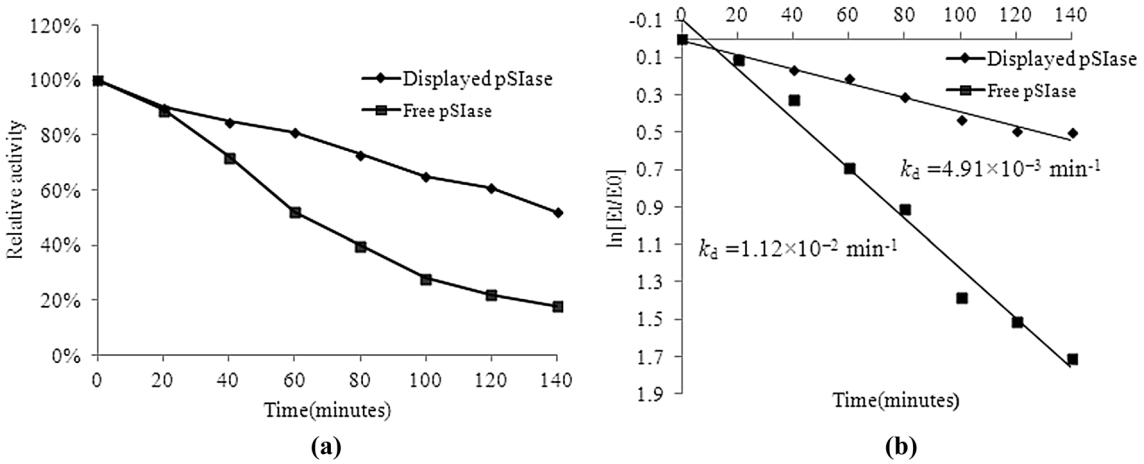

Fig. 4 Thermal deactivation curves (**a**) and thermal deactivation constants (**b**) of displayed *p*SIase and free *p*SIase. The thermal inactivation at 40 °C of enzyme was determined up to 0 to 140 min. The residual activities after heat treatment were measured immediately. The optimal enzyme activity was taken as 100%

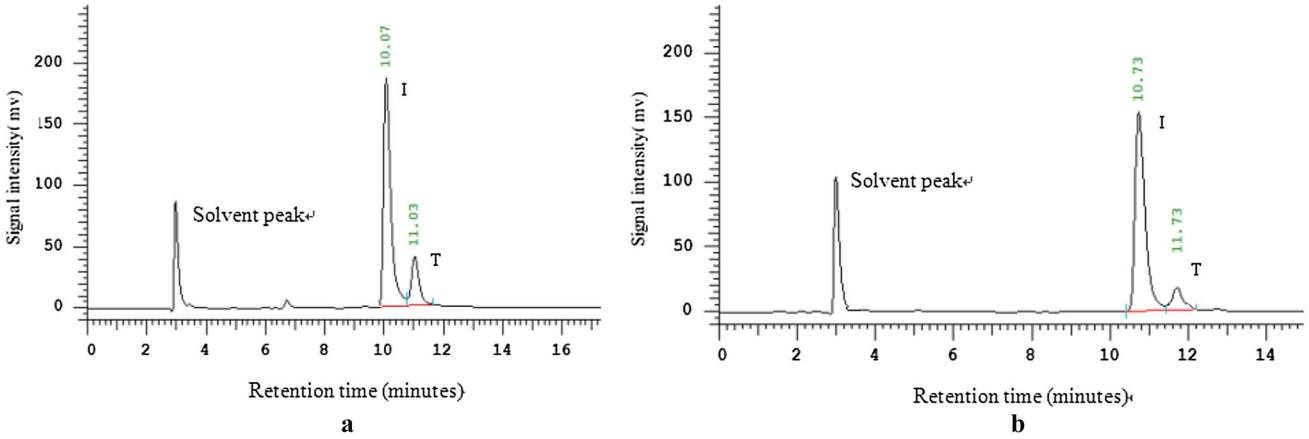

Fig. 5 HPLC analysis of products by displayed *p*SIase. Trehalulose (T), Isomaltulose (I). The effect of reaction temperatures on products was tested by 10 ml wet cells solution harboring *p*SIase were mixed with 100 ml 20% (m/v) sucrose solutions as a substrate at 40 °C (**a**) and 30 °C (**b**) for 2 h

Isomaltulose synthesis by a whole-cell biocatalyst with *p*SIase

A typical conversion curve of sucrose to isomaltulose and trehalulose using the whole-cells biocatalyst under optimal conditions (30 °C) was determined (Fig. 6). The conversion velocity of sucrose was boosted during the initial l40 min and the conversion peaked at approximately 100 min. The isomaltulose concentration reached 184.8 g/L, indicating that the isomaltulose yield was 92.4% when the sucrose was almost completely consumed. From the perspective of industrial catalysts, continued reusability of an enzyme is very important. To address this issue, we used the pretreated cane molasses as a substrate and the batch catalysis reaction of sucrose was repeated to determine the practical reusability of the whole-cell biocatalyst. The displayed *p*SIase showed

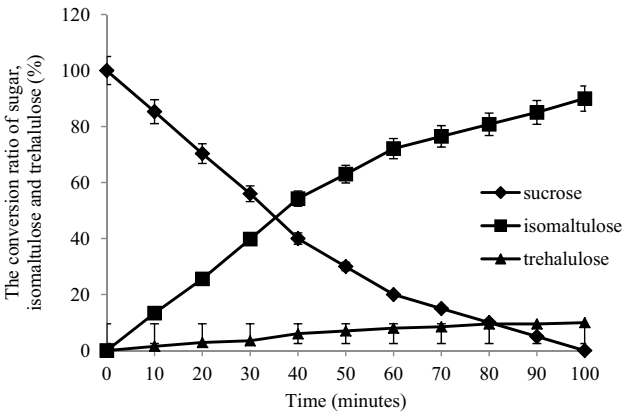

Fig. 6 The conversion curve of sucrose. 10 g wet *Y. lipolytica* harboring *p*SIase were mixed with 100 ml 20% (m/v) sucrose solutions under 30 °C

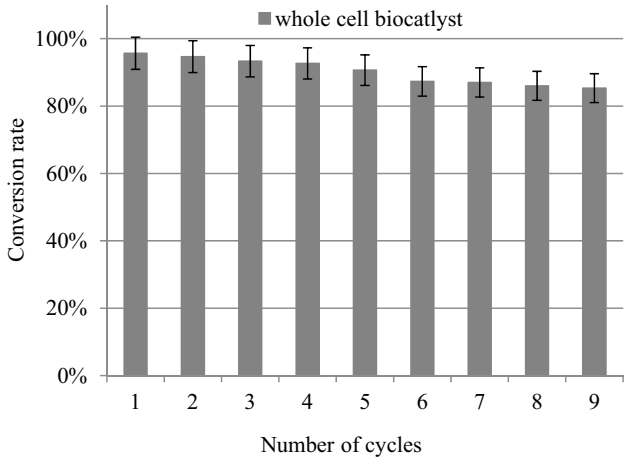

Fig. 7 Number of cycles of the whole cell biocatalyst

an outstanding operational stability, the isomaltulose conversion rate still remained more than 85% of original even after 9 batches catalysis (Fig. 7).

Conclusions

In this study, we demonstrated how to display a *p*SIaseb on the *Y.lipolytica* and its reusability on continuous production of isomaltulose. Compared with the traditional microbiological conversions, displayed *p*SIase showed three advantages: First, the sucrose isomerization can be achieved at high substrate concentration, which greatly enhances the production efficiency of isomaltulose. Second, there are almost no microbial metabolites except for the small amounts of monosaccharides in the enzymatic products; simpler product purification and cost effective downstream processing are convenient for industrial production. Thirdly, the displayed enzyme was anchored in a matrix, in this case, the cell envelope stabilizes the enzyme and prevents it from proteolytic degradation resulting in continuous higher activities and stability.

Although surface display shows great properties and applications, there are still some uncertainties affecting its universality. First, the amount of enzyme produced is limited due to the unavailability of membrane area. In general, the number of displayed enzymes is between 10^3 and 10^4 (Feldhaus et al. 2004; Kuroda et al. 2009). Second, some catalysis reactions need endogenous coenzymes for enzymatic reactions, which limit the kind of displayed enzymes. Therefore, if we want to develop a commercial displayed enzyme technology, the types, structures and applications of enzyme should be considered.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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