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研究报告

# Expression and characterization of surface-displayed sucrose:sucrose 1-fructosyltransferase on *Yarrowia lipolytica* cells

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**Abstract: [Objective]** Sucrose:sucrose 1-fructosyltransferase (1-SST) catalyzes the transfer of a fructosyl residue from one sucrose to another sucrose, forming 1-kestose and glucose. 1-Kestose has the highest prebiotic activity among fructooligosaccharides. In this study, 1-SST displayed on the cell-surface of *Yarrowia lipolytica* was used to prepare 1-kestose. **[Methods]** 1-*SST* gene from *Lactuca sativa* was cloned into the surface-display vector and expressed in the cells of *Y. lipolytica*. Biochemical characteristics of the displayed 1-SST were investigated with sucrose as its substrate. **[Results]** Immunofluorescence microscopy assay and high performance liquid chromatography (HPLC) indicated that the expressed protein was displayed on the cell-surface of *Y. lipolytica* and possessed 1-SST activity. The displayed 1-SST showed the highest activity at 45 °C and pH 7.5. The activity of the displayed 1-SST was inhibited by $Zn^{2+}$ and $Cu^{2+}$, while stimulated by $Ca^{2+}$. The enzyme activity reduced 50% of initial activity after the displayed 1-SST was repeatedly used for seven times. The highest content of 1-kestose reached 20.8 mmol/L after the reaction mixture containing the displayed 1-SST and 3% sucrose was incubated at 40 °C for 30 min. **[Conclusion]** 1-SST was successfully expressed and displayed on the cells of *Y. lipolytica*, and the fructosyltransferase activity was detected. The surface-displayed 1-SST as a whole-cell catalyst can be applied to the 1-kestose preparation.

**Keywords:** Sucrose:sucrose 1-fructosyltransferase, Surface-display, *Yarrowia lipolytica*, 1-Kestose, Enzyme characterization

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# 蔗糖:蔗糖-1-果糖基转移酶的表面展示及酶学性质分析

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**摘 要:** 【目的】蔗糖:蔗糖-1-果糖基转移酶催化 1 分子蔗糖上的果糖基转移到另一个蔗糖分子上，形成 1-蔗果三糖和葡萄糖。在低聚果糖中，1-蔗果三糖益生素活性最高。本研究将该酶展示在酵母菌细胞表面上，并用于 1-蔗果三糖的制备。【方法】将来自莴苣的蔗糖:蔗糖-1-果糖基转移酶基因克隆到用于酵母细胞表面展示的表达载体上，并在解脂亚罗酵母菌中进行异源表达，表达的酶展示在该细胞表面上，然后以蔗糖为底物，研究表面展示的蔗糖:蔗糖-1-果糖基转移酶的性质。【结果】免疫荧光实验结果表明蔗糖:蔗糖-1-果糖基转移酶已展示在酵母菌的细胞表面上，高效液相色谱结果表明酵母表面展示的该酶具有转移酶的催化活性。该酶的最适作用温度、最适作用 pH 分别为 45 °C 和 7.5；该酶的催化活性受 $Zn^{2+}$和 $Cu^{2+}$的抑制，受 $Ca^{2+}$激活；该酶重复使用 7 次后，酶活下降 50%。表面展示的蔗糖:蔗糖-1-果糖基转移酶和 3%蔗糖混合后在 40 °C 条件下孵育 30 min 后，所产 1-蔗果三糖含量最高为 20.8 mmol/L。【结论】蔗糖:蔗糖-1-果糖基转移酶在解脂亚罗酵母菌中得到成功表达，并展示在其细胞表面上，生化研究表明该重组蛋白具有果糖基转移酶活性，且催化蔗果三糖的生成。表面展示的蔗糖:蔗糖-1-果糖基转移酶作为一种全细胞催化剂能够用于 1-蔗果三糖的制备。

**关键词:** 蔗糖；蔗糖-1-果糖基转移酶，表面展示，解脂亚罗酵母菌，1-蔗果三糖，酶学性质

Fructooligosaccharide (FOS) consists of fructose oligomers with one, two or three fructose units bound to the β (2→1) position of sucrose, including 1-kestose, nystose, fructofranosyl-nystose and their mixtures[1]. FOS is widely used as functional food because of its low caloric value, non-cariogenic sweeteners and prebiotic effects[2]. FOS is usually produced by β-D-fructofuranosidases (FFase, EC 3.2.1.26) or fructosyltransferase (FTase, EC 2.4.1.9) from bacteria and fungi. The compositions of FOS were found to be related to the catalytic properties of enzymes[3]. It has been demonstrated that 1-kestose has the highest prebiotic activity among the FOS, which can selectively stimulate the growth of beneficial bacteria (bifidobacteria and lactobacillus) and inhibit the growth of detrimental pathogens (clostridia)[4-6]. Sucrose:sucrose 1-fructosyltransferase (1-SST, EC 2.4.1.99) catalyzes the transfer of a fructosyl moiety from one sucrose to another sucrose to form 1-kestose[7-8]. Therefore, the preparation of 1-kestose could be performed using 1-SST as a catalyst and sucrose as a raw material. However, 1-SST from plant was limited in industrial application because of its low yield and the difficulty in enzyme extraction. It had been reported that 1-*SST* gene from plant was expressed in *E. coli* and *Pichia pastoris*[8-11]. But, the expression of 1-SST in *E. coli* or *P. pastoris* has to be induced with IPTG (isopropyl β-D-1-thiogalactopyranoside) or methanol, which will limit their application on a large scale. In addition, it is difficult to separate the enzyme from products. In recent years, genetic engineering techniques have been used to display heterologous protein on the yeast cell surface, which have many applications in biotechnological and industrial fields[12-13]. The enzyme displayed on the cell surface can directly react with its substrate. The intact cells without enzyme purification or concentration can be used for catalyst, and then harvest and concentrate the yeast cells by low speed centrifugation. The displayed enzyme can be easily separated from other products, and be repeatedly used like immobilized enzyme. Therefore, surface-displayed enzymes have

received increasing attentions[14]. It has not been reported that 1-SST is displayed on the cell-surface of yeast so far.

In this study, 1-*SST* gene from *L. sativa* was isolated and ligated into the multiple cloning sites of the surface display vector pINA1317-Y1CWP110[15], and expressed in the cells of *Y. lipolytica*. Biochemical characteristics of the 1-SST immobilized on cell-surface of the yeast were investigated with sucrose as its substrate. The preparation of 1-kestose was performed using the displayed 1-SST and sucrose.

## 1 Materials and Methods

### 1.1 Strains, media and plasmids

*E. coli* DH5α used for recombinant DNA manipulation was cultured in Luria-Bertani (LB) medium with 100 mg/L ampicillin or 30 mg/L kanamycin. *Y. lipolytica* Po1h used for cell-surface expression was grown in YPD liquid medium. The yeast transformants were selected on YNB-N5000 medium plate and surface-engineered *Y. lipolytica* was cultured on the PPB liquid medium[12,16]. The plasmid pGEMT-*SST*[7] was used as the template for amplifying 1-*SST* gene by polymerase chain reaction (PCR). The surface display vector was kindly donated by Professor Zhen-Ming Chi from Ocean University of China.

### 1.2 1-SST display on cells of *Y. lipolytica*

Two specific primers were designed to amplify 1-*SST* gene according to the sequence of the gene (accession number: ABX90019) and the multiple cloning site of the expression vector pINA1317-YlCWP110. The forward primer SB was 5′-CGGGGCCGTTCTGGCCGGATCTCCGGTGGGTCGGC-3′ (the underlined bases represent *Sfi* I restriction site) and the reverse primer SB-His was 5′-CCCAAGCTT*GTGATGGTGATGGTGATG*AGAACTCCACCCAGAAAGAGGGAAA-3′ (the underlined bases represent *Hin*d III restriction site, the italic bases encode 6-His tag). The amplified PCR products obtained by PCR were digested with *Sfi* I and *Hin*d III. The digested products were ligated into the vector pINA1317-YlCWP110 digested with the same enzymes. The resulting plasmid, which was named pINA1317-YlCWP110-*SST*, was extracted from *E. coli* transformants. The positive plasmid was digested with *Not* I. The DNA fragment carrying pINA1317-YlCWP110-*SST* was transformed into the competent cells *Y. Lipolytica* Polh by lithium acetate method[17]. The transformants were screened on the YNB-N5000 medium plates as described by Yue et al[15]. The cells of *Y. Lipolytica* Polh carrying Y1CWP110 DNA fragment without 1-*SST* gene was used as a control. After the determination of 1-SST activity as described below, it was found that the transformant a2 had the highest activity of 1-SST among the transformants. Therefore, transformant a2 was used in subsequent investigation.

### 1.3 Determination of 1-SST activity

The transformant a2 obtained above was cultured in the PPB medium for 70 h at 28 °C. The cells were collected and washed two times with 50 mmol/L phosphate buffer solution (PBS buffer) (pH 8.0) by centrifugation at 6 000×*g* at 4 °C for 10 min. The washed cells were suspended in 50 mmol/L PBS buffer (pH 8.0). The cell suspensions were mixed with 2% sucrose and the mixture was incubated at 30 °C for 30 min. The reaction was stopped in boiling water for 5 min and the mixture was centrifuged at 10 000×*g* at 4 °C for 30 min. The glucose content of the supernatant was measured using glucose kit (Shanghai Rongsheng Biotechnology Corporation, China). One unit (U) of 1-SST activity is defined as the amount of the displayed enzyme that produces 1 μmol glucose in 1 min.

### 1.4 Immunofluorescence microscopy

The immunofluorescence microscopy of the transformant a2 was performed to confirm the cell-surface display of 1-SST, according to the methods described by Adams et al[18], that using the 6×His monoclonal antibody (Clontech, USA) as the primary antibody and immunoglobulin G/fluorescein isothiocyanate (IgG/FITC) as the secondary antibody (ZSGB-BIO, China). *Y. Lipolytica* Polh and the transformant a2 were cultured in the PPB medium at 28 °C for 70 h, respectively. The collected cells were washed with 50 mmol/L PBS buffer (pH 8.0) for three times. The pellets were suspended in 3.7% formaldehyde, then the mixture was incubated overnight at 28 °C. The treated cells were labeled using 6×His monoclonal antibody and IgG/FITC. Finally, the labeled cells were observed under the

fluorescence microscope (Olympus BH-2) and photographed.

### 1.5 High performance liquid chromatography

HPLC analysis of carbohydrates of the supernatant obtained was performed using a LC-10AD instrument equipped with a differential refraction detector (RID-10A) (Shimadzu, Japan). The column used for separation was an Aminex HPX-42C sugar column (Bio-Rad, 300 mm×7.8 mm). The HPLC was operated at 85 °C with 0.3 mL/min of Milli-Q water as mobile phase. Glucose, fructose, sucrose and 1-kestose (Sigma) were used as standard chemicals.

### 1.6 Characterization of the surface-displayed 1-SST

The effect of temperature on the displayed 1-SST activity was evaluated with the standard method at various temperatures ranging from 25 °C to 55 °C. Thermal stability of the displayed 1-SST was tested by pre-incubating the enzyme in 50 mmol/L PBS buffer (pH 8.0) at 40, 50, 60 °C for 180 min to assay the residual activity as the standard method. The effect of pH on the displayed 1-SST activity was estimated in different buffer systems at the optimal temperature. For pH stability of the enzyme, the cells containing the displayed 1-SST were suspended in 50 mmol/L PBS buffer (pH 5.0−8.0), sodium carbonate and sodium bicarbonate buffer (pH 9.0−10.0), and the cell suspensions in different buffer systems were incubated at 4 °C for 24 h. The relative activity of the pre-incubated sample was considered as 100%.

The effects of different metal ions (at a concentration of 10 mmol/L) on the enzyme activity were determined by dissolving the chloride salts of metallic ions ($Ca^{2+}$, $Mg^{2+}$, $Zn^{2+}$, $Cu^{2+}$ and $Al^{3+}$) into the reaction system as described above. The reaction system was incubated at 40 °C for 30 min. The activity of the displayed 1-SST without metallic ions was considered as 100%.

To investigate reusability of the displayed 1-SST, enzyme activity of the first reaction was detected according to the standard method. The yeast cells were collected and washed with 50 mmol/L PBS buffer (pH 8.0) by centrifugation after the first reaction. The cells obtained were again applied for the second reaction and so on. The initial activity of the displayed 1-SST was defined as 100%.

### 1.7 Preparation of 1-kestose using the displayed 1-SST

Seed cultures were prepared by inoculating the transformant a2 cells into 50 mL PPB liquid medium in 250 mL Erlenmeyer flasks and cultivating at 30 °C for 24 h. 50 mL of the seed cultures was transferred into 1 L PPB medium in a Biostat B2 2-L fermentor (B. Braun, Germany). The fermentation was carried out under the conditions of an agitation speed of 300 r/min, an aeration rate of 5 L/min, a temperature of 30 °C. The samples were harvested every 24 h. Cells were collected by centrifugation and suspended with 50 mmol/L PBS buffer (pH 7.5). The reaction mixture of cell suspensions and 3% sucrose was incubated at 40 °C for 30 min. The content of 1-kestose was determined by HPLC as described above.

## 2 Results and Discussion

### 2.1 Expression of 1-SST gene

Structure of the recombinant plasmid pINA1317-Y1CWP110-*SST* is shown in Figure 1. The recombinant plasmid was digested by *Not* I and the lineared DNA fragments carrying the 1-*SST* gene were transformed into the competent cells of *Y. Lipolytica* Polh. After the transformants were grown on YNB-N5000 medium plates without uracil, the 69 transformants were obtained and their enzyme activities were determined using glucose kit. Among these transformants, the transformant a2 was found to possess the highest 1-SST activity (15.6 U/mL) for further experiments. The surface display vector pINA1317-Y1CWP110 used in this study has many advantages. The vector uses a strong recombinant growth-phase-dependent promoter (hp4d). The target gene can be expressed when the positive transformants are grown in the medium without adding any inducer. Besides, only a "yeast expression cassette" is transformed into the recipient strain and the DNA fragments with antibiotics resistant gene are avoided, so that the spreading of antibiotic resistance gene in bacterial DNA can be stopped. Moreover, *Y. Lipolytica* is generally regarded as safety[15-16].

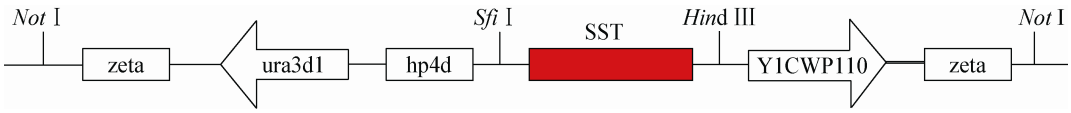


**Figure 1 The plasmid pINA1317-Y1CWP110-*SST* for 1-SST display on *Yarrowia Lipolytica* cells**
**图 1 表面展示质粒 pINA1317-Y1CWP110-*SST* 的示意图**

## 2.2 Confirmation of the recombinant 1-SST displayed on the yeast cells surface

To confirm the presence of $His_6$-*SST*-Y1CWP110 fusion protein on the cell-surface of the yeast, the immunofluorescence labeling of the transformant a2 cells was conducted with the 6×His monoclonal antibody as the primary antibody and IgG/FITC as the secondary antibody. The green fluorescence was observed on the cells of the cultivated transformant a2 under the fluorescent microscope, while no fluorescence was detected on the yeast cells harboring the control plasmid Y1CWP110 without 1-*SST* gene (Figure 2). These results indicated that 6×His-*SST*-YICWP110 fusion protein was indeed displayed on the cell-surface of *Y. lipolytica* Polh.

After the displayed 1-SST was incubated with 2% sucrose at 30 °C for 30 min, the supernatant of the reaction mixture was analyzed by HPLC. The results in Figure 3B showed that the equal proportion of 1-kestose (retention time 11.517) and glucose (retention time 16.175) was produced, which indicated that the expressed protein had fructosyltransferase activity. No 1-kestose and glucose was detected as control samples from *Y. lipolytica* Polh only carrying the Y1CWP110 DNA fragment (data not shown). The results confirmed that the displayed 1-SST could transform sucrose into 1-kestose.

## 2.3 Characterization of the surface displayed 1-SST

The characterization of the displayed 1-SST was conducted as described in materials and methods. The optimum temperature range of the displayed 1-SST was found to be between 40 °C and 55 °C, with the maximum activity at 45 °C (Figure 4A), which was higher than the optimum temperature (25−30 °C) of the MSSTAG (1-SST) from *Agave tequilana*[8], similar to the FST-1 (1-SST) from onion[9], but lower than the optimum temperature (65−70 °C) reported for the native fructosyltransferase (FTA) from *Rhodotorula* and *Cryptococcus*[19-20]. The results in Figure 4B showed that the displayed 1-SST kept 70% of the initial activity after pre-incubating enzyme at 40 °C for 180 min. The influence of pH on the displayed 1-SST activity was studied in different buffer systems. The displayed 1-SST exhibited 80% relative activity at pH 6.5−8.0, with the maximum activity at pH 7.5 (Figure 4C and 4D). The results in the Table 1 showed that $Zn^{2+}$ and $Cu^{2+}$ strongly

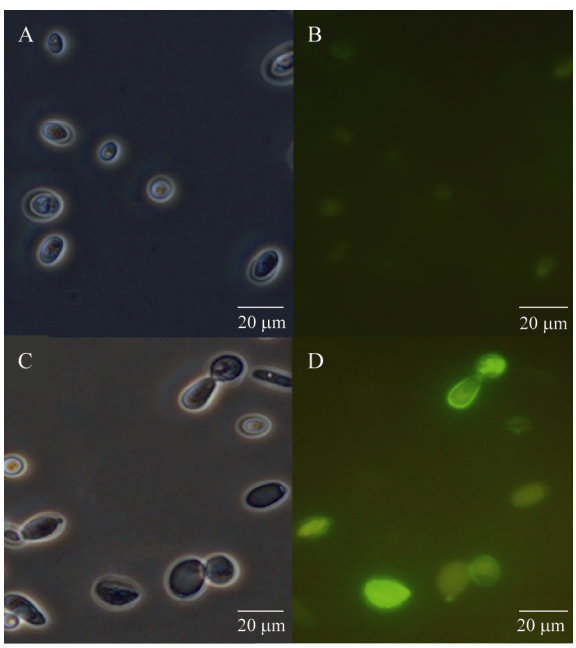


**Figure 2 Immunofluorescent labeling of recombinant 1-SST engineering strain using 6×His monoclonal antibody as the primary antibody and IgG/FITC as the secondary antibody**
**图 2 重组 1-SST 基因工程菌的免疫荧光检测**
Note: A, C: Microphotographs under visible light; B, D: Immunofluorescence microphotographs under emission 500 nm. A, B: *Y. lipolytica* cells harboring YlCWP110; C, D: *Y. lipolytica* cells harboring 6×His-*SST*-YlCWP110. Magnification: 40×10.
注：A，C：可见光下的酵母细胞；B，D：荧光下的酵母细胞；A，B：转入空质粒的对照酵母细胞；C，D：转入含有目的蛋白基因的酵母细胞. 放大倍数：40×10.

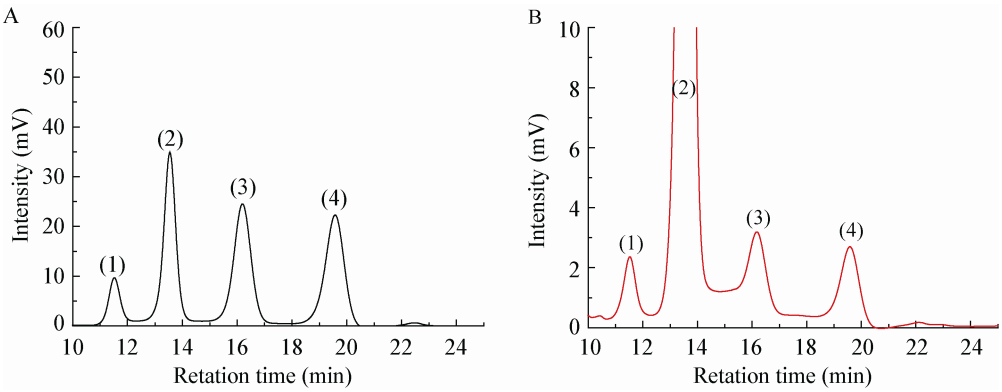


**Figure 3 HPLC analysis of the reaction products**

**图 3 反应产物的高效液相色谱分析结果**

Note: A: The standard samples containing 1-kestose (1), sucrose (2), glucose (3) and fructose (4); B: The supernatant from the reaction mixture. HPLC condition: Aminex HPX-42C sugar column, Milli-Q water as mobile phase, flow rate 0.3 mL/min, column temperature 85 °C, differential refraction detector (RID-10A).

注：A：标准样品的 HPLC 结果为 1-蔗果三糖(1)、蔗糖(2)、葡萄糖(3)和果糖(4)；B：反应产物的 HPLC 结果. HPLC 条件：Aminex HPX-42C 型糖柱，以 Milli-Q 水为流动相，流速 0.3 mL/min，柱温 85 °C，示差检测器(RID-10A).

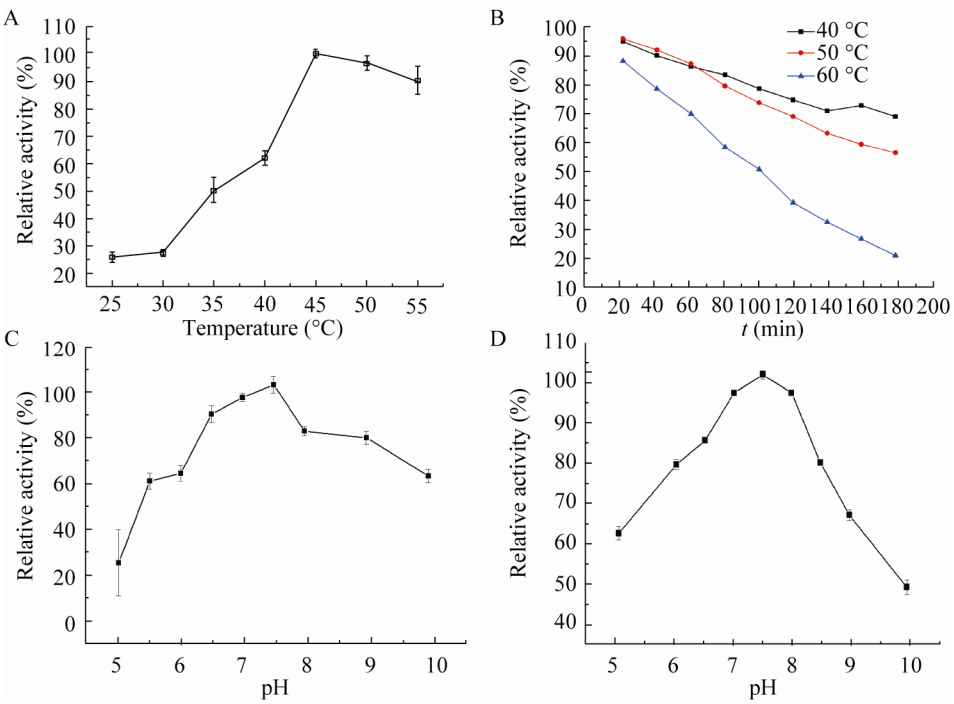


**Figure 4 Effects of temperature and pH on the surface displayed 1-SST activity**

**图 4 温度和 pH 对表面展示 1-SST 的酶活性影响**

Note: A: The optimal temperature of the displayed 1-SST. The displayed 1-SST activities under different temperature (25−55 °C) were measured with the method described in "materials and methods". The enzyme activity at 45 °C was considered as 100%. B: The thermal stability of 1-SST. The surface displayed 1-SST was incubated at 40 °C (square), 50 °C (circle) and 60 °C (triangle) for 180 min, the residual activities were measured every 20 min interval. C: The optimal pH of the displayed 1-SST. The displayed 1-SST was incubated in different buffer systems (PBS buffer (pH 5.0−8.0), sodium carbonate and sodium bicarbonate buffer (pH 9.0−10.0)) at 45 °C. The highest activity at pH 7.5 was taken as 100%. D: pH stability of the displayed 1-SST. The residual activities were determined at 45 °C after 1-SST was incubated into above buffers at 4 °C for 24 h. 100% activity=15.6 U/mL.

注：A：1-SST 的最适温度. 在不同温度(25−55 °C)下测定 1-SST 的酶活，定义 45 °C 条件下 1-SST 酶活为 100%. B：1-SST 的温度稳定性. 将 1-SST 分别在 40 °C (方形)，50 °C (环形)和 60 °C (三角形)下孵育 180 min，每 20 min 取样测定剩余酶活. C：1-SST 的最适 pH. 在 45 °C 条件下测定不同 pH 缓冲液下的 1-SST 酶活，定义 pH 7.5 缓冲液下的 1-SST 酶活为 100%. D：1-SST 的 pH 稳定性. 不同 pH 缓冲液下的 1-SST 在 4 °C 放置 24 h 后测定剩余酶活.

inhibited the enzyme activity, as reported by Chevalier and Rupp[21]. The $Ca^{2+}$ obviously elevated the activity of the recombinant enzyme. However, previous results indicated that $Ca^{2+}$ reduced 1-SST activity[21]. The significant difference remains unclear. Using the expression system, 1-SST was displayed on the cell surface of yeast after expression and reused like immobilized enzyme. The enzyme activity eliminated step by step with the increase of recycle number. The enzyme activity reduced 50% of the initial activity at the seventh cycle (Figure 5).

**Table 1 Effect of different metal ions on activity of the displayed 1-SST**
**表 1 不同金属离子对表面展示 1-SST 的酶活性影响**

| Metal ions (10 mmol/L) | Relative activity (%) |
|---|---|
| Control | 100.0±2.0 |
| $Mg^{2+}$ | 102.0±1.6 |
| $Cu^{2+}$ | 60.0±2.0 |
| $Zn^{2+}$ | 61.0±2.1 |
| $Al^{3+}$ | 112.0±1.2 |
| $Ca^{2+}$ | 168.0±3.1 |

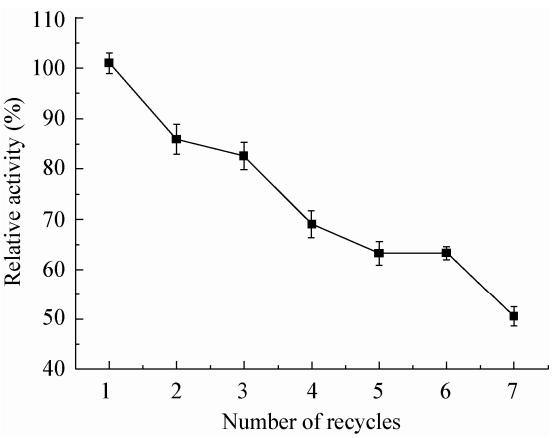


**Figure 5 Effect of reuse times on the activity of the displayed 1-SST**
**图 5 表面展示 1-SST 的使用次数对 1-SST 酶活性的影响**

Note: The cell suspensions of the displayed 1-SST were washed with 50 mmol/L PBS buffer (pH 8.0) for two times and measured enzyme activity before every cycle. The displayed 1-SST activity was measured at 40 °C. Initial activity of 1-SST was defined as 100%. 100% activity=15.6 U/mL.

注：1-SST 在每次循环使用前用 50 mmol/L PBS 缓冲液洗两次后测定剩余酶活. 定义第一次使用时 1-SST 的酶活为 100%.

## 2.4 Fermentation and 1-kestose preparation

The transformant a2 was cultured in 2 L fermentation tank containing 1 L of the PPB liquid medium. The cell suspensions with the displayed 1-SST were applied to prepare 1-kestose. The results shown in Figure 6 indicated that the maximum concentration of 1-kestose reached 20.8 mmol/L using the cell suspensions fermented 48 h and 3% sucrose, which was 4-fold higher than the concentration of 1-kestose as reported by Lüscher et al[10], slightly lower than that reported by Fernádez et al[8].

In conclusion, 1-*SST* gene was isolated, ligated into the surface display vector pINA1317-Y1CWP110, and successively expressed and displayed on the cell-surface of *Y. Lipolytica*. The displayed enzyme showed 1-SST activity. Compared with free enzyme, the surface displayed enzyme may be repeatedly utilized like immobilized enzymes, and easily collected by centrifugation of the cell culture at low speed without purification and concentration. This is a new and safe technique for 1-kestose preparation. Further studies are required to increase the production of 1-kestose.

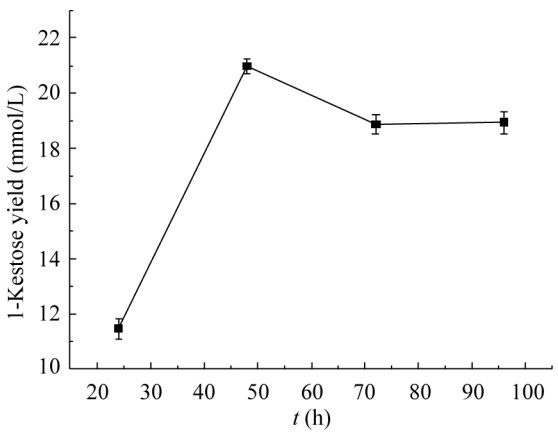


**Figure 6 Time course of the produced 1-kestose**
**图 6 1-蔗果三糖产量随时间变化曲线**

Note: The transformant a2 was cultivated in a Biostat B2 2 L fermentor. The fermentation conditions were as follows, an agitation speed of 300 r/min, an aeration rate of 5 L/min and a temperature of 30 °C. The mixture of the displayed 1-SST and 3% sucrose was incubated at 40 °C for 30 min.

注：转化子 a2 在 Biostat B2 2 L 发酵罐中发酵培养. 培养条件如下：转速 300 r/min、通气量 5 L/min、发酵温度 30 °C. 1-SST 和 3%蔗糖在 40 °C 孵育 30 min 后测定 1-蔗果三糖的含量.

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