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Research Article

Synthesis of Trehalose by the Erythritol-Producing Yeast *Yarrowia lipolytica* Co-Displaying Maltooligosyltrehalose Synthase and Maltooligosyltrehalose Trehalohydrolase

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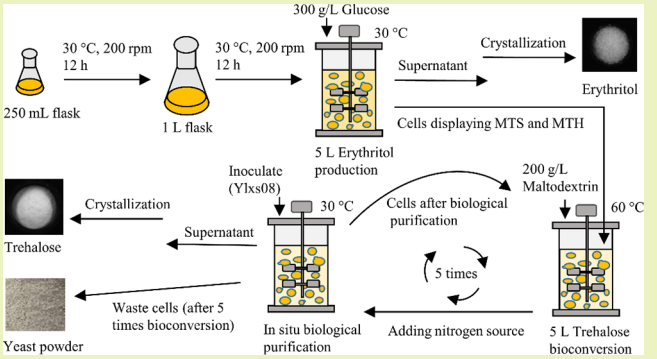
ABSTRACT: Industrial trehalose production faces economic challenges with costly enzyme preparations, prompting the exploration of eco-friendly alternatives. Here, we established a coupled functional sugar production line leveraging erythritol-producing cells as an innovative enzyme preparation for trehalose synthesis. The erythritol-producing *Yarrowia lipolytica* was modified to express a fusion protein consisting of maltooligosyltrehalose synthase and maltooligosyltrehalose trehalohydrolase from *Sulfolobus solfataricus* ATCC35092. The engineered strain YlxsT08 was used for the simultaneous synthesis of erythritol and the fusion protein. The yield, production, and productivity of erythritol reached 0.55 g/g of glucose, 166.18 g/L, and 1.98 g/(L·h), respectively, in a 5 L bioreactor. The recycled cells (49.77 U/mg of DCW) exhibited a high catalytic efficiency in the bioconversion of maltodextrin to generate trehalose. The yield, production, and productivity of trehalose reached 0.67 g/g maltodextrin, 134.40 g/L, and 5.60 g/(L·h), respectively, in a 5 L bioreactor. The byproducts were removed by biological purification, and the resulting cells were recycled as catalysts for at least the next 5 rounds of trehalose production. Our novel strategy provides an ecologically sustainable and economically feasible alternative to enzyme combinations in trehalose production, offering a cost-effective and eco-friendly enzyme manufacturing method.



KEYWORDS: *trehalose, erythritol, coproduction, recycle, Yarrowia lipolytica*

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INTRODUCTION

Trehalose is a commonly occurring nonreducing disaccharide, composed of two glucose molecules linked by a stable α-1,1-glycosidic bond.[1] It has an excellent protective effect on proteins, biofilms, and other macromolecules, which makes trehalose a highly effective and nontoxic cryoprotectant for enzymes, animal cells, and plant cells in the medical field.[2] Before the 1990s, trehalose was mostly extracted from *Saccharomyces cerevisiae*, which has a high cost.[3] Although trehalose can be chemically synthesized, its low yield and byproducts prevent it from being used in industrial production.[4] The successful discovery of several trehalose biosynthesis methods has enabled the development of trehalose production by biocatalysis. Nevertheless, three of them involve the use of or the formation of phosphate compounds, which depend on expensive mediators and are thus considered inappropriate for commercial development.[5] A one-step trehalose production from maltose can be catalyzed by trehalose synthase (TreS), which involves the direct conversion of maltose into trehalose.[6] Nevertheless, this approach has a high substrate cost, and the reaction may be inhibited by the glucose byproduct.[7]

The double-enzyme approach is the predominant way for industrial trehalose production.[8] First, maltooligosyltrehalose synthase (MTS) catalyzes an intramolecular transglycosylation to produce maltooligosyltrehalose by converting the α-1,4-glucosidic bond at the reducing end of maltooligosaccharide to an α-1,1-glucosidic bond.[9] Then, the maltooligosyltrehalose trehalohydrolase (MTH) mainly cleaves the α-1,4-glucosidic bond next to the α-1,1-linked terminal disaccharide of maltooligosyltrehalose to produce trehalose.[10] In order to increase conversion, α-amylase was first added to starch for partial liquefaction, followed by MTS, MTH, pullulanase, and cyclodextrin glycosyltransferase for biocatalysis to produce trehalose.[8,11] However, enzyme production and the subsequent separation of sugars are expensive steps in this procedure.

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Thus, the development of low-cost enzyme preparation and trehalose separation technology is required.

Previously, a low-cost fructosyltransferase was successfully prepared using an erythritol-producing yeast *Y. lipolytica*.[12] Erythritol is a four-carbon sugar alcohol sweetener used as a functional sugar substitute in specialty foods for people with diabetes and obesity.[13] Erythritol is produced by microbial methods using mostly osmophilic yeasts.[14] Among them, *Y. lipolytica* is a nontraditional yeast widely used in producing food ingredients such as organic acids[15] and erythritol.[16] Due to its excellent protein production capacity, it has been used to prepare various enzymes, such as lipase[17] and β-glucosidase.[18] *Y. lipolytica* can also be used as an immobilized carrier to produce enzymes for whole-cell catalysts, which can replace the laborious process of protein purification and enzyme immobilization.[19] Thousands of tons of yeast cells are produced every year in the fermentation process of *Y. lipolytica* to produce erythritol.[12] Yeast cells are widely utilized as a source of nitrogen or feed additives.[20] Further development of yeast cells is beneficial to reduce the cost of the fermentation industry and the risk of biological waste.

In this study, we developed a cascade process that integrated erythritol fermentation, trehalose biotransformation, and biological purification to increase the valorization of *Y. lipolytica* cells produced in the erythritol production, thus reducing the trehalose production cost. First, the optimal expression mode of MTS and MTH was screened in an erythritol-producing yeast, *Y. lipolytica*, and the expression level of engineered enzymes was increased by multiround gene integration. The engineered yeast first produced erythritol through the fermentation process with 300 g/L glucose, which was accompanied by the formation of a fusion enzyme (MTH-L-MTS). The enzyme production did not affect the fermentation process of erythritol production. The supernatant was used to crystallize erythritol, and the recovered enzyme-containing cells were used to produce trehalose from maltodextrin in the whole-cell biocatalyst. The residual cell biomass can still be employed as a protein-rich animal feed resource. Instead of using complex and expensive simulated moving bed chromatography, the reaction mixture was biologically purified to facilitate trehalose crystallization, which is a cost-effective alternative.

MATERIALS AND METHODS

Chemicals and Biological Reagents. Trehalose and maltotriose standards were purchased from Sigma-Aldrich (St. Louis, United States). Maltodextrin (DE17.5) was supplied by Xi Wang Sugar Industry Co., Ltd. (Shandong, China). Maltodextrin (DE7.7) was generously provided by Ao Gu Biotech Co., Ltd. (Jiangsu, China). Pullulanase (≥1000 U/mL) was purchased from Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Amyloglucosidase (≥100,000 U/g) was purchased from Solarbio science & technology Co., Ltd. (Beijing, China). All restriction enzymes were purchased from New England Biolabs. The Phanta Super- DNA Polymerase, Taq Master Mix, and One Step Cloning Kit were obtained from Vazyme Biotech Co., Ltd. (Nanjing, China). The HiPure Plasmid Micro Kit for plasmid recycling and the HiPure Gel Pure DNA Mini Kit for DNA fragment recycling were purchased from Magen (Guangdong, China). Oligonucleotides were synthesized by GENEWIZ, Inc. (Beijing, China). The corresponding genes were chemically synthesized by General Biol (Anhui) Co., Ltd. (Anhui, China). Sequences of genes (*mts*, *mth*, and *hph*) are listed in Table S1. Anhydrous glucose was used in this study. All other chemicals were of analytical grade.

Strains, Media, and Culture Conditions. All strains used in this study are listed in Table 1. The *E. coli* strains were grown at 37 °C and 200 rpm in Luria−Bertani (LB) medium supplemented with either ampicillin (100 mg/L) or kanamycin sulfate (50 mg/L). The *Y. lipolytica* strains were grown at 30 °C and 200 rpm in YPD (10 g/L yeast extract, 5 g/L tryptone, and 10 g/L glucose) or YNB medium (6.70 g/L yeast nitrogen base without amino acids, 3.75 g/L ammonia sulfate) supplemented with glucose or sucrose (20 g/L, YNBG/YNBS). For solid media, agar (20 g/L) was added. Hygromycin (400 mg/L) was added to the YPD, and mycophenolic acid (200 mg/L) was added to the YNBG when necessary to screen transformants. The carbon and nitrogen sources were sterilized separately at 115 °C for 25 min. For enzyme production coupled with erythritol fermentation, 20 mL of YPNP medium (8 g/L yeast extract, 2 g/L tryptone, 4 g/L ammonium citrate, and 3 g/L diammonium hydrogen phosphate) supplemented with glucose (300 g/L) was used and cultivated at 30 °C, 200 rpm in 250 mL baffled flasks.

Table 1. Strains Used in This Study

strains	description	reference
E. coli		
TOP10	F-*mcr*AΔ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (*Str*^{r}) *end*A1 *nup*G	Thermo Fisher Scientific
Y. lipolytica		
CGMCC7326 (YlxsWT)	*Suc-, Lac-, Mal-*	Wang et al.,[16]
YlxsT01	CGMCC7326 derivative, △*KU70*△*MDH2*△*EYD1*	this work
YlxsT02	YlxsT01 derivative, 26S rDNA:: *mts-guaB* 26S rDNA:: *mth-hph*	this work
YlxsT03	YlxsT01 derivative, 26S rDNA:: *lipmts-guaB* 26S rDNA:: *lipmth-hph*	this work
YlxsT04	YlxsT01 derivative, 26S rDNA:: *lipmts-guaB* 26S rDNA:: *mthpir-hph*	this work
YlxsT05	YlxsT01 derivative, 26S rDNA:: *mts-l-mth-guaB*	this work
YlxsT06	YlxsT01 derivative, 26S rDNA:: *mth-l-mts-guaB*	this work
YlxsT07	YlxsT06 derivative, 26S rDNA:: *mth-l-mts-guaB* 26S rDNA:: *mth-l-mts-hph*	this work
YlxsT08	YlxsT07 derivative, 26S rDNA:: *mth-l-mts-guaB* 26S rDNA:: *mth-l-mts-hph* 26S rDNA:: *mth-l-mts-suc2*	this work
YlxsT09	YlxsT01 derivative, 26S rDNA:: *mth-hph*	this work
YlxsT10	YlxsT01 derivative, 26S rDNA:: *lipmth-hph*	this work
YlxsT11	YlxsT01 derivative, 26S rDNA:: *mthpir-hph*	this work
YlxsT12	YlxsT01 derivative, 26S rDNA:: *mts-guaB*	this work
YlxsT13	YlxsT01 derivative, 26S rDNA:: *mtspir-hph*	this work
YlxsT14	YlxsT01 derivative, 26S rDNA:: *pirmts-hph*	this work
YlxsT15	YlxsT01 derivative, 26S rDNA:: *pirmth-hph*	this work

DNA Manipulations. All plasmids and primers used in this study are listed in Tables S2 and S3, respectively. The codon-optimized *mth* (*treZ*) and *mts* (*treY*) genes from the *Sulfolobus solfataricus* ATCC35092 (GenBank: AE006641.1) were chemically synthesized and cloned downstream of the promoter P_{hp4d} into pX-*hph* or pX-*guaB*[21] at *Kpn*I restriction enzyme site, yielding the plasmid pX-*hph-mth* and pX-*guaB-mts*. A linker "SGSG" was used for fusion protein, yielding the plasmids pX-*hph-mth-l-mts*, pX-*guaB-mth-l-mts*, and pX-*guaB-mts-l-mth*. To construct pX-*suc2-mth-l-mts*, the *Nde*I restriction enzyme site was used. The DNA for the anchor protein YlPir1 was amplified using the pINA-Pir1-Ftase[12] as the template. The DNA for the secretory signaling protein YlLip2 was amplified using the pINA1313[17] as the template. DNA fragments were assembled using a One Step Cloning Kit according to the instructions and cloned downstream of the promoter P_{hp4d} into pX-*hph* or pX-*guaB* at the *Kpn*I restriction enzyme site, yielding the plasmids pX-*guaB-lipmts*,

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pX-*hph-lipmth*, pX-*hph-mthpir*, pX-*hph-pirmth*, pX-*hph-mtspir*, and pX-*hph-pirmts*. More details about plasmids and strain construction are shown in supplemental files - Methods.

Identification and Screening of *Y. lipolytica* Transformants. The transformed single colony was verified by yeast colony PCR. The genomic DNA of the target *Y. lipolytica* was extracted using the method described before,[16] and further verified by PCR. Corresponding validation primers are listed in Table S3. To avoid the effect of gene copy number and gene insertion position on enzyme activity, 10 clones of each expression pattern were selected and cultured in 20 mL of YPD medium at 30 °C and 200 rpm for 48 h (Figure S1). The cells were collected by centrifugation and washed with phosphate buffer solution (PBS) three times. To detect the enzymatic activity of the cells and supernatants, 20 g/L maltodextrin (DE17.5) was used to resuspend the cells and adjust the OD_{600} to 20. The supernatants were treated with maltodextrin (DE17.5) at 20 g/L. One milliliter of the cell suspensions and supernatants were placed in a metal bath and catalyzed at 60 °C, 1000 rpm for 48 h. For the following experiment, the phenotype that produces and contains the highest amount of trehalose was selected. To detect the enzymatic activity of the cell lysate slurry, 20 g/L maltodextrin (DE17.5) was used to resuspend the cells and adjusted the OD_{600} to 20 before biocatalysis; the suspension was treated by ultrasound.[22]

Characterization of the Produced Enzyme. The engineered yeast YlxsT08 was cultivated in YPD medium at 30 °C for 48 h, collected by centrifugation, and washed with PBS three times. To determine the effect of pH, the cells were suspended with 20 g/L dextrin solution (DE17.5) to adjust the OD_{600} to 20 and incubated in 1.5 mL tubes (1 mL) for 48 h at 60 °C with pH 3.0, 4.0, 5,0, 6.0, 7.0, and 8,0 in a metal bath shaker at a speed of 1000 rpm. To determine the effect of temperature, the cell suspensions were incubated at 30, 40, 50, 60, 65, 75, 80, and 85 °C. To determine the effect of the concentration of substrate, the cells were suspended in 100, 150, 200, and 250 g/L of dextrin solution (DE17.5). To analyze the stability of the enzymes produced by the strain YlxsT08, the cells were suspended with 20 g/L dextrin solution (DE17.5) to adjust the OD_{600} to 20 and incubated in 1.5 mL tubes (1 mL) for 24 h at 60 °C without adjusting the pH in a metal bath shaker at a speed of 1000 rpm. The cells were then recycled until the relative activity of the enzyme was below 50%. One unit of activity is defined as the quantity of yeast cells per milligram of dry cell weight (DCW) that promotes the formation of 1 μmol of trehalose per minute. One milliliter of cells at an OD_{600} of 1.0 was equivalent to 0.25 mg of DCW.[16]

Optimization of Pullulanase Dosage. The engineered yeast YlxsT08 was cultivated in YPNP medium at 30 °C for 96 h, collected by centrifugation, and washed with PBS three times. The cells were suspended with 20 g/L dextrin solution (DE17.5) to adjust the OD_{600} to 20, and the final pullulanase concentrations were 0, 1.25, 2.5, 5, 7.5, 10, and 20 U/g maltodextrin and incubated in 1.5 mL tubes (1 mL) for 48 h at 60 °C in a metal bath shaker at a speed of 1000 rpm. The supernatants were taken after centrifugation, and trehalose was then analyzed by high-performance liquid chromatography (HPLC). To identify pullulanase types, 20 g/L maltose with pullulanase (100 U/g maltose) was incubated at 60 °C for 72 h.

Production of Trehalose Coupled with Erythritol Production in the 5 L Bioreactor. The erythritol production process was carried out in a 5 L bioreactor containing a total of 3.5 L of YPNP (300 g/L glucose) after inoculation. The fermentation seed culture was prepared by transferring single colonies from YPD plates to 20 mL of YPNP (50 g/L glucose) medium at 30 °C for 12 h in a shaker at 200 rpm. Next, 20 mL of the culture was transferred into 180 mL of YPNP (200 g/L glucose) medium in a 1 L baffled shake flask with 200 rpm and grown at 30 °C for approximately 12 h to an OD_{600} of 30−40. Then seed culture was used to inoculate the 5 L bioreactor with 3.3 L of YPNP (320 g/L glucose) medium. Fermentation was carried out at 30 °C with an agitation of 600 rpm without adjusting the pH. The aeration rate stayed at 1 air volume/culture volume/min (vvm) during fermentation. Glucose and erythritol were analyzed by HPLC.[16] After fermentation, the cells were collected by centrifugation and washed twice with deionized water. Then the cells were suspended with 2 L of dextrin solution (DE17.5 or 7.7, 200 g/L) to adjust the OD_{600} to 40, and pullulanase 2.5 U/g maltodextrin was added. The bioconversion of dextrin to trehalose was carried out at 60 °C and 400 rpm (stirring rate) without aeration in a 5 L bioreactor.

Purification of Trehalose Bioconversion Solution. After bioconversion, 1 g/L amyloglucosidase (100,000 U/g) was added for a 2 h reaction at 60 °C and 400 rpm. After a rapid drop in temperature to 30 °C, 100 mL of nitrogen source (5 g/L yeast extract and 5 g/L diammonium hydrogen phosphate) and 100 mL of seed culture (YlxsT08) were added into the 5 L bioreactor. The fermentation process was carried out at 600 rpm at 30 °C, without pH adjustment. Throughout fermentation, the aeration rate remained at 1 vvm. Fermentation was terminated upon the exhaustion of glucose. The seed culture was prepared by transferring single colonies (YlxsT08) from YPD plates to 100 mL of YPNP (50 g/L glucose) medium at 30 °C for 12 h in a shaker with 200 rpm. The cells after bioconversion were employed as catalysts for the next round of trehalose production with 200 g/L dextrin solution (DE 7.7) at 60 °C and 400 rpm without aeration for 24 h until the trehalose yield dropped below 0.60 g/g dextrin.

Crystallization of Trehalose. After biological purification, the yeast cells were removed by centrifugation and used for the next cycle of bioreaction for trehalose production (5 times). The supernatant was decolorized with activated carbon and concentrated with a rotary evaporator; then, trehalose seeds were added to crystallize at room temperature (25 °C). The crystals were centrifuged, washed with 95% ethanol, and dried at 60 °C in a drying oven. The trehalose crystals were then dissolved in deionized water and estimated by ^{1}H NMR spectroscopy.

Determination of Protein and Lipid Content of Waste Yeast Cells. After dextrin was catalyzed to produce trehalose, the final waste yeast cells were separated by centrifugation, washed with deionized water three times, frozen with liquid nitrogen, and freeze-dried to a constant weight in a lyophilizer. The protein content of lyophilized yeast cells was determined by the Kjeldahl method[23] and lipid content by the Soxhlet extraction method.[24]

Analytical Methods. Optical density was measured at a wavelength of 600 nm (OD_{600}) with a UV-7504 spectrophotometer. Glucose, maltose, maltotriose, and trehalose were quantified by HPLC using a refractive index detector (Shodex RI101) and a Thermo Fisher Scientific Hypersil APS-2 column (250 × 4.6 mm). Elution was performed at 30 °C using acetonitrile−water (8:2) at a flow rate of 1.2 mL/min. The determination of erythritol and parameter calculation of erythritol and trehalose refer to our previously published method.[16]

RESULTS AND DISCUSSION

Selection of Suitable Enzyme Expression Mode in Yeast. Starch, or dextrin, was used as a substrate for trehalose production by the double-enzyme method.[25] Thermophilic enzymes encoded by *mts* and *mth* from *S. solfataricus* ATCC 35092 were chosen to prevent starch retrogradation, enhance substrate concentration, increase the reaction rate, and reduce the risk of microbial contamination. Previous studies have shown that MTS and MTH catalyze the generation of glucose during side reactions.[10] In order to ensure gene expression in an erythritol-producing *Y. lipolytica* and reduce the formation of byproducts, the *mts* (P402Q, F405Y)[26] and *mth* (A259S)[10] were synthesized artificially as the template for the next vector construction after codon optimization.

In order to display MTS and MTH on the cell surface, we first attempted to fuse the cell wall protein YlPir1 at the N-terminus or C-terminus of MTH and MTS. Unfortunately, the MTH-YlPir1 was the only active among the MTS-YlPir1, YlPir1-MTS, MTH-YlPir1, and YlPir1-MTH, and the relative enzyme activity was only 64.84% compared to MTH (Figure S2A). The enzyme inactivation may be because the addition of

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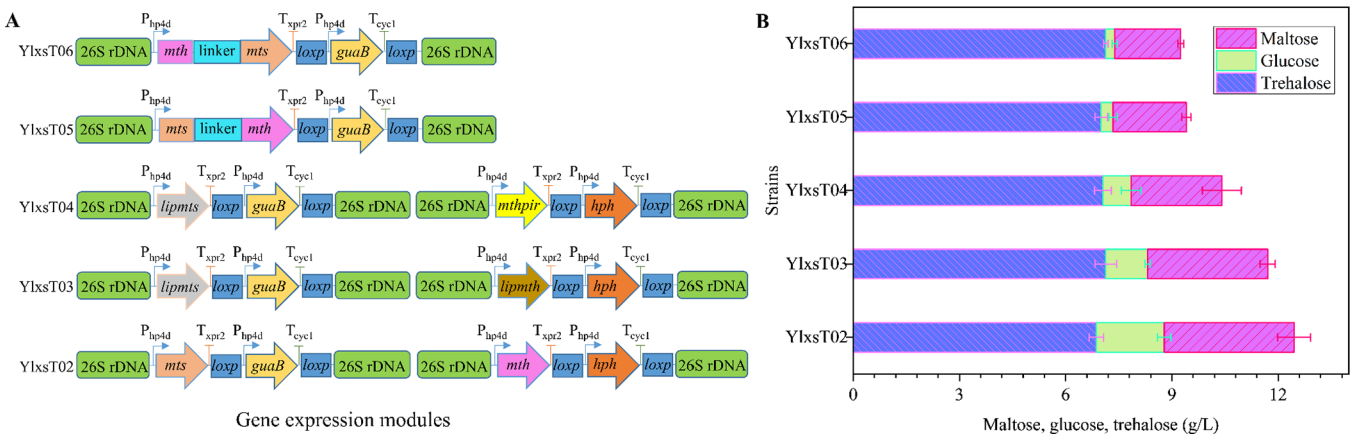

Figure 1. Screening the expression patterns of *mth* and *mts* in the erythritol-producing yeast *Y. lipolytica*. (A) The linearized functional elements for the integration of *mth* and *mts* into *Y. lipolytica*. *hph*, encoding hygromycin-B-phosphotransferase for *Y. lipolytica* selection using hygromycin B. *guaB*, encoding inosine 5′-monophosphate dehydrogenase for *Y. lipolytica* selection using mycophenolic acid. A linker "SGSG" was used for fusion protein. (B) Evaluation of YlxsT02-06 with 20 g/L maltodextrin (DE17.5) and sugar composition after the enzymatic transformation. Other polysaccharides, such as maltotriose, are not presented. Error bars represent standard deviation (n = 3).

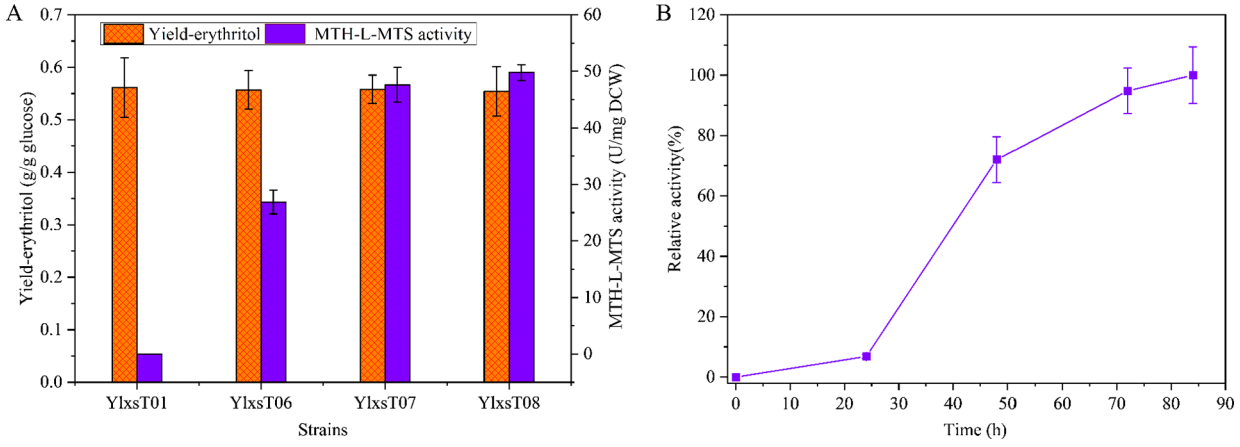

Figure 2. Effects of multiround integration *mth-l-mts* on erythritol production and enzyme activity. (A) Production capacity of erythritol and MTH-L-MTS in YlxsT06-08. (B) Time courses of the relative activity of MTH-L-MTS during erythritol production by the engineered *Y. lipolytica* YlxsT08. Error bars represent standard deviation (n = 3).

YlPir1 affects the normal structure, as the YlPir1 is relatively large (288 AA) in anchored proteins. YlLip2, a relatively small secretory signal peptide (33 AA), has been used for the secretion of foreign proteins in *Y. lipolytica*.[18]

Then, in order to promote enzyme extracellular translocation, YlLip2 was strategically added at the N-terminus of MTS and MTH. The fused YlLip2-MTS and YlLip2-MTH clearly exhibited functional enzymatic activity. Interestingly, the relative enzyme activity of the strains YlxsT03 (expressed YlLip2-MTS and YlLip2-MTH) and YlxsT04 (expressed YlLip2-MTS and MTH-YlPir1) was less than 10% in the culture medium, with most of the enzymes concentrated on the cell surface (Figure S2B). This phenomenon is similar to the expression of invertase in *Y. lipolytica*; most of the enzyme activity is localized in the periplasmic space.[22] Therefore, we hypothesized that MTS and MTH might be released to the cell surface and trapped in the periplasmic cavity. The MTS and MTH were expressed without modification, and the relative enzyme activity of the cell lysate slurry was only 7.88% higher than that of the whole-cell catalysts (Figure S2C). This indicated that MTS and MTH retained the highest activity under unmodified conditions and could be directly applied to trehalose production after expression in *Y. lipolytica*.

To screen for suitable enzyme expression patterns, we constructed different strains containing different forms of MTS and MTH (Figure 1A). For YlxsT02, the MTS and MTH were expressed separately with the P_{hp4d} promoter,[27] and the genes were inserted into the 26S rDNA polyclonal site in *Y. lipolytica*. For YlxsT03, the same pattern as YlxsT02 was used with YlLip2 to fuse at the N-terminus of MTH and MTS. The MTS of N-terminal fusion YlLip2 and the MTH of C-terminal fusion YlPir1 were expressed in *Y. lipolytica*, resulting in the YlxsT04. The YlxsT05 and the YlxsT06 expressed MTS and MTH as a fusion protein using a linker "SGSG" with a different sort. To avoid the influence of gene insertion site location and gene copy number on enzyme activity, 50 correct transformants were verified by PCR for each expression pattern of the strains, and then 10 of them were randomly selected for the enzyme activity test (Figure S1). The high trehalose-producing strains were selected in each of the different enzyme expression patterns and compared to determine the final expression pattern under the same conditions. As shown in Figure 1B, all the strains except YlxsT02 showed similar trehalose production

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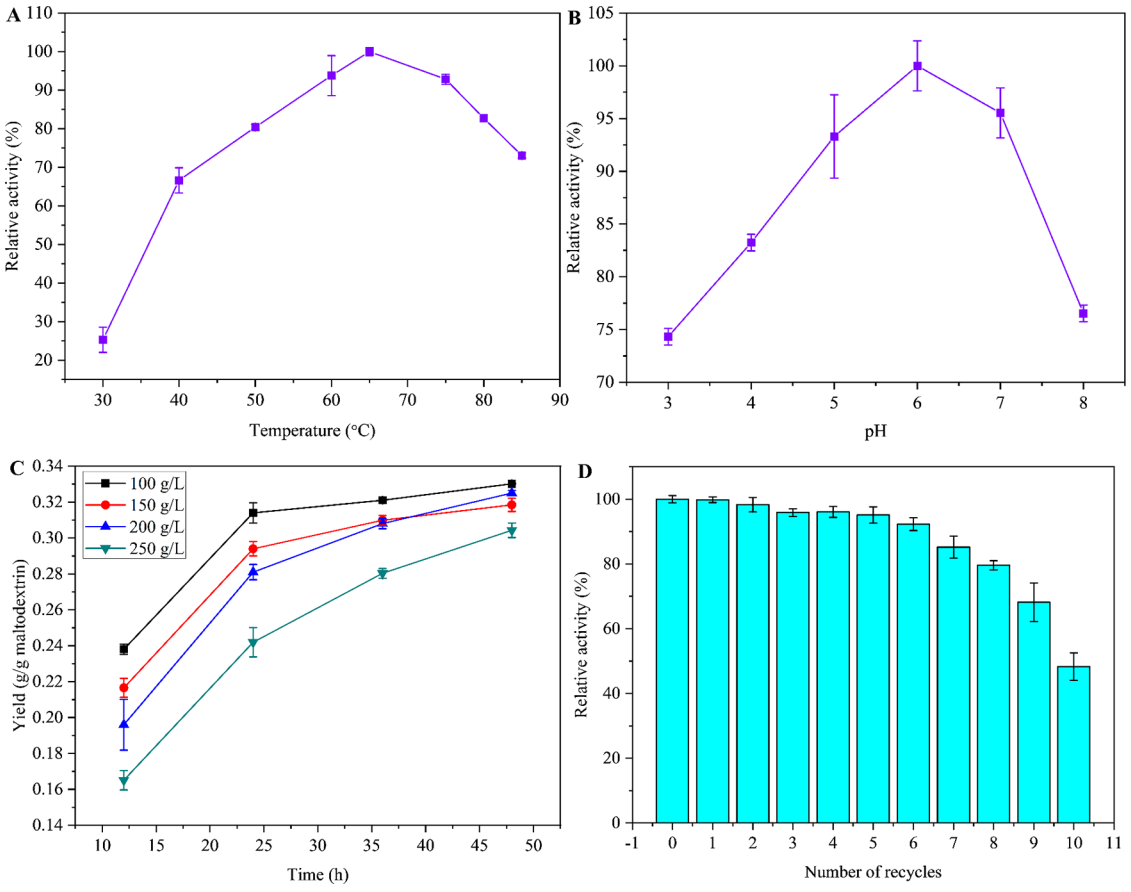

Figure 3. Characterization of MTH-L-MTS produced by engineered *Y. lipolytica* YlxsT08. (A) Effect of temperature on the activity of MTH-L-MTS. Relative activity was calculated by assuming the activity obtained at 65 °C to be 100%. (B) Effect of pH on the activity of MTH-L-MTS. Relative activity was calculated by assuming the activity obtained at pH 6.0 to be 100%. (C) Effect of maltodextrin (DE17.5) concentration on the trehalose production by the YlxsT08. (D) Effect of reuse times on the activity of MTH-L-MTS. Relative activity was calculated by assuming the first round activity as being 100%. The values that corresponded to 100% relative activity for the above cases were 49.77 ± 1.42 U/mg DCW. Error bars represent standard deviation (n = 3).

(about 7 g/L), but the YlxsT05 and the YlxsT06 produced less glucose and maltose byproducts (total 2 g/L). The YlxsT03 and YlxsT04 produced similar trehalose as the YlxsT05 and the YlxsT06, but the glucose and maltose byproducts were higher (total up to 3 g/L). The YlxsT02 had the lowest trehalose production (6.87 g/L) and the highest byproduct content (1.92 g/L glucose, 3.66 g/L maltose, and 7.55 g/L residual substrate). Given the very low hydrolysis activity of the MTS, this result could be explained by the distinct hydrolytic activities of the various MTH forms.[28] As shown in Figure S2A, MTH had the highest activity, followed by YlLip2-MTH (92.06%) and MTH-YlPir1 (64.84%). The high activity of the MTH competed with the substrate of the MTS to produce more byproducts. Another possibility is that the uneven distribution of the two enzymes on the cell surface increases substrate exposure to MTH. According to earlier research, the fusion enzyme catalyzed the subsequent reactions more effectively than an equimolar combination of the two separate enzymes.[29,30] Side effects may also be lessened by the close proximity effect on the catalytic sites of the enzymes. Considering the production of trehalose and the amount of byproducts (glucose and maltose), we finally chose the form of fusion expression. The YlxsT06 was selected as the strain for further modification.

Multicopy Fusion Gene Integration to Enhance Enzyme Production. The enzyme content on the cell surface will affect the reaction time of biocatalysis. Increasing the amount of enzyme will reduce the reaction time and improve the production efficiency.[18] The experiments presented above indicated that the expression pattern of the fusion protein is more conducive to the high yield of trehalose. Hence, to further improve the content of fusion protein, more *mth-l-mts* gene copies were obtained by iterative transformation and integration at the 26S rDNA sites. Strain screening is shown in Figure S1F,G. The final recombinant strains YlxsT07 and YlxsT08 with two and three copies, respectively, were cultured in 20 mL of YPNP (300 g/L glucose) medium, and the erythritol and enzyme production were studied for 96 h. The primary strain, YlxsT01, and the single copy, YlxsT06, were used as controls. As shown in Figure 2A, the YlxsT06, YlxsT07, and YlxsT08 produced erythritol like the YlxsT01 (0.56 g/g glucose), and the enzyme content increased with the increase of gene copy number (from 26.89 U/mg DCW to 49.77 U/mg DCW). The enzyme content of YlxsT08 (49.77 U/mg DCW) is slightly higher than that of YlxsT07 (47.61 U/mg DCW). This may be because the amount of enzymes on the cell surface has reached saturation. Figure S3 displays the time courses of the engineered *Y. lipolytica* YlxsT01 and YlxsT08 producing erythritol in 250 mL shake flasks. The experimental

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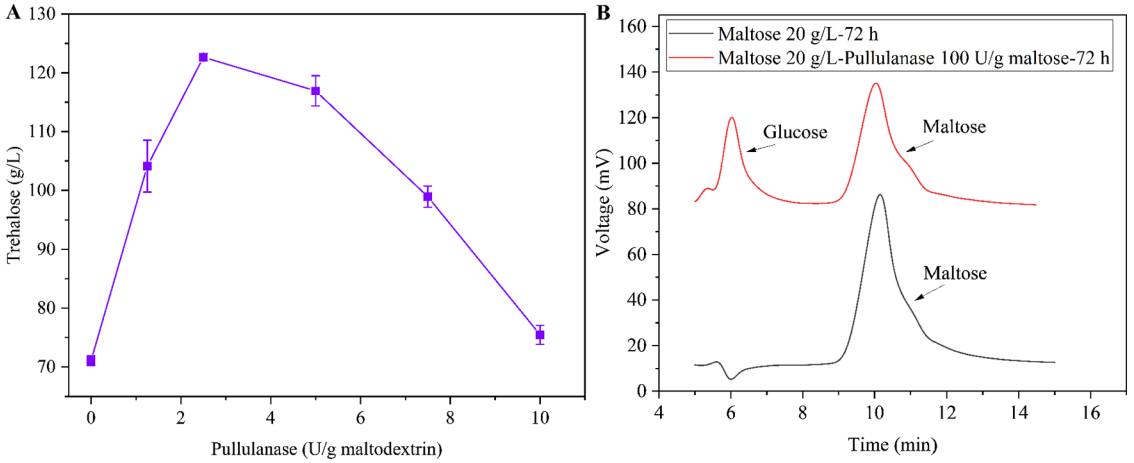

Figure 4. Optimization of trehalose production from maltodextrin (DE17.5) catalyzed by multiple enzymes. (A) Optimization of pullulanase concentration in the reaction mixture containing 200 g/L maltodextrin (DE17.5) 200 g/L and 200 U MTH-L-MTS at 60 °C for 48 h. (B) The chromatography profiles for identification of pullulanase types. The reaction was incubated at 60 °C for 72 h. All plots were shown as means of three independent experiments.

results suggested similar growth, cell biomass, and erythritol yields for strains YlxsT01 and YlxsT08. Subsequently, we observed changes in enzyme activity of YlxsT08 with the production process of erythritol. As shown in Figure 2B, the enzyme content increased with erythritol production progress, reaching the maximum at the end of fermentation (49.31 U/mg DCW). Controlled gene expression and balanced utilization of enzyme resources in the biosynthesis pathway are key issues for the effective production of high-value-added chemicals by microorganisms.[31] As a commonly used tool for heterologous protein expression in *Y. lipolytica*,[27,32] the P_{hp4d} promoter used here successfully balanced exogenous enzyme and erythritol production. The results showed that it was practical to synthesize trehalose by whole-cell transformation coupled with erythritol production by the engineered yeast *Y. lipolytica*.

Characterization of the Fusion Protein Produced by the Strain YlxsT08. In order to further optimize the catalytic conditions, the optimum temperature, pH, substrate concentration, and enzyme stability of the fusion protein were investigated. The optimal temperature for the fusion protein on the cell surface was 65 °C, and up to 90% of the activity remained at 60−75 °C (Figure 3A). Figure 3B demonstrates that the optimal pH for the fusion protein on the cell surface was 6.0, and at least 90% activity persisted at pH 5.0−7.0. The ideal pH for the wild-type MTS and MTH from *S. solfataricus* ATCC 35092 purified from *E. coli* is 5.0.,[28,33] and their optimum temperatures are 75 and 85 °C, respectively. Differences in optimal conditions may be due to mutations in core sites and different expression environments.[34] The cell-surface display of *β*-galactosidase on *Y. lipolytica* had the maximum activity at 60 °C, 20 °C higher than that of free enzyme prepared by *Aspergillus oryzae*.[19] The influence of different substrate concentrations on trehalose yield over time is shown in Figure 3C. In the first 24 h, the substrate maltodextrin was rapidly converted to trehalose [1.31−2.52 g/(L·h)], and then the conversion rate decreased [0.06−0.83 g/(L·h)]. This is due to the substrate being suitable for the enzymatic reaction of high molecular weight gradually into the complicated low molecular weight reaction.[33] As the substrate concentration increased from 200 to 250 g/L, the yield of trehalose decreased from approximately 0.33 to 0.30 g/g maltodextrin. The high substrate concentration caused the solution viscosity to increase, which may affect the enzyme's reaction rate and structure. Another possibility is that there was insufficient enzyme present to catalyze the end of the reaction within 48 h. Considering the balance between the final trehalose production and yield, we finally chose the substrate concentration of 200 g/L. The engineered *Y. lipolytica* cells lost only 10% activity after recycling for 6 times (Figure 3D). The results indicated that the fusion protein on the cell surface was stable and could be easily recycled and used as a whole-cell biocatalyst for the efficient production of trehalose.

Combination of Enzymes Improved Trehalose Production. Through the above condition optimization experiment, the maximum yield of trehalose was 0.33 g/g maltodextrin at 65 °C, pH 6.0, with 200 g/L maltodextrin (DE17.5) for 48 h. Previous studies have shown that the yield of trehalose from starch was 81.50% using the two enzymes from *S. solfataricus* KM1 and a thermostable debranching enzyme. Trehalose production was carried out at 60 °C, pH 5.5, with 100 g/L waxy corn starch (DE0.3) for 72 h.[35] Similarly, more than 80% trehalose yield in 10 h was reached at both a high reaction temperature (75 °C) and a high substrate concentration (100 g/L soluble starch) using the two enzymes from *S. solfataricus* ATCC 35092 and a thermophilic isoamylase.[9] Maltodextrin (15%, DE16) has recently been employed as a substrate, the MTS and MTH from *Arthrobacter ramosus* S34 with cyclodextrin glycosyltransferase (CGTase) and pullulanase at 45 °C, pH 5.5 for 36 h, yielding 0.72 g/g maltodextrin.[8] There were several reasons for the low yield of trehalose here. (i) There are *α*-1,6 bonds in the substrate; without the addition of a debranching enzyme, the yield of trehalose was low (40.90%), because the MTS and MTH could not hydrolyze the *α*-1,6 bonds.[35] (ii) The hydrolysis degree of substrate maltodextrin was relatively high; the yield increased with substrate length.[25] (iii) Residual low molecular weight maltooligosaccharides are challenging to produce trehalose in a short time.[33] Hence, we increased the level of trehalose production by adding pullulanase to the biocatalysis system.

The addition of pullulanase at a concentration of 2.5 U/g of maltodextrin resulted in a significant increase in trehalose production, reaching 0.61 g/g of maltodextrin (Figure 4A).

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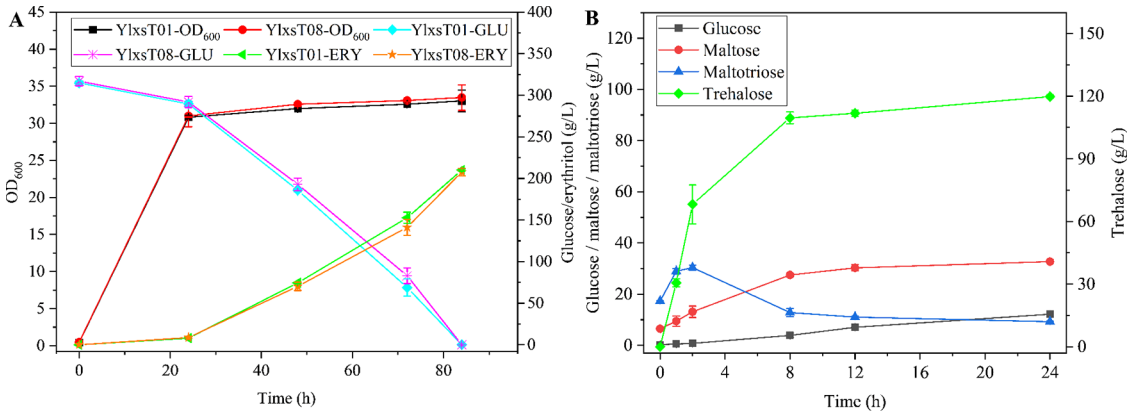

Figure 5. Scale-up of the erythritol and trehalose production in a 5 L bioreactor. (A) Time course of erythritol production by the engineered *Y. lipolytica* YlxsT08, and the strain YlxsT01 was used as a control. (B) Time course of trehalose production by the engineered *Y. lipolytica* YlxsT08.

Table 2. Trehalose Production under Different Maltodextrin DEs[a]

substrate 200 (g/L)	glucose (g/L)	maltose (g/L)	trehalose (g/L)	other sugars (g/L)	Y_{TRE} (g/g maltodextrin)	Q_{TRE} [g/(L·h)]
maltodextrin (DE17.5)	12.15 ± 0.63	32.72 ± 0.74	119.80 ± 0.85	35.32 ± 0.53	0.60 ± 0.02	4.99 ± 0.04
maltodextrin (DE7.7)	2.85 ± 0.21	22.22 ± 0.74	134.40 ± 1.13	40.52 ± 0.61	0.67 ± 0.01	5.60 ± 0.05

[a]Q_{TRE}: trehalose productivity; Y_{TRE}: trehalose yield; and other sugars: maltotriose and residual substrates. The enzyme-containing cells were co-produced with erythritol by the engineered *Y. lipolytica* YlxsT08. The data represent the mean values and standard deviations of three independent experiments.

Table 3. Characterization of Recombinant MTS and MTH from Different Sources and Its Biotechnical Production of Trehalose[a]

sources	enzyme preparation	productive process	P_{TRE} (g/L)	Y_{TRE} (g/g)	Q_{TRE} [g/(L·h]	reference
Sulfolobus solfataricus KM 1	*E. coli* JM109	waxy corn starch (10%, DE0.3), 60 °C, pH 5.5, 72 h, MTS/MTH, and a thermostable debranching enzyme.	81.00	0.82	1.13	Kobayashi et al.[35]
Sulfolobus acidocaldarius ATCC 33909	*S. acidocaldarius* ATCC 33909	starch (30%, hydrolysis 2.9%), 55−57 °C, pH 5.5, 48 h, MTS/MTH/CGTase and isoamylase as a debranching enzyme.	246.00	0.82	5.12	Mukai et al.[11]
Brevibacterium helvolum ATCC 11822	*E. coli* BL21(DE3)	soluble starch (1.0%), 37 °C, pH 7.0, 24 h, α-amylase and the BvMTSH fusion enzyme.	7.04	0.70	0.29	Kim et al.[29]
S. solfataricus ATCC 35092	*E. coli* Rosetta (DE3)	soluble starch (10%), 75 °C, pH 5.0, within 10 h, MTS/MTH and a thermophilic isoamylase.	≥80.00	≥0.80	≥11.80	Fang et al.[9]
Brevibacterium helvolum ATCC 11822	*E. coli* BL21(DE3)	sucrose (20 mM), 37 °C, pH 7.0, 6 h, amylosucrase and the BvMTSH fusion enzyme.	1.08	0.16	0.18	Kim et al.[37]
Arthrobacter ramosus S34	*E. coli* BL21 (DE3)	maltodextrin (15%, DE16), 45 °C, pH 5.5, 36 h, MTS/MTH/CGTase and pullulanase as a debranching enzyme.	107.00	0.72	2.98	Chen et al.[8]
S. solfataricus ATCC 35092	*Y. lipolytica* YlxsT08	maltodextrin (20%, DE7.7), 60 °C, 24 h, MTS-L-MTH and pullulanase as a debranching enzyme.	134.40	0.67	5.60	this study

[a]P_{TRE}: trehalose production titer; Q_{TRE}: trehalose productivity; and Y_{TRE}: trehalose yield.

Nevertheless, excessive use of pullulanase has an adverse effect on the creation of trehalose. The enzymatic activity of pullulanase type II, which is capable of hydrolyzing α-1,4 glycosidic linkages, led to the decrease in substrate chain length, reducing the total trehalose production (Figure 4B).[36]

Trehalose and Erythritol Production in the 5 L Bioreactor. To verify the stability and industrial feasibility of the method, trehalose production coupled with the fermentation process of erythritol production was carried out in a 5 L bioreactor. The YlxsT08 was first used to produce the fusion protein for trehalose biocatalysis production at the same time as erythritol production.

Compared with YlxsT01, enzyme production did not affect the fermentation process of erythritol production (Figure 5A). The results were consistent with the level in 250 mL baffled flasks (Figure S3). The yield, production, and productivity of erythritol reached 0.55 g/g glucose, 166.18 g/L, and 1.98 g/(L·h), respectively. At the end of fermentation, the cells were collected by centrifugation and used to whole-cell catalyze trehalose production from maltodextrin. The supernatant was used to crystallize erythritol. As shown in Figure 5B, most of maltodextrin (DE17.5) was converted to trehalose within 8 h. After 24 h of biocatalysis, trehalose reached 119.80 g/L, yielding 0.60 g/g maltodextrin.

The lower the hydrolysis degree of the maltodextrin substrate, the higher the yield of trehalose.[25] To further increase trehalose production, maltodextrin (DE7.7) was used as a substrate, and the yield, production, and productivity of trehalose reached 0.67 g/g maltodextrin, 134.40 g/L, and 5.60 g/(L·h), respectively. Compared with maltodextrin (DE17.5) as substrate, the byproduct glucose and maltose decreased (Table 2). Compared with some previous studies (Table 3),

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this method is attractive for trehalose production, considering the low cost of enzyme production and the high efficiency of trehalose productivity.

Based on the above experimental results, we developed a green and sustainable enzyme production process coupled with erythritol production for whole-cell catalytic maltodextrin production of trehalose. Briefly, erythritol was produced by fermentation with glucose as a carbon source and YlxsT08 as the strain. The fermentation was finished when glucose was consumed. After centrifugation, the supernatant was used for erythritol crystallization. The obtained cells were cleaned and used for whole-cell biocatalysis of maltodextrin trehalose production. The transformation solution contains a variety of byproducts, such as glucose and maltose (Figure S6A). All byproducts were then converted into glucose by amyloglucosidase (Figure S6B). In industry, glucose and trehalose are further separated by chromatography before crystallization, which is costly and complicated to operate.[38] Biological purification is a low-cost and easy-to-operate method for the purification of sugar-containing liquid.[39] Meanwhile, YlxsT08 could not use trehalose, so we produced cells for the next round of trehalose production by adding a nitrogen source to the mixture of glucose and trehalose in situ.

The purified fermentation broth contained only trehalose due to its low glucose content and sufficient nitrogen supply, without detection of erythritol (Figure S6C). After centrifugation, the cells were used as catalysts for an additional five cycles of trehalose synthesis (Figure S4), and the supernatant was used to purify trehalose. The supernatant was concentrated after decolorization with activated carbon and crystallized after cooling. The purity of trehalose reached 99.24%. Figure S5 displays the product identification for erythritol and trehalose. The nutrients of residual cells obtained by centrifugation after the catalytic process were also determined. The yeast powder contains 18.01% protein and 16.47% lipid content and is rich in a variety of amino acids (Table S4), especially aspartic acid (Asp) and glutamic acid (Glu). Hence, it may be used as an advantageous constituent in animal feed.

CONCLUSIONS

This study proposes a novel and cost-effective strategy for green enzyme preparation used for whole-cell biocatalyzed trehalose production from maltodextrin, coupled with erythritol production by engineered *Y. lipolytica*. The engineered yeast was first evaluated for erythritol production accompanied by the generation of cell-surface enzymes, with results of 0.55, 166.18, and 1.98 g/(L·h) in a 5 L bioreactor for yield, production, and productivity, respectively. The yeast cells accumulated after erythritol production were employed as an enzyme to catalyze trehalose synthesis from maltodextrin. Compared with the traditional *E. coli* enzyme production method, protein purification, and physical immobilization were not required, thus further reducing the production costs. The yield, production, and productivity of trehalose reached 0.67 g/g maltodextrin, 134.40 g/L, and 5.60 g/(L·h) in a 5 L bioreactor. After biological purification, the purity of trehalose obtained from crystallization reached 99.24%. In addition, residual cells that remain after biocatalysis may be reused as a cost-effective and valuable supplement for animal feed.

ASSOCIATED CONTENT

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.3c07191.

Sequences of genes used in this study; plasmids used in this study; primers used in this study; contents of main amino acids in waste yeast powder; selection of *Y. lipolytica* transformants after PCR check; evaluation of the relative enzyme activity; time courses of erythritol production by the engineered *Y. lipolytica* YlxsT01 and YlxsT08 in 250 mL shake flasks; effect of reuse times on the yield of trehalose production in the 5 L bioreactor; product identification of erythritol and trehalose; chromatographic diagram of products analysis during the trehalose production and biological purification; and methods (construction of strains and integrative plasmids) (PDF)

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Notes

The authors declare no competing financial interest.

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