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BIOTECHNOLOGY

Biosynthesis of Raffinose and Stachyose from Sucrose via an *In Vitro* Multienzyme System

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ABSTRACT Herein, we present a biocatalytic method to produce raffinose and stachyose using sucrose as the substrate. An *in vitro* multienzyme system was developed using five enzymes, namely, sucrose synthase (SUS), UDP-glucose 4-epimerase (GalE), galactinol synthase (GS), raffinose synthase (RS), and stachyose synthase (STS), and two intermedia, namely, UDP and inositol, which can be recycled. This reaction system produced 11.1 mM raffinose using purified enzymes under optimal reaction conditions and substrate concentrations. Thereafter, a stepwise cascade reaction strategy was employed to circumvent the instability of RS and STS in this system, and a 4.2-fold increase in raffinose production was observed. The enzymatic cascade reactions were then conducted using cell extracts to avoid the need for enzyme purification and supplementation with UDP. Such modification further increased raffinose production to 86.6 mM and enabled the synthesis of 61.1 mM stachyose. The UDP turnover number reached 337. Finally, inositol in the reaction system was recycled five times, and 255.8 mM raffinose (128.9 g/liter) was obtained.

IMPORTANCE Soybean oligosaccharides (SBOS) have elicited considerable attention because of their potential applications in the pharmaceutical, cosmetics, and food industries. This study demonstrates an alternative method to produce raffinose and stachyose, which are the major bioactive components of SBOS, from sucrose via an *in vitro* enzyme system. High concentrations of galactinol, raffinose, and stachyose were synthesized with the aid of a stepwise cascade reaction process, which can successfully address the issue of mismatched enzyme characteristics of an *in vitro* metabolic engineering platform. The biocatalytic approach presented in this work may enable the synthesis of other valuable galactosyl oligosaccharides, such as verbascose and higher homologs, which are difficult to obtain through plant extraction.

KEYWORDS *in vitro* metabolic engineering, multienzyme system, soybean oligosaccharides, stepwise cascade reaction, sucrose

In vitro metabolic engineering comprising numerous enzymes and/or coenzymes has emerged as a promising biomanufacturing platform for producing the desired products. This technology features several compelling advantages over living microorganisms, such as high product yields, fast reaction rates, easy process control and optimization, easy product separation, and the construction of nonnatural synthetic pathways (1–3). The manufacturing potential of this process has recently come under investigation, and its applications have been evaluated in, for example, the synthesis of rare sugars from formaldehyde (4), hydrogen from numerous sugars (5, 6), higher alcohols from methanol (7), fructose 1,6-diphosphate and *myo*-inositol from starch (8, 9), α-ketoglutarate from glucuronate (10), and amorpha-4,11-diene from mevalonic acid (11). This excellent cell-free synthetic biology platform demonstrates a remarkable

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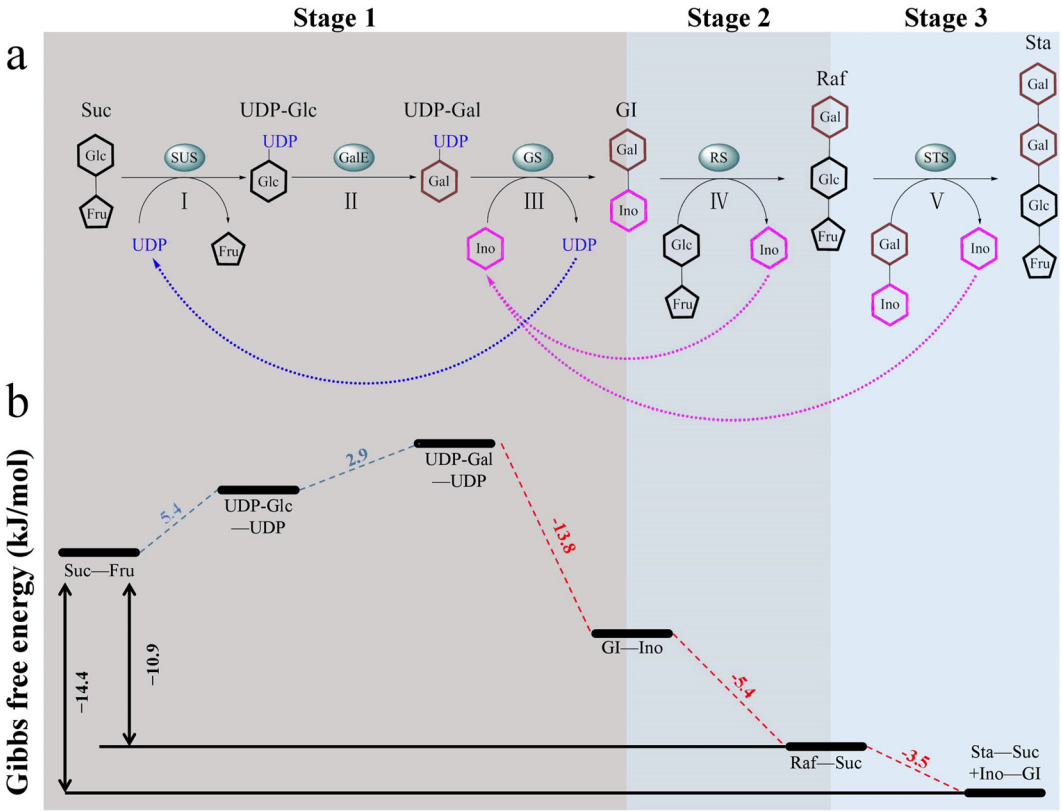

FIG 1 (a) *In vitro* enzymatic pathways for the synthesis of raffinose and stachyose from sucrose. (b) Standard Gibbs free energy change of each and the overall reaction. Suc, sucrose; UDP-Glc, UDP-glucose; UDP-Gal, UDP-galactose; GI, galactinol; Raf, raffinose; Sta, stachyose; Glc, glucose; Fru, fructose; Gal, galactose; Ino, inositol. The ΔG° represents the change of Gibbs free energy and was freely available on the website http://equilibrator.weizmann.ac.il.

potential in the synthesis of artificial and valuable oligosaccharides. For example, one-pot enzymatic synthesis of galacto-*N*-biose from sucrose and *N*-acetylgalactosamine has been achieved by the concomitant action of four enzymes (12). Yu and Chen developed 12 effective one-pot multienzyme (OPME) systems for the direct production of eight sugar nucleotides, including UDP-Glc, UDP-GlcNAc, UDP-GalNAc, UDP-Gal, UDP-GlcA, GDP-Man, GDP-Fuc, and CMP-Sia, from their corresponding simple monosaccharides (13). Sequential assemblies of those OPME systems have synthesized the lacto-*N*-neotetraose and its sialyl and fucosyl derivatives in the preparative scale (14, 15).

Soybean oligosaccharides (SBOS) represent a mixture of carbohydrates extracted from soybeans. These short-chain carbohydrates exhibit numerous biological functions, such as prebiotics, hypotensive action, immunity activity, and liver protection (16, 17). Raffinose and stachyose are the major bioactive components of SBOS (18). These oligosaccharides are traditionally extracted from plants, but such extraction processes usually suffer from low yields and high production costs (19). Whole-cell transformation of a recombinant cell expressing a levansucrase has recently been developed to produce raffinose from melibiose and sucrose with high productivity. Nevertheless, melibiose is rare and expensive (20, 21). In addition, few reports have thoroughly described a biosynthetic approach for producing stachyose or higher homologs.

In this study, we present a biocatalytic method to produce raffinose and stachyose by constructing two *in vitro* enzymatic systems (Fig. 1a). The cascade reactions were conducted using the cell lysate supernatant without the addition of the expensive UDP. A stepwise reaction strategy was employed to address mismatches in enzyme characteristics. Multiple batch reaction processes were performed to yield high concentrations of raffinose.

TABLE 1 Properties of enzymes and reactions

Enzyme	Source	UniProt accession no.	MW[a] (kDa)	Activity (U/mg)[b]	
				Cell extract	Purified enzyme
SUS	*A. thaliana*	NP_197583.1	113.0	0.18 ± 0.05	0.47 ± 0.12
GalE	*E. coli*	WP_074488048.1	37.3	6.61 ± 0.30	24.21 ± 0.41
GS	*A. thaliana*	NP_172406.1	38.7	9.25 ± 1.29 ($\times 10^{-3}$)	22.25 ± 4.29 ($\times 10^{-3}$)
RS	*A. thaliana*	NP_198855.1	106.2	5.58 ± 0.95 ($\times 10^{-3}$)	16.75 ± 1.56 ($\times 10^{-3}$)
STS	*A. thaliana*	NP_192106.3	108.1	0.02 ± 0.03	0.21 ± 0.04

[a]MW, molecular weight.
[b]One unit of enzymatic activity was defined as the amount of enzyme synthesizing 1 μmol of product per minute.

RESULTS

***In vitro* pathway design to synthesize raffinose and stachyose.** The synthesis of SBOS in plants requires galactinol as a glycosyl donor, which can be obtained from UDP-galactose and inositol catalyzed by galactinol synthase (GS; EC 2.4.1.123). The sequential transfer of galactosyl from galactinol to sucrose catalyzed by raffinose synthase (RS; EC 2.4.1.82) and stachyose synthase (STS; EC 2.4.1.67) yields raffinose and stachyose, respectively (Fig. 1a). Previous studies have reported the production of UDP-galactose with UTP and galactose-1-phosphate as the substrates (22, 23). However, UTP and galactose-1-phosphate are expensive. Sucrose synthase (SUS; EC 2.4.1.13) catalyzed the reversible conversion of sucrose and UDP into fructose and UDP-glucose through a one-step activation process (24). UDP-glucose can be easily isomerized to the desired UDP-galactose using UDP-galactose 4-epimerase (GalE; EC 2.4.1.13). This mechanism indicates that the use of SUS and GalE could provide a cost-effective approach for preparing UDP-galactose using sucrose and UDP as the substrates. The consumed UDP can be regenerated during galactinol formation. Therefore, in this work, we designed two *in vitro* enzymatic pathways using five enzymes, namely, SUS, GalE, GS, RS, and STS, to synthesize raffinose and stachyose from sucrose (Fig. 1a). The overall reactions can be written as the following stoichiometric reactions: (i) 2 sucrose $\rightarrow$ fructose + raffinose; (ii) 3 sucrose $\rightarrow$ 2 fructose + stachyose.

In this multienzyme system, UDP and inositol can be recycled in one vessel. The standard Gibbs energy changes ($\Delta G°$) of reactions I to V were calculated (25). Although reactions I and II were thermodynamically unfavorable under standard conditions, the $\Delta G°$ calculated for the overall routes of raffinose and stachyose synthesis were -10.9 and -14.4 kJ/mol (Fig. 1b), respectively, pushing the overall reaction forward.

Proof-of-concept biosynthesis of raffinose. To conduct the multienzyme cascade reaction for raffinose and stachyose synthesis, the corresponding enzymes should be prepared. Raffinose family oligosaccharides play important roles in the tolerance of *Arabidopsis thaliana* plants to drought, high salinity, and cold stress (26). The corresponding GS, RS, and STS from *A. thaliana* have been identified, and the SUS and STS from *A. thaliana* and GalE from *Escherichia coli* have been successfully expressed and characterized in *E. coli* (DE3) (27–29). Therefore, these enzymes were selected to construct a multienzyme system in this work. In a search of the NCBI database, the gene sequences of *SUS1*, *GolS3*, *SIP1*, and *STS* from *A. thaliana* encoding SUS, GS, RS, and STS, respectively, were obtained, codon-optimized according to their usage bias in *E. coli* (gene sequences are presented in the supplemental material), and inserted into the expression plasmid pET28 or pET32. The GalE gene was directly amplified from the genome of *E. coli*. All enzymes were expressed functionally in *E. coli* BL21(DE3) (see Fig. S1). The soluble portions of RS and STS obtained were extremely low, and SDS-PAGE of the final purified enzymes showed that the molecular masses of SUS (113.0 kDa), GalE (37.3 kDa), GS (38.7 kDa), RS (106.2 kDa), and STS (108.1 kDa) were in agreement with the sizes predicted by their amino acid sequences. The specific activities of purified enzymes for SUS, GalE, GS, RS, and STS at 30°C were 0.47, 24.21, 0.022, 0.016, and 0.21 U/mg, respectively (Table 1), and the V_{max} values of RS and GS for sucrose were 0.025 and 0.019 μmol/(min · mg), respectively (Table 2).

TABLE 2 Kinetic parameters of relative enzymes

Enzyme	Substrate	K_m (mM)	V_{max} (μmol · min^{-1} · mg^{-1})	k_{cat} (s^{-1})	Reference or source
SUS	UDP	0.39 ± 0.03	0.59 ± 0.05	1.10	27
GalE	UDP-glucose	1.2 ± 0.05	28.95 ± 2.42	18.01	28
GS	Inositol	5.70 ± 1.65	25.36 ± 1.29 ($\times 10^{-3}$)	15.36 $\times 10^{-3}$	This paper
RS	Sucrose	3.22 ± 0.93	18.59 ± 2.29 ($\times 10^{-3}$)	36.42 $\times 10^{-3}$	This paper
STS	Raffinose	0.26 ± 0.02	0.28 ± 0.01	0.510	29

The proof-of-concept biosynthesis of raffinose was conducted by mixing purified SUS (0.24 U, 0.5 mg), GalE (0.24 U, 0.1 mg), GS (0.2 U, 10 mg), and RS (0.32 U, 20 mg) with 50 mM sucrose, 3 mM inositol, and 5 mM UDP. This platform successfully produced 1.33 mM raffinose. The effects of temperature, pH, and metal ions on raffinose production were then investigated to determine the optimal reaction conditions. The optimal temperature and pH for the cascade reactions were 30°C and 7, respectively (Fig. 2a and b). The metal ions used inhibited raffinose production at different levels (Fig. 2c), thereby indicating that the production was independent of metal ions. The impacts of the concentrations of UDP and sucrose on raffinose production were measured. Raffinose was produced at 5 mM UDP, but production was inhibited when the concentration of UDP was increased to 10 mM or higher (Fig. 2d). An increase in the initial sucrose concentration in the medium resulted in improved raffinose production (Fig. 2e). When the sucrose concentration was 600 mM, for example, 11.1 mM raffinose was produced (Fig. 2f).

Improvement of raffinose production using a one-pot two-stage reaction process. Although raffinose was synthesized via a one-pot one-stage reaction process, the production efficiency observed was far from satisfactory. We found that the enzyme activity of RS and STS significantly decreased when these enzymes were reacted at 30°C for 36 h (see Fig. S3). This result indicates that the instability of RS and STS may lead to the low production efficiency of raffinose. To circumvent this problem, a stepwise reaction process containing two reaction stages was employed. In stage 1, the first

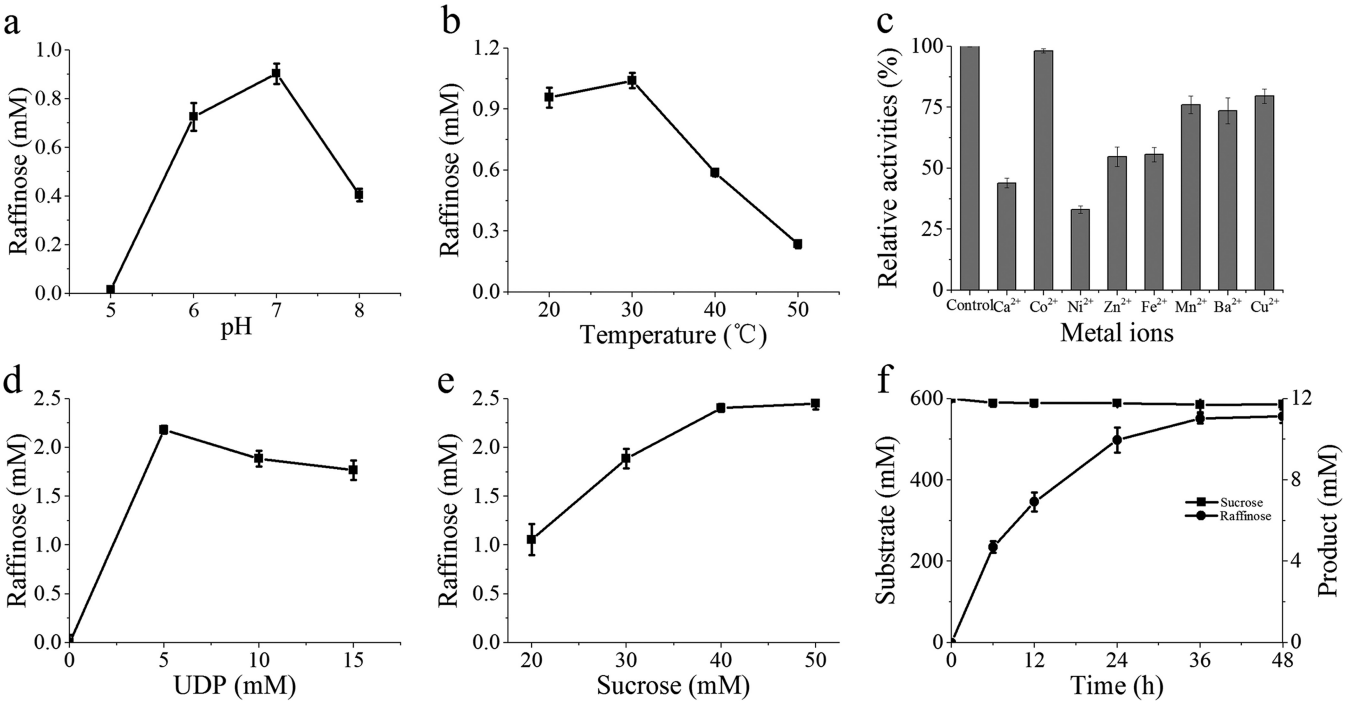

FIG 2 Optimization of reaction conditions for raffinose production by the one-pot one-stage reaction. (a) Effects of temperature on raffinose production. (b) Effects of pH on raffinose production. (c) Effects of metal ions on enzymatic activity. (d) Effects of UDP concentration on raffinose production. (e) Effects of sucrose concentration on raffinose production. (f) Biosynthesis of raffinose with 600 mM sucrose under optimal conditions.

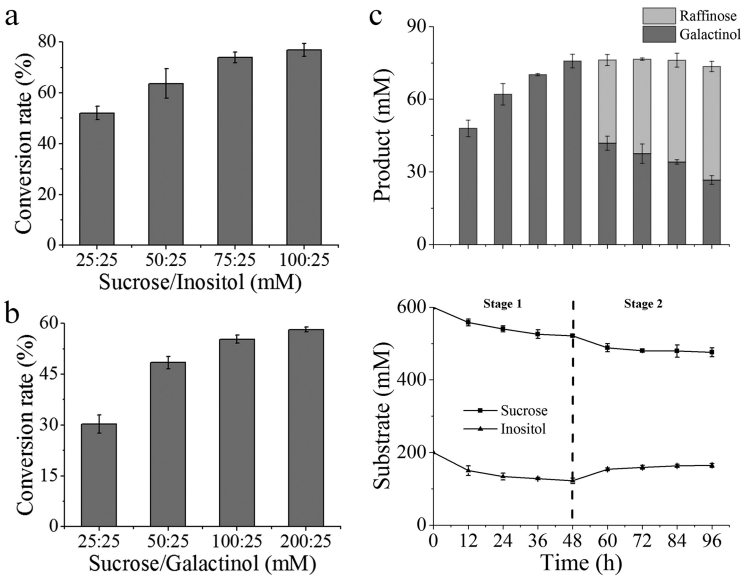

FIG 3 (a) Effects of sucrose/inositol ratio on galactinol production. (b) Effects of sucrose/galactinol ratio on raffinose production. (c) Time courses of raffinose production with purified enzymes using a one-pot two-stage reaction process. In stage 1, purified SUS, GalE, GS, sucrose, and inositol were added to the reaction medium. In stage 2, purified RS powder was supplemented.

three enzymes (SUS, GalE, and GS) were arranged to convert sucrose to galactinol efficiently. In stage 2, the obtained galactinol was supplemented with RS and/or STS to produce raffinose or stachyose (Fig. 1a).

According to the stepwise cascade reaction approach, high galactinol production in stage 1 was necessary for raffinose synthesis. This objective could be achieved by increasing the conversion rate of galactinol and using high concentrations of inositol. Then, the concentration ratio of sucrose to inositol in the reaction medium was optimized. When the concentration ratio (mM/mM) was improved from 25:25 to 75:25, the conversion rate of galactinol production increased to 74% (Fig. 3a). Further adjustment of the sucrose/inositol ratio to 100:25 only slightly increased the conversion rate. On the basis of the optimized concentration ratio, we implemented a reaction system using SUS, GalE, GS, sucrose (600 mM), and inositol (200 mM) in stage 1 and obtained 76 mM galactinol with a conversion rate of 38% after reacting for 48 h (Fig. 3c; see also Fig. S2).

The raffinose synthesis reaction catalyzed by RS was reversible, and the reaction equilibrium inevitably restricted the conversion rate. To circumvent this problem, the substrate concentration ratio of sucrose to galactinol was optimized. When this ratio was improved from 25:25 to 100:25, the conversion rate increased from 31% to 55%. Further improvement of the ratio from 100:25 to 200:25 only resulted in a slight increase in the conversion rate (Fig. 3b). We obtained nearly 76 mM galactinol in stage 1 and detected 420 mM residual sucrose, which means the concentration ratio of sucrose to galactinol was 5.5:1. Therefore, the protein powder of purified RS (0.32 U, 10 mg) was directly supplemented into the reaction system in stage 2 to produce 46.8 mM raffinose with a conversion rate of 60.5% (Fig. 3c and Fig. S2). Raffinose production in this optimized system was 4.2-fold higher than that using the one-pot one-stage reaction process (Table 3).

Production of raffinose using cell extracts. In our multienzyme system, UDP serves as an intermedium to transfer galactosyl and can be recycled. We detected small amounts of UDP and UDP-glucose (total near 20 μmol/g cell dry weight) in the cell lysate supernatant of *E. coli* and measured the crude enzyme activities of SUS, GalE, GS, RS, and STS to be 0.18, 6.61, 0.009, 0.006, and 0.02 U/mg, respectively (Table 1). We thus believe that raffinose synthesis using cell extracts is a feasible and cost-effective

TABLE 3 Raffinose and stachyose production under different reaction conditions

No.	Substrate(s)	Product	Reaction process and medium	Production (mM)	Yield (mol/mol)	Conversion (%)
1	Sucrose/inositol	Galactinol	One stage, purified enzyme	138.3 ± 3.8	0.96[c]	69[c]
2	Sucrose	Raffinose	One stage, purified enzyme	11.1 ± 0.3	0.42[d]	1.6[d]
3	Sucrose	Raffinose	Two stages, purified enzyme	46.8 ± 1.9	0.36[d]	7.8[d]
3	Sucrose	Raffinose	Two stages, crude enzyme[a]	72.4 ± 2.8	0.39[d]	12.1[d]
3	Sucrose	Raffinose	Two stages, crude enzyme[b]	86.6 ± 2.8	0.39[d]	14.4[d]
4	Sucrose/raffinose	Stachyose	Two stages, crude enzyme	61.1 ± 2.9	0.71[e]	30.6[e]
5	Sucrose	Stachyose	Three stages, crude enzyme	22.5 ± 1.2	0.11[d]	3.8[d]
6	Sucrose	Raffinose	Inositol recycling, crude enzyme	255.8 ± 10.8	0.40[d]	

[a]In stage 2, enzyme RS was supplemented in the reaction medium.
[b]In stage 2, enzyme RS and fresh sucrose (150 mM) were supplemented in the reaction medium. The yield was calculated with the ratio of product formation to substrate consumption. The conversion was calculated with the ratio of product amount to substrate used.
[c]The conversion and yield were calculated with the ratio of galactinol concentration to inositol.
[d]The conversion and yield were calculated with the ratio of product concentration to sucrose.
[e]The conversion and yield were calculated with the ratio of stachyose concentration to raffinose.

approach, because such a process would avoid the need for enzyme purification and additional supplementation with UDP. The multienzyme cascade reaction was performed using cell extract-based lyophilized powders of SUS (0.36 U, 2 mg), GalE (0.33 U, 0.5 mg), and GS (0.36 U, 40 mg), 600 mM sucrose, and 200 mM inositol. In stage 1, 138.0 mM galactinol was produced with a conversion rate of 69% (Table 3), which is 1.8-fold higher than the value using purified enzymes. This increase in raffinose production is likely due to the higher enzyme amount used in the present reaction system compared with that applied in the previous system. Then, cell extract-based lyophilized powders of RS (0.36 U, 60 mg) were added to the reaction system for raffinose production in stage 2. After another 48 h of reaction, 72.4 mM raffinose was produced with a yield of 0.39 mol/mol sucrose, representing 78% of the theoretical value (Fig. 4a). We increased the scale of the reaction system to 50 ml to prepare galactinol and raffinose. The amount of each component was increased accordingly, and at the end of the reaction, we obtained 146 mM galactinol and 76.4 mM raffinose. We then purified these two compounds and determined their structures using nuclear magnetic resonance (NMR) analysis (see Fig. S4 to S5). The isolated yields for galactinol and raffinose were 61.4% and 30.3%, respectively.

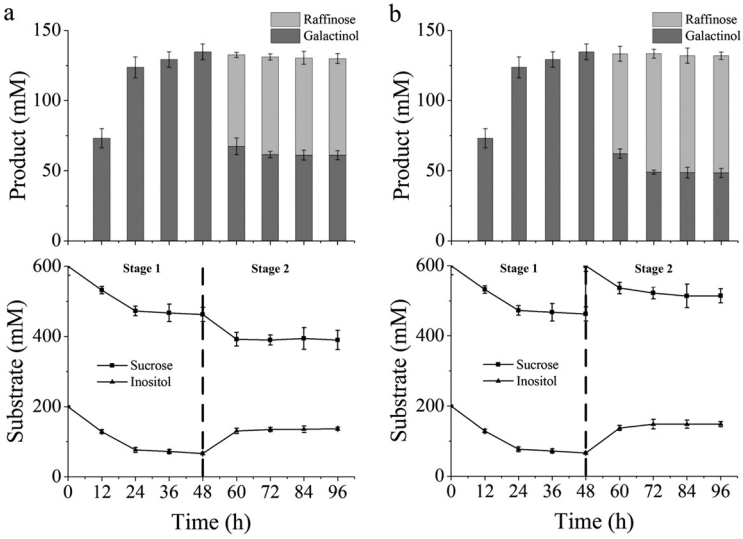

FIG 4 Time courses of raffinose production with cell extracts by two different stepwise reaction strategies. (a) Without supplement of sucrose in stage 2. In stage 1, cell extract-based lyophilized powders of SUS, GalE, GS, sucrose, and inositol were added to the reaction medium. In stage 2, cell extract-based lyophilized powders of RS were supplemented. (b) Supplement of sucrose in stage 2. The materials in stage 1 were similar to that for panel a. In stage 2, cell extract-based lyophilized powder of RS and fresh sucrose powder were supplemented.

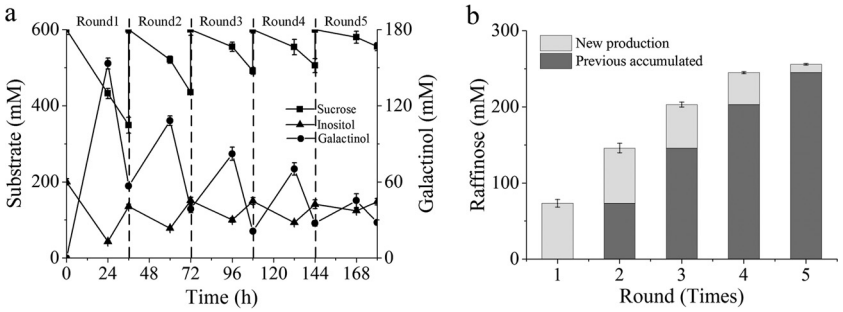

FIG 5 Raffinose production via recycling of inositol. (a and b) Inositol was recycled five times, and 73.2, 72.5, 57.2, 42.0, and 10.9 mM raffinose was synthesized from reactions in batches 1, 2, 3, 4, and 5, respectively. New production indicates that the raffinose production in every batch reaction; previous accumulated indicates total raffinose production from previous batch reactions relative to the current batch reaction.

To shift the reaction equilibrium to raffinose production, we carried out experiments by supplementing RS and fresh sucrose (150 mM, 51.3 mg) powders into the reaction medium in stage 2. This modification enhanced raffinose production to 86.6 mM (43.6 g/liter) with a conversion rate of 14.4%, which is 7.8-fold higher than that observed using the one-pot one-stage reaction process (Fig. 4b). The turnover number of UDP also increased to 337.

Inositol recycling to further increase raffinose production. During the raffinose production process, inositol serves as a partner to transfer galactosyl and is regenerated in the last reaction step. We then designed a cyclic reaction process to investigate the feasibility of inositol recycling. In the first batch reaction, the cell extract-based lyophilized powders of SUS, GalE, and GS, as well as sucrose (600 mM, 205 mg) and inositol (200 mM, 36 mg) powders, were mixed in stage 1, and RS was supplemented in stage 2. After reacting for 36 h, 73.2 mM raffinose was produced. The reaction system was centrifuged, and the supernatant was transferred to a new tube reactor. Then, the same amounts of sucrose and enzyme powder were supplemented for the next batch reaction. Inositol was recycled five times. This system produced 72.5, 57.2, 42.0, and 10.9 mM raffinose in batches 2, 3, 4, and 5, respectively (Fig. 5a and b). The significant decrease in raffinose production during the last round of the reaction may be due to product inhibition and the reaction equilibrium. If raffinose is promptly separated from the reaction system, the yield would further increase. The present system synthesized 255.8 mM (128.9 g/liter) raffinose with a yield of 0.4 mol/mol sucrose (Table 3), and 555 mM sucrose, 154 mM inositol, and 33.6 mM galactinol remained in the medium. The total concentrations of inositol and galactinol were 6.2% lower than the initial concentration of 200 mM. This loss may have resulted from decreases in the sample volume over consecutive batches with a sampling frequency of 11 (11×5).

Improvement of stachyose production using stepwise cascade reaction process. Stachyose was synthesized by transferring the galactosyl moiety from galactinol to raffinose. We initially mixed lyophilized cell extract powders of SUS, GalE, GS, RS, and STS with sucrose and inositol to synthesize stachyose via the one-pot one-stage reaction process. However, no desired product was detected. Then, the stepwise cascade reaction process comprising three stages was used (Fig. 1a). The reaction media in stages 1 and 2 were similar to those used for raffinose synthesis. After reacting for 24 h, the system produced 55.1 mM raffinose and retained 81.6 mM galactinol (Fig. 6a). The crude enzyme powder of STS (0.4 U, 20 mg) was supplemented in stage 3 to convert raffinose and galactinol to stachyose. This system produced 22.5 mM (15.0 g/liter) stachyose with a yield of 0.11 mol/mol sucrose, representing 33% of the theoretical value (Table 3). We conducted larger-scale experiments (50 ml) to test the extent of stachyose production. The reaction system resulted in 25.6 mM stachyose. The purified product was obtained with an isolated yield of 9.8%, and its structure was determined using NMR analysis (see Fig. S6).

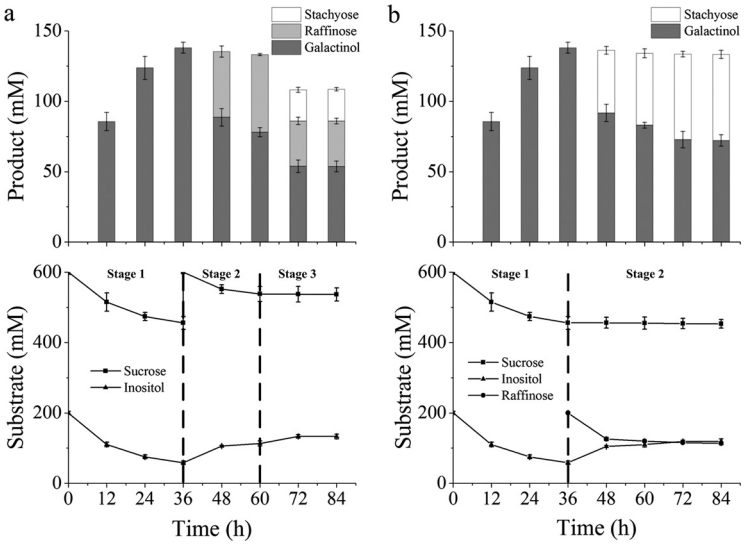

FIG 6 Time courses of stachyose production by two different stepwise reaction strategies. (a) Synthesis of stachyose by the one-pot three-stage reaction. In stage 1, cell extract-based lyophilized powders of SUS, GalE, GS, sucrose, and inositol were added to the reaction medium. In stage 2, cell extract-based lyophilized powders of RS were added. In stage 3, cell extract-based lyophilized powders of STS were supplemented. (b) Synthesis of stachyose by the one-pot two-stage reaction. The materials in stage 1 were similar to that for panel a. In stage 2, cell extract-based lyophilized powders of STS and fresh raffinose powders were supplemented.

We also developed a two-stage reaction process for stachyose production. In stage 1, the reaction media were identical to those used for raffinose synthesis. In stage 2, the crude enzyme powders of STS and fresh raffinose (200 mM, 100.8 mg) were supplemented into the reaction system. Stachyose production reached 61.1 mM (40.7 g/liter) with a yield of 0.71 mol/mol sucrose (Fig. 6b).

DISCUSSION

Transglycosylation-based reaction routes play a major role in the synthesis of valuable oligosaccharides and their derivatives (24, 30). Several one-pot multienzyme systems have been developed to synthesize artificial oligosaccharides by transferring the glycosyl from activated nucleotide sugars to mono- or oligosaccharides (14, 15). However, these systems usually suffer from low product concentrations and high production costs resulting from the use of expensive nucleotides, such as UDP, ATP, and GTP. Herein, we presented an *in vitro* metabolic engineering platform for the low-cost production of raffinose and stachyose from sucrose. In the proposed system, UDP and inositol were reused and regenerated to push sucrose to form the desired product. To decrease production costs, cell extracts containing expressed enzymes and a small amount of UDP were used in the reaction medium to avoid enzyme purification and additional supplementation with UDP (31). Stachyose was produced by *in vitro* enzymatic catalysis, a process that has not been reported in previous studies.

One of the major concerns related to the *in vitro* metabolic engineering platform is mismatched or undesirable enzyme characteristics, such as poor enzyme activity, stability, nonideal reaction conditions, substrate specificity, and substrate or product inhibition (32). A "two stages in one pot" strategy was thus proposed to address these problems (33, 34). We previously employed a similar strategy, namely, the stepwise cascade reaction approach, to decrease by-product formation resulting from the low substrate specificity of the enzyme component (4). In the raffinose and stachyose synthesis platform presented in this work, RS and GS exhibited low enzyme activity and instability, which restricted raffinose production during the one-pot one-stage reaction process. Therefore, the stepwise cascade reaction strategy was used to boost production. Galactinol synthesis is the key node in raffinose and stachyose production. Then,

the reaction process could be divided into two parts, namely, galactinol synthesis and raffinose and stachyose production. In the first stage, an optimal production of galactinol is necessary. In the second stage, galactinol serves as the galactosyl donor to synthesize the desired product. Interestingly, the stepwise reaction approach facilitated a 7.8-fold increase in raffinose production and yielded 61.1 mM stachyose from zero.

Raffinose and stachyose are traditionally extracted from plants such as soybean, cottonseed, and sugar beet; however, the yields of these metabolites are usually low (19). In addition, such extraction obtains the mixture of several types of SBOS, and the preparation of a single component involves complex purification steps. The biocatalytic method presented in the present work provides an alternative means to prepare SBOS. Levansucrase and galactosidase have been developed for the biosynthesis of raffinose; however, processes employing these metabolites suffer from high substrate costs and low conversion rates (20, 35). In the present study, we developed an efficient and cyclic reaction process to obtain high concentrations of galactinol (138.0 mM), raffinose (225.8 mM), and stachyose (61.1 mM). The galactinol may serve as a galactosyl carrier and facilitate the synthesis of other SBOS, such as verbascose and higher homologs. The obtained raffinose can be used as feedstock to produce melibiose with the aid of recombinant *Saccharomyces cerevisiae* cells (36). Our reaction system also meets the problem of low enzyme activity and stability of RS, GS, and STS; reaction systems employing these materials often require larger amounts of enzymes and longer reaction times to circumvent these disadvantages. Therefore, improvements in the enzymatic activity, expression level, and stability of RS, GS, and STS through database mining or a protein engineering strategy may be attempted in future researches.

In conclusion, we demonstrated an efficient and cost-effective approach to biosynthesize galactinol, raffinose, and stachyose via an *in vitro* multienzyme system and a stepwise cascade reaction process. The biocatalytic approach opens new avenues for the synthesis of other valuable galactosyl oligosaccharides that are not easily obtained through plant extraction.

MATERIALS AND METHODS

Materials. Standard raffinose and stachyose were purchased from Sigma (St. Louis, MO, USA). Sucrose, inositol, and galactinol were obtained from Fisher Scientific (Pittsburgh, PA). Ampicillin, kanamycin, and isopropyl-β-D-1-thiogalactopyranoside (IPTG) were purchased from Solarbio (Beijing, China). A nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography column was obtained from Qiagen (Hilden, Germany).

Strains and plasmids. *E. coli* DH5α was used as the host for DNA manipulation, and *E. coli* BL21(DE3) was employed for protein expression. The vectors pET28 and pET32 were used for gene expression. The gene sequences of *SUS1*, *GolS3*, *SIP1*, and *STS* from *Arabidopsis thaliana* were codon optimized according to usage bias in *E. coli* (see the gene sequences presented in the supplemental material) and synthesized by GenScript (Nanjing, China). The *galE* gene was amplified from *E. coli* MG1655. The *SUS1*, *SIP1*, and *STS* genes were linked to pET32 to produce recombinant plasmids pETHisSUS, pETHisRS, and pETHisSTS, respectively. In addition, *galE* and *GolS3* were ligated to pET28 to obtain pETHisGalE and pETHisGS, respectively.

Protein expression and purification. *E. coli* BL21(DE3) strains harboring the expression plasmids were cultured at 37°C in 1 liter of LB medium (10 g/liter tryptone, 5 g/liter yeast extract, and 10 g/liter sodium chloride) to an optical density and 600 nm (OD_{600}) of 0.6. IPTG (1 mM) was added to the culture to induce protein expression, and the temperature was adjusted to 16°C to avoid the formation of inclusion bodies. After incubating for an additional 20 h, the cells were harvested, washed twice, and suspended in 50 mM phosphate-buffered saline (PBS; pH 7.0). Then, the cells were disrupted by sonication and centrifuged at 14,000 $\times$ *g* at 4°C for 10 min to obtain the crude enzymes. The clear supernatant was collected and loaded onto a Ni^{2+}-NTA-agarose column preequilibrated with binding buffer (50 mM PBS, 300 mM NaCl, 20 mM imidazole, pH 7.0). The retained proteins were recovered with elution buffer (50 mM PBS, 300 mM NaCl, 300 mM imidazole, pH 7.0). The purified proteins and crude enzymes were freeze-dried using a vacuum pump and stored at −20°C. Protein concentrations were determined by the method of Bradford using bovine serum albumin as a standard.

Enzymatic activity assays. Activities of the five enzymes (SUS, GalE, GS, RS, and STS) were determined in a reaction mixture (1 ml) containing 50 mM PBS (pH 7.0), 30 mM substrates, and 5 mg/ml of the corresponding enzymes. The reactions were performed at 30°C for 30 min and stopped by boiling for 10 min. The product was measured by high-performance liquid chromatography (HPLC). One unit of enzymatic activity was defined as the amount of enzyme synthesizing 1 μmol of product per minute.

The kinetic parameters K_m and V_{max} of GS were determined at 30°C with 50 mM PBS (pH 7.0), 1 mM UDP-galactose, various concentrations of inositol (2 to 15 mM), and 0.19 mg of purified GS for 1 h. The K_m and V_{max} values of RS were determined at 30°C with 50 mM PBS (pH 7.0), 1 mM galactinol, various

concentrations of sucrose (2 to 25 mM), and 0.57 mg of purified RS for 1 h. Then, these reactions were stopped by boiling for 10 min and analyzed by HPLC.

Optimization of reaction conditions in raffinose synthesis process. The reaction medium (1 ml) containing 50 mM PBS (pH 7.0), 50 mM sucrose, 3 mM inositol, 5 mM UDP, purified SUS (0.24 U, 0.5 mg), GalE (0.24 U, 0.1mg), GS (0.2 U, 10 mg), and RS (0.32 U, 20 mg) was performed at 30°C for 48 h. Then, the reaction was stopped by boiling for 10 min and analyzed by HPLC. The effects of temperature (20°C, 30°C, 40°C, and 50°C), pH values (5, 6, 7, and 8), and 1 mM metal ions ($CaCl_2$, $CoCl_2$, $NiCl_2$, $ZnSO_4$, $FeSO_4$, $MnCl_2$, $BaCl_2$, and $CuSO_4$) on raffinose production were investigated to obtain the optimal reaction conditions. In addition, different concentrations of sucrose (20, 30, 40, and 50 mM) and UDP (0, 5, 10, and 15 mM) in the reaction medium were used to measure the synthesis of raffinose.

To optimize the concentration ratio of sucrose to inositol, different concentration ratios of sucrose (mM) to inositol (mM) (25:25 [1:1], 50:25 [2:1], 75:25 [3:1], and 100:25 [4:1]) and purified enzymes of SUS (0.24 U, 0.5 mg), GalE (0.24 U, 0.1mg), and GS (0.2 U, 10 mg) were used at 30°C for 48 h. To optimize the concentration ratio of sucrose to galactinol, the ratios of sucrose (mM)/galactinol (mM) were set to 25:25 (1:1), 50:25 (2:1), 100:25 (4:1), and 200:25 (8:1), and RS (0.32 U, 20 mg) was used. The reaction was performed at 30°C for 48 h.

Synthesis of raffinose using purified enzymes. For the one-pot two-stage reaction process, the reaction medium (1 ml) contained PBS (50 mM, pH 7.0), 600 mM sucrose, 200 mM inositol, 5 mM UDP, purified SUS (0.24 U, 0.5 mg), GalE (0.24 U, 0.1mg), and GS (0.2 U, 10 mg), and the reactions were conducted at 30°C for 48 h in stage 1. Then, purified RS (0.32 U, 20 mg) was directly supplemented into the reaction medium, and the reaction was performed for another 48 h in stage 2. The samples were collected every 12 h, stopped by boiling for 10 min, and analyzed by HPLC.

Synthesis of raffinose using cell extracts. In stage 1, the reaction medium (1 ml) contained PBS (50 mM, pH 7.0), cell extract-based lyophilized powders of SUS (0.36 U, 2 mg), GalE (0.33 U, 0.5 mg), and GS (0.36 U, 40 mg), 600 mM sucrose, and 200 mM inositol, and the reactions were conducted at 30°C for 48 h. Then, cell extract-based lyophilized powders of RS (0.36 U, 60 mg) were directly supplemented into the reaction medium, and the reaction was performed for another 48 h in stage 2. The samples were collected every 12 h, and the reactions were stopped by boiling for 10 min and then analyzed by HPLC.

To produce galactinol in a 50-ml-scale reaction system, the medium contained PBS (50 mM, pH 7.0), cell extract-based lyophilized powders of SUS (18 U, 100 mg), GalE (16.5 U, 25 mg), and GS (18 U, 400 mg), 600 mM sucrose, and 200 mM inositol, and the reactions were conducted at 30°C at 100 rpm for 48 h. To produce raffinose in a 50-ml-scale reaction system, the components PBS (50 mM, pH 7.0), cell extract-based lyophilized powders of SUS (18 U, 100 mg), GalE (16.5 U, 25 mg), GS (18 U, 200 mg), and RS (18 U, 300 mg), 600 mM sucrose, and 200 mM inositol were used. The reaction process was similar to that mentioned above.

To purify the galactinol and raffinose, the 50-ml samples were boiled for 15 min and centrifuged at 14,000 $\times$ *g* at 4°C for 30 min. Then, the supernatants were concentrated to a volume of 2 ml using rotary evaporators. The product was purified using a semipreparative HPLC system, which was equipped with a pump (6000 LDS), a chromatographic column filled with Ca^{2+} ion exchange resin, a refractive index detector (RID; RI-2000), and a fraction collector (LF-300B). The supernatant was pumped into the system and separated by the chromatographic column. The desired product was identified by the RID detector and collected by the collector. Deionized water was used as the mobile phase. The solution was then freeze-dried using a vacuum pump (SCIENTZ-10N) to obtain the desired product. We obtained 2.1 g galactinol and 1.5 g raffinose using this purification method. The purified product was then dissolved in D_2O and analyzed by NMR.

Inositol recycling for raffinose synthesis. Five-round cascade reactions were designed for cycling the use of inositol. Every batch reaction involved a two-stage cascade reaction. In stage 1, PBS (pH 7.0), the cell extract-based lyophilized powders of SUS (0.36 U, 2 mg), GalE (0.33 U, 0.5 mg), and GS (0.36 U, 40 mg), and sucrose (600 mM, 205 mg) and inositol (200 mM, 36 mg) powders were reacted at 30°C for 24 h. In stage 2, RS (0.36 U, 60 mg) was added, and the reaction was conducted at 30°C for another 12 h. The reaction system was then centrifuged at 14,000 $\times$ *g* at 4°C for 20 min and the supernatant was transferred to another 1.5-ml Eppendorf tube. To continue the next round, the same amounts of sucrose and enzyme powder were added again for the next batch reaction. Every 12 h, 5 μl of sample was collected and diluted to 100 μl. Then, the diluted sample was boiled in water for 10 min and analyzed by HPLC.

Stachyose synthesis via one-pot two/three-stage reaction process. For the stachyose synthesis in the one-pot two-stage reaction process, the reaction medium in stage 1 contained PBS (pH 7.0), the cell extract-based lyophilized powders of SUS (0.36 U, 2 mg), GalE (0.33 U, 0.5 mg), and GS (0.36 U, 40 mg), and sucrose (600 mM, 205 mg) and inositol (200 mM, 36 mg) powders, and the reaction was performed at 30°C for 36 h. Then, cell extract-based lyophilized powders of STS (0.4 U, 20 mg) and fresh raffinose powder (200 mM, 100.8 mg) were supplemented into the reaction in stage 2, and the reaction was performed for another 48 h. To produce stachyose in a 50-ml-scale reaction system, the components PBS (50 mM, pH 7.0), cell extract-based lyophilized powders of SUS (18 U, 100 mg), GalE (16.5 U, 25 mg), GS (18 U, 200 mg), RS (18 U, 300 mg), and STS (20 U, 100 mg), 600 mM sucrose, and 200 mM inositol were used. The reaction process was similar to that mentioned above. The product purification process for stachyose was identical to that for galactinol and raffinose. We obtained 656 mg stachyose after the purification process. The purified stachyose was then dissolved in D_2O and analyzed by NMR.

For the stachyose synthesis in the one-pot three-stage reaction process, the reaction in stage 1 was the same as that mentioned above. In stage 2, crude enzyme powders of RS (0.36 U, 60 mg) were added, and the reaction continued for another 24 h. In stage 3, crude enzyme powders of STS (0.4 U, 20 mg)

were supplemented, and the reaction was performed for the last 24 h. The samples were collected every 12 h, and the reactions were stopped by boiling for 10 min and analyzed by HPLC.

Analytical methods. All substrates and products were analyzed by an HPLC system (Agilent 1200 series; Agilent Technologies, Inc., Santa Clara, CA, USA). The experiments were performed with a Sugar-Pak chromatographic column (6.5 mm by 300 mm) and a refractive index detector, with deionized water as the mobile phase at a flow rate of 0.4 ml/min and a column temperature of 80°C.

UDP concentration was measured by reversed-phase HPLC using a Kinetex C_{18} column (5 μm, 100 Å, 50 mm by 4.6 mm; Phenomenex, Germany) in reversed-phase ion-pairing mode. HPLC analysis was performed at 35°C with a mobile phase of 87.5% 20 mM potassium phosphate buffer (pH 5.9) containing 40 mM tetra-*n*-butylammonium bromide (TBAB) and 12.5% acetonitrile at an isocratic flow rate of 2 ml/min. UV detection at 262 nm was used.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02306-18.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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REFERENCES

1. Dudley QM, Karim AS, Jewett MC. 2015. Cell-free metabolic engineering: biomanufacturing beyond the cell. Biotechnol J 10:69–82. https://doi.org/10.1002/biot.201400330.
2. Kwon SJ, Mora-Pale M, Lee MY, Dordick JS. 2012. Expanding nature's small molecule diversity via *in vitro* biosynthetic pathway engineering. Curr Opin Chem Biol 16:186–195. https://doi.org/10.1016/j.cbpa.2012.02.001.
3. Zhang YHP. 2015. Production of biofuels and biochemicals by in vitro synthetic biosystems: opportunities and challenges. Biotechnol Adv 33:1467–1483. https://doi.org/10.1016/j.biotechadv.2014.10.009.
4. Yang J, Sun S, Men Y, Zeng Y, Zhu Y, Sun Y, Ma Y. 2017. Transformation of formaldehyde into functional sugars via multi-enzyme stepwise cascade catalysis. Catal Sci Technol 7:3459–3463. https://doi.org/10.1039/C7CY01062A.
5. Myung S, Rollin J, You C, Sun F, Chandrayan S, Adams MWW, Zhan YHP. 2014. *In vitro* metabolic engineering of hydrogen production at theoretical yield from sucrose. Metab Eng 24:70–77. https://doi.org/10.1016/j.ymben.2014.05.006.
6. Rollin JA, Martin del Campo J, Myung S, Sun F, You C, Bakovic A, Castro R, Chandrayan SK, Wu C-H, Adams MWW, Senger RS, Zhang Y-HP. 2015. High-yield hydrogen production from biomass by *in vitro* metabolic engineering: mixed sugars coutilization and kinetic modeling. Proc Natl Acad Sci U S A 112:4964–4969. https://doi.org/10.1073/pnas.1417719112.
7. Bogorad IW, Chen CT, Theisen MK, Wu TY, Schlenz AR, Lam AT, Liao JC. 2014. Building carbon–carbon bonds using a biocatalytic methanol condensation cycle. Proc Natl Acad Sci U S A 111:15928–15933. https://doi.org/10.1073/pnas.1413470111.
8. Wang W, Liu M, You C, Li Z, Zhang YHP. 2017. ATP-free biosynthesis of a high-energy phosphate metabolite fructose 1,6-diphosphate by in vitro metabolic engineering. Metab Eng 42:168–174. https://doi.org/10.1016/j.ymben.2017.06.006.
9. Fujisawa T, Fujinaga S, Atomi H. 2017. An *in vitro* enzyme system for the production of *myo*-inositol from starch. Appl Environ Microbiol 83:e00550-17. https://doi.org/10.1128/AEM.00550-17.
10. Beer B, Pick A, Sieber V. 2017. *In vitro* metabolic engineering for the production of α-ketoglutarate. Metab Eng 40:5–13. https://doi.org/10.1016/j.ymben.2017.02.011.
11. Chen X, Zhang C, Zou R, Stephanopoulos G, Too HP. 2017. *In vitro* metabolic engineering of amorpha-4,11-diene biosynthesis at enhanced rate and specific yield of production. ACS Synth Biol 6:1691–1700. https://doi.org/10.1021/acssynbio.6b00377.
12. Nishimoto M, Kitaoka M. 2009. One-pot enzymatic production of β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-galactose (galacto-*N*-biose) from sucrose and 2-acetamido-2-deoxy-D-galactose (*N*-acetylgalactosamine). Carbohydr Res 344:2573–2576. https://doi.org/10.1016/j.carres.2009.09.031.
13. Yu H, Chen X. 2016. One-pot multienzyme (OPME) systems for chemoenzymatic synthesis of carbohydrates. Org Biomol Chem 14:2809–2818. https://doi.org/10.1039/C6OB00058D.
14. Chen C, Zhang Y, Xue M, Liu X, Li Y, Chen X, Wang PG, Wang F, Cao H. 2015. Sequential one-pot multienzyme (OPME) synthesis of lacto-*N*-neotetraose and its sialyl and fucosyl derivatives. Chem Commun (Camb) 51:7689–7692. https://doi.org/10.1039/C5CC01330E.
15. Zhao C, Wu Y, Yu H, Shah IM, Li Y, Zeng J, Liu B, Mills DA, Chen X. 2016. One-pot multienzyme (OPME) synthesis of human blood group H antigens and a human milk oligosaccharide (HMOS) with highly active *Thermosynechococcus elongatus* α1–2-fucosyltransferase. Chem Commun (Camb) 52:3899–3902. https://doi.org/10.1039/C5CC10646J.
16. Chen H, Liu L, Zhu J, Xu B, Li R. 2010. Effect of soybean oligosaccharides on blood lipid, glucose levels and antioxidant enzymes activity in high fat rats. Food Chem 119:1633–1636. https://doi.org/10.1016/j.foodchem.2009.09.056.
17. Deng J, Mai K, Ai Q, Zhang W. 2007. Effects of soybean oligosaccharides on lipid metabolism of Japanese flounder (*Paralichthys olivaceus* Temminck et Schlegel) fed animal or plant protein source-based diets. Front Agric China 1:315–323. https://doi.org/10.1007/s11703-007-0053-4.
18. Espinosa-Martos I, Rupérez P. 2006. Soybean oligosaccharides. Potential as new ingredients in functional food. Nutr Hosp 21:92–96.
19. Zhong X, Huang G, Chen Y, Li C, Deng Z, Ma X. 2013. Optimization of extracting stachyose from *Stachys floridana* Schuttl. ex Benth by response surface methodology. J Food Sci Technol 505:942–949. https://doi.org/10.1007/s13197-011-0413-1.
20. Li W, Yu S, Zhang T, Jiang B, Mu W. 2017. Synthesis of raffinose by transfructosylation using recombinant levansucrase from *Clostridium arbusti* SL 206. J Sci Food Agric 97:43–49. https://doi.org/10.1002/jsfa.7903.
21. Xu W, Yu S, Liu Q, Zhang T, Jiang B, Mu W. 2017. Enzymatic production of melibiose from raffinose by the levansucrase from *Leuconostoc mesenteroides* B-512 FMC. J Agric Food Chem 65:3910–3918. https://doi.org/10.1021/acs.jafc.7b01265.
22. Bourne EJ, Walter MW, Pridham JB. 1965. The biosynthesis of raffinose. Biochem J 97:802–806. https://doi.org/10.1042/bj0970802.
23. Pridham JB, Hassid WZ. 1965. Biosynthesis of raffinose. Plant Physiol 40:984–986. https://doi.org/10.1104/pp.40.6.984.
24. Schmölzer K, Gutmann A, Diricks M, Desmet T, Nidetzky B. 2016. Sucrose synthase: a unique glycosyltransferase for biocatalytic glycosylation pro-

cess development. Biotechnol Adv 34:88–111. https://doi.org/10.1016/j.biotechadv.2015.11.003.
25. Flamholz A, Noor E, Bar-Even A, Milo R. 2012. eQuilibrator–the biochemical thermodynamics calculator. Nucleic Acids Res 40:D770–D775. https://doi.org/10.1093/nar/gkr874.
26. Taji T, Ohsumi C, Iuchi S, Seki M, Kasuga M, Kobayashi M, Yamaguchi-Shinozaki K, Shinozaki K. 2002. Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. Plant J 29:417–426. https://doi.org/10.1046/j.0960-7412.2001.01227.x.
27. Baroja-Fernandez E, Munoz FJ, Li J, Bahaji A, Almagro G, Montero M, Etxeberria E, Hidalgo M, Sesma MT, Pozueta-Romero J. 2012. Sucrose synthase activity in the sus1/sus2/sus3/sus4 *Arabidopsis* mutant is sufficient to support normal cellulose and starch production. Proc Natl Acad Sci U S A 109:321–326. https://doi.org/10.1073/pnas.1117099109.
28. Chen X, Kowal P, Hamad S, Fan H, Wang P. 1999. Cloning, expression and characterization of a UDP-galactose 4-epimerase from *Escherichia coli*. Biotechnol Lett 21:1131–1135. https://doi.org/10.1023/A:1005678225031.
29. Gangl R, Behmüller R, Tenhaken R. 2015. Molecular cloning of *AtRS4*, a seed specific multifunctional RFO synthase/galactosyl hydrolase in *Arabidopsis thaliana*. Front Plant Sci 6:1–15. https://doi.org/10.3389/fpls.2015.00789.
30. Bruyn F, Maertens J, Beauprez J, Soetaert W, De MM. 2015. Biotechnological advances in UDP-sugar based glycosylation of small molecules. Biotechnol Adv 33:288–302. https://doi.org/10.1016/j.biotechadv.2015.02.005.
31. Chen L, Sun P, Zhou F, Li Y, Chen K, Jia H, Yan M, Gong D, Ouyang P. 2018. Synthesis of rebaudioside D, using glycosyltransferase UGTSL2 and *in situ* UDP-glucose regeneration. Food Chem 259:286–291. https://doi.org/10.1016/j.foodchem.2018.03.126.
32. Zhong C, You C, Wei P, Zhang YHP. 2017. Thermal cycling cascade biocatalysis of *myo*-inositol synthesis from sucrose. ACS Catal 7:5992–5999. https://doi.org/10.1021/acscatal.7b01929.
33. Parmeggiani F, Ahmed ST, Weise NJ, Turner NJ. 2016. Telescopic one-pot condensation-hydroamination strategy for the synthesis of optically pure L-phenylalanines from benzaldehydes. Tetrahedron 72:7256–7262. https://doi.org/10.1016/j.tet.2015.12.063.
34. Yu H, Li T, Chen F, Luo X, Li A, Yang C, Zheng G, Xu J. 2018. Bioamination of alkane with ammonium by an artificially designed multienzyme cascade. Metab Eng 47:184–189. https://doi.org/10.1016/j.ymben.2018.02.009.
35. Hiroaki N, Hiroaki S, Taizo N, Michiko T, Teruo S. 2013. Raffinose production using α-galactosidase from *Paraphaeosphaeria* sp. Mycoscience 54:247–251. https://doi.org/10.1016/j.myc.2012.09.016.
36. Zhou Y, Zhu Y, Men Y, Dong C, Sun Y, Zhang J. 2017. Construction of engineered *Saccharomyces cerevisiae*, strain to improve that whole-cell biocatalytic production of melibiose from raffinose. J Ind Microbiol Biotechnol 44:489–501. https://doi.org/10.1007/s10295-017-1901-8.