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Engineering cyanobacteria for converting carbon dioxide into isomaltulose

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ABSTRACT

Isomaltulose is a promising functional sweetener with broad application prospects in the food industry. Currently, isomaltulose is mainly produced through bioconversion processes based on the isomerization of sucrose, the economic feasibility of which is influenced by the cost of sucrose feedstocks, the biocatalyst preparation, and product purification. Cyanobacterial photosynthetic production utilizing solar energy and carbon dioxide represents a promising route for the supply of sugar products, which can promote both carbon reduction and green production. Previously, some cyanobacteria strains have been successfully engineered for synthesis of sucrose, the main feedstock for isomaltulose production. In this work, we introduced different sucrose isomerases into *Synechococcus elongatus* PCC 7942 and successfully achieved the isomaltulose synthesis and accumulation in the recombinant strains. Combinatory expression of an *Escherichia coli* sourced sucrose permease CscB with the sucrose isomerases led to efficient secretion of isomaltulose and significantly elevated the final titer. During a 6-day cultivation, 777 mg/L of isomaltulose was produced by the engineered *Synechococcus* cell factory. This work demonstrated a new route for isomaltulose biosynthesis utilizing carbon dioxide as the substrate, and provided novel understandings for the plasticity of cyanobacterial photosynthetic metabolism network.

As a natural isomer of sucrose, isomaltulose (α-D-glucosylpyranosyl-1,6-D-fructofuranose; also referred to as palatinose) has a similar appearance and taste as sucrose, and possesses a lower energy value, non-carcinogenicity and a lower insulin index, making it useful for mitigating diabetes mellitus (Mu et al., 2014; Sawale et al., 2017; Fletcher et al., 2020). Currently, isomaltulose is mainly manufactured through bioconversion of sucrose with sucrose isomerase (SIase) in industry, with trehalulose (α-D-glucosylpyranosyl-1,1-D-fructofuranose) as the main by-product, which has similar properties and functions to isomaltulose (Fu et al., 2021). Glucose and fructose would also be synthesized as hydrolysis by-products in the sucrose isomerization system (Cha et al., 2009; Zhang et al., 2021). For efficient isomaltulose production, SIase from a variety of sources have been mined, characterized, optimized, and applied (Wu and Birch, 2004; Watzlawick and Mattes, 2009; Duan et al., 2016). By overexpression of these enzymes in microbial chassis, e.g. *E. coli* (Li et al., 2013), *Saccharomyces cerevisiae* (Lee et al., 2011), *Bacillus subtilis* (Wu et al., 2017), *Yarrowia lipolytica* (Zhang et al., 2019), and *Corynebacterium glutamicum* (Guo et al., 2022), efficient biocatalysts have been developed, facilitating sucrose conversion rate of over 90% to the final isomaltulose product (Cho et al., 2007; Liu et al., 2021). However, the economic competitiveness of the SIase based sucrose bioconversion route for isomaltulose production is still influenced by several factors: the stability of sucrose feedstock supply, the cost of biocatalysts preparation, and the cost of extracting

Abbreviations: WT, wild-type; NS1 and NS3, neutral site I and neutral site III in the genome of *Synechococcus elongatus* PCC 7942; SIase, sucrose isomerase.

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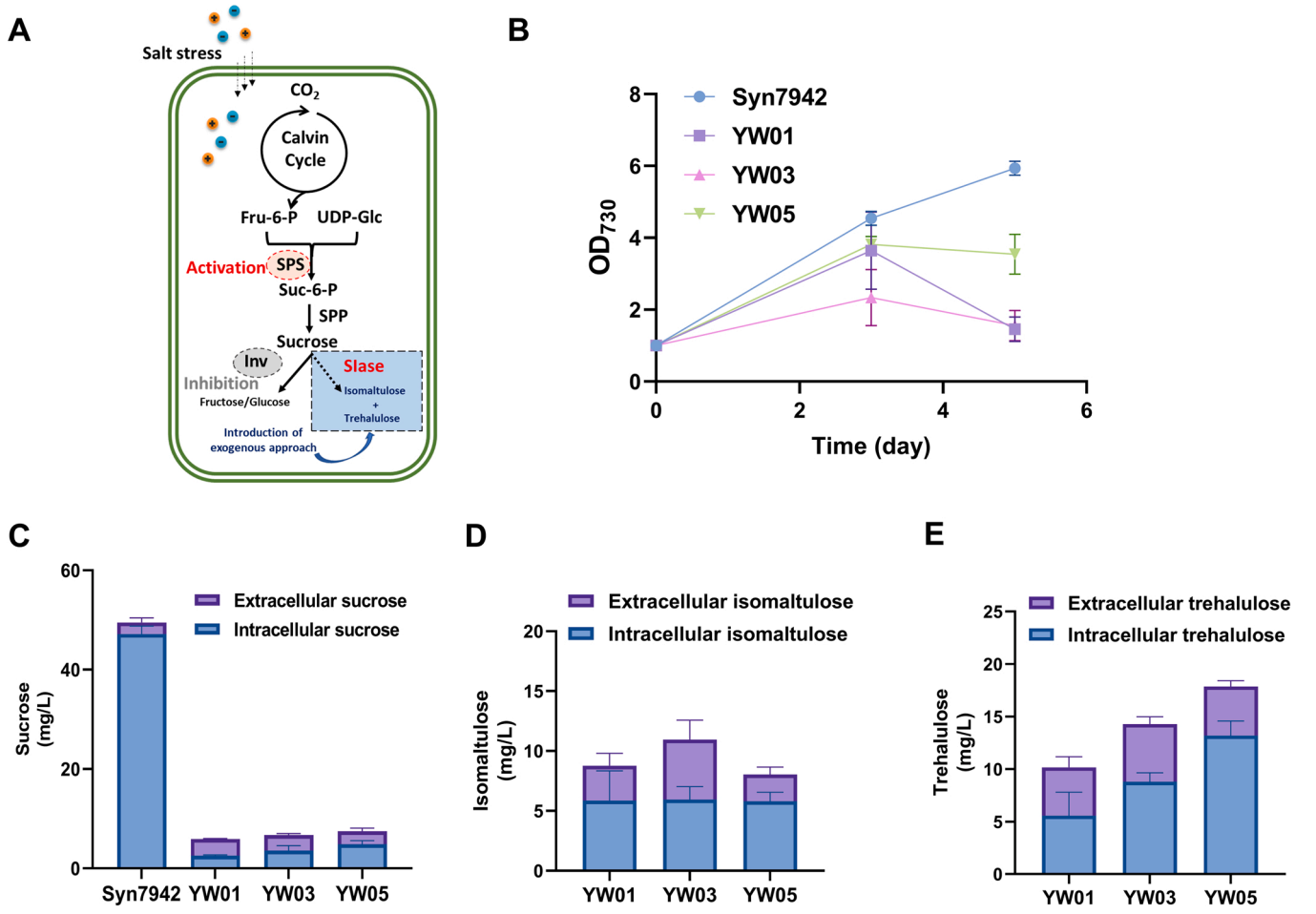

Fig. 1. Expression of heterologous sucrose isomerases in Syn7942 for production of isomaltulose and trehalulose induced by high salinity stress. Isomaltulose synthesis of *Synechococcus* was designed to be achieved by introduction of heterologous SIases and induction of NaCl stress (A). The growth curve of the strains with 150 mM salt induction (B). Sucrose production (C), isomaltulose production (D), trehalulose production (E) in the recombinant strains were calculated after 5 days cultivation in BG11 medium supplemented with 150 mM NaCl. The plasmids and strains constructed in this study are listed in Table S1 and S2, respectively. The construction strategy and the genotype verification in this study are shown in Fig. S1 and S2, respectively. The methods for cultivation and the detection of sugar products are described in Methods S1 and S2, respectively. The HPLC profiles of isomaltulose and trehalulose standards and samples are shown in Fig. S3. The graphs illustrate representative experimental data, and error bars represent standard deviations from three biological replicates.

isomaltulose from the system containing multiple sugars (Agbor et al., 2011; Sydney et al., 2021). Thus, it is of great significance to explore novel alternative routes for isomaltulose synthesis.

Photosynthetic production is a novel mode of biomanufacturing, channeling CO_2 and solar energy into biofuels, biochemicals, and biomaterials. As the only group of prokaryotes performing oxygenic photosynthesis, cyanobacteria possess multiple desirable properties for photosynthetic production, including simpler cellular structure, more rapid growth, and better genetic tractability than other photoautotrophs (Hays and Ducat, 2015; Savakis and Hellingwerf, 2015; Luan et al., 2019). Benefiting from the developments in synthetic biology, cyanobacteria have been successfully engineered to produce dozens of natural or non-natural metabolites, of which sugar products (carbohydrates) are a highly representative group. Sucrose, the direct bioconversion substrate for isomaltulose synthesis, has been synthesized by cyanobacterial cell factories. In a large portion of cyanobacteria species, sucrose could be naturally synthesized and accumulated as compatible substances to resist the extracellular high salinity stress (Hagemann, 2011; Klahn and Hagemann, 2011). With systematic metabolic modifications, photosynthetic sucrose production could be further enhanced (Ducat et al., 2012; Song et al., 2016). Inspired by that, we supposed that sucrose-synthesizing cyanobacteria could be utilized as a chassis for engineering isomaltulose producing cell factories.

Theoretically, isomaltulose synthesis could be achieved by expression of heterologous SIases into sucrose synthesizing cyanobacterial strains (Fig. 1A). In this work, we selected *Synechococcus elongatus* PCC 7942 (hereafter termed as Syn7942 for short), one of the most representative cyanobacteria strains for sucrose synthesis research, as the initial chassis. Three SIase genes from *Erwinia rhapontici* NX-5, *Raoultella terrigena*, and *Klebsiella* sp. LX3, which are reported to have high conversion ratio (>80%) of sucrose to isomaltulose (Zhang et al., 2003; Li et al., 2011; Liu et al., 2021), were put under control of a strong promoter P_{cpcB} (Qiao et al., 2018) and inserted into the neutral site I of the Syn7942 chromosome by homologous recombination (Liu et al., 1996), getting the strains YW01, YW03, and YW05, respectively (Fig. S1 & S2). Naturally, sucrose would be synthesized and accumulated in cells facing extracellular high salinity stress; thus, NaCl was added to the culture medium to induce the synthesis of sucrose as the substrate of the SIases. As shown in Fig. 1B, all the strains overexpressing SIase genes showed growth retardation, and this might result from the decreased intracellular sucrose contents (by about 63%–84% comparing with that of the wild-type Syn7942) (Fig. 1C), which might impair the cellular capacities to resist extracellular osmo-pressure. With decreased sucrose concentrations, significant amounts of isomaltulose and trehalulose were synthesized by recombinant strains, indicating that all the three heterologous SIases were functionally expressed and could catalyze the

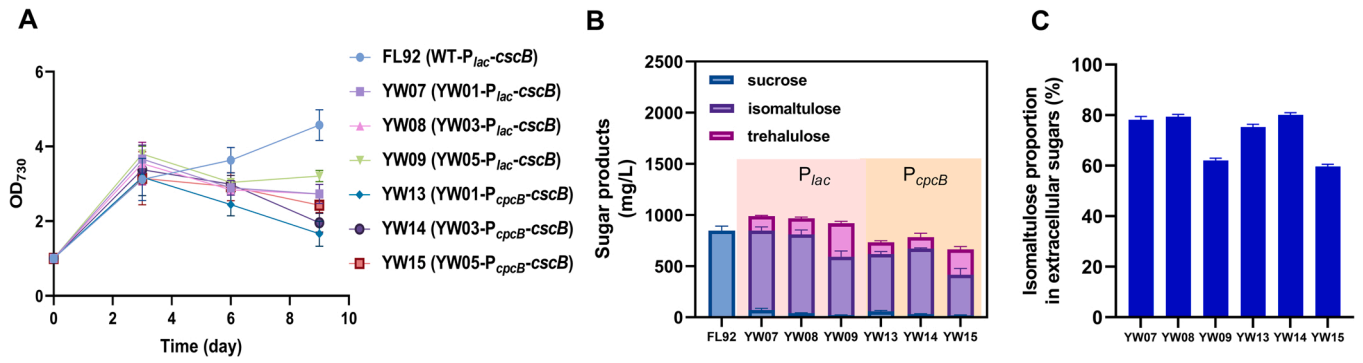

Fig. 2. Growth and sugar production of recombinant *Synechococcus* strains carrying SIases and CscB. The growth (A) of serial engineered strains were evaluated during the cultivation in BG11 supplemented with 150 mM NaCl. The extracellular sugars accumulation (B), and the proportion of isomaltulose (C) were calculated after 6 days cultivation in BG11 medium supplemented with 150 mM NaCl. The P$_{lac}$-*cscB* was introduced into strains carrying SIase genes (YW01, YW03, YW05) and the wild-type Syn7942, to get the strains YW07, YW08, YW09, and FL92, respectively. The P$_{cpcB}$-*cscB* was introduced into strains carrying SIase genes (YW01, YW03, YW05), to get the strains YW13, YW14, and YW15, respectively. The graphs illustrate representative experimental data, and error bars represent standard deviations from three biological replicates.

reactions as expected (Fig. 1D & 1E; Fig. S3). After 5 days cultivation, about 10 mg/L of isomaltulose and 10–15 mg/L of trehalulose were synthesized in the recombinant strains. The ratios of isomaltulose to trehalulose were not in consistence with previously reported enzymatic properties of the selected SIases, which possess conversion rates of sucrose to isomaltulose reaching > 80% (Zhang et al., 2003; Li et al., 2011; Liu et al., 2021). Besides, a significant portion (42% for YW01, 36% for YW03, and 27% for YW05) of the synthesized isomaltulose and trehalulose (here the two sugars were calculated in sum) was detected in the culture medium (Fig. 1D & E), which might be caused by potential native secretory mechanisms or by cell lysis and leakage of intracellular contents. Considering that the extracellular sucrose was detected with a higher ratio (versus the total concentration) in the recombinant strains than that of the wild-type control, we supposed that the cell lysis/leakage might contribute more to this phenomenon.

The above results confirmed that synthesis of isomaltulose in Syn7942 could be achieved by introduction of heterologous SIases. However, the accumulation of sucrose in Syn7942 cells facing high salinity conditions is quite limited, which might restrict the yield of isomaltulose. A strategy for improving the yield of sugar products in cyanobacteria is to introduce specific transporters, promoting the secretion of the desired metabolites, which could alleviate the potential feedback inhibition and drive more carbon flow to the synthesis pathway (Ducat et al., 2012; Qiao et al., 2020). However, there has not been any reported isomaltulose-specific transporters in literature or industry; thus, we selected the *E. coli* sourced sucrose permease CscB as a candidate (Vadyvaloo et al., 2006), which have been found effective for transporting sucrose (Lin et al., 2020), trehalose (Qiao et al., 2020) and maltose (Peng et al., 2009) in different chassis. Under control of the previously used IPTG-inducible promoter P$_{lac}$ (Song et al., 2016), the *cscB* was introduced into strains carrying SIase genes, to get the strains YW07, YW08, YW09, respectively. A wild-type Syn7942 derived strain carrying *cscB* (named FL92) (Qiao et al., 2018) was used as a control. Comparing with FL92, the recombinant cells showed growth retardation (Fig. 2A), which might still be caused by reduced sucrose contents (as osmo-protectant). The expression of *cscB* enabled extracellular secretion of sucrose and significantly improved the final yield (847 mg/L extracellular sucrose in FL92, 447 folds than that in wild-type). The secretion and synthesis of isomaltulose was also activated by CscB. For the strains carrying SIase genes, extracellular sucrose contents were reduced by over 92%, meanwhile significant amounts of isomaltulose/trehalulose were synthesized and secreted extracellularly, and the YW07 strain (NS1::P$_{cpcB}$-*sIase,* which is from *Erwinia rhapontici* NX-5; NS3::P$_{lac}$-*cscB*) possessed the best performances, accumulating 777 mg/L isomaltulose and 140 mg/L trehalulose in the culture medium after 6 days induction (Fig. 2B). In all the recombinant strains, more than 96% of the products were efficiently transported extracellularly, and the secreted sucrose were sharply decreased (Fig. S4), indicating that the CscB showed high affinity to isomaltulose and trehalulose, and the efficient export of isomaltulose/trehalulose might further drive the conversion of sucrose. In addition, the ratios of isomaltulose to trehalulose in the recombinant strains were significantly improved by the introduction of CscB (Fig. 2C & Fig. S4), reaching up to 5.5:1 in YW07. And this may be a result of the removal of intracellular products over-accumulation, which further relieved the effects on the enzymatic properties of SIases. To the best of our knowledge, this is reported for the first time that the well-known sucrose permease CscB can be used as effective transporter for two isomers, isomaltulose and trehalulose.

To enhance the expression of CscB, we replaced the P$_{lac}$ promoter with a constitutive strong promoter P$_{cpcB}$. However, the decrease of yields of isomaltulose and trehalulose were observed in YW13, YW14, and YW15, comparing with the respective control carrying the same SIase genes and P$_{lac}$-*cscB* (Fig. 2B). A possible explanation could be that the over-expression of CscB driven by the strong promoter P$_{cpcB}$ would cause over-consumption of energy and materials in the recombinant strains, inhibit the homeostasis of intracellular environment, and bring in adverse effects on cell growth and isomaltulose production in *Synechococcus* (Song et al., 2016). It is noteworthy that the isomaltulose production was also influenced by the timepoint of extracellular NaCl induction. When NaCl was supplemented in Day 3 rather than the initial stage, synthesis and secretion of isomaltulose would be sharply impaired, which might be resulted from the weakened physiological activities of *Synechococcus* cells gradually entering the stationary phase (Fig. S5).

In summary, an alternative route for isomaltulose synthesis based on cyanobacteria photosynthetic production was explored and confirmed in this work. Combinatory introduction of heterologous SIase genes and sucrose permease gene CscB successfully remodeled the salt stress activated sucrose metabolism network in *Synechococcus*, and resulted in 777 mg/L extracellular accumulation of isomaltulose. This work demonstrated novel attempts to engineer photoautotrophs for direct photosynthetic sugar production and improved our understandings of a previously well-known and widely-utilized sugar transporter.

CRediT authorship contribution statement

Yannan Wu: Investigation, Formal analysis, Visualization, Writing – original draft. **Jiahui Sun** : Investigation, Formal analysis, Visualization, Writing – original draft. **Xuejing Xu**: Investigation. **Shaoming Mao**: Writing – review & editing, Supervision. **Guodong Luan:**

Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Xuefeng Lu**: Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Yannan Wu, Jiahui Sun, Guodong Luan, Xuefeng Lu has patent pending to CN 202210878243.X.

Data Availability

Data will be made available on request.

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Author contributions

Y. W., J. S., and X. X. performed the research project. M. S., G. L. and X. L. supervised the research project and guided the design of experiments. Y. W., J. S., X. X., S. M., G. L., and X. L. drafted and revised the manuscript.

Declaration of Competing Interest

The authors have no competing interests to declare.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jbiotec.2023.01.007.

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