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Construction of homologous and heterologous synthetic sucrose utilizing modules and their application for carotenoid production in recombinant *Escherichia coli*

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HIGHLIGHTS

- Switchable modules expressing sucrose pathway genes were constructed.
- Heterologous and homologous modules conferred sucrose-utilizing ability on *E. coli*.
- *E. coli* expressing synthetic modules was further engineered to produce carotenoid.

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ABSTRACT

Sucrose is one of the most promising carbon sources for industrial fermentation. We expressed synthetic modules expressing genes of the PEP-PTS and non-PTS pathways in *Escherichia coli* K12 for comparison. We selected PEP-PTS pathway genes of *Lactobacillus plantarum* and *Staphylococcus xylosus* and non-PTS pathway genes of sucrose-utilizing (Scr^+) *E. coli* EC3132. Switchable Scr^+ modules expressing *E. coli* EC3132 non-PTS genes conferred better sucrose-utilizing ability on Scr^- *E. coli* K12 than *E. coli* EC3132. Scr^+ modules expressing *S. xylosus* PEP-PTS genes conferred a sucrose-utilizing ability on *E. coli* K12. Among *L. plantarum* PEP-PTS genes, $SacA_{LP}$ and $SacK_{LP}$ were functional in *E. coli* K12. $CscA_{EC}$–$CscB_{EC}$–$CscK_{EC}$ (non-PEP-PTS module) or $ScrA_{SX}$–$SacA_{LP}$–$SacK_{LP}$ (PEP-PTS module) was introduced to a diapolycopene-producing *E. coli* strain. In both Scr^+ *E. coli* K12, the sucrose-utilizing ability of the modules was not affected by diapolycopene formation, indicating that the modular Scr^+ systems could be employed for developing sustainable bioprocesses using sucrose.

1. Introduction

Among the various carbohydrates on earth, sucrose is the most abundant disaccharide, extracted from sugarcane and sugar beet (approximately 35–40% of the total mass) (de Oliveira et al., 2007). Many gram-positive and gram-negative bacteria are known to readily utilize sucrose as a carbon source (Wagner et al., 1993), as exemplified by the prevalence of over 90% of wild-type *Klebsiella* strains, which can utilize sucrose as the sole carbon source, thus making it a highly suitable substrate in the microbial fermentation for industrial production of biofuels (Ruanglek et al., 2006), amino acids (Lee et al., 2010), and biochemicals (Chan et al., 2012). However, amongst related microorganisms like *Escherichia coli*, strains such as K-12, B, and C are unable to utilize sucrose as a carbon source, except for *E. coli* EC3132 and some enteropathogenic *E.coli* strains (e.g., *E. coli* 0157:H7) (Jahreis et al., 2002; Trevino-Quintanilla et al., 2007). Despite this drawback, *E. coli* K-12 strains have been extensively used in the production of biofuels, amino acids, biochemicals, secondary metabolites, etc. because of increasing environmental concerns, rising petroleum prices, bio-based chemicals, and biofuel production from inexpensive carbon sources (such as sucrose). However, most studies have focused on simple transfer of a sucrose operon into *E. coli* (Chan et al., 2012; Sahin-Tóth et al., 1999) or the use of different gene sources for construction of sucrose-utilizing (Scr^+) *E. coli* (Scholle et al., 1989; Trindade et al., 2003).

Bacteria have 2 main catabolic pathways for sucrose utilization (Reid and Abratt, 2005) (Fig. 1). Most sucrose-utilizing bacteria take up and then phosphorylate sucrose by a phosphoenolpyruvate

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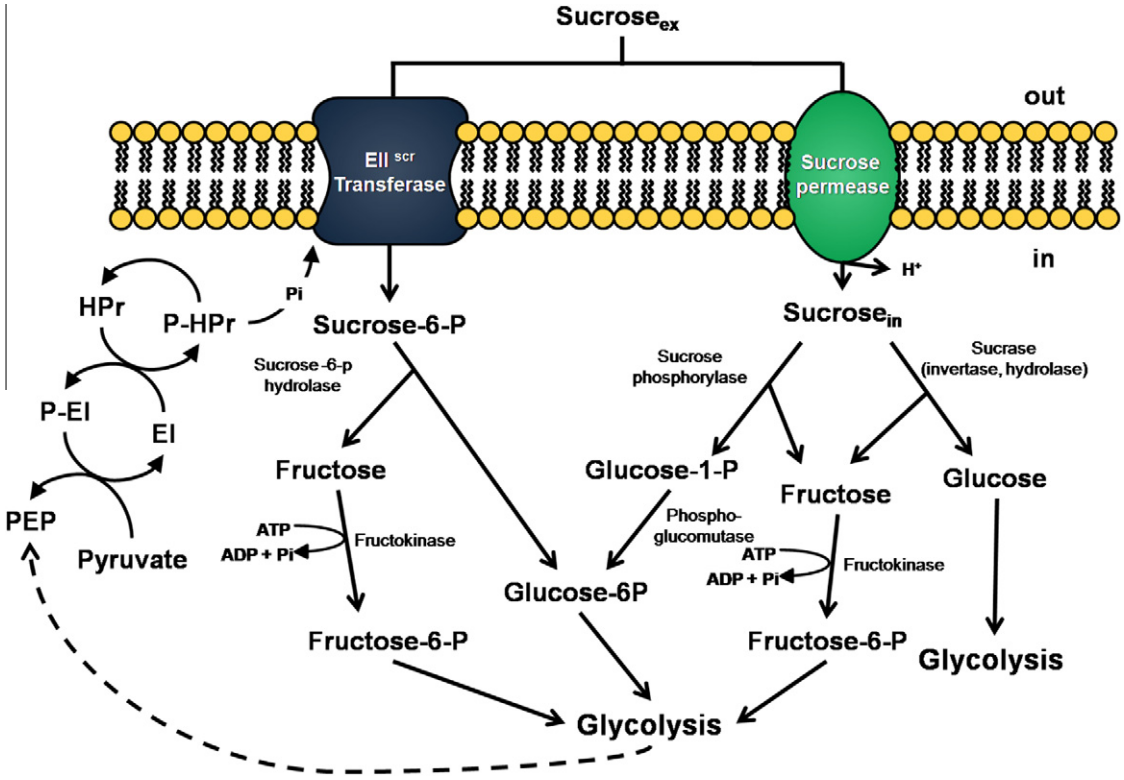

Fig. 1. Schematic representation of the 2 sucrose-utilization systems (PEP-PTS and non-PTS) Sucrose$_{ex}$, extracellular sucrose; Sucrose$_{in}$, intracellular sucrose; sucrose-6-P, sucrose-6-phosphate; glucose-6-P, glucose-6-phosphate; glucose-1-P, glucose-1-phosphate; fructose-6-P, fructose-6-phosphate; PEP, phosphoenolpyruvate.

(PEP)-dependent phosphotransferase (PTS) system (PEP-PTS system), which generates intracellular sucrose-6-phosphate. Sucrose-6-phosphate is cleaved by a sucrose-6-phosphate hydrolase into D-glucose-6-phosphate and D-fructose, which is phosphorylated by fructokinase. Non-phosphotransferase-dependent sucrose metabolic system (non-PTS system) involves facilitated diffusion of sucrose via a sucrose-H^+ symport system. Then, an intracellular sucrose hydrolase phosphorylates the sucrose taken up to provide D-glucose-1-phosphate and D-fructose (Bockmann et al., 1992; Gunasekaran et al., 1990).Subsequently, similar to in the PEP-PTS system, D-fructose is phosphorylated by fructokinase.

A few studies have compared the performances of a heterologous PEP-PTS system and a non-PTS system for sucrose transport and utilization through switchable synthetic expression module approach by constructing a Scr^+ *E. coli*.

Thus, in this study, we constructed and expressed synthetic modules with the PEP-PTS and non-PTS pathway genes in a modular fashion in *E. coli* K12 strain for comparative analysis. Furthermore, we utilized these constructed PEP-PTS and non-PTS pathway gene modules for the production of the secondary metabolite, carotenoid.

2. Methods

2.1. Bacterial strains, plasmids, and genetic manipulation

Bacterial strains and plasmids used in this study are listed in Table 1. All cloning and expression experiments were performed using the *E. coli* K12 SURE strain except for using pBBR1MCS-2-derived vectors. *E. coli* JM109 was used for cloning and expressing pBBR1MCS-2-derived vectors (Peterson, 1995). Genomic DNAs of *E.coli* EC3132, *Lactobacillus plantarum*, and *Staphylococcus xylosus* were isolated by the gDNA extraction kit (Intron, Korea) and used for cloning the genes that code for the sucrose-utilizing pathway. For this purpose, PCR primers were designed according to corresponding gene sequences from the GenBank database (Table 2). The PCR products were digested by restriction enzymes and cloned into the corresponding sites in the constitutive expression vector, pUCM (Kim et al., 2010b), where 8 genes encoding sucrose hydrolase ($CscA_{EC}$) of *E. coli* EC3132, sucrose-specific permease ($CscB_{EC}$) of *E. coli* EC3132, d-fructokinase ($CscK_{EC}$) of *E. coli* EC3132, sucrose phosphotransferase ($Pts1BCA_{LP}$) of *L. plantarum*, sucrose hydrolase ($SacA_{LP}$) of *L. plantarum*, D-fructokinase ($SacK_{LP}$) of *L. plantarum*, sucrose phosphotransferase ($ScrA_{SX}$) of *S. xylosus*, or sucrose hydrolase ($ScrB_{SX}$) of *S. xylosus* were engineered to be constitutively expressed and modularly exchangeable. To assemble the sucrose-utilizing modules in a plasmid, each module was subcloned from pUCM-X (X, a pathway gene) into pACYC184 by amplifying the gene together with a modified constitutive *lac*-promoter, forming pAC-X. Additionally, 2 modules encoding sucrose phosphotransferases ($Pts1BCA_{LP}$ and $ScrA_{SX}$) were subcloned into pBBR1MCS-2, a vector that is compatible with pUCM and pACYC184 in *E. coli*. For expression of His-tagged $CscA_{EC}$, a gene encoding $CscA_{EC}$ was fused to 6× extra histidine codon at the 3′-terminus by PCR by using specific PCR primers (Table 2). For cloning, recombinant *E. coli* cells were grown in Luria–Bertani (LB) media supplemented with ampicillin (Ap, 100 μg/mL), chloramphenicol (Cm, 50 μg/mL), or kanamycin (km, 45 μg/mL) as needed.

2.2. SDS–PAGE and Western blotting

For Western blotting, the *E. coli* K12 strain expressing His-tagged $CscA_{EC}$ was cultured and harvested by centrifugation at 4 °C. The cell pellet was washed and disrupted on ice using Sonifiers® (Sonics, USA) according to the manufacturer's protocol. The cell-free culture media was re-centrifuged at 4 °C, and 50 mL of

Table 1
Bacterial strains and plasmids used in the study.

Strains and plasmids	Relevant properties	Source or reference
Strains		
Lactobacillus plantarum	Gram-positive, PEP-PTS for sucrose	KCTC 13093
Staphylococcus xylosus	Gram-positive, PEP-PTS for sucrose	KCTC 3342
Escherichia coli str.EC3132	F^-, csc^+, non-PEP-PTS for sucrose	Jahreis et al. (2002))
Escherichia coli str. SURE	*endA*1 *glnV*44 *thi*-1 *gyrA*96 *relA*1 *lac recBrecJsbcCumuC*::Tn5 *uvrC e*14-Δ (*mcrCB-hsdSMR-mrr*)171 F′[*proAB*$^+$*lacI*^{q} *lacZ*ΔM15 Tn10]	Stratagene
Escherichia coli str. JM109	*endA*1 *glnV*44 *thi*-1 *relA*1 *gyrA*96 recA1 *mcrB* + Δ(lac-*proAB*) e14-[F′ traD36 proAB + lacIqlacZ ΔM15] hsdR17 (*rK-mK*$^+$)	Stratagene
Plasmids		
pUCM	Ap^R, cloning vector modified from pUC19. Constitutive *lac* promoter	Kim et al. (2010b)
pBBR1MCS-2	Km^R, cloning vector. SC101 origin. Inducible *lac* promoter	Peterson (1995)
pACYC184	Cm^R, expression vector. p15A origin.	NEB
pUC-crtMN	Ap^R, constitutively expressed *crtM* and *crtN* genes to produce diapolycopene	This study
pJBL101	Ap^R, Csc gene cluster	Jahreis et al. (2002)
pUC-cscA	Ap^R, constitutively expressed *cscA* of *E. coli* EC3132	This study
pUC-cscA-His_6	Ap^R, constitutively expressed 6×His-tagged *cscA*	This study
pAC-cscA	Cm^R, constitutively expressed *cscA* of *E. coli* EC3132	This study
pUC-cscB	Ap^R, constitutively expressed *cscB* of *E. coli* EC3132	This study
pAC-cscB	Cm^R, constitutively expressed *cscB* of *E. coli* EC3132	This study
pUC-cscK	Ap^R, constitutively expressed *cscK* of *E. coli* EC3132	This study
pAC-cscK	Cm^R, constitutively expressed *cscK* of *E. coli* EC3132	This study
pAC-cscAK	Cm^R, constitutively expressed *cscA* and *cscK* of *E. coli* EC3132	This study
pAC-cscAB	Cm^R, constitutively expressed *cscA* and *cscB* of *E. coli*EC3132	This study
pAC-cscBK	Cm^R, constitutively expressed *cscB* and *cscK* of *E. coli* EC3132	This study
pAC-cscABK	Cm^R, constitutively expressed *cscA*, *cscB*, and *cscK* of *E. Coli* EC3132	This study
pUC-pts1BCA	Ap^R, constitutively expressed *pts1BCA* of *L. plantarum*	This study
pAC-pts1BCA	Cm^R, constitutively expressed *pts1BCA* of *L. plantarum*	This study
pBBR-pts1BCA	Km^R, constitutively expressed *pts1BCA* of *L. plantarum*	This study
pUC-sacA	Ap^R, constitutively expressed *sacA* of *L. plantarum*	This study
pAC-sacA	Cm^R, constitutively expressed *sacA* of *L. plantarum*	This study
pUC-sacK	Ap^R, constitutively expressed *sacK* of *L. plantarum*	This study
pAC-sacK	Cm^R, constitutively expressed *sacK* of *L. plantarum*	This study
pAC-sacAK	Cm^R, constitutively expressed *sacA* and *sacK* of *L. plantarum*	This study
pUC-scrA	Ap^R, constitutively expressed *scrA* of *S. xylosus*	This study
pAC-scrA	Cm^R, constitutively expressed *scrA* of *S. xylosus*	This study
pBBR-scrA	Km^R, constitutively expressed *scrA* of *S. xylosus*	This study
pUC-scrB	Ap^R, constitutively expressed*scrB* of *S. xylosus*	This study
pAC-scrB	Cm^R, constitutively expressed *scrB* of *S. xylosus*	This study
pAC-scrAB	Cm^R, constitutively expressed *scrA* and *scrB* of *S. xylosus*	This study
pAC-AAK	Cm^R, constitutively expressed *scrA* of *S. xylosus*, *sacA* of *L. plantarum*, and *sacK* of *L. plantarum*	This study

the supernatant was collected and then concentrated to 1 mL by Centricon® (10,000MWCO; Millipore) at 4 °C. The crude extract of *E. coli* K12 and the concentrated cell-free culture media were separated on pre-formed SDS polyacrylamide gels (Bio-Rad), and then transferred onto PVDF membranes (Bio-Rad). PVDF membranes were treated with blocking solution (PBS, 5% BSA, and 0.1% Tween-20) for 1 h, and washed with PBS containing 0.1% Tween-20 for 30 min. PVDF membranes were treated with an anti-His antibody and alkaline phosphatase-conjugated anti-mouse IgG (Sigma, St. Louis, MO). Bound antibodies were visualized using the chromogenic substrate for alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate/*p*-nitrobluetetrazolium chloride (BCIP/NBT; Sigma).

2.3. Synthetic module construction of sucrose-utilizing pathway genes

In order to construct sucrose-utilizing modules in *E. coli* K12, a synthetic module constitutively expressing $CscA_{EC}$, $CscB_{EC}$, $CscK_{EC}$, $Pts1BCA_{LP}$, $SacA_{LP}$, $SacK_{LP}$, $ScrA_{SX}$, or $ScrB_{SX}$ was assembled as described above, generating a series of plasmids expressing sucrose-utilizing modules as shown in Table 1.

2.4. Sucrose fermentation

Sucrose utilization of recombinant *E. coli* K12 strains was initially screened on modified MacConkey agar plates (20 g/L peptone, 1.5 g/L bile salts no. 3, 5.0 g/L NaCl, 0.03 g/L Neutral Red, 1 mg/L Crystal Violet, and 13.5 g/L agar, pH 7.1 ± 0.2) supplemented with filter-sterilized 1% sucrose (Jahreis et al., 2002; Olson et al., 2007; Shukla et al., 2004). M9 minimal medium (12.8 g/LNa_2HPO_4, 3.0 g/LKH_2PO_4, 0.5 g/L NaCl, 1.0 g/L NH_4Cl, 0.5 g/L$MgSO_4$, 0.015 g/L$CaCl_2$, 0.01 g/L thiamine, and 0.01 g/L $FeSO_4$) supplemented with 2% sucrose was used to grow recombinant *E. coli* K12 cells (Da Silva et al., 2005). Ap, Cm, and/or Km were supplemented in previously mentioned concentrations as needed. Recombinant *E. coli* K12 cells were grown in 300-mL flasks containing 100 mL medium in a rotary shaker set at 250 rpm and 37 °C. Cell growth was monitored by measuring the optical density of cells at a wavelength of 600 nm (OD_{600}) using SPECTRAmax PLUS384 (MD, USA). Dry cell weight (DCW) was calculated from a curve relating the OD_{600} to DCW: an OD_{600} of 1.0 represented 0.3 g-DCW/L. The cell mass yield (g-DCW/g-sucrose) was defined as the amount of DCW (g) produced from one gram of the sucrose consumed.

2.5. Analysis of sucrose consumption

Recombinant *E. coli* strains were periodically harvested by centrifugation at 13,000 rpm and filtration with a 0.2-μm syringe filter. A 5- to 10-μL aliquot of cell-free culture media was applied to Agilent Technologies 1200 HPLC system equipped with a Aminex HPX-87C column (Bio-Rad, 250 × 4 mm) and a reflective index

Table 2
PCR primers used in the study.

Gene	Sequence[1]	Enzyme site
*pts*1BCA	F; GC T CTAGA AGGAGGATTACAAA ATG AAT CAT CAA GAA GTT GC	*Xba* I
	R; CCG G AATTC TTA TAT CGC TGT AGC AGC	*Eco*R I
sacA	F; GC T CTAGA AGGAGGATTACAAA ATG ATA TGG AAT CGT AAA ACC	*Xba* I
	R; TTCCCTT GC GGCCGC TCA TTT AAT TTT GGT TTC ATT G	*Not* I
sacK	F; GC T CTAGA AGGAGGATTACAAA ATG CTT TTA GGT GCA ATT GA	*Xba* I
	R; TTCCCTT G AATTC TTA AGC GTT CTT TAA AGC AG	*Eco*R I
scrA	F; GC T CTAGA AGGAGGATTACAAA ATG AAT TAT AAA AAG TCT GCA	*Xba* I
	R; CCG G AATTC CTA TGC TTC TAT ATT TCT ATA TTT CTT TCT ATA TGA	*Eco*R I
scrB	F; GC T CTAGA AGGAGGATTACAAA ATG TCG GAG TGG ACA AAA G	*Xba* I
	R; TTCCCTT GC GGCCGC TCA TAT AGT GTCA CCT TTC A	*Not* I
cscB	F; GC T CTAGA AGGAGGATTACAAA ATG GCA CTG AAT ATT CCA TT	*Xba* I
	R; CCG G AATTC CTA TAT TGC TGA AGG TAC AG	*Eco*R I
cscA	F; GC T CTAGA AGGAGGATTACAAA ATG ACG CAA TCT CGA TTG C	*Xba* I
	R; CCG G AATTC TTA ACC CAG TTG CCA GAG	*Eco*R I
cscK	F; GC T CTAGA AGGAGGATTACAAA ATG TCA GCC AAA GTA TGG G	*Xba* I
	R; CCG G AATTC TTA CTT CTC ACT TTC CAG TTC	*Eco*R I

[1] Underlined sequences indicate restriction enzyme sites.

detector (Agilent Technologies, Santa Clara). Amounts of sucrose, glucose, and fructose in the culture media were calculated by known concentrations of standard carbohydrates (Sigma, USA).

2.6. Production, extraction and analysis of carotenoids

For carotenoid production, recombinant *E. coli* K12 cells were cultivated for 72–90 h in the dark at 30 °C with shaking at 250 rpm in M9 medium + 2% sucrose (200 mL medium in a 1-L flask) supplemented with 100 μg/mL Ap and 50 μg/mL Cm. Carotenoids were extracted from cell pellets by using 15 or 30 mL of acetone or methanol until all visible pigments were removed. Colored supernatants were pooled after centrifugation (4 °C at 4000 rpm) and concentrated to a small volume using an EZ2-Plus centrifugal evaporator (Genevac, New York). Five-milliliters of ethyl acetate was added to the concentrated solution and re-extracted after adding 5 mL NaCl (5 N) solution for salting out. The upper organic phase containing carotenoids was collected, washed with distilled water, and completely dried using the EZ2-Plus evaporator. Until further analysis, the dried samples were stored at −70 °C. A 5- to 10-μL aliquot of the crude extract was applied to a Zorbax eclipse XDB-C18 column (4.6 × 150 mm, 5.0 μm; Agilent Technologies) and eluted under isocratic conditions with a solvent system comprising acetonitrile:methanol:isopropanol (80:15:5) at a flow rate of 1 mL/min using an Agilent 1200 HPLC system equipped with a photodiode array detector (Agilent Technologies). The carotenoids in the extracts were quantified by measuring the absorbance at a specific wavelength (469 nm) by using SPECTRAmax PLUS384 (MD, USA). Finally, the quantification of the carotenoids was calculated by a theoretical equation (Kim et al., 2010a). The equation for total carotenoid content is represented by.

$$\frac{A \times Vol\,(mL) \times 10^4}{E^{1\%}_{1cm} \times \text{cell weight}\,(g)} \quad (1)$$

where A is absorbance, $E^{1\%}_{1cm}$ is specific absorption coefficient ($E^{1\%}_{1cm}$ of diapolycopene = 3410). (George Britton, 2004; Rodriguez-Amaya and Kimura, 2004). The carotenoid yield was defined as the amount of carotenoid (μg) produced from one gram of the sucrose consumed.

3. Results and discussion

3.1. Construction of sucrose-utilizing modules

Sucrose can be utilized by microorganisms via 2 distinguished pathways: non-PTS and PEP-PTS pathways (Fig. 1). To investigate the preferred sucrose-utilizing pathway in an engineered *E. coli* K12 strain for the production of heterologous carotenoids from sucrose, the PEP-PTS pathway genes of *L. plantarum* and *S. xylosus* and the non-PTS pathway genes of Scr$^+$ *E. coli* EC3132 strain were selected. Three genes encoding $CscA_{EC}$, $CscB_{EC}$, and $CscK_{EC}$ from *E. coli* EC3132, 3 genes encoding sucrose phosphotransferase ($Pts1BCA_{LP}$), sucrose hydrolase ($SacA_{LP}$), and D-fructokinase ($SacK_{LP}$) of *L. plantarum*, and 2 genes encoding sucrose phosphotransferase ($ScrA_{SX}$) and sucrose hydrolase ($ScrB_{SX}$) of *S. xylosus* were cloned and engineered for constitutive expression in a modular way in Scr$^-$ *E. coli* K12 SURE strain (Table 3). After introduction of the engineered Scr$^+$ expression modules into Scr$^-$ *E. coli* K12, sucrose-utilization of the modules was initially screened on MacConkey agar plates supplemented with 1% sucrose (MacConkey + 1% sucrose) (Sahin-Tóth et al., 1999). In this screening system, Scr$^+$ *E. coli* K12 strains were expected to turn red or deep purple due to sucrose fermentation, while Scr$^-$ *E. coli* K12 strains remained white (Fig. S1a). The selected recombinant Scr$^+$ *E. coli* K12 strains were then grown in M9 minimal medium supplemented with 1%

Table 3
Functionality of reconstructed sucrose-utilizing modules in *E. coli*.

Reconstructed homogeneous modules	Growth	Reconstructed heterogeneous modules	Growth
$CscA_{EC}$/6×His-tagged $CscA_{EC}$	+[1]	$ScrA_{SX}$ + $ScrB_{SX}$ + $CscK_{EC}$	+
$CscA_{EC}$ + $CscB_{EC}$	+	$ScrA_{SX}$ + $ScrB_{SX}$ + $SacK_{LP}$	+
$CscA_{EC}$ + $CscK_{EC}$	+	$ScrA_{SX}$ + $SacA_{LP}$ + $SacK_{LP}$	+
$CscA_{EC}$ + $CscB_{EC}$ + $CscK_{EC}$	+		
$Pts1BCA_{LP}$	–		
$Pts1BCA_{LP}$ + $SacA_{LP}$	–	$Pts1BCA_{LP}$ + $ScrB_{SX}$	–
$Pts1BCA_{LP}$ + $SacA_{LP}$ + $SacK_{LP}$	–	$Pts1BCA_{LP}$ + $ScrB_{SX}$ + $SacK_{LP}$	–
$ScrA_{SX}$	–		
$ScrA_{SX}$ + $ScrB_{SX}$	+	$ScrA_{SX}$ + $SacK_{LP}$	–

[1] –: no growth; +: growth.

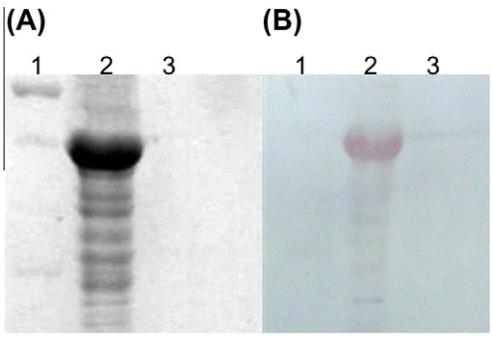

Fig. 2. Analysis of localization of overexpressed His-tagged $CscA_{EC}$ in *E. coli* K12. Cellular 6×His-tagged $CscA_{EC}$ and extracellular 6×His-tagged $CscA_{EC}$ expressed in *E. coli* K12 were analyzed on SDS–PAGE (A) and by Western blotting (B). Lane 1: size marker, 2: crude cell extract, 3: concentrated cell-free culture media.

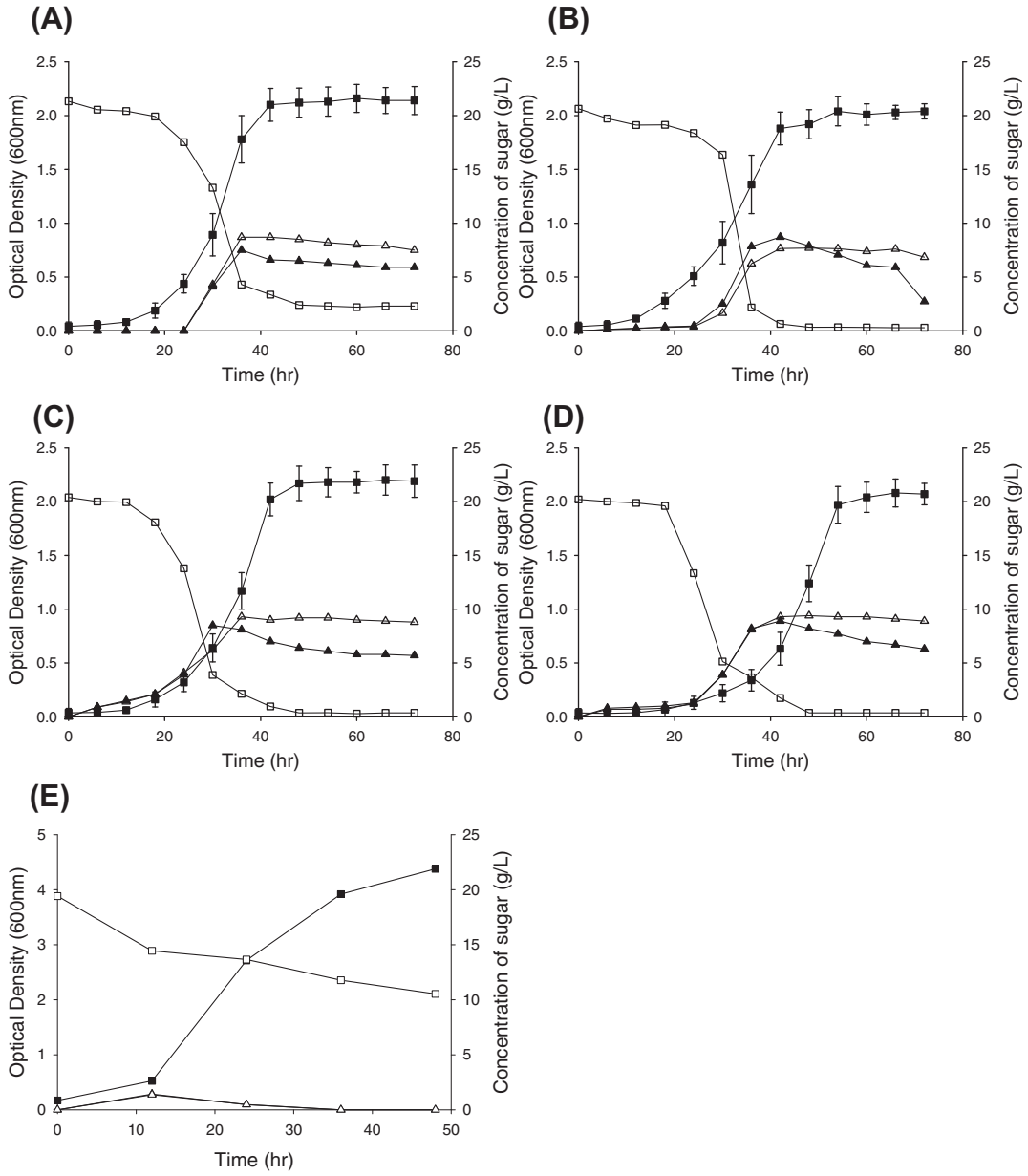

Fig. 3. Growth kinetics of *E. coli* K12 cells harboring non-PTS sucrose-utilizing modules and wild-type Scr$^+$ *E. coli* 3132 grown on M9 minimal medium containing 2% sucrose as a carbon source. (A) *E. coli* K12 cell harboring pAC-cscA, (B) *E. coli* K12 cell harboring pAC-cscAK, (C) *E. coli* K12 cell harboring pAC-cscAB, (D) *E. coli* K12 cell harboring pAC-cscABK, and (E) wild-type *E. coli* EC3132. Symbols represent ■: cell, □: sucrose, ▲: glucose, and △: fructose.

Table 4
Comparison of cell mass and sucrose consumption of recombinant *E. coli* cells expressing sucrose-utilizing modules in M9 minimal medium + 2% sucrose.

Reconstructed Sucrose-utilizing modules	Cell mass (g-DCW/L)[1]	Cell mass yield (g-DCW/g-sucrose)
	M9	M9
EC3132 wildtype	1.374	0.155
$CscA_{EC}$	0.645 ± 0.059	0.148 ± 0.014
$CscA_{EC}$ + $CscK_{EC}$	0.613 ± 0.032	0.109 ± 0.006
$CscA_{EC}$ + $CscB_{EC}$	0.662 ± 0.069	0.131 ± 0.014
$CscA_{EC}$ + $CscB_{EC}$ + $CscK_{EC}$	0.639 ± 0.023	0.141 ± 0.005
$ScrA_{SX}$ + $ScrB_{SX}$	0.811 ± 0.092	0.163 ± 0.018
$ScrA_{SX}$ + $SacA_{LP}$ + $SacK_{LP}$	0.587 ± 0.092	0.139 ± 0.022
$ScrA_{SX}$ + $ScrB_{SX}$ + $CscK_{EC}$	0.623 ± 0.078	0.156 ± 0.02
$ScrA_{SX}$ + $ScrB_{SX}$ + $SacK_{LP}$	0.820 ± 0.092	0.175 ± 0.02

[1] DCW: dry cell weight.

sucrose (M9 + 1% sucrose) (Fig. S1b), and the performance of the engineered Scr modules was evaluated on the basis of cell growth.

3.2. Cell growth and sucrose utilization of E. coli K12 strains expressing non-PTS sucrose-utilizing modules

The sucrose-utilizing abilities of 4 recombinant *E. coli* K12 strains expressing $CscA_{EC}$, $CscA_{EC}$–$CscB_{EC}$, $CscA_{EC}$–$CscK_{EC}$, and $CscA_{EC}$–$CscB_{EC}$–$CscK_{EC}$ were examined by evaluating their growth on MacConkey + 1% sucrose. As seen in Table 3, all 4 recombinant *E. coli* K12 strains expressing the 4 modules were able to grow in M9 + 1% sucrose media, indicating that all the 4 Scr expression modules were functional in Scr$^-$ *E. coli* K12. Interestingly, a single gene module expressing sucrose hydrolase ($CscA_{EC}$) conferred sucrose-utilization ability on Scr$^-$ *E. coli* K12. $CscA_{EC}$ is known to be

localized inside a cell and involved in hydrolyzing transported sucrose from an extracellular culture medium (Fig. 1); therefore, it was necessary to investigate how the expression of $CscA_{EC}$ alone conferred a sucrose-utilizing ability on Scr$^-$ *E. coli* K12. For this, we constructed 6×His-tagged $CscA_{EC}$ and then analyzed the cellular localization of His-tagged $CscA_{EC}$ by immunobloting. The His-tagged $CscA_{EC}$ also bestowed a sucrose-utilizing ability on Scr$^-$ *E. coli* K12 like the wild-type $CscA_{EC}$ (Table 3). As seen from Fig. 2, most of the expressed His-tagged $CscA_{EC}$ was detected inside the cell, but not in the cell-free culture medium, strongly suggesting that the site of sucrose hydrolysis is located intracellularly and not in the extracellular medium. This observation is in agreement with other reports on heterologously expressed sucrose hydrolases in Scr$^-$ *E. coli* K-12 strain where the expressed sucrose hydrolases localized in both cytoplasmic and periplasmic regions of Scr$^+$ *E. coli* K12 strains because of a high overexpression causing the recombinant *E. coli* expressing sucrose hydrolase alone to grow on sucrose (Sahin-Tóth et al., 1999; Scholle et al., 1989). Therefore, it is highly plausible that periplasmic release of $CscA_{EC}$ accounts for the sucrose-utilizing ability of transformed *E. coli* K12 expressing $CscA_{EC}$ alone. In this case, *E. coli* K12 expressing $CscA_{EC}$ alone does not need an ancillary permease $CscB_{EC}$. A contrasting report stated that only the sucrose 6-phosphate hydrolase of *Mannheimia succiniciproducens* was secreted into the culture media, which then hydrolyzed extracellular sucrose to glucose and fructose and led to their uptake and metabolism by *E. coli* K12 (Lee et al., 2010).

The effectiveness of the 4 sucrose-utilizing modules was compared with wild-type Scr$^+$ *E. coli* E3132 grown in batch cultures on M9 medium + 2% sucrose. As indicated in Fig. 3A–D, all 4 recombinant *E. coli* K12 strains expressing the Scr$^+$ modules reached approximately OD_{600} = 2.3 in 48–54 h with a doubling time of approximately 5 h. Even though glucose and fructose accumulated in the culture media, sucrose was not detected in 3 recombinant Scr$^+$ *E. coli* K12 expressing $CscA_{EC}$–$CscB_{EC}$, $CscA_{EC}$–$CscK_{EC}$, and $CscA_{EC}$–$CscB_{EC}$–$CscK_{EC}$ except for Scr$^+$ *E. coli* K12 expressing $CscA_{EC}$ alone (Fig. 3A).This result supports the coexpression of ancillary pathway enzymes $CscB_{EC}$ and/or $CscK_{EC}$ with $CscA_{EC}$ for enhancing sucrose-utilization ability in Scr$^+$ *E. coli* K-12 strains (Chan et al., 2012; Wang et al., 2011). The 4 recombinant Scr$^+$ *E. coli* K12 strains reached similar cell masses (0.61–0.66 g-DCW/L) with similar cell mass yields (0.11–0.15 g-DCW/g-sucrose) (Table 4). In comparison, wild-type Scr$^+$ *E. coli* E3132 (Jahreis et al., 2002) showed relatively slow consumption of sucrose, less accumulation of glucose and fructose, but higher cell growth (Fig. 3E). It seems that more carbon flux was directed into cell mass of the wild-type Scr$^+$ *E. coli* E3132 than those of the 4 recombinant 4 *E. coli* K12 strains overexpressing sucrose-utilizing pathway enzymes. Based on this result, we suggested that Scr$^+$ *E. coli* K12 cells expressing the synthetic sucrose-utilizing modules area better system than wild-type Scr$^+$ *E. coli* E3132.

3.3. Cell growth and sucrose utilization of *E. coli* cells expressing PTS-dependent sucrose-utilizing modules

There is little information on functionality comparison between non-PTS and PEP-PTS pathways for sucrose utilization in a modular way in *E. coli*. Therefore, we selected PEP-PTS sucrose pathway genes of *S. xylosus* or *L. plantarum* for comparison. When synthetic modules expressing the PEP-PTS sucrose pathway genes of *S. xylosus* or *L. plantarum* were analyzed in Scr$^-$ *E. coli* K12, only one mod-

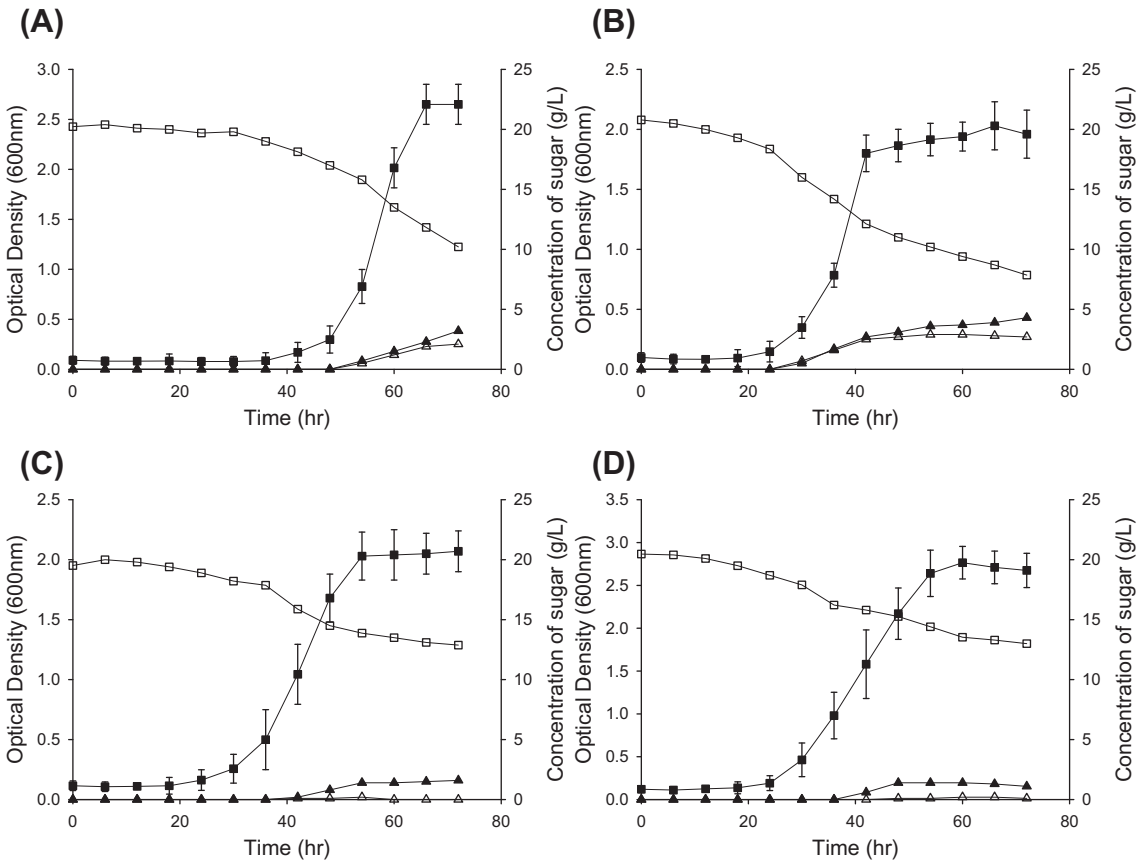

Fig. 4. Growth kinetics of *E. coli* K12 cells expressing homologous or heterologous PEP-PTS-sucrose-utilizing modules on M9 minimal medium containing 2% sucrose as a carbon source. (A) *E. coli* K12 cell harboring pAC-scrAB, (B) *E. coli* K12 cell harboring pAC-AAK, (C) *E. coli* K12 cell harboring pAC-scrAB + pUC-cscK, and (D) *E. coli* K12 cell harboring pAC-scrAB + pUC-sacK. Symbols represent ■: cell, □: sucrose, ▲: glucose, and △: fructose.

ule expressing $ScrA_{SX}$ and $ScrB_{SX}$ of *S. xylosus* was functional in Scr^- *E. coli* K12 strain (Table 3). The recombinant Scr^+ *E. coli* K12 strain expressing $ScrA_{SX}$ and $ScrB_{SX}$ reached approximately OD_{600} = 2.5 (0.8 g-DCW/L) with a large cell mass yield (0.16 g-DCW/g-sucrose) after a relatively long lag period (Fig. 4A and Table 4). Unlike the *S. xylosus* module expressing $ScrA_{SX}$–$ScrB_{SX}$, the *L. plantarum* modules expressing $Pts1BCA_{LP}$, $Pts1BCA_{LP}$-$SacA_{LP}$, or $Pts1BCA_{LP}$-$SacA_{LP}$-$SacK_{LP}$ were not functional in *E. coli* K12 (Table 3). To determine which one of the *L. plantarum* sucrose-utilizing module was responsible for non-functionality in *E. coli* K12, functional *S. xylosus* heterologous modules were complemented. When heterologous $ScrA_{SX}$ was complemented with a module expressing $SacA_{LP}$–$SacK_{LP}$, the resulting hybrid module expressing $ScrA_{SX}$–$SacA_{LP}$–$SacK_{LP}$ conferred a sucrose-utilizing ability on Scr^-*E. coli* K12 (Table 3). The growth of recombinant Scr^+ *E. coli* K12 strain expressing $ScrA_{SX}$–$SacA_{LP}$–$SacK_{LP}$ attained approximately OD_{600} = 2.0 (0.61 g-DCW/L) having a cell mass yield of 0.14 g-DCW/g-sucrose on M9 medium + 2% sucrose (Fig. 4B). The growth of recombinant *E. coli* K12 strain expressing $Pts1BCA_{LP}$ was retarded compared to a control strain expressing an empty plasmid, indicating that $Pts1BCA_{LP}$ was expressed in *E. coli* K12. This finding supports that both $SacA_{LP}$ and $SacK_{LP}$ were functional in *E. coli* K12, whereas $Pts1BCA_{LP}$ was not functional. It appeared that unlike $ScrA_{SX}$, $Pts1BCA_{LP}$ is unable to complement a histidine-rich protein (PtsH)/enzyme I (PtsI) system of *E. coli*.

It is known that sucrose utilization in *S. xylosus* depends on $ScrA_{SX}$ and $ScrB_{SX}$ (Jankovic and Brückner, 2007), the recombinant Scr^+ *E. coli* K12 strain expressing $ScrA_{SX}$–$ScrB_{SX}$ was able to grow on sucrose (Fig. 4A). The effect of expression of the additional pathway enzyme, fructokinase, on sucrose-utilization ability of the $ScrA_{SX}$–$ScrB_{SX}$ module was evaluated by coexpressing a heterologous fructokinase $CscK_{EC}$ or $SacK_{LP}$ with a module expressing $ScrA_{SX}$–$ScrB_{SX}$ in *E. coli* K12. The additional heterologous fructokinase shortened lag phases of the recombinant Scr^+ *E. coli* K12 strains expressing $ScrA_{SX}$–$ScrB_{SX}$, but it could not enhance sucrose consumption (Fig. 4C and D).

3.4. Carotenoid production by reconstructed sucrose metabolic system in E. coli

A diapolycopene-producing *E. coli* K12 strain harboring pAC-crtMN (Kim and Lee, 2012) was transformed with pAC-cscABK or pAC-AAK, which expressed $CscA_{EC}$–$CscB_{EC}$–$CscK_{EC}$ (non-PEP-PTS module) or $ScrA_{SX}$–$SacA_{LP}$–$SacK_{LP}$ (PEP-PTS module), respectively, and were cultivated on M9 medium + 2% sucrose. In both Scr^+ *E. coli* K12 strains, diapolycopene formation showed a close correlation with cell growth and sucrose consumption, and the sucrose-utilizing ability of the modules was not affected (Fig. 5A and B). The Scr^+ *E. coli* K12 strain harboring pAC-crtMN and pAC-cscABK produced diapolycopene at 1.5 mg/g-DCW and 281 μg/g-sucrose at 78-h culture (Fig. 5A). However, as seen from Fig. 3D, glucose and fructose accumulated in the culture media of the Scr^+ *E. coli* K12 strain harboring pAC-crtMN and pAC-cscABK even though sucrose was not detected in the medium. Unlike the Scr^+ *E. coli* K12 strain having pAC-crtMN and pAC-cscABK, the Scr^+ *E. coli* K12 strain containing pAC-crtMN and pAC-AAK produced less diapolycopene (1.3 mg/g-DCW and 108 μg/g-sucrose) at 48-h culture (Fig. 5B). Longer incubation time did not improve diapolycopene formation and even reduced carotenoid formation in both diapolycopene producing Scr^+ *E. coli* K12 strains. This could be mainly attributable to the age or physiological change of *E. coli* because carotenoids are known to protect host microorganisms from reactive oxygen species generated during late stationary growth or aging (Lee et al., 2008; Lee and Schmidt-Dannert, 2003).

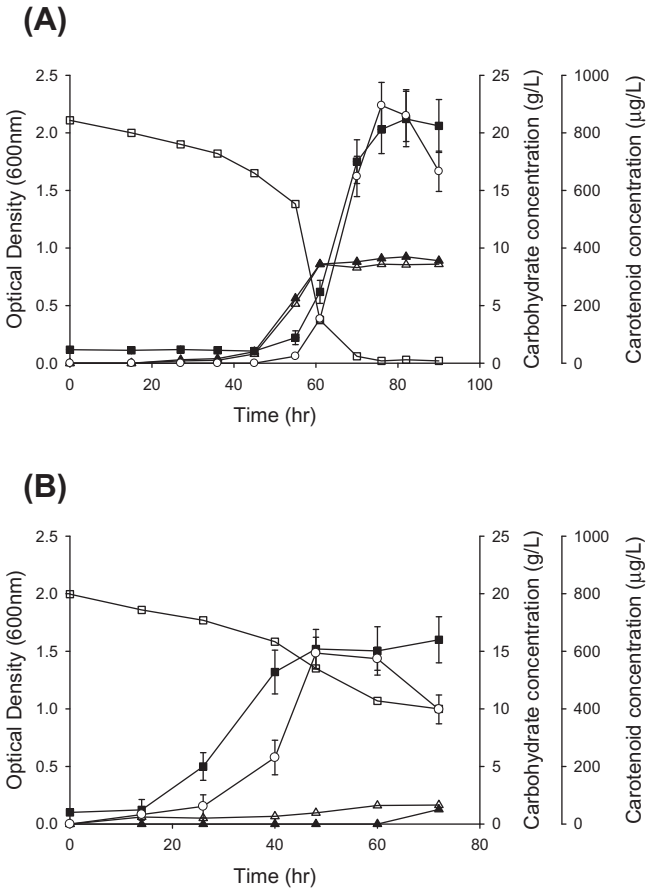

Fig. 5. Diapolycopene production in recombinant *E. coli* K12 cells expressing 2 sucrose-utilizing modules ($CscA_{EC}$–$CscB_{EC}$–$CscK_{EC}$ and $ScrA_{SX}$–$SacA_{LP}$–$SacK_{LP}$) on M9 minimal medium containing 2% sucrose as a carbon source. (A) *E. coli* K12 cells expressing the $CscA_{EC}$–$CscB_{EC}$–$CscK_{EC}$ module (as pAC-cscABK) + diapolycopene module (as pUC-crtMN) and (B) the $ScrA_{SX}$–$SacA_{LP}$–$SacK_{LP}$ module (as pAC-AAK) + diapolycopene module (as pUC-crtMN) were grown in M9 minimal medium containing 2% sucrose as a carbon source. Symbols represent ■: cell, □: sucrose, ▲: glucose, △: fructose, and ⊙: carotenoid.

4. Conclusions

In this study, we demonstrated that sucrose-utilizing ability could be conferred on the *E. coli* K12 strain by introducing switchable synthetic modules of sucrose-utilizing genes from various sources (PEP-PTS pathway genes of *L. plantarum* and *S. xylosus* and non-PTS pathway genes of Scr^+ *E. coli* EC3132). Furthermore, the experiments with the carotenoid-producing *E. coli* K12 strain proved that the reconstructed modular Scr^+ systems can be transferred to various industrial *E. coli* K12 strains developed to produce bio-based chemicals, fuels, and other nutritional compounds.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2012.11.148.

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