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RESEARCH PAPER

Production of Tagatose by Whole-cell Bioconversion from Fructose Using *Corynebacterium glutamicum*

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Abstracts Tagatose is a rarely occurring sugar found in food and sweet fruits. It is a potential sweetener with low calories but a sweetness similar to that of sucrose. In this study, we developed a whole-cell biocatalyst using *Corynebacterium glutamicum* for direct bioconversion of fructose to tagatose. First, we constructed a biological conversion pathway for the conversion of fructose to tagatose by expressing tagatose 4-epimerase (TN) from *Thermotoga neapolitana* in *C. glutamicum*. Next, to increase the expression level of the enzyme, we engineered copy number of plasmid. Plasmid library was constructed by randomizing the *cgrI* antisense RNA region in the plasmid, and the plasmid with high-copy number was isolated using fluorescence-activated single cell sorting (FACS)-based high-throughput screening. The isolated plasmid, pHCP7, had 2-fold higher copy numbers than the original plasmid. A higher expression level and conversion yield could be achieved using pHCP7. Finally, we examined a novel tagatose 4-epimerase TN(KNF4E) isolated from *Kosmotoga olearia*. The gene expression level was further increased (33.9% of total fraction) through codon optimization and expression in pHCP7, and a conversion yield as high as 21.7% was achieved.

Keywords: *Corynebacterium glutamicum*, tagatose, fructose, whole-cell biocatalyst, high copy plasmids

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1. Introduction

Tagatose is a rarely occurring sugar present in food and sweet fruits such as apples, tangerines, and cacao. It has a low calorie (1.5 kcal/g), which is one third that of sucrose, and a low glycemic index of 3, which is 5% that of sucrose; however, its sweetness is similar to that of sucrose [1,2]. In addition, tagatose has various health benefits due to its antidiabetic, antiobesity, antioxidant, noncariogenic, and prebiotic properties [3-5]. Thus, tagatose is a potential sweetener that can satisfy both health and the taste buds—and demand for tagatose is increasing.

Tagatose is produced by chemical and biological methods using galactose as the main raw material. In the chemical method, tagatose is isomerized from galactose using calcium hydroxide and calcium chloride [6]. In the biological method, tagatose is produced from galactose using L-arabinose isomerase or through recombinant cells expressing L-arabinose isomerase [7,8]. However, the use of galactose, which is usually obtained from lactose through β-galactosidase-mediated hydrolysis, is limited by price fluctuations according to the supply and demand of dairy products in global markets [9]. Thus, a huge demand exists for the development of a new, economic, and stable bioprocess capable of producing tagatose using common

saccharides (sucrose, glucose, fructose, *etc*.) that have a more stable supply and lower cost in global markets than that of lactose and galactose [10].

Among the various saccharides, several efforts have been made to develop new biological processes using fructose derived from the starch industry. Lee *et al*. [11] developed a three-step enzymatic reaction, using hexokinase, fructose-1,6-biphosphate aldolase, and phytase, for the bioconversion of fructose into tagatose with high conversion yield (77%) and productivity of 9 g/L/h. More recently, Shin *et al*. [12] reported the direct conversion of fructose to tagatose using an engineered tagatose 4-epimerase (TN) derived from *Thermotoga petrophila*. The enzyme, which showed a 184-fold higher 4-epimerization activity toward D-fructose, was successfully engineered using structure-based engineering and random mutagenesis. However, in both studies, the enzymes were obtained by culturing *Escherichia coli* and multiple purification processes were required. This significantly increased the production costs, particularly at an industrial scale. Compared with enzymatic or chemical processes, whole-cell biocatalyst process, which use microorganisms, has several advantages, such as: (i) relatively inexpensive processes for enzyme purification, (ii) easy regeneration of cofactors (*e.g*., NADPH) for multi-step reactions, and (iii) simple establishment of the recycling process [13-15]. The critical factor while developing a whole-cell biocatalysts process is the choice of host cell as it affects, the overall production yields, and cost. The general attributes to be considered while choosing a host are: (i) ease of high-density cell cultivation, (ii) various genetic tools for high expression of genes encoding target enzymes, (iii) high-tolerance to harsh conditions due to pH, temperature, and salts, and (iv) recognition as a safe host. In this regard, *Corynebacterium glutamicum,* a non-sporulating gram-positive bacterium, is an attractive option as a host and has been widely used for the production of amino acids and value-added chemicals [16,17]. *C. glutamicum* is generally recognized as safe (GRAS), and recent advances in genetic engineering have enabled diverse applications of *C. glutamicum,* including as a whole-cell biocatalyst to produce various chemicals such as cinnamaldehyde, flavanones, cinnamic acid, keto-fatty acids, and undec-9-enoic acid [18-22].

In this study, we developed a whole-cell biocatalyst using *C. glutamicum* for the direct bioconversion of fructose to tagatose (Fig. 1). We first constructed an expression system of TN from *Thermotoga neapolitana* [23] and confirmed the successful conversion of fructose to tagatose using *C. glutamicum* as a whole-cell biocatalyst. Next, to further increase gene expression, we developed a high-copy number plasmid through high-throughput screening of a promoter library in which the *cgrI* antisense RNA region in the plasmid was randomly mutated. Using the isolated plasmid, much improved gene expression and conversion yield could be achieved. Finally, we examined a novel tagatose 4-epimerase TN(KNF4E) isolated from *Kosmotoga olearia* [24]. Through codon optimization and use of plasmid with high copy number, gene expression level (33.9% in total fraction) could be further increased, and a conversion yield as high as 21.7% could be achieved.

2. Materials and Methods

2.1. Bacterial strains and cultivation conditions

The bacterial strains and plasmids used in the present study are listed in Table 1. *E. coli* XL1-Blue was used for gene cloning and plasmid maintenance, and *C. glutamicum* ATCC13032 was used as a host for the expression of conversion enzymes and whole-cell biocatalyst reactions. *E. coli* was cultivated in Luria-Bertani (LB) medium (BD, Franklin Lakes, NJ, USA) at 37°C with shaking at 200 rpm. *C. glutamicum* was cultivated in brain heart infusion (BHI) medium (BD) at 30°C with shaking at 200 rpm. Kanamycin

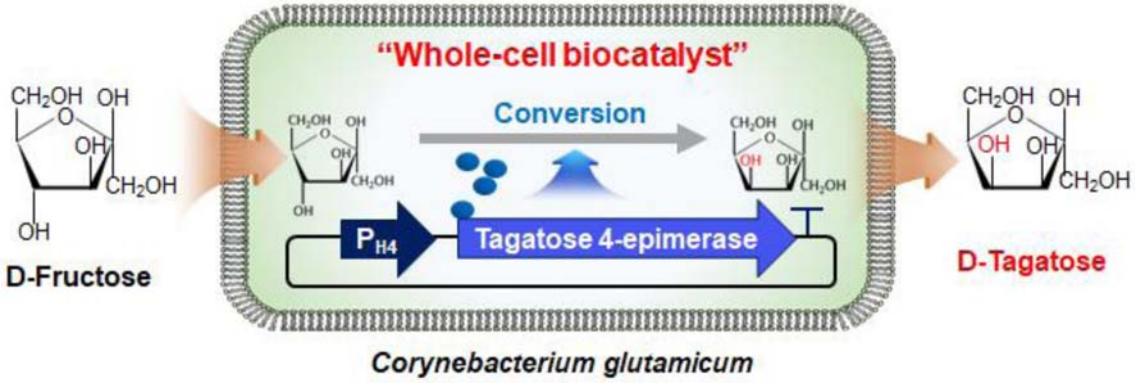

Fig. 1. Overall scheme of fructose to tagatose bioconversion reaction by whole-cell biocatalysts platform.

Table 1. List of strains and plasmids

Strains and plasmids	Relevant characteristics	Reference or source
Strains		
Escherichia coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacI^q ZΔM15 Tn10 (Tet^r)]*	Stratagene[a]
Corynebacterium glutamicum ATCC 13032	Biotin-auxotrophic wild type	ATCC
Plasmids		
pCES_PLPV	*E. coli - C. glutamicum* shuttle vector, pCES208 derivative; MCS and rrnB terminator, Km^r	[25]
pHCP	pCES_PLPV derivative, pHCMS; *parB* nonsense mutation, Km^r, P_{H4}, *sfGFP*	[32]
pCES_TN	pCES_PLPV derivative; P_{H4}, *TN* gene, 6xHis tag	This study
pHCP_TN	pHCP derivative; P_{H4}, *TN*, 6xHis tag	This study
pHCP_TNsfGFP	pHCP derivative; P_{H4}, 99 bp of *TN* fused with *sfGFP*, 6xHis tag	This study
pHCP_TNsfGFP_L	pHCP_TNsfGFP derivative; Library of anti-sense RNA mutation	This study
pHCP7_TNsfGFP	pHCP_TNsfGFP_L derivative	This study
pHCP7_TN	pHCP 7 derivative; P_{H4}, *TN*, 6xHis tag	This study
pHCP_TN(KNF4E)	pHCP derivative;P_{H4}, *TN(KNF4E)* gene, 6xHis tag	This study
pHCP7_TN(KNF4E)	pHCP 7 derivative; P_{H4}, *TN(KNF4E)*, 6xHis tag	This study
pHCP_TN(KNF4E)_Opti	pHCP derivative; P_{H4}, *TN(KNF4E)_Opti*	This study
pHCP7_TN(KNF4E)_Opti	pHCP 7 derivative; P_{H4}, *TN (KNF4E)_Opti*, 6xHis tag	This study

[a]Stratagene California, La Jolla, CA, USA.

(Km, 25 μg/mL) was added to all culture media as the sole antibiotic.

2.2. Plasmid construction

All restriction enzymes used for gene manipulation were purchased from Enzynomics (Daejeon, Korea). Polymerase chain reaction (PCR) was performed in a C1000^{TM} Thermal Cycler (Bio-Rad, Hercules, CA, USA) using Prime STAR HS polymerase (Takara Bio Inc., Shiga, Japan). All primers used for PCR are listed in Table S1. Two tagatose 4-epimerases genes, the *TN* gene from *T. neapolitana* and *TN(KNF4E)* gene from *K. olearia*, were synthesized by General Biosystems (Durham, NC, USA). PCR was performed to amplify the *TN* gene and *TN(KNF4E)* using the primers NdeI_TN_F and NotI_His_R, and NdeI_TN (KNF4E)_F, and NotI_His_TN(KNF4E)_R, respectively. All PCR products were digested with *Nde*I and *Not*I and cloned into pCES_PLPV [25], pHCP, and pHCP7_TNsfGFP at the same restriction enzyme sites. The codon-optimized *TN(KNF4E)* gene, named *TN(KNF4E)_Opti*, was synthesized and cloned into pHCP and pHCP7_TNsfGFP to yield pHCP_TN(KNF4E)_Opti and pHCP7_TN(KNF4E)_Opti, respectively. In all expression systems, genes were expressed under the synthetic constitutive promoter (P_{H4}) [26], and the 6xHis tag was fused to the 3′end of the *TN* and *TN(KNF4E)* genes. After construction, each plasmid was transformed into *C. glutamicum* ATCC13032 via electroporation using a Gene Pulser (Bio-Rad, Hercules, CA, USA).

2.3. Antisense RNA library construction

The *sfGFP* gene fused to 99 bp of the *TN* gene sequence was amplified by PCR with the primer sets pCES208_confirm_F/TN_R and TNsfGFP_F/pCES208_no Ter_R and amplified again by overlap PCR with pCES208_confirm_F/pCES208_no Ter_R. The PCR products were cloned into the pHCP vector, yielding pHCP_TNsfGFP. For constructing the library, the antisense RNA region was amplified with ctRNA_F and ctRNA_R primers by error-prone PCR using Taq polymerase (Takara Bio Inc.) and a pHCP plasmid was used as a template. The PCR products were amplified again by overlap PCR using two other PCR products amplified with primer sets HC_F/ctRNA_F_RR and ctRNA_R_FF/HC_R. Overlap PCR was performed with primers HC_F and HC_R, and the PCR product was digested with *Nco*I and *Eco*RI and cloned into pHCP_TNsfGFP, yielding pHCP_TNsfGFP_L.

2.4. Fluorescence-activated single cell sorting (FACS) screening

For FACS screening, an antisense RNA library (*C. glutamicum* harboring pHCP_TNsfGFP_L) was cultivated in BHI medium overnight and transferred into 50 mL of fresh BHI medium in flasks. After cultivation for 24 h, cells were harvested by centrifugation at 6,000 rpm for 10 min at 4°C. The harvested cells were washed with phosphate-buffered saline (PBS, 135 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 14 mM KH_2PO_4; pH 7.2), and the fluorescence intensity

was analyzed using FACS (MoFlo XDP; Beckman Coulter, Inc., Miami, FL, USA). For FACS screening, the cells were excited with a laser at 488 nm and detected with a 530/40 band-pass filter for the green fluorescent protein (GFP) emission spectrum. Cells with high fluorescence intensity (top 1% of the cell population) were sorted, and the sorted cells were grown overnight and transferred into 50 mL of BHI medium in a 250 mL flask for the next round of screening.

2.5. Real-time quantitative PCR (RT-qPCR)

Relative quantification of plasmid copy number (PCN) was done using the RT-qPCR method [27,28]. First, genomic DNA, including plasmids, was extracted from the cells using the MasterPure™ DNA Purification Kit (Epicenter®; Illumine Company, Madison, WI, USA). RT-qPCR was performed with two primer sets: i) p15A ori_F and p15A ori_R for the amplification of the p15A origin of the plasmid and ii) SigB_RT_F and SigB_RT_R for the amplification of the *sigB* gene in the chromosomal DNA of *C. glutamicum*. The value of the quantification cycle (Cq) of the target sequence was analyzed using CFX Real-Time PCR (Bio-Rad) with the One-Step SYBR PrimeScript RT-PCR kit (Takara Bio Inc.). The amplification efficiency (E) of each gene was determined using a tenfold dilution of the plasmids and genomic DNA between 0.002 ng/μL to 20 ng/μL. The PCN was calculated using the method described by Lee *et al*. [27].

2.6. RNA secondary structure prediction

The secondary structures of the antisense RNA molecules and the leader mRNA sequence of *repA* gene were predicted using the RNAfold webserver (http://rna.tbi.univie.ac.at/) [29]. RNA secondary structure diagrams were obtained using the Forna (force-directed RNA) online tool [30].

2.7. Protein preparation and analysis

After cultivation, cells were harvested by centrifugation (13,000 rpm for 10 min at 4°C). The cell pellets were resuspended in PBS and were disrupted by sonication for 7 min at 50% pulse and 20% amplitude (Sonics, Newtown, CT, USA). Total protein fractions were collected after sonication. After centrifugation of cell lysates at 13,000 rpm for 10 min at 4°C, the soluble proteins were collected from the supernatant. The fractionated protein samples were analyzed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting [31]. GelAnalyzer2010a software was used to perform protein densitometry.

2.8. One-step whole-cell biocatalyst reaction and analysis of tagatose

After flask cultivation at 30°C for 24 h, the cells were harvested by centrifugation (3,000 rpm for 10 min at 4°C), and the harvested cells were washed twice with a 50 mM Tris-HCl buffer. Then, 20% (*w/w*) of the washed cells and 30 g/L fructose (Sigma-Aldrich, St. Louis, MO, USA) were mixed in 50 mM Tris-HCl buffer. For the reaction with TN, $NiSO_4$ (1 mM) was added, and for that with TN(KNF4E), $CoSO_4$ (3 mM) was added. After pre-incubation of the reaction mixture at 60°C for 30 min, the reaction was allowed to take place at 60°C for 2 h. After centrifugation (13,000 rpm for 10 min at 4°C), the reaction samples were collected from the supernatant and were analyzed using high-performance liquid chromatography (HPLC). The residual sugar concentrations were measured using an HPLC system (LC-20 AD, CTO-20A, SPD-20A; Shimadzu, Kyoto, Japan) with a UV/Vis detector at 210 nm. Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad) was used with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.5 mL/min, and the oven temperature was maintained at 40°C. Standard materials for HPLC analysis were 0.3125-10 g/L of fructose and 0.03125-1 g/L of tagatose (CJ CheilJedang, Suwon, Korea) after serial dilution. The tagatose conversion yield was calculated using the following equation.

$$\text{Convestion yield (\%)} = \frac{\textit{conc. of tagatose synthesized by reaction}\left(\frac{g}{L}\right)}{\textit{Total conc. of fructose used for reaction}\left(\frac{g}{L}\right)} \quad (1)$$

3. Results and Discussion

3.1. Construction of TN expression system

To convert fructose to tagatose, we first expressed TN from *Thermotoga neapolitana* in *C. glutamicum*. We constructed an expression system for TN fused with a 6xHis tag at the C-terminus in two plasmids, pCES_PLPV (low copy) and pHCP (high copy), which have different copy numbers [32], yielding pCES_TN and pHCP_TN, respectively (Fig. 2A). After flask cultivation, the expression levels of TN in each construct were analyzed using SDS-PAGE and western blotting. In both constructs, His-tag-fused TN (56.6 kDa) was successfully produced, and as expected, the expression level in pHCP_TN was much higher than that in pCES_TN (Fig. 2B).

3.2. Development of higher PCN for increase of gene expression

As shown in Fig. 2, gene expression levels were correlated with PCNs; therefore, to further increase the expression level, we tried to increase the copy number of pHCP. However, excessively high PCNs frequently cause metabolic

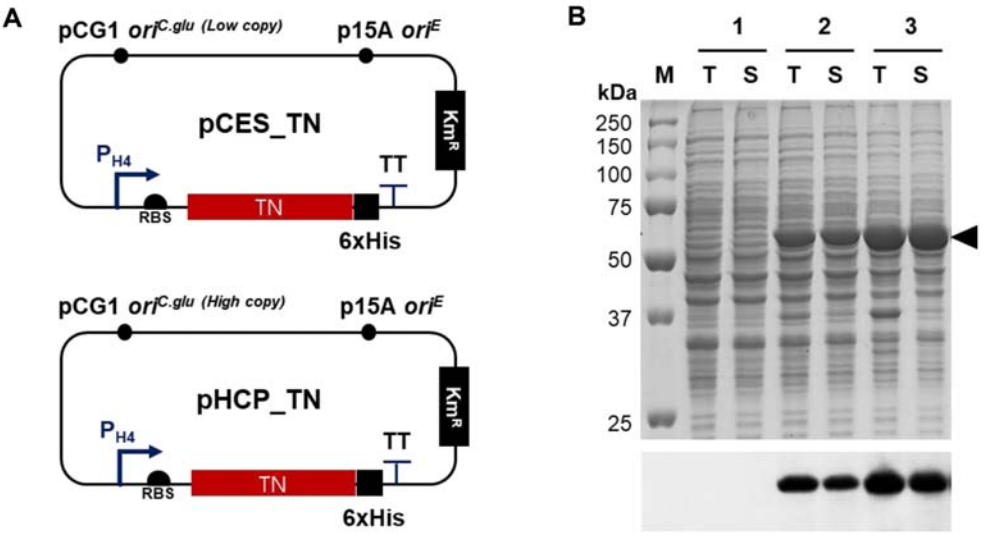

Fig. 2. Expression of *TN* gene in *Corynebacterium glutamicum*. (A) Schematic illustration of expression plasmid, pCES_TN (upper) and pHCP_TN (lower). (B) Analysis of the expression level of TN by SDS-PAGE (upper panel) and western blotting (lower panel). Lane M represent protein size markers (kDa). Lanes 1 to 3 represent a protein sample of cells harboring pHCP as a negative control, pCES_TN, and pHCP_TN, respectively. Lanes T and S represent total and soluble fraction of protein samples, respectively. Arrowhead (◀) indicates TN band. P_{H4}: H4 synthetic promoter, TT: transcription terminator, ori^E and $ori^{C.glu}$: origin of replication for *E. coli* and *C. glutamicum*.

burden and poor cell growth, which is not suitable for heterologous gene expression [33-35]. To maximize the expression level of heterologous genes, PCN needs to be increased to an optimal level, and for this purpose, we constructed a plasmid library with different copy numbers and tried to isolate the plasmid with optimal copy number for *TN* gene expression in *C. glutamicum*. It is known that antisense RNAs, such as *crrI* and *cgrI*, are major control factors of plasmid replication and copy number in the pCG1 plasmid family [36-38]. Okibe *et al*. [37] showed that abolishing *cgrI* expression resulted in a remarkable increase in PCN, and the loop structure between antisense RNAs and their target mRNAs is essential for the control of PCN. This implies that the change in antisense RNA modifies the copy number of plasmids and could be a target for mutagenesis. First, similar to above report [39], we tried the deletion of *cgrI* gene in plasmid, and we also found that PCN could increase remarkably (higher than 200 copies). However, we recognized PCN could not be maintained at high level during the cultivation, and gene expression level were not increased (data not shown). Next, instead of *cgrI* deletion, we tried its modification by random mutation. The *cgrI* antisense RNA region of pHCP belonging to the pCG1 family was randomly mutated by error-prone PCR, and the PCR products were cloned into pHCP_TNsfGFP, yielding a plasmid library (pHCP_TNsfGFP_L) (Fig. 3A). The library was first constructed in *E. coli* XL1-Blue with a size of 1×10^7 cells. After recovery of the library constructs, they were retransformed into

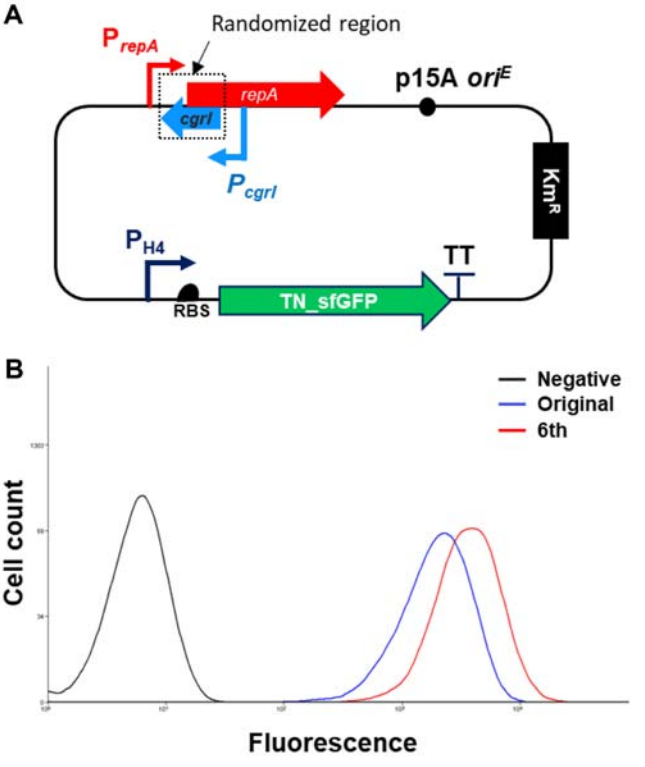

Fig. 3. Engineering of the expression system by fluorescence-activated single cell sorting (FACS). (A) Map of plasmid library, pHCP_TNsfGFP_L. Randomization region of the library was indicated by a dotted box. (B) FACS screening after 6th rounds of library sorting. pHCP and pHCP_TNsfGFP were used as negative and original controls. P_{H4}: H4 synthetic promoter, TT: transcription terminator, ori^E: origin of replication for *E. coli.*

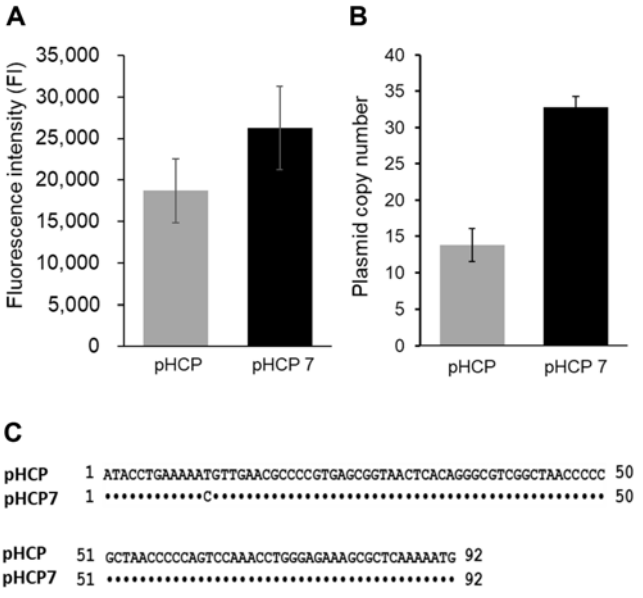

Fig. 4. Analysis of fluorescence intensity and plasmid copy number. (A) Fluorescence intensity of pHCP_TNsfGFP and pHCP7_TNsfGFP. (B) Plasmid copy numbers of pHCP_TN and pHCP7_TN.

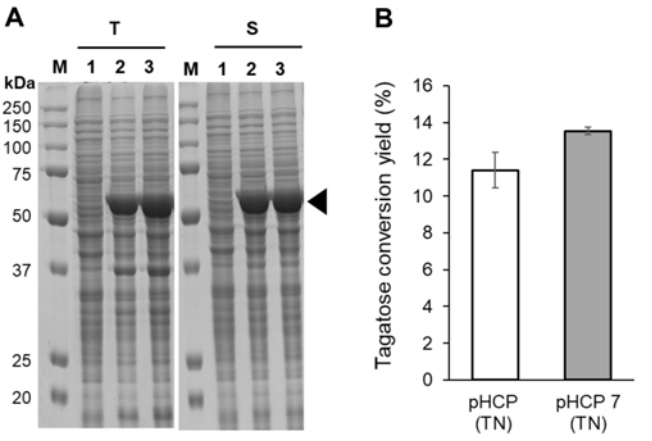

Fig. 5. Comparison of the expression level and tagatose conversion yield of TN. (A) SDS-PAGE analysis of pHCP_TN. Lane M represents protein size markers (kDa). Lanes 1 to 3 represent a protein sample of cells harboring pHCP as a negative control, pHCP_TN, pHCP7_TN, respectively. Arrowhead (◀) indicates TN band. Lanes T and S represent total and soluble fraction of protein samples. (B) Tagatose conversion yield of each system.

C. glutamicum with a size of 2×10^4 cells and used for screening. In this library, the *sfGFP* gene was fused with the first 99 bp of the *TN* gene, which was used as a reporter for FACS-based high-throughput screening (Fig. 3A). In every round of FACS screening, the top 1% of cells were selectively sorted. During the six rounds of sorting, the fluorescence intensities were gradually enriched, indicating that cells with higher PCNs were successfully enriched (Fig. 3B). After the sixth round of sorting, individual clones grown on agar plates were analyzed, and we selected one clone that showed the highest fluorescence intensity, which was 1.5-fold higher than that of the original plasmid (pHCP_TNsfGFP) (Fig. 4A). The plasmid pHCP7 was purified from the isolated clone and its copy number was determined using RT-qPCR (Fig. 4B). The copy number of pHCP7 was approximately 33, which was 2.4-fold higher than that of the original plasmid (pHCP).

Next, to identify the mutation, the sequences of the antisense RNA region in pHCP7 were determined, and we found that pHCP7 contains only one base mutation (T to C) located at the 3'-end of the *cgrI* antisense RNA region and the 5'-untranslated region (+10 region) of the *repA* gene (Fig. 4C). The secondary structures of *repA* and *cgrI* antisense RNA are critical in the regulation of PCN. Hence, to elucidate the effect of a single mutation on the *cgrI* antisense RNA secondary structures, we compared the predicted secondary structures of the original and mutated *cgrI*. Despite both *cgrI* regions having almost the same secondary structures, we found that minimum free energy (MFE) of the antisense RNA(*CgrI*) in pHCP7 was slightly lower; the original and mutated *cgrI* regions have MFE of -37.7 kcal/mol and -41.2 kcal/mol, respectively (Fig. S1A and S1B). A lower MFE value indicates a more stable antisense RNA structure; however, we do not know how it contributes to the increase of PCN.

Using the isolated pHCP7, we examined the TN expression and the conversion of fructose to tagatose during flask cultivation. Using SDS-PAGE analysis, we confirmed that the expression level of *TN* gene in pHCP7 constituted 32.4% of total proteins, which was higher than that in pHCP (27.1%) (Fig. 5A). Also, the use of pHCP7 exhibited higher tagatose conversion yield (13.8%) than that of pHCP (11.4%) (Fig. 5B).

3.3. Tagatose production by expression of new enzyme TN(KNF4E)

In addition to TN, we attempted bioconversion using cells expressing a novel tagatose 4-epimerase gene (*TN(KNF4E)*) isolated from *K. olearia* [24]. The *TN(KNF4E)* gene was cloned into pHCP and pHCP7, into which the *TN(KNF4E)* gene was fused using a 6xHis tag at the C-terminus. After flask cultivation, the expression level of the *TN(KNF4E)* gene in each plasmid was determined using SDS-PAGE and western blotting. In both expression systems, TN(KNF4E) (50.2 kDa) was successfully produced. As expected, the gene expression level in pHCP7 (28.3% of the total fraction) was higher than that in pHCP (19.3%) (Fig. 6A). The tagatose conversion yield in pHCP7 was 14.4%, which was 1.25-fold higher than that in pHCP (11.5%) (Fig. 6B). Also, we confirmed that the expression of novel enzyme

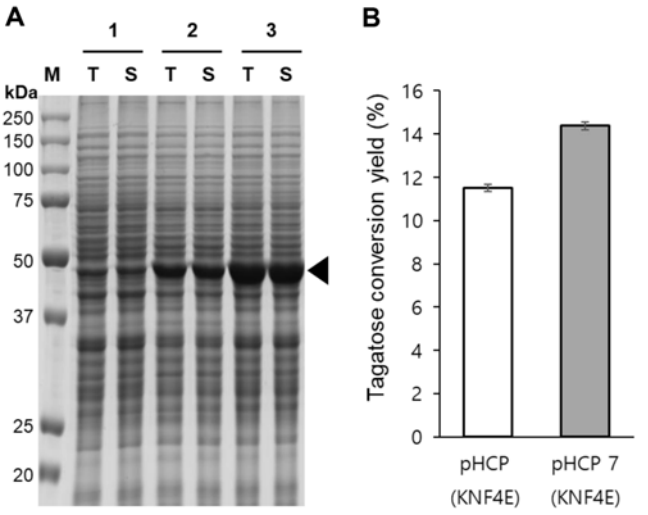

Fig. 6. Comparison of the expression level and tagatose conversion yield of TN(KNF4E). (A) SDS-PAGE analysis of TN(KNF4E). Arrowhead (◀) indicates TN(KNF4E) band. Lanes T and S represent total and soluble fraction of protein samples, respectively. Lane 1 to 3 represent a protein sample of cells harboring pHCP as a negative control, pHCP_TN(KNF4E), and pHCP7_TN(KNF4E), respectively. (B) Tagatose conversion yield of each system.

(TN(KNF4E)) in pHCP7 exhibited a mildly higher conversion yield than that of TN expression in pHCP7 (13.8%) (Fig. 5B).

3.4. Tagatose production by expression of codon-optimized *TN(KNF4E)* gene in *C. glutamicum*

To further increase the expression of the *TN(KNF4E)* gene in *C. glutamicum*, the *TN(KNF4E)* gene from *K. olearia* was modified to *C. glutamicum*-preferable codons. The codon-optimized gene (*TN (KNF4E)_Opti*) fused with a 6xHis tag at the C-terminus was cloned into pHCP7, and the expression level in *C. glutamicum* was compared with that of the original gene using SDS-PAGE. As shown in Fig. 7A, the expression level of the optimized gene (33.9% of total fraction) was higher than that of original gene (28.5%). Also, the bioconversion yields from fructose to tagatose in each expression system were compared, and we found that *C. glutamicum* harboring pHCP7(KNF4E)_Opti showed 21.7% bioconversion yield which was 1.5-fold higher than that of original gene (14.4%) (Fig. 7B).

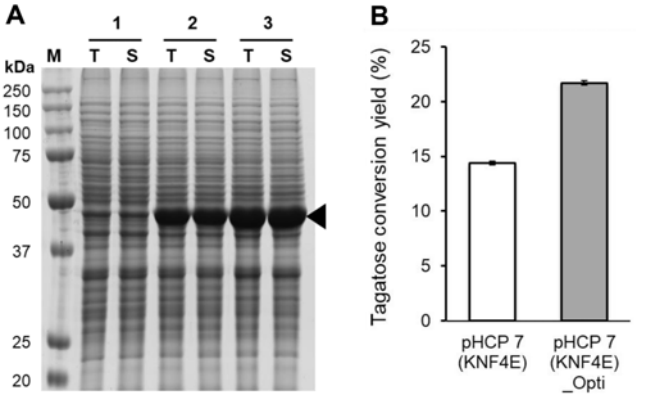

Fig. 7. Analysis of the expression level and tagatose conversion yield of TN(KNF4E) and TN(KNF4E)_Opti. (A) SDS-PAGE analysis. Lane M represents protein size markers (kDa). Lanes 1 to 3 represent a protein sample of cells harboring pHCP as a negative control, pHCP_TN(KNF4E), and pHCP7_TN(KNF4E)_Opti. Lanes T and S represent total and soluble fraction of protein samples, respectively. Arrowhead (◀) indicates TN(KNF4E) band. (B) Tagatose conversion yield of pHCP_TN(KNF4E) and pHCP7_TN(KNF4E)_Opti.

4. Conclusion

In this study, we successfully developed a *C. glutamicum*-based whole-cell biocatalyst platform for the bioconversion of inexpensive fructose to high-value tagatose. In particular, for high-level gene expression of the synthesis gene, we developed a high-copy number plasmid (pHCP7) and achieved a much-improved conversion yield with this plasmid system. Also, using the codon-optimized tagatose 4-epimerase (*TN(KNF4E)*), we optimized the expression level to 33.9% of total proteins and achieved conversion yield as high as 21.7%. To the best of our knowledge, this is the first whole-cell biocatalyst for the bioconversion of fructose to tagatose with the highest recorded productivity among microbial production systems to date. As described in the introduction, this whole-cell biocatalyst has several advantages over previous systems such as the use of immobilized enzymes. Particularly, *C. glutamicum* has very rigid cell wall structure, so it is more tolerant to toxic environment and more suitable for repeated use. Previously, we successfully demonstrated the repeated use of *C. glutamicum*-based whole-cell biocatalyst for the conversion of L-Phenylalanine to *trans*-cinnamic acid [22]. Although we did not demonstrate here, the current system is also applied for the repeated use, which would allow more economic production and contribute greatly to the commercialization of high-value tagatose production. The bioconversion efficiency of current system can be further improved. In this work, we examined only two enzymes (*TN* and *TN(KNF4E)*) because few enzymes have been reported for the conversion of fructose to tagatose. The bioconversion efficiency can be improved by employing more active enzymes, so, in this regard, it is necessary to screen more potential enzyme or to engineer the existing enzymes [12]. In addition, it is highly critical to optimize reaction condition (temp, pH, salts *etc.*) in enzyme reaction. In this work, we examined one temperature (60°C) condition with corresponding metal ion ($NiSO_4$ for TN and $CoSO_4$

for TN(KNF4E)) for each enzyme, which were previously optimized [23,24]. However, the optimal condition for free enzyme reaction and whole cell bioconversion reaction may not be same, so we think it is necessary to examine more conditions to find optimal condition for whole cell bioconversion reaction.

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Authors' Contributions

EJJ designed the study, performed the experiments, analyzed the data and drafted the manuscript. EJC, YML, SBK participated in the execution of the experiments and data interpretation. KJJ initiated and supervised the study, and revised manuscript. All authors read and approved the final manuscript.

Ethical Statements

EJJ, SBK, and KJJ declare no direct and indirect conflict of interest that relate to this manuscript. YMJ and EJC are full-time employees of CJ Cheiljedang, however the funding sponsors had no role in the performance of the experiments and in the collection, analysis and interpretation of the data. Neither ethical approval required nor informed consent was required for this study.

Electronic Supplementary Material (ESM)

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