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Large Increase in *Leuconostoc citreum* KM20 Dextransucrase Activity Achieved by Changing the Strain/Inducer Combination in an *E. coli* Expression System

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A recombinant putative dextransucrase (DexT) was produced from *Leuconostoc citreum* KM20 as a 160 kDa protein, but its productivity was very low (264 U/l). For optimization, we examined enzyme activity in 7 *Escherichia coli* strains with inducer molecules such as lactose or IPTG. *E. coli* BL21-CodonPlus(DE3)-RIL exhibited the highest enzyme activity with lactose. Finally, DexT activity was remarkably increased by 12-fold under the optimized culture conditions of a cell density to start induction (OD_{600}) of 0.95, a lactose concentration of 7.5 mM, and an induction temperature of 17°C. These results may effectively apply to the heterologous expression of other large DexT genes.

Keywords: Dextransucrase, optimization, lactose, response surface methodology, *Leuconostoc citreum* KM20

Dextransucrase (DexT, E.C. 2.4.1.5), belonging to glycoside family 70 [5], catalyzes the polymerization of sucrose to dextran *via* a transglucosyl reaction. Dextran from *L. mesenteroides* NRRL-B-512F was the first commercialized biopolymer, with applications in medicine (blood plasma substitutes, iron dextan) [15], epichlorohydrin cross-linked molecular sieves (Sephadex) [6], and various oligosaccharides [9]. Furthermore, its strong transglucosylation reaction has been used for synthesizing new functional carbohydrate materials such as high value-added food additives or pharmaceuticals [15]. Because of its high industrial values, heterologous expression of DexT has been studied in *E. coli* [14], *Bacillus megaterium* [10], and *Lactococcus lactis* [13]. However, the lab-scale productivity of DexT (2–5,000 mU/ml) is insufficient for industrial applications [18]. It is important to combine *E. coli* host strains and inducers to enhance the activity of the enzyme. In general, isopropyl-1-thio-β-D-galactopyranoside (IPTG) and lactose are widely used as inducers for target protein activity in *E. coli*. IPTG is an inducer suitable only for small-scale fermentation because of its costs and high toxicity in cells. Conversely, lactose is not only cheaper than IPTG, but is also not toxic to humans and does not have any negative influence on cell growth. As a cost-effective inducer without harmful effects, lactose is a suitable replacement for IPTG [7]. Nevertheless, few laboratory studies have investigated lactose induction of heterogeneous protein activity in *E. coli* [19]. Furthermore, the process of enzyme expression has been optimized using a combination of factorial design and response surface methodology (RSM). RSM is an effective statistical tool that is widely applied in the optimization of fermentation processes. RSM comprises many reported designs such as the Box–Behnken design, Graeco–Latin design, and central composite design (CCD) [1].

Leuconostoc citreum KM20 is one of the most prevalent lactic acid bacteria in the Korean *kimchi* fermentation process [4]. A recent genome analysis of *L. citreum* KM20 revealed that the bacterium expresses 4 types of putative glucansucrase genes that are important for the flavor and taste of *kimchi* [8]. However, there is no report regarding its expression and product specificity. In this study, DexT

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activity was optimized under the best combination of *E. coli* BL21-CodonPlus(DE3)-RIL with lactose and the biochemical properties of DexT were investigated.

Materials and Methods

Gene Cloning and Investigation of Variable Induction Conditions

The gene encoding DexT was amplified by PCR using the genomic DNA of *L. citreum* KM20 as a template. The primer sequences used for gene cloning were based on the DNA sequence of *L. citreum* DexT (GenBank Accession No. ACA83218). The forward (5'-AAACCATGGAAAACGGCGAAGTGTGTCAGCG-3') and reverse primers (5'-TTTCTCGAGGTATGTTATTTTTTTCATTTCACC-3') were designed to introduce *Nco*I and *Xho*I restriction sites (underlined), respectively. The PCR product was subcloned into the pET23d (+) plasmid (Novagen, Madison, WI, USA), digested with the same restriction enzymes, and then transformed into *E. coli* BL21-CodonPlus(DE3)-RIL.

DexT production was investigated with the following host strains harboring the expression vector for DexT: *E. coli* BL21 (DE3), *E. coli* BL21-CodonPlus(DE3)-RP, *E. coli* BL21-CodonPlus(DE3)-RIL, *E. coli* Origami (DE3), *E. coli* Tuner (DE3), and *E. coli* C41 (DE3). DexT was generated in *E. coli* BL21 (DE3)-CodonPlus RIL to optimize the induction conditions. The recombinant *E. coli* cells for protein expression were cultivated by shaking at 180 rpm in a 1 L flask containing 250 ml of LB medium at 37°C with 50 μg/ml ampicillin until the OD_{600} equaled 0.2, 0.5, 1.0, 1.5, or 1.8. IPTG or lactose was added at a concentration of 0.2 mM to induce enzyme expression, and the culture was grown at 20°C for 16 h. The effects of induction temperature (6, 10, 20, or 23°C) and inducer concentration (0.6−12.4 mM) were experimentally investigated.

Optimization Procedure and Experimental Design

A 3-level Box−Behnken design with 3 factors was applied to the DexT optimization procedure using Design Expert 8.0 software, including the 5 replicates at the central point, which were utilized in the fitting of a second-order response surface. Temperature (X_1), lactose concentration (X_2), and inoculum cell density to start induction (X_3) were utilized in the preparation of each of the 17 cultivation conditions summarized in Table 1. Optimization was conducted using a desirability function to determine the effects of X_1, X_2, and X_3 on enzyme activity. A total of 17 experiments were conducted to determine the 10 coefficients of the model as follows:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 \quad (1)$$

where Y is the predicted response; β_0 is the intercept; β_1, β_2, and β_3 are linear coefficients; β_{11}, β_{22}, and β_{33} are squared coefficients; and β_{12}, β_{13}, and β_{23} are interaction coefficients. Once an appropriate model was obtained, this model was used to determine the predicted optimum conditions for the process.

Purification of DexT

Wet cells collected by centrifugation at 4,000 ×*g* for 5 min were suspended in 20 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl (buffer A). The suspended cells were sonicated and centrifuged at 10,000 ×*g* for 20 min, and the supernatant was used as a crude enzyme solution. Purification of the DexT was performed with Ni-chelating Sepharose resin equilibrated with buffer A. The

Table 1. Central composite design of 3 independent variables with experimental and predicted enzyme activity responses.

Run	Coded level			Actual level			Activity (U/ml)	
	X_1	X_2	X_3	Temp. (°C)	Lactose (mM)	Cell OD	Actual	Predicted
1	−1	−1	−1	10.0	3.0	0.5	1.650	1.427
2	1	−1	−1	20.0	3.0	0.5	2.246	2.267
3	−1	1	−1	10.0	10.0	0.5	1.471	1.108
4	1	1	−1	20.0	10.0	0.5	2.889	2.701
5	−1	−1	1	10.0	3.0	1.5	1.812	1.856
6	1	−1	1	20.0	3.0	1.5	1.577	1.795
7	−1	1	1	10.0	10.0	1.5	1.867	1.702
8	1	1	1	20.0	10.0	1.5	2.316	2.394
9	−1.682	0	0	6.6	6.5	1.0	0.201	0.551
10	1.682	0	0	23.4	6.5	1.0	1.985	1.839
11	0	−1.682	0	15.0	0.6	1.0	2.295	2.190
12	0	1.682	0	15.0	12.4	1.0	2.115	2.425
13	0	0	−1.682	15.0	6.5	0.2	1.970	2.348
14	0	0	1.682	15.0	6.5	1.8	2.624	2.451
15	0	0	0	15.0	6.5	1.0	2.974	2.975
16	0	0	0	15.0	6.5	1.0	2.857	2.975
17	0	0	0	15.0	6.5	1.0	2.578	2.975
18	0	0	0	15.0	6.5	1.0	3.280	2.975
19	0	0	0	15.0	6.5	1.0	3.164	2.975
20	0	0	0	15.0	6.5	1.0	3.031	2.975

$Y = -5.80279 + 0.85164 \times X_1 + 0.085513 \times X_2 + 2.88553 \times X_3 + 0.010757 \times X_1 \times X_2 - 0.090000 \times X_1 \times X_3 + 0.23571 \times X_2 \times X_3 - 0.025166 \times X_1^2 - 0.019266 \times X_2^2 - 0.81392 \times X_3^2$

column was washed with buffer A containing 20 mM imidazole, followed by elution with a linear gradient of 20–300 mM imidazole in buffer A. The fraction containing the purified DexT was dialyzed against 20 mM sodium acetate buffer (NaAcB, pH 5.5) and concentrated by ultrafiltration using an Amicon Ultra 10,000 Da molecular weight cut-off (Millipore, Billerica, MA, USA). Approximately 17 mg of purified protein was obtained from 1,000 ml of the culture medium. Each protein concentration was determined by the Bradford's method [2] with bovine serum albumin as the standard, and enzyme purity was measured by SDS-PAGE.

Biochemical Properties of DexT

The enzyme activity was examined with 1% (w/v) sucrose in 40 mM NaAcB (pH 5.5) in the presence of 0.1 mM $CaCl_2$ at 30°C. The amount of fructose liberated from sucrose was measured using the 3,5-dinitrosalicylic acid assay [11]. One unit of activity was defined as the amount of enzyme that released 1 μmol reducing power per minute under the assay conditions. To determine the optimal pH, DexT (12 μg/ml) was incubated at 30°C (pH 2–12) [3]. To assess the pH stability, the enzyme (30 μg/ml) was kept at 4°C for 20 h in 40 mM Britton–Robinson buffer (pH 2–12), and the residual enzyme activity was examined with 1% sucrose in 40 mM NaAcB (pH 5.5) in the presence of 0.1 mM $CaCl_2$ at 30°C. To determine the optimal temperature, the enzyme (12 μg/ml) was incubated at 20–70°C for 15 min in 40 mM NaAcB (pH 5.5) with 1% sucrose. To determine the thermal stability, the enzyme (12 μg/ml) was kept at 20–70°C for 1 h in 40 mM NaAcB (pH 5.5), and the residual enzyme activity was examined at 30°C.

Product Analysis

The products of sucrose hydrolysis and dextran formation were analyzed by thin layer chromatography (TLC) by using a silica gel 60 plate (Merck, Darmstadt, Germany) in a solvent system consisting of nitromethane, 1-propanol, and water at a ratio of 4:10:3 (v/v/v), with isomaltooligosaccharide standards, and carbohydrates were visualized on the TLC plate using the method described previously by Su and Robyt [16]. For the structure determination of produced glucan, NMR analysis was performed as follows: 40 mg of each glucan was exchanged 3 times with DMSO (Sigma), dissolved in 0.5 ml of DMSO, and transferred to a 20 mm NMR microtube. ^{1}H- and ^{13}C-NMR spectra were measured at 500 and 125 MHz, respectively, using sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 as an internal standard.

RESULTS AND DISCUSSION

Optimization of Cultivation Conditions for DexT Activity

A 4,488 bp gene encoding a putative DexT from *L. citreum* KM20 was expressed in the periplasmic space of *E. coli*. However, IPTG-induced DexT activity was very low (0.264 U/ml) at 37°C. To evaluate the factors affecting the activity of DexT, the enzyme production process was investigated using 7 expression host strains harboring the same expression vector at 25°C. The result revealed that the DexT activity has 3 different patterns depending on whether IPTG or lactose is used as the inducer (Table 2). In the first group, 3 *E. coli* strains, Origami (DE3), Tuner (DE3), and C41 (DE3), exhibited high IPTG-induced DexT activity. In the second induction pattern, exhibited by *E. coli* BL21 (DE3), DexT activity was similar irrespective of the type of inducer. In the third group, consisting of *E. coli* BL21-CodonPlus(DE3)-RIL, BL21-CodonPlus(DE3)-RP, and Rosetta (DE3), lactose-induced DexT activity was 1.35–3.5-fold higher than that of IPTG. The pET expression vectors with strong T7 RNA polymerase promoter activity are known to cause the production of insoluble inclusion bodies in *E. coli* cells [7]. The rapid expression or over-expression of DexT induced by IPTG may result in the production of toxic inclusion bodies in cells. Conversely, the high DexT activity appears attributable to the mild expression rate and increased cell growth caused by lactose induction. Similar to the *E. coli* BL21-CodonPlus(DE3)-RIL and RP strains, the additional copies of specific tRNA genes (*argU*, *ileY*, and *leuW*) in rare codons in *E. coli* simulate the production of high-molecular-weight DexT. In fact, DexT contained 0.93% of rare codons in the total ORF. These combinations with lactose and selected host cells may enhance the production of other high-molecular-weight DexTs in *E. coli*. Finally, *E. coli* BL21-CodonPlus(DE3)-RIL strains that exhibited the highest production with lactose were selected for further study under different cultivation conditions.

RSM was used to study the interaction of these variables in relation to DexT production. From the preliminary

Table 2. *E. coli* host-dependent DexT production induced by lactose or IPTG.

E. coli strains	Dextransucrase activity (U/ml)		Ratio of lactose/IPTG induction
	Lactose induction	IPTG induction	
Origami (DE3)	0.44 (± 0.02)	1.12 (± 0.02)	0.39
Tuner (DE3)	0.71 (± 0.03)	1.69 (± 0.04)	0.42
C41 (DE3)	0.45 (± 0.02)	0.94 (± 0.03)	0.48
BL21 (DE3)	0.23 (± 0.01)	0.24 (± 0.01)	0.95
BL21-CodonPlus(DE3)-RP	2.05 (± 0.07)	1.52 (± 0.04)	1.35
BL21-CodonPlus(DE3)-RIL	2.55 (± 0.05)	1.57 (± 0.03)	1.62
Rosetta (DE3)	2.38 (± 0.08)	0.68 (± 0.03)	3.5

E. coli transformants were grown in 100 ml of LB medium containing 50 μg/ml ampicillin in a 250 ml flask at 37°C until the OD_{600} was 0.5, and the proteins were induced with 0.2 mM IPTG or lactose.

Table 3. Analysis of variance for the response surface quadratic model.

	Sum of square	Degree of freedom	Mean square	F-value	P-value
Regression	9.16	9	1.02	9.90	0.0007
Residual	1.03	10	0.10		
Lack of fit	0.72	5	0.14	2.39	0.1806
Pure error	0.30	5	0.061		
Corrected total	10.18	19			

$R^2 = 0.8991$; adjusted-$R^2 = 0.8083$; CV = 14.28%

experiments, the 3 important factors of induction temperature (6.6–23.4°C), lactose concentration (0.6–12.4 mM), and the inoculum cell density to start induction (0.2–1.8) were selected to optimize the DexT cultivation conditions for 16 h. The CCD design with DexT activity is shown in Table 3. As ascertained from the central points of the corresponding contour plots (Fig. 1), the 3 variables that proved optimal for DexT activity included a cell density (OD_{600}) of 0.95, a temperature of 17°C, and a lactose concentration of 7.5 mM. DexT activity was measured to be 3,053 U/l. The results indicated that depending on the induction conditions, DexT activity could be expressed in terms of the following regression equation:

$$Y = -5.80279 + 0.85164 \times X_1 + 0.085513 \times X_2 + 2.88553 \times X_3 + 0.010757 \times X_1 \times X_2 - 0.090000 \times X_1 \times X_3 + 0.23571 \times X_2 \times X_3 - 0.025166 \times X_1^2 - 0.019266 \times X_2^2 - 0.81392 \times X_3^2$$

Table 3 lists the results of the second-order response surface model fitting in the model equation fitting in the form of ANOVA. To test the fit of the model equation, if the regression-based determination coefficient R^2 was close to 1, the model would better explain the variability of experimental values to the predicted values. The model had a high determination coefficient ($R^2 = 0.8991$), which explained 89% of the variability in the response (Table 3). DexT is currently being mass-produced under optimized culture conditions using, a 3 L bench-top-scale laboratory fermentor (working volume, 3 L) under the above-described optimized conditions. DexT activity was 3,319 ± 27 U/l, which is consistent with the predicted value of 3,053 U/l. These findings led us to conclude that DexT activity can be efficiently induced in *E. coli* by lactose.

Purification and Biochemical Characterization of DexT from *L. citreum* KM20

The expressed enzyme exhibited high identity (86%) to the *L. mesenteroides* NRRL B-1299-originated DsrA [12]. DexT also possessed well-known structures such as conserved regions I–IV, a glucan-binding domain, and YG repeats commonly found in other DexTs [17]. In contrast to DsrA, DexT contained a signal peptide. The enzyme was purified with a final purification of 20-fold, a yield of 45% (17 mg), and a specific activity of 98 U/mg. The final purified enzyme exhibited a single band with a molecular mass of approximately 160 kDa in SDS-PAGE (data not shown), consistent with the calculated value of 167 kDa based on an amino acid length of 1,495. DexT was stable until the temperature reached 35°C, and the optimum temperature for its activity was 30°C. The enzyme was stable in the pH range of 4.0–7.5, and the optimum pH was approximately pH 5.5 (Fig. 2). These data agreed well with the literature findings, in which DexT activity in the *L. citreum* KM20 strain was optimized in the reaction temperature and pH ranges of 28–35°C and 5.0–6.6, respectively [8].

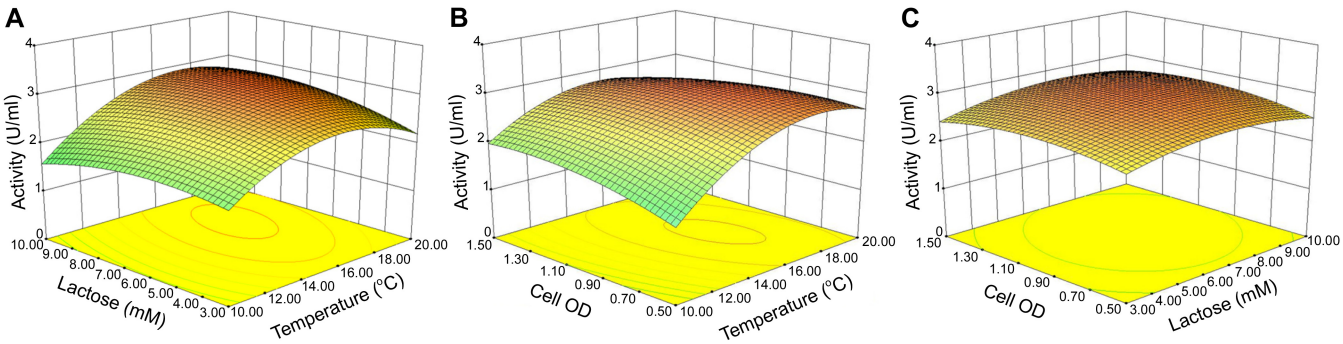

Fig. 1. Response surface and contour plots of DexT activity induced by lactose revealing mutual interactions between (**A**) temperature and lactose, (**B**) temperature and cell OD_{600}, and (**C**) lactose and cell OD_{600}.
Other parameters, excluding the 2 parameters in each figure, are set at the level of 0 in coded units.

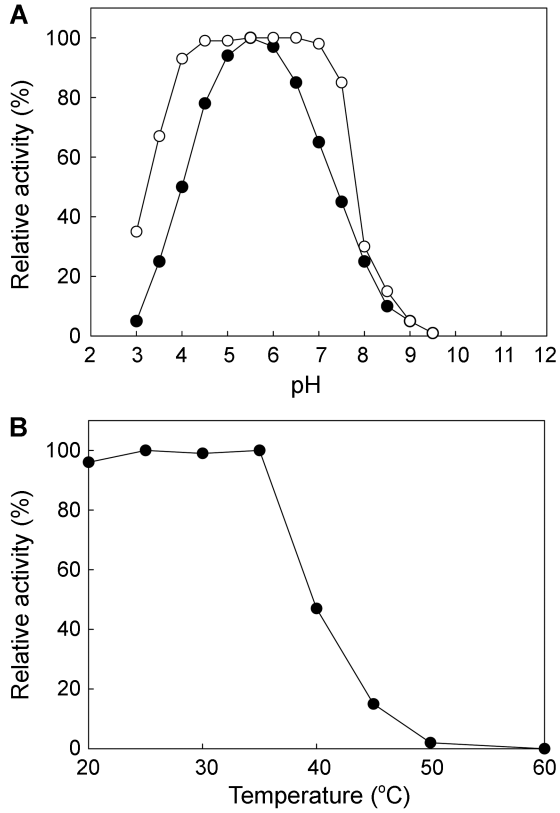

Fig. 2. Optimum pH (**A**) and temperature (**B**) for activity and stability of DexT.
Activity profile for various pHs (●): the enzyme (12 μg/ml) and 1% (w/v) sucrose were incubated in Britton–Robinson buffer at 30°C for 20 min; pH stability profile (○): the enzyme (30 μg/ml) was incubated in Britton–Robinson buffer at 4°C for 20 h; thermal stability profile: the enzyme (12 μg/ml) was maintained at 20–70°C for 1 h in 40 mM NaAcB (pH 5.5). The residual enzyme activity was examined with 1% sucrose in 40 mM NaAcB (pH 5.5) containing 0.1 mM $CaCl_2$ at 30°C.

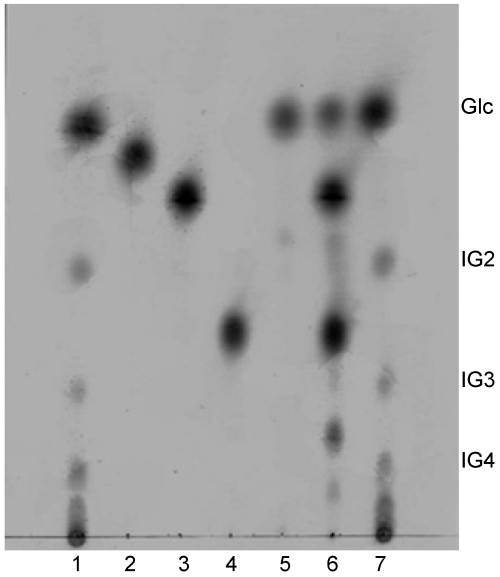

Fig. 3. Characterization of the products synthesized by DexT.
A reaction mixture containing DexT (12 μg/ml) and 50 mM sucrose with/without 75 mM maltose was incubated in 40 mM NaAcB (pH 5.5) at 30°C for 8 h. After heating for 5 min to inactivate the enzyme, the reaction products were analyzed by thin-layer chromatography using nitromethane/1-propanol/H_2O in a volume ratio of 4:10:3. Lane 1, isomaltooligosaccharide standards (isomaltose to isomaltoheptaose); lane 2, sucrose; lane 3, maltose; lane 4, panose; lane 5, reaction aliquots of DexT with 50 mM sucrose; lane 6, reaction aliquots with 50 mM sucrose and 75 mM maltose; lane 7, isomaltooligosaccharide standards (isomaltose to isomaltoheptaose).

The reaction products or acceptor products formed by DexT were compared with those produced by DsrA [12]. In the presence of sucrose, DexT catalyzed the synthesis of insoluble dextran. The ^{13}C-NMR analysis of formed glucan indicated that DexT only synthesized polymers containing α-1,6-glucosidic linkages (data not shown), which is a different type of glucan from the insoluble dextran containing 5% α-1,3-branch linkages that was produced by DsrA. This difference in branching linkage formation in dextran was caused by molecular levels between DexT and DsrA. In the presence of maltose as the acceptor molecule, DexT synthesized a series of isomaltodextrin analogs very similar to those produced by DsrA. These products had a degree of polymerization from the trisaccharide-panose to homologous saccharides harboring 2–3 units of glucose (Fig. 3). The biochemical properties suggest that DexT has a very high regioselectivity to only α-1,6-glucosidic linkages without any branched linkages, and that it can be applied to the production of new functional carbohydrate materials such as high value-added food additives or pharmaceuticals [15].

In the present study, we used RSM to optimize DexT activity and characterized its biochemical properties for the first time. DexT activity exhibited a remarkable 12-fold increase under cultivation conditions involving a cell density (OD_{600}) of 0.95, a lactose concentration of 7.5 mM, and an induction temperature of 17°C. Consequently, *E. coli* BL21-CodonPlus(DE3)-RIL and lactose proved to be an efficient host strain and inducer, respectively, for DexT activity by simultaneously enhancing both the biomass and the enzyme production. These cultivation conditions may effectively apply to the heterologous expression of other large DexT genes. For industrial application, various DexT-stimulated transglucosylation productions are currently in progress with several healthcare materials from natural sources.

Acknowledgments

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