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NOTE

Development of a High-Throughput Screening Method for Recombinant *Escherichia coli* with Intracellular Dextransucrase Activity

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To efficiently engineer intracellular dextransucrase (DSase) expression in *Escherichia coli*, a high-throughput screening method was developed based on the polymer-forming activity of the enzyme. Recombinant *E. coli* containing the *Leuconostoc citreum* DSase (LcDS) gene was grown on Luria-Bertani agar plates, containing 2% sucrose, at 37°C for 8 h. The plates were then evenly overlaid with 0.6% soft agar, containing 1.2 mg/ml D-cycloserine, and incubated at 30°C to allow gradual cell disruption until a dextran polymer grew through the overlaid layer. A significant correlation between dextran size and enzyme activity was established and applied for screening truncated mutants with LcDS activity.

***Keywords*:** high-throughput screening, D-cycloserine, dextransucrase, recombinant *E. coli*

Lactic acid bacteria (LAB) have traditionally been associated with the fermentation of foods world-wide and such characteristics have gained them a growing importance in the food industry. *Leuconostoc mesenteroides* is a well-known LAB that produces dextransucrases (DSases; EC 2.4.1.5), which can transfer the glucose residue of sucrose to sugar acceptors principally by forming α-(1, 6)-glycosidic linkages (Monchois *et al*., 1999). Dextran is a high-molecular weight polysaccharide synthesized by DSases. Because of its physicochemical characteristics, dextran is often used in the pharmaceutical field, such as blood plasma substitutes, iron-carriers, and anticoagulants (Naessens *et al*., 2005). In addition, it is possible to develop new carbohydrate materials by exploiting the transglycosylation activity of DSases (Remaud-Simeon *et al*., 2000; Iliev *et al*., 2008). Over the past few decades, a number of DSase genes have been cloned and characterized, mainly from *L. mesenteroides.* In a previous study, we successfully cloned and overexpressed the *L. citreum* HJ-P4 DSase (LcDS; Eom *et al*., 2007) gene in *E. coli* by low-temperature induction (Yi *et al.*, 2009). The LcDS gene consists of 4,431 nucleotides encoding 1,477 amino acid residues, sharing 63-98% amino acid sequence identity with other known *L. mesenteroides* DSase genes. However, it is still difficult to use DSases for industrial purposes, due to their large molecular mass and extremely low expression level in any microbial system, including *E. coli*. In order to overcome these limitations, highly active DSases with reduced molecular weight should be developed to maximize enzyme production. Recently developed protein engineering technologies, including directed evolution, are promising alternatives to reach these goals. However, the most crucial step in directed evolution experiments is the development of an efficient procedure for screening large libraries. In contrast to glycosylhydrolases, it is extremely tricky to detect or assay for glycosyltransferase activity, due to their complex reaction mechanisms and subtle absorbance changes during sugar-transferring reactions. Thus, it is highly desirable to develop more powerful high-throughput screening (HTS) procedures to facilitate *in vitro* evolution work for glycosyltransferases with polymerization activities. In the present study, therefore, we tried to develop a rapid and economical HTS tool for the direct screening of high-molecular weight polymer-forming, intracellular enzyme activity in recombinant *E. coli* using DSase as a model system.

DSase genes are expressed and transported to the medium via the secretory pathways in LABs. Extracellular DSases subsequently react with a sucrose substrate in the medium, and synthesize a dextran polymer around the bacterial colony. When *L. citreum* HJ-P4 was grown on PE (Phenylethanol medium, Difco Laboratories Inc., USA) or PES (PE with 2% sucrose) agar plates for 24 h, the glassy and viscous dextran polymer was only produced by the cells grown on PES (Figs. 1A and B). Accordingly, DSase-producing LABs can easily be screened on sucrose-containing medium on the basis of their dextran-forming activity. However, foreign enzyme genes are commonly expressed inside *E. coli* cells. As a result, intracellular enzymes cannot interact with the extracellular sucrose substrate until the cell is disrupted. The most popular mechanical methods for cell disruption, such as ultrasonication, work most efficiently when there is a sufficient amount of liquid cell culture, thus making the HTS procedure with *E. coli* complicated and indirect. Here, a chemical cell disruption method was investigated as an alternative to the physical methods.

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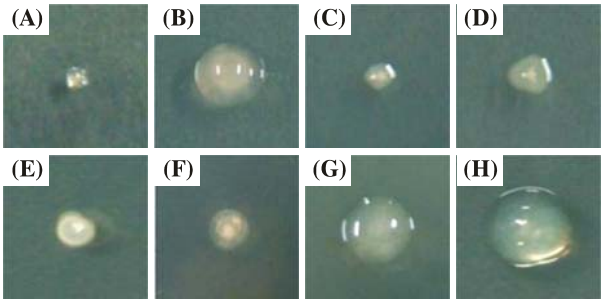

Fig. 1. Detection of dextran polymer synthesized by *L. citreum* dextransucrase (LcDS) activity. *L. citreum* HJ-P4 was grown on (A) Phenylethanol (PE) medium and (B) PES (PE with 2% sucrose) medium, respectively, for 24 h. Recombinant *E. coli* harboring pHCLcDS was grown on (C) LBA (Luria-Bertani medium with 100 μg/ml ampicillin), (D) LBAS (LBA with sucrose), (E) LBA overlaid by 0.6% soft agar with 1.2 mg/ml D-cycloserine, (F) LBA overlaid by soft agar with D-cycloserine and sucrose, (G) LBAS overlaid by soft agar with D-cycloserine, and (H) LBAS overlaid by soft agar with D-cycloserine and sucrose, respectively.

For example, the antibiotic D-cycloserine has been known to block bacterial cell wall synthesis through the inhibition of both D-alanine racemase and synthetase (Lambert and Neuhaus,

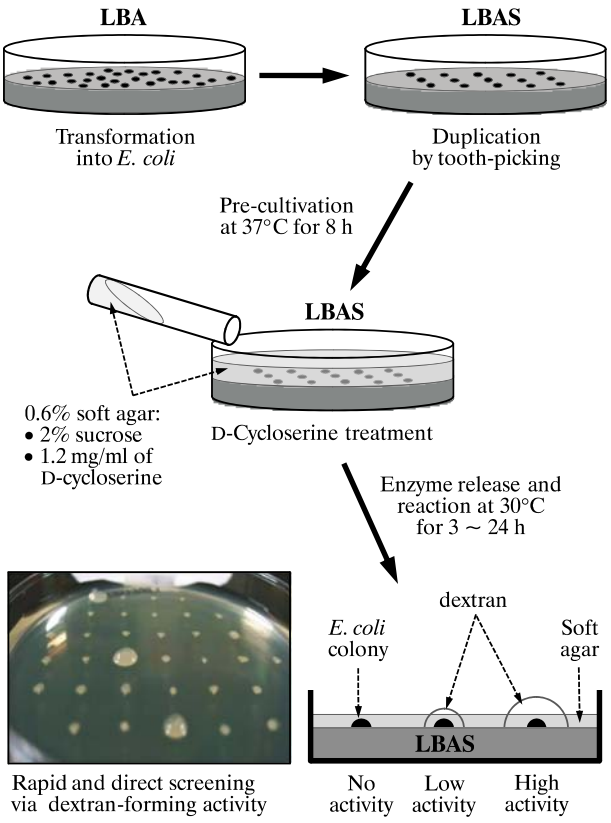

Fig. 2. Strategy describing the high-throughput dextransucrase activity screening (HTDS) method for recombinant *E. coli* harboring a dextran-synthesizing enzyme gene. LBA plate contains Luria-Bertani medium with 1.5% agar and 100 μg/ml ampicillin, while LBAS contains 2% sucrose as a substrate.

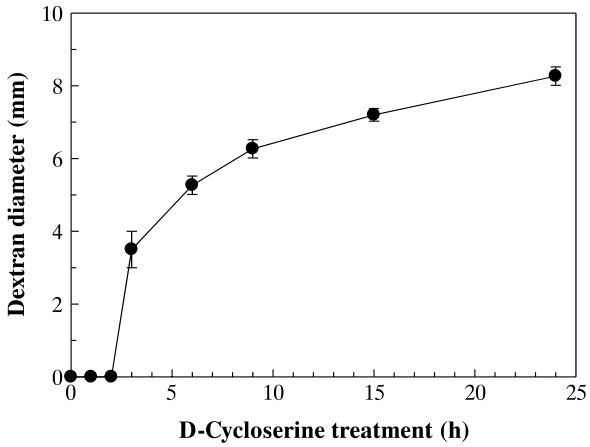

Fig. 3. Time course study between apparent dextran diameter and incubation time after D-cycloserine treatment. Five randomly chosen recombinant *E. coli* clones harboring pHCLcDS were tooth-picked onto a LBAS plate and pre-cultured for 8 h. After D-cycloserine treatment, the change in average dextran diameter was monitored for 24 h.

1972), which can result in the gradual disruption of cells during cell growth. Kim *et al*. (1992) used this method for the detection of intracellular amylolytic enzyme activity from *E. coli*, followed by iodine staining on starch-containing agar plates. In the same way, recombinant *E. coli* MC1061 transformants harboring pHCLcDS (Yi *et al.*, 2009) were tooth-picked onto LBA (Luria-Bertani medium with 100 μg/ml ampicillin) or LBAS (LBA with 2% sucrose) plates containing 1.5% agar. When each colony was pre-cultured at 37°C for 8 h and their diameter reached 1.5-2.0 mm, approximately 10 ml of soft agar (LB with 0.6% agar and 1.2 mg/ml D-cycloserine), with or without 2% sucrose substrate, was carefully overlaid on top of the colonies. After solidification, the agar plates were incubated further at 30°C for the enzyme reaction to take place. As shown in Figs. 1C and D, no polymer formation was observed on either the LBA or LBAS plates that had not been treated with D-cycloserine. Even though D-cycloserine treatment was applied, no detectable dextran was formed from colonies grown on LBA plates (Fig. 1E). The other cycloserine-treated colonies, which used sucrose as a substrate, could form detectable dextrans, but their apparent diameters varied significantly. The largest dextran polymer was synthesized when both the medium and soft agar was supplemented with 2% sucrose substrate (Fig. 1H). In contrast, even though soft agar containing 2% sucrose was overlaid, a barely detectable amount of polymer was found on the LBA plate (Fig. 1F). With regard to these results, it was suggested that D-cycloserine treatment could facilitate the slow and steady release of high-molecular weight intracellular LcDS into the medium. Interestingly, the dextran synthesized by extracellular LcDS was continuously accumulated over the colony and finally grew up through the micropores of the soft agar layer. Although sucrose in the LBAS medium could play a critical role in dextran synthesis, the substrate should be present in both the medium and in the soft agar for maximum results. The high-throughput dextransucrase activity screening (HTDS) method

Table 1. Comparison of *L. citreum* DSase and its N-terminally truncated mutant enzymes expressed in recombinant *E. coli*

Enzyme[a]	DSase activity[b] (U/ml)	Dextran diameter[c] (mm)	Detectable period[d] (h)
LcDS	5.0	8.5	3.0
LcDSΔN42	2.6	6.5	12.0
LcDSΔN194	5.9	8.3	3.0
LcDSΔN199	3.4	7.5	6.0
LcDSΔN332	2.3	6.5	8.0

[a] Deleted amino acid residues are indicated in each enzyme name along with its corresponding number
[b] DSase activity from 1 ml of *E. coli* culture broth was determined by DNS assay
[c] Diameter of dextran polymer was compared at 15 h after D-cycloserine treatment
[d] Time period for the first appearance of detectable dextran polymer

established here is schematically summarized in Fig. 2.

For the time-course study, 5 randomly chosen *E. coli* transformants harboring pHCLcDS were incubated at 30°C and the growth of dextran monitored for 24 h following D-cycloserine treatment. The production of the dextran polymer was first detected after 3 h incubation, with its diameter increasing rapidly up to 9 h, followed by a significant decrease in growth rate from 9 to 24 h after treatment (Fig. 3). As a result, the growth of dextran, as assessed by the change in diameter during a limited time period, can be semi-quantitatively correlated to the DSase activity in the recombinant *E. coli*. Interestingly, it was observed that the dextran polymer could grow up through the micropores within the soft agar layer and that the amount of polymer placed over the surface is significantly correlated to the enzyme activity. Even though only DSase was tested as a model system in the present work, these results strongly suggest that this HTDS method can be applied to the direct detection of various other types of visible polymeric materials synthesized by glycosyltransferase reactions.

There has been an increased interest into the mechanisms of various glucansucrases due to their versatile transferring activities in the production of useful polymeric materials (Robyt *et al*., 2008). Moulis *et al*. (2006) utilized typical site-directed mutagenesis techniques to elucidate the reaction mechanism of *L. mesenteroides* DSase, and Monchois *et al*. (1998) attempted to understand the roles of the enzyme carboxy-terminal domain using PCR-based site-specific truncation. In order to improve enzymatic properties, random mutagenesis approaches, including directed evolution, are more powerful than traditional site-specific mutagenesis techniques based on rational design. However, very few adequate tools have been prepared for the HTS screening of randomly engineered glycosyltransferases. We attempted to apply this HTDS method in practice for the direct activity screening of LcDS mutant libraries generated by serial truncation. The LcDS gene encoding 1,477 amino acids, was degraded in one direction, starting from the amino-terminus, by exonuclease III treatment (Erase-A-Base system; Promega Co., USA). The resulting truncated mutant library was transformed into *E. coli* and directly screened using the dextran-forming activity on LBAS agar plates as a readout. As a result, 107 dextran-forming clones were identified as active clones from a total of 4,000 screened transformants. Among them, 4 active mutant clones, LcDSΔN42 (42 amino acid residues deleted from N-terminus), LcDSΔN194, LcDSΔN199, and LcDSΔN332, were chosen and verified by DNA sequencing analyses. Wild-type LcDS and truncated LcDS genes were expressed in *E. coli* MC1061 and their enzymatic activity compared on the basis of the DNS reducing sugar assay (Yi *et al*., 2009) using sucrose as a substrate (Table 1). As expected, there was a significant correlation between dextran diameter and DSase activity for most of the clones. For example, the diameters of the highly active LcDS (wild-type) and LcDSΔN194 clones were clearly larger than those of the less active clones, LcDSΔN42 and LcDSΔN332. Moreover, the time required for the first dextran polymer to appear was closely related to the enzyme activity. While LcDSΔN42 and LcDSΔN332 shared similar factors, in terms of activity and dextran size, interestingly, the time periods before they were detected were quite different from each other. This indicates that some mutants with extremely weak activity can be detected by using an alternative, time-dependent screening as well. In order to obtain more detailed information about enzymatic characteristics, further investigations into the remaining truncated clones are currently in progress.

Recently, some additional carbohydrate-modifying enzymes with transglycosylation activity in bifidobacteria have been used for the production of prebiotic oligosaccharides (van den Broek *et al*., 2008). Therefore, more intensive engineering of these versatile glycosyltransferases is essential for the industrial synthesis of carbohydrates. To date, a small number of HTS methodologies for the evolution of glycosyltransferases have been developed and successfully applied to enzymes for the synthesis of oligosaccharides (Aharoni *et al*., 2006; Ben-David *et al*., 2008). Nevertheless, alternative methods are required, as most HTS tools are based on the synthesis of oligosaccharides rather than polysaccharides. Additionally, most polymerization reactions have been detected indirectly using simultaneous hydrolysis reactions.

In conclusion, the high-throughput DSase activity screening (HTDS) method described in this study has some distinguishable advantages over previous HTS tools by virtue of its capacity to synthesize oligosaccharides, even though it seems to be a less sensitive and less quantitative method. The method can be utilized to detect direct polymerization activity, but not alternative hydrolysis activity or indirect chemical reactions. In addition, chemicals or equipment are not required for the detection procedure. Therefore, this HTDS tool is expected to facilitate protein engineering of glycosyltransferases for the synthesis of not only dextrans, but also a variety of polymeric saccharides.

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