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A Universal Screening Assay for Glycosynthases: Directed Evolution of Glycosynthase XynB2(E335G) Suggests a General Path to Enhance Activity

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SUMMARY

Glycosynthases are catalytic mutants of mainly retaining glycoside hydrolases that catalyze the synthesis of oligosaccharides from their corresponding glycosyl-fluoride donors and suitable acceptors. Here we describe the development of a general, high-throughput screening procedure for glycosynthase activity, which is based on the release of hydrofluoric acid, a by-product of all glycosynthase reactions. This assay is sensitive, does not require the synthesis of special chromophoric or modified substrates, and, most importantly, is applicable for all glycosynthases. We used this screening procedure on error-prone PCR libraries to isolate improved glycosynthase variants of XynB2(E335G) glycosynthase, a family 52 β-xylosidase from *Geobacillus stearothermophilus*. The improved variants exhibited higher K_M values toward the acceptor and the donor, suggesting that enzyme-product release is rate determining for k_{cat}.

INTRODUCTION

One of the most striking characteristics of carbohydrates is their extreme diversity in stereochemistry and the enormous number of their possible structural combinations. For example, there are more than 10^{12} possible oligosaccharide isomers for a reducing hexasaccharide (Laine, 1994). This feature enables carbohydrates not only to serve in structural and storage roles, but also to function as highly specific molecular recognition elements that mediate biological processes (Varki, 1993). This diversity, however, poses a significant challenge for the synthesis of oligosaccharides. The main difficulties stem from the need to control both the regio- and stereochemistry of bond formation in order to obtain well-defined products. To overcome these hurdles, protecting group manipulation sequences are required, which hamper the overall yield. In addition, there are no general reaction conditions for all oligosaccharides; therefore, each synthesis route has to be tailored specifically, thus requiring considerable research efforts (Paulsen, 1982). For these reasons, enzymatic synthesis of oligosaccharides provides an attractive alternative to the classical chemical methods. The specificity of enzymes enables complete control over newly generated anomeric centers, thus eliminating the need for extensive protection-deprotection operations. Moreover, the reactions can be performed under mild conditions (Palcic, 1999; Sears and Wong, 2001). Enzymes for organic synthesis are widely used in a variety of applications (Koeller and Wong, 2001; Schmid et al., 2001). The most frequently used enzymes are lipases, which often can function well in organic media, and their substrates/products are soluble in organic solvents. Under these conditions, it is possible to reverse their natural hydrolytic activity toward synthesis (Davis and Boyer, 2001). Unfortunately, this approach cannot be utilized with glycoside hydrolases due to the poor solubility of the substrates and the limited functionality of these enzymes in organic solvents. Therefore, there are only a few examples in which these enzymes were found useful for such syntheses (Lang et al., 2006; Yoon and McKenzie, 2005).

In nature, synthesis of oligo- and polysaccharides is performed by glycosyltransferases. Use of these enzymes for carbohydrate synthesis, although widespread (Blixt and Razi, 2006), is currently rather limited, due to the need for expensive sugar nucleotide donors and the high specificity of glycosyltransferases, which often restricts the use of nonnatural donors and acceptors (Aharoni et al., 2006; Hoffmeister et al., 2002; Williams et al., 2007; Williams and Withers, 2002). Another enzymatic approach utilizes the natural transglycosylation capability of some retaining glycoside hydrolases. The main drawback of this strategy is that the formed product is necessarily a substrate for the enzyme, and, therefore, low yields are obtained (Feng et al., 2005; Sears and Wong, 2001). Several years ago, Withers and coworkers introduced a third enzymatic approach that relies on a catalytic mutant glycosidase, coined glycosynthase (Hancock et al., 2006). In these enzymes, mostly retaining glycosidases, the acidic catalytic nucleophile is replaced by a small, nonnucleophilic residue, resulting in practically inactive hydrolase. However, in the presence of glycosyl-fluorides of the opposite anomeric configuration (to that of their natural substrates), these enzymes completely change their nature, and transfer the activated sugars to suitable acceptors (Figure 1). Because the hydrolytic activity of the glycosidase is abolished, the glycosynthase technology benefits from high product yields. In addition, the use of robust and available enzymes that can accommodate nonnatural substrates, and the use of inexpensive glycosyl-fluoride donors, made the glycosynthase approach an attractive option for oligosaccharide synthesis.

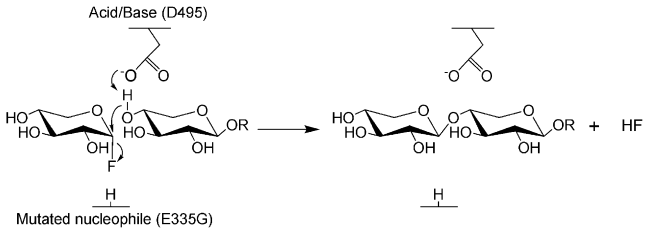

Figure 1. The Proposed Mechanism of Glycosynthase XynB2(E335G)

Since the report of the first glycosynthase, the glycoside hydrolase family 1 *Agrobacterium* sp. β-glucosidase (Mackenzie et al., 1998), considerable research has been undertaken in this field. To date, glycosynthases have been generated from 11 more glycoside hydrolase families (Ben-David et al., 2007; Hancock et al., 2006; Perugino et al., 2004; Umekawa et al., 2008), producing products ranging in length from di- and trisaccharides to polysaccharides (Faijes and Planas, 2007). However, reactions catalyzed by glycosynthases are rather slow in comparison to their glycosidase activity, requiring large amounts of enzymes and extended incubation times (Kim et al., 2004; Mayer et al., 2001). Moreover, not all retaining glycoside hydrolases can function as glycosynthase, and the structural elements required for effective glycosynthesis remain unclear. For these reasons, directed evolution has been the method of choice for improving glycosynthase activity. An essential requirement for the success of directed evolution experiments is the ability to screen large libraries for the desired property. In the case of glycosynthases, the development of a screening assay is challenging, because the reaction products do not produce a distinct, screenable signal, such as absorbance or fluorescence. To date, two elegant approaches have been developed for screening glycosynthase activity. Withers and coworkers used a coupled enzyme assay with an endocellulase that releases a synthetic chromophore group only from the product that is generated by the glycosynthase (Kim et al., 2004; Williams and Withers, 2002). They used this screening assay to isolate a β-glucosidase variant that exhibits a 27-fold improvement in k_{cat}/K_m value. This assay, however, has its limitations: for each glycosynthase reaction, a specific chromophore-containing acceptor has to be synthesized, and an appropriate endoglycosidase, if it exists at all, should be found. In the second approach, a yeast three-hybrid assay system was used to link glycosynthase activity to the transcription of an *LEU2* reporter gene, making cell growth dependent on glycosynthase activity in the absence of leucine. Lin et al. (2004) used this strategy with the endo-β-glucanase Cel7B from *Humicola insolens* to obtain a variant exhibiting a 5-fold increase in glycosynthase activity. The main limitation of this approach is the need for substrates, the synthesis of which is a multistep endeavor (Tao et al., 2006). In addition, the bulky groups that should be attached both to the donor and the acceptor might prevent the substrates from entering the active sites due to steric hindrance. This is especially true for exoglycosynthases having an active site that possesses pocket topology.

Here we describe the development of a general, high-throughput screening procedure for glycosynthase activity. The assay is based on the release of hydrofluoric acid, a by-product of all glycosynthase reactions. This procedure should be applicable for all glycosynthases, does not require the synthesis of special chromophoric or modified substrates, and is suitable for directed evolution experiments. As a model enzyme, we have used the family 52 β-xylosidase XynB2(E335G) glycosynthase from *Geobacillus stearothermophilus*. This glycosynthase can promote self-condensation of α-D-xylopyranosyl-fluoride (XylF) to produce α-D-xylobiosyl- and α-D-xylotriosyl-fluoride (Ben-David et al., 2007). Screening error-prone PCR libraries of the enzyme resulted in the isolation of variants displaying improved oligosaccharide synthesis activity. Moreover, the improved activity appeared to be associated with mutations that reduced the affinity of the product to the enzyme, suggesting a general approach for improving glycosynthase activity.

RESULTS

Developing a Screening Assay for Glycosynthase Activity

As outlined in Figure 1, the formation of glycosidic linkage via the glycosynthase mechanism results in the release of hydrofluoric acid. The principle underlying the screening assay is to translate the liberation of this by-product into a screenable signal by using common pH indicators. The feasibility of this principle was first tested on crude cell extracts. *Escherichia coli* BL21 cells carrying either a plasmid for the expression of XynB2(E335G) or an empty vector were grown overnight and treated with lysozyme. The cell debris was removed by centrifugation, and the crude extracts were incubated with the donor XylF, as well as various pH indicators, such as bromocresol purple, methyl red, and bromophenol blue. After a few minutes of incubation at room temperature, the indicators in the test tubes that contained XynB2(E335G) altered their colors, whereas, in the control samples, the color remained unchanged. These results indicated that the pH changes resulting from the glycosynthase activity can be readily detected. Next, we sought to develop an "on-agar" assay that would allow us to examine a large number of variants. *E. coli* BL21 cells harboring either pET9d*xynB2*(E335G) or empty vector were streaked on agar plates and grown overnight. The colonies were then covered with a 1% soft agar layer containing 20 mM XylF and 0.001% methyl red. After 2 days of incubation at room temperature, it was possible to distinguish between colonies expressing the glycosynthase XynB2(E335G), which turned red, and the control colonies, which remained yellow. However, although this assay gave indication of the desired activity, it suffered from several disadvantages. The assay required an extended incubation period and, therefore, the acid around the colonies diffused, resulting in low sensitivity. Moreover, relatively large amounts of reagents were required for each plate. The main reason for these difficulties was probably the high buffer capacity of the growth medium.

To overcome the buffering problem, we modified the assay and tested the activity on a filter paper. Briefly, *E. coli* BL21 cells expressing XynB2(E335G) were first plated on an LB agar plate and grown overnight. Next, the colonies were lifted onto 8.5 cm-diameter Whatman no. 1 filter paper and the paper was immersed in liquid nitrogen for a few seconds to facilitate cell disruption. Subsequently, the filter paper was parched for 1 hr in an incubator at 37°C and then soaked with 1 ml of low-strength

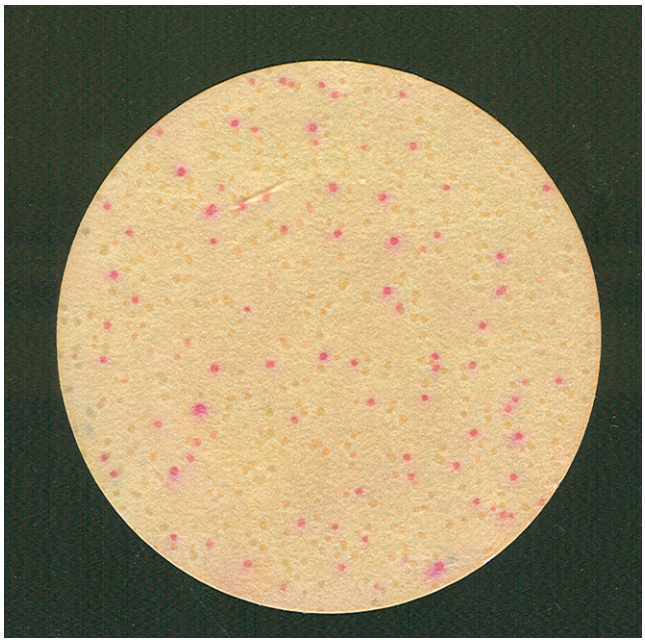

Figure 2. Primary Screening Assay
E. coli BL21 cells expressing mutant variants of XynB2(E335G) were grown on agar plate and lifted onto filter paper. The paper was treated with liquid nitrogen, parched, and finally soaked with a XylF and methyl red mixture. Colonies expressing active glycosynthase turned red following 20 min at room temperature. The differences in the colonies' color intensities (presumably glycosynthase activity) can be easily seen.

sodium phosphate buffer solution (3 mM [pH 6.4]) containing XylF (15 mM) and methyl red (0.001%). After 20 min of incubation, the colonies turned red (control colonies containing empty vector remained yellow). Thus, performing the assay on filter paper not only reduced the time needed for incubation from days to minutes, but also increased the sensitivity and reduced the amount of reagents used. A representative example of the filter assay is shown in Figure 2.

Library Construction and Screening for Improved Glycosynthase Activity

A mutant library of XynB2(E335G) was generated by error-prone PCR with high Mg^{2+} (7 mM) and an unbalanced dNTP concentration. Sequence analysis of four randomly picked clones revealed a mutation rate of 4–7 bp substitutions per gene, resulting in two to four amino acid substitutions per variant. The error-prone PCR DNA library was transformed into *E. coli* BL21, and the colonies were subjected to the primary filter paper assay as described above. Following soaking of the filter paper with XylF and methyl red, the first colonies to become red were picked for further analysis (about two to three colonies per plate containing about 600 variants). Screening over 10,000 colonies yielded 35 candidates for improved activity. To further refine this pool of variants, we used a secondary 96-well assay, which provided a more quantitative estimation for glycosynthase activity. In this assay, the donor and the pH indicator were dispensed into a 96-well plate containing equal amounts of toluene-treated cells. The activity was estimated by monitoring the increase in absorbance at 535 nm (methyl red in its protonated state) with time (Figure 3). Variants that increased the absorbance faster than the control reaction with XynB2(E335G) were defined as improved variants. The enzymes from the four most promising variants were purified, and their activity was tested quantitatively with a fluoride electrode at 15 mM XylF. The fluoride releasing rate of all four variants was 1.2- to 3-fold higher than XynB2(E335G). TLC analysis of the products indicated that all four variants produced the two expected products (α-D-xylobiosyl- and α-D-xylotriosyl-fluoride). However, one of the variants also promoted the hydrolysis of the donor XylF, resulting in detectable amounts of xylose. The most improved mutant, variant 27I, gave a k_{cat} value of 11.2 s^{-1}, which is 8.6-fold higher than that of the parental enzyme, XynB2(E335G). Sequence analysis revealed that this mutant contained four amino acid substitutions: F206L, I211T, C253R, and N342K. To further improve the glycosynthase activity, the gene encoding mutant 27I was used as a template for the construction of a second-generation error-prone PCR library. In this round, over 11,000 colonies were screened by the filter paper primary screen, of which 40 were further examined by

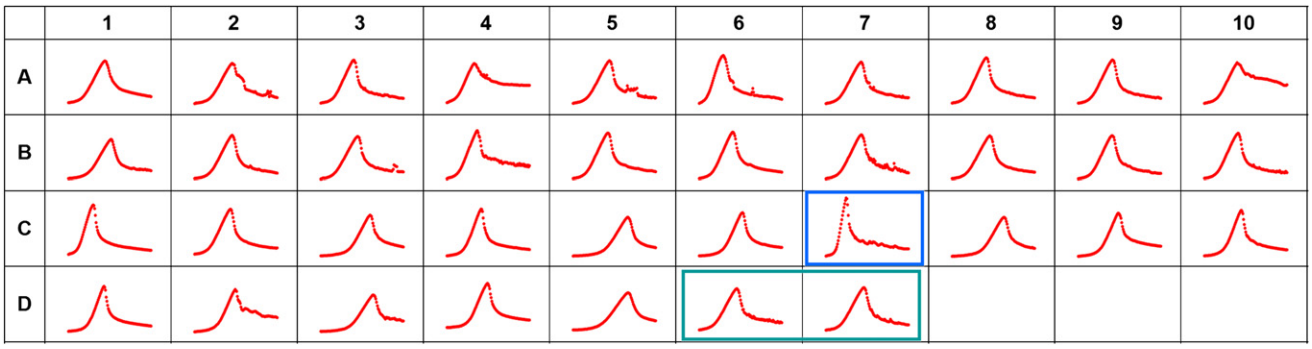

Figure 3. Secondary 96-Well Screening Assay
XylF and methyl red were dispensed into wells containing equal amounts of toluene-treated *E. coli* BL21 cells carrying potentially improved variants. The extent of hydrofluoric acid formation was monitored at 535 nm (the absorbance of methyl red in its protonated state). Each box represents an individual reaction in which the absorbance at 535 nm (y axis, ranging from 0 to 1.1 OD) is plotted as a function of time (x axis, ranging from 0–190 min). Reactions of XynB2(E335G) (boxes D6 and D7) and variant 27I (box C7) are denoted in green and blue, respectively. The observed decrease in absorbance at the later stages of the reaction results from precipitation of the indicator in its protonated state.

the secondary 96-well assay. Small aliquots from reaction wells of the six most promising candidates were removed and subjected to TLC analysis to verify that they do not promote hydrolysis. None of the tested candidates indicated hydrolysis. Next, the enzymes from these improved variants were purified, and their synthesis rates at 15 mM XylF were found to be 2- to 3-fold higher than the rate of 27I. The most improved mutant of this round was 29II, with a k_{cat} value of 45.7 s^{-1} (35-fold higher than XynB2(E335G)). This variant contained six additional amino acid substitutions: V123E, A282T, R291H, T343P, D470G, and D597G. TLC analysis of 27I and 29II confirmed that both variants produce only α-D-xylobiosyl- and α-D-xylotriosyl-fluoride without detectable hydrolysis products.

Kinetic Behavior of the Improved Variants

We have previously demonstrated that GH52 β-xylosidase XynB2(E335G) is an efficient glycosynthase (Ben-David et al., 2007). The native enzyme functions as an exo-type glycosidase, releasing single xylose units from the nonreducing termini of short xylooligomers (Bravman et al., 2003b). It is therefore likely that the active site of the enzyme possesses a pocket-type topology (Davies and Henrissat, 1995). This architecture of the active site dictates sequential binding of the donor and acceptor when the mutant enzyme functions as glycosynthase. On this basis, we suggested earlier that the kinetic model for the self condensation of XylF by XynB2(E335G) proceeds according to the expression (Ben-David et al., 2007):

$$E + S \underset{}{\overset{K_{M(donor)}}{\rightleftharpoons}} SE + S \underset{}{\overset{K_{M(acceptor)}}{\rightleftharpoons}} SES \xrightarrow{k_{cat}} E + P,$$

where E is a free enzyme, S is XylF, SE is an enzyme with a substrate molecule at the glycon site (donor site), and SES represents an enzyme with both glycon and aglycon (acceptor) sites occupied by XylF molecules. The derived rate expression for this model (Equation 1) shows that the reaction rate depends on the squared substrate concentration ($[S]^2$), suggesting a sigmoid type of behavior:

$$v = \frac{k_{cat}[E]_t[S]^2}{K_{M(acceptor)}K_{M(donor)} + K_{M(acceptor)}[S] + [S]^2}. \quad (1)$$

When the initial rates of the glycosynthase activity were measured at different substrate concentrations, a classical Michaelis-Menten behavior was observed (Ben-David et al., 2007). This can be explained if $K_{m(donor)}$ is much smaller than $K_{m(acceptor)}$ and the [S] used in the assay. In this case, Equation 1 can be reduced to Equation 2, which is analogous to the Michaelis-Menten rate expression:

$$v = \frac{k_{cat}[E]_t[S]}{K_{M(acceptor)} + [S]}. \quad (2)$$

Since many glycoside hydrolases, including XynB2 (Bravman et al., 2003a, 2003b), exhibit high selectivity for the sugar that occupies the glycon site, and remarkable plasticity for the aglycon, it is likely that the enzyme affinity for the donor is much higher than that of the acceptor (i.e., $K_{m(acceptor)} > > K_{m(donor)}$).

Interestingly, plotting the initial reaction rates of variants 27I and 29II as a function of XylF concentrations resulted in sigmoidal curves that resemble the expected behavior given in Equation 1. Indeed, modeling the experimental data to Equation 1 resulted in a good fit (Figure 4). The kinetic parameters for the self-condensation of XylF by XynB2(E335G) and the two improved variants, 27I and 29II, are summarized in Table 1. Remarkably, the increase in k_{cat} was accompanied by a large increase in the K_m values for both the donor and the acceptor. For 29II, the $K_{m(donor)}$ value increased over three orders of magnitude compared with the parental XynB2(E335G) glycosynthase. This result may suggest that the rate-limiting step in the XynB2(E335G) glycosynthase reaction is the release of the product (which is the true substrate of the native enzyme) from the active site. It should be noted that the "improved variants" may have, in fact, a lower specificity constant value (k_{cat}/K_m), which is the most common criteria for enzyme performance. However, for many applications, including enzymatic synthesis, k_{cat} is the crucial parameter for judging an enzyme, since the reactions are carried out in high substrate concentrations.

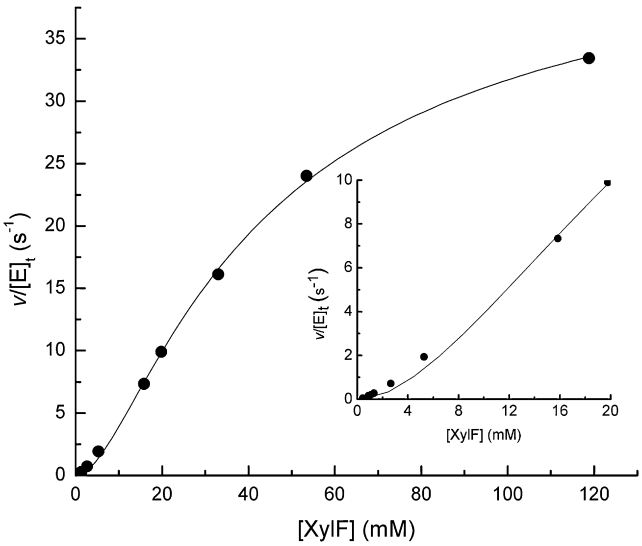

Figure 4. Kinetics for the Self-Condensation of XylF by 29II
The curve exhibits sigmoidal behavior resulting from the increased $K_{m(donor)}$ value. The inset focuses on the sigmoid toe. The initial rates of the reactions at the indicated XylF concentration were determined with a fluoride electrode. The reactions were performed in phosphate buffer (100 mM [pH 7.0]) at 30°C.

DISCUSSION

We describe here the development of a general screening methodology for glycosynthase activity, allowing the identification of improved enzymes from large libraries of variants. The principle

Table 1. Kinetic Constants for the Self-Condensation of XylF by XynB2(E335G), 27I, and 29II

Enzyme	k_{cat} (s^{-1})	$K_{m(acceptor)}$ (mM)	$K_{m(donor)}$ (mM)
XynB2(E335G)	1.3	2.2	<0.02
27I	11.2	26.6	5.3
29II	45.7	37.2	18.6

See Figure 4 for experimental details.

underlying the screening assay relies on a general feature of the glycosynthesis reaction; namely, the release of hydrofluoric acid. A primary assay is conducted on a filter paper and allows the screening of 600 colonies per filter or over 10,000 colonies per day. This assay effectively reduced the number of variants that should be tested from more than 10,000 to less than 100. A secondary 96-well plate-based assay allows quantitative measurements, and can be used to compare promising clones coming from different filters. It is noteworthy that the most active candidates in the 96-well assay originated from colonies that were the first to turn red on their filter paper. This correlation between the assays minimizes the number of mutants that have to be screened in the secondary assay, which is more time consuming.

The main advantages of our methodology are: (1) it is simple, cost effective, and requires no special instrumentation or specialties; (2) it has the potential to be universal for any glycosynthase reaction (moreover, it can be applied for any reactions that involve subtle pH changes); (3) it allows screening of a large number of variants; (4) it is sensitive and enables detection of small improvements (20%); (5) it does not require synthesis of special substrates, and any existing protein expression system can be used without prior DNA manipulations. A potential drawback of this screening procedure is that it does not distinguish between true glycosynthase activity (bond formation) and hydrolysis, in which the donor sugar is transferred to a water molecule. However, this shortcoming can be easily circumvented. Since glycosynthase activity is, in many cases, acceptor dependent, hydrolysis-promoting mutants can be easily detected by performing the assay in the absence of an acceptor molecule. Alternatively, performing a simple TLC analysis can be used to detect the hydrolysis product. Therefore, although hydrolysis-prone variants can be selected at an initial stage, they can be eliminated very early on by performing a simple control assay.

We demonstrated the strength of this methodology by obtaining improved glycosynthase mutants of XynB2(E335G) via directed evolution. Following two successive rounds of mutations and screening, a variant that exhibits 35-fold improvement in k_{cat} was isolated. Interestingly, the increase in k_{cat} was accompanied by a large increase in the K_m values for both the donor and the acceptor. In glycosynthesis, the direction of the natural hydrolysis reaction is reversed, resulting in a product that is, or resembles, the true substrate of the native enzyme. Thus, it is possible that enzyme product release is the rate-determining step for k_{cat} (Fersht, 1999), because the product is, in fact, the natural substrate of the native glycosidase. Therefore, mutations that reduce the affinity of the glycosynthase to the product may improve the enzyme turnover at high substrate concentrations. This general conclusion can serve as a guideline for improving glycosynthesis activity with a more rational approach.

SIGNIFICANCE

The glycosynthase approach is an attractive route for the efficient synthesis of oligosaccharides, as it utilizes inexpensive glycosyl-fluoride donors, together with robust and available enzymes, and results in high overall yields. For this methodology to be widely accepted, many more glycosynthases must be developed to allow the synthesis of a broad spectrum of oligosaccharides. Currently, it is unclear why not all retaining glycosidases can function as glycosynthases, and what enzyme structural elements are required for efficient glycosynthesis. Directed evolution approaches should provide insights from improved glycosynthases. We report here the development of a generic, high-throughput screening assay for the directed evolution of glycosynthases. The assay is simple, sensitive, allows the screening of a large number of variants, and, most importantly, has the potential to be universal and utilized for any glycosynthase reaction. We demonstrated the potential of this methodology by performing directed evolution on XynB2(E335G), a GH52 β-xylosidase glycosynthase. The screening procedure allowed us to obtain improved mutants following two rounds of mutagenesis and screening. The improved variants exhibited higher K_m values toward the donor and acceptor, suggesting that enzyme-product release can be rate determining for k_{cat}. This novel screening methodology should facilitate the improvement of glycosynthases by directed evolution approaches, and provides basic knowledge regarding the structure-function relationships of glycosynthases.

EXPERIMENTAL PROCEDURES

General

The glycosyl-fluoride donor XylF was prepared as previously reported (Ben-David et al., 2007). All other chemicals were purchased from Sigma Chemical Co. or Fluka, unless otherwise stated. DNA manipulation reagents were purchased from New England Biolabs. TLC analysis was performed as previously described (Ben-David et al., 2007).

Construction of Error-Prone Mutant Library

The cloning of the *xyn*B2 gene to pET9d (Novagen) and the preparation of the E335G mutant have been reported elsewhere (Bravman et al., 2003b). A mutant library of *xyn*B2(E335G) was generated by error-prone PCR according to established protocols (Cadwell and Joyce, 1992). The PCR reaction (200 μl in four separate tubes) contained 10 mM Tris (pH 8.3 at 25°C), 50 mM KCl, 7 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.2 mM of dGTP and dATP, 1 mM of dCTP and dTTP, 2 ng/μl of each primer (T7 promoter universal primer and a C-terminal primer 5′-CGATCTAGATCTTCATTCCCCCTCCTCCAACC-3′), 0.1 ng/μl of template, and 5 U of *Taq* DNA polymerase. The PCR program consisted of 30 cycles of 94°C, 45 s; 50°C, 60 s; 72°C, 150 s. The resulting PCR product was digested with NcoI and BglII and ligated with PCR-amplified pET9d that was digested with NcoI and BamHI. The use of PCR-amplified linear vector reduces the background of colonies carrying empty vectors and overcomes the need for gel purification. The ligation mixture was drop dialyzed against 0.5 × TE buffer and transformed to *E. coli* BL21 electrocompetent cells. Transformants were plated on Luria-Bertani (LB) agar media containing 30 μg/ml kanamycin and incubated overnight at 37°C.

Primary Screening Assay

Following overnight incubation of the transformants, colonies (about 600/plate) were lifted onto a sterile Whatman no. 1 filter paper (8.5 cm diameter). The colony residuals on the plate were left to recover at 37°C for a few hours. The filter paper was immersed in liquid nitrogen, parched for 1 hr in a 37°C incubator, and subsequently soaked with 1 ml of a reaction solution containing 15 mM XylF and 0.001% (w/v) methyl red sodium salt in a weak phosphate buffer (3 mM [pH 6.4]). Colonies that turned red faster than their neighbors were picked from their corresponding agar plates and tested in the secondary screening assay (about two to three colonies per plate).

Secondary 96-Well Screening Assay

Promising candidates obtained in the primary screen were grown overnight in 3 ml LB medium supplemented with kanamycin. Bacterial growth was

quantified by reading the absorbance at 600 nm. Cells from each culture were taken for the assay according to the following ratio: 500 μl from 5 OD cultures (it was important to assay similar amounts of cells from each culture, since the cells contribute buffering agents that can influence the results). The cells were centrifuged and the pellet was suspended with 500 μl phosphate buffer (10 mM [pH 6.4]). The suspensions were then treated with 15 μl of toluene and incubated for 10 min at room temperature. Subsequently, the cells were diluted 10-fold with distilled water, and 26 μl of each sample was transferred to a 96-well plate. The plate was incubated in a Synergy HT microplate reader set at 30°C and, after 10 min of incubation, 44 μl of reaction mixture was dispensed to each well (30 mM XylF, 0.001% methyl red). The rate of the reactions was monitored at 535 nm, which corresponds to the red color of the protonated state of the indicator. Variants were defined as improved if they changed the absorbance from 0.3 to 0.7 OD faster than XynB2(E335G). To ensure that these mutants were not false positives that hydrolyze the donor, aliquots from the wells were taken for TLC analysis.

Kinetic Analysis

Protein purification and the kinetics of self-condensation of XylF by XynB2 derivatives were performed as previously described (Ben-David et al., 2007). Kinetic parameters were determined by nonlinear fit with OriginPro 7.5 (OriginLab Corp., Northampton, MA).

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