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Rational design of an improved transglucosylase for production of the rare sugar nigerose†

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The sucrose phosphorylase from *Bifidobacterium adolescentis* (BaSP) can be used as a transglucosylase for the production of rare sugars. We designed variants of BaSP for the efficient synthesis of nigerose from sucrose and glucose, thereby adding to the inventory of rare sugars that can conveniently be produced from bulk sugars.

Rare sugars hold tremendous potential for practical applications in various industries.[1] Regardless, few of them have been exploited commercially due to their scarcity in nature which prevents them from being isolated in large quantities. These compounds have consequently become attractive targets for biocatalytic production processes starting from affordable and widely available carbohydrates.[2]

The sucrose phosphorylase from *Bifidobacterium adolescentis* (BaSP; carbohydrate-active enzyme database family GH13) is a particularly interesting candidate enzyme for the production of such rare sugars. *In vivo*, BaSP catalyses the reversible phosphorolysis of sucrose into α-D-glucose 1-phosphate and D-fructose (EC 2.4.1.7). However, the enzyme can be applied as a versatile transglycosylase when phosphate is replaced by a different acceptor substrate thanks to its renowned substrate promiscuity.[3–6] When presented with glucose as acceptor, for example, BaSP synthesises a mixture of the α-(1,4)-bonded glucobiose maltose and the rare α-(1,2)-bonded kojibiose. We previously exploited this property by engineering a mutant enzyme that preferentially forms kojibiose, enabling large-scale production and evaluation of this prebiotic sugar.[7,8] More recently, Kraus *et al.* reported that the regioselectivity of BaSP towards glucose can be altered by introducing a Q345F mutation. This variant is no longer capable of forming kojibiose, but instead produces an equimolar mixture of maltose and nigerose (Fig. 1).[9–12]

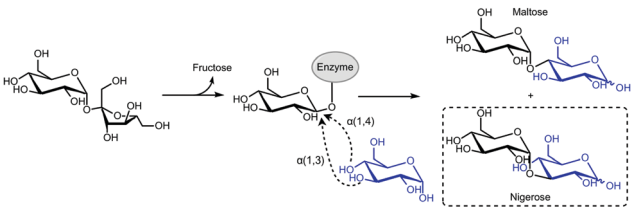

Fig. 1 Transglucosylation of glucose by mutant Q345F of the *B. adolescentis* sucrose phosphorylase, resulting in the synthesis of maltose and nigerose.

Nigerose is the rare α-(1,3)-bonded disaccharide of glucose that occurs in nature as a constituent of polysaccharides such as nigeran. It is also found in Japanese rice wine or sake, hence its alternative name, sakebiose. Despite its limited availability, a range of possible applications for this sugar have already been described over the years. Nigerose has been shown to exert immunopotentiating activity in mice,[13] it has potential as a prebiotic,[14] it has been evaluated as an additive in cryopreservation media for human cell lines[15] and it has been applied as a building block for promising biocompatible nanocarriers for sustained drug delivery.[16]

Although the Q345F exchange allows BaSP to synthesise nigerose, the applicability of this biocatalyst remains low due to its poor activity and regioselectivity. Curiously, this problem can be alleviated somewhat by adding DMSO as cosolvent. Increasing the DMSO concentration from 0% to 40% shifts the nigerose/maltose ratio from 0.98 to 3.04 and improves product yield sixfold.[10] However, the use of DMSO is far from desirable in an industrial context since it will impede the downstream processing (DSP), resulting in elevated DSP costs and thus also increased overall production costs. Furthermore, the use of DMSO in production processes envisaging food applications, where rare sugars show potential as alternative sweeteners, is strongly undesired. Therefore, we began a semi-rational engineering effort to improve the activity of BaSP towards nigerose formation in aqueous solution.

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Table 1 Specific activity and nigerose/maltose ratio of several variants of BaSP (100 mM sucrose, 200 mM glucose, 50 mM MOPS pH 7, 52 °C)

BaSP mutant	Relative activity[a] (%)	Nig/malt ratio
Q345F	100 ± 4	0.95
D342G/Q345F	120 ± 5	4.10
Y344Q/Q345F	165 ± 4	5.60
D342G/Y344Q/Q345F	910 ± 45	5.25
R135W/D342G/Y344Q/Q345F	2550 ± 90	5.40
R135Y/D342G/Y344Q/Q345F	3620 ± 98	7.35

[a] Relative to variant Q345F (100% = 0.004 U mg^{-1}).

The +1 subsite of BaSP is mainly shaped by two highly dynamic loops that undergo crucial conformational changes throughout the catalytic cycle.[17] It is one of these loops (^{341}LDLYQ^{345}) that houses the Q345F mutation necessary for enabling nigerose synthesis. Starting from this variant, the remaining loop positions were individually targeted for NNK-based saturation mutagenesis (Fig. S1 and Experimental details; ESI†) and the resulting libraries were screened with high-performance anion exchange chromatography in search for mutations that compensate for the impaired activity of the starting point (Fig. S2, ESI†). From the ~400 transformants screened on crude cell extract, two hits (D342G/Q345F and Y344Q/Q345F) were purified and analyzed in more detail. Both showed a modest increase in activity, but also exhibited a far more favorable regioselectivity, with at least a fourfold improvement of the nigerose/maltose ratio (Table 1).

Encouraged by the better properties of the obtained variants, the mutations were joined together to examine whether their positive effects could be combined. While the regioselectivity of the resulting mutant (D342G/Y344Q/Q345F) was not enhanced any further, its transglucosylation activity towards nigerose synthesis went up ninefold when compared to the Q345F variant, indicating a strongly cooperative non-additive effect.

The other dynamic acceptor site loop (^{132}YRPR^{135}) was targeted in a second round of site randomizations, using the triple mutant from the first round as template. Two distinctly beneficial mutations were uncovered in the library created at position 135, the best one being R135Y which triggers another fourfold increase in specific activity (Table 1). The libraries created at the other positions offered no further improvements. Compared to the Q345F variant that was used as starting point for mutagenesis, final mutant R135Y/D342G/Y344Q/Q345F (BaSP-YGQF) shows a 68-fold increase in catalytic efficiency (k_{cat}/K_m) for the synthesis of nigerose (Table 2 and Fig. S3, ESI†). Most of this positive effect can be attributed to the turnover number which went up 24-fold. Interestingly, the change in regioselectivity barely appears to be caused by a higher affinity for glucose in its nigerose-forming binding mode, rather by the turnover number for maltose synthesis benefiting less from the introduced mutations.

These additional mutations eliminated the need for DMSO in production processes for nigerose by sucrose phosphorylase. Indeed, the mutant BaSP-YGQF presented here achieves a far higher activity in aqueous solution than the Q345F variant does in 40% DMSO. It is furthermore worth noting that the presence of DMSO in reactions catalyzed by BaSP-YGQF no longer has any significant effect on activity or regioselectivity at all.

Peculiarly, all hits are located at those positions that undergo the most striking rearrangements throughout the catalytic itinerary of BaSP. As the wild-type enzyme switches from sucrose-binding mode to phosphate-binding mode, residue Asp342 moves out of the active site while residues Arg135 and Tyr344 move in.[17] For this reason, we hypothesized that the beneficial effects of the mutations in BaSP-YGQF might stem from a change in acceptor loop dynamics. Molecular dynamics simulations were performed and differences in backbone flexibility were assessed by comparing the root mean square fluctuations of all residues (Fig. 2 and Fig. S4, Supplementary movies, ESI†).

Subsite +1 was indeed observed to be pronouncedly more flexible in the novel mutant, particularly in and around loop ^{341}LGLQF^{345}, while the motility of subsite −1 and the catalytic residues remained unaffected. The rise in k_{cat} might therefore find its origin in a higher backbone plasticity, allowing the acceptor site to more easily adopt a conformation that is favorable for the synthesis of nigerose.

BaSP-YGQF was put to use for producing nigerose on 0.5 L scale using higher substrate concentrations, *i.e.* 1.5 M of both sucrose and glucose (Fig. 3 and Fig. S5, ESI†). Nigerose was synthesized with a yield of 645 mM (221 g l^{-1}) or 43% (n/n) relative to the donor substrate, and a transglucosylation efficiency of 73%. The hydrolytic side reaction, inherent to the double displacement mechanism of sucrose phosphorylases, accounted for an acceptable sucrose loss of 15%. The product was isolated by means of enzymatic degradation of the contaminating sugars and subsequent preparative liquid chromatography. However, purification can alternatively be performed by adding baker's yeast which can metabolize sucrose, glucose, fructose and maltose while leaving nigerose untouched, as described earlier.[7,10] A total of 98 g of nigerose was finally obtained and its structure was confirmed by NMR spectroscopy, which was in agreement with data reported in previous studies (Fig. S6, ESI†).[10,18] The production of nigerose using BaSP-YGQF thus appears to outperform methods reported earlier, as those typically require expensive

Table 2 Apparent kinetic parameters of *B. adolescentis* sucrose phosphorylase mutants in aqueous solution

	Glucose$_{nig}$[a]			Glucose$_{malt}$[b]		
BaSP mutant	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} M^{-1})	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} M^{-1})
Q345F	855 ± 45	0.007 ± 0.001	0.008	750 ± 80	0.008 ± 0.001	0.011
R135Y/D342G/Y344Q/Q345F	305 ± 20	0.17 ± 0.01	0.55	370 ± 31	0.03 ± 0.01	0.08

[a] D-Glucose in binding mode that leads to formation of nigerose. [b] D-Glucose in binding mode that leads to formation of maltose.

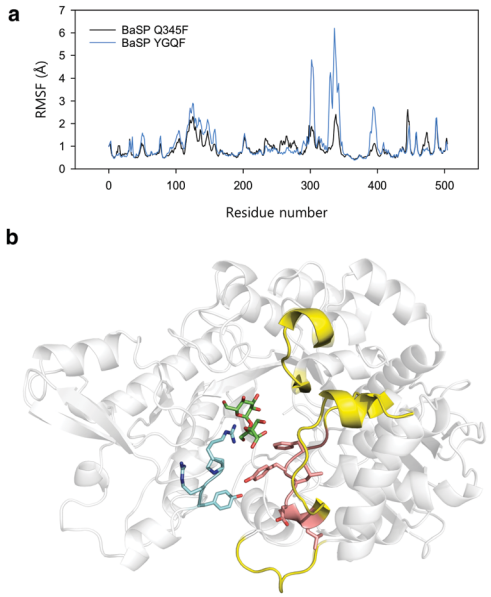

Fig. 2 Average root mean square fluctuation (RMSF) of the backbone at all positions of BaSP Q345F and the novel R135Y/D342G/Y344Q/Q345F (YGQF) mutant (a). Structure of BaSP (b). Dynamic acceptor site loops are shown in sticks and colored blue (residues 132–135) or red (residues 341–345). Other regions with increased flexibility in mutant BaSP-YGQF are colored yellow.

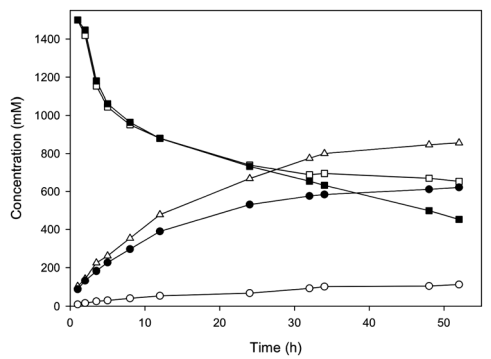

Fig. 3 Production of nigerose with BaSP-YGQF (1.5 M sucrose, 1.5 M glucose, 52 °C, pH 7.0; data obtained by HPAEC) (● nigerose, ○ maltose, ■ sucrose, □ glucose, △ fructose).

substrates or multiple enzymes, suffer from lower yields, or result in complex product mixtures.[19–23]

In summary, a mutant transglycosylase was designed that allows the rare disaccharide nigerose to be produced efficiently and conveniently, starting from cheap, renewable and abundant bulk sugars in aqueous solution. Because nigerose can now easily be produced on multi-gram scale as was demonstrated in this work, its properties can be thoroughly evaluated moving forward. Furthermore, the process can readily be scaled up for potential industrial purposes. Additionally, our findings demonstrate how the natural promiscuity of the *B. adolescentis* sucrose phosphorylase can be finetuned by targeting merely two short loops, and how the dynamics of these loops may play a larger than expected role in that regard.

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Conflicts of interest

There are no conflicts to declare.

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