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High-yield production of completely linear dextrans and isomalto-oligosaccharides by a truncated dextransucrase from *Ligilactobacillus animalis* TMW 1.971

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ABSTRACT

Several lactic acid bacteria are capable of producing water-soluble exopolysaccharides such as dextran from sucrose by using glucansucrases. Several recombinant glucansucrases were described, however, yields were often limited and most dextrans were branched at position *O*3. In this study, the dextransucrase from *Ligilactobacillus animalis* TMW 1.971 was recombinantly produced without its *N*-terminal variable region and used for dextran synthesis. The enzyme expressed well and showed very high total as well as transferase activities compared to other glucansucrases. It was able to transfer nearly all glucose from sucrose to oligo- and polymeric products under certain conditions (about 95 % of glucose transferred). The high efficiency of the enzyme made it possible to obtain absolute dextran yields of up to 214.9 g/L from a 1.5 M sucrose solution. Structural characterization of the products showed that the dextrans produced have a rather low molecular weight, a narrow size distribution, and are completely linear. Furthermore, we showed that various low molecular weight dextrans or 1,6-linked isomalto-oligosaccharides can be efficiently produced by acid hydrolysis. Overall, we demonstrated that *Ligilactobacillus animalis* TMW 1.971 dextransucrase can be used to efficiently synthesize dextrans with a quite unique structural composition. The dextrans produced have a high potential for further applications such as synthesis of copolymers or size standards with a very defined molecular structure.

1. Introduction

Lactic acid bacteria are widely spread in nature and several species are able to produce exopolysaccharides (EPS). Generally, EPS have a wide range of properties and compositions [1,2]. Therefore, they are of great interest for the pharmaceutical, biomedical and food industries. Lactic acid bacteria can produce heteroexopolysaccharides which are composed of several monosaccharide units and are usually obtained in comparably low yields. Furthermore, they can produce homoexopolysaccharides such as α-glucans or β-fructans. The most important bacterial α-glucan is dextran, which is produced by extracellular dextransucrases (EC. 2.4.1.5) from sucrose. The enzymes cleave the disaccharide, liberate fructose and transfer glucose to an acceptor. Water as well as other oligo- and polysaccharides can subsequently serve as acceptor which leads to a hydrolytic activity and a transferase activity, respectively [3]. Thus, an enzyme should completely convert sucrose and have a high transferase activity to achieve an efficient dextran synthesis.

Dextrans are composed of a backbone with α-1,6-linked glucose units, which can be branched at positions *O*2, *O*3, and/or *O*4 [4]. The degree of branching varies depending on the origin of the dextransucrase [5]. Commercially available dextran is fermentatively produced by *Leuconostoc mesenteroides* (*Leu. mesenteroides*) NRRL B–512F. This dextran is composed of about 95 % α-1,6 linkages, however, it also contains low portions of mono- and oligomeric side chains [5–8]. So far, there are only a few reports on completely linear, solely 1,6-linked dextran. Du et al. [9] used *Leu. mesenteroides* from Chinese sauerkraut to produce high yields of a dextran which was described as completely linear. However, the corresponding dextrans were only studied by ^{1}H NMR spectroscopy and small signals which could correspond to side

Abbreviations: DP, degree of polymerization; EPS, exopolysaccharides; HPAEC-PAD, high-performance anion exchange chromatography with pulsed amperometric detection; HPSEC, high-performance size exclusion chromatography; IMOs, isomalto-oligosaccharides; MALLS, multiangle laser light scattering; TFA, trifluoroacetic acid.

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chains can be observed in the spectrum. The other linear dextrans described in the literature were produced by recombinant glucansucrases: Meng et al. [10] created a mutant from *Limosilactobacillus* (*Li.*) *reuteri* dextransucrase (GTF180-ΔN L940W) and detected 100 % 1, 6-linkages in the produced glucan by using methylation analysis. The dextransucrase DSR-M from *Leu. citreum* NRRL B-1299 is the only native glucansucrase which was described to produce linear dextrans (99 % based on NMR spectroscopy) [11,12]. The molecular weight of dextran is generally very variable, ranging from 10 kDa to 1770 MDa, depending on the production conditions [13,14]. However, most dextrans show a rather high molecular weight above 10^6 Da [13]. While the linear dextran produced by GTF180-ΔN L940W showed a rather high molecular weight (>10^6 Da) [10], DSR-M produced low molecular weight dextrans (11–28 kDa) [11,12].

The yield of dextran depends on the production system. Fermentative production with *Leu. mesenteroides* NRRL B–512F provides absolute yields of dextran up to 44 g/L with a sucrose concentration of 0.47 M [15]. High dextran yields of up to 22 g/L can also be obtained by using other *Leu. mesenteroides* strains and a sucrose concentration of 0.29 M [16]. Fermentative production with *Leu. pseudomesenteroides* strains as well as various *Weissella confusa* and *Weissella cibaria* strains also results in high dextran yields between 24 g/L and 38.4 g/L by using sucrose concentrations between 0.058 M and 0.37 M [17–20]. A very high yield of dextran was obtained with the strain *Leu. mesenteroides* B-5481: By using a fermentative approach with immobilized cells, a dextran yield of 63 g/L was achieved with a sucrose concentration of 0.58 M [21]. Furthermore, Du et al. [9] described a yield of 70 g/L for their *Leu. mesenteroides* strain using 0.34 M sucrose, however, this yield was higher than the yield theoretically achievable with the sucrose concentration used. Therefore, impurities from the culture medium may contribute to the dextran yield, which is a general problem of fermentative production. Dextran production with recombinant dextransucrases can be carried out in buffers and thus provides high purity dextrans. For example, DSR-M is a good candidate for high yield production, as this enzyme showed a high transferase activity and 81–84 % of the glucosyl units from sucrose were recovered in the polymeric fraction by using a 0.292 M sucrose solution and incubation time between 8 and 16 h. The application of these conditions would result in a yield of 39.7 g/L [11,12]. Other promising candidates are the engineered dextransucrase variants of *Leu. mesenteroides* NRRL B–512F which also exhibited a high transferase activity. These enzymes incorporated 65–75 % of the glucosyl units into polymeric structures when 0.3 M − 0.73 M sucrose solutions were incubated. This would correspond to absolute dextran yields of up to 81.6 g/L [22]. Other dextransucrases described in the literature exhibited lower dextran yields between 0.03 and 3 g/L (with 0.15/0.2 M sucrose solutions) [23,24]. Dextran has a wide range of applications, for example, the texturizing and stabilizing properties are of interest for applications in food [3]. Dextran is also very interesting for the development of biodegradable and environmentally friendly biopolymers [25,26]. Furthermore, dextran is often partially hydrolyzed to produce low molecular weight dextrans (e.g. for the use as size standards) or even isomalto-oligosaccharides (IMOs) by using diluted acid. However, commercially available dextrans, which are usually obtained from *Leu. mesenteroides*, are branched at position *O*3 [27]. Because of the presence of different degrees of branching as well as side chain distributions and lengths, commercial dextrans and their derivatives have a complex and in part undefined composition. This is for example detrimental in the synthesis of copolymers, in which functionalities such as the primary hydroxyl group (present at the non-reducing end and side chains) can be important. Furthermore, the investigation of structure-function relationships is hindered if the complex dextran structure cannot be controlled completely. Linear dextrans have a completely defined structure and can be further tailored for example by using branching sucrases [12,28]. Therefore, enzymes which produce high yields of completely linear dextrans are of great interest. The dextransucrase from *Ligilactobacillus* (*L.*) *animalis* TMW 1.971 could potentially be used for this purpose. This enzyme was already described to produce solely linear dextrans [29,30] and only shows 44 % identity with DSR-M and 40 % identity with GTF180. Some variations of the amino acids in the conserved motifs were also observed. Thus, it is an interesting candidate for the high yield production of linear dextrans. However, the yields in previous studies were quite low and the products were water-insoluble [29,30].

Therefore, we expressed the dextransucrase from *L. animalis* TMW 1.971 in *N*-terminally truncated, recombinant form and used it for dextran synthesis. The *N*-terminal truncation was carried out because a significantly increased transferase activity was described for other *N*-terminally truncated glucansucrases. The reason for this is a possible steric hindrance of the growing glucan chain [31,32]. The aim of this study was to evaluate the yields which can be obtained and to characterize the dextrans formed by the enzymatic synthesis and after partial acid hydrolysis.

2. Experimental

2.1. Materials

If not stated otherwise, all chemicals used were “p.a.” grade or better and were purchased from Carl Roth (Karlsruhe, Germany), Merk (Darmstadt, Germany), Thermo Fisher Scientific (Waltham, MA, USA) and VWR (Darmstadt, Germany). *Endo*-dextranase (EC 3.2.1.11) from *Chaetomium* sp., 8000 U/mL was purchased from Megazyme (Bray, Ireland).

2.2. Molecular cloning and heterologous expression

The gene encoding the truncated version of the *L. animalis* TMW 1.971 dextransucrase (accession number: CCK33644.1) was amplified from genomic DNA and cloned into the pLIC-SGC1 vector by a ligation independent cloning approach as described previously [30]. By using the primers listed in Table S1, the *N*-terminal region (Glu1 – Tyr507) of the dextransucrase was truncated. For protein expression, the plasmid was transformed into OneShot BL21 Star (DE3) cells by heat shock. A single colony was transferred to 10 mL of LB medium (100 μg ampicillin/mL) and grown for 5 h at 37 °C and 225 rpm. Subsequently, the suspension was transferred to 800 mL of fresh LB medium (100 μg ampicillin/mL) and incubated for 3 h at 37 °C and 225 rpm. Subsequently, isopropyl-β-D-thiogalactopyranoside (0.1 mM) was added to induce protein expression. After incubating overnight at 20 °C, cells were collected by centrifugation, resuspended in phosphate buffered saline (50 mM sodium phosphate, 300 mM NaCl, pH 7.5) and lysed by sonication (amplitude 50 %, 3 × 20 s pulse, 59.9 s pause) using a sonifier (SFX250, Branson Ultrasonic Corporation USA). Cell debris were removed by centrifugation (30 min, 4 °C, 14,000×*g*) and the clear supernatant was transferred to HisPur Ni-NTA resin (Thermo Fisher Scientific) with wash buffer (50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, pH 7.5). The suspension was incubated for 1 h at 4 °C, washed twice with 4 mL of wash buffer and the recombinant enzymes were eluted with elution buffer (50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole, pH 7.5). The enzyme was then transferred into a Vivaspin 20 spin concentrator (30 kDa cut-off) and centrifuged for 5 min. Then 20 mL of sodium acetate buffer (50 mM, pH 6.0) were added and centrifuged until only 0.5 mL remained. The solution was filled up to 1 mL with sodium acetate buffer. The molecular weight and purity of *L. animalis* dextransucrase was determined by SDS-PAGE. The protein concentration was determined by using a microplate reader (Infinite M200 PRO, Tecan, Männedorf, Switzerland) at 280 nm and the molecular weight and extinction coefficient calculated by the ExPASy ProtParam tool.

2.3. Analysis of enzyme activity

To standardize the enzyme amount added in different experiments, the total enzyme activity was calculated: The enzyme was incubated with a 0.25 M sucrose solution for 2 h, the enzyme was inactivated by heating the solution to 95 °C for 15 min, and the amount of released fructose was determined by using a HPAEC-PAD ICS-3000 system (Thermo Fisher Scientific, Sunnyvale, CA, USA) equipped with a CarboPac PA20 column (150 × 3 mm i.d., 6.5 μm particle size, Thermo Fisher Scientific). Quantification was performed by using an external calibration. The column temperature was 30 °C, the detector temperature was 25 °C, and a flow rate of 0.4 mL/min was applied. The following gradient consisting of four eluents (A: ultrapure water, B: 10 mM NaOH, C: 200 mM NaOH, D: 200 mM NaOH + 200 mM sodium acetate) was used for separation: column equilibration with 100 % B for 15 min prior every run, 0–20 min isocratic 100 % B, 20–30 min: linear gradient to 100 % C; 30–40 min: linear gradient to 100 % D; 40–50 min: isocratic 100 % C. The total activity was calculated from the amount of free fructose as follows: total activity (U/mg protein) = fructose released (μmol/mg protein * min).

To determine total, hydrolytic, and transferase activity with sucrose, 0.01 U, 0.05 U, 0.10 U, 0.15 U, 0.20 U, 0.30 U, 1 U, or 10 U dextransucrase were added to 1 mL of a 0.25 M and a 1.5 M sucrose solution (50 mM sodium acetate buffer; 1 mM $CaCl_2$, pH 6.0). After incubation for 24 h at 40 °C, the reaction was stopped by inactivating the enzyme at 95 °C for 15 min. To monitor sucrose conversion over time, the enzyme (0.1 U/mL, 0.3 U/mL, 1 U/mL or 10 U/mL) was added to a 0.25 M and a 1.5 M sucrose solution. After 2 h, 4 h, 8 h, 24 h and 48 h, a sample was taken from the solution and inactivated by heating to 95 °C for 15 min. Sucrose, glucose, and fructose concentrations were then measured by HPAEC-PAD on an ICS-3000 system as stated above. The different activities were calculated as follows from the amounts of glucose and fructose: total activity (U/mg protein) = fructose released (μmol/mg protein * min); hydrolysis activity (U/mg protein) = glucose released (μmol/mg protein * min); transferase activity (U/mg protein) = total activity – hydrolysis activity.

2.4. Dextran production and isolation

Four sucrose solutions (1.5 M, 1.0 M, 0.5 M, and 0.25 M sucrose in 50 mM sodium acetate, 1 mM $CaCl_2$, pH 6.0) were used for dextran synthesis. After the addition of different amounts of enzyme (0.15, 0.3, 1.0, and 10.0 U *L. animalis* dextransucrase/mL), the solution was incubated at 40 °C for 24 h. To stop the reaction, the solution was heated to 95 °C for 15 min. Subsequently, the solution was dialyzed against ultrapure water for 48 h (molecular weight cut-off: 3.5 kDa) and lyophilized to obtain water-soluble dextran. The absolute yield of each reaction was determined gravimetrically by weighing the residue obtained after lyophilization.

2.5. Structural analysis

To analyze the dextran structure, the polysaccharides were dissolved in ultrapure water (1 mg/mL) and incubated with *endo*-dextranase (5 U/mg) at 40 °C for 24 h. After incubation, the enzyme was inactivated by heating to 95 °C for 15 min and the obtained solution was used for HPAEC analysis. Furthermore, the untreated dextrans were analyzed and compared with commercial dextran standards (Sigma Aldrich). HPAEC-PAD analysis of enzymatically liberated IMOs as well as the unmodified dextrans was carried out on an ICS-6000 system (Thermo Fisher Scientific). A CarboPac PA200 column (250 × 3 mm i.d., 5,5 μm particle size, Thermo Fisher Scientific) was used at 0.4 mL/min and a column temperature of 30 °C. The detector temperature was 25 °C. The following gradient comprised of four eluents (A: ultrapure water, B: 10 mM NaOH, C: 200 mM NaOH, D: 200 mM NaOH + 500 mM sodium acetate) was used for separation: column equilibration with 100 % B for 20 min prior every run, 0–10 min isocratic 100 % B, 10–20 min linear gradient to 50 % B + 50 % C, 20–85 min linear gradient to 50 % B + 50 % D, 85–95 min linear gradient to 100 % D, 95–110 min isocratic 100 % D, 110–125 min isocratic 100 % B.

2.6. Partial acid hydrolysis of the L. animalis dextran

The *L. animalis* dextran was partially hydrolyzed with trifluoroacetic acid (TFA) hydrolysis to obtain different mixtures of low molecular weight dextrans and/or isomalto-oligosaccharides. Different TFA concentrations (0.2 M, 0.4 M, and 0.6 M) were used for hydrolysis and the hydrolysis time was varied from 2 h to 24 h. For hydrolysis, dextran was dissolved in the TFA solution (final concentration 10 mg/mL) and heated to 95 °C in a shaking water bath (WSB shaking water bath, witeg Labortechnik GmbH, Wertheim, Germany) at 160 rpm. All hydrolyses were carried out in 100 mL or 50 mL Duran flasks. After the hydrolysis, the solution was lyophilized and analyzed by HPAEC-PAD as described in section 2.5.

3. Results and discussion

3.1. Enzyme production and characterization

The non-truncated version of *L. animalis* TMW 1.971 dextransucrase was first described by Rühmkorf et al. [29]. The authors analyzed the total, hydrolysis, and transferase activities under different conditions, evaluated the influence of EDTA and different cations on the activity, and determined optimal pH and incubation temperatures. However, the transferase activity as well as the total activity was comparably low. The use of an enzyme truncated at the *N*-terminus may offer advantages compared to the full-length enzyme. The function of the *N*-terminal variable region is unclear, but increased transferase activities were observed for glucansucrases from *Li. reuteri* after removing the *N*-terminal region [31,32]. In the case of the dextransucrase DSR-E from *Leu. mesenteroides* NRRL B-1299, it was also shown that the enzyme activity could be increased by removing the *N*-terminal region [33]. Therefore, a truncated variant of the dextransucrase of *L. animalis* TMW 1.971

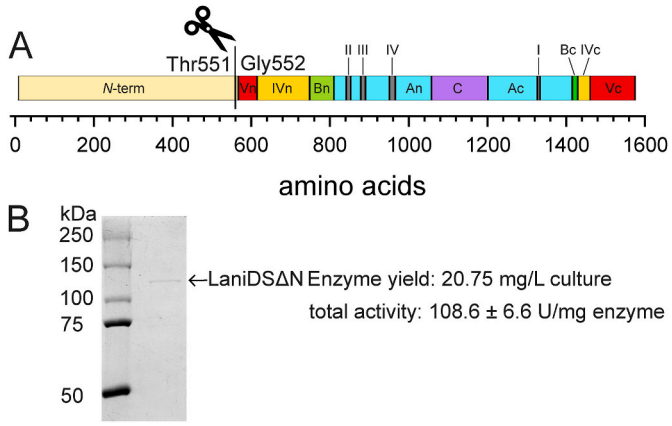

Fig. 1. A: Domain architecture of the truncated *L. animalis* TMW 1.971 dextransucrase (LaniDSΔN). The *N*-terminal region was truncated at Thr551. *N*-terminal variable region (residues 1–558), light yellow; domain V (residues 559–607; 1454–1567), red; domain IV (residues 608–740, 1422–1453), yellow; domain B (residues 741–802, 1422–1453), green; domain A (residues 803–1050, 1194–1407), light blue; domain C (residues 1051–1193), purple; conserved regions (motif II residues 834–844, motif III residues 871–882, motif IV residues 944–958, motif I residues 1320–1326), grey. Domains A, B, C, IV and V as well as the conserved regions were assigned by sequence comparison with *Li. reuteri* 180 GTF180 [34]. B: SDS-PAGE of the recombinant truncated *L. animalis* TMW 1.971 dextransucrase as well as enzyme yield and enzyme activity on sucrose.

(LaniDSΔN) was used in this study. The variable part of the *N*-terminus was truncated before domain Vn. The truncation site (Fig. 1A) was selected based on a sequence comparison with the *N*-terminally truncated dextransucrase from *Li. reuteri* TMW 1.106 [29]. LaniDSΔN was very well expressed in *E. coli* and obtained in high purity (Fig. 1B). The SDS-PAGE migration is in good agreement with the expected size range, as the predicted molecular weight of LaniDSΔN is 118.13 kDa. The protein yield was comparably high and the enzyme showed a high activity on sucrose.

Different activities of the enzyme were added to a 0.25 M and a 1.5 M sucrose solution to determine a suitable amount of enzyme for dextran production. For these experiments, the optimal pH and temperature determined by Rühmkorf et al. [29] were used. Fig. 2 shows the portions of sucrose, glucose, and fructose after incubation of a 0.25 M and a 1.5 M sucrose solution with different enzyme activities.

After 24 h, almost all sucrose in the 0.25 M sucrose solution was utilized by LaniDSΔN when 0.3 U/mL were used. In contrast, 0.3 U/mL of the enzyme utilized only about 32 % of the sucrose in the 1.5 M sucrose solution after 24 h. As expected, the addition of more enzyme led to an increased conversion of sucrose: While some sucrose remained after incubation with 1 U/mL, complete sucrose conversion was achieved by using 10 U/mL. From the low portions of monomeric glucose (4 %), it can be concluded that the major part of glucose is incorporated into oligo- and polymeric products. Thus, the conditions used in these experiments are well suited for the enzymatic synthesis of dextran in high yields.

For a more quantitative assessment of sucrose conversion, the yield of glucose which is incorporated into oligo- and polymeric products was calculated (Table S2). An incubation of 0.25 M sucrose solution with 0.3 U/mL resulted in the transfer of 91.2 % of the glucose moieties into oligo- and polymeric products. In contrast, adding 10 U/mL to a 1.5 M sucrose solution yielded a conversion rate of 95.5 %. Therefore, the enzyme shows a comparable percentual yield like DSR-M which transferred 81%–84 % of the glucosyl units into polymeric products, 8 % into leucrose, 5 % into oligosaccharides, and only 3 % into glucose [11,12]. The engineered variants of *Leu. mesenteroides* NRRL B–512F dextransucrase were found to transfer 65%–75 % of the glucosyl units into oligo- and polymeric products [22]. This demonstrates the efficiency of LaniDSΔN.

To analyze the course of the enzyme reaction, the concentrations of sucrose, glucose, and fructose were determined at different points of the reaction. For this purpose, a 0.25 M and a 1.5 M sucrose solution were incubated for a period of 48 h. Based on the previous results, an enzyme concentration of 0.1 U/mL or 0.3 U/mL was added to the 0.25 M sucrose solution, whereas the 1.5 M sucrose solution was incubated with 1 U/mL and 10 U/mL. The results are shown in Fig. 3. The continuously changing sugar profile demonstrates that the enzyme is active over this time period. For example, the sucrose content is further depleted in the incubation of the 0.25 M sucrose solution with 0.1 U/mL. Only about 10 % of sucrose are left after 48 h. As expected, sucrose was completely consumed by raising the amount of enzyme, however, an incubation time of about 24 h is necessary for that (about 40 % of sucrose are present after 8 h of incubation). For the 1.5 M sucrose solution, the remaining sucrose in the incubation mixture with 1 U enzyme/mL can be converted by elongating the reaction to 48 h. The application of 10 U/mL results in a very fast consumption of sucrose, which is completely converted after 4 h.

To quantitatively assess the activity of the enzyme with its natural substrate, total activity, hydrolysis activity, and transferase activity were calculated from the amounts of the individual released sugars (Table 1).

Generally, the enzyme showed high total activities at all conditions and time points (as long as its substrate was present), with a maximum total activity of about 125 U/mg (between 2 and 4 h with 1.5 M sucrose and 1 U/mL). At the beginning of the reaction (between 0 and 2 h), LaniDSΔN showed a high total activity, but also a comparably high hydrolysis activity. Although the total activity in 0.25 M and 1.5 M sucrose solution was similar in this time period, the hydrolysis activity was higher for the reactions with 0.25 M sucrose than for the reactions with 1.5 M sucrose. The higher hydrolytic activity at the beginning of the reaction could be attributed to the possibility that only sucrose is initially available as acceptor and transferase activity increases after some acceptor oligosaccharides are formed. However, this hypothesis needs to be confirmed by further experiments. During the course of the reaction, the hydrolysis activity declined, and the transferase activity increased at both sucrose concentrations. Notably, the transferase activities are roughly comparable among all reactions, as long as enough sucrose is present. As expected, the activity rapidly declines at lower sucrose concentrations. This can already be observed for the reaction with 0.25 M sucrose and 0.10 U enzyme/mL, although sucrose is still

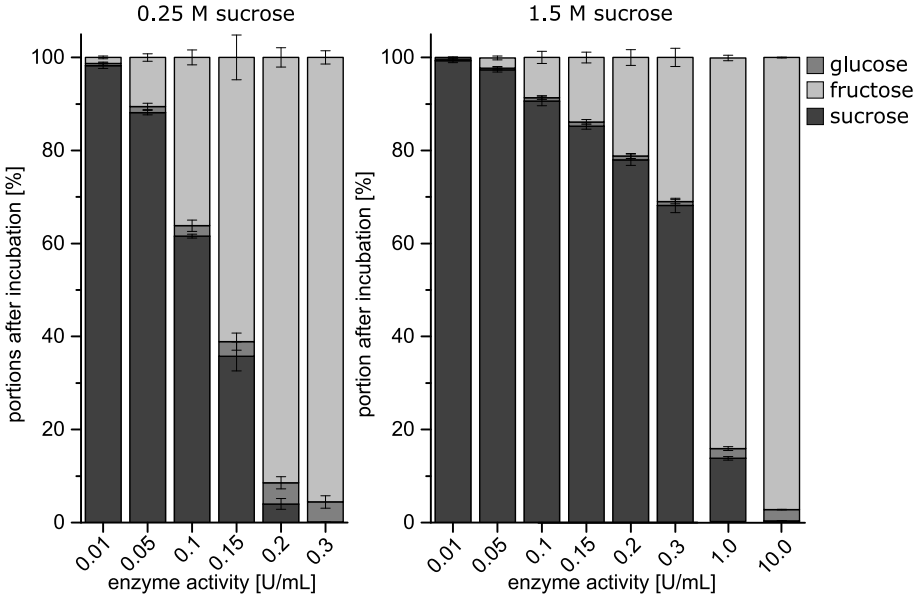

Fig. 2. Portions of fructose, sucrose, and glucose after incubation of 0.25 M and 1.5 M sucrose solutions for 24 h with different activities of LaniDSΔN. Sugars were analyzed by HPAEC-PAD. Analyses were performed in triplicate.

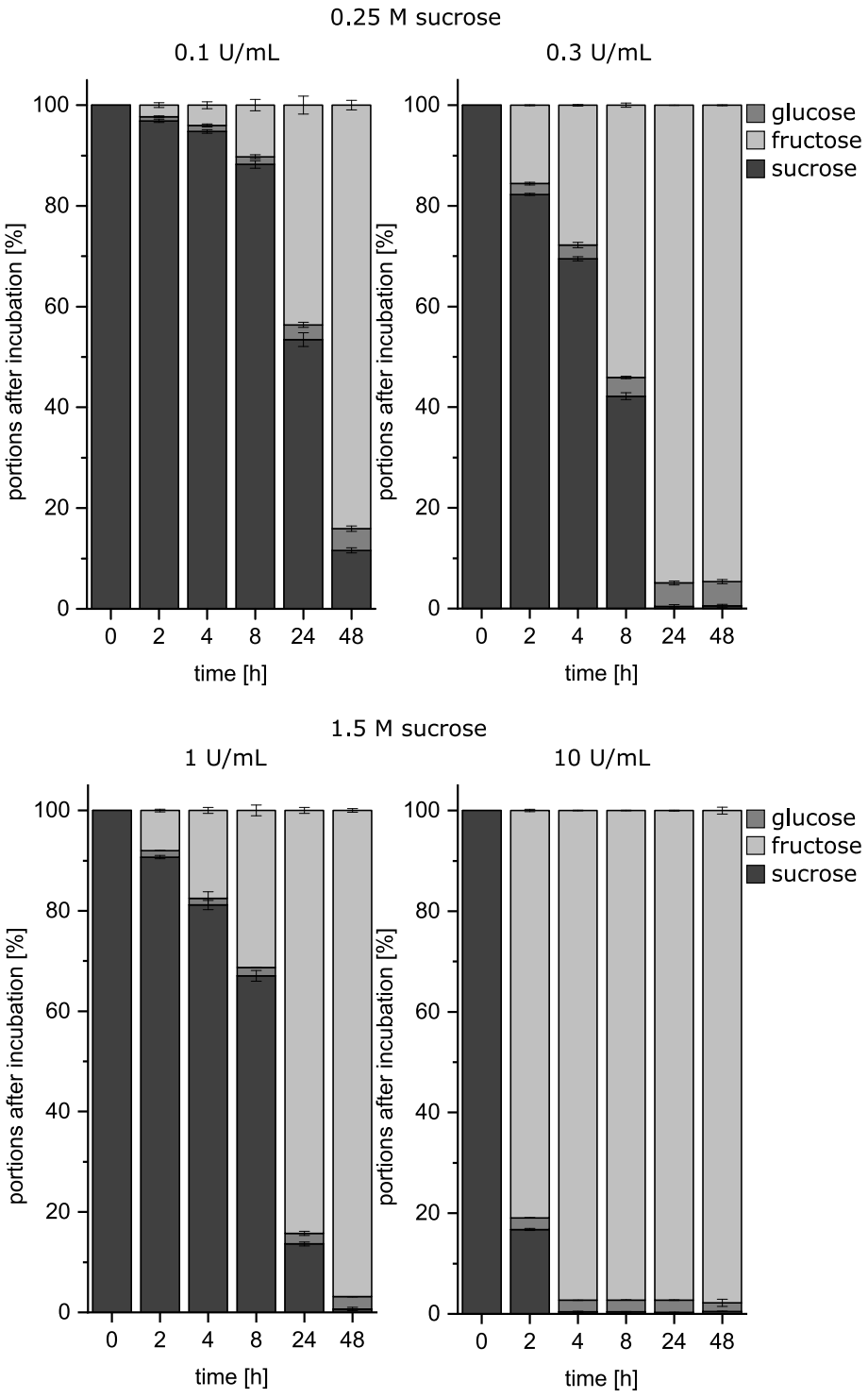

Fig. 3. Portions of fructose, sucrose, and glucose after incubation of a 0.25 M sucrose solution with 0.1 U/mL or 0.3 U/mL and after incubation of a 1.5 M sucrose solution with 1 U/mL and 10 U/mL of LaniDSΔN. Sugar analysis after different time periods was carried out by HPAEC-PAD. Analyses were performed in triplicate.

present after 48 h. Compared to the full-length *L. animalis* TMW 1.971 dextransucrase studied by Rühmkorf et al. [29], the truncated enzyme shows a much higher total activity (108 U/mg vs. 0.5 U/mg). Compared to DSR-M which also showed a comparably high activity of 60 U/mg protein [11], the activity of LaniDSΔN was moderately higher (although differences in the activity assays may impact comparability). Other dextransucrases in the literature showed a clearly lower total activity [29,35–37].

3.2. Dextran production and characterization

For the application of dextrans for copolymer synthesis or investigations on structure-function relationships, the amount of dextran which can be isolated from the reaction mixture is very important. To study the absolute polysaccharide yields, dextrans were prepared by using four different sucrose concentrations (0.25 M, 0.5 M, 1.0 M, and 1.5 M) and different enzyme activities. The additional concentrations were used to evaluate if the dextran yield increases or decreases if more

Table 1
Total activity, hydrolysis activity, and transferase activity of LaniDSΔN calculated from different time periods of a continuous incubation. Reactions were carried out by adding 0.1 U/mL and 0.3 U/mL LaniDSΔN to a 50 mM acetate buffer (pH 6.0) with 0.25 M sucrose and adding 1 U/mL and 10 U/mL LaniDSΔN to a 50 mM acetate buffer (pH 6.0) with 1.5 M sucrose. The different activities were calculated as follows from the amounts of glucose and fructose: total activity (U/mg protein) = fructose released (μmol/mg protein * min); hydrolysis activity (U/mg protein) = glucose released (μmol/mg protein * min); transferase activity (U/mg protein) = total activity – hydrolysis activity. Analyses were performed in triplicate.

Time period (h)	Total activity (U/mg)	Hydrolysis activity (U/mg)	Transferase activity (U/mg)
0.25 M sucrose (0.1 U/mL)			
0–2	46.2 ± 9.7	15.8 ± 3.8	30.4 ± 13.4
2–4	34.1 ± 5.0	7.4 ± 2.6	26.8 ± 7.5
4–8	62.0 ± 4.6	3.6 ± 1.1	58.3 ± 5.5
8–24	83.2 ± 2.1	3.2 ± 0.2	80.0 ± 2.3
24–48	67.3 ± 1.6	2.3 ± 0.3	65.0 ± 1.8
0.25 M sucrose (0.3 U/mL)			
0–2	108.2 ± 6.6	15.1 ± 2.6	93.1 ± 4.4
2–4	84.76 ± 5.0	3.9 ± 1.3	80.8 ± 5.8
4–8	100.1 ± 5.9	4.1 ± 0.7	96.1 ± 6.6
8–24	34.9 ± 2.2	0.7 ± 0.1	34.2 ± 2.2
24–48	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
1.5 M sucrose (1 U/mL)			
0–2	109.9 ± 1.5	17.5 ± 0.7	92.4 ± 1.5
2–4	125.7 ± 10.7	0.1 ± 5.9	125.6 ± 4.9
4–8	92.5 ± 9.4	2.6 ± 2.7	90.0 ± 6.7
8–24	66.3 ± 1.5	0.3 ± 1.2	66.0 ± 0.7
24–48	15.1 ± 2.9	0.4 ± 0.5	14.7 ± 2.6
1.5 M sucrose (10 U/mL)			
0–2	91.6 ± 0.6	2.6 ± 0.1	89.0 ± 0.5
2–4	4.5 ± 7.0	0.0 ± 0.0	4.5 ± 7.0
4–8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
8–24	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
24–48	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

substrate is available. Different enzyme activities were examined to determine if the results of the sugar analysis correspond to the actual yields of dextran (low molecular weight compounds might be removed by dialysis). Fig. 4 summarizes the yields of the dextrans from the different reaction mixtures.

The results confirmed that LaniDSΔN is suited to produce dextrans in very high absolute yields. The highest absolute yield (214.9 g/L) was obtained from an incubation of a 1.5 M sucrose solution with 10 U/mL enzyme. When 0.25 M, 0.5 M and 1.5 M sucrose solutions were used, the theoretically possible absolute yields were almost reached. Although some fructose may be present at the reducing end of the dextrans [11],

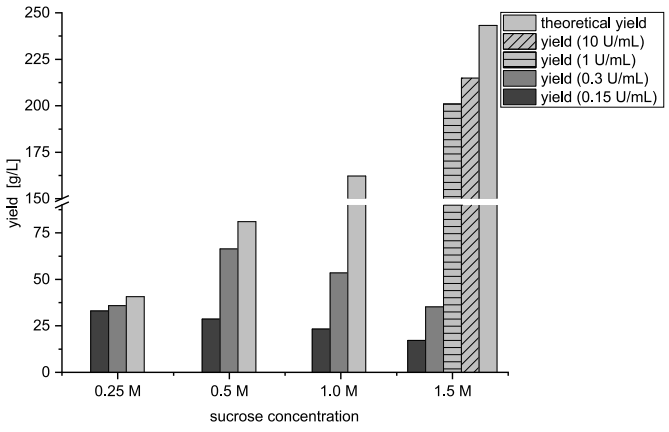

Fig. 4. Yields of dextrans obtained after incubation of 0.25 M, 0.5 M, 1.0 M, and 1.5 M sucrose solutions for 24 h with different activities of LaniDSΔN. Absolute yields were determined gravimetrically. The numerical values as well as the yields based on sucrose conversion are shown in Table S3.

the data on the molecular weights (see next section) suggest that this should not significantly alter the dextran amounts. Assuming that the isolated polysaccharides exclusively contain glucose, the products contain 7.0–88.4 % of the glucose initially present in sucrose (Table S3). The small difference between the actual and the theoretical yield can be attributed to the hydrolysis of sucrose (see also Fig. 3). Furthermore, smaller oligo- and polysaccharides could have been lost as a result of the dialysis with a molecular weight cut-off of 3.5 kDa. Notably, applying 1 U enzyme/mL also results in a yield of 201 g/L. Therefore, only 10 % of the enzyme still provide a high yield and it is likely that a higher yield could be achieved by extending the incubation period. For the 0.5 M, 1.0 M, and 1.5 M sucrose solution, doubling the enzyme amount from 0.15 U/mL to 0.3 U/mL roughly resulted in doubled yields. However, the amount of dextran obtained with these enzyme activities decreased with increasing sucrose concentrations and the lower sucrose concentrations resulted in higher dextran yields. Therefore, the higher sucrose concentration may lead to substrate or product inhibition [38,39]. However, it is also possible that increased amounts of low molecular weight products are formed at higher sucrose concentrations which are subsequently removed during the sample workup. Nevertheless, a complete conversion of sucrose can be achieved by adjusting the enzyme activity. As described in the introduction, absolute dextran yields up to 63 g/L were described in the literature by using fermentative production and sucrose concentrations of 0.03–0.87 M [21,40]. Higher dextran yields can be obtained by using recombinant dextransucrases such as the *Leu. mesenteroides* NRRL B–512F dextransucrase variants which showed yields of up to 81.6 g/L (glucose conversion up to 75 %) with 0.73 M sucrose solution [22]. However, LaniDSΔN showed an even higher dextran yield. Only DSR-M showed comparable although slightly lower absolute/relative yield than LaniDSΔN (DSR-M: transfer of 84 % glucose which equals 39.7 g/L with 0.292 M sucrose solution; LaniDSΔN: transfer of 88 % glucose, 36.0 g/L with 0.25 M sucrose solution) [11,12]. It is of course possible that this or the other enzymes with a high transglycosylation activity could give similar absolute yields if the incubation conditions are adjusted. Nevertheless, LaniDSΔN is a very promising enzyme to produce dextran in very high yields.

In preliminary experiments, we observed that dextrans produced by LaniDSΔN cannot be redissolved in water if they are precipitated with ethanol after enzyme incubation. Therefore, the sample preparation was modified, and it was shown that the most suitable procedure is to directly dialyze the entire incubation mixture and then freeze-dry the obtained solution. The dextran obtained from this sample workup was readily soluble in water. However, we observed that solubility decreased after prolonged storage of the dried dextran which is most likely caused by the formation of ordered structures. Therefore, further analysis of the dextran samples was carried out directly after production.

The structure of LaniDSΔN dextrans was analyzed by using partial enzymatic hydrolysis with *endo*-dextranase. This enzyme cleaves the 1,6-linked backbone of dextrans to isomaltose and glucose. However, the presence of branched sections leads to the formation of branched oligosaccharides which can be separated and detected by HPAEC-PAD [30,41]. For comparison, a commercial dextran from *Leuconostoc* spp. as well as the dextran produced by *Li. reuteri* TMW 1.106 dextransucrase were also hydrolyzed and analyzed. The corresponding hydrolysates contained different previously characterized oligosaccharides derived from ramifications at position *O*3 and *O*4, respectively. The HPAEC-PAD chromatograms obtained from *endo*-dextranase hydrolysis of LaniDSΔN dextrans and the reference dextrans are shown in Fig. 5.

In contrast to the branched reference dextrans, branched oligosaccharides were not detected in the hydrolysate of LaniDSΔN dextrans. The detection of isomaltose as well as the complete degradation of the high molecular weight material clearly demonstrated that completely linear dextrans are produced by LaniDSΔN. These results were also confirmed by NMR spectroscopy (Fig. S1) and methylation analysis (Fig. S2).

To obtain information about the molecular size of the dextrans

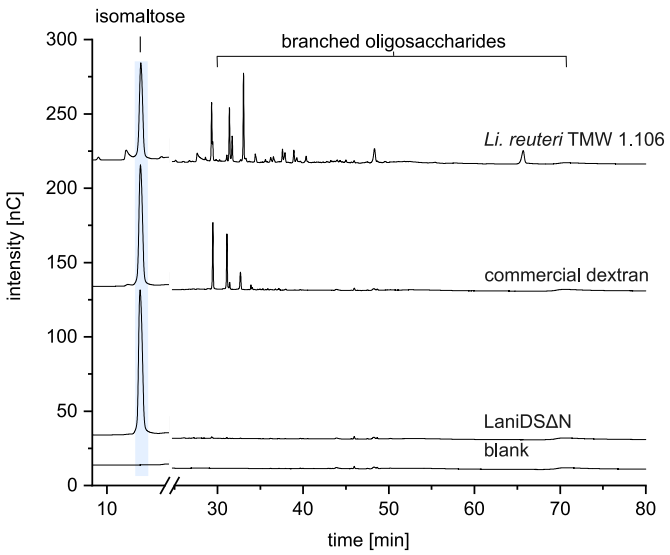

Fig. 5. HPAEC-PAD chromatograms of commercial dextran from *Leuconostoc* spp., dextrans produced by the dextransucrase from *Li.* reuteri TMW 1.106, and dextrans produced by LaniDSΔN after 24 h of incubation with 5 U/mg *endo*-dextranase.

formed, they were analyzed by using HPAEC-PAD and dextran size was estimated by using molecular weight standards (Fig. 6).

In preliminary experiments, we observed that this technique is suitable to resolve low molecular weight dextrans up to a degree of polymerization of 60 and that low molecular weight dextrans show varying retention times (which increases with the degree of polymerization). A molecular weight determination by using HPSEC-RI/MALLS was not possible, because the dextrans only yielded a very low signal intensity due to their (in part) low molecular weight. Even the application of HPSEC columns with a suitable molecular weight range cannot provide a comparable separation range and resolution.

The HPAEC-PAD results clearly show that dextran size increased with decreasing sucrose concentration when 0.15 U/mL were used. Other studies on dextransucrases already showed that the sucrose concentration is an important factor for the size of the produced dextrans [39,42,43]. The dextran formed from a 0.25 M sucrose solution contained only low portions of compounds with a comparably low molecular weight and had a rather narrow molecular weight distribution. Distinct peaks became more prominent with increasing sucrose concentrations, and it was possible to estimate that the dextran produced from 1.5 M sucrose solution had an approximate DP of 20–55. By comparing the elution profiles to the commercial dextran size standards, it can also be concluded that the dextran formed from the 0.25 M sucrose solution is larger than 12 kDa and reaches almost 25 kDa. The dextrans produced from 0.5 M to 1.0 M sucrose solution eluted closer to the 12 kDa standard. However, the enzymatically produced dextrans showed a more narrow elution profile than the commercial standard. Therefore, varying sucrose concentrations can be used to tailor the molecular weight of the dextrans produced by LaniDSΔN. However, sucrose is not completely converted by using 0.15 U/mL, thus, the dextrans obtained with 0.3 U/mL and 0.25 M sucrose as well as the dextrans obtained with 10 U/mL and 1.5 M sucrose were analyzed (complete conversion of sucrose, see Fig. 2). Although roughly comparable elution profiles were observed, the dextrans obtained with 1.5 M sucrose showed slightly higher portions of low molecular weight compounds and the peak maximum was shifted to lower retention times. Consequently, these results confirm that dextran size is influenced by the sucrose concentration. Our results demonstrate that dextrans produced by LaniDSΔN show a rather low molecular weight compared to non-linear dextrans from the literature [10,14,43–46]. In contrast, the full-length LaniDS enzyme was observed to form dextrans with a size range of 90–110 kDa [29]. This discrepancy in size can be attributed, at least in part, to the differing conditions employed in glucan formation. Rühmkorf et al. [29] produced the glucans by using 146 mM sucrose solution and as described above, the dextran size increases with decreasing sucrose concentration. However, the enzyme itself and its activity is most likely to be another important factor: The *N*-terminal domain of LaniDSΔN could lead to a better release of transferase products, while its higher enzyme activity compared to the native LaniDS could lead to the formation of a high number of free oligo- and polymeric products at the beginning of the reaction. In this case, the subsequent elongation of a higher number of initially formed products (compared to the wild type of the enzyme at the same sucrose concentration) would lead to a lower molecular weight. Notably, the molecular weights obtained for LaniDSΔN dextrans were comparable with the data obtained for dextrans from truncated variants of DSR-M which produced dextrans with molecular weights from 11 to 23 kDa [11]. Therefore, the two enzymes produced comparable products. It is noteworthy that Claverie et al. [17] demonstrated that the full-length DSR-M forms a dextran with a molecular weight of 28 kDa, while the *N*-terminally truncated variant DSR-MΔ2 formed a dextran with a molecular weight of 23 kDa. Therefore, the *N*-terminal truncation also led to a molecular weight reduction. Altogether, the defined structure, the limited size of 12–25 kDa, and the narrow size distribution of the LaniDSΔN dextrans makes them suitable candidates for the use in copolymers or as size standards.

3.3. Partial acid hydrolysis of L. animalis dextrans

Due to their completely linear structure, LaniDSΔN dextrans can be hydrolyzed with acid to obtain completely linear dextrans with an even lower molecular weight or IMOs. To investigate the potential of dextrans produced in this study, they were hydrolyzed by using varying TFA concentrations (0.2 M, 0.4 M, 0.6 M) at 95 °C. In order to obtain

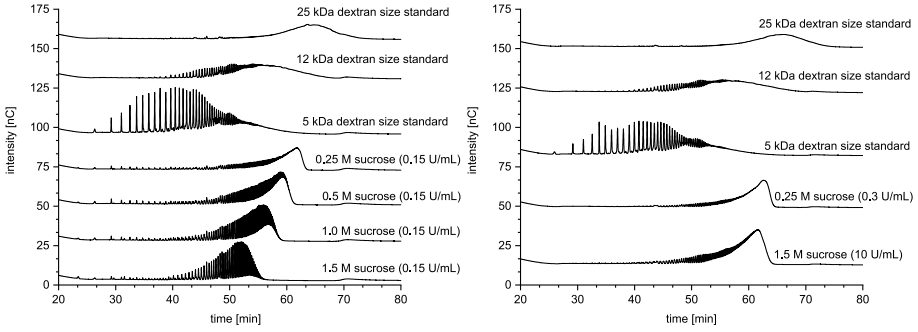

Fig. 6. Left: HPAEC-PAD chromatograms of different commercial dextran size standards as well as the dextrans obtained from incubating 0.15 U/mL LaniDSΔN for 24 h with different sucrose solutions. Right: HPAEC-PAD chromatograms of different commercial dextran size standards as well as of the dextran obtained by incubating a 0.25 M sucrose solution with 0.3 U/mL and by incubating a 1.5 M sucrose solution with 10 U/mL.

comparable results, all hydrolyses were carried out in Schott Duran bottles (50 mL or 100 mL) and in a shaking water bath. Due to the continuous mixing and the good heat transfer provided by the water, this method proved to be preferable to a drying oven. The products formed by partial acid hydrolysis were again analyzed by HPAEC-PAD. To compare the hydrolysis profiles, the peaks corresponding to low molecular weight dextrans and isomalto-oligosaccharides were integrated and the obtained areas were used to describe the product spectra. However, it must be emphasized that the results do not quantitatively reflect the oligo-/polysaccharide composition, because the individual compounds have different PAD response factors. A comparison of the products obtained from the different acid concentrations demonstrated that using 0.2 M TFA is well suited to obtain mixtures of low molecular weight dextrans/IMOs without the excessive formation of glucose or side products (Fig. 7).

The latter were most likely formed due to reversion reactions under the acidic solution. The application of 0.4 M TFA yielded rather small products after short hydrolysis times and significant portions of degradation products and glucose after longer incubation times (Fig. S3). As expected, 0.6 M TFA solution led to an even stronger hydrolysis and undesirable degradation products (Fig. S4). Furthermore, significant amounts of glucose were already formed after 6 h. For the hydrolysates obtained with 0.2 M TFA, the portions of dextrans decreased with increasing incubation time, while the portions of IMOs increased. Therefore, a hydrolysis time of 2 h is well suited to obtain low molecular weight dextrans, while high amounts of IMOs can be produced by using a hydrolysis time of 6 h or 8 h. During partial acid hydrolysis, the LaniDSΔN dextrans were completely converted into low molecular weight products or IMOs. However, this was only achieved as long as the dextran sample used was water-soluble. Although significant amounts of hydrolysis products were obtained, an insoluble residue remained when insoluble dextrans were used. Therefore, the ordered structures formed during prolonged storage cannot be broken up by using diluted acid. Consequently, it is important to ensure that the LaniDSΔN dextrans are as soluble as possible (e.g. by using them directly after the synthesis) to achieve high yields of hydrolysis products. To investigate whether the length of the dextrans has an influence on the composition of the hydrolysates, LaniDSΔN dextrans synthesized by using different sucrose solutions were hydrolyzed under identical conditions. The results are summarized in Fig. S5. The results clearly demonstrate that the different molecular weights of the dextrans do not influence the composition of the hydrolysate. To investigate if the partial acid hydrolysis can be scaled up to a higher volume (up to this point, 25 mL solution were hydrolyzed in a 50 mL flask), a higher volume (50 mL) and different flasks were used (Fig. S6). The composition of the individual hydrolysates was almost identical, thus, partial acid hydrolysis of LaniDSΔN dextrans is scalable and a higher yield can be obtained.

4. Conclusion

The initial hypothesis that *N*-terminal truncation of *L. animalis* dextransucrase leads to increased activity was confirmed. The enzyme also showed a very high transferase activity. Not only in solutions with comparably low sucrose concentrations (0.25 M) but also with high concentrations (1.5 M), sucrose was completely consumed, and glucose was almost completely transferred into dextrans (yield with regards to the transfer of glucose: up to 95.5 %). Thus, the theoretically possible yield was almost reached in some cases. The absolute yields (up to 215 g/L) were higher than the fermentative and enzymatical yields described in the literature, making LaniDSΔN a very promising candidate for dextran production. This is also supported by its good expression in *E. coli* and its high specific activity. The amount of enzyme obtained from 1 L of *E. coli* culture in this study could be used to incubate about 7.5 L of 0.5 M sucrose solution. Assuming the yields obtained in this study, this would result in 498 g dextran. Furthermore, it must be emphasized that the size distribution can be easily modulated by using different sucrose concentrations. In addition, a completely linear dextran yields a very defined structure and may be used in new applications. One of them is the production of defined low molecular weight dextrans and IMOs by partial acid hydrolysis which was successfully applied in this study. By adjusting the hydrolysis times, it was possible to control the molecular weight distribution of the products and a subsequent separation by GPC could be used to obtain standard substances or dextrans with a very low polydispersity. Altogether, future studies could focus on further applications of LaniDSΔN dextrans.

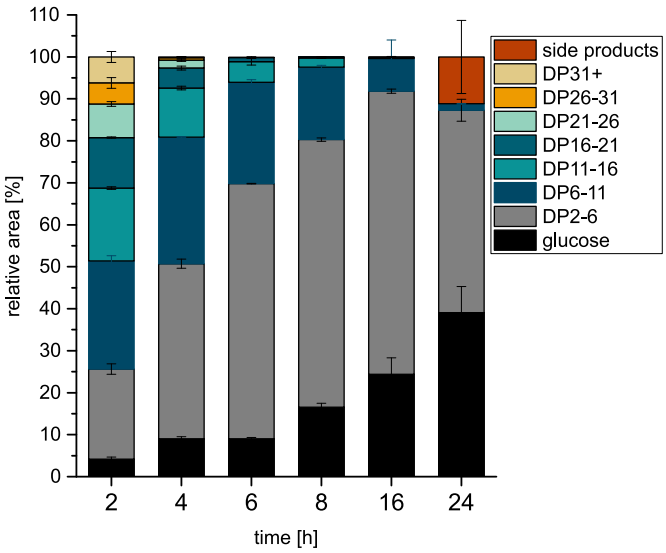

Fig. 7. Relative areas of low molecular weight dextrans and isomalto-oligosaccharides after hydrolysis of a 10 mg/mL LaniDSΔN dextran solution with 0.2 M TFA for different times. Oligo- and polysaccharides were analyzed by HPAEC-PAD and areas were determined by integration of the individual signals. The individual areas do not quantitatively reflect the oligo-/polysaccharide composition because the individual compounds have different PAD response factors. All analyses were carried out in duplicate.

CRediT authorship contribution statement

Oliver Müller: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Daniel Wefers:** Writing – review & editing, Supervision, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carres.2024.109284.

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