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REVIEW PAPER

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Dextran dextrinase and dextran of *Gluconobacter oxydans*

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Abstract Certain strains of *Gluconobacter oxydans* have been known since the 1940s to produce the enzyme dextran dextrinase (DDase; EC2.4.1.2)—a transglucosidase converting maltodextrins into (oligo)dextran. The enzyme catalyses the transfer of an α1,4 linked glucosyl unit from a donor to an acceptor molecule, forming an α1,6 linkage: consecutive glucosyl transfers result in the formation of high molecular weight dextran from maltodextrins. In the early 1990s, the group of K. Yamamoto in Japan revived research on DDase, focussing on the purification and characterisation of the intracellular DDase produced by *G. oxydans* ATCC 11894. More recently, this was taken further by Y. Suzuki and coworkers, who investigated the properties and kinetics of the extracellular DDase formed by the same strain. Our group further elaborated on fermentation processes to optimise DDase production and dextran formation, DDase characterisation and its use as a biocatalyst, and the physiological link between intracellular and extracellular DDase. Here, we present a condensed overview of the current scientific status and the application potential of *G. oxydans* DDase and its products, (oligo)dextrans. The production of DDase as well as of dextran is first described via optimised fermentation processes. Specific assays for measuring DDase activity are also outlined. The general characteristics, substrate specificity, and mode of action of DDase as a transglucosidase are described in detail. Two forms of DDase are produced by *G. oxydans* depending on nutritional fermentation conditions: an intracellular and an extracellular form. The relationship between the two enzyme forms is also discussed. Furthermore, applications of DDase, e.g. production of (oligo)dextran, transglucosylated products and speciality oligosaccharides, are summarized.

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Introduction

Brewing bacteriologists have recognised that certain varieties of acetic acid bacteria, known in the 1940s as "*Acetobacter viscosum*" and " *Acetobacter capsulatum*", were often associated with the type of spoilage of beer known as "ropiness". The production of ropiness by these bacteria apparently depends upon their capacity to form slime from dextrin, a natural constituent of beer. It was found that cultures of *A. viscosus* and *A. capsulatus* became highly viscous in dextrin-rich beer or in a medium of yeast extract containing dextrin, but not in beer devoid of dextrin or in yeast extract media in which dextrin was omitted or was replaced by glucose, fructose, maltose (G2) or sucrose. Slime formation in dextrin-rich media was first reported in 1898, as a differential feature of the related *Bacterium* (*Acetobacter*) *industrium* strain [21]. Little had been recorded of the nature of the slimy material produced by these acetic acid bacteria associated with ropy beer, apart from an early statement that the material was "of the nature of a dextran", i.e. a polyglucoside [21].

In 1949, Hehre and Hamilton [7] reported that the viscous materials were similar to the dextran synthesised by dextransucrase (DSase) of *Leuconostoc mesenteroides*. Hehre [6] also reported that a cell-free enzyme, prepared from an *A. capsulatus* culture by ammonium sulphate precipitation and removal of cells, could synthesise dextran from maltoheptaose, maltoheptaoate, or partial hydrolysates of amylose, amylopectin and glycogen. For this unique enzyme system of *A. capsulatus*,

which was able to convert chains of α(1,4)-linked glucose units into new chains of α(1,6)-linked units, the name "dextran dextrinase" (DDase) seemed most appropriate [6, 10]. The reason why this logical name was altered to "dextrin dextranase" in certain more recent publications remains unclear. From the 1950s until about 1990 few scientific reports dealing with this unusual bacterial enzyme (EC 2.4.1.2) were published.

Production of DDase

We have studied DDase production in shake flask cultures and in laboratory fermentors up to the 60 L scale. Conventional and statistical optimisation procedures were used successfully for the improvement of intracellular DDase ($DDase_{int}$) production by *Gluconobacter oxydans* ATCC 11894 [14, 18]. The extent of growth and enzyme formation was studied in fermentations with various carbon and nitrogen sources. Glycerol or mannitol, and mycological peptone resulted in highest enzyme yields (Fig. 1). As early as 1958, a patent already stated that polyhydric alcohols such as sorbitol, mannitol and glycerol are excellent substrates for DDase production by *G. oxydans* [10]. A typical fermentation profile on glycerol/peptone medium standard fermentation (SF) is presented in Fig. 2. *Gluconobacter* growth reaches a maximum within 24 h, while the initial pH of 5.5 drops gradually to 4.0 over the same timespan. Glycerol is used for growth, but considerable levels of dihydroxyacetone (DHA) are formed; glyceric acid is also a typical product, accumulating at the onset of the log phase.

The $DDase_{int}$ formation profile indicates that the enzyme is produced from the onset of the log phase and further parallels the active growth phase (Fig. 2).

The effect of glycerol concentration, mycological peptone concentration, and initial pH, on $DDase_{int}$ production was investigated by means of a five-level three-factor central composite rotatable design, and optimal fermentation parameters were determined. The optimised fermentation process showed a 3-fold increase in enzyme yield, and could be the starting point for scale-up purposes or enzyme purification experiments [18].

The uncontrolled pH course in the standard $DDase_{int}$ fermentation was demonstrated to be beneficial for $DDase_{int}$ production by *G. oxydans*, as fermentations with buffered pH values all resulted in inferior enzyme recoveries. Bacterial growth and the resulting $DDase_{int}$ yield were improved by increased culture aeration and agitation. The effect of maltooligosaccharide and isomaltooligosaccharide addition to the SF medium was most intriguing: intracellular DDase yields significantly and suddenly decreased in the presence of these oligosaccharides, while the enzyme was detected mainly extracellularly (Fig. 3). Whether, under these conditions, *G. oxydans* shifts from intracellular to extracellular DDase production, or whether the intracellular stock of DDase is rapidly secreted into the culture medium, is still a matter of debate [14].

Assays for DDase activity

The usefulness of several activity assays for the quantification of $DDase_{int}$ has been evaluated by the authors [15]. The conventional dinitrosalicylic acid (DNS) assay, and the viscosity build-up assay, are based on the conversion of maltodextrins into dextran by the action of $DDase_{int}$. The sensitivity of the DNS assay was shown to be low, and the measurements only of poor accuracy. This general carbohydrate determination method was especially hampered by the interference of unreacted maltodextrins in the reaction mixtures. A rheological $DDase_{int}$ quantification assay could not be used routinely as the maltodextrin/dextran mixtures displayed complex non-Newtonian and time-dependent flow behaviour.

A new DDase activity assay based on discrete transglucosylation reactions catalysed by the enzyme

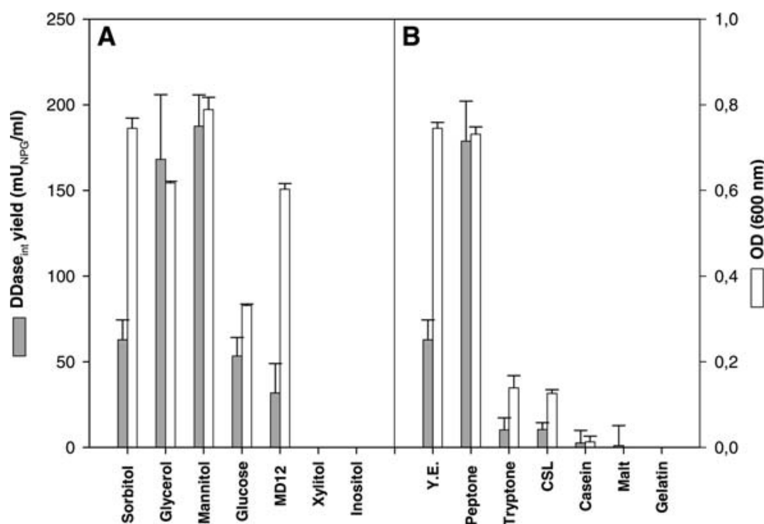


Fig. 1 **a** Influence of the carbon source on intracellular dextran dextrinase ($DDase_{int}$) yield and on *Gluconobacter oxydans* growth. **b** Influence of the nitrogen source on $DDase_{int}$ yield and on *G. oxydans* growth with glycerol as carbon source. *MD12* Maltodextrins with a dextrose equivalent (DE) of 12, *YE* yeast extract, *CSL* corn steep liquor

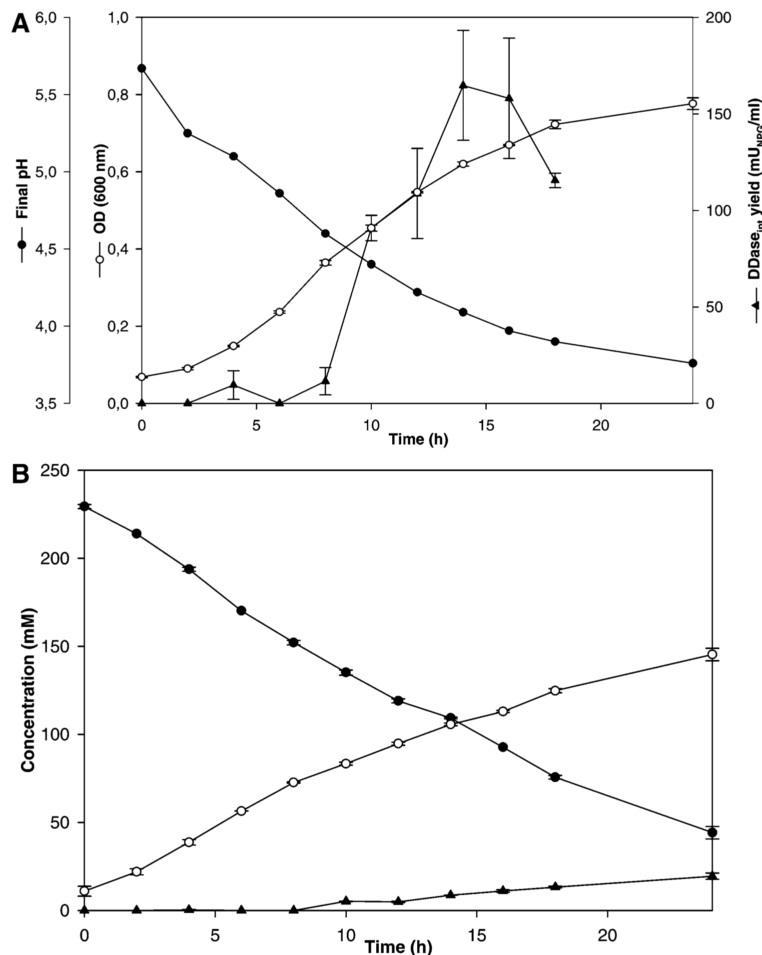


Fig. 2 **a** Medium acidification (*filled circles*), DDase$_{int}$ yield (*filled triangles*) and *G. oxydans* growth (*open circles*) during glycerol/peptone fermentation. **b** Oxidation of glycerol (*filled circles*) to dihydroxyacetone (*open circles*), and formation of glyceric acid (*filled triangles*) during glycerol/peptone fermentation

upon incubation with maltose (G2) as a substrate was developed [14, 15]. The DDase$_{int}$ converted G2 into glucose, panose [α-D-glucose-(1,6)-α-D-glucose-(1,4)α-D-glucose] and higher oligosaccharides. Panose was shown to be a reliable indicator for DDase$_{int}$ transglucosylation activity. The non-linear increase in the trisaccharide concentration during DDase$_{int}$ action suggested an activity assay based on the measurement of the initial rate of panose synthesis. This assay enabled clear distinction between the transglucosylation activities of different DDase$_{int}$ solutions. The G2 was transglucosylated to panose and higher oligosaccharides during incubation with *G. oxydans* cell extracts; a major fraction of the disaccharide was also converted to glucose through an unidentified hydrolytic activity also present in the crude enzyme preparation initially used. The DDase$_{int}$ quantification based on panose formation would have been of low value if DDase$_{int}$ was also the enzyme responsible for G2 hydrolysis. When the G2 concentration in the reaction mixture was increased, higher transglucosylation/hydrolysis ratios were observed. This indicated that the two activities were interdependent, and that it was improbable that the hydrolysis activity originated from a contaminating (amylo)glucosidase in the *G. oxydans* cell extracts. The DDase nature of the hydrolytic activity was further confirmed by starch zymogram analysis of our enzyme preparation. None of the proteins present in the cell extract, apart from DDase$_{int}$, was capable of starch and G2 degradation requiring α(1,4) linkage hydrolysis.

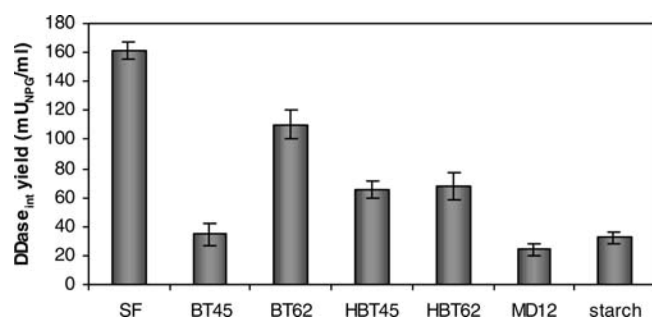


Fig. 3 DDase$_{int}$ yield in standard fermentation (*SF*) and in fermentations supplemented with different oligosaccharides or starch. *BT45* Isomaltooligosaccharides containing panose (30%), maltose (16%), isomaltotriose (5%), isomaltotetraose (3%), glucose (0.3%) and unspecified branched oligosaccharides and higher oligomers; *BT62* isomaltooligosaccharides containing panose (2%), isomaltose (34%), maltose (5%), isomaltotriose (19.8%), isomaltotetraose (8.5%), glucose (0.4%) and unspecified branched oligosaccharides and higher oligomers; *HBT45* hydrogenated BT45 (70% dry matter); *HBT62* hydrogenated BT62 (70% dry matter), *MD12* maltodextrins with a DE of 12, *starch* soluble starch (Merck, Darmstadt, Germany) degraded by acid hydrolysis

A second reliable $DDase_{int}$ assay based on the release of nitrophenol from *p*-nitrophenyl-α-D-glucopyranoside (NPG), mimicking G2 utilisation by the transglucosidase, was developed [15]. The $DDase_{int}$ specificity of the test was proven via an NPG zymogram. The NPG assay was preferred to the panose assay, although both were capable of accurately distinguishing between $DDase_{int}$ preparations of varying activity. The release of nitrophenol from NPG gave a more complete indication of $DDase_{int}$ action by quantifying transglucosylation as well as hydrolysis, and was far less time-consuming and laborious than the panose assay. The assay used for extracellular DDase ($DDase_{ex}$) is discussed below in Extracellular DDase of *G. oxydans*.

General characteristics of $DDase_{int}$, substrate specificity and mode of action

$DDase_{int}$ purfication

Yamamoto et al. [28] initially found that *A. capsulatus* ATCC 11894 produced DDase mainly intracellularly, and purified the enzyme with a yield of 49.4% by sequentially applying, *n*-butanol extraction, Phenyl-Toyopearl chromatography, and Toyopearl HW-65S gel filtration with ethylene glycol as the solvent of elution. This suggests that the enzyme is hydrophobic and stable in these solvents. The molecular weight of the enzyme was estimated at 300 kDa, by SDS-PAGE. The optimum temperature and pH were 37–45°C and 4.0–4.2, respectively. The enzyme retained its original activity up to 45°C, and was stable within the pH range of 3.5–5.2 at 30°C for 30 min. Fe^{3+} was found to strongly inhibit enzyme activity.

Substrate specificity of $DDase_{int}$

Dextran synthesis has been tested with glucose, various maltooligosaccharides, short chain amylose and soluble starch as substrates [14, 28]. Intracellular DDase could synthesise dextran from all substrates except glucose and G2 (Table 1). The level of dextran synthesised by DDase increased with expanding substrate chain length. The DDase reacted with G2 slightly, and produced glucose and panose, but no polymer formation was obtained [14, 15, 28].

The donor specificity of DDase was examined using salicin [2-(hydroxymethyl)phenyl-β-D-glucopyranoside] as an acceptor compound [35]. Salicin contains a β-glucosidic residue, and could not be acted on solely by DDase. Glucosyl residues were transferred by DDase action to salicin from G2, isomaltose, starch and dextran, which have non-reducing terminal α(1,4)-linked or α (1,6)-linked glucosyl residues (Table 2). When the acceptor characteristics of various sugars were investigated using starch as a glucosyl donor compound, DDase transferred glucosyl residues to saccharides that had glucosyl residues or xylosyl residues at non-reducing termini. When D-glucose was used as an acceptor, G2 and isomaltose were formed; when D-xylose was used, DDase formed only α(1,4) linkages. In both cases, the yields of these transfer products were low. Furthermore, DDase transferred glucosyl residues from starch to various derivatives of D-glucose, which were substituted at the C-2, C-3 or C-6-hydroxyl group or deleted at the C-5-hydroxymethyl group. Epimers of D-glucose, such as D-mannose, D-allose and D-galactose, did not act as acceptors.

Table 1 Dextran formation from various substrates by purified *Gluconobacter oxydans* intracellular dextran dextrinase ($DDase_{int}$) [28]

Substrate	Dextran yield (%)
Glucose	0
Maltose	0
Maltotriose	11.0
Maltotetraose	13.4
Maltopentaose	25.0
Maltohexaose	30.2
Short chain amylose	57.6
Soluble starch	21.4

Mode of action of $DDase_{int}$ on maltooligosaccharides

The detailed action mechanism of DDase of *G. oxydans* ATCC 11894 was also investigated by Yamamoto et al.

Table 2 Donor and acceptor specificity of *G. oxydans* $DDase_{int}$ on various oligosaccharides, methyl-,α-, and β-D-glucosides, starch and dextran [35]

Compound	Transfer products[b]	
	With salicin	With starch
Kojibiose	–	+
Sophorose	–	+
Nigerose	–	+
Laminaribiose	–	+
Maltose	+	+
Cellobiose	–	+
Isomaltose	+	+
Gentiobiose	–	+
Trehalose	–	–
D-Glucosyl-α(1,4)-D-xylose	–	+
Sucrose	–	+
Raffinose	–	–
Xylosucrose	–	+
Isoprimeverose[a]	–	+
Lactose	–	–
Melibiose	–	–
Methyl-α-D-glucoside	–	+
Methyl-β-D-glucosides	–	+
Salicin	Not tested	+
Starch	+	Not tested
Dextran	+	Not tested

[a] Isoprimeverose = D-xylosyl-α(1,6)-D-glucose
[b] + Transglucosylation products formed, – no transfer product formed

[30]. By examination of the reactivity of DDase on maltotetraitol (G4H) and *O*-6-deoxy-6-[2-pyridyl-amino]-α-D-glucopyranosyl-(1,4)-maltotriose [a derivative of maltotetraose (G4), of which only the non-reducing terminal glucosyl residue is modified], DDase was demonstrated to react with non-reducing terminal glucosyl residues of substrates.

These data supported the mode of action of DDase suggested in 1951 by Hehre [6]: non-reducing terminal α(1,4)-glucosyl residues are transferred to dextran, forming α(1,6)-linkages. Naessens and Vandamme [15] confirmed these transglucosylation and hydrolysis activities of $DDase_{int}$ with several donor and acceptor substrates. This is actually the main mode of action. The formation of glucose and panose from G2 is catalysed via the same mechanism; however, this reaction is rather slow. In addition to the main mode of action, secondary modes of action of DDase have also been proven, as illustrated in Fig. 4.

Thus, DDase functions in three main transglucosylation modes:

1. Transfer of an α(1,4)-linked glucosyl group to an acceptor, with formation of an α(1,6)-linkage (main mode of action).
2. Transfer of an α(1,4)-linked glucosyl group to an acceptor, with formation of an α(1,4)-linkage (disproportionation action on maltooligosaccharides).
3. Transfer of an α(1,6)-linked glucosyl group to an acceptor, with formation of an α(1,6)-linkage (disproportionation action on isomaltooligosaccharides).

Transfer of an α(1,6)-linked glucosyl residue to an acceptor, with formation of an α(1,4)-linkage could not be detected. However, this action might have occurred in a masked fashion, counteracted by the faster reverse action mechanism. The α(1,4)-linkage is thought to form as frequently as the α(1,6)-linkage. However, when G4 was used as a substrate, the maltooligosaccharides produced by action (2) were soon reverted to G4 by action (1), so that the maltooligosaccharides produced by action (2) could be detected only in the initial stages of the DDase reaction. Eventually, products with α(1,4)-linked glucosyl termini were rapidly consumed, and products with α(1,6)-linked glucosyl termini accumulated. Thus, during the DDase reaction, α(1,6)-linked glucosyl residues accumulate so that DDase eventually synthesises dextran from maltodextrins [30].

The initiator to which a glucosyl residue is first transglucosylated, forming an α(1,6)-linkage to begin dextran synthesis, has not yet been confirmed. For example, in the case when G4 was used as a substrate for dextran synthesis, Glc-α(1,6)-G4 might be produced, or various maltooligosaccharides produced from G4 by action (2) might be transglucosylated to Glc-α(1,6)-maltooligosaccharides, and elongation of these molecules to dextran could take place. Thus, the initiator of DDase has not yet been specified, irrespective of whether the substrate used is defined, and in fact several initiators could be used simultaneously in dextran synthesis.

The transglucosylation action of glucosyl units by DDase from non-reducing termini on the substrates to non-reducing termini on the acceptors, would indicate that the structure of "*Gluconobacter* dextran" is a linear glucan, consisting chiefly of α(1,6)-linkages with minor α(1,4)-linkages. However, *Gluconobacter* dextran is in fact frequently branched, as indicated by methylation analysis [33], in spite of its preparation from G4. This fact indicated that transglucosylations from donor substrates to glucosyl residues *not* positioned at non-reducing termini of acceptors, in addition to the transglucosylation to glucosyl residues at non-reducing termini, were catalysed by DDase. However, it remains unclear whether the branching action to form α(1,4):α(1,6)-linked branching points is the result of glucosyl residues being transferred to isopanosyl residues forming α(1,6)-linkages or to isomaltooligosyl residues forming α(1,4)-linkages.

Mode of action of $DDase_{int}$ on reduced maltooligosaccharides

Dextran yield from reduced maltooligosaccharides was higher as compared to yields achieved using the

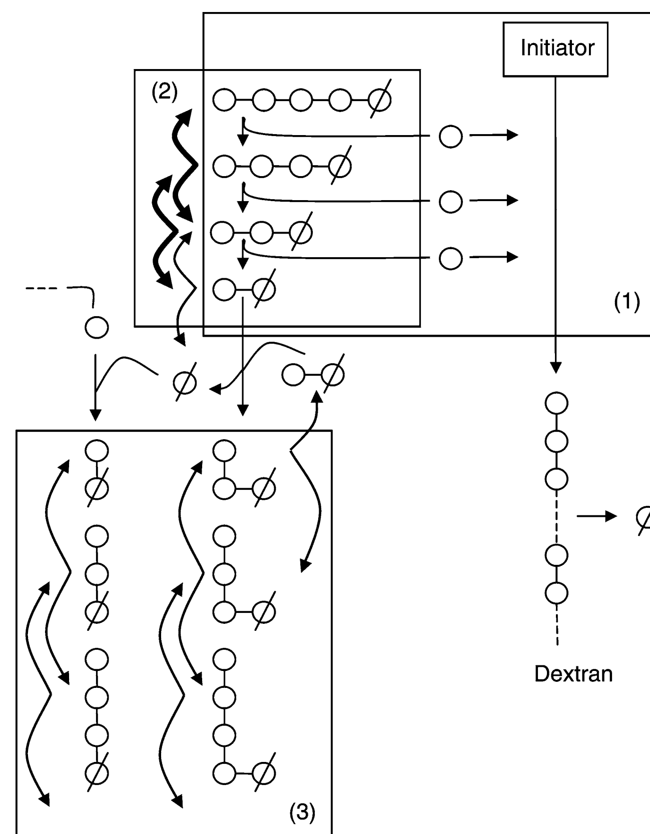


Fig. 4 Summary of $DDase_{int}$ actions: *1* From α(1,4)-linkage to α(1,6)-linkage. Non-reducing terminal glucosyl residues are transferred to acceptors, forming dextran. *2* From α(1,4)-linkage to α(1,4)-linkage. *3* From α(1,6)-linkage to α(1,6)-linkage [30]. ○ Glycosyl residues; ∅ glucose or reducing glucosyl residues; — α(1,4) glucosidic linkages; |α(1,6) glucosidic linkages; *thick* and *thin arrows* indicate fast and slow reactions, respectively

corresponding maltooligosaccharides as starting material [31]. As shown in Table 3, dextrans were formed from maltotriose (G3), G4, maltotritol (G3H) and G4H, and dextran yields from G3H or G4H were clearly higher than from G3 or G4, respectively, in spite of the identical chain length of the substrates. Also, DDase produced some transfer products from G2 or G2H in the absence of dextran. Moreover, the amount of transfer products from G2 was higher than from G2H, and transfer products from G2 or G2H were increased by the addition of dextran. It was thought that G2 or G2H, and especially the transfer products from G2 or G2H, were transglucosylated by DDase action with glucosyl units originating from the added dextran, so that the transfer products were elongated and their level was increased. This coincided with the degradation of dextran and a decrease in dextran yield. Thus, when using maltooligosaccharides as substrates (Fig. 4), dextran is synthesised and G2 accumulates. G2, in turn is transglucosylated, yielding transfer products. This reaction causes the distribution of glucosyl residues among the transfer products and dextran molecules so that the level of transfer products is increased, dextran is degraded and dextran yield, obtained by ethanol precipitation, is decreased.

During the reaction with reduced maltooligosaccharides as substrates (Fig. 5), dextran is likewise synthesised and G2H accumulated. However, transfer products are only rarely produced from G2H, since G2H is not as reactive as G2. Therefore the increase in the level of transfer products from reduced maltooligosaccharides is less than that from maltooligosaccharides. Thus, the difference of DDase reactivity towards G2 and G2H means that dextran yields from maltooligosaccharides and reduced maltooligosaccharides are varied in spite of the identical chain lengths of the substrates. In conclusion, reduced maltooligosaccharides are useful in obtaining higher levels of dextran as compared to maltooligosaccharides [31].

Extracellular DDase of *G. oxydans*

Assay, purification and general characteristics of extracellular DDase

G. oxydans ATCC 11894 also secretes an extracellular dextran dextrinase ($DDase_{ex}$) in culture medium with

Table 3 Dextran yields from maltooligosaccharides and reduced maltooligosaccharides as substrates [31]

Substrate	Dextran yield (%)
Maltose (G2)	0
Maltotriose (G3)	11.0
Maltotetraose (G4)	13.4
Maltitol (G2H)	0
Maltotritol (G3H)	22.4
Maltotetraitol (G4H)	36.8

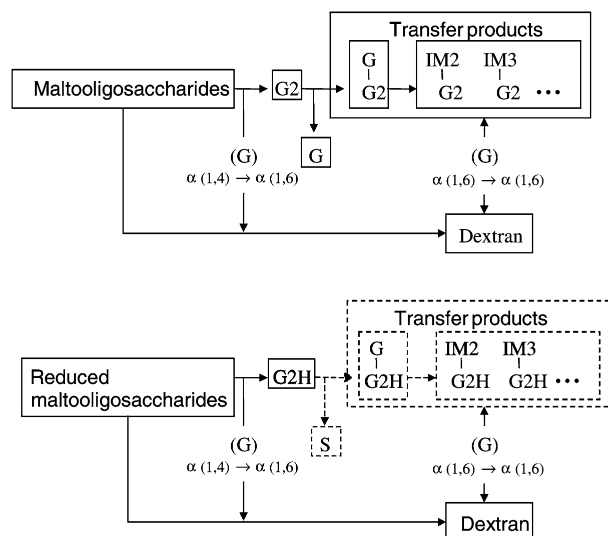


Fig. 5 Comparison of pathways for dextran synthesis by DDase from reduced maltooligosaccharides and from maltooligosaccharides [31]. *G2* Maltose, *G3* maltotriose, *G4* maltotetraose, *G2H* maltitol, *G3H* maltotritol, *G4H* maltotetraitol, *G* glucose, *(G)* transferring glucosyl unit, *S* sorbitol, | α(1,6) glucosidic linkage, *IM2* isomaltose

glucose and a low level (0.05%) of maltodextrins as carbon source. Suzuki et al. [24] purified this enzyme by a simple one-step centrifugation at 20,000 *g* for 20 min at 4°C. The enzyme was tightly bound to the dextran formed. The molecular mass of $DDase_{ex}$ was estimated to be 150 kDa by SDS-PAGE, just one-half of the size of the intracellular variant [24, 28]. The optimum pH and temperature of the purified $DDase_{ex}$ was 5.2 and 38°C, respectively. The enzyme retained its original activity up to 45°C, and was stable in the pH range of 4.1–5.4 at 4°C for 24 h. The $DDase_{ex}$ was completely inactivated by 1 mM Hg^{2+}, Pb^{2+} or $KMnO_4$, and partly inhibited by 1 mM Zn^{2+}, Cd^{2+} or Cu^{2+}. The effect of Fe^{3+} on this enzyme has not been investigated. Quantification of $DDase_{ex}$ activity with the above mentioned NPG assay is problematic, due to the high initial absorbance values of enzyme solutions (lowering the sensitivity of the assay) and dextran interference (lowering enzyme affinity for NPG). These conditions made it difficult to detect the low levels of $DDase_{ex}$ present in culture samples. An alternative $DDase_{ex}$ assay was developed by the authors [14, 17], based on the increase in opalescence (dextran formation) of a maltodextrin solution upon incubation with $DDase_{ex}$. Culture supernatant or enzyme samples could be directly subjected to this turbidity assay, which resulted in accurate and reproducible measurements of enzyme activity as long as the turbidity increase did not exceed 0.2 absorbance units, and as long as pH effects were circumvented. A unit conversion factor was calculated, allowing comparison of activity measurements performed by means of the NPG assay with activities obtained with the turbidity assay.

The general properties of purified $DDase_{ex}$ resemble well those of the intracellular enzyme from *G. oxydans* ATCC 11894, apart from the clearly different molecular masses of the enzymes.

Substrate specificity of $DDase_{ex}$

As early as 1951, Hehre [6] tested the *Gluconobacter* enzyme for its ability to synthesise dextran from a variety of carbohydrates. Products of partial hydrolysis of amylose, amylopectin and glycogen by acid or salivary amylase proved to be suitable substrates, but dextran formation could not be detected from unhydrolysed amylose, amylopectin or glycogen, or from products of their partial hydrolysis by β-amylase. Cycloheptaamylose was not converted to dextran, while the seven-membered linear dextrin (amyloheptaose) derived from it by opening of the cyclic ring by acid, as well as the corresponding dextrinic acid, were active as substrates. None of the potential α-D-glucose donating sugars tested, including G2, sucrose and glucose-1-P, were converted to dextran.

The affinity of purified $DDase_{ex}$ for maltooligosaccharides as glucosyl donors was shown to increase with increasing degrees of polymerisation (DP) of the substrates [25]. The K_m and V_{max} values of $DDase_{ex}$ for different maltooligosaccharides are summarised in Table 4, as are dextran yield increases for oligosaccharides of higher DP.

The conversion of maltooligosaccharides (DP≥6) into dextran by DDase clearly outweighs the conversion efficiency displayed by DSase (Table 4). The maximum dextran yield obtained with *L. mesenteroides* DSase is theoretically 50%, since only the glucosyl moiety of sucrose is utilised for polymer synthesis. Use of DDase could thus be an interesting alternative for industrial dextran production [25].

The acceptor specificity of $DDase_{ex}$ was also examined by Suzuki et al. [26]. $DDase_{ex}$ demonstrated a strong affinity for sugars having non-reducing terminal glucosyl residues linked in either an α(1,4) or α(1,6) fashion. The amount of transfer products increased for oligosaccharides of increasing DP, as shown in Table 5.

Table 4 Kinetic parameters of *G. oxydans* extracellular DDase ($DDase_{ex}$) for maltooligosaccharides, and dextran yields obtained with these maltooligosaccharides as substrates [25]

Oligosaccharide	K_m (mM)	V_{max} (mg dextran mg protein^{-1} min^{-1})	Dextran yield (% (v/v))
Maltotriose	10.2	1.74	22.1
Maltotetraose	6.41	2.56	34.4
Maltopentaose	3.34	2.64	46.2
Maltohexaose	2.59	2.39	52.3
Maltoheptaose	1.66	2.17	58.6
Short chain amylose	0.12	2.23	74.0

Mode of action of $DDase_{ex}$ on maltooligosaccharides

Upon incubation of maltopentaose with $DDase_{ex}$, dextran was synthesised, along with a series of oligosaccharides with α(1,6) linked glucosyl units and an acceptor maltooligosaccharide at the end of the molecule. The $DDase_{ex}$ converted maltooligosaccharides to dextran and isomaltooligosaccharides, and also displayed disproportionation activity towards newly synthesised isomaltooligosaccharides [26]. The transglucosylation actions of $DDase_{ex}$, as proposed by Suzuki et al. [26] were revealed to be quite similar to the action patterns described for the intracellular enzyme. However, $DDase_{ex}$ apparently does not catalyse the disproportionation of maltooligosaccharides.

Relationship between intra-and extracellular DDases of *G. oxydans*

We have examined the relationship between $DDase_{int}$ and $DDase_{ex}$ from *G. oxydans* ATCC 11894 to some extent [16, 17]. The addition of even very low concentrations of maltodextrins (50 mg/L) to the culture medium of *G. oxydans*, rapidly led to only a basal level of $DDase_{int}$. The rapid response of the culture to increasing levels of added maltooligosaccharides in producing $DDase_{ex}$ indicated that the intracellular enzyme became secreted under these conditions. Secretion was not prevented by addition of energy-uncoupling agents to the fermentation medium, suggesting a non-energy requiring secretion mechanism [16, 17].

The level of $DDase_{ex}$ increased to a certain extent with increasing concentrations of added maltodextrins

Table 5 Acceptor specificity of *G. oxydans* $DDase_{ex}$ (expressed relative to the reactivity towards isomaltose) [26]

Sugar	Acceptor specificity
Maltose	7.0
Maltotriose	7.0
Maltotetraose	7.0
Maltopentaose	6.9
Maltohexaose	7.3
Maltoheptaose	7.9
Isomaltose	1.0
Isomaltotriose	2.2
Isomaltotetraose	3.2
Isomaltopentaose	3.7
Isomaltohexaose	4.6
Isomaltoheptaose	5.5
Isomaltulose	3.1
Trehalose	0
Kojibiose	0
Nigerose	0
Sucrose	0
Lactose	0
Melibiose	0
Panose	2.5
Isopanose	5.1
Raffinose	0

in the medium (Fig. 6). Quantification of $DDase_{ex}$ activity in media with a maltodextrin concentration exceeding 20.0 g/L was believed to be inaccurate due to co-precipitating dextran/$DDase_{ex}$ complexes during cell removal. Fermentations with a maltodextrin concentration exceeding 10.0 g/L displayed a higher $DDase_{ex}$ yield than the $DDase_{int}$ yield of SF. Further optimisation or maximisation of DDase production by *G. oxydans* should therefore focus on $DDase_{ex}$ rather than $DDase_{int}$. The real relationship between the intracellular and extracellular DDase variants remains unclear: decreases in $DDase_{int}$ activity in SF supplemented with maltodextrins were not reflected in equally high increases in $DDase_{ex}$ activity.

The different DDase forms elaborated by *G. oxydans* could be assigned to different cellular locations by means of chemical and physical extraction procedures, and by spheroplast examination. Strain ATCC 11894 is endowed not only with an extracellular DDase and a cytoplasmic $DDase_{int}$, but also with an exocellular wall-bound DDase variant and an (amylo)glucosidase, located in the periplasmic space [14, 16, 17].

In vitro experiments to further elucidate the relationship between $DDase_{int}$ and $DDase_{ex}$ revealed that *G. oxydans* cell suspensions liberate $DDase_{int}$ within 10 min in the cellular environment, when incubated in Na-acetate buffer (10 mM, pH 4.8). This process could be a consequence of the low molarity of the buffer used. Here again, the level of activity determined in the cell suspension supernatant was lower than what could have been expected from the decrease in $DDase_{int}$ activity. It seems that the secretion mechanism, or processing of the intracellular enzyme, negatively affect its activity and/or stability. In the absence of maltodextrins and endogenous dextran, the liberated enzyme apparently adsorbed to *G. oxydans* cells. Adhesion was not due to ionic interactions between the enzyme and the cells. *G. oxydans* is thus capable of rapidly secreting $DDase_{int}$ under certain reaction conditions (maltodextrin presence, low molarity environment). However, $DDase_{int}$ and $DDase_{ex}$ are never directly associated with each other, as their profiles are neither parallel nor complementary. It remains unclear whether the high $DDase_{ex}$ activities observed in a fermentation supplemented with 20.0 g/L maltodextrins was indeed a secreted form of the $DDase_{int}$ observed in a maltodextrin-free fermentation. The relationship between the intracellular and extracellular DDases of *G. oxydans* should be further elucidated by determination and comparison of the amino acid sequences of both enzymes and by identification of the encoding gene(s) [14, 16, 17].

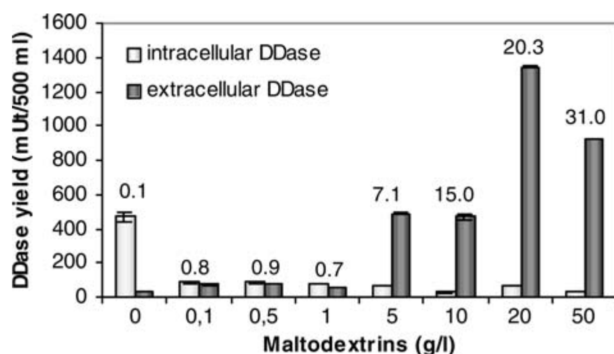


Fig. 6 $DDase_{int}$ and $DDase_{ex}$ yields in fermentations with increasing concentrations of maltodextrins; data labels indicate the ratio $DDase_{ex}$ yield/$DDase_{int}$ yield for each fermentation

Applications of DDase

Production of *G. oxydans* dextran and oligodextrans

Dextran cannot be produced from unhydrolysed starch by DDase. Hehre [6] observed that the arrangements of glucosyl residues as part of a macromolecule made them unsuitable for conversion to dextran by the *Gluconobacter* enzyme. However, when short-chain amylose was used as a substrate, a rather high level of dextran was obtained. These facts suggested that DDase could act on the non-reducing terminal residues of α(1,4)-linked glucosyl linear structures, but not on structures close to branch points in starch or soluble starch.

Patents from the 1950s [8, 9] describe a whole-cell-culture process for dextran production from an acid hydrolysate of starch by an organism of the "*A. capsulatus*" and "*A. viscosus*" group. The rate of dextran formation was increased by agitation and aeration of the culture broth, and by maintaining the pH at about 5.5–6.5 for the first 10–24 h, and thereafter lowering it to about 3.5–5.0 until the fermentation was complete. An additional means of ensuring rapid dextran formation was to start the fermentation at a carbohydrate concentration of not more than 10% and adding a concentrated solution of the carbohydrate material after active fermentation had begun.

Naessens [14] obtained a maltodextrin conversion into dextran of 55% (w/w) with a similar fermentation procedure. Figure 7 shows a typical *G. oxydans* growth profile and the corresponding formation of dextran. The growth of *G. oxydans* stagnated after approximately 50 h of fermentation, most probably because of the acidic culture pH at that stage. It is known that *G. oxydans* growth is hampered at pH values below 3.5–4.0 by an almost complete inhibition of the enzymes of the pentose phosphate pathway. Culture samples were shown to contain gluconic acid, 2-keto-D-gluconic acid, and acetic acid. The problem of culture acidification by *G. oxydans* growth on partially hydrolysed amylaceous material has also been described by Kooi [9]. Thus, when *G. oxydans* is grown on partially hydrolysed amylaceous material, three enzyme systems are produced: (1) DDase, (2) an amylase, which hydrolyses glucose polymers to glucose, and (3) a glucose dehydrogenase, which converts glucose to gluconic acid. During the conversion of amylaceous material to dextran by DDase, the two other enzyme systems are also operative. This results in

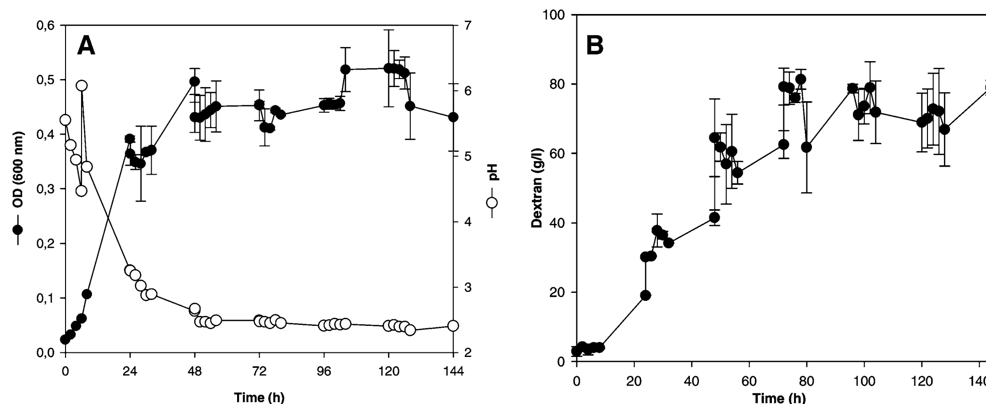


Fig. 7 **a** Growth profile of *G. oxydans* and culture acidification in a submerged dextran fermentation (*Arrow* pH adjustment to 6.0). **b** Dextran formation profile

an accumulation of considerable quantities of gluconic acid, and, unless this acid is continuously neutralised, the pH value of the fermentation broth falls below the optimum value for the synthesis of dextran. This problem can be overcome by the addition of compounds such as sodium cyanide, calcium hypochlorite or sodium bisulfite, which specifically inhibit the glucose dehydrogenase, but which apparently do not inhibit DDase activity [9].

Yamamoto et al. [32] succeeded in improving the dextran yield from starch and from low-degree hydrolysed starch by addition of a debranching enzyme (pullulanase or isoamylase). The debranching enzyme hydrolysed starch at α(1,6) branching points, releasing short-chain amyloses. These amyloses were then converted to dextran by purified intracellular DDase. This cooperation between $DDase_{int}$ and a debranching enzyme resulted in dextran yields of 55 and 60% from starch and low-degree hydrolysed starch, respectively. A Japanese patent mentions yields of 65% and 70% when isoamylase and pullulanase, respectively, were used [4].

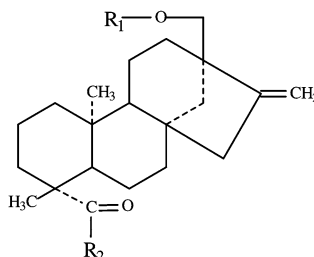
Mountzouris et al. [13] optimised the conversion of maltodextrins to dextran by *G. oxydans* cell suspensions by means of a central composite statistical design. A maximum conversion of 42% was achieved and dextran yield was significantly affected by cell concentration and incubation time.

Suzuki et al. [26] investigated dextran-producing capability using $DDase_{ex}$. The repeated incubation of $DDase_{ex}$ with G3, followed by centrifugal recovery of the biocatalyst with subsequent addition of fresh substrate solutions, resulted in continuous production of polymeric material. Since $DDase_{ex}$ did not display any product inhibition, the authors concluded that large-scale continuous dextran production with $DDase_{ex}$ would be possible.

In addition to the synthesis of dextran, *G. oxydans* cultures supplemented with maltodextrins also result in the formation of oligosaccharides, which might have prebiotic properties [22]. These oligosaccharides contain varying proportions of α(1,4)- and α(1,6)-linked glucosyl residues, depending on the molecular size of the initial substrate and the culture time.

Properties of *Gluconobacter* dextran

Gluconobacter dextran, recovered from submerged fermentation, displays unusual rheological behaviour. We have evaluated several methods for the removal of contaminating residual maltodextrins from the polysaccharide preparation [14]. Amyloglucosidase treatment of a *Gluconobacter* dextran solution, in an attempt to obtain in situ degradation of maltodextrins, was



Compound	R1	R2
Stevioside	$G_\beta \overset{}{\underset{2}{—}} G_\beta$ —	G_β —
SG1a	G_α (6) on first G_β; $G_\beta \underset{2}{—} G_\beta$ —	G_β —
SG1b	G_α (6) on second G_β; $G_\beta \underset{2}{—} G_\beta$ —	G_β —
SG2	G_α (6) on each G_β; $G_\beta \underset{2}{—} G_\beta$ —	G_β —

Fig. 8 Structures of stevioside, SG1a, SG1b and SG2 [34]. *SG1a*/*SG1b* Mono-glucosyl-steviosides, *SG2* di-glucosyl-stevioside

found to be useless as the enzyme was suspected to significantly alter the rheological characteristics of the polysaccharide itself. Ultrafiltration resulted in maltodextrin washout, and normalised the viscosity profile of the dextran solution. Production of dextran by means of a solid-state fermentation process yielded a maltodextrin-free polysaccharide of 8-fold higher molecular weight. The difference in molecular weight of dextran from submerged culture versus solid-state fermentation indicated the pronounced negative effect of culture agitation on the length or branching of the resulting polymer chains; it could also be due partially to incomplete removal of residual maltodextrins by ultrafiltration of the polysaccharide from submerged culture.

Gluconobacter dextran displays shear-thinning flow behaviour [14]. The polysaccharide displayed lower viscosity than *L. mesenteroides* dextran of similar molecular weight as a consequence of its higher degree of branching. *Gluconobacter* dextran might thus be suitable for certain food use applications not associated with thickening functionality, such as use as a source of dietary fibre, as a cryostabiliser, as a fat substitute, or as a low-calorie bulking agent for sweeteners.

Production of transglucosylated products

Stevioside [13- *O*-(2-β-glucosyl-β-glucosyl)-19-*O*-β-glucosyl-steviol] is the major glycoside isolated from the leaves of *Stevia rebaudania* Bertoni (Compositae). The sweetness of stevioside is about 100 times higher than that of sucrose, and the compound is used as a low-calorie sweetener. However, it also has a slight bitterness and a bad aftertaste. To improve the taste of stevioside for food applications, enzymatic transglycosylations by various enzymes have been investigated [12]. As stevioside has two non-reducing terminal glucosyl residues, transglucosylation of stevioside by DDase action was attempted by Yamamoto et al. [34]. The mixture of glycosyl-steviosides produced by DDase in the presence of starch hydrolysate and isoamylase was composed of three major products: two mono-glucosyl-steviosides, SG1a, SG1b, and one di-glucosyl-stevioside, SG2 (Fig. 8).

During the glycosyl-steviosides production reaction, SG1a and SG1b were initially produced from stevioside at almost the same rate; SG1b subsequently decreased, with concurrent accumulation of SG2. This result indicated that transglucosylation to SG1a rarely occurred, although stevioside was effectively transglucosylated to become SG1a and SG1b, SG1b was destined to become SG2.

A possible conversion pathway between stevioside and these glucosyl-steviosides, proposed by Yamamoto et al. [34], is shown in Fig. 9. The DDase catalyses transglucosylation from glucosyl donor compounds to stevioside, yielding SG1a and SG1b, and to SG1b to yield SG2 by rapidly forming α(1,6)-linkages. DDase also catalyses conversions among stevioside and these glucosyl-steviosides by the transfer of α(1,6)-linked glucosyl residues, with the formation of new α(1,6)-linkages, although the action is rather slow compared to the transfer of α(1,4)-linked glucosyl residues to form an α(1,6)-linkage. An increase in the concentration of glucosyl donor did not efficiently increase the level of tri-glucosylated and further glucosylated steviosides. Although the capacity of DDase action to efficiently produce SG1 and SG2 remains unclear, the transglucosylation rates of DDase to glucosyl residues of acceptor compounds are thought to vary depending on the glucosidic linkage forms of acceptors; high affinity to stevioside and low affinities to SG1a and SG2 could induce such a phenomenon [34].

The manufacturing of glycosylated stevioside by DDase has been patented by Ezaki Glico Company [29]. An analogous production process has been patented for palatinose [27] and thiamine sugar derivatives [23], which can be used as food additives.

Conclusions

The above studies indicate that both the intracellular and extracellular forms of *G. oxydans* DDase could be promising alternatives to *L. mesenteroides* DSase as biocatalysts for the synthesis of dextran and oligodextrans. The enzyme system of *G. oxydans* has been far less extensively studied than the glucosyltransferase of *L. mesenteroides*; further strain optimisation via mutation and rDNA techniques remains essential. A notable lack in the research carried out to date is the absence of molecular and structural information regarding the enzyme, as well as genomics of *Gluconobacter* DDase and the metabolic regulation of its production. The structural difference between the extracellular and intracellular enzymes from the same strain needs further study. Is the primary structure of DDase similar to that of other starch transforming enzymes? Does its tertiary structure have active site features similar to other dextran-forming enzymes? What are the molecular regulatory mechanisms of transcription/translation/secretion? These molecular aspects have only recently been elucidated in

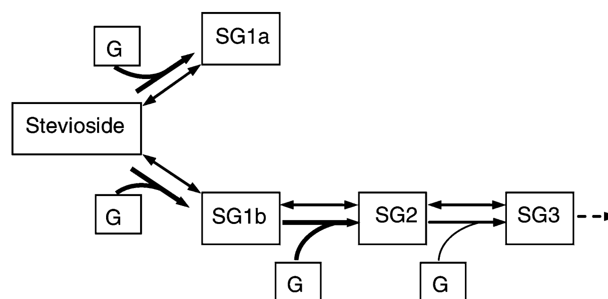


Fig. 9 Hypothetical bioconversion pathway between stevioside and glycosylsteviosides [34]. *Thick* and *thin arrows* indicate fast and slow reactions, respectively. *G* Transferring α(1,4) linked glucosyl unit, forming α(1,6) linkage; *SG1a*/*SG1b* mono-glucosyl-steviosides, *SG2* di-glucosyl-stevioside, *SG3* tri-glucosyl-stevioside

the case of *Leuconostoc* DSase, an enzyme that has been the subject of numerous studies since the 1960s [1–3, 5, 11, 19, 20]. This basic information is essential in order to arrive at a clear understanding of the molecular mode of action of DDase, a really neglected enzyme; it will also contribute to higher yielding fermentation processes with respect to DDase enzyme levels and/or *Gluconobacter* (oligo)dextran production. Moreover, the structural characteristics of *Gluconobacter* dextran are significantly different from those of commercial dextran, and an array of new potential application fields needs to be explored. *G. oxydans* seems to be an interesting microorganism for the development of a range of novel food ingredients or specialty sugars based on starch. *Gluconobacter* dextran could be used as a dietary fibre, based on its low digestibility by intestinal enzymes [33]. Furthermore, preliminary trials indicate the potential applicability of the polymer as a fat replacer in acceptable low-fat foods [22]. The intentional production of a range of *Gluconobacter* oligodextrans and other transglucosylation products also warrants further attention.

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