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Characterisation of polysaccharides synthesised by *Gluconobacter oxydans* NCIMB 4943

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Abstract

Fermentation of *Gluconobacter oxydans* NCIMB 4943 using malto-oligosaccharides of differing average degrees of polymerisation (DP) as substrates resulted in the synthesis of dextrans consistent with the action of the enzyme dextran–dextrinase (DDase). These dextrans could be precipitated from the fermentation broth using Fehling's solution. The glycosyl linkage composition of the dextrans was largely independent of the size of the initial substrate used and fermentation time. They contained an average of 83 mol% 6-linked, 8 mol% 4-linked, 6 mol% terminal and 3 mol% 4,6-linked glucopyranosyl residues. The dextrans gave viscous solutions that displayed shear-thinning behaviour. In addition to the synthesis of dextrans, the fermentations of *G. oxydans* with malto-oligosaccharides also resulted in the formation of oligosaccharides that were soluble in Fehling's solution. These oligosaccharides contained varying proportions of 4-Glc*p* and 6-Glc*p* residues depending on the size of the initial substrate and the culture time. These oligosaccharides may have prebiotic properties.

Keywords: *Gluconobacter oxydans*; Fermentation; Linkage analysis; Viscosity; Dextran

1. Introduction

The spoilage of beer by production of slimes has been known for almost a century. The origin of these slimes was shown to be due to the production of capsular material from malto-oligosaccharides and dextrins by species of *Acetobacter* (Shimwell, 1947). This material was shown to have serological properties similar to dextrans produced by cultures of the bacteria *Leuconostoc mesenteroides* (Hehre & Hamilton, 1949). Conversion of malto-oligosaccharides to dextrans by cell-free extracts of *A. capsulatum* (re-named *Gluconobacter oxydans*) led to the enzyme system responsible being named dextran–dextrinase (Hehre, 1951, 1953) or dextrin–dextranase (e.g. Yamamoto, Yoshikawa, Kitahata & Okada, 1992) (DDase, α-1,6-glucan-6-glucosyltransferase, EC 2.4.1.2)

Synthesis of dextran from sucrose by the action of dextransucrase (EC 2.4.1.5) from *L. mesenteroides* has been studied extensively (Robyt, 1995 and references therein). Polymerisation occurs by the transfer of glucosyl residues to the non-reducing end of an extending dextran molecule, with the precise structure of the dextran produced dependent on the bacterial strain used and on the culture conditions employed (Robyt, 1995). The synthesis of dextran when malto-oligosaccharides are incubated with DDase isolated from *G. oxydans* has been shown to occur by reversible transfer of either α-1,4-glucopyranosyl (4-Glc*p*) or α-1,6-glucopyranosyl (6-Glc*p*) residues from the non-reducing end of one oligosaccharide to glucosyl residues at the non-reducing end of another oligosaccharide forming either an α-1,4- or an α-1,6-linkage (Yamamoto, Yoshikawa & Okada, 1993a). The rate of formation of α-1,4- or α-1,6-linkages is thought to be similar, and the accumulation of dextran is due to α-1,6-linkages being less susceptible to cleavage than α-1,4-linkages (Yamamoto et al., 1993a).

The majority of the more recent studies on the action of DDase have investigated the substrate specificity of partially purified extracts of this enzyme. Sucrose is not a substrate for dextran production by DDase, while maltose reacts slowly and produces only glucose and panose (α-D-glucopyranosyl-[1 → 6]-D-maltose) (Yamamoto et al., 1992). Maltose, isomaltose, starch and dextran can act as glucosyl donors, and glucosyl residues can be transferred to D-glucose and D-xylose, and to various oligosaccharides with glucosyl, or glucosyl derivatives such as 2-acetamido-2-deoxy-, 6-deoxy- and 3-O-methyl-glucosyl residues, or

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xylosyl residues at their non-reducing ends (Yamamoto, Yoshikawa & Okada, 1994a). The use of reduced malto-oligosaccharides as substrates resulted in a higher yield of dextran and lower amounts of malto-oligosaccharide transfer products, than when non-reduced malto-oligosaccharides were used (Yamamoto, Yoshikawa & Okada, 1993b). DDase has also been used in the production of glucosyl derivatives of the bitter-sweet compound, stevioside (Yamamoto, Yoshikawa & Okada, 1994b).

We were attracted to the potential food use of the viscous polysaccharide that was produced by the direct culture of *G. oxydans* on inexpensive malto-oligosaccharides and recovered by alcohol precipitation. Preliminary trials indicated that this material could replace fat in the preparation of acceptable low-fat foods. The polysaccharide was shown by ^{13}C-NMR spectroscopy to contain a mixture of dextran- and amylose-like resonances, but it was unclear if these were on the same or separate polymer molecules. Thus, we undertook further studies of this culture system and its glucan products. The synthesis of polysaccharides from malto-oligosaccharide substrates with a degree of polymerisation (DP) ranging from DP 3 to DP 85 by cultures of *G. oxydans* was investigated. The polymeric material recovered by alcohol precipitation was fractionated by selective precipitation using Fehling's solution. The sizes of the polysaccharides obtained were estimated by gas chromatography (GC) and size-exclusion HPLC techniques, and their structures determined by glycosyl linkage analysis.

2. Experimental

2.1. Preparation of malto-oligosaccharide substrates

Malto-oligosaccharide substrates (A–C) with varying average DP were prepared by fractionating corn maltodextrins (Maltrin 100, NZ Starch Ltd, Auckland, NZ) with ethanol. Thus, Maltrin 100 was dissolved in distilled water (5%, w/v) at 60°C, the solution cooled to room temperature and cold ethanol added to 50% v/v while stirring. After 1 h at 4°C, the precipitated polysaccharide was collected by centrifugation (1600 g, 10 min), redissolved in distilled water and freeze-dried to give substrate A. The supernatant was then brought to 60% v/v ethanol, the precipitated polysaccharide pelleted by centrifugation and discarded, and this new supernatant was then brought to 65% v/v ethanol and the precipitated polysaccharide collected and freeze-dried as previously to give substrate B. Substrate C was the material that precipitated between 70 and 75% v/v ethanol. The resulting malto-oligosaccharides had an average DP of 85 (substrate A), 28 (B) and 17 (C), respectively, as estimated by GC analysis (see later).

Maltotriose (substrate D) was obtained from ICN Biomedicals Inc. (OH, USA).

2.2. Fermentation of malto-oligosaccharides with G. oxydans

Freeze-dried *G. oxydans* NCIMB 4943 was inoculated onto agar (15 mg mL^{-1}) slopes containing sorbitol (50 mg mL^{-1}), yeast extract (20 mg mL^{-1}, Difco) and K_2HPO_4 (4 mg mL^{-1}) and incubated for 24 h at 30°C. A loopful of this culture was used to inoculate a baffled flask (100 mL) of liquid medium (25 mL) containing the same components as the slopes without agar and incubated on an orbital shaker (120 rpm) for 24 h at 30°C.

Fermentations for the biosynthesis of polysaccharides were grown in 100 mL baffled conical flasks in 25 mL of medium containing malto-oligosaccharides (50 mg mL^{-1}), polypeptone (3 mg mL^{-1}), yeast extract (5 mg mL^{-1}) and ethanol (1% v/v). Media were inoculated with 0.5 mL of culture and incubated on an orbital shaker (120 rpm) for up to 7 days at 30°C. At harvest, fermentations were heated at 70°C for 1 h to kill the bacteria. Polysaccharides were precipitated from the medium by the addition of two volumes of absolute ethanol, recovered by centrifugation (1600 g, 10 min), redissolved in distilled water and freeze-dried.

2.3. Determination of viscosity

The viscosity properties of *G. oxydans* culture products were measured on a Haake VT500 viscometer (Karlsruhe, Germany) using a concentric cylinder system (NV) with a gap of 0.35 mm for the determination of viscosity of ethanol-insoluble culture products or a cone and plate geometry (28 mm diameter cone, 1° angle) for the determination of viscosity of Fehling's insoluble material. Viscosity measurements with increasing shear-stress were made on solutions of ethanol precipitated *G. oxydans* culture products dissolved in distilled water at 5.0% w/v at 20°C.

2.4. Determination of degree of polymerisation

Average DP was determined by a GC-MS technique, similar to that described by Courtin, Van den Broeck & Declour (2000). Samples (100–200 μg) were reduced with 1.0 M NaB^2H_4 in 2.0 M ammonia (200 μl) for 1 h at 60°C, with myo-inositol (20 μg) added as an internal standard. The reaction was stopped by the addition of glacial acetic acid (50 μl), and borate removed as volatile trimethylborate by five evaporations with 5% v/v acetic acid in methanol (0.5 ml) and three evaporations with methanol (0.5 ml). The samples were then hydrolysed with 2.5 M trifluoroacetic acid (TFA, 200 μl) for 1 h at 120°C, evaporated to dryness and acetylated in acetic anhydride (100 μl) and TFA (100 μl) for 10 min at 50°C. The derivatives were separated by GC on a capillary column (HP Ultra 2, 50 m × 0.20 mm internal diameter, film thickness 0.33 μm, Hewlett Packard) and analysed using a Hewlett Packard 5970 GC-MS. The GC oven was programmed from 160°C (held for 1 min) to 230°C at a rate of 3°C min^{-1}. Identifications were based on

Table 1
Yields of products insoluble in 66% (v/v) ethanol from cultures of *G. oxydans* with malto-oligosaccharide substrates of differing average degree of polymerisation (DP)

Substrate	Average DP	Yield of ethanol-insoluble products					
		Day 3		Day 5		Day 7	
		mg	%	mg	%	mg	%
A	85	990	79	1060	85	1200	96
B	28	700	56	825	66	816	65
C	17	485	39	640	51	666	53
D	3	200	16	202	16	170	14

peak retention times and electron impact mass spectra compared with standards, and quantified by integration of the total ion chromatogram scanning the range m/z 40–400.

In this procedure, reducing end glucose residues were first reduced to their respective alditols, and then following hydrolysis were acetylated to give glucitol hexaacetate (retention time relative to myo-inositol hexaacetate, rRt — 1.023; molecular weight 435). Non-reducing glucose residues were not reduced and following hydrolysis were acetylated resulting in the formation of the α- and β-anomers of glucopyranose pentaacetate (rRt — 0.924 and 0.935, respectively; molecular weight 390) and glucofuranose pentaacetate (rRt — 0.971 and 0.991, respectively; molecular weight 390). The average DP of the samples was calculated from the molar ratio of glucitol hexaacetate (total ion chromatogram peak area/435) to total glucose acetates (total ion chromatogram peak area/390), assuming that the various derivatives had equal mass responses in the GC-MS. The reproducibility of this GC-MS method was similar to that obtained for maltodextrins by Courtin et al. (2000), who showed that the determination of DP by GLC gave similar results to those obtained by colorimetric and NMR spectroscopic techniques.

2.5. Fractionation with Fehling's solution

Maltodextrin substrates and polysaccharide culture products were treated with Fehling's solution according to the method of Jones and Stoodley (1965). Samples (~100 mg) were dissolved in distilled water (10 mg mL^{-1}) and one volume of Fehling's solution was added. The mixtures were allowed to stand at room temperature for 2 h and centrifuged at 200*g* for 5 min at 15°C. The pellets were macerated in chilled ethanol that contained 5% (v/v) conc. HCl and then centrifuged (500*g*, 10 min), and the supernatants removed. Maceration of the pellets in ethanol was continued until no more chloride could be detected; addition of acetone to the pellet showed the presence of $CuCl_2$ as an intense yellow colour. The pellets were then dissolved in distilled water, dialysed extensively against deionised water and freeze dried. The Fehling's supernatants were neutralised with glacial acetic acid, dialysed extensively against deionised water and freeze dried.

2.6. Size-exclusion HPLC

Maltodextrin substrates and polysaccharide fermentation products fractionated using Fehling's solution (250 μg) were dissolved in 0.25 M NaCl (5 μg μL^{-1}) at 60°C and chromatographed on a Sephadex G75 (300×7.8 mm^2) eluted with 0.25 M NaCl at 1 mL min^{-1} at 60°C. The columns were calibrated with a series of pullulans (molecular weights 0.58, 1.22, 2.37, 4.8, 10.0 and 18.6×10^4 Da; Showa Denko K.K., Tokyo, Japan) and glucose. Eluted material was detected by differential refractometry.

2.7. Glycosyl linkage analysis

Samples (100–200 μg) were methylated using NaOH and CH_3I in DMSO (Ciucanu & Kerek, 1984) as described by McConville, Homans, Thomas-Oates, Dell and Bacic (1990). The methylated polysaccharides were hydrolysed with 2.5 M TFA (4 h, 100°C), reduced with 1.0 M NaB^2H_4 in 2.0 M NH_4OH (200 μl) overnight at 35°C and borate removed as described earlier. The resulting alditols were acetylated in acetic anhydride (100 μl) and TFA (100 μl) for 10 min at 50°C. The partially methylated alditol acetates were analysed by GC-MS with the GC oven programmed from 130°C (held for 1 min) to 230°C at a rate of 3°C min^{-1}.

3. Results and discussion

3.1. Yield of culture polysaccharides

Preliminary experiments demonstrated that the cell cultures had reached stationary phase after 7 days of incubation and thus cultures were harvested at day 3, 5 and 7. Table 1 shows the weight of insoluble material recovered by the addition of two volumes of ethanol to the *G. oxydans* cultures as a function of culture time. The yields as a percentage of the initial weight of the malto-oligosaccharide substrate are also indicated. The yield of ethanol-insoluble material from substrate A (DP 85) increased with each harvest, whereas the yields from substrates B and C (DP 28 and 17, respectively) increased from day 3 to day 5, but changed only slightly after that. The yield of ethanol-insoluble material from substrate D (maltotriose) was almost identical from day 3 to day 5 and subsequently decreased.

The results indicated that the yield of ethanol-insoluble material was greater when substrates with larger DP were used. For fermentation of substrates A and B, interpretation of these results was confused by the substrates themselves being completely insoluble in 66% v/v ethanol. Thus, the material recovered by the addition of ethanol to the culture broth was a mixture of newly synthesised polymeric material and unreacted substrate. After 3 days culture the yield of

Table 2
Average degree of polymerisation (DP) of products insoluble in 66% v/v ethanol from cultures of *G. oxydans* with malto-oligosaccharides substrates of differing size

Substrate	Average DP			
	Substrate	Day 3	Day 5	Day 7
A	85	198	>250	>250
B	28	31	45	48
C	17	26	53	55
D	3	19	26	22

ethanol-insoluble material from substrates A and B was 79 and 56% w/w of the initial substrate added, respectively, indicating that at least 21 and 44% of substrates A and B, respectively, had been altered to make them soluble in 66% v/v ethanol. The subsequent increase in the ethanol-insoluble products from the culture of these two substrates suggested that new polymeric material has been synthesised. Thus, the yield of newly synthesised polymeric material on day 7 from culture of substrate A was at least 17% w/w (96–79%). Substrates C and D were completely soluble in 66% v/v ethanol and the ethanol-insoluble material recovered from the culture broth was thus new polymeric material resulting from the culture of the substrates by *G. oxydans*.

Table 3
Linkage composition of ethanol-insoluble products from culture of *G. oxydans* with malto-oligosaccharides of different sizes

Substrate (DP)	Deduced linkage of Glc*p* residues[a]	Composition of culture products (mol%)[b]			
		Substrate	Day 3	Day 5	Day 7
A (85)	Terminal	7	6	7	9
	4-	91	89	86	81
	6-	–[c]	3	5	9
	4,6-	2	2	2	1
B (28)	Terminal	7	8	11	10
	4-	92	74	46	32
	6-	–	17	41	56
	4,6-	1	1	2	2
C (17)	Terminal	7	8	18	19
	4-	93	75	14	15
	6-	–	16	68	64
	4,6-	–	1	–	1
D (3)	Terminal	n.d.[d]	16	16	15
	4-	n.d.	13	13	13
	6-	n.d.	68	68	69
	4,6-	n.d.	2	2	2

[a] Terminal Glc*p* deduced from 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-glucitol, etc.
[b] Average of duplicate determinations.
[c] Not detected.
[d] Not determined.

3.2. Degree of polymerisation of culture products

The DP of the substrates and culture products was determined by reducing terminal residues to their respective alditols, hydrolysis to constituents, acetylation and GC-MS analysis. Maltotriose (substrate D) gave a 2.2:1 molar ratio of glucose pentaacetate (derived from non-reducing sugar residues) to glucitol hexacetate (derived from the reducing terminus), close to the expected 2.0:1 molar ratio corresponding to DP 3 (Table 2). The average DP of substrates A–C produced by ethanol fractionation of a commercial malto-oligosaccharide mixture (Maltrin 100) was 85, 28 and 17, respectively (Table 2). The average DP of ethanol-insoluble culture products generally increased with harvest time. The ethanol-insoluble products from culture of substrate A harvested on day 3 had a DP of 198 and the products harvested on days 5 and 7 were larger than could be determined reliably by GC-MS, but were estimated to be greater than DP 250. The average DP of the ethanol-insoluble products from substrates B and C increased markedly from day 3 to day 5, but changed little after that, indicating that the reaction had reached equilibrium by day 5. The average DP of the ethanol-insoluble products from maltotriose (substrate D) increased from day 3 to day 5, but then decreased from day 5 to day 7.

The DP of the ethanol-insoluble products from culture of substrates C and D indicated that larger substrates resulted in the synthesis of larger polymeric products. Interpretation of the results of the analysis of DP of the ethanol-insoluble material from fermentation of substrates A and B was again made more difficult by potential precipitation of unreacted substrates.

3.3. Glycosyl linkage composition of ethanol-insoluble products

Glycosyl linkage analysis showed that, as expected, the malto-oligosaccharide substrates A–C contained mostly 4-Glc*p* and that there was no 6-Glc*p* present (Table 3); substrate D (maltotriose) was not analysed.

The ethanol-insoluble culture products from substrate A contained mostly 4-Glc*p* together with a small amount of 6-Glc*p* that increased with longer culture time, indicating that there was a large amount of unreacted substrate present. The slow increase in 6-Glc*p* with culture time suggested that a small amount dextran-like material was synthesised. The products from substrate B contained much higher amounts of 6-Glc*p* than those from substrate A and the proportion of 6-Glc*p* increased with culture time. The ratio of 6-Glc*p* to 4-Glc*p* in the products from substrate B increased from 0.2 on day 3 to 1.8 on day 7. The presence of 4-Glc*p* once again indicated that there was some unreacted substrate remaining. Thus, from these results it was not possible to conclude if the newly synthesised material contained both 4-Glc*p* and 6-Glc*p* or just 6-Glc*p*. The ethanol-insoluble culture product from substrate C harvested on day 3 had an almost identical

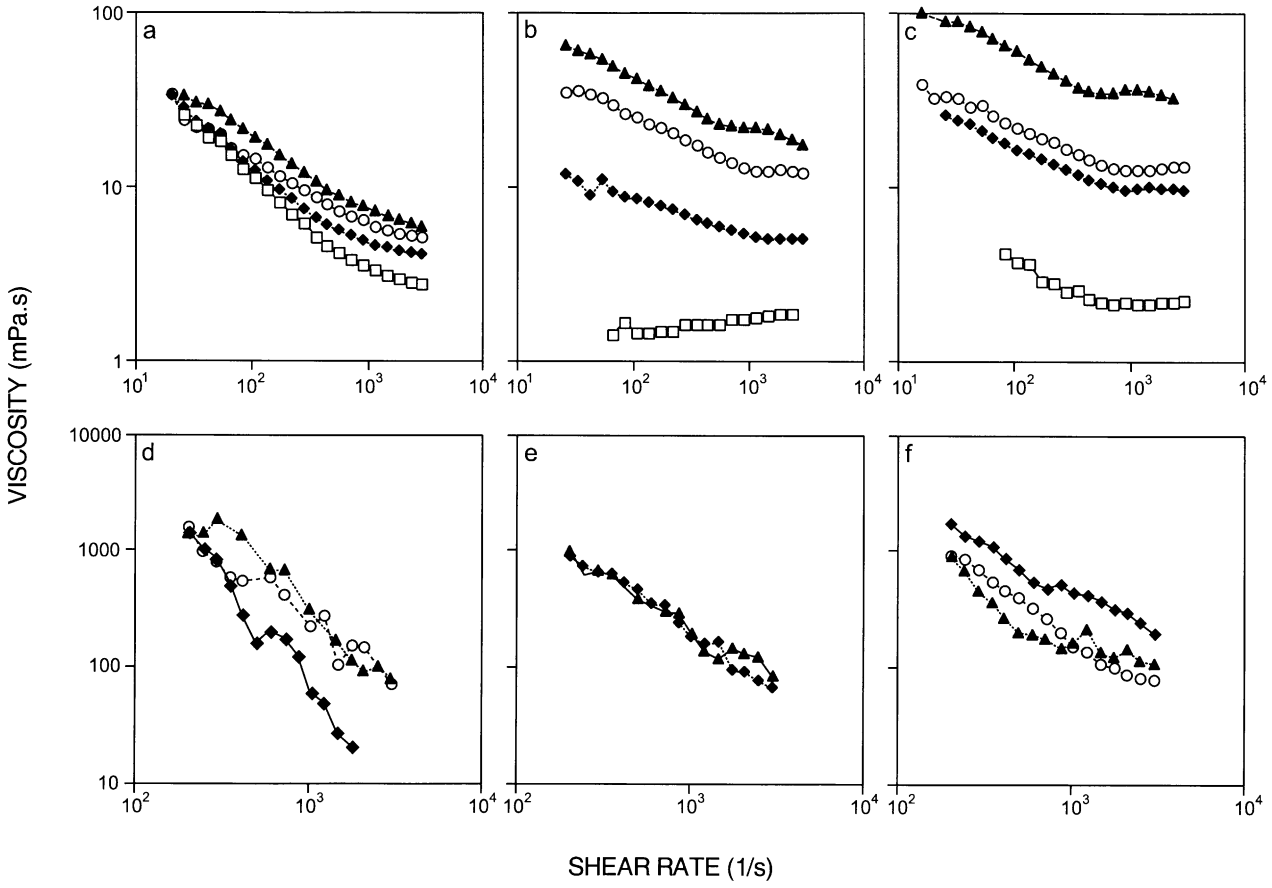

Fig. 1. Viscosity with increasing shear of 5% (w/w) aqueous solutions of ethanol-insoluble products (a, b and c) and material recovered from precipitate after treatment with Fehling's solution (d, e and f) from cultures of *G. oxydans* with substrate A (DP 85; a and d), substrate B (DP 28; b and e) and substrate C (DP 17; c and f). Substrate (□) and fermentation products harvested on day 3 (◆), 5 (○) and 7(▲).

linkage composition to the day 3 culture product from substrate B with a ratio of 6-Glc*p* to 4-Glc*p* of 0.2. However, as substrate C was soluble in 66% v/v ethanol it appeared that the newly synthesised polysaccharide contained both amylose-like 4-Glc*p* and dextran-like 6-Glc*p* linkages. The day 5 and 7 products from substrate C were similar to each other and the ratio of 6-Glc*p* to 4-Glc*p* had increased to 4.9 and 4.6, respectively. The linkage compositions of the ethanol-insoluble culture products from substrate D were almost identical to each other with a ratio of 6-Glc*p* to 4-Glc*p* of 5.2–5.3, which was similar to the ratio of 6-Glc*p* to 4-Glc*p* at day 5 from substrate C.

These results indicated that synthesis of 6-Glc*p* linkages was faster with the smaller substrate. The decrease of 4-Glc*p* residues and corresponding increase in 6-Glc*p* residues continued until day 7 for substrates A and B, but had been completed for substrates C and D by day 5 and 3, respectively.

3.4. Viscosity properties of ethanol-insoluble culture products

Fig. 1 shows the viscosity at 20°C of aqueous solutions (5% w/v) of the ethanol-insoluble culture products from substrate A (Fig. 1a), B (Fig. 1b) and C (Fig. 1c) harvested at days 3, 5 and 7, together with their respective substrates. There was insufficient material available for testing the viscosity of the ethanol-insoluble culture products from substrate D. The products tested all displayed non-Newtonian behaviour. The viscosities of each of the products decreased as shear-rate increased, and thus the mixtures of components in each of the fermentation products displayed shear-thinning behaviour. The viscosities of the products ranged from 100 mPa.s for the day 7 product from substrate C at 20 s^{-1} (Fig. 1c) to 4 mPa.s for day 3 product from substrate A at 3000 s^{-1}(Fig. 1a).

Substrate A displayed shear-thinning behaviour ranging from 25 mPa.s at 25 s^{-1} to 3 mPa.s at 3000 s^{-1} (Fig. 1a). The ethanol-insoluble culture products from this substrate all displayed similar viscosity behaviour to the substrate, with only small increases in viscosity with time of harvest. The day 3 culture product had an almost identical viscosity to the substrate at the lower shear-rates tested, but had a slightly higher viscosity as the shear rate increased to more than 200 s^{-1}. The day 5 and day 7 culture products showed further small increases in their viscosities.

The viscosities of substrates B and C (2–3 mPa.s, Fig. 1b and c) were much lower than that of substrate A and showed

Table 4
Yield of Fehling's precipitated products from ethanol-insoluble culture products from cultures of *G. oxydans* with malto-oligosaccharide substrates of differing average DP

Substrate	Yield of Fehling's-insoluble products					
	Day 3		Day 5		Day 7	
	%EtOH[a]	% Total[b]	%EtOH	% Total	% EtOH	% Total
A	13	10	21	18	15	17
B	20	11	57	38	52	34
C	38	15	53	27	51	27
D	41	6	40	6	49	7

[a] Weight of ethanol-insoluble fraction.
[b] Weight of initial substrate.

little shear-thinning behaviour. However, the viscosities of the ethanol-insoluble culture products were much greater than the substrates and they were shear-thinning. The day 3 culture product from substrate B ranged in viscosity from 12 mPa.s at 25 s^{-1} to 5 mPa.s at 3000 s^{-1} (Fig 1b). The day 5 and day 7 culture products showed progressively higher viscosities with the day 7 product ranging from 63 mPa.s at 25 s^{-1} to 17 mPa.s at 3000 s^{-1}. The ethanol-insoluble culture products from substrate C (Fig. 1c) showed higher viscosities than those from substrate B (Fig.1c), with the day 3 product showing similar viscosity behaviour to that of the day 5 product from substrate B and the day 7 products ranging from 88 mPa.s at 25 s^{-1} to 32 mPa.s at 3000 s^{-1}.

The changes observed in the viscosity properties of the recovered polymeric materials indicated that the structural changes occurring were greater for cultures with substrates B and C than substrate A. These changes in viscosity did not appear to be consistent with changes observed in the average DP and glycosyl linkage composition of the ethanol-insoluble products. Although the relatively minor changes in the linkage composition of the ethanol-insoluble products from culture of substrate A was consistent with the small increases observed in viscosity, the large increase in average DP from the initial substrate to the day 3 product indicated that a much larger increase in viscosity may have been expected. The increased viscosity of the ethanol-insoluble products from the culture of substrates B and C could have been consistent with the increase observed in the average DP if these results were considered in isolation. However, comparison of the viscosity of these products with those from culture of substrate A suggested that these results were more complex. Based on the average DP of the products, the lower average DP of the ethanol-insoluble products from substrates B and C would be expected to give solutions with lower viscosities than those from the ethanol-insoluble products from substrate A. In fact, the products from substrates B and C had higher viscosities than those from substrate A. Thus, it appeared that the changes in the viscosity of the culture products of substrates B and C were the result of alterations to the structure of the polysaccharides.

The presence of a large amount of unreacted substrate especially in cultures with substrate A made interpretation of the results complex. Attempts to separate newly synthesised polysaccharide material from unreacted substrate by selective precipitation using a number of reagents ($Ba(OH)_2$, $(NH_4)_2SO_4$, CTAB) were unsuccessful. However, treatment of the ethanol-insoluble culture products with Fehling's solution was able to separate these two components.

Table 5
Linkage composition of ethanol-insoluble *G. oxydans* culture products fractionated with Fehling's solution

Substrate (DP)	Deduced linkage of Glc*p* residues[a]	Composition of culture products (mol%)[b]					
		Day 3		Day 5		Day 7	
		PPT[c]	S/N[d]	PPT	S/N	PPT	S/N
A (85)	Terminal	6	5	4	5	7	7
	4-	7	92	4	91	7	89
	6-	84	0	87	0	84	1
	4,6-	3	3	3	4	2	3
	2,6- + 3,6-	–[e]	–	2	–	–	–
B (28)	Terminal	6	6	7	8	6	9
	4-	7	90	6	83	6	69
	6-	83	1	83	6	85	14
	4,6-	4	2	4	3	3	5
	3,4-	–	–	–	–	–	3
C (17)	Terminal	7	10	8	11	6	7
	4-	11	82	12	77	8	67
	6-	79	5	76	11	84	26
	4,6-	3	2	4	–	2	–
D (3)	Terminal	5	9	6	16	6	18
	4-	6	73	4	28	8	29
	6-	86	15	87	51	81	49
	4,6-	3	3	3	2	5	4

[a] Terminal Glc*p* deduced from 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol, etc.
[b] Average of duplicate determinations.
[c] PPT, material recovered from precipitate after treatment with Fehling's solution.
[d] S/N, material recovered from supernatant after treatment with Fehling's solution.
[e] Not detected.

3.5. Fractionation of culture products using Fehling's solution

Addition of one volume of Fehling's solution to aqueous solutions of each of the ethanol-insoluble culture products from culture of *G. oxydans* with malto-oligosaccharides of different sizes gave Fehling's insoluble and Fehling's soluble fractions. The amounts of the Fehling's insoluble fractions expressed as percentages of the amounts of the ethanol-insoluble culture products and as percentages of the initial weights of substrates added to the culture broth are shown in Table 4. The yields of Fehling's insoluble

material for culture products from substrates A, B and C increased greatly from day 3 to 5, but then decreased slightly on day 7. The maximum yield of Fehling's insoluble material (38% of the initial weight of substrate) was obtained from substrate B on day 5. The Fehling's insoluble fraction accounted for 40–49% w/w of the ethanol-insoluble products from substrate D. However, the yields of Fehling's insoluble material from substrate D represented only 6–7% of the initial weight of substrate.

Gel-filtration HPLC showed that the Fehling's insoluble material eluted at the V_0 (molecular weight >150,000 Da) of the column and that the Fehling's soluble material had a similar elution profile to that of the malto-oligosaccharide substrate used (data not shown). These data suggested that culture of malto-oligosaccharides with *G. oxydans* resulted in the synthesis of high-molecular-weight polymeric materials that were distinct from the initial substrates.

3.6. Glycosyl linkage composition of Fehling's fractions

Glycosyl linkage analysis of the two fractions obtained by selective precipitation with Fehling's solution clearly showed that the insoluble materials, which eluted at the V_0 of the gel-filtration column, contained mostly 6-Glc*p* residues, while the soluble materials generally contained mostly 4-Glc*p* residues (Table 5). The glycosyl linkage compositions of the Fehling's insoluble fractions from culture of each of the substrates A–D were all in the range 76–87 mol% 6-Glc*p*, 4–12 mol% 4-Glc*p*, 2–5 mol% 4,6-Glc*p* and 4– 8 mol% terminal Glc*p*. This suggested that the polysaccharide synthesised by culture of malto-oligosaccharides with *G. oxydans* was a dextran, which contained a small proportion of amylose-like linkages. The structure of the dextran synthesised appeared to be independent of the size of the malto-oligosaccharide fermented.

The Fehling's soluble fractions from culture of substrate A contained predominantly 4-Glc*p*, which suggested that this material was mostly unreacted substrate (Table 5). Thus, a considerable proportion of this substrate remained largely unaltered even after 7 days culture with *G. oxydans*. The Fehling's soluble fractions from culture of substrate B contained an increasing amount of 6-Glc*p* and a decreasing amount of 4-Glc*p* as a function of culture time. Similar, but progressively more pronounced changes in the relative proportions of 6-Glc*p* and 4-Glc*p* were observed for the Fehling's soluble fractions from culture of substrates C and D (Table 5). Thus, it appears that the Fehling's soluble carbohydrates from culture of substrates B–D are composed of oligosaccharides of similar size to the malto-oligosaccharide substrates used, but contain varying proportions of dextran- and amylose-like regions.

3.7. Viscosity properties of Fehling's precipitated polysaccharides

The viscosity properties at 20°C of aqueous solutions (5% w/v) of the Fehling's insoluble materials from fermentation of substrates A (Fig. 1d), B (Fig. 1e) and C (Fig. 1f) were determined using cone and plate geometry. All the Fehling's insoluble fractions displayed similar shear-thinning behaviour over the shear-rate range tested (200–3000 s^{-1}), ranging from approximately 900–1400 mPa.s at 200 s^{-1} to 50–190 mPa.s at 3000 s^{-1}. There were only relatively minor differences in the viscosity of the Fehling's insoluble fractions as a function of either substrate size or culture time. These data again indicated that the dextrans synthesised were structurally similar and did not alter with the size of the substrate used.

4. Conclusions

The *G. oxydans* culture of malto-oligosaccharides with an average DP of up to 85 resulted in the synthesis of dextran, which could be precipitated from the culture broth using Fehling's solution. The synthesis of dextran from malto-oligosaccharides is consistent with the action of the enzyme DDase first described by Hehre (1951). The viscosity properties of the dextran were largely independent of the size of the malto-oligosaccharide substrate and culture time. The linkage composition of the dextran was largely independent of the size of the initial substrate and contained an average of 83 mol% 6-Glc*p*, 8 mol% 4-Glc*p*, 6 mol% terminal Glc*p* and 3 mol% 4,6-Glc*p*. The structure of the dextran was similar to that of dextran synthesised from maltotetraose by purified DDase, which was calculated to contain 7% 4-Glc*p* and 6% 4,6-Glc*p* (Yamamoto, Yoshikawa & Okada, 1993c). From the linkage composition data it was not possible to deduce the distribution of 6-Glc*p* and 4-Glc*p* in the dextran molecules. From the action of DDase proposed by Yamamoto et al. (1993a) it would seem most likely that the 4-Glc*p* residues were present in a block at that reducing end of the dextran with the 6-Glc*p* residues extending from the non-reducing terminus.

Although the structure of the dextran synthesised by *G. oxydans* was independent of substrate size, the yield of dextran was influenced by the size of the malto-oligosaccharide substrate. Dextran yield increased with increasing substrate size from DP 3 to DP 28, but then decreased with substrate of DP 85. Dextran yield (7% w/w initial substrate) from substrate D (maltotriose) was slightly lower than that obtained from incubation of maltotriose with purified DDase (11%; Yamamoto et al., 1992). However, the method they used for determination of dextran, by determining total sugar content after enzymic digestion of residual substrate with α-amylase and precipitation of undigested material with ethanol, would detect not only the Fehling's precipitated dextran, but also the 6-Glc*p* residues present in the Fehling's soluble fraction in this present study. Thus, the maximum yield of 6-Glc*p* from culture of *G. oxydans* with maltotriose obtained in this present study was 11% (16% w/w yield, 69 mol% 6-Glc*p*), identical to that obtained by Yamamoto et al. (1992).

The maximum yield of dextran obtained was 38% w/w of the initial substrate for substrate B (DP 28) harvested at day 5. The yield of dextran on day 7 from substrates B and C, 34 and 27%, respectively, was similar to that obtained from incubation of maltopentaose and maltohexaose with purified DDase (25–30%; Yamamoto et al., 1992). Also, the dextran yield on day 5 from substrate A (18%) was similar to that obtained from incubation of hydrolysed starch with purified DDase (21%), but much lower than that obtained from short-chain amylose (58%; Yamamoto et al., 1992). These data suggested that branching of the maltodextrin substrate reduced the yield of dextran by cultures of *G. oxydans*. The yield of dextran from the action of DDase on starch or low-degree-hydrolysed starch was improved by the presence of debranching enzymes in the incubation mixture (Yamamoto, Yoshikawa & Okada, 1993d). Comparison of the yield of dextrans with substrates with specified degrees of branching would provide valuable data on the effect of 4,6-Glc*p* residues on dextran synthesis.

In addition to the synthesis of dextran, the cultures of *G. oxydans* with malto-oligosaccharides also resulted in the formation of oligosaccharides that were soluble in Fehling's solution. These oligosaccharides contained varying proportions of 4-Glc*p* and 6-Glc*p* residues depending on the size of the initial substrate and the culture time. The Fehling's soluble fractions from cultures with substrates A and B contained unreacted substrate and were not considered further, whereas the Fehling's soluble fractions from cultures with substrates C and D were newly synthesised products. The ratio of 6-Glc*p*:4-Glc*p* of the Fehling's soluble products from substrates C and D was 0.06 and 0.2, respectively, at day 3 and increased to 0.4 and 1.7, respectively, at day 7. The synthesis of oligosaccharides with both 4-Glc*p* and 6-Glc*p* residues is a consequence of the action mode of DDase, and extension of the culture time would be expected to result in further consumption of 4-Glc*p* residues (Yamamoto et al., 1993a). Various gluco-oligosaccharides, which have prebiotic properties, are produced on an industrial scale by the action of *L. mesenteroides* dextransucrase through reaction with sucrose in the presence of maltose as an acceptor (Dols, Remaud-Simeon, Willemot, Vignon, & Monsan, 1998). These oligosaccharides have a maltose moiety at the reducing end, varying numbers of 6-Glc*p* residues and may possess a single 2-Glc*p* residue either as a non-reducing terminus or as a branch point. The oligosaccharides produced by cultures of *G. oxydans* with malto-oligosaccharides in this present study may also have prebiotic properties.

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