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Productivity of Four α-D-Glucosyltransferases Released by *Streptococcus sobrinus* under Defined Conditions in Continuous Culture

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ABSTRACT

The distribution and activity of four different α-D-glucosyltransferases (GTF) were determined in culture filtrates of Streptococcus sobrinus *grown in the chemostat. The rate of production of each enzyme depended on the growth rate, the pH of the medium, the limiting nutrient and on the presence of Tween 80. Changes in the relative proportion of GTF were reflected in the altered structures and adhesive qualities of the water-insoluble α-D-glucans synthesized from sucrose by the culture filtrates.*

INTRODUCTION

The structure of the extracellular α-D-glucans synthesized by the mutans group of streptococci is closely related to the growth conditions of the microorganisms (Walker *et al.*, 1983). This dependency is due to the variation in production of several D-glucosyltransferases (GTF) involved in the conversion of sucrose into α-D-glucan (Walker *et al.*, 1983, 1984). For example, the activity of the enzyme designated GTF-S, that catalyzes the synthesis of the highly branched dextran-like chains in *Streptococcus sobrinus* strain OMZ176 glucans, is highest at low growth rates, and accounts for 97% of the total GTF activity. By contrast, another enzyme,

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designated GTF-I, that catalyzes the synthesis of sequences of α-(1→3)-linked glucose residues, becomes prominent only at high growth rates, under which conditions its activity becomes approximately equal to that of GTF-S. Similar results have been obtained for the proportion and activity of GTF-S and GTF-I released by *S. sobrinus* strain 6715 and its mutants grown in continuous culture at low and high growth rate. Such changes in the relative activities of GTF-S and GTF-I lead to variations in the structure and solubility of their glucan product.

It has long been known that growth of oral streptococci in media containing the non-ionic surfactant Tween 80 can result in markedly enhanced levels of GTF (Umesaki *et al.*, 1977; Wittenberger *et al.*, 1978; Shimamura *et al.*, 1983). Two studies with batch cultures of *S. sobrinus* strain 6715 showed that extracellular GTF activity in Tween 80-medium was higher by 7- and 6-fold respectively, than in unsupplemented medium. In the absence of Tween 80, two enzymes, GTF-S and GTF-I, were separated from batch cultures of strain 6715 (Ciardi *et al.*, 1977). The enhanced activity in Tween 80 cultures of the same strain was shown by analytical isoelectric focusing to be mainly due to production of a new GTF that catalyzed the synthesis of a soluble dextran having few branch linkages. An increased production of GTF-I was also indicated from the higher intensity of the white bands of water insoluble glucan that appeared when the gels were incubated in sucrose solution (Shimamura *et al.*, 1983).

In the present work, the streptococci were grown in the chemostat, thereby avoiding the limitations of batch culture. Growth in continuous culture, under defined and reproducible conditions, has permitted the effects of different fermentable carbohydrates, growth rate, pH and Tween 80 to be fully documented. The authors have separated several distinct GTF that are released by two related *S. sobrinus* strains, K1-R and 6715-13-201, grown in the presence and absence of Tween 80. One of the major effects of Tween 80 was to promote the release of *two* new GTF capable of synthesizing soluble dextran.

MATERIALS AND METHODS

Growth conditions

Continuous culture was carried out at 37°C in a 1-liter Bio-Flo chemostat (Model C30, New Brunswick Scientific Co. Inc., NJ, USA). The fermentation vessel contained 325 ml of complex medium (Van Houte & Saxton, 1971) supplemented with D-glucose, either 0·5% or

4%. Anaerobic conditions were maintained with a gas mixture of nitrogen and carbon dioxide (19:1 v/v) sparged at 200 ml min^{-1}. The pH was kept constant to within ± 0·05 pH unit by the automatic addition of 2 M sodium hydroxide with pH-control equipment (Model pH-21) supplied by New Brunswick Scientific Co. Inc. At least five culture volumes were allowed to flow from the fermentation vessel before samples were taken for analysis at steady state condition (that is, after seven or more mean generation-times). Each dilution rate was maintained within a variation of ± 0·01 h^{-1}. The overflow was collected in a refrigerated bath, and culture filtrates were obtained by centrifuging for 10 min at 12 000 *g*. The dry weight of the cells was determined on duplicate volumes (5 ml) of culture, by centrifuging and washing the cells, and drying them to constant weight at 90°C.

Determination of D-glucosyltransferase activity

Cells from freshly centrifuged broth (5 ml) were washed twice with sodium citrate buffer (50 mM, pH 6·0) containing sodium fluoride (10 mM), and resuspended in the same buffer (3·75 ml). Portions of the freshly prepared cell-free filtrate (usually containing 5–30 mU of GTF activity), or washed cell suspension (0·15 ml), were incubated at 35°C in duplicate reaction mixtures containing sucrose [glucose-^{14}C(U)] (5 mg, 6 mCi/mol), Sigma type 2000 dextran (0·25 mg) and sodium citrate buffer (25 mM, pH 6·0) in a final volume of 0·5 ml; the assays for cell-bound GTF also contained sodium fluoride (10 mM). At appropriate intervals of time (from 5 min to 1 h), ethanol (2 vol) was added to precipitate polysaccharide. After 1 h at 4°C the glucans were sedimented by centrifuging, and washed twice with methanol. The precipitates were suspended in methanol (1 ml), and filtered through a Whatman 3 MM paper disc (25 mm). After washing with methanol (20 ml) the papers were transferred into scintillation vials, and the radioactivity of the glucans was counted. One unit (U) was defined as the amount of enzyme either free or cell-bound, that incorporated 1 μmol of D-glucose per min into glucan under the foregoing conditions.

Separation of D-glucosyltransferases

One liter of culture supernatant was concentrated to 100 ml in a hollow fiber concentrator fitted with an H1P10 cartridge (Amicon Corporation). Saturated ammonium sulphate (pH 6·3) was added to precipitate proteins between 0 and 75% saturation, and the mixture was allowed to stand at 4°C for 4 h, and then centrifuged for 10 min at 12 000 *g*. The

pellet was washed with 75% saturated ammonium sulphate, and then dissolved in 12 ml of sodium citrate buffer (50 mM, pH 6·0), and dialyzed for 4 h against the same buffer.

A qualitative analysis of the distribution of GTF in the samples was obtained by isoelectric focusing in thin-layer 5% polyacrylamide gel slabs (1 × 245 × 110 mm) containing 2·2% (w/v) ampholytes in pH ranges 3·5 to 9·5 and 4·0 to 6·5 (LKB). The gels were cooled to 7·5°C (Hoefer Isobox), the samples (0·1 U GTF in 2–10 μl) were applied, and the focusing was programmed with a computer controlled electrophoresis power supply (Bio-Rad Model 3000 xi). The gels were rinsed with 0·05 M sodium phosphate buffer (pH 6·0), and then incubated overnight at 35°C in the same buffer containing 1% sucrose, 1% Triton X-100 and 0·002% dextran T10 (Pharmacia). The opaque white zones indicated the position of water-insoluble glucans, and water-soluble glucans were located at transparent, swollen zones which became white when the slabs were fixed for 30 min in 75% ethanol. Further, minors zones of dextrans were revealed after oxidation with periodic acid and staining with Schiff's reagent (Kapitany & Zebrowski, 1973).

More — quantitative and preparative separations of the major GTF were obtained by liquid chromatography. For this a sample containing *c.* 20 U of GTF activity was dialyzed for 1 h against potassium phosphate buffer (10 mM, pH 6·5), and then applied to a column (1·5 × 26·5 cm) of hydroxyapatite (Bio-Gel HTP) prepared in the same buffer. The column was washed with 40 ml of the same buffer, and then the proteins were eluted with a linear gradient of potassium phosphate buffer (pH 6·5) at a flow rate of 10 ml h^{-1}. Variation in the growth conditions slightly altered the phosphate concentration required to elute some of the GTF. The separations were carried out at 5°C.

Glucosyltransferase activity in the fractions (4 ml) eluted from the column was determined by incubating portions (0·1 ml) with sucrose (1·7 mg) and Sigma type 2000 dextran (125 μg) in duplicate 0·25 ml digests at 35°C. After incubation for 2 h and 20 h the utilization of sucrose was determined (Van Handel, 1968) and then aliquots of the fractions from each peak of activity were pooled. The total GTF activity in each pool was determined by incubation with sucrose [glucose-^{14}C(U)] as described above, and also by the sucrose utilization test. The results permitted the proportion of the different types of GTF produced under each growth condition to be calculated. Both methods gave essentially similar results, showing that the utilization of sucrose was due to GTF activity. A sample (1 ml) from each pool was dialyzed against water, and the protein content was determined with Bio-Rad protein assay (Bradford, 1976), using bovine serum albumin as standard.

The enzyme formerly called GTF-S, which was released by all the strains we tested, was the first glucosyltransferase to be eluted off hydroxyapatite, and has now been renamed GTF-S1. Because the pool of GTF-S1 contained two active proteins, with pI 3·9 and 4·1 respectively, the next GTF-S enzymes eluted have been designated GTF-S3 and GTF-S4. This nomenclature then agrees with that adopted by McCabe *et al.* (1983) for GTF-S4, released by strain 6715-13-27.

Carbohydrates

Sucrose [glucose-^{14}C(U)] was purchased from NEN Research Products, Boston, USA. Water-insoluble α-D-glucans were prepared by incubating cell-free filtrates with sucrose (Hare *et al.*, 1978). The glucans were methylated and analyzed by capillary gas–liquid chromatography-mass spectrometry (Slodki *et al.*, 1986).

RESULTS AND DISCUSSION

The well-known sensitivity of microorganisms to their environments means that small differences in the growth conditions of *S. sobrinus* are likely to lead to changes in the production of extracellular enzymes. A study of the factors affecting the production and distribution of GTF therefore requires strict adherence to constant growth conditions so that reproducible results can be obtained. In batch culture the streptococci are in a continuously transient situation because the environment changes progressively with time. The concentration of nutrients and products alters throughout the exponential phase, and pronounced changes in the properties of a culture occur even in the stationary phase. Because the microorganisms are never in a steady state, enzyme activity tests on samples taken from successive batch cultures of the same organism do not give consistent values.

In the present study, time has been eliminated as a factor by growing *S. sobrinus* in continuous culture. Microbial activity has been measured in a steady state system, and one variable has been changed at a time, without affecting the others. It has been possible to show which of the variables such as generation time, pH and carbohydrate source can affect the production of GTF.

Effect of growth rate and carbohydrate source

The specific growth rate in the chemostat is equal to the dilution rate (D), which is the rate of input of fresh medium per volume of culture. The mean graduation time (t_d), which is related to D by $D=(\log_e 2)/t_d$,

was controlled by adjusting the rate of supply of the medium, which, in most of the experiments, contained sugar (0·5%) as the growth-limiting nutrient. When D was set at values between 0·35 and 0·50 h^{-1}, the growth rate was high, with mean generation times in the range of 1·4 to 2·0 h. By contrast, values of D between 0·05 and 0·10 h^{-1} gave low growth rates with doubling times (t_d) of 7–14 h. In dental plaque, as in biological systems in general, the streptococci grow slowly (*c.* two mean generations per day) under nutrient limitation. Carbohydrate usually limits growth, although surface organisms are periodically subject to windfalls of food. Accordingly, the authors have examined the effect on GTF output of culturing *S. sobrinus* strains at both high and low growth rates, and under conditions where the sugar concentration was limiting or sufficient.

In the initial studies the pH of the cultures was maintained at 6·0. Three strains of *S. sobrinus* were grown in glucose- and in fructose-limited medium (Table 1), and two more strains were grown in glucose-limited and in glucose-sufficient medium (Table 2). When the dilution rate was increased sequentially from 0·05 h^{-1} to 0·50 h^{-1}, extracellular GTF activity (U $liter^{-1}$) was highest at the lowest dilution rate for all strains except 6715-13-201, and decreased with increasing growth rate. In contrast, the rates of production of GTF (U g^{-1} h^{-1}) by all strains increased with increasing dilution rate. The replacement of D-glucose with D-fructose as the limiting nutrient generally resulted in decreased productivity (14–33%) but only at low growth rate (D 0·1 h^{-1}), perhaps due to the longer residence times of the bacteria in the culture vessel under this condition. The difference was not significant at high growth rate (D 0·5 h^{-1}).

Under conditions of glucose sufficiency, the rate of production of GTF by *S. sobrinus* K1-R was lower than in cultures where glucose was limiting, irrespective of growth rate (Table 2). This might result from catabolite repression by glucose, which was present at a concentration of 0·2% at D 0·05 h^{-1} and 1·9% at D 0·45 h^{-1}. By contrast, filtrates from glucose-limited cultures never contained more than 0·0005% glucose. The reduction of enzyme production in glucose-excess medium varied between 36% and 50%, and a somewhat smaller reduction (12·5%) was found for *S. sobrinus* K1-R grown at D 0·12 h^{-1} by Wenham *et al.* (1981). Strain 6715-13-201 washed out of the culture vessel during attempts to grow at dilution rates higher than D 0·35 h^{-1} in glucose-excess medium. At low growth rate there was no evidence for repression of GTF production, and a comparison of slopes from plots (not shown) of productivity versus dilution rate indicated that excess D-glucose reduced productivity by no more than 20% as the growth rate increased. These results with *S. sobrinus* are in complete contrast to those obtained with two other organisms from the mutans groups of streptococci

TABLE 1

Effect of Growth Rate and Carbohydrate Source on the Activity and Productivity of Extracellular D-Glucosyltransferase of *S. sobrinus* Strains Cultured at pH 6·0

Strain	*Growth conditions*		*Culture density dry wt (g liter^{-1})*	*Glucosyltransferase*	
	Limiting sugar (0·5%)	*Dilution rate (h^{-1})*		*Activity (U liter^{-1})*	*Productivity (U g^{-1} h^{-1})*
B13	Glc	0·1	0·86	56	6·5
		0·5	0·94	46	24·5
	Fru	0·1	1·00	49	4·9
		0·2	0·98	36	7·3
		0·5	0·78	37	24
6715	Glc	0·1	0·81	192	24
		0·2	0·86	143	33
		0·5	0·82	97	59
	Fru	0·5	0·98	110	56
6715-1119R	Glc	0·1	1·04	28	2·7
		0·2	1·10	20	3·6
		0·5	1·07	17	7·9
	Fru	0·1	0·88	15	1·8
		0·2	0·92	14	3·1
		0·5	0·88	11	6·2

TABLE 2

Effect of Growth Rate on the Activity and Productivity of Extracellular D-Glucosyltransferase of *S. sobrinus* Strains grown in Glucose-Limited and Glucose-Sufficient Medium at pH 6·0

Strain	*Growth conditions*		*Culture density dry wt (g liter^{-1})*	*Glucosyltransferase*	
	Glucose (%)	*Dilution rate (h^{-1})*		*Activity (U liter^{-1})*	*Productivity (U g^{-1} h^{-1})*
6715-13-201	0·5	0·05	0·85	210	12·4
		0·44	0·83	266	141
		0·50	0·81	277	171
	4·0	0·05	1·71	420	12·3
		0·25	1·74	454	65·2
		0·35	1·58	446	98·8
K1-R	0·5	0·05	0·82	57	3·5
		0·30	1·09	31	8·5
		0·50	1·13	33	14·6
	4·0	0·05	2·09	70	1·7
		0·30	2·02	36	5·3
		0·50	1·56	29	9·3

(Wenham *et al.*, 1979, 1981). *Streptococcus mutans* strains Ingbritt and 3209 released 3-fold and 10-fold more GTF activity respectively, in glucose-sufficient medium than in glucose-deficient medium, when they were grown at D 0·12 h^{-1}.

Effect of variation in pH

Three strains of *S. sobrinus* were grown in glucose-limited medium at D 0·10 h^{-1} and at different values of pH. The cell free filtrates were all dialyzed for 4 h against 50 mM sodium citrate buffer (pH 6·0) in preparation for the assay. Maximum production of GTF occurred at pH 6·5 for all strains (Table 3). The effect of growth rate on the variation in GTF productivity with pH was then tested by growing strain K1-R at D 0·49 h^{-1} and at pH 6·0, 6·5 and 7·0. The rate of production of GTF was 14·3, 29·3 and 24·2 U g^{-1} h^{-1} respectively, showing that despite the changes in distribution of component GTF enzymes that could result from the increase in growth rate, a pH of 6·5 was optimal at both low and high values of D; accordingly in subsequent studies the pH was maintained at 6·5. The marked loss of productivity found when the pH was decreased to 5·5 (Table 3) illustrates that a significant reduction in GTF activity must occur in batch cultures without pH control, where the pH can fall to 4·5. The extracellular GTF activity of *S. sobrinus* strain 6715 was unusually low in batch cultures when the final pH growth was 4·6 to 5·0 (Shimamura *et al.*, 1982, 1983). The isoelectric points of the major *S. sobrinus* glucosyltransferases occur below pH 6·5, with most of the enzymes having pI values in the range of 4·0 to 5·8 (Tsumori *et al.*, 1983). It is clearly advisable to control the pH of growth above 6·0, to prevent the partial inactivation of the GTF that may occur at their isoelectric points.

Effects of Tween 80

The authors have confirmed previous reports that Tween 80 has little or no effect on the cell density in batch cultures of *S. sobrinus* (Shimamura *et al.*, 1983), or on the determination of GTF activity in the standard assay (Umesaki *et al.*, 1977). When strain K1-R was grown in medium supplemented with D-glucose (0·5%), the dry cell weights remained at 1 g $liter^{-1}$, but GTF activity was 362 and 21 U $liter^{-1}$, in batch cultures grown at pH 6·5 with and without Tween 80 (0·5%) respectively. However, in continuous cultures, the presence of Tween 80 resulted in detectable increases in cell density at high growth rates (Tables 5 and 6). The effect of Tween 80 on the rate of production of GTF was deter-

mined at low and high dilution rates (Table 4). In glucose-limited cultures, GTF productivity of strain 6715-13-201 was enhanced *c.* 2-fold at each growth rate, whereas the increase in productivity of strain K1-R GTF at high dilution rate (13-fold) was greater than that at low dilution rate (5-fold). Addition of Tween 80 to the medium therefore had more effect on the wild-type strain that produced the lesser amount of enzyme, and brought the rate of GTF production by strain K1-R closer to that of strain 6715-13-201. For instance, in normal unsupplemented medium, strain 6715-13-201 produced over 10-fold more GTF than strain K1-R at D 0·45 h^{-1} (cf. 172 with 16 U g^{-1} h^{-1}), whereas in Tween 80 medium, the strain difference was less than 2-fold (cf. 363 with 206 U g^{-1} h^{-1}). This effect has previously been observed with other micro-organisms. Surfactant stimulation of extracellular cellulase secretion in

TABLE 3

Effect of the pH of Growth of *S. sobrinus* Strains on the Productivity of D-Glucosyltransferase in Sugar-Limited Continuous Culture at a Dilution Rate of 0·10 h^{-1}

Strain	*Sugar (0·5%)*	*Productivity (U g^{-1} h^{-1})*			
		pH 5·5	*pH 6·0*	*pH 6·5*	*pH 7·5*
B13	Glc	3·7	6·3	8·4	7·3
6715	Glc	—	20	66	20
	Sucr	—	—	175	41
6715-1119R	Glc	1·4	2·7	6·2	5·5

TABLE 4

Effect of Tween 80 on the Rate of Production of D-Glucosyltransferases by *S. sobrinus* Strains in Continuous Culture Under Various Growth Conditions at pH 6·5

Growth conditions		*Rate of GTF production (U g^{-1} h^{-1})*			
D (h^{-1})	*Tween 80 (%)*	*Strain K1-R*		*Strain 6715-13-201*	
		Glc-lim.	*Sucr-lim.*	*Glc-lim.*	*Sucr-lim.*
0·075	0	4·5	4·2	28	20
	0·5	22	19	57	35
0·30	0	10	—	145	—
	0·5	—	—	270	—
0·45	0	16	23	172	70
	0·5	206	125	363	249

Trichoderma viride (Reese, 1972) and *Thermomonospora curvata* (Stutzenberger, 1987) was maximal in strains that normally produced little enzyme, and minimal in strains that produce high concentrations. Another mutant, *S. sobrinus* 6715-13-27, with a high natural production of GTF, was little affected by growth in Tween 80 medium, the total activity at D 0·075 h^{-1} remaining unchanged at 175 U g^{-1}. By contrast, *Streptococcus salivarius* ATCC 25975, an oral streptococcus with a low natural production of GTF, was stimulated by growth in Tween 80 medium to produce high concentrations of GTF, the activity at D 0·40 h^{-1} being raised from 13 U g^{-1} up to 1180 U g^{-1} in glucose-limited continuous culture at pH 6·5 (Pitty & Jacques, 1987).

When strain K1-R was grown under conditions of glucose sufficiency, supplementation with Tween 80 stimulated GTF productivity only at low dilution rate, the value being increased from 1·7 to 31 U g^{-1} h^{-1}. At high growth rate the increase in productivity was not significant, showing that the effect of Tween 80 may be governed by other cultural conditions. A decreased influence of Tween 80 on catabolite-repressed cells has been noted previously (Stutzenberger, 1987).

Because the predominant dietary sugar is sucrose, strains K1-R and 6715-13-201 were also grown in sucrose-limited cultures at low and high dilution rate. The results (Table 4) were similar to those found for glucose-limited cultures, namely, Tween 80 enhanced GTF productivity of strains K1-R and 6715-13-201 by 5-fold and 2-fold respectively, again reducing the difference in the productivity between the two strains. To avoid the problems (e.g. wall growth) associated with excess sucrose in the culture during the log phase, the strains were inoculated into glucose-medium, and the flow of sucrose-medium began after all the glucose was utilized. Although sucrose could not be detected in culture filtrates, sufficient sucrose was available in the culture to cause a significant amount of GTF to become cell-associated (Spinell & Gibbons, 1974). Therefore, the results in Table 4 refer to the sum of cell-associated and extracellular GTF activity. Under most conditions, the rate of GTF production decreased when sucrose replaced glucose as the limiting nutrient. This could be explained by the cell-associated component being incompletely expressed or becoming irreversibly inactivated (McCabe & Smith, 1973).

Separation of *S. sobrinus* α-D-glucosyltransferases

It could not be assumed that the variations in total GTF activity shown to occur under different growth conditions meant that there were equivalent changes in the production of each type of glucosyltransferase. It

was possible that quite small changes in total activity could conceal large alterations in the proportion and productivity of the component GTFs. The distribution and activity of glucosyltransferases were therefore determined in culture filtrates from a variety of growth conditions. The aim was to adopt a simple procedure that gave high yields and reproducible separations of each GTF; complex schemes that result in low yields of highly purified enzymes were unacceptable for this purpose. Hydroxyapatite column chromatography was considered to be the most suitable means of separating the enzymes, because the sensitivity of hydroxyapatite to subtle differences in stereochemical structure permits the separation of proteins even when they have similar shapes, isoelectric points and aminoacid composition (Kawasaki *et al.*, 1986). Although early work with culture filtrates treated directly with hydroxyapatite failed to demonstrate the production of GTF-S by mutans streptococci (Ceska *et al.*, 1972), column chromatography has become a most successful procedure for separating GTF-S and GTF-I. Now, the high resolving power of hydroxyapatite has enabled the separation, in one step, of up to four stable GTF (Figs 1–6) that catalyze the synthesis of structurally distinct α-D-glucans. Enzyme recoveries were often higher than 90% and the distribution of the GTF at any given growth condition was highly reproducible. The results depicted in Figs 1–6 give an indication of the variety of profiles obtained with different strains grown under different conditions; more data are presented in Tables 5 and 6.

Effect of growth rate on the distribution of glucosyltransferases

S. sobrinus strain 6715 released no detectable GTF-I when it was grown at low dilution rate (D 0·1 h^{-1}) under glucose limitation in dialyzed medium (Fig. 1), and the culture filtrate produced no water-insoluble glucan from sucrose. Several other strains, grown in nondialyzed medium, were capable of releasing minor amounts of GTF-I under this condition. For instance, with streptococci grown at D 0·05 h^{-1} and pH 6·0, the activity in the GTF-I pool, expressed as a percentage of total GTF activity eluted from the HTP column, was 1·5% for strain 6715-1119R, 3% for strain OMZ176, 9% for strain K1-R and 11% for strain 6715-13-201. These results were reproducible from one chemostat culture to another, and did not alter when the chromatography was repeated after preparations had been stored at 5°C for several weeks. The only other glucosyltransferase eluted was GTF-S1. This enzyme, which was eluted with 0·07 M potassium phosphate, corresponded to the enzyme previously designated GTF-S. GTF-I was well separated from GTF-S1, being eluted with 0·25 M potassium phosphate. Culture filtrates

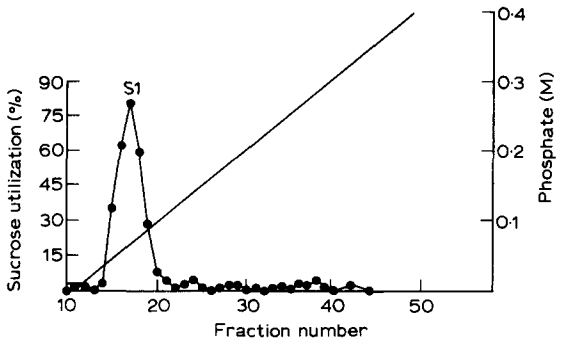

Fig. 1. Hydroxyapatite column chromatography of a single extracellular D-GTF (●) of *S. sobrinus* 6715. The organism was grown at pH 6·5 in glucose-limited dialyzed medium at a dilution rate of 0·10 h^{-1}. Culture filtrate (43 ml) containing GTF activity (605 U $liter^{-1}$) and no dextranase, was prepared as described in 'Methods' and applied to the column. GTF activity in the fractions was assayed by the sucrose utilization test after incubation for 2 h. All the activity was associated with GTF-S1.

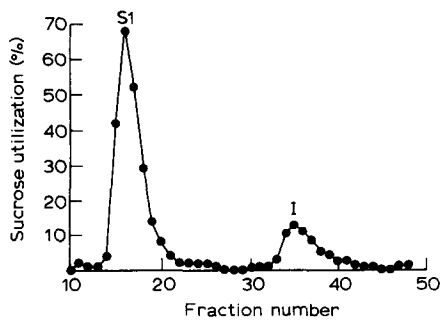

Fig. 2. Separation on hydroxyapatite of two extracellular GTF (●) of *S. sobrinus* 6715-13-201 (dextranase negative). The organism was grown at pH 6·0 under glucose limitation at a dilution rate of 0·05 h^{-1}. Culture filtrate (0·1 liter) containing GTF activity (160 U $liter^{-1}$) was prepared and applied to the column. GTF activity in the fractions was assayed by the sucrose utilization test after incubation for 1 h. The proportion of activity associated with the two peaks was GTF-S1, 89%; GTF-I, 11%.

from low growth rates never exhibited dextranase activity, and none was detected in column eluates. The water-insoluble glucans produced from sucrose, when such culture filtrates contained small amounts of GTF-I, were therefore synthesized in the absence of dextranase. The yields of water-insoluble glucans obtained from strains OMZ176, K1-R and 6715-13-201 were 2·3, 2·4 and 9·1 g $liter^{-1}$ of culture filtrates that contained GTF-I activity of 5·6, 5·1 and 23·1 U $liter^{-1}$, respectively. These glucans did not adhere to the walls of the glass flasks during synthesis from sucrose.

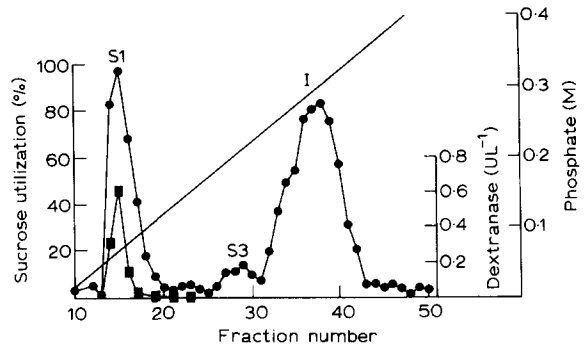

Fig. 3. Separation on hydroxyapatite of three extracellular D-glucosyltransferases (●) of *S. sobrinus* K1-R. The organism was grown at pH 6·0 in glucose-sufficient medium at a dilution rate of 0·50 h^{-1}. Culture filtrates (0·25 liter) containing GTF activity (29 U $liter^{-1}$) and dextranase activity (19 U $liter^{-1}$) were prepared and applied to the column. GTF activity in the fractions was assayed by the sucrose utilization test after incubation for 24 h. The proportion of activity associated with the peaks was GTF-S1, 39%; GTF-S3, 1%; and GTF-I, 60%. Dextranase activity (■) eluted with GTF-S1.

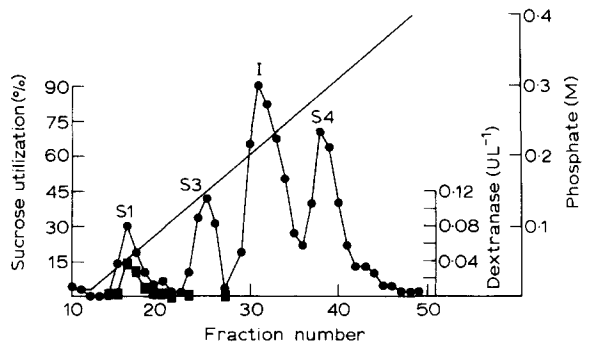

Fig. 4. Separation on hydroxyapatite of four extracellular GTF (●) of *S. sobrinus* K1-R. The organism was grown at pH 6·5 at a dilution rate of 0·45 h^{-1} in glucose-limited medium supplemented with Tween 80 (0·5%). Culture filtrate (0·18 liter) containing GTF activity (478 U $liter^{-1}$) and dextranase activity (2 U $liter^{-1}$) was prepared and applied to the column. GTF activity in the fractions was assayed by the sucrose utilization test after incubation for 45 min. The proportion of activity associated with the four peaks was GTF-S1, 7%; GTF-S3, 7%; GTF-I, 60% and GTF-S4, 26%. Dextranase activity (■) eluted together with GTF-S1.

Though GTF-S1 was the predominant GTF at low growth rate, GTF-I became a significant enzyme when the growth rate was high. The results in Table 5 show that when the dilution rate was increased from 0·05 to 0·50 h^{-1}, the proportion of GTF-I in K1-R culture filtrates increased over 7-fold from 9% to 67%. Although the total GTF activity fell from 57 to 33 U $liter^{-1}$, the productivity of GTF-I rose from 0·31 to 9·8 U g^{-1}

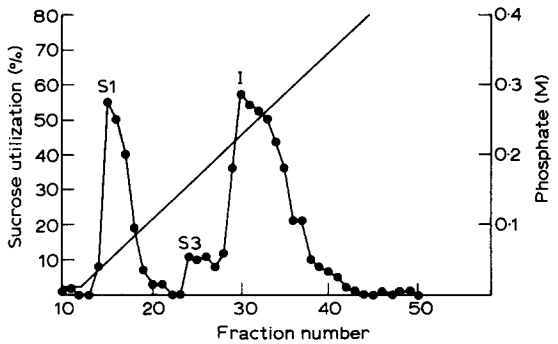

Fig. 5. Separation on hydroxyapatite of three extracellular GTF (●) of *S. sobrinus* 6715-13-201. The organism was grown at pH 6·5 under glucose limitation at a dilution rate of 0·45 h^{-1}. Culture filtrate (63 ml) containing GTF activity (336 U $liter^{-1}$) was prepared and applied to the column. GTF activity in the fractions was assayed by the sucrose utilization test after incubation for 2 h. The proportion of activity associated with the three peaks was GTF-S1, 42%; GTF-S3, 4%; GTF-I, 54%.

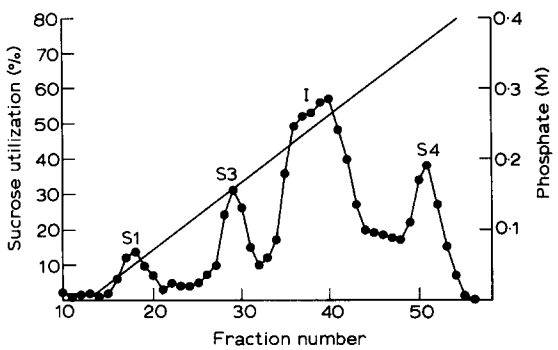

Fig. 6. Separation on hydroxyapatite of four extracellular GTF (●) of *S. sobrinus* 6715-13-201. Culture conditions were the same as those shown in Fig. 5, but the medium was supplemented with Tween 80 (0·5%). Culture filtrate (25 ml) containing GTF activity (912 U $liter^{-1}$) was prepared and applied to the column. GTF activity in the fractions was assayed by the sucrose utilization test after incubation for 2 h. The proportion of activity associated with the four peaks was GTF-S1, 4%; GTF-S3, 8%; GTF-I, 70%; GTF-S4, 14%.

h^{-1}, a 31-fold increase. A high growth rate also favored the production of dextranase (Walker *et al.*, 1982), and the traces of this enzyme found in the GTF-S1 pool could either inhibit synthesis by GTF-S1 and/or partially degrade the S1-dextran product (Pulkownik & Walker, 1977), giving an apparent reduction in the contribution of GTF-S1 to the total GTF activity. This is not however a likely explanation for the pre-

TABLE 5
Effect of Growth Conditions on the Activity and Distribution of *S. sobrinus* K1-R Extracellular D-Glucosyltransferases

Growth conditions				*Culture density dry wt*	*Extracellular glucosyltransferases*				
Glc (%)	*pH*	*Tween 80 (%)*	*D (h^{-1})*	*(g $liter^{-1}$)*	*Activity (U $liter^{-1}$)*	*Distribution (%)*			
						S1[a]	*S3*	*S4*	*I*[b]
0·5	6·0	0	0·05	0·82	57	91	0	0	9
0·5	6·0	0	0·50	1·13	33	33	0	0	67
0·5	6·5	0·5	0·075	1·09	315	12	6	22	60[c]
0·5	6·4	0	0·49	0·98	59	63	1	0	36
0·5	6·6	0	0·45	0·86	25	70	1	0	29
0·5	6·5	0·5	0·45	1·05	478	7	7	26	60
0·5	7·0	0	0·50	0·79	39	80	1	0	19[c]
4·0	6·0	0	0·05	2·09	70	73	0	0	27
4·0	6·0	0	0·50	1·56	29	39	1	0	60
4·0	6·5	0·5	0·075	1·70	704	17	10	±	73[c]
4·0	6·4	0	0·45	0·97	92	71	1	0	28
4·0	6·5	0·5	0·45	1·36	164	4	10	7	79

[a]S1, S3, S4 represent GTF-S1, −S3, −S4 respectively.
[b]I represents GTF-I.
[c]Double peak.

dominance of GTF-I at high growth rate, because a dextranase inhibitor (Hamelik & McCabe, 1982) is produced by *S. sobrinus* at all growth rates (Pearce *et al.*, 1988), and this protein also partly coelutes with GTF-S1 and dextranase, suppressing the latter activity. Thus, the results obtained with the dextranase-positive strain K1-R and the 'dextranase-negative' mutant 6715-13-201 were similar (Tables 5, 6), and the proportion of GTF-I in culture filtrates of the latter increased 6- and 15-fold when the dilution rate was raised from 0·05 to 0·45 h^{-1} at pH 6·0 and 6·5 respectively. The productivity of GTF-I was over 100 U g^{-1} h^{-1} when strain 6715-13-201 was grown at *D* 0·45 h^{-1} at both values of pH. This mutant did not grow well at pH 7·0, and began to wash out after five culture volumes at *D* 0·45 h^{-1}.

Effect of pH on the distribution of glucosyltransferases

S. sobrinus strain K1-R was grown under glucose limitation at high dilution rate with the pH of the cultures maintained at four different

values. This resulted in a marked alteration in the distribution of GTF-S and GTF-I, with the proportion of GTF-I falling from 67% at pH 6·0 down to 19% at pH 7·0 (Table 5). There was no significant change in the release of endodextranase, the activity at pH 6·0 and 7·0 being 6·9 and 7·2 U liter^{-1}, respectively. The water-insoluble glucans synthesized by the culture filtrates differed greatly in their adhesion to glass. Culture filtrates from growths between pH 6·4 and 7·0 produced glucans that remained in suspension, whereas the filtrate from growths controlled at pH 6·0 synthesized glucan that adhered so strongly to the wall of the flask that it was difficult to dislodge. Many other strains of mutans streptococci gave similar results, and the mutant 6715-13-201 was the only strain able to synthesize adherent glucan when growth had occurred at pH 6·5. The adhesive property of *S. sobrinus* water-insoluble glucan was clearly related to the distribution of GTF-S and GTF-I, and the glucans became soft, gelatinous and non-adhesive when the proportion of GTF-S began to exceed *c.* 40%. A high value for total GTF activity was of no importance for the production of adhesive glucan unless GTF-I was the predominant enzyme. However, the contribution of the dextran chains synthesized by GTF-S is essential for adhesion, pure α-(1→3)-D-glucan being non-adhesive. Inoue *et al.* (1982), who synthesized strain OMZ176 glucans with GTF-I in the presence of increasing amounts of GTF-S, found that the water-insoluble glucans were most adhesive when the proportion of GTF-I to GTF-S was 63:37, and became non-adhesive when the ratio was 20:80. At high growth rate, the predominance of GTF-I, which accounted for over 60% of the total glucosyltransferase activity of strain 6715-13-201 culture filtrates at all values of pH, explains why the water-insoluble glucans synthesized from sucrose were always adhesive.

Influence of glucose concentration in the medium on the distribution of glucosyltransferases

When *S. sobrinus* strains K1-R and 6715-13-201 were grown at low dilution rate and pH 6·0 in medium containing an excess of glucose rather than limited glucose, the proportion of the total GTF activity associated with GTF-I rose 3- to 6-fold (Tables 5, 6). At high growth rates (D 0·45–0·50 h^{-1}) the glucose concentration in glucose-limited continuous cultures is close to being cell saturating, and the organisms may therefore express a state in common with glucose-sufficient cultures and also with those growing exponentially in batch culture (Tempest, 1978). Thus, the concentration of glucose in the medium had little effect on the distribution of GTF released by strain K1-R cells growing at high

dilution rates. In glucose-sufficient medium the proportion of GTF-I released at pH 6·0 was again much higher than that released at pH 6·4. Strain 6715-13-201 would not grow in glucose-excess medium at dilution rates higher than 0·36 h^{-1}, but GTF-I was the major GTF under the conditions that could be tested. Most culture filtrates obtained from high growth rates in both glucose-limited and glucose-sufficient medium contained small amounts (1–3%) of a GTF that eluted between GTF-S1 and GTF-I.

Influence of the distribution of glucosyltransferases in culture filtrates on the structure of water-insoluble glucans

The authors have shown that variations in the environmental conditions result in quantitative changes in total GTF activity, and also in radical qualitative changes in the distribution of GTF-S types and GTF-I. These changes were expected to affect the structure of the water-insoluble glucans synthesized by culture filtrates from sucrose, with those from conditions that elicit a higher proportion of GTF-I producing glucans with higher proportions of (1→3)-linked chains. Methylation analysis of selected glucans proved this to be the case. The molar proportion of 2,4,6-tri-o-methyl ether was lowest from glucans synthesized with culture filtrates from low dilution rate where GTF-I is a minor component (Table 7). An increase in the dilution rate, or a change in the pH of growth from 7·0 to 6·0 at D 0·49 h^{-1} brought about an increase in the proportion of GTF-I and a concomitant increase in the percentage of (1→3)-linked chains in the glucans. With strain OMZ176, growth at pH 6·0 either in glucose-deficient and glucose-sufficient continuous culture at D 0·50 h^{-1}, or in batch culture, gave a glucan product containing similar proportions of (1→3)-linked chains (63%, 64% and 68%, respectively). The results also demonstrate the more-highly branched nature of the dextran-like regions of the water-insoluble glucans synthesized when GTF-S1 was the predominant enzyme. Culture from low growth rates at pH 6·0, or from high growth rate at pH 7·0, released from 73–97% of their GTF activity as GTF-S1, and the extent of branching (range 20–22%) was 75% higher than the average value (12%) for water-insoluble glucans (Table 7).

Distribution of D-glucosyltransferases in medium containing Tween 80

The profile of GTF activity in column eluates was greatly altered when culture filtrates from growth in Tween 80-supplemented medium were applied to hydroxyapatite columns. This was due to the selectivity of the

TABLE 6
Effect of Growth Conditions on the Activity and Distribution of *S. sobrinus* 6715-13-201 Extracellular D-Glucosyltransferases

Growth conditions				*Culture density*	*Extracellular glucosyltransferases*				
Glc (%)	*pH*	*Tween 80 (%)*	*D (h^{-1})*	*dry wt (g $liter^{-1}$)*	*Activity (U $liter^{-1}$)*	*Distribution (%)*			
						S1	*S3*	*S4*	*I*
0·5	6·0	0	0·05	0·85	210	89	0	0	11
0·5	6·0	0	0·39	0·83	266	25	2	0	73
0·5	6·0	0·5	0·39	0·96	703	28	13	14	45
0·5	6·5	0	0·05	0·85	274	96	0	0	4
0·5	6·5	0·5	0·05	0·98	666	31	3	16	50
0·5	6·5	0	0·45	0·88	336	36	3	0	61
0·5	6·5	0·5	0·45	1·13	912	4	8	14	74
0·5	7·0	0	0·05	0·76	211	87	2	0	11
0·5	7·0	0	0·39	0·96	491	32	1	3	64
0·5	7·0	0·5	0·39	1·03	903	26	6	11	57
4·0	6·0	0	0·05	1·71	420	34	0	0	66
4·0	6·5	0	0·05	1·78	474	27	2	0	71
4·0	6·1	0	0·35	1·58	446	20	3	0	76
4·0	6·3	0	0·36	1·37	568	28	3	0	69

surfactant in stimulating the release of certain components rather than facilitating an increased production of the complete array of GTF. Addition of Tween 80 to culture filtrate obtained from growth in the absence of Tween 80 failed to promote the separation of any new peaks of GTF activity on hydroxyapatite. Whereas growth in normal medium led to the release of two major enzymes, GTF-S1 and GTF-I, with minor amounts of GTF-S3 being produced under certain conditions, the effect of growth in Tween 80-medium was to permit the release of four major GTF which were well separated on hydroxyapatite (Figs 4 and 6). The two new enzymes, now released in significant amounts, were primer-independent, and they both synthesized soluble dextrans (Taylor *et al.*, 1990).

One of these enzymes, GTF-S3, was eluted with 0·15 M potassium phosphate, and its contribution to the total GTF activity of strain 6715-13-201 rose from the previous maximum of 3% up to 13%, depending on the growth condition (Table 6). This resulted in the productivity of GTF-S3 being increased over 10-fold. The response found with strain K1-R was even higher. When growth occurred at D 0·45 h^{-1}, the pres-

TABLE 7
Effect of Growth Conditions of *S. sobrinus* on the Structure of the Water-Insoluble Glucans Synthesized from Sucrose[a]

Strain	*Growth conditions*			*Glucosyltransferase*		*Analysis of glucan*			
	Glc (%)	*pH*	*D (h^{-1})*	*Total (U $liter^{-1}$)*	*GTF-I (%)*	*o-Methyl ether of D-glucose (%)*			
						2,4,6-	*2,3,4-*	*2,4-*	*2,3,4,6-*
K1-R	0·5	6·0	0·49	33	67	67	11	12	10
	0·5	6·4	0·49	59	36	62	13	13	12
	0·5	7·0	0·49	39	19	47	14	19	20
K1-R	4·0	6·0	0·05	70	27	41	17	22	20
	4·0	6·0	0·50	20	60	62	13	13	11
	2·0	6·0	Batch			64	11	13	12
OMZ176	0·5	6·0	0·05	181	3	39	17	21	22
	0·5	6·0	0·53	31	46	64	11	13	12
	0·5	6·0	0·69			66	10	12	12
OMZ176	4·0	6·0	0·05	73		58	11	16	15
	4·0	6·0	0·50	37		63	15	11	11
	2·0	6·0	Batch		42	68	12	9	11
	2·0	6·0	Batch		—[b]	88	7	2	3

[a]Sucrose was incubated with cell-free culture filtrate (CFF).
[b]Sucrose was incubated with GTF-I isolated from CFF.

ence of Tween 80 in the medium brought about an increase in GTF-S3 activity from 1% of 25 U $liter^{-1}$ to 7% of 478 U $liter^{-1}$, corresponding to an increase in productivity of well over 100-fold. Under comparable conditions, strain 6715-13-201 released twice as much GTF-S3 as strain K1-R (73 and 33 U $liter^{-1}$, respectively), and from the growth conditions examined (Tables 5, 6) maximum values were 70 U $liter^{-1}$ for strain K1-R and 91 U $liter^{-1}$ for strain 6715-13-201.

The second new enzyme, GTF-S4, was eluted with 0·35 M potassium phosphate buffer, and emerged from the hydroxyapatite column after GTF-I. Incubation of peak fractions of GTF-S4 with sucrose resulted in the synthesis of a highly viscous glucan. Although this enzyme had not previously been detected in culture filtrates from growth at pH 6·0 in the absence of Tween 80, subsequent studies revealed that a small amount of enzyme corresponding to GTF-S4 was released by strain 6715-13-201 in continuous culture at D 0·39 h^{-1} and pH 7·0 (Table 6). Moreover, the double peak for GTF-I recorded when strain K1-R was grown under a

similar condition (Table 5), might have resulted from a partial separation of GTF-I and GTF-S4. In Tween 80 medium, GTF-S4 became the predominant type of GTF-S for both strains when growth occurred at D 0·45 h^{-1} and pH 6·5. Values for the two strains were the same, the activity and productivity of GTF-S4 being 125 U $liter^{-1}$ and 52 U g^{-1} h^{-1}, respectively.

Under many conditions, the activity of GTF-S3 and GTF-S4, either alone or in combination, exceeded that of GTF-S1, when the medium contained Tween 80. However, the actual production of GTF-S1 was not significantly altered by the presence of Tween 80. By far the major effect of the surfactant was to stimulate the release of large amounts of GTF-I, the effect on strain K1-R being the more pronounced, with *c.* 60% of the total effect of Tween 80 being due to increased production of GTF-I. The consequences were particularly significant under conditions of low growth rate, when *S. sobrinus* strains normally produced low concentrations of GTF-I. For example, when strain K1-R was grown under glucose limitation or glucose sufficiency, the proportion of GTF-I, which was normally 9% and 27% respectively, for growth at pH 6·0, rose to 60% and above, giving a distribution of GTF favorable for the synthesis of adhesive water-insoluble glucan. At high growth rate in glucose-limited medium, the activity of strain K1-R GTF-I rose from 29% of 25 U $liter^{-1}$ to 60% of 478 U $liter^{-1}$, a 40-fold stimulation by Tween 80. Similar calculations indicated that the release of GTF-I activity from strain 6715-13-201, which was already high at D 0·45 h^{-1} (61% of 336) was increased only 3·3-fold by growth in Tween 80. Thus, although this strain released nearly 30-fold more GTF-I than did K1-R in normal medium, the difference between the strains fell to 2·3 when the medium was supplemented with Tween 80.

Examination of the GTF in concentrated culture filtrates by isoelectric focusing revealed the presence of up to five zones with pI values ranging from 3·9 to 7·0. The *S. sobrinus* strains released GTF-S1, S2 under all growth conditions, with the two zones resolving at pH 3·9 and 4·1. Similar zones were observed when *S. sobrinus* strains had grown in Tween 80 medium and, in addition, there were multiple zones with pI 6·5–7·0 which appeared to be identical with the major GTF-S, GTF-S4, released by mutant 6715-13-27. The pI values were in general similar to those obtained by several investigators (McCabe, 1985; Musaka, 1986), which enabled comparisons to be made between this and other studies. For example, Tween 80 was previously observed to stimulate the synthesis by *S. sobrinus* of only one extra GTF-S. Isoelectric focusing pointed to this being GTF-S4, but absolute identification on the basis of pI alone is never possible because the GTF are prone to give multiple

bands, and their pI values change during purification and storage. A more satisfactory approach, which served to distinguish the three types of GTF-S without doubt, was to allow each enzyme to catalyze the synthesis of soluble dextran. Analysis of these products has furnished unequivocal evidence that GTF-S3 and GTF-S4 synthesize glucans with a different structure from the well-known highly-branched dextran synthesized by GTF-S1 (Taylor *et al.*, 1990; Cheetham *et al.*, 1990).

A number of conflicting results have been published from different laboratories regarding the number, type and activity of the GTF released by different strains of *S. sobrinus*. The present studies have shown that when the *same* strain is grown in the *same* laboratory under defined conditions, the variations can be just as wide, because such factors as growth rate, pH of growth, the limiting nutrient, and supplementation of the medium with Tween 80, can all exercise a marked effect on the activity and relative proportion of each component GTF. Chromatography on hydroxyapatite, which distinguishes subtle structural differences, can separate proteins with similar pI values, and has provided a satisfactory separation of GTF for these studies. The specific activities of the four *S. sobrinus* GTF (Table 8) were as high as those published for GTF components isolated in low yield from strain K1-R and its derivatives following several chromatographic procedures. The high yields obtained from the single chromatography on hydroxyapatite permit a better evaluation than was possible hitherto, of the contribution of each GTF type to the total activity of the culture under each growth condition.

TABLE 8

The Specific Activity of Extracellular D-Glucosyltransferases Separated by Column Chromatography on Hydroxyapatite

S. sobrinus *strain*	*Growth conditions*			*Specific activity (U mg^{-1})*			
	D (h^{-1})	*Glc (%)*	*Tween 80 (%)*	*S1*	*S3*	*S4*	*I*
K1-R	0·075	4·0	0·5	9	14	—	56
	0·45	0·5	0	4	—	—	24
	0·45	0·5	0·5	10	41	88	38
	0·45	4·0	0	24	—	—	70
6715-13-201	0·05	0·5	0·5	18	9	26	33
	0·39	0·5	0	9	—	—	88
	0·39	0·5	0·5	—	2	23	31
	0·45	0·5	0·5	10	34	70	49
6715-13-27	0·075	0·5	0	19	—	14	—
	0·42	0·5	0	33	28	7	3

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