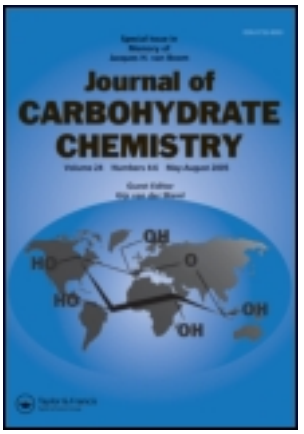

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ENZYMATIC SYNTHESIS OF FRUCTOSYL GLYCEROL

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ABSTRACT

The enzymatic synthesis of ß2-2' and ß2-1'-D- fructopyranosyl glycerol was carried out with levansucrase from *Bacillus circulans* or *B.subtilis*, using sucrose as fructosyl donor and glycerol. The specificity and efficiency of the enzyme was modified by controlling both the water and the total substrate concentrations. The products were purified by HPLC and analyzed by ^{1}H , ^{13}C NMR and GC-MS.

INTRODUCTION

Levansucrase (EC 2.4.1.1) is an enzyme known for its transfructosylase activity product levan, a ß2-6 fructan polymer. However, under particular reaction conditions transfructosylases are capable of transferring fructose to water and to other nucleophiles, producing oligosaccharides in the presence of mono- or disaccharides. It has been known that levansucrase from *B.subtilis* may transfer fructose to sugars such as D-glucose, D-xylose, L-arabinose, D-galactose, melibiose,

and lactose.[1] Levansucrase from *Rhahnella aquatilis* has also been succesfully applied to the synthesis of oligosaccharides using mono and disaccharides as acceptors. However, the enzyme was not able to transfer fructose to sugar alcohols such as xylitol, sorbitol or maltitol among others.

Lilosides and regalosides are glycerol glucosides isolated from different plants of the genus Lilium or from *Sporobolus stapfianus*, a typical desiccation plant.[2] The glycosidic bond between glucose and glycerol may involve the 2-position (liloside A and B) or a primary hydroxyl such as lilosides C, D, and E. The chemical synthesis of glycerol glycosides is complex, requiring protection/deprotection steps. For instance, the synthesis of glycerol glucosides was carried out, using a variation of the classical Koenigs-Knorr reaction, from 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide with 1,3-dibenzyloxy-2-propanol in dry acetonitrile in the presence of $Hg(CN)_2$ and $HgBr_2$ as catalysts.[3] Similar processes have been reported for the synthesis of galactosyl[4] and lactosyl glycerols.[5]

Various enzymes are capable of transferring sugars from oligosaccharides to glycerol but very low affinities have been reported. For instance, Graber and Combes [6] working with α-amylase from *A. niger* and using maltotetraose as substrate concluded that alcohols are better acceptors than polyols as only small amounts of glycerol are consumed. Dextrasucrase, a classical glycosyltransferase capable of transferring glucose from sucrose to a wide variety of sugars, has not been reported as capable of transferring glucose to alcohols or polyols. Fogarty and Benson,[7] found that a transglucosidase from *A.niger* transfers glucose to glycerol, and although no mention is made about the structure of the acceptor reaction product, they report that the transfer is made only from certain substrates such as α-methyl-D-glucoside, but not from maltose. Recently, we have reported the transferring properties of levansucrase from *B.circulans*.[8] The enzyme is capable of transferring fructose to glycerol although in low yields due to its hydrolytic and levan-synthesizing activities. In the present paper we report an efficient process for the enzymatic synthesis of fructosyl glycerol using levansucrase.

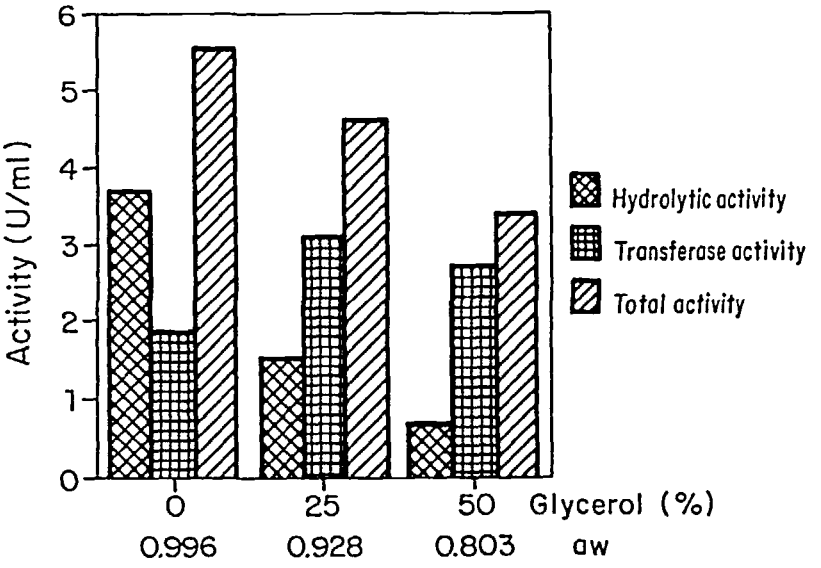


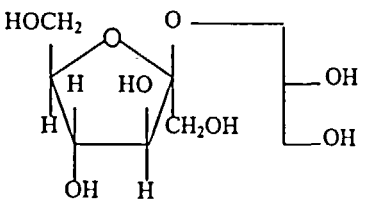
Figure 1. Effect of glycerol on the activities of *B.circulans* levansucrase. (Reaction conditions: sucrose 60 g/L, pH 7, 30 °C and 0.57 U/mL of levansucrase activity).

RESULTS AND DISCUSSION

The effect of reaction conditions on the relative hydrolase and transferase activities of levansucrase was studied. It was observed that in sucrose aqueous solutions, more than 50% of the enzyme activity is hydrolytic, producing glucose and fructose, while the rest of the activity is involved in the transfer of fructose to the growing fructose polymer chain (levan). When the water concentration is reduced, in this case by addition of glycerol which is also an acceptor of the transferred fructose, the hydrolytic activity is considerably reduced. These results are shown in Figure 1.

The same behavior obtained with levansucrase from *B.circulans* was obtained with levansucrase from *B.subtilis* (results not shown). It was observed that a very low hydrolytic activity is obtained when 500 g/L of glycerol (50%) are used in the reaction medium. Therefore, reaction conditions were chosen at 500 g/L of glycerol (50%), with the highest sucrose concentration. In Figure 2, the evolution of a

batch reaction containing 500 g/L of glycerol and 280 g/L of sucrose is shown. Under these conditions it was found that hydrolysis is reduced to a minimum, 78% of fructose being transferred to glycerol, the remaining being found as free fructose, in levan, or as traces of byproducts with the longest elution times in HPLC. An example of an HPLC chromatogram of the reaction products formed after 40 hours of reaction is shown in Figure 3. After elution of water and residual glycerol, two peaks are obtained (referred to as peak 1 and peak 2 in the figure). Peak 1 represents a new product, while peak 2 has the same retention time as fructose. However, the fructose concentration measured enzymaticaly is very much lower than the corresponding area in the HPLC chromatogram, suggesting the presence of a second reaction product with the same elution time. All compounds of the mixture were separated by preparative HPLC as described in the Experimental. The product in peak 1 corresponds to a transferase activity product and was identified as 2,2'-*O*-ß-D-fructopyranosyl glycerol (structure 2) by NMR ^{1}H, ^{13}C, and ^{13}C DEPT experiments. The chemical shift in the ^{13}C spectrum indicates that glycerol is linked by the hydroxyl of C'_2 which corresponds to a "methyne" (DEPT) found at 77.1 ppm due to the ether linkage. The other two glycerol methylene groups are found at 62.1 and 62.2 ppm, probably due to the new chemical environment in which the nuclei of C'_1 and C'_3 are diastereotopic and therefore differ in chemical shift.[9] The β configuration of the linkage was confirmed by comparing the chemical shifts of these signals with those of some β-D-fructofuranose derivatives described in the literature.[10,11] The chemical shift resonances were more consistent with those of β-D -fructofuranose itself with its derivatives (Table 1). The yield of the product with structure **2** was 38%.



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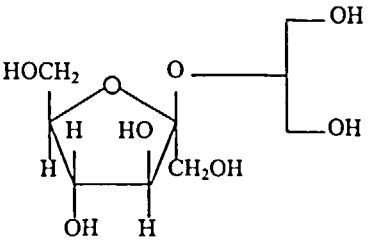

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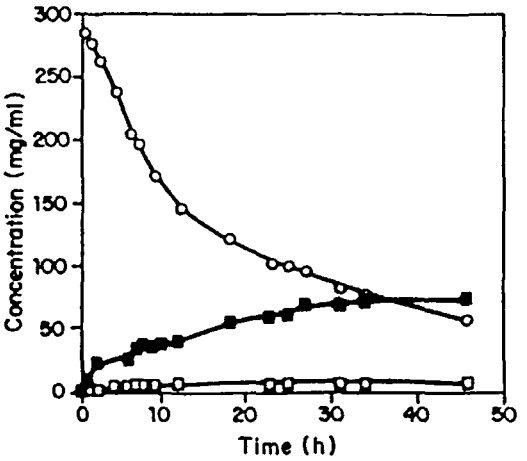

Figure 2. Change in concentration with time of (■) transferred fructose, (□)fructose from hydrolysis and (○) sucrose from a levansucrase reaction in the presence of 500 g/L w/v glycerol and 280 g/L sucrose; at pH 7 and 30 °C, with 0.7 U/mL of total levansucrase activity.

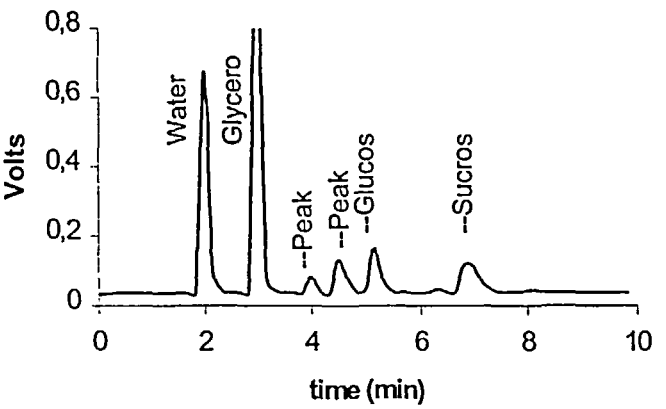

Figure 3. HPLC Chromatogram of reaction products of levansucrase in the presence of sucrose and glycerol after 40 h of reaction. (Nova-Pack C_{18} carbohydrate column with 75:25 acetonitrile-water as eluent).

Table 1. ^{13}C chemical shifts of D-fructose, sucrose, levan and **2.**

Compound	Carbon atom					
	1	2	3	4	5	6
β- D-fructofuranose[10]	63.6	102.6	76.4	75.4	81.6	63.2
α- D-fructofuranose[10]	63.8	105.5	82.9	77.0	82.2	61.9
Sucrose(β-D-fructofuranosyl moiety)[12]	62.6	104.9	77.7	75.3	82.5	63.5
Sucrose(α-D-glucopyranosyl moiety)[12]	93.3	73.8	73.6	70.4	72.2	61.2
Levan (β-2,6)-linked D-fructofuranose)[12]	61.3	105.4	77.6	76.5	81.5	64.6
2,2'-*O*-β- D-fructopyranosyl glycerol (**2**)	62[a] C1' 62.2 or 62.1	104.1 C2' 77.1	74.6 C3' 62.2 or 62.1	73.5	81.88	61.3[a]

a. The assignments for C_1 and C_6 may be reversed.

In the first elution product, structure **1** may be discarded as the signals corresponding to methylene groups of glycerol would be separated by approximately 5 ppm, a situation that is not observed. Finally, the signal at 104.1 ppm is characteristic of a linked C_2 carbon of ß-fructose.

As already mentioned, peak 2 contains a mixture of fructose and a second transfer product. This was also concluded from the NMR spectra of the products in the peak. However, the presence of fructose, made difficult the analysis of the product structure from the complex spectrum of the mixture. Therefore, the products contained in peaks 1 and 2, as well as the corresponding standards, were transformed to silylated derivatives with *N,O*-bis(trimethylsilyl)acetamide, and analyzed by GC-MS.

Mass spectral data of the transferase products were quite similar, indicating that the product in the first and the second HPLC peaks are isomers (Table 2). Both products showed a low intensity molecular ion (*m/z* = 687) and a mass ion distribution in agreement with a D-fructose 1,3,4,6-tetrakis-*O*-(trimethylsilyl)-dimethyl-silyl-glycerol. As the product from peak 1 was identified as 2,2'-*O*-β-D-

Table 2. Mass spectral data of the TMS-derivative products formed by the transferase activity of levansucrase from *Bacillus subtilis* and separated by HPLC.

Products	Mass spectral ions (*m/z*)[a]
Product from peak 2 2,2'-*O*-β-D-fructopyranosyl glycerol	687 [M^+] (1), 583 (1), 438 (3), 437 (8), 230 (4), 219 (11), 218 (14), 217 (63), 191 (3), 149 (3), 148 (4), 147 (23), 133 (6), 131 (4), 129 (9), 104 (4), 103 (44), 101 (3), 75 (7), 74 (9), 73 (100), 59 (3).
Product from peak 1 2,1'- *O*-β-D-fructopyranosyl glycerol	687 [M^+] (1), 583 (1), 438 (4), 437 (11), 257 (3), 230 (5), 220 (3), 219 (18), 218 (20), 217 (97), 215 (3), 191 (3), 189 (3), 149(3), 148 (4), 147 (26), 131 (6), 129 (11), 117 (5), 116 (3), 104 (3), 103 (36), 101 (5), 75 (9), 74 (9), 73 (100), 59 (3).

a. Values in parenthesis are relative intensities (in percentage). [M^+], molecular ion.

fructopyranosyl glycerol, product from peak 2 most likely corresponds to the isomer 2,1'-*O*-β-D-fructopyranosyl glycerol, in agreement with the different chromatographic behavior showed by both products on HPLC and GC. The yield of fructose transferred to the product of structure **1** was 40%. It is interesting to observe that the enzyme has only a slight preference for the 1,3 hydroxyl positions in glycerol over the secondary hydroxyl in C_2.

EXPERIMENTAL

Enzyme Production. LS was produced from two strains. A strain derived from *B.subtilis* Marburg 168 (kindly provided by Dr. F. Valle from IBt-UNAM), designated with the genotype *Δnpr*, *Δapr*, Cm^R, *degU32* (Hy), which has the property of overexpressing the LS gene. A second strain was isolated from cane

sugar, identified and characterized as *Bacillus circulans*. Fermentations were carried out in a 2L Fermbach with 450 mL of fermentation media as already described.[8] Once the stationary phase is reached the culture is centrifuged and the extracellular enzyme precipitated with 25% w/v PEG (Mw 1500) and recovered in phosphate buffer 50mM pH 6.0 after centrifugation. This a simple process which provides an adequate LS preparation from both microorganisms for the synthesis reactions.

Enzyme Activity. The total enzymatic activity was determined measuring the release of reducing sugars from a 6% sucrose solution in phosphate buffer 50mM pH 6.0 during the first 15 min, using the DNS[13] and glucose as standard. In order to differentiate among the various activities, glucose and fructose produced were measured using an enzymatic kit (Boehringer-Mannheim). One LS activity unit is defined as the amount of enzyme liberating one μmol of glucose per minute.

Enzyme Reactions. Reactions were carried out with 0.5-1.0 U/mL of LS and various concentrations and ratios of sucrose and glycerol in 10 mL volume reactions at pH 6.0 and 37 °C. The substrate/product concentrations of batch reactions were measured in the same volume with 0.7 U/mL of the enzyme, 280 g/L of sucrose and 500 g/L of glycerol. In all cases, 250 μL samples were inactivated and the products and substrate quantified by HPLC using a Waters 600E System Controller equipped with a μ-Bondapack-NH_2 carbohydrate column, with acetonitrile-water (75:25) as eluent and a Waters 410 differential refractometer detector.

Product Characterization. The main reaction products were recovered after the enzymatic reaction. Samples (100-500 μL) were applied to a μ-Bondapack-NH_2 preparative column with an eluent rate of 8 mL/min, and fractions eluted between 16-18 min collected, vacuum concentrated and freeze dried. The products were analyzed by NMR. ^{1}H NMR spectra were recorded on a Varian VXR-300 instrument in DMSO-D_6 (TMS as internal standard). Chemical shifts are expressed in δ (ppm) relatives to TMS, splitting patterns are designed as follows: s, singlet; d, doublet; t, triplet; m, multiplet. Coupling constants (J) are reported in Hz.

2-2'-*O*-ß-D-fructopyranosyl glycerol (2); ^{1}H NMR (DMSO) 5,1 (1H, *d* , J 5.1 Hz, H-3), 4.7, 4.5 (2H, 2 *t*, J 6, 5.1, 5.7, 6 Hz, H-2') 4.6 (1H, *d*, J 8.7 Hz, H-6a-

6b) 3.8-3.7 (1H, m, H-5) 3.6-3.3 (4H, m, H-1'a, 1'b, 3'a, 3'b) 3.3 (2H, *t*, J 6, 4.5 Hz, H-1). ^{13}C-NMR(DMSO), δ (ppm): 104.1 C_2, 81.85 C_5, 77.1 C_2', 74.6 C_3, 73.5 C_4, 62.2 C_1' or C_3',62.1 C_1' or C_3', 62 C_1 or C_6, 61.3 C_1 or C_6. The products were silylated with *N,O*-bis(trimethylsilyl) acetamide (BSA) for GC-MS analysis. Dry products from HPLC purification were dissolved in 100 mL of pyridine and 400 mL of BSA reagent were added in a stoppered 4 mL vial, then the mixture was shaken and warmed to 60 °C for 60 min. The mixture containing the silylated product was analyzed by GC-MS in a Hewlett-Packard CG (model 6890) MS (model 5972), with a 30 m x 0.25 mm SPB-20 column (Supelco). The GC temperature program started at 90 °C for 2 min, then raised to 290 °C at 8 °C/min rate and held for 10 min.

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