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Characterization of a cell-associated inulosucrase from a novel source: A *Leuconostoc citreum* strain isolated from *Pozol*, a fermented corn beverage of Mayan origin

V Olivares-Illana[1], C Wacher-Rodarte[2], S Le Borgne[1] and A López-Munguía[1]

[1]*Instituto de Biotecnología, Universidad Nacional Autónoma de México, Apdo. Postal 510-3, Cuernavaca, Morelos, CP 62271, Mexico;* [2]*Facultad de Química, Universidad Nacional Autónoma de México, Cd. Universitaria, CP 04510, México, D.F., Mexico*

A cell-associated fructosyltransferase was extracted from a novel source, a strain of *Leuconostoc citreum* isolated from *Pozol*, a Mexican traditional fermented corn beverage, where lactic microflora are partially responsible for the transformation process. The enzyme is associated with the cell wall. It was characterized both in its cell-associated insoluble form and after separation by urea treatment. The fructosyltransferase has a molecular mass of 170 kDa, the highest reported for this type of enzyme, and in its insoluble form is highly specific for polymer synthesis, with low fructose transferred to maltose and lactose added to the reaction medium (acceptor reactions). The synthesized polymer has an inulin-like structure with β2-1 glycosidic linkages, as demonstrated by ^{13}C nuclear magnetic resonance (NMR). Bacterial inulosucrases have only been reported in *Streptococcus mutans*.
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Introduction

Fructosyltransferases such as levansucrase (LS), as well as glucosyltransferases like dextransucrase (DS), are examples of glycosyltransferases that catalyze the transfer of a fructose or a glucose residue from sucrose to a growing polysaccharide chain, resulting in the synthesis of high molecular weight polymers of fructose (fructans) or glucose (glucans), respectively. The polymers produced by these enzymes have different sizes and structures, depending on the enzyme-producing strain. Dextrans are glucans containing a main linear chain with α1-6 glucosidic linkages [23], while mutans are distinguished by the α1-3 bonds in the main chain "alternate" α1-6 and α1-3 linkages in their structure. Branching may occur in different positions such as α1-2, α1-3, α1-4 and α1-6, depending on the source of the enzyme [5]. On the other hand, levans are fructans containing β2-6 linkages in the main linear chain with β2-1 branch points, while the reverse is the case for inulin (β2-1 in the main chain and β2-6 branch points) [24]. The corresponding glycosyltransferases do not require any cofactors or high-energy phosphorylated intermediates, as they use the free energy liberated by the cleavage of sucrose for the synthesis [19]. All these polymers have found a variety of applications in the fields of cosmetics, foods, separation technology and medicine.

An interesting feature of these enzymes is their ability to catalyze the synthesis of low molecular weight oligosaccharides from sucrose when efficient acceptor molecules like maltose are added to the reaction medium [13]. Some of these oligosaccharides are used as prebiotics in both cosmetic and food applications as they are highly resistant to attack by digestive enzymes, being substrates only for beneficial native probiotic flora [30].

The main producers of fructosyltransferases are Gram-positive bacteria such as several *Bacillus* species (including *Bacillus subtilis* [4], *Bacillus circulans* [19], *Bacillus polymyxa* [3], *Bacillus amyloliquefaciens* [29]), *Rahnella aquatilis* [18], and *Lactobacillus reuteri* [32]. They are also found in some Gram-negative bacteria such as *Zymomonas mobilis* [14], *Erwinia herbicola* [5], *Pseudomonas syringae* pv. *glycinea* and *P. syringae* pv. *phaseolicola* [9] and *Acetobacter diazotrophicus* [1,2]. All the fructosyltransferases from these microorganisms are levansucrases (LSs), with levan as the main enzymic product. Inulosucrases have been isolated only from plants and fungi, with the exception of a similar activity reported in *Streptococcus mutans* by Shiroza *et al* [26]. Both *Leuconostoc mesenteroides* as well *Streptococci* from oral flora have been reported as dextran and levan producers [7,8]. Differences among bacterial fructosyltransferases are illustrated in Table 1.

Dextransucrases (DSs) are extracellular enzymes, cell-associated or both, depending on the producing strain while LSs are generally extracellular with the exception of LS from *Actinomyces viscosus*, which produces extracellular and cell-associated forms [31] and that from *Streptococcus salivarius*, which is cell-associated in the absence of sucrose, but released from the cell and secreted into the culture medium in its presence [12]. DSs are large enzymes, with a reported molecular mass from 64 to 184 kDa [21], while LSs are smaller with a molecular mass between 45 and 64 kDa, with the exception of the *S. salivarius* and *S. mutans* enzymes, which have molecular masses of 140 (fructosyltransferase) and 87 kDa (fructosyltransferase with inulin-like activity), respectively, as reported in Table 1 [11,26]. There are few reports documenting LS activity in *L. mesenteroides*. Actually, due to very low expression levels, LS activity in this source had previously been considered as a minor contaminant of DS [17,20,33]. Up to now

Correspondence: Dr A López-Munguía, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Apdo. Postal 510-3, Cuernavaca, Morelos, CP 62271, México
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Table 1 Comparison of the properties of fructosyltransferases produced by several bacteria

Microorganisms	Cellular localization	Molecular mass (kDa)	Optimal pH	Optimal temperature (°C)	K_m (mM)	Production	Acceptor reactions	References
B. subtilis	extracellular	50	6.5	45	18	induced	yes	[4]
B. circulans	extracellular	52	6.5	40	20	–	yes	[19]
A. diazotrophicus	extracellular	58, 65	5.0	–	11.8±1.4	constitutive	yes	[1,2]
R. aquatilis	extracellular	64	6.0	55–60	50	–	–	[18]
Z. mobilis	extracellular	47	5.0	50	122	–	–	[14]
E. herbicola	extracellular	–	–	–	–	constitutive	–	[5]
P. syringae pv. *phaseolicola*	extracellular	45	6.2	18	160	constitutive	–	[9,10]
S. mutans	extracellular	74, 80, 87, 90	–	–	12±1	constitutive	–	[11,12,25]
S. salivarius	extracellular[a]	140	–	–	–	constitutive	–	[11,26]
***L. citreum* CW28**	**cell-associated**	**170**	**6.5**	**45**	**66**	**induced**	**no**	**this work**

[a]Extracellular in the presence of sucrose.
– None reported.

the fructosyltransferase from *S. mutans* is the only inulosucrase reported in bacteria.

In this work, we report on a novel *Leuconostoc citreum* strain (CW28) identified by RNA 16S analysis performed by Midi Labs (Newark, DE), and previously identified as *L. mesenteroides* by API 50-CH, a strain that produces large amounts of a cell-associated fructosyltransferase in the presence of sucrose. This organism was detected in a collection of *L. mesenteroides* strains isolated from *Pozol*, a nonalcoholic fermented beverage produced by lactic acid fermentation of lime-treated corn, which is consumed in the southeast of México, in the Maya region located in the states of Chiapas, Tobasco, Campeche and Yucatán. This beverage dates back to the Maya culture and its production has been transferred by tradition and confined mainly to the rural regions. *L. citreum* was first described by Farrow *et al* in 1989 [6], followed by *Leuconostoc amelibiosum* by Schillinger *et al* in 1989 [22]. In 1992 Takahashi *et al* [28] concluded that *L. amelibiosum* is a later subjective synonym of *L. citreum*, and proposed that the name *Leuconostoc citreum* should be retained. This is the first report concerning the isolation of fructosyltransferase from *L. citreum* with inulin-synthesizing capability.

Materials and methods

Materials

Sucrose, glucose, lactose, raffinose and 3,5-dinitrosalicylic acid were purchased from Sigma Chemical (St. Louis, MO). The electrophoresis reagents were from Bio-Rad Laboratories (CA). Urea and Tween 80 were from Merck (Darmstadt, Germany). Yeast extract, was from Difco Laboratories (Detroit, MI). K_2HPO_4, $MgSO_4$, $CaCl_2$, NaCl, $MnSO_4$ and $FeSO_4$ were from J.T. Baker (S.A. de C.V. Edo. De México, México). Two enzymes capable of polymer digestion were used for identification purposes: dextranase (Amano Chemical, Yokohama, Japan) and inulinase from *Kluyveromyces marxianus*, a gift from Dr. M. García-Garibay from UAM-I, Mexico.

Bacterial strains and culture conditions

The *Leuconostoc* strains used in this work were isolated from *Pozol* by Dr. Carmen Wacher (Facultad de Química of the UNAM, México). These strains were cultured in 250-ml Erlenmeyer flasks containing 50 ml of culture medium. The cultures were incubated in a New Brunswick incubator shaker Series 25 (Edison, NJ) at 30°C, pH 6.9 for 12 h; aeration was provided by agitation at 200 rpm. The culture medium was composed of (in g/l): sucrose 20, yeast extract 20, K_2HPO_4 20, $MgSO_4$ 0.2, $CaCl_2$ 0.05, NaCl 0.01, $MnSO_4$ 0.01 and $FeSO_4$ 0.01. After 8 h, when the culture reached an optical density of 7–10, the culture was centrifuged for 10 min at 10,000×*g*. Both supernatant and cell pellet were assayed for fructosyltransferase activity. The same medium and culture conditions were used later to produce the enzyme in 500 ml Erlenmeyer flasks with 200 ml of culture medium.

The selected strain was identified as *Leuconostoc mesenteroides* by the API 50-CH test (Biomerieux, Marcy l'Etoile, France), a classification kit based on the strain fermentation profile for 50 different carbohydrates. However, the strain was later sent for classification to Midi Labs (Newark, DE) who identified it as *L. citreum* by 16S rRNA analysis.

Enzyme assays

Enzyme assays were carried out at 30°C in 12 ml of 50 mM phosphate buffer, pH 6.5, containing 27 mM sucrose and using complete cells or an appropriate dilution of the culture supernatant as the enzyme source. Total glycosyltransferase activity was measured by following the initial release of reducing sugars by the 3,5-dinitrosalicylic acid method (DNS) [27]. One activity unit (U) for all glycosyltransferases is defined as the amount of enzyme that produces 1 μmol of either fructose or glucose per minute. In the

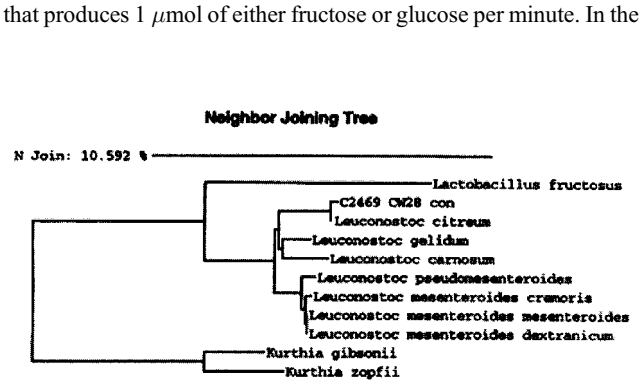

Figure 1 Phylogenetic analysis of the 16S rRNA from *Leuconostoc* strain CW28.

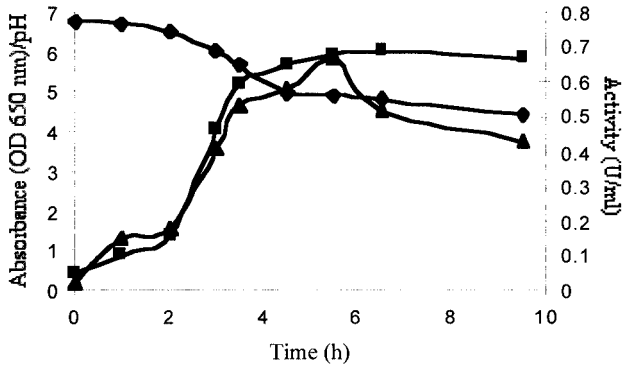

Figure 2 Time course of *L. citreum* CW28 growth, LS production and pH change in culture. (■) OD 650 nm; (▲) IS activity; (◆) pH.

case of IS the activity was also determined by measuring the initial rate of glucose release from sucrose with a glucose/fructose kit (Roche Biochemicals, Mannheim, Germany). The release of fructose in the reaction medium indicated DS activity while the release of glucose indicated LS or IS activity. Both sugars could also be formed by sucrose hydrolysis due to the transfer of glycosyl residues to water molecules. The relative amount of each activity could be determined by a sugar balance (HLPC), while in order to discriminate between LS and IS ^{13}C nuclear magnetic resonance (NMR) analysis of the product was required.

Enzyme solubilization

L. citreum CW28 cells grown in 200 ml culture for 4–6 h were centrifuged for 10 min at 10,000×*g* and extensively washed with 50 mM phosphate buffer, pH 6.5. Afterwards, several methods were applied to extract the cell-associated enzyme. Best results were obtained when the washed pellet was suspended in 5 ml 8 M urea at 25°C for 1 h with occasional gentle shaking [8]. The extract was then dialyzed overnight against 10 mM phosphate buffer, pH 6.5, centrifuged for 10 min at 10,000×*g* and the supernatant was assayed for IS activity.

Electrophoresis

SDS-PAGE was performed as reported by Laemmli [15]. Samples were mixed with an equal volume of 2× sample buffer (0.125 M Tris–HCl pH 6.8, 1% SDS, 20% glycerol, 10% 2-mercaptoethanol) and incubated at 90°C for 5 min. The samples were electrophoretically separated on 6% polyacrylamide gels and stained with Coomassie Brilliant Blue.

Glycosyltransferase activity gels

DS, IS and LS activities were detected on gels after SDS-PAGE by *in situ* production of dextran inulin or levan from sucrose. For activity *in situ*, the samples were mixed with an equal volume of 2× sample buffer (without 2-mercaptoethanol), but were not incubated at 90°C. After electrophoresis the gel was washed twice for 30 min with 50 mM phosphate buffer pH 6.5 containing 1% Tween 80, followed by incubation with 5% sucrose or 5% raffinose in the same buffer. Zones of enzyme activity were evident to the eye due to the formation of polymer bands. While sucrose was a substrate of DS, LS and IS, raffinose was specific for LS and IS. In some cases, the gels were treated with dextranase (Amano Chemical) after polymer synthesis in order to provide further evidence for the nature of the polymers.

Table 2 Chemical shifts for C NMR spectra of inulin, levan and the *L. citreum* CW28 polymer

Carbon atom	Chemical shift (ppm)		
	L. citreum CW28 polymer	Levan[a]	Inulin[a]
C-1	61.4	60.7	60.9
C-2	103.6	104.2	103.3
C-3	77.5	76.3	77.0
C-4	74.9	75.2	74.3
C-5	81.8	80.3	81.1
C-6	62.7	63.4	62.2

[a]Shimamura *et al* [25].

Polymer production

Polymer production was performed with whole *L. citreum* cells at 30°C in 50 mM phosphate buffer pH 6.5 containing 10% sucrose for a reaction period of 5 h. Cells were removed by centrifugation and the polymer was precipitated with two volumes of ethanol, dialyzed against 10 mM phosphate buffer pH 6.5, lyophilized and analyzed by ^{13}C NMR.

Results and discussion

Identification of the isolated strains

Fourteen strains isolated from *Pozol* in the collection of C. Wacher were identified on the basis of their morphological and biochemical properties. The strains were aerobic, nonmotile, non-spore-forming Gram-positive bacteria. The cells were rod shaped and organized in chains of three to four cocci. All 14 strains grew as viscous colonies in sucrose solid media and were primarily identified as *L. mesenteroides* strains. Afterwards, a 16S rRNA analysis revealed that strain CW28 was closer to *L. citreum* as illustrated in Figure 1.

Glycosyltransferase activity

The 14 strains isolated from *Pozol* were assayed for glycosyltransferase activity by measuring total reducing sugars, glucose and fructose liberated in the reaction medium. Six of the 14 strains exhibited extracellular DS activity, two cell-associated DS activity

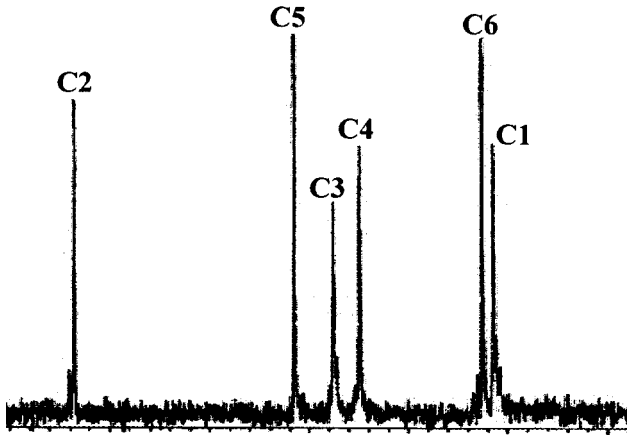

Figure 3 ^{13}C-NMR spectra of *L. citreum* CW28 polymer.

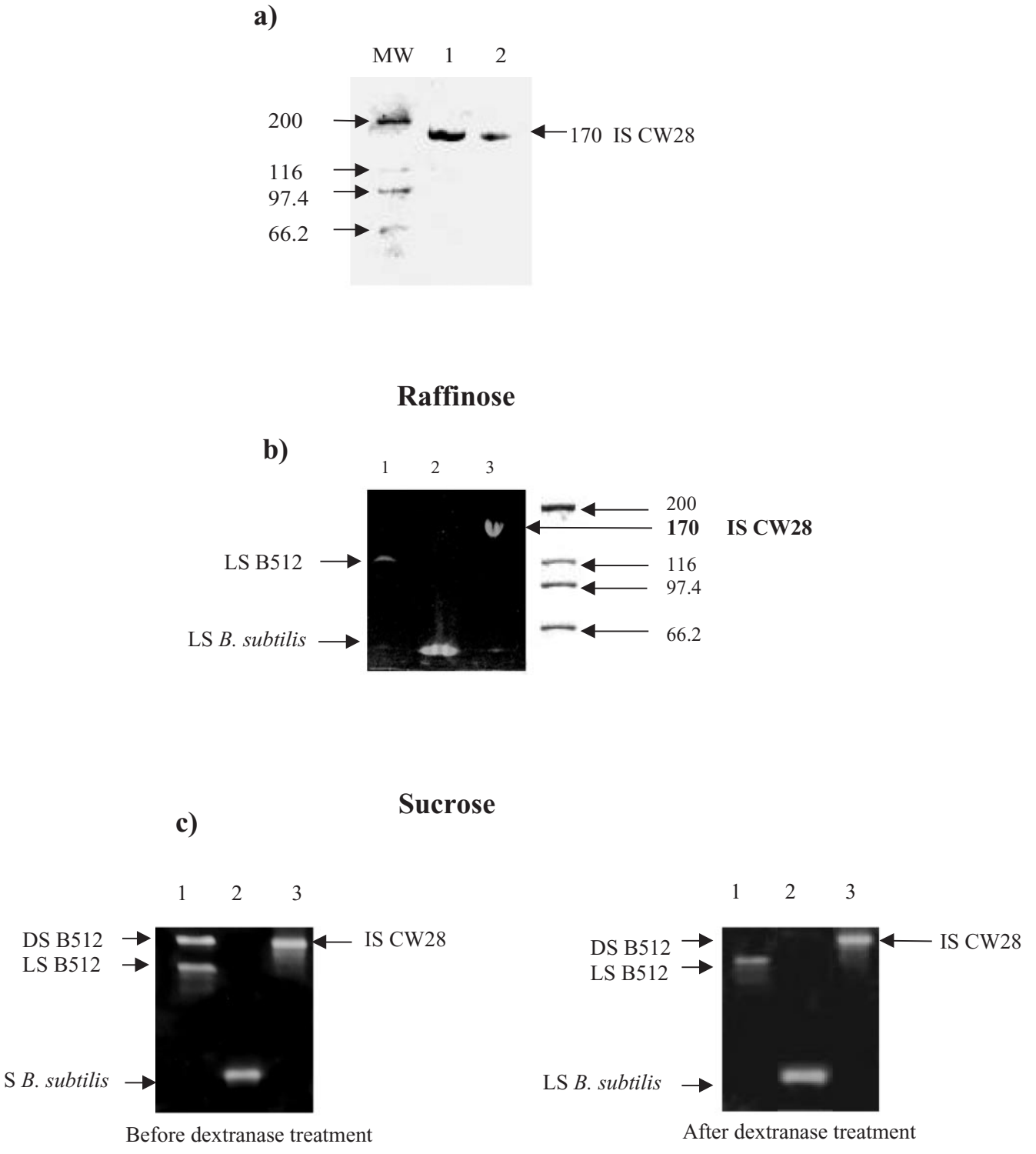

Figure 4 SDS-PAGE analysis of *L. citreum* CW28 cell-associated and solubilized IS. (a) SDS-PAGE gel stained with Coomassie blue. Lane 1, *L. citreum* whole cells in lytic solution; lane 2, 8 M urea solubilized *L. citreum* IS. (b) Activity gel. *In situ* polymer production after incubation with raffinose. Lane 1, *L. mesenteroides* B-512F; lane 2, *B. subtilis* LS; lane 3, *L. citreum* CW28 IS. (c) Activity gel. *In situ* digestion of dextran by dextranase after incubation with sucrose. Lane 1, *L. mesenteroides* B-512F; lane 2, *B. subtilis* LS; lane 3, *L. citreum* CW28 IS.

and one strain had cell-associated fructosyltransferase activity and was registered as CW28. The rest of the strains presented very low glycosyltransferase activity.

Fructosyltransferase production

L. citreum CW28 was cultured as described earlier. A typical growth curve is shown in Figure 2, where it may be observed that

enzyme production is cell associated. The enzyme was induced by sucrose and no fructosyltransferase activity was detected in cells grown in the presence of 125 mM glucose.

Production, identification and purification of the fructose polymer produced by the CW28 strain

Both enzymes, LS and IS produce fructose polymers in the presence of sucrose or raffinose, liberating glucose in the reaction medium. Therefore, in order to differentiate between both activities the structure of the polymer must be determined. Polymer was performed with whole *L. citreum* cells as described earlier. The ^{13}C NMR spectrum of the polymer corresponded to a fructose polymer with β2-1 and β2-6 linkages, which is the usual structure found in inulin as shown in Figure 3. Six main resonances at 103.6, 81.8, 77.5, 74.9, 62.7 and 61.4 ppm were found, which are similar to the peak positions reported for inulin [25]. In our sample, the anomeric carbon C2, and the primary carbons C1 and C6 are closely grouped as in inulin, while of the ring carbons C3, C4 and C5, only C4 is closer to the levan group (Table 2). Furthermore, the polymer was digested with inulinase providing additional proof that the polymer produced by *L. citreum* CW28 is an inulin-like polysaccharide.

IS solubilization

L. citreum CW28 cells were treated with urea as already described in order to solubilize the cell-associated activity. After the treatment most of the IS was lost; only 10% of the initial activity in whole cells was recovered in the supernatant, with the remaining activity found in the cellular debris (30% of the original activity). Whole cells and the supernatant proteins obtained after this treatment were analyzed by SDS PAGE. Surprisingly, in whole cells only one protein, IS, was solubilized by the lytic solution in the sample buffer used for SDS-PAGE, as shown in Figure 4a and demonstrated by electrophoresis (size) and the zymogram analysis (*in situ* gel activity).

Properties of soluble and cell-associated IS

Molecular weight determination by SDS-PAGE was carried out with whole cells directly applied to the gel and with the solubilized IS. After SDS treatment, the gels were extensively washed to remove residual SDS and incubated in the presence of sucrose to observe the bands of activity as a result of polymer synthesis. LS from *B. subtilis* and DS from *L. mesenteroides* B-512F were used as controls. The single-band activity observed in the CW28 whole cells gel lane corresponds to a protein of a molecular mass of approximately 170 kDa. In addition, an activity gel was performed using raffinose as the fructosyl residue donor (Figure 4b). In this figure, activity bands were observed for *B. subtilis* and *L. citreum* CW28, supporting the conclusion that both strains present fructosyltransferase activity. An activity band of low molecular mass (approximately 90 kDa) was also observed in Figure 4b for *L. mesenteroides* B-512F corresponding to LS produced by this strain, as already reported [16]. The molecular weight of the *L. citreum* IS was much higher than values reported for other fructosyltransferases (45 and 64 kDa) and for almost all LSs, except for the enzyme from *S. salivarius*, which produces a 140-kDa LS; a value of 87 kDa has been documented for IS from *S. mutans* as indicated in Table 1. Further evidence for IS activity is presented in Figure 4c, where the polymer band from DS is completely digested by dextranase, while activity bands obtained with enzymes from *B. subtilis* and *L. citreum*, containing levan and the inulin-like polymers, respectively, are not degraded (differences between the two gels shown in Figure 4c are also due to the treatment of the right-hand gel with dextranase for 2 h at 50°C).

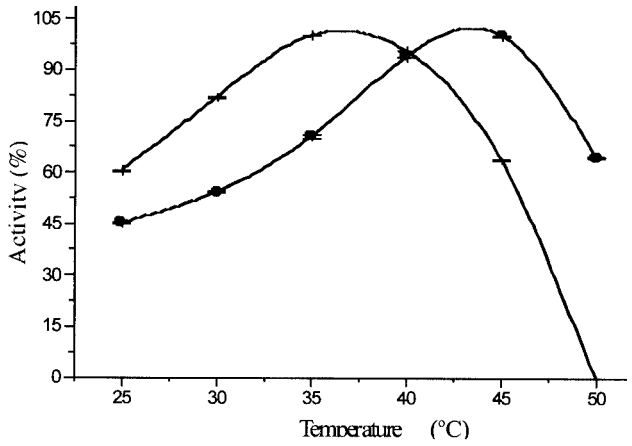

Figure 5 Effect of temperature on IS activity. The effect was determined in the presence of phosphate buffer pH 6.5. (–) solubilized IS; (●) cell-associated IS.

The optimum reaction conditions for CW28 IS were investigated. Optimum pH values for cell-associated and free IS were 6.5 in both cases (data not shown). The optimum temperature for cell-associated IS was higher than the optimum found for the soluble form, as shown in Figure 5. However, the enzymatic activity in both preparations decreased rapidly at temperatures above 45°C, with complete deactivation after 30 min at 50°C. A stability study was carried out at three different temperatures. Both cell-associated and soluble IS were stable at 30°C in a 1-h experiment. However, at 50°C the enzyme in both forms was deactivated in 30 min (results not shown). At 30°C the enzyme followed Michaelis–Menten kinetics, with K_m values of 66 and 38 mM for the cell-associated and the solubilized IS, respectively, as calculated by linear regression analysis from Lineweaver–Burk type plots in a sucrose concentration range of 0–550 mM. The specific activity of the IS was 0.25 U/mg of cells and 5.6 U/mg of urea-solubilized protein.

A common property of fructosyltransferases is their specificity to transfer the fructosyl moiety of sucrose to several acceptors, with sugars such as maltose and lactose reported among the most efficient [18]. We observed that both forms of IS from *L. citreum* had very low efficiency towards maltose and lactose. No acceptor products were obtained with the cell-associated IS, as most of the fructose in the substrate was directed towards polymer synthesis. This result, as well as the increase in the K_m to twice its original value, may be due to the effect of steric hindrances on the cell-associated enzyme. Therefore, sites potentially implicated in acceptor reactions, might become accessible when IS is released from the cell wall.

Inulin is a polysaccharide of growing industrial interest obtained solely from vegetal sources. Enzymatic synthesis from sucrose may become an attractive alternative source if successful scale-up and process economics with inulosucrases show feasibility.

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