Journal of Molecular Catalysis B: Enzymatic 70 (2011) 41–48

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Enzymatic fructosylation of aromatic and aliphatic alcohols by *Bacillus subtilis* levansucrase: Reactivity of acceptors

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ARTICLE INFO

Article history:
Received 7 December 2010
Received in revised form 25 January 2011
Accepted 1 February 2011
Available online 26 February 2011

Keywords:
Levansucrase
Activity coefficients
COSMO-RS
Organic solvents
Acceptor reaction
Phenols
Phenolic fructosides

ABSTRACT

Levansucrases from *Bacillus subtilis* (BS-LVS) and *Leuconostoc mesenteroides* ssp. mesenteroides ATCC 8293 (LevC), inulosucrase from *Leuconostoc citreum* (IslA) and an invertase from *Saccharomyces cerevisiae* (Inv) were evaluated in acceptor reactions with non-sugar acceptors. Among them, BS-LVS was selected for the fructosylation of aromatic or aliphatic alcohols due to its high activity and stability. The effects of acceptor concentration, enzyme concentration and the presence of a co-solvent in the fructosylation efficiency of hydroquinone were evaluated. It was demonstrated that this reaction is kinetically controlled, producing the best yields of phenolic fructosides when 500 mM of acceptor and 5 U mL^{-1} of enzyme were employed. Higher enzyme loads resulted in the rapid hydrolysis of the products. Increased amounts of organic co-solvent up to 50% (v/v) reduced fructoside yield due to a concomitant decrease in the thermodynamic activity of the acceptor, as confirmed by theoretical calculations using COSMO-RS; moreover, increased fructose transfer to levan and reduced hydrolysis were observed. It was found that BS-LVS preferentially fructosylates aromatic over aliphatic alcohols. A maximum fructoside production (19–35 mM) was obtained with dihydroxybenzene acceptors such as hydroquinone, whereas reactions with primary alcohols, such as benzyl alcohol resulted in lower fructosylation efficiency. This selectivity was also demonstrated by the fact that 4-hydroxybenzylalcohol, a bifunctional acceptor, was fructosylated at a rate ten times faster on its aromatic hydroxyl group. BS-LVS selectivity over phenol fructosylation was inversely correlated with the acceptor pK_a value.

1. Introduction

Glycosides are compounds formed by a carbohydrate covalently linked to a non-sugar moiety (aglycon). They are widely distributed in nature and engaged in diverse functions, mostly as secondary metabolites [1,2]. In recent years, natural phenolic glycosides have generated industrial interest due to their diverse biological activities in humans. For instance, glycosides of flavonoids are widely used as radical-scavenging molecules [3–6], whereas β-arbutin is used as a skin-whitening agent [7]. Glycosylation strategies can also be useful to modify the physicochemical properties of the aglycon (*i.e.*, to increase its water solubility or stability), modulating its pharmacological properties [8,9]. Despite the great interest in using phenolic glycosides as therapeutic agents, antioxidants or free radical scavengers, the most important limitation in their industrial application is their low content in plants and the complexity of their synthesis [10–12]. Indeed, chemical strategies involve multi-step reactions for the protection and deprotection of hydroxyl groups as well as the use of heavy metal salts [13,14], which result in a complex mixture of products and toxic by-products [1].

To overcome these limitations, enzymatic glycosylation has emerged as an interesting alternative, taking advantage of the

Abbreviations: BS-LVS, *B. subtilis* levansucrase; Inv, *S. cerevisiae* β-D-fructofuranosidase; IslA, *L. citreum* inulosucrase; LevC, *L. mesenteroides* ssp. *mesenteroides* ATCC 8293 levansucrase; GlyTFs, glycosyltransferases; FTFs, fructosyltransferases; GTFs, glucosyltransferases; 2M2P, 2-methyl-2-propanol; **Hq**, hydroquinone; **Rsr**, resorcinol; **Cat**, catechol; **4mPh**, 4-methoxyphenol; **Ph**, phenol; **Bnz**, benzyl alcohol; **Bu**, butanol; **4HB**, 4-hydroxybenzyl alcohol; **Fru-Hq**, 4-hydroxyphenyl-β-D-fructofuranoside; **Fru-Rsr**, 3-hydroxyphenyl-β-D-fructofuranoside; **Fru-Cat**, 2-hydroxyphenyl-β-D-fructofuranoside; **Fru-4mPh**, 4-methoxyphenyl-β-D-fructofuranoside; **Fru-Ph**, phenyl-β-D-fructofuranoside; **Fru-Bnz**, benzyl-β-D-fructofuranoside; **Fru-Bu**, *n*-butyl-β-D-fructofuranoside; **Fru_1-4HB**, 4-(hydroxy)-benzyl-β-D-fructofuranoside; **Fru_2-4HB**, 4-(hydroxymethyl)phenyl-β-D-fructofuranoside.

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doi:10.1016/j.molcatb.2011.02.002

intrinsic selectivity of enzymes. Throughout the past decades, glycosidases have been the most frequently reported enzymes for the glycosylation of primary and secondary alcohols, whereas the glycosylation of phenols has been seldom addressed [8,15]. In general, low yields of phenol glycosides have been reported [16,17], a result that has been attributed to the low nucleophilicity of these acceptor molecules [8,15].

Alternatively, non-Leloir glycosyltransferases (GlyTFs) have been successfully applied for glycosylation reactions using sucrose as a donor for the transfer of the glycosyl moiety to the acceptor molecule [18]. GlyTFs include fructosyltransferases (FTFs) and glucosyltransferases (GTFs), which are enzymes capable of transferring fructose or glucose, respectively, to an acceptor such as a glycan growing chain (polymerisation), water (hydrolysis) or another suitable compound added to the reaction medium (acceptor reaction), resulting in a glycoside

Although there are some studies reporting the glycosylation of several phenolic molecules such as flavonoids and dihydroxybenzenes, this has only been achieved at low acceptor concentrations (from 3 to 10 mM). In consequence, productivity remains low and limited by the acceptor solubility. This is the case for flavonoids such as catechin, luteolin, quercetin, myricetin and epigallocatechin, which were glucosylated in the phenolic group using glucansucrases from *Streptococcus* sp. and *Leuconostoc* sp. [19–22]. To cope with this limitation, different water-miscible organic solvents have been used to enhance acceptor solubility, but low amounts of glucosides were observed [20].

For small phenolic molecules, such as catechol, 4-methylcatechol and 3-methoxycatechol, which have higher solubility in water, glucosylation was successful using the glucosyltransferase-D from *S. mutans* GS-5 in an aqueous medium, with yields of 65%, 50% and 75%, respectively, using 40 mM of acceptor [23]. However, high concentrations of these compounds (200 mM) led to a dramatic loss in enzyme activity. More recently, the fructosylation of hydroquinone was reported using a levansucrase from *Leuconostoc mesenteroides* at a high initial concentration of the acceptor (350 mM) but with a rather low fructoside final concentration (4 mM) [24].

In this context, the availability of biocatalysts with high transferase activity and specificity as well as high stability in the presence of high concentrations of phenolic compounds is still required to enhance the glycosylation yields of hydroxylated aromatic compounds. Levansucrase from *Bacillus subtilis* (BS-LVS) has been proved to be a robust biocatalyst, showing high activity and stability in non-conventional media, such as organic solvents [25], and high efficiency in acceptor reactions [26]. In this work, the enzymatic fructosylation of phenolic compounds such as hydroquinone, catechol and resorcinol catalysed by FTFs is reported. The selectivity of BS-LVS for the fructosylation of different aromatic or aliphatic alcohols was studied and explained in terms of the chemical and thermodynamic properties of the acceptors.

2. Experimental

2.1. Materials

Hydroquinone, benzyl alcohol, 4-hydroxybenzyl alcohol, 4-methoxyphenol, sucrose, fructose, glucose and arbutin were supplied by Sigma–Aldrich Inc. (MO, USA). Phenol was obtained from Merck (Edo. de México, México). Catechol and resorcinol were purchased from Fluka (Buchs, Switzerland). Butanol, 2-methyl-2-propanol, diisopropyl ether and methyl *tert*-butyl ether were acquired from J.T. Baker (Edo. de México, México). All substances were of high purity grade (≥98%). Acetonitrile and acetone were of HPLC grade and were supplied by Burdick & Jackson (MI, USA). Silica gel 0.04–0.063 mm was obtained from Macherey–Nagel (Düren, Germany); Sephadex™ G10 and Lipophilic Sephadex™ LH20 were purchased from Sigma–Aldrich, Inc. (MO, USA). β-D-Fructofuranosidase (EC 3.2.1.26) from *S. cerevisiae* (Inv) was purchased from Sigma Chemical Co. (MO, USA).

2.2. Enzymes

Levansucrase (E.C. 2.4.1.10) from *B. subtilis* (BS-LVS) was produced from a strain derived from *B. subtilis* Marburg 168 as previously reported [25]. This strain, designated with the genotype Δnpr, Δapr, CmR, degU32 (Hy), overexpresses the BS-LVS gene (sacB). After fermentation, the extracellular enzyme was precipitated using 25% (w/v) PEG (Mw: 5000), centrifuged (22,100 × *g*, 25 min) and recovered in phosphate buffer 50 mM, pH 6.0 [26,27]. Inulosucrase (E.C. 2.4.1.9) from *L. citreum* (IslA) in cell-associated form was used. The microorganism was grown as previously reported [28], the culture was centrifuged (15,300 × *g*, 10 min), and cells containing IslA activity were washed and recovered in phosphate buffer 50 mM, pH 6.0. The gene coding for LevC, a levansucrase from *L. mesenteroides* ssp. *mesenteroides* ATCC 8293, was expressed in *Escherichia coli* TOP 10 under the conditions previously reported [29]. The culture was centrifuged (8630 × *g*, 10 min), and cells were recovered in 50 mM phosphate buffer, pH 6.0. After cell rupture (French press, 1125 psi) and centrifugation (22,100 × *g*, 10 min), the supernatant containing levansucrase activity was directly used in the reaction. A commercial β-D-fructofuranosidase (EC 3.2.1.26) from *S. cerevisiae* (Inv) was employed.

2.3. Analytical methods

2.3.1. Enzyme activity

The activity of the enzymes studied was determined from the initial rate of reducing sugar release (fructose and glucose) from a 292 mM sucrose solution in phosphate buffer 50 mM, pH 6.0 for FTFs, or acetate buffer 50 mM pH 4.5 for β-D-fructofuranosidase using the dinitrosalicylic acid (DNS) method with glucose as a standard [30]. One activity unit (U) was defined as the amount of enzyme releasing the equivalent of 1 μmol of reducing sugar per minute (μmol min^{-1}). The global enzyme activity in reactions containing phenolic acceptors was evaluated as the initial rate of sucrose consumption by high-performance liquid chromatography (HPLC). Samples from these reactions were inactivated in boiling water for 10 min and analysed. Fructoside yields were calculated from sucrose conversion as determined by HPLC. All of these assays and further experiments were performed in triplicate and error bars are included in graphs.

2.3.2. TLC analysis of fructosylation reactions

TLC was performed using HPTLC silica gel 60 glass plates (Merck, Darmstadt, Germany) and 85:15 (v/v) acetonitrile/water as the mobile phase. Plates were eluted twice, and the aromatic substrates and product spots were visualised with iodine vapour. Subsequently, sugars and products were revealed by spraying α-naphtol solution (2.4% α-naphtol in an ethanol/water/H_2SO_4 83:7:11 mixture) and heating at 100 °C for 1 min.

2.3.3. HPLC analysis of fructosylation reactions

HPLC analyses were performed using a Waters HPLC system (Waters Corp., MA, USA) equipped with a 600E system controller, using an Alltech Prevail Carbohydrate ES column (5 μm, 250 mm × 4.6 mm) (Alltech Associates, Inc., IL, USA) or a Waters Spherisorb ODS-2 column (5 μm, 250 mm × 4.6 mm, Waters Corp., MA, USA). System was coupled to a 410 refractive index (RI) detector and a 996 photodiode array detector (Waters Corp., MA, USA) at 280 nm (or 223 nm). Fructosylation products were quantified using

the corresponding purified fructosides as standards. See Supporting Information (SI) file for details.

2.4. Enzyme selection

To select the most active and efficient enzyme for these acceptor reactions, the already described enzymes (three FTFs and one fructosidase) were evaluated in reactions containing different amounts of hydroquinone, benzyl alcohol or butanol as acceptors. Reactions containing 0–400 mM acceptor, sucrose 292 mM and enzyme 1 U mL^{-1} in the appropriate buffer were incubated at 30 °C using an Eppendorf Thermomixer comfort (Hamburg, Germany). In all cases, reactions without an acceptor were carried out as controls. Enzyme performance was quantitatively evaluated in terms of the enzyme activity described in Section 2.3.1; the substrates and products were identified by TLC.

2.5. Effect of substrate concentration on acceptor reactions

Hydroquinone was used as a model substrate to select the reaction conditions for BS-LVS. Reactions containing 400 mM sucrose and 100–500 mM hydroquinone as an acceptor with 1 U mL^{-1} of BS-LVS in phosphate buffer 50 mM and pH 6.0 were incubated for 20 h at 30 °C. Samples were withdrawn, heat-inactivated and diluted for product and sugar analysis by HPLC.

Stability of BS-LVS during storage in hydroquinone was evaluated by incubation of the enzyme in a 500 mM hydroquinone in phosphate buffer 50 mM, pH 6.0. Aliquots were withdrawn from 0 to 24 h, and the global enzyme activity measured as already described. The size of the samples from the incubation solution was calculated to dilute the hydroquinone content in the reaction medium to 25 mM. This concentration was considered low enough to avoid any effect on the enzyme activity. A control reaction was carried out using the enzyme without previous contact with hydroquinone. Residual enzyme activity was measured from the initial rate of sucrose conversion by HPLC during 45 min of reaction.

2.6. Effect of enzyme concentration on acceptor reactions

The effect of enzyme concentration (1–20 U mL^{-1} BS-LVS) on fructoside production was evaluated in reactions containing 400 mM sucrose and 500 mM hydroquinone in phosphate buffer 50 mM, pH 6.0. Reactions were incubated 20 h at 30 °C. The fructoside concentration in the samples was determined by HPLC.

2.7. Acceptor reaction in aqueous–organic mixtures

The effect of 2-methyl-2-propanol as an organic co-solvent in the acceptor reactions was evaluated in solutions containing up to 50% (v/v) of the solvent, 400 mM sucrose, 500 mM hydroquinone and 5 U mL^{-1} BS-LVS in phosphate buffer 50 mM pH 6.0 incubated at 30 °C.

2.8. Isolation and purification of fructosides

Fructosides were produced by BS-LVS (5 U mL^{-1}) in reactions with 400 mM sucrose and 500 mM acceptor (850 mM for butanol and 200 mM for 4-hydroxybenzyl alcohol and phenol). Purification techniques consisted in gel chromatography, flash chromatography or preparative HPLC using a Waters Spherisorb®S5 ODS2 semi-prep column (5 μm, 250 mm × 20 mm, Waters Corp., MA, USA). See SI file for details.

2.9. Structural analysis (RMN)

NMR spectra were acquired either on a Varian Unity NMR Spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C nuclei or on an Eclipse JEOL (E) NMR spectrometer operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C nuclei. Chemical shifts are reported in parts per million (ppm) relative to H_2O and were made on the basis of ^{1}H-^{1}H COSY, HMBC and HSQC spectral analysis as required. HRFABMS spectra in a matrix of *m*-nitrobenzyl alcohol were recorded on a JEOL JMX-AX 505 HA mass spectrometer. Purified fructosides structure was elucidated on the basis of the 1D (^{1}H, ^{13}C) and 2D (COSY, HSQC and HMBC) NMR experiments. All products corresponded to monofructosides. The 4-(hydroxy)-benzyl-β-D-fructofuranoside (**Fru$_1$-4HB**) was used as a model for structural description. Structural details of all synthesised compounds are included in SI file.

2.10. Nucleophilicity index calculation

Calculations were carried out using the GAUSSIAN 09 program. The geometry of the neutral and anionic species were optimised at the B3LYP/6-311+G (d, p) level; the inclusion of diffuse functions has been shown to be important for anionic species [31]. The nucleophilicity index (ω^-) was evaluated from the frontier molecular orbital values, according to Jaramillo et al. [32,33]:

$$\omega^- = \frac{1(\mu_a - \mu_b)^2}{2(\eta_a + \eta_b)^2}\eta_a$$

where $\mu \approx (\varepsilon_H + \varepsilon_L)/2$ is the electronic chemical potential; $\eta \approx (\varepsilon_L - \varepsilon_H)$ is the hardness; the subscript a is the nucleophile, either neutral or anionic; b the electrophile (fructose). ε_H and ε_L correspond to the HOMO and LUMO energies, respectively.

2.11. COSMO-RS calculations

A full description of COSMO-RS (COnductor-like Screening MOdel for Realistic Solvents) theory developed by Klamt et al. is provided elsewhere [34–36]. In this work, Turbomol 5.9.1 and COSMOtherm version C2.1 release 01.07 software were employed (COSMOlogic GmbH & Co. KG, Leverkusen, Germany, 2007). Three-dimensional molecular structures of the compounds and solvents, as well as a minimisation of their molecular conformations, were implemented with Marvin Sketch 5.3.8 software (v.2010 ChemAxon, http://www.chemaxon.com/). Structural conformers were not calculated due to the structural simplicity of the evaluated compounds. The generation of molecular COSMO files was performed with the Turbomole 5.9.1 program package at the density functional theory level using the BP (B88-VWN-P86) functional with a triple-z valence polarised basis set (TZVP). The infinite dilution activity coefficients estimation was computed with a non-iterative mode carried out on COSMOtherm version C2.1 release 01.07.

3. Results and discussion

3.1. Enzyme selection

The enzyme activities of three FTFs (BS-LVS, IslA and LevC) and one β-fructofuranosidase (Inv) were evaluated in the presence of sucrose and different concentrations of hydroquinone (**Hq**), butanol (**Bu**) and benzyl alcohol (**Bnz**) as fructosyl acceptors. These were compared to the activity of the control reaction without an acceptor (Fig. 1). The higher water solubility of **Bu** allowed the use of higher concentrations of this acceptor.

Under these conditions, BS-LVS was the enzyme retaining the highest activity at high acceptor concentration. Indeed, BS-LVS

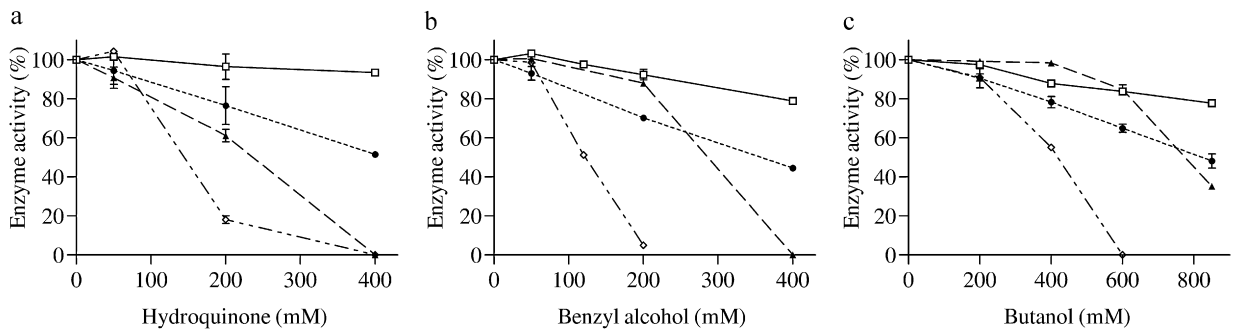

Fig. 1. Relative enzyme activity of FTFs and Inv in acceptor reactions with (a) hydroquinone, (b) benzyl alcohol and (c) butanol. (□) BS-LVS; (●) Inv; (▲) IslA; (◊) LevC.

retained more than 95% of its activity in the presence of 400 mM **Hq** (Fig. 1a) and more than 80% in 400 mM **Bnz** (Fig. 1b) and 850 mM **Bu** (Fig. 1c).

Conversely, a drastic decrease in activity was observed for Inv, IslA and LevC when concentrations of **Bnz** and **Hq** were increased. Previous reports have dealt with the low stability of some glycosyltransferases and glycosidases in the presence of phenolic compounds as acceptors [16,23]. The high activity of BS-LVS in the presence of these phenolic compounds is in agreement with its high activity and stability in the presence of organic solvents [25].

To evaluate the fructosylation efficiency, all four enzymes were evaluated in the presence of 200 mM **Hq** and analysed by TLC, using arbutin, a monoglucosylated hydroquinone derivative, as a standard. These results are shown in Fig. 2, where it is observed in the TLC plates that all four enzymes retained a fraction of their activity; different extents of glucose, fructose and polymer formation can be observed.

All enzymes were capable of forming a fructosylated product that elutes at the same Rf as arbutin, which corresponds to monofructosylated **Hq** (**Fru-Hq**). However, a qualitative comparison with fructoside spots showed that the largest amount of **Fru-Hq** was obtained with BS-LVS. On the other hand, very low amounts of fructoside were observed for FTFs IslA and LevC, in agreement with low amounts of fructose and/or glucose released. It is notable that, although Inv is rather stable in 200 mM **Hq**, the acceptor reaction leading to the formation of **Fru-Hq** takes place at the lowest efficiency among the assayed enzymes. It may therefore be concluded that among these enzymes, BS-LVS presents the highest stability in the presence of phenolic compounds and the highest **Hq** fructosylation efficiency. In addition, these results demonstrate that, in general, FTFs are better catalysts for **Hq** fructosylation than β-fructofuranosidase. As a consequence, BS-LVS was selected for further analysis in acceptor reactions.

3.2. Effect of substrate concentration on acceptor reactions

It has been previously reported that the efficiency of GTFs on acceptor reactions is highly influenced by the nature and concentration of the acceptor [37,38]. Considering that **Fru-Hq** is an efficient tyrosinase inhibitor [24], the enzymatic fructosylation of hydroquinone mediated by BS-LVS was studied as a reference reaction to establish suitable conditions for acceptor reactions. For this purpose, the sucrose concentration was set at 400 mM: high concentrations are reported to favour transfer over hydrolysis reactions [26,28]. **Hq** concentration was varied from 100 to 500 mM, which is close to the limit of **Hq** solubility. After 20 h of reaction, sucrose was converted, and the fructosylated products were quantified by HPLC.

In all of the reactions studied, only one fructosylated product was identified as **Fru-Hq**, with the highest concentration (22 mM) obtained from 500 mM **Hq** (black bars in Fig. 3).

It is worth mentioning that at this acceptor concentration sucrose conversion was the lowest (gray bars in Fig. 3), suggesting low stability of BS-LVS at high concentrations of **Hq**. Therefore, BS-LVS storage stability in 500 mM **Hq** at 30 °C was evaluated over 24 h. Samples were withdrawn at different time intervals, and the initial rate of sucrose conversion in the absence of an acceptor was assayed as described (see Section 2.5). During the first two hours of incubation in **Hq**, more than half of the BS-LVS activity was lost (Fig. S1 in Supporting Information file), and all of the activity disappeared after 24 h. This time-dependent inactivation of BS-LVS was

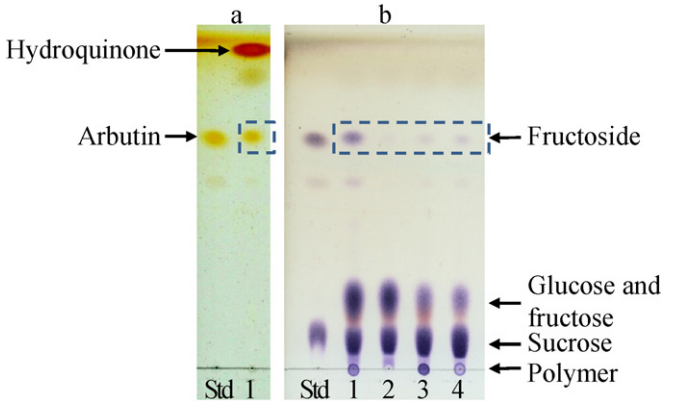

Fig. 2. TLC analysis of the fructosylation reaction catalysed by FTFs and Inv using **Hq** as acceptor. (a) Layer was visualized with iodine (only two lines are shown); (b) the same layer was visualized with α-naphtol. (Std) Sucrose and arbutin standards. (1) BS-LVS, (2) Inv, (3) IslA, and (4) LevC.

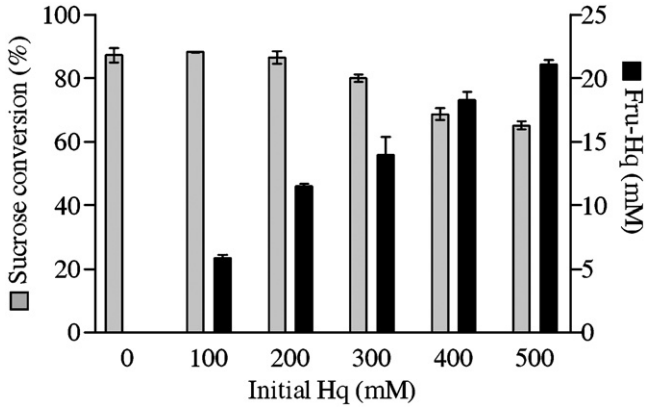

Fig. 3. Fructoside synthesised and sucrose converted by BS-LVS after 20 h of reaction as a function of the initial concentration of **Hq**. The concentrations of **Fru-Hq** produced are shown as black bars; the percentage of sucrose converted is shown as gray bars. Reactions were carried out in 400 mM sucrose and 1 U mL^{-1} of enzyme at 30 °C.

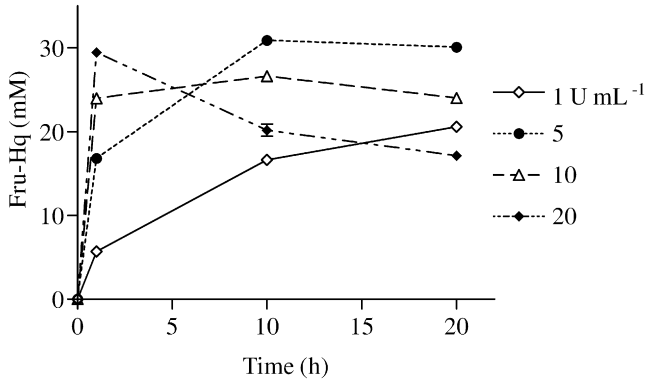

Fig. 4. Effect of enzyme concentration on the kinetics of fructoside production. Reactions were carried out in 400 mM sucrose and 500 mM **Hq** at 30 °C.

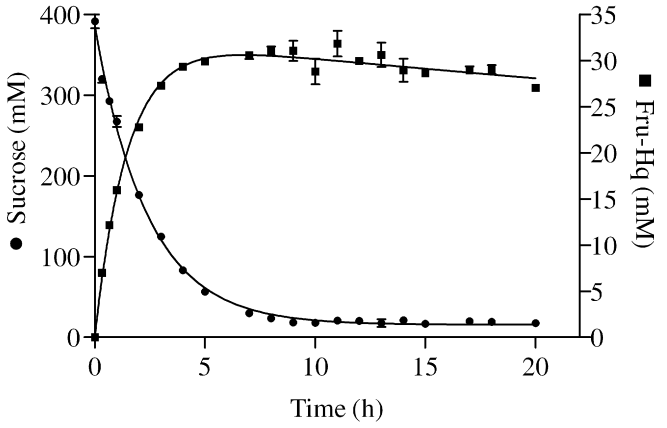

Fig. 5. Evolution of hydroquinone fructoside (**Fru-Hq**) synthesis and sucrose consumption by BS-LVS in reactions containing 400 mM sucrose, 500 mM **Hq** and 5 U mL^{-1} of enzyme at 30 °C.

not reversible because after incubation, the enzyme was diluted to very low **Hq** concentrations to measure the activity. Although these results show an unfavourable effect of **Hq** on BS-LVS, it should be noted that the efficiency of the enzyme under operational conditions is usually higher due to the sucrose stabilisation effect on the enzyme [39]. This can be observed in Fig. 3, where in operational conditions a relatively high conversion of sucrose with the concomitant production of **Fru-Hq** is observed.

3.3. Effect of enzyme concentration

The effect of enzyme concentration on sucrose conversion and fructoside production was studied in reactions containing 400 mM sucrose and 500 mM **Hq**. As shown in Fig. 4, an important improvement on **Fru-Hq** initial production rate is observed with increasing BS-LVS concentration, with the highest **Fru-Hq** production obtained at 5 U mL^{-1}. Although at higher concentrations of enzyme (10 and 20 U mL^{-1}) a high initial **Fru-Hq** synthesis rate is observed, it is followed by a decrease in rate proportional to enzyme activity, suggesting that **Fru-Hq** synthesis is subjected to kinetic control.

From these results, it is clear that the selection of enzyme concentration in the reaction medium plays a major role in defining **Fru-Hq** yield and productivity. In fact, the higher the enzyme activity in the reaction, the higher the initial fructosylation rate and sucrose conversion, but the faster **Fru-Hq** is lost by hydrolysis. The effect of enzyme concentration was further studied following the evolution of the acceptor reaction with a BS-LVS activity of 5 U mL^{-1} (Fig. 5). In this case, **Fru-Hq** concentration reaches 30 mM with an almost total conversion of sucrose after 10 h of reaction, as compared to 22 mM **Fru-Hq** and 65% sucrose conversion obtained with 1 U mL^{-1} (Fig. 3). In all cases, when sucrose is exhausted, the fructoside concentration decreases. When purified **Fru-Hq** was treated with BS-LVS in the absence of sucrose, it was readily hydrolysed (results not shown). However, because the affinity of the enzyme for sucrose is higher, to maximise the **Fru-Hq** synthesis and avoid hydrolysis, further experiments were performed at 500 mM acceptor, 400 mM sucrose and 5 U mL^{-1} BS-LVS.

3.4. Effect of organic co-solvent on the acceptor reaction

One of the most frequently used strategies to enhance yields in enzymatic processes involving hydrophobic substrates in aqueous media is the use of organic co-solvents. The general idea when using this approach is that these organic solvents may increase the solubility of the substrates and, therefore, their availability for the enzymatic reaction [16,20,40]. An additional effect of co-solvents has been reported in reactions with BS-LVS, where high 2M2P concentrations enhance the initial rate and lead to a higher transfer/hydrolysis ratio due to the depletion of thermodynamic water activity [25]. Therefore, the reactions with phenolic acceptors were carried out in 0–50% 2M2P in the reaction medium and evaluated in terms of **Fru-Hq** yield and sucrose conversion.

Unexpectedly, while sucrose conversion was practically equivalent for all of the 2M2P concentrations evaluated (data not shown), **Fru-Hq** synthesis was drastically reduced as 2M2P concentration increased (black bars in Fig. 6). It is worth mentioning that the fructose released decreased with increasing 2M2P content (results not shown), indicating that the co-solvent favours the transfer of fructosyl groups to the growing levan polymer chain, reducing the transfer to water (hydrolysis).

The effect of solvent mixtures on enzymatic processes has been evaluated in terms of reaction rates and these, in turn, have been evaluated as a function of thermodynamic activity coefficients (γ) of the substrates [41]. Actually, this coefficient may be considered as a measure of how much the substrate interacts with the medium and, consequently, of how it is available for the reaction. A robust and reliable way to predict thermophysical data for liquid systems, such as γ coefficients, has recently been reported [42]. The COnductor-like Screening MOdel for Realistic Solvation (COSMO-RS) method is based on a combination of quantum chemical calculations for solutes and solvents and statistical thermodynamics procedures. This method allows for the estimation

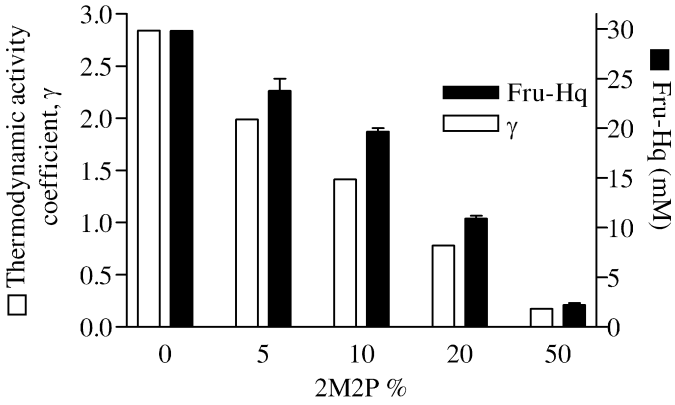

Fig. 6. Fructosylation of **Hq** with BS-LVS in aqueous–organic mixtures: the predicted thermodynamic activity coefficients (γ) of **Hq** are shown as white bars. Reactions were carried out in 400 mM sucrose, 500 mM **Hq** and 5 U mL^{-1} of enzyme in the appropriate aqueous–organic mixture. In all reactions, conversion was higher than 85%.

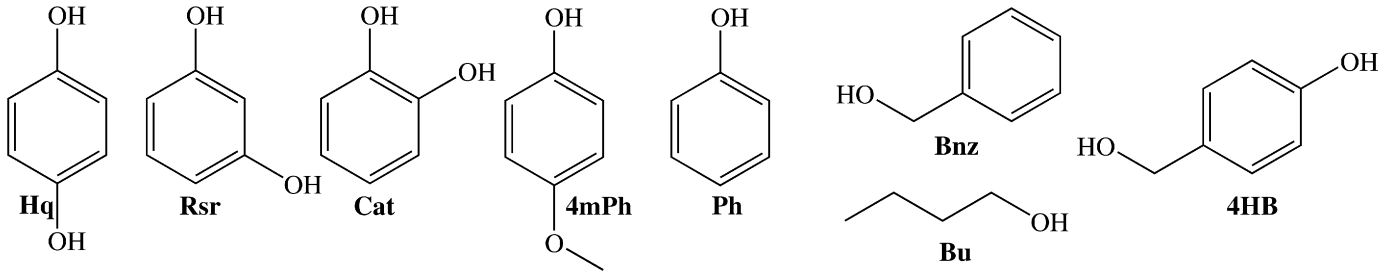

Fig. 7. Acceptors used in fructosylation reactions with BS-LVS: **Hq**, hydroquinone; **Rsr**, resorcinol; **Cat**, catechol; **4mPh**, 4-methoxyphenol; **Ph**, phenol; **Bnz**, benzyl alcohol; **Bu**, butanol; **4HB**, bifunctional acceptor 4-hydroxybenzyl alcohol.

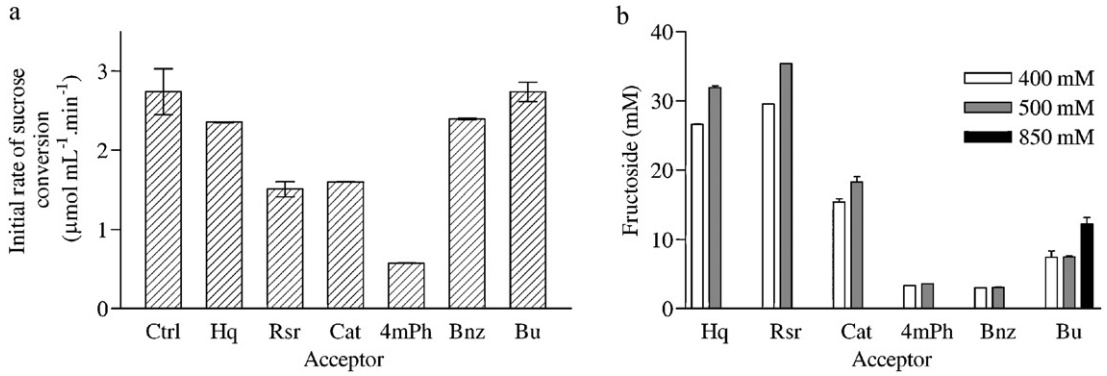

Fig. 8. Fructosylation of aromatic and primary alcohol acceptors with BS-LVS. (a) Initial rate of sucrose conversion with 500 mM acceptor. (b) Total fructoside produced at 400 mM (white bars), 500 mM (gray bars) and 850 mM (black bars) initial acceptor concentration. All reactions were carried out with 400 mM sucrose and 5 U mL^{-1} of enzyme at 30 °C.

of solvent–solute molecular surface interactions and, from these estimations, the prediction of thermodynamic properties for liquid systems. Thus, to explain why the presence of 2M2P results in a negative effect on fructosylation, γ values of **Hq** in water/2M2P mixtures were predicted using the COSMO-RS method and are reported in Fig. 6, where it may be observed that γ values decrease almost linearly with increasing 2M2P concentration (white bars in Fig. 6).

Indeed, the hydrophobic character of **Hq** leads to a stronger molecular interaction with 2M2P than with water, decreasing the γ value as the amount of 2M2P increases. Hence, it may be concluded that the reduction in **Hq** fructosylation is due to the lower availability of the acceptor in this co-solvent. Conversely, the presence of 2M2P decreases the water activity, favouring the transfer of fructose to levan with the concomitant decrease of hydrolysis of sucrose. Therefore, the addition of a hydrophobic organic co-solvent in acceptor reactions with **Hq** is not an adequate strategy to improve fructosylation, water being a better solvent.

3.5. Acceptor reactions with hydrophobic substrates

To extend the fructosylation strategy to other aromatic or aliphatic alcohols, resorcinol (**Rsr**), catechol (**Cat**), 4-methoxyphenol (**4mPh**), phenol (**Ph**), benzyl alcohol (**Bnz**) and butanol (**Bu**) were also evaluated in acceptor reactions with BS-LVS (Fig. 7). These reactions were carried out under the previously selected conditions, *i.e.*, 400 mM sucrose, 5 U mL^{-1} of enzyme and an adequate concentration of the acceptor. All substrates are soluble at 500 mM, except **4mPh** and **Bnz**, whose maximum solubility in water is 320 mM and 370 mM (at 25 °C), respectively; the high water solubility of **Bu** allowed for the evaluation of the effect of higher concentrations of this acceptor (850 mM).

In all cases, the highest fructoside concentrations were obtained at the highest concentration of acceptor (500 mM for dihydroxybenzenes or 850 mM **Bu**). Surprisingly, high initial rates of sucrose conversion (Fig. 8a) and high fructoside concentration (Fig. 8b) were obtained in reactions with **Hq**, **Cat** and **Rsr**, dihydroxybenzenes of reported low nucleophilicity, whereas reactions with **Bu** and **Bnz**, which are reportedly better nucleophiles [15,43], resulted in lower fructoside concentrations. In these conditions, negligible initial rates were observed when phenol was used as the acceptor.

It is important to note that, although the initial rates of sucrose conversion for **Bu** and **Bnz** are similar to those observed for dihydroxybenzenes (Fig. 8a), the final fructoside concentrations were considerably lower (Fig. 8b). This suggests that even if **Bu** and **Bnz** do not seem to affect BS-LVS activity, these primary alcohols are not good acceptors: all sucrose converted is either hydrolysed or transferred to levan.

Interestingly, even when the activity of BS-LVS in the presence of **4mPh** was greatly reduced, the fructoside yield relative to sucrose converted was higher than that observed for **Bnz**, leading to the conclusion that **4mPh** is a better acceptor than primary alcohols, as shown in Table 1 for 500 mM of **4mPh**. In this context, BS-LVS may be considered an interesting catalyst for fructosylation and other synthetic applications, as fructoside yields (*i.e.*, 30 mM **Fru-Hq**)

Table 1
Nucleophilicity (ω^-) and pK_a values for the different acceptors evaluated.

Acceptor	pK_a	Nucleophilicity (ω^-)[a]	Fructosylation yield (%)[b]
Butanol	16.95	0.0194	1.91
Benzyl alcohol	15.20	0.0102	0.81
4-Methoxyphenol	9.94	0.0065	6.25
Hydroquinone	9.68	0.0104	9.51
Catechol	9.34	0.0080	6.86
Resorcinol	8.91	0.0066	11.70

[a] Nucleophilicity calculated for the deprotonated species.
[b] Yield relative to sucrose conversion, using 500 mM acceptor and 400 mM sucrose.

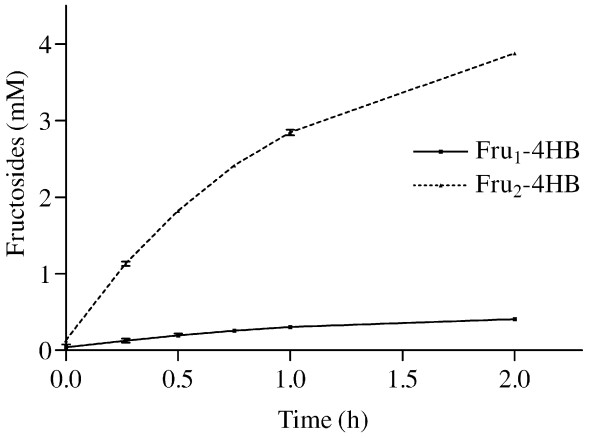

Fig. 9. Evolution of the fructosylation products in acceptor reactions of BS-LSV with **4HB**. Reaction conditions: 400 mM sucrose, 200 mM **4HB**, 5 U mL^{-1} at 30 °C.

are higher than those previously reported for phenolic compounds (4 mM) [24].

3.6. Influence of acceptor nucleophilicity on the fructosylation reaction

To explain the better acceptor selectivity shown by BS-LVS for phenols over primary alcohols, a bifunctional compound such as 4-hydroxybenzyl alcohol (**4HB**) was used as an acceptor (Fig. 7). This substrate allows a direct selectivity comparison because it holds a phenol and a primary hydroxyl within the same molecule. The reaction was carried out in a medium saturated with 200 mM **4HB** (water solubility of **4HB** is around 54 mM at 20 °C).

Two different fructosylated products **Fru$_1$-4HB** and **Fru$_2$-4HB** were identified, corresponding to the fructosylation of **4HB** on the primary and aromatic –OH, respectively, as confirmed by NMR and HPLC.

When evaluating the reaction kinetics, it was observed that the initial rate of **Fru$_1$-4HB** formation was 10 times lower (5.03 μmol L^{-1} min^{-1}) than the initial rate of **Fru$_2$-4HB** formation (50.64 μmol L^{-1} min^{-1}), demonstrating the higher specificity of BS-LVS for aromatic hydroxyls (Fig. 9).

According to the acid/base reaction mechanism proposed for BS-LVS [44,45], in the first step, the Glu342 residue donates a proton to the glycosyl oxygen atom of the leaving glucosyl residue, whereas the nucleophile Asp86 residue forms a typical glycosyl-enzyme intermediate. In the second step, the deprotonated Glu342 acts as a general base to activate (deprotonate) the acceptor molecule, making it able to attack the fructose C2 to form the new glycosidic linkage (Fig. S2 in SI file).

Deprotonated hydroxyls in the acceptor molecules are regarded as better nucleophiles than their non-deprotonated species. Therefore, a critical step in the acceptor reaction is the ability of a hydroxylated acceptor molecule to donate a proton and become a better nucleophile. This property may be associated with the pK_a of the acceptor.

To explain the different reactivity of primary and aromatic alcohols in acceptor reactions with BS-LVS, the nucleophilicity (ω^-) values of the deprotonated species of some of the acceptors studied were calculated according to Jaramillo et al. [32,33] and compared to pK_a values reported for different aromatic and primary alcohols. These values are given in Table 1, where it is clear that the values of ω^- are in the same order of magnitude for both aromatic and primary deprotonated alcohols, indicating that once the aromatic and primary alcohols are deprotonated, both species may behave as good nucleophiles. Nevertheless, the same data revealed an inverse relationship between fructosylation yield and pK_a values. Indeed, the fructosylation of **4HB** on the aromatic hydroxyl (pK_a = 9.48; ω^- = 0.0041) proceeds 10 times faster than the fructosylation of its primary hydroxyl (pK_a = 15.21; ω^- = 0.009). This indicates that the higher reactivity of phenolic groups may be explained by their ability to be deprotonated and become activated nucleophiles.

In terms of BS-LVS catalytic behaviour, these facts suggest that the higher acidity of phenolic compounds facilitates the loss of their acidic proton, especially when they are confronted with the basic environment of the catalytic site in BS-LVS, that is, when Glu342 is deprotonated. Therefore, phenolic molecules become better acceptors, as higher efficiencies in enzymatic fructosylation reactions with BS-LVS were observed.

4. Conclusions

Among several enzymes that are able to fructosylate hydrophobic compounds, BS-LVS was selected due to its activity in the presence of aromatic or aliphatic alcohol acceptors. In particular, BS-LVS was very efficient in fructosylation reactions due to its higher stability in the presence of hydrophobic compounds, allowing concentrations of up to 500 mM in the reaction medium. Three different activities are commonly present in BS-LVS with sucrose: transfer of fructose to levan, transfer of fructose to an acceptor and sucrose hydrolysis. Surprisingly, the common strategy to improve glycosylation of hydrophobic molecules by the addition of organic co-solvents resulted in an important reduction of sucrose hydrolysis and also a decrease in the fructosylation yield, favouring the fructose transfer to levan. This behaviour was theoretically explained in terms of the effect of the solvent on the acceptor thermodynamic activity, and this concept may become a useful tool in predicting and designing acceptor reactions in organic media.

Several fructosides were produced and purified through acceptor reactions in aqueous media; the synthesis of **Fru-Cat**, **Fru-Rsr**, **Fru-4mPh**, **Fru$_2$-4HB** and **Fru-Ph** is reported for the first time. Indeed, to our knowledge, we report the highest concentration of **Fru-Hq** (30 mM) using a fructosyltransferase as catalyst. It was found that phenolic compounds are fructosylated with higher efficiencies than primary alcohols, and this reaction selectivity correlates with the pK_a value of the acceptor, with higher yields at low pK_a values, where the amount of deprotonated acceptor is higher. The present enzymatic procedures and design tools represent a good reference for the synthesis of novel glycosylated products, with potential use in cosmetics and pharmaceuticals.

Acknowledgments

E. Castillo thanks DGAPA-UNAM for a sabbatical fellowship and INSA-Toulouse for an invited researcher fellowship. A. Mena-Arizmendi thanks CONACyT for Ph.D. fellowship 169648. This work has been supported by the grants IN226706-3 PAPIIT-UNAM and CONACyT 40609-Z. Authors also thank M.E. Rodríguez and F. Gonzalez from IBt-UNAM for technical assistance, as well as Victoria Labastida and Gabriela Vargas from CIQ-UAEM, for mass spectroscopy and NMR analysis, and J.M. Hurtado from IBt-UNAM for assistance with computing resources.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2011.02.002.

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