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Exploration of transfructosylation activity in cell walls from *Cryptococcus laurentii* for production of functionalised β-D-fructofuranosides

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Abstract

Cell wall preparations from a strain of the yeast *Cryptococcus laurentii* catalyse formation of β-D-fructofuranosides from sucrose. The enzyme preparation exhibits high stability and broad substrate specificity enabling use of a variety of aliphatic and phenolic primary alcohols as fructofuranosyl acceptors. Chemical yields range from 3 to 38% depending on reaction conditions and chemical nature of acceptor.

Keywords: *Cryptococcus laurentii*; β-D-Fructofuranosidase; β-D-Fructofuranosides; Transfructosylation

1. Introduction

Saccharides, due to their chirality and other properties, offer many advantages for construction of various chemical precursors. They are generally available in large quantities from renewable resources and may confer properties of biodegradability. Sucrose is one of the most accessible candidates for industrial use, as it is produced in pure form on the order of millions of tonnes annually.

The availability of glycosides with useful functional groups would permit development of strategies for synthesis of new sugar derivatives. The development of stereoselective methods for the synthesis of glycosidic linkages presents a considerable challenge to synthetic chemists, requiring protection, activation, stereoselective and chemoselective coupling and deprotection steps. Therefore, simple, cheap and effective enzymatic approaches are highly desirable [1].

Nowadays, there is a growing effort to apply hydrolases as biocatalysts in transglycosylation and reverse hydrolytic processes. Regio- and stereoselective syntheses of glycosides mediated by glycohydrolases [EC 3.2.1.x] are becoming widely used in the functionalisation of saccharides. The greatest commercial potential of these biocatalysts is in the production of different chemicals which are not easily synthesized by chemical means [1,2].

Our current work is directed towards chemical or enzymatic syntheses of monosaccharide components with tailored functional diversity and amphiphilic behaviour. In this context, we are interested in preparation of D-fructofuranosides and in utilisation of their two primary hydroxyl groups in a specific orientation for subsequent study. Anomerically pure fructofuranosides are, however, difficult to synthesize by a simple chemical methods [2]. Many recent reports describe the enzymatic synthesis of fructooligosaccharides by invertase (β-D-fructofuranosidase, EC 3.2.1.26) [3–6]. In addition, there exist several papers concerning enzymatic preparation of short-chain alkyl β-D-fructofuranosides [7–13] from sucrose or enzymatic β-D-fructofuranosylation of less conventional acceptors like 2-mercaptoethanol [14], glycerol [15] and ergot alkaloids [16], mainly using invertase from baker's yeast (*Saccharomyces cerevisiae*).

According to our screening results, the enzyme system of crude cell walls from the yeast *Cryptococcus laurentii* (acapsular strain) catalysed fructofuranosyl transfer from sucrose to various acceptors with a broad range of structural features. Miscellaneous alkyl alcohols (methanol, ethanol, *n*-propanol, 2-propanol, *n*-butanol, *n*-pentanol, *n*-hexanol, *n*-heptanol, *n*-octanol), phenols (4-hydroxybenzyl, vanillyl and coniferyl alcohol) and other alcohols (allyl, propargyl and furfuryl alcohol) were used as acceptors to test transfructosylation activity of the enzyme system (Scheme 1).

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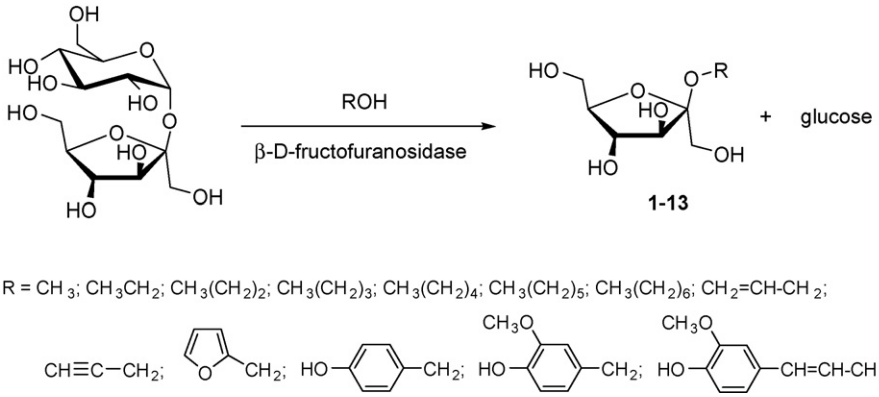

Scheme 1. Enzymatic synthesis of β-D-fructofuranosides by crude β-fructofuranosidase from cell walls of *Cryptoccocus laurentii*.

2. Experimental

2.1. Analytical methods

All reactions were monitored by TLC on silica gel plates with chloroform/methanol (3/1, v/v) as eluent. The compounds were detected under UV light (254 nm) as well as by charring the plates with 10% (v/v) ethanolic solution of H_2SO_4 and heating at ca. 200 °C. Optical rotations were measured with a Perkin-Elmer 241 polarimeter at 20 °C. ^{1}H NMR spectra were recorded at 300 MHz with Bruker AM 300 (Me_4Si as internal standard). ^{13}C NMR spectra were recorded at 75 MHz and chemical shifts are referenced to CD_3OD as internal standard.

2.2. Materials

Sucrose and alcohols were purchased from Lachema, Brno, Czech Republic, Acrōs Organics, Geel, Belgium and Merck, Darmstadt, Germany. Thin-layer chromatography was performed on precoated silica gel 60 F_{254} plates (0.25 mm, Merck). Silica gel (0.035–0.070 mm, pore diameter ca. 6 nm, Acrōs Organics) was employed for column chromatography.

2.3. Microorganism and culture conditions

C. laurentii CCY 17-3-6 (acapsular strain) was purchased from the Culture Collection of Yeasts (Institute of Chemistry, SAS, Bratislava, Slovakia). Cultures were grown at 28 °C in 1000 ml Erlenmeyer flasks on the orbital shaker (100 rpm). Each flask contained 400 ml of liquid medium of following composition (in grams per liter): $MgSO_4{\cdot}7H_2O$, 0.3; KH_2PO_4, 1.36; urea, 1.29; sodium glutamate, 1.0; thiamine, 0.002; biotin, 1×10^{-5}; lactose 20.0 and 1 ml microelement solution. Composition of microelement solution (in milligrams per liter): H_3BO_4, 1.25; $CuSO_4{\cdot}5H_2O$, 0.1; KI, 0.25; $MnSO_4{\cdot}5H_2O$, 1.0; $FeCl_3{\cdot}6H_2O$, 0.5; $(NH_4)_2Mo_7O_{24}{\cdot}4H_2O$, 0.5; $ZnSO_4{\cdot}7H_2O$, 1.0.

2.4. Preparation of cell walls

All operations were carried out at 4 °C. Cells in stationary phase were harvested by centrifugation at 8000 × *g* for 15 min and washed twice with 0.05 M TRIS–HCl pH 7.5 and once with 0.05 M TRIS–HCl pH 7.5 containing 0.1 mM PMSF. Cells were resuspended in 0.1 M TRIS–HCl pH 7.5 containing 0.46 mM PMSF, 2 mM EGTA and 1 mg/ml ovalbumin and lysed by homogenzing the suspension with glass beads (0.5–1 μm) in Novotny rotary homogenizer for 10 2-min intervals, alternately cooling at equal intervals on ice. Lysis of cells was assessed by microscopic examination. Broken cells were centrifuged at 1300 × *g* for 10 min to remove debris and unbroken cells. Pellet was washed five times with cold distilled water and resuspended in 0.1 M McIlvaine buffer (pH 4.8). Cell wall associated fructofuranosidase activity in the suspension (1 g/2 ml) was determined according to method described below.

2.5. β-Fructofuranosidase assay

A reaction mixture containing 600 μl 1% sucrose (w/v) in 0.1 M McIlvaine buffer (pH 4.8) and 20 μl enzyme suspension was incubated at 37 °C for 20 min. Enzyme was inactivated by boiling the samples in a water-bath for 5 min. Inactivated samples were centrifuged at 12,000 rpm for 10 min. The amount of released glucose was estimated by glucose-oxidase test [17].

2.6. Screening experiments and determination of transfructosylation activity

2.6.1. Liquid acceptors

The standard reaction mixture (2 ml overall) containing sucrose (0.1 g, 0.292 mmol) and cell wall preparation (80 mg, 24 U of β-fructofuranosidase) in appropriate volumes of 0.1 M McIlvaine buffer (pH 4.8) and alcohol (methanol, ethanol, *n*-propanol, 2-propanol, *n*-butanol, *n*-pentanol, *n*-hexanol, *n*-heptanol, *n*-octanol, allyl, propargyl or furyl alcohol, 30, 50, or 90%) was shaken on VIBRAMAX 100 shaker (Heidolf) at 750 rpm and 37 °C. The course of the reaction was monitored by thin layer chromatography (Table 1).

2.6.2. Solid acceptors

2.6.2.1. Organic solvent concentration. The standard reaction mixtures (2 ml) comprising (0.1 g, 0.292 mmol) sucrose, 0.4 M vanillyl alcohol and cell wall preparation (80 mg, 24 U of β-fructofuranosidase) in 10, 30 or 50% solvent/water mixtures

Table 1
Product yields in transfructosylation of liquid alcohols catalysed by *Cryptococcus laurentii* cell walls preparations

Alcohol	Alcohol content (%, v/v) (approximate time of maximum concentration (h))		
	30%	50%	90%
Methyl	+++ (3)	++ (6)	n.d.
Ethyl	+++ (3)	+++ (6)	+++ (23)
n-Propyl	++ (3)	++ (7)	+++ (22)
n-Butyl	++ (6)	++ (14)	+++ (25)
n-Pentyl	–	–	+++ (20)
n-Hexyl	–	–	++ (28)
n-Heptyl	–	–	+ (20)
n-Octyl	–	–	n.d.
2-Propyl	+ (2)	tr. (4)	n.d.
Allyl	++ (3)	+ (4)	+ (12)
Propargyl	++ (2)	n.d.	n.d.
Furfuryl	+ (4)	+ (4)	++ (12)

+, (5–10%); ++, (10–20%); +++, (20–30%); n.d., not detected; tr., traces.

were shaken on VIBRAMAX 100 (Heidolf) shaker at 750 rpm and 37 °C. Different water-immiscible or miscible organic solvents were examined, namely toluene, 1,2-dimethoxyethane, methyl isobutyl ketone, acetonitrile, *tert*-butanol, acetone, dimethylformamide and dimethylsulfoxide. Formation of the product was monitored by thin layer chromatography (chloroform/methanol, 3/1) at 1 h intervals. Only reactions in media containing water-miscible aprotic solvents (DMSO, DMF and acetone) were positive for glycoside production (Table 2).

2.6.2.2. Sucrose concentration. Reaction mixtures (2 ml) comprising 0.146, 0.3 or 0.5 M sucrose, 0.4 M vanillyl alcohol and cell wall preparation (80 mg, 24 U of β-fructofuranosidase) in 30% acetone/water mixture were shaken on VIBRAMAX 100 shaker at 750 rpm and 37 °C. Formation of the product was monitored by thin layer chromatography (chloroform/methanol, 3/1) in 1 h intervals (Table 2).

Table 2
Screening of conditions in *Cryptoccocus laurentii* cell walls catalysed transfructosylation of vanillyl alcohol (0.4 M) at 37 °C

Sucrose concentration (M)	Organic co-solvent content (%, v/v)	Approximate time of maximum concentration (h)	Results
0.146	Toluene (30)	–	–
0.146	DME (30)	–	–
0.146	*i*-BuMeCO (30)	–	–
0.146	CH_3CN (30)	1	tr.
0.146	*t*-BuOH (30)	1	tr.
0.146	DMF (30)	4	++
0.146	DMSO (30)	4	+++
0.146	DMSO (10)	2	+++
0.146	Acetone (30)	2	++
0.146	Acetone (60)	4	+
0.146	Acetone (90)	23	tr.
0.3	Acetone (30)	3	++
0.5	Acetone (30)	4	+++

+, (1–2%); ++, (2–3%); +++, (3–5%); tr., traces; DME, 1,2-dimethoxyethane; *i*-BuMeCO, isobutyl methyl ketone.

2.7. Preparative transfructosylation reactions using Cryptoccocus laurentii cell walls

2.7.1. Fructosylations of liquid alcohols (serving both as acceptors and cosolvents)

Fructosylations of liquid alcohols in preparative scale were executed under the same conditions as the screening reactions, using 10-fold greater reaction volumes (20 ml). Reaction mixtures possessing selected water content (Table 3) were stirred at 37 °C for different times (Table 3) and monitored by TLC (chloroform/methanol, 3/1). Reactions were stopped by filtration on Celite to remove biocatalyst, concentrated by evaporation under reduced pressure and purified by column chromatography on silica gel using appropriate eluents.

2.7.2. Fructosylations of solid alcohols

Preparative fructosylations of solid alcohols were accomplished by following procedure: reaction mixtures comprising cell walls (150 U/ml β-fructosidase), 0.5 M sucrose, 30% acetone and vanillyl alcohol, coniferyl alcohol or p-hydroxybenzyl alcohol (0.3, 0.2 or 0.2 M, respectively) were shaken at 37 °C and monitored by TLC (chloroform/methanol, 3/1). After the appropriate time (2, 3.5 and 3.5 h for vanillyl alcohol, coniferyl alcohol or *p*-hydroxybenzyl alcohol, respectively) (Table 3), the reaction mixture was filtered through Celite and evaporated under reduced pressure. The residue was chromatographed on column of silica gel.

*2.7.2.1. Methyl β-D-fructofuranoside (**1**).* The residue after solvent evaporation was chromatographed on column of silica gel with chloroform/methanol (4/1, v/v) as eluent to afford **1** (96.7 mg, 17%) as colorless oil. R_f (chloroform/methanol, 3/1) 0.27, $[\alpha]_D^{20}$ −26.0 (c = 1, MeOH), ^{1}H NMR (CD_3OD): δ 3.33 (s, 3H, CH_3), 3.51–3.78 (m, 5H, H-5, H-6, H-6′, H-1, H-1′), 3.93 (dd, 1H, *J* = 7.6, 7.8 Hz, H-4), 4.11 (d, 1H, *J* = 8.0 Hz, H-3); ^{13}C NMR (CD_3OD): δ 49.5 (CH_3), 61.5, 64.8 (C-1, C-6), 77.2 (C-4), 78.7 (C-3), 83.5 (C-5), 105.3 (C-2).

Table 3
Reactivity of alcohols in *Cryptoccocus laurentii* cell walls catalysed transfructosylations

Alcohols	Organic co-solvent content (%, v/v)	Time (h)	Product	Isolated yields (%)
Methyl	30[a]	4	**1**	17
Ethyl	30[a]	4	**2**	19
n-Propyl	90[a]	14	**3**	28
n-Butyl	90[a]	14	**4**	38
n-Pentyl	90[a]	20	**5**	26
n-Hexyl	90[a]	20	**6**	12
n-Heptyl	90[a]	20	**7**	6
Allyl	30[a]	3	**8**	4
Propargyl	30[a]	3.5	**9**	3
Furfuryl	90[a]	12	**10**	12
4-Hydroxybenzyl	30[b]	3.5	**11**	4
Vanillyl	30[b]	2	**12**	4
Coniferyl	30[b]	3.5	**13**	4

[a] Glycosylated alcohol serves as co-solvent.
[b] Acetone as co-solvent.

*2.7.2.2. Ethyl β-D-fructofuranoside (**2**).* The rest was purified on column of silica gel with chloroform/methanol (4/1, v/v) to afford **2** (117.1 mg, 19%) as colourless oil. R_f (chloroform/methanol, 3/1) 0.39, $[\alpha]_D^{20}$ −30.8 (c = 1, MeOH), ^{1}H NMR (CD_3OD): δ 1.15 (t, 3H, CH_3), 3.50–3.81 (m, 7H, H-5, H-6, H-6′, H-1, H-1′, OCH_2), 3.94 (dd, 1H, J = 7.6, 7.8 Hz, H-4), 4.10 (d, 1H, J = 8.0 Hz, H-3); ^{13}C NMR (CD_3OD): δ 16.1 (CH_3), 57.9, 62.0, 65.0 (C-1, C-6, OCH_2), 77.3 (C-4), 78.4 (C-3), 83.4 (C-5), 105.3 (C-2).

*2.7.2.3. n-Propyl β-D-fructofuranoside (**3**).* The residue was chromatographed on column of silica gel with ethyl acetate as eluent to afford **3** (180 mg, 28%) as colourless oil. R_f (chloroform/methanol, 3/1) 0.40, $[\alpha]_D^{20}$ −22.5 (c = 1, MeOH), ^{1}H NMR (CD_3OD): δ 0.92 (t, 3H, CH_3), 1.56 (sextet, 2H, CH_2), 3.43–3.74 (m, 7H, H-5, H-6, H-6′, H-1, H-1′, OCH_2), 3.95 (dd, 1H, J = 7.7, 7.9 Hz, H-4), 4.11 (d, 1H, J = 8.1 Hz, H-3); ^{13}C NMR (CD_3OD): δ 11.0 (CH_3), 24.5 (CH_2), 62.0, 64.1, 65.0 (C-1, OCH_2, C-6), 77.4 (C-4), 78.5 (C-3), 83.4 (C-5), 105.2 (C-2).

*2.7.2.4. n-Butyl β-D-fructofuranoside (**4**).* The rest after concentration was chromatographed on column of silica gel with ethyl acetate as eluent to afford **4** (265.6 mg, 38%) as colourless oil. **4**: R_f (chloroform/methanol, 3/1) 0.43, $[\alpha]_D^{20}$ −29.0 (c = 1, MeOH), ^{1}H NMR (CD_3OD): δ 0.92 (t, 3H, CH_3), 1.37 (sextet, 2H, CH_2), 1.51 (quintet, 2H, CH_2), 3.47–3.77 (m, 7H, H-5, H-6, H-6′, H-1, H-1′, OCH_2), 3.92 (dd, 1H, J = 7.7, 7.8 Hz, H-4), 4.11 (d, 1H, J = 8.1 Hz, H-3); ^{13}C NMR (CD_3OD): δ 14.4 (CH_3), 20.3 (CH_2), 33.5 (CH_2), 62.0, 64.2, 65.0 (C-1, OCH_2, C-6), 77.3 (C-4), 78.4 (C-3), 83.3 (C-5), 105.2 (C-2).

*2.7.2.5. n-Pentyl β-D-fructofuranoside (**5**).* Ethyl acetate as chromatography eluent was used to give **5** as the product (193 mg, 26%). R_f (chloroform/methanol, 3/1) 0.47, $[\alpha]_D^{20}$ −23.0 (c = 1, MeOH), ^{1}H NMR (CD_3OD): δ 0.91 (t, 3H, CH_3), 1.31-1.35 (m, 4H, CH_2), 1.54 (quintet, 2H, CH_2), 3.46–3.76 (m, 7H, H-5, H-6, H-6′, H-1, H-1′, OCH_2), 3.92 (dd, 1H, J = 7.7, 7.8 Hz, H-4), 4.10 (d, 1H, J = 8.1 Hz, H-3); ^{13}C NMR (CD_3OD): δ 14.5 (CH_3), 23.7 (CH_2), 29.5 (CH_2), 31.1 (CH_2), 62.0, 62.5, 65.1 (C-1, OCH_2, C-6), 77.4 (C-4), 78.5 (C-3), 83.4 (C-5), 105.3 (C-2).

*2.7.2.6. n-Hexyl β-D-fructofuranoside (**6**).* Ethyl acetate as eluent was used to afford **6** as the product (93.5 mg, 12%). R_f (chloroform/methanol, 3/1) 0.48, $[\alpha]_D^{20}$ −16.0 (c = 1, MeOH), ^{1}H NMR (CD_3OD): δ 0.90 (t, 3H, CH_3), 1.32 (m, 6H, CH_2), 1.54 (quintet, 2H, CH_2), 3.44–3.78 (m, 7H, H-5, H-6, H-6′, H-1, H-1′, OCH_2), 3.92 (dd, 1H, J = 7.7, 7.8 Hz, H-4), 4.10 (d, 1H, J = 8.1 Hz, H-3); ^{13}C NMR (CD_3OD): δ 14.5 (CH_3), 23.7 (CH_2), 27.0 (CH_2), 31.3 (CH_2), 32.9 (CH_2), 62.0, 62.5, 65.0 (C-1, OCH_2, C-6), 77.3 (C-4), 78.4 (C-3), 83.4 (C-5), 105.2 (C-2).

*2.7.2.7. n-Heptyl β-D-fructofuranoside (**7**).* The chromatography purification was realised by ethyl acetate as eluent to afford **7** as the product (48 mg, 6%). R_f (chloroform/methanol, 3/1) 0.49, $[\alpha]_D^{20}$ −16.7 (c = 1, MeOH), ^{1}H NMR (CD_3OD): δ 0.90 (t, 3H, CH_3), 1.31 (m, 8H, CH_2), 1.54 (quintet, 2H, CH_2), 3.46–3.74 (m, 7H, H-5, H-6, H-6′, H-1, H-1′, OCH_2), 3.92 (dd, 1H, J = 7.6, 7.9 Hz, H-4), 4.10 (d, 1H, J = 8.1 Hz, H-3); ^{13}C NMR (CD_3OD): δ 14.4 (CH_3), 23.7 (CH_2), 27.2 (CH_2), 30.4 (CH_2), 31.3 (CH_2), 33.1 (CH_2), 62.0, 62.5, 65.0 (C-1, OCH_2, C-6), 77.3 (C-4), 78.4 (C-3), 83.4 (C-5), 105.2 (C-2).

*2.7.2.8. Allyl β-D-fructofuranoside (**8**).* The mixture of chloroform/methanol (4/1, v/v) as eluent was used to afford **8** as the product (23.2 mg, 4 %). R_f (chloroform/methanol, 3/1) 0.37, $[\alpha]_D^{20}$ −15.1 (c = 1, MeOH), ^{1}H NMR (CD_3OD): δ 3.51–3.79 (m, 5H, H-5, H-6, H-6′, H-1, H-1′), 3.96 (dd, 1H, J = 7.6, 7.9 Hz, H-4), 4.12 (d, 1H, J = 8.1 Hz, H-3), 4.15 (ddt, 2H, J = 12.9, 5.4, 1.6, 1.4 Hz, OCH_2), 5.08 (dd, 1H, J = 10.5, 1.6 Hz, $=CH_{cis}$), 5.27 (dd, 1H, J = 17.2, 1.7 Hz, $=CH_{trans}$), 5.92 (ddd, 1H, J = 17.2, 10.5, 5.3 Hz, –CH=); ^{13}C NMR (CD_3OD): δ 61.9, 63.5, 64.8 (C-1, OCH_2, C-6), 77.1 (C-4), 78.4 (C-3), 83.5 (C-5), 105.4 (C-2), 116.0 (=CH_2), 136.8 (CH=).

*2.7.2.9. Propargyl β-D-fructofuranoside (**9**).* The mixture chloroform/methanol (4/1, v/v) as eluent was used to give **9** as the product (18,5 mg, 3%). R_f (chloroform/methanol, 3/1) 0.32, $[\alpha]_D^{20}$ −16.0 (c = 1, MeOH), ^{1}H NMR (CD_3OD): δ 2.76 (t, 1H, J = 2.4 Hz, ≡C–H), 3.53–3.79 (m, 5H, H-5, H-6, H-6′, H-1, H-1′), 3.96 (dd, 1H, J = 7.8, 8.0 Hz, H-4), 4.12 (d, 1H, J = 8.2 Hz, H-3), 4.32 (t, 2H, OCH_2–C≡); ^{13}C NMR (CD_3OD): δ 50.5 (OCH_2–C≡), 61.8, 64.5 (C-1, C-6), 74.8 (≡CH), 76.6 (C-4), 78.2 (C-3), 82.0 (–C≡), 83.7 (C-5), 105.9 (C-2).

*2.7.2.10. 2-Furanylmethyl β-D-fructofuranoside (**10**).* Ethyl acetate as eluent was used to afford **10** as the product (91.2 mg, 12%). R_f (chloroform/methanol, 3/1) 0.39, $[\alpha]_D^{20}$ −19.8 (c = 1, MeOH), ^{1}H NMR (CD_3OD): δ 3.55–3.84 (m, 5H, H-5, H-6, H-6′, H-1, H-1′), 3.97 (dd, 1H, J = 7.5, 8.0 Hz, H-4), 4.14 (d, 1H, J = 8.2 Hz, H-3), 4.64 (dd, 2H, J = 12.2, OCH_2), 6.33–6.35 (m, 2H, H-3″, H-4″), 7.45 (dd, 1H, J = 1.2, 1.3 Hz, H-5″); ^{13}C NMR (CD_3OD): δ 56.9, 61.9, 64.7 (OCH_2, C-1, C-6), 76.9 (C-4), 78.4 (C-3), 83.7 (C-5), 105.6 (C-2), 109.8, 111.3 (C-4′,C-3′), 143.7 (C-5′), 153.6 (C-2′).

*2.7.2.11. 4-Hydroxybenzyl β-D-fructofuranoside (**11**).* Product **11** was purified by chromatography with mixture of chloroform/methanol (9/1 → 4/1, v/v) as eluent to give 49.0 mg (4%). R_f (chloroform/methanol, 3/1) 0.36, white solid, t. t. 116–118 °C, $[\alpha]_D^{20}$ −22.0 (c = 1, MeOH), ^{1}H NMR (CD_3OD): δ 3.56–3.82 (m, 5H, H-5, H-6, H-6′, H-1, H-1′), 3.98 (dd, 1H, J = 7.7, 8.0 Hz, H-4), 4.15 (d, 1H, J = 8.2 Hz, H-3), 4.58 (dd, 2H, J = 10.9, OCH_2), 7.19 (dd, 4H, H-2″, H-3″, H-5″, H-6″); ^{13}C NMR (CD_3OD): δ 64.5, 62.2, 64.8 (OCH_2, C-1, C-6,), 77.1 (C-4), 78.5 (C-3), 83.5 (C-5), 105.6 (C-2), 116.0, 130.5 (C-2′, C-6′ and C-3′, C-5′), 131.2 (C-1′), 157.9 (C-4′).

*2.7.2.12. 4-Hydroxy-3-methoxybenzyl β-D-fructofuranoside (**12**).* Product **12** (73.6 mg, 4%) was obtained by chromatography with chloroform/methanol (9/1 → 4/1, v/v) as eluents. R_f (chloroform/methanol, 3/1) 0.38, white solid, t. t. 117–119 °C, $[\alpha]_D^{20}$ −25.0 (c = 1, MeOH), ^{1}H NMR (CD_3OD): δ 3.55–3.82

(m, 5H, H-5, H-6, H-6′, H-1, H-1′), 3.85 (s, 3H, OCH_3), 4.01 (dd, 1H, $J = 7.8$, 8.0 Hz, H-4), 4.16 (d, 1H, $J = 8.2$ Hz, H-3), 4.59 (dd, 2H, $J = 11.0$, OCH_2), 6.73–7.00 (m, 3H, H-2″, H-5″, H-6″); ^{13}C NMR (CD_3OD): δ 56.4 (OCH_3), 62.2, 2 × 64.6 (C-1, C-6, OCH_2,), 77.0 (C-4), 78.6 (C-3), 83.5 (C-5), 105.5 (C-2), 112.9, 115.9, 121.8 (C-2′, C-5′, C-6′), 131.8 (C-1′), 147.0, 148.9 (C-4′, C-3′).

*2.7.2.13. (E)-3-(4-Hydroxy-3-methoxyphenyl)prop-2-enyl β-*D*-fructofuranoside (**13**).* The reaction mixture after evaporation off the solvent was chromatographed by gradient of chloroform/methanol (9/1 → 4/1, v/v) to afford **13** (49.3 mg, 4%). R_f (chloroform/methanol, 3/1) 0.45, white foam, $[\alpha]_D^{20}$ −13.0 (c = 1, MeOH), ^{1}H NMR (CD_3OD): δ 3.57–3.84 (m, 5H, H-5, H-6, H-6′, H-1, H-1′), 3.85 (s, 3H, OCH_3), 4.01 (dd, 1H, $J = 7.6$, 7.9 Hz, H-4), 4.15 (d, 1H, $J = 8.0$ Hz, H-3), 4.29 (ddd, 2H, $J = 12.3$, 6.0, ∼1 Hz, OCH_2–C=), 6.14 (dt, 1H, =CH_A, $J = 6.0$, 15.9 Hz), 6.69 (d, 1H, =CH_B, $J = 15.9$ Hz), 6.69–7.00 (m, 3H, H-2″, H-5″, H-6″); ^{13}C NMR (CD_3OD): δ 56.4 (OCH_3), 62.0, 63.6, 64.8 (C-6, C-1, OCH_2), 77.2 (C-4), 78.6 (C-3), 83.6 (C-5), 105.6 (C-2), 110.6, 115.9, 121.0 (C-2′, C-5′, C-6′), 125.0 (C-A), 130.7 (C-1′), 132.9 (C-B), 146.9, 149.0 (C-3′, C-4′).

2.8. Test of fructofuranosidase stability in repeated glycosylations

The ability to reuse/recycle the biocatalyst was tested. Repeated glycosylations of *n*-butanol, methanol and vanillyl alcohol were accomplished under the same reaction conditions as the aforementioned screening procedures, at scaled-up reaction volumes of 500, 100 and 100 ml, respectively. Reaction mixtures were stirred at 37 °C for different times (Table 4) and monitored by TLC. Each reaction cycle was stopped by centrifugation at 3000 rpm for 15 min to remove biocatalyst. The sedimented cell wall pellets were used in additional repeat cycles by resuspending under the same experimental conditions. Supernatants, which contained the reaction products, were concentrated under reduced pressure and products purified by column chromatography. To conserve resources, unreacted butanol was recovered by vacuum distillation and reused. Neither methanol nor acetone were reused in repeated experiments (Table 4).

Table 4
Transfructosylation ability of walls in repeated experiments

Alcohols	Cycle number	Cycle duration (h)	Product	Isolated yields (%)
Methyl	1	4	**1**	17
Methyl	10	4	**1**	14
n-Butyl	1	14	**4**	36
n-Butyl	20	14	**4**	24
Vanillyl	1	3	**11**	4
Vanillyl	2	3	**11**	4
Vanillyl	5	3	**11**	3

3. Results and discussion

3.1. Reactivity of cell walls toward acceptors

3.1.1. Liquid alcohols

To initially study the fructofuranosyl-transfer potential of cell walls of *C. laurentii* with sucrose as fructofuranosyl donor, different alcohols were used as acceptors. Since the alcohols differ in their structure, reactivity and cost [18], reaction conditions were examined to maximise the yield of particular reactions and results were evaluated by TLC (Tables 1 and 2). High acceptor concentration is practical only if the acceptor is cheap or recoverable from the reaction mixture. Short and medium chain-length aliphatic alcohols (either miscible or immiscible with water) were used both as an acceptor and a solvent. Due to enzyme inactivation and decreased solubility of saccharides in anhydrous organic solvents, the influence of added amount of water was also studied (Table 1). High methanol concentrations inactivated the enzyme [10]. Optimal enzyme activity occured at 30% methanol. When ethanol and propanol were used, the cell wall preparation was more stable and reactive in higher alcohol concentrations. The rate of conversion decreased in low-water media, but hydrolysis was suppressed and kinetic control was easier. In fructosylations of higher alcohols, which were not completely miscible with water, e.g., *n*-butanol and higher, more complex two-phase reaction rules prevailed. The reaction efficiency in terms of equilibrium and yield depended on the extraction of the product into the organic (i.e. acceptor) phase and the sugar derivatives possess surfactant properties. Reactions with less than 90% concentration of the less polar alcohols were not carried out, since a greater proportion of alcohol phase is necessary [19]. No product was observed in fructosylation of *n*-octanol. Conditions similar to those used in reactions with short aliphatic alcohols were adopted in fructosylations of other acceptors like 2-propyl, allyl, propargyl and furfuryl alcohol. Low reactivity with the more sterically hindered 2-propanol indicate that the fructofuranosidase is selective for primary hydroxyl groups. However, our attempts to prepare glycosides suitable for chemical polymerisation [20,21], i.e., allyl and propargyl fructofuranosides, gave satisfactory results, even though the product formation was lower (Table 1).

Furfuryl alcohol, a dienic compound, represents an important building block in chemical syntheses. Thus, it was chosen as a representative functional alcohol. Since furans are labile under acidic conditions, mild enzymatic catalysis may be useful for their derivatisation. Recently, Martin et al. reported a very successful glycosylation of furfuryl alcohol by β-glucuronidase from bovine liver [22]. Compared to fructosylation of allyl and propargyl alcohols, furfuryl alcohol was more reactive at higher concentrations, suggesting lower inactivation of cell wall fructofuranosidase (Table 1).

3.1.2. Solid alcohols

There have been relatively few reports of enzymatic preparation of 4-hydroxybenzyl or 4-hydroxyphenylpropenyl glycosides. Almond β-D-glucosidase appears to be the only

effective enzyme for the synthesis of hydroxyphenylalkyl β-D-glucopyranosides [23–25].

In our attempt to glycosylate phenolic alcohols, addition of organic co-solvents was studied to overcome their insolubility and product hydrolysis. The effect of co-solvents on the transfructosylation was examined in experiments using 0.146 M sucrose (concentration relative to entire reaction volume) and 0.4 M vanillyl alcohol in mixtures of water with organic solvents (7:3, v/v). Toluene (water-immiscible), 1,2-dimethoxyethane (partially miscible) and highly miscible organic solvents like isobutyl methyl ketone, acetone, acetonitrile, *tert*-butyl alcohol, dimethylformamide and dimethylsulphoxide were tested for this purpose (Table 2). The reaction did not proceed in toluene, 1,2-dimethoxyethane and isobutyl methyl ketone. We found that water-miscible aprotic solvents (DMSO, DMF and acetone) in aqueous medium were more effective for our reaction than the traditional *tert*-butanol–water (buffer) or CH_3CN–water (buffer) systems. Acetone was chosen as the co-solvent for further testing in order to avoid laborious removal of DMF or DMSO from the reaction mixture. Increasing the proportion of acetone to concentrations greater than 30% (i.e. 60% and 90%) suppressed product formation. Increasing the concentration of sucrose positively influenced the rate and extent of the reaction (Table 2). Conditions giving best results were subsequently used for glycosylations of 4-hydroxybenzyl and coniferyl alcohol.

3.2. Preparative transfructosylation of liquid alcohols

The best screening results were repeated in preparative scale and the products **1**–**10** (Table 3) were isolated and characterised. Despite moderate chemical yields, all products were obtained selectively as β-anomers in the furanoside form. In order to favour the formation of β-fructofuranosides in higher yields and to suppress the competitive hydrolysis, liquid alcohols were preferentially used both as substrates and as cosolvents with McIlvaine buffer (pH 4.8). Reactions with primary aliphatic alcohols gave best results for *n*-butanol (38 % yield). 'Minimum water' procedures comparable to that used for higher aliphatic alcohols have been adopted in the fructosylation of succesfully screened functionalised alcohols as furfuryl alcohol to afford **10** in satisfactory yield. Similarly to methanol, fructosylations of allyl and propargyl alcohols were most effective at their concentration as low as 30%.

3.3. Preparative transfructosylations of solid alcohols

Generally, the arylalkyl glycosides are more sensitive to acidic conditions and higher temperature. For preparative purposes we therefore preferred to use water instead of buffer, with acetone serving as an easily removable solvent. The acetone concentration affected both reaction rate and equilibrium, the highest yield being at 3:7 (v/v) ratio of acetone to water. When 4-hydroxybenzyl, vanillyl and coniferyl alcohols were used as acceptors, only the primary hydroxyl was glycosylated, while the phenolic hydroxyl remained unchanged. Under the optimal conditions (using 0.5 M sucrose as fructosyl donor), three phenolic β-D-fructofuranosides **11**–**13** were successfully synthesized in moderate yields (Table 3).

3.4. Stability of cell walls in repeated fructosylations

Glycosidases often show a lack of activity in organic media. Cell walls of acapsular *Cryptoccocus laurentii* with β-fructofuranosidase activity showed satisfactory stability in high concentrations of organic solvents (Table 3). We tested the stability of this enzyme system in repeated glycosylations of methanol, *n*-butanol and vanillyl alcohol. Table 4 shows that the recovered enzyme worked excellently in several repeated cycles without significant loss of activity. The ability to reuse catalyst, moderate yields, and cost reduction by regenerating the acceptors alcohols, indicate great promise for the multigram biocatalytic preparation of β-fructofuranosides.

4. Conclusion

A variety of β-D-fructofuranosides **1**–**13** from sucrose and different alcohols (alkyl, functionalised and phenolic) was prepared through transfructosylation by cell walls from acapsular strain of *Cryptoccocus laurentii* as a new biocatalyst. Different reaction conditions have been examined to increase chemical yields of produced β-fructofuranosides. A facile protocol was developed for enzymatic fructofuranosylation of 4-hydroxylated arylalkyl alcohols in a monophasic aqueous-acetone mixture.

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