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Investigating the Potential of Phenolic Compounds and Carbohydrates as Acceptor Substrates for Levansucrase**-Catalyzed Transfructosylation Reaction

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This study characterizes the acceptor specificity of levansucrases (LSs) from *Gluconobacter oxydans* (LS1), *Vibrio natriegens* (LS2), *Novosphingobium aromaticivorans* (LS3), and *Paraburkholderia graminis* (LS4) using sucrose as fructosyl donor and selected phenolic compounds and carbohydrates as acceptors. Overall, *V. natriegens* LS2 proved to be the best biocatalyst for the transfructosylation of phenolic compounds. More than one fructosyl unit could be attached to fructosylated phenolic compounds. The transfructosylation of epicatechin by *P. graminis* LS4 resulted in the most diversified products, with up to five fructosyl units transferred. In addition to the LS source, the acceptor specificity of LS towards phenolic compounds and their transfructosylation products were found to greatly depend on their chemical structure: the number of phenolic rings, the reactivity of hydroxyl groups and the presence of aliphatic chains or methoxy groups. Similarly, for carbohydrates, the transfructosylation yield was dependent on both the LS source and the acceptor type. The highest yield of fructosylated-trisaccharides was Erlose from the transfructosylation of maltose catalyzed by LS2, with production reaching 200 g/L. LS2 was more selective towards the transfructosylation of phenolic compounds and carbohydrates, while reactions catalyzed by LS1, LS3 and LS4 also produced fructooligosaccharides. This study shows the high potential for the application of LSs in the glycosylation of phenolic compounds and carbohydrates.

Introduction

Due to the promising physiological effects of levan and levan-type fructooligosaccharides (FOSs), levansucrase (LS, EC 2.4.1.10) has garnered much interest in the pharmaceutical, cosmetics and food industries. LS is a fructosyl-transferase that can catalyze the synthesis of complex oligosaccharides, by acquiring a fructosyl residue from a donor molecule and performing a non-leloir transfer to an acceptor molecule.[1] The catalytic mechanism of selected LSs and their donor/acceptor specificities toward various carbohydrates have been documented.[2] However, given that the microbial source of enzymes heavily impacts their catalytic properties, constant research is necessary for newly discovered LSs.

The differences in the reaction selectivity of LSs are attributed to their amino acid sequences, which dictate their active subsite structure.[1b,3] The cavities of LS from *G. oxydans* (LS1), *V. natriegens* (LS2) and *P. graminis* (LS4) were compared to that of the deep negatively charged pocket of *Bacillus subtilis* LS. The cavity of the LS1 had a similar shape but with less depth, and with many charged residues concentrated together on the exterior of the active site. The active site of LS2 was wider and shallower and had more positive and less negative electrostatic potential. LS4's active site was deeper and had more positive electrostatic potential. Comparing the least binding energy conformation of sucrose with the LSs, sucrose was rotated 45° downwards with LS1 but was orientated in the same way as the *B. subtilis* LS with LS2 and LS4.[1b] In addition, while previous studies have reported that the fructose residue of the donor sucrose molecule binds the −1 subsite with high affinity, it was suggested that the docking of acceptor molecules could be variable because of the +1 subsite's relaxed binding nature. The +1 subsite can participate in both donor and acceptor binding.[4]

In addition to the carbohydrate acceptors, aromatic compounds and aliphatic alcohols can be utilised as acceptor molecules. Butanol, hydroquinone and benzyl alcohol were successfully used as fructosyl acceptors by LS from *B. subtilis*.[5] The fructosylation of hydroquinone was also investigated with LS from *Leuconostoc mesenteroides*, synthesizing a hydroquinone fructoside, 4-hydroxyphenyl-β-D-fructofuranoside.[6] Then, isopropanol and 1- pentanol were as well able to act as fructosyl acceptors using *Bacillus licheniformis* 8-37-0-1, produc-

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ing isopropyl and pentyl fructosides respectively.[7] In-depth studies are needed to investigate and modulate the ability of phenolic compounds to act as acceptor substrates for LS-catalyzed transfructosylation reactions. The functional benefits of phenolic compounds have been intensely studied over the years and are prized for their antitumor, antioxidant, antibacterial, and anti-inflammatory properties, while also showing great potential in preventing and treating cardiovascular or cerebrovascular diseases.[8] Yet, despite the validated importance of phenolic compounds, their poor solubility in water and easy degradation by light irradiation in aqueous solutions have limited their applications.[9] One approach suggested to cater for these drawbacks is glycosylation.[8b] The positive influence of glycosylation of phenolic compounds on drug properties, including pharmacokinetics, pharmacodynamics, solubility, mechanism and potency have been confirmed.[10] Enzymatic glycosylation has further shown to be an interesting alternative to chemical methods that are often labour-intensive procedures requiring multistep synthetic routes that produce low overall yields. Furthermore, the use of toxic catalysts and solvents, and the production of significant waste could be avoided using an enzymatic approach.[11] The enzymatic glycosylation of phenolic compounds is indeed still of great interest. For instance, a self-sufficient biocatalyst was developed by co-immobilization of a glycosyltransferase, a sucrose synthase and the cofactor UDP and it successfully glycosylated piceid, phloretin and quercetin, with in situ regeneration of the UDP-glucose.[12] Another study enzymatically glucosylated citrus flavonoids, via a cyclodextrin glucosyltransferase, to enhance their bioactivity and taste as new food additives.[13] Various classes of carbohydrate-active enzymes can catalyze glycosylation reactions: 'Leloir' glycosyl transferase, transglycosidase, glycoside phosphorylase and glycoside hydrolase (GH).[11a] Nonetheless, few studies have investigated the potential of LSs to fructosylate phenolic compounds.

The objective of the present study was to characterize the acceptor specificity of LSs from *Gluconobacter oxydans* (strain 621H) (LS1), *Vibrio natriegens* NBRC 15636 (LS2), *Novosphingobium aromaticivorans* (LS3), and *Paraburkholderia graminis* C4D1 M (LS4), towards various phenolic compounds and carbohydrates using sucrose as a fructosyl donor. LS from these strains were selected following a genome mining carried out in our previous study.[1a] Both the bioconversion of substrates and end-product profiles were investigated. Furthermore, the reaction selectivity of selected LSs towards transfructosylation and hydrolysis were assessed.

Results and Discussion

The Transfructosylation of Phenolic Acceptors Catalysed by Selected Levansucrases

Time Courses for the Bioconversion of Phenolic Acceptor Substrates

Figure 1 depicts the time courses for the bioconversion of phenolic compounds through LS-catalyzed transfructosylation reactions. *G. oxydans* LS1, *V. natriegens* LS2, *N. aromaticivorans* LS3, and *P. graminis* LS4 exhibited different acceptor specificities towards the investigated phenolic compounds. Supplementary Figure 1 shows the HPLC profiles of some phenolic compounds and corresponding fructosylated products. Besides vanillic acid, all tested phenolic compounds, including catechol, catechin, epicatechin, coniferyl alcohol, gallic acid, caffeic acid, chlorogenic acid and rosmarinic acid, were successfully fructosylated by at least one of the selected LSs. The stability of the phenolic compounds in the reaction mixtures was proven, as shown in Supplementary Table 1.

Except for *G. oxydans* LS1, all three other LSs were able to catalyse the transfructosylation of catechol, leading to a maximum bioconversion yield of 58% with *V. natriegens* LS2, 36% with *N. aromaticivorans* LS3 and 18% with *P. graminis* LS4. The results also show that the time courses for the transfructosylation of catechol were similar for all LSs with a low increasing rate after 1 h, before reaching a steady state at 24 h. The glycosylation of catechol was previously achieved by other enzymes including glucansucrase from *Lactobacillus reuteri*,[14] β-galactosidase from site-mutated *Lactobacillus bulgaricus* L3,[15] glucansucrase Gtf180-ΔN of *L. reuteri* 180[16] and β-xylosidase BxTW1 from *Talaromyces amestolkiae*.[17] Catechin was converted to a maximum yield of up to 85% with *V. natriegens* LS2, 50% with *N. aromaticivorans* LS3 and 25% with *P. graminis* LS4. No reaction equilibrium was observed in the 48 h time courses of LS2 and LS3-catalysed transfructosylation of catechin. However, a slight decrease in the bioconversion yield of catechin from 25% at 8 h to 22% at 48 h occurred in LS4-catalysed transfructosylation of catechin. The glycosylation of catechin was reported by other enzymes like *Leuconostoc mesenteroides* sucrose phosphorylase (81%),[18] *Aspergillus niger* cellulase (26%) and *Bacillus stearothermophilus* α-glucosidase (20%),[19] amylosucrase from *Deinococcus geothermalis* DSM 11300[20] (97%) and β-glucosidase from *Novosphingobium* sp. GX9 (100%).[21]

With Epicatechin, *V. natriegens* LS2, followed by *N. aromaticivorans* LS3, showed to be the best biocatalyst, reaching a steady rate at 24 h and a maximum bioconversion yield of 93% at 48 h. No equilibrium was reached with LS3 which achieved a final bioconversion yield of 68%. Low bioconversion yields were recorded with *P. graminis* LS4, with a maximum yield of 9%. Previously, a bioconversion of around 69% of epicatechin was reported with *L. mesenteroides* sucrose phosphorylase.[18] *V. natriegens* LS2 exhibited the highest catalytic transfructosylation efficiency on catechol, catechin and epicatechin, followed by *N. aromaticivorans* LS3 and *P. graminis* LS4. The higher acceptor

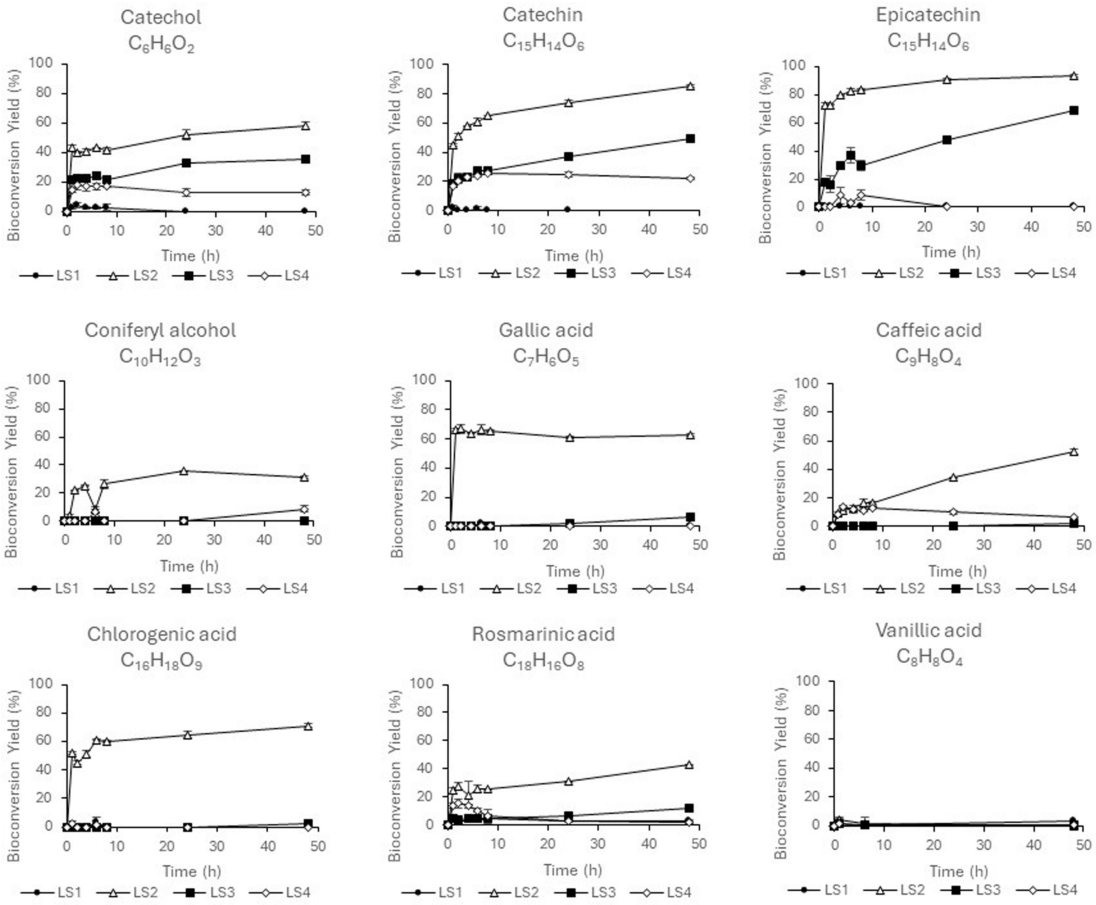

Figure 1. Time course for the bioconversion of phenolic compounds through levansucrase-catalysed transfructosylation (*G. oxydans* LS1, *V. natriegens* LS2, *N. aromaticivorans* LS3, and *P. graminis* LS4).

specificity of LS2 and LS3 towards catechin and epicatechin than catechol may be attributed to the presence of multiple hydroxyl groups on the dihydropyran heterocycle, catechol and resorcinol moieties of catechin and epicatechin providing many potential sites for the transfructosylation. Contrary to LS2 and LS3, *P. graminis* LS4 showed more or less similar acceptor specificity towards catechin and catechol, but significantly lower bioconversion yield with epicatechin. The difference in the configuration of catechin and epicatechin might partly explain their difference in acceptor specificity. Indeed, catechin has a trans configuration, unlike epicatechin, making the hydroxyl group on its dihydropyran heterocycle moiety potentially more accessible for transfructosylation. When coniferyl alcohol was used as an acceptor, *V. natriegens* LS2 and *P. graminis* LS4 were able to transfer fructosyl groups from sucrose at a peak conversion of 35% and 9%, respectively. LS2 recorded a drop in conversion at 6 h but had an overall increasing trend that stabilized after 24 h. LS4 had negligible bioconversion yield except at 6 h and 48 h. The fluctuation in the bioconversion yield can be attributed to the reversible nature of the transfructosylation reaction. A shift toward the forward reaction can cause the transfer of fructosyl units to the phenolic compound, while a shift toward the reverse reaction can lead to the loss of fructosyl units from the fructosylated phenolic compound. Fructosides of coniferyl alcohol were previously reported by reactions catalyzed by β-fructofuranosidase from cell walls of *Cryptoccocus laurentii*.[22] Then, gallic acid fructosides were produced at a significant level with *V. natriegens* LS2, with a bioconversion yield of up to 66%. Only few studies have investigated the enzymatic production of gallic acid fructosides. For instance, the glycosylation of gallic acid by *Leuconostoc* dextransucrase at a bioconversion yield of 35.7% was reported.[23]

Caffeic acid was also successfully transfructosylated by *V. natriegens* LS2 at a bioconversion yield reaching 52%. No reaction equilibrium was observed for the transfructosylation of caffeic acid by LS2; while it was achieved after 1 h in the presence of gallic acid acceptor after a high transfructosylation rate. These results reveal the high acceptor substrate affinity of LS2 towards gallic acid compared to caffeic acid. It can be hypothesized that the aliphatic chain of the caffeic acid may have hindered its binding on the active site of LS2. *P. graminis* LS4 slightly converted caffeic acid at a maximum yield of 14%. *N. aromaticivorans* LS3 exhibited low activity on gallic acid and caffeic acid acceptors with a maximum bioconversion yield of 6% and 2%, respectively. Caffeic acid glucosides have been produced by other enzymes such as glucansucrases from *Leuconostoc* and *Weissella* species,[24] glycosyltransferases from

Arnebia euchroma[25] and glycosyltransferase from *Paenibacillus polymyxa* NJPI29.[26]

As for chlorogenic acid, only *V. natriegens* LS2 was significantly favourable in fructosylating the acceptor substrate at a maximum bioconversion yield of 71% with no reaction equilibrium achieved. The esterification of caffeic acid with (−) quinic acid to yield chlorogenic acid enhanced the transfructosylation rate and yield catalysed by LS2; this result can be attributed to the number and location of the hydroxyl groups of the chlorogenic acid. The enzymatic synthesis of chlorogenic acid glucoside was performed using dextransucrase from *L. mesenteroides* with a production yield of 44.0%.[27]

With rosmarinic acid, the highest transfructosylation activity was obtained with *V. natriegens LS2* at a maximum bioconversion yield of 40%. At the initial stage of the transfructosylation reaction catalysed by LS2, the rosmarinic acid acceptor, caffeic acid ester with tyrosine, led to a higher bioconversion rate than caffeic acid; however, at the later stage, the reaction progressed at a lower rate with rosmarinic acid acceptor. On the other hand, *N. aromaticivorans* LS3 and *P. graminis* LS4 recorded a maximum bioconversion yield of 12% and 16%, respectively, for the transfructosylation of rosmarinic acid. *P. graminis* LS4 exhibited more or less the same acceptor specificity towards rosmarinic acid and caffeic acid with reversible reaction trend at the later stage. Contrary to LS2 and LS4, insignificant transfructosylation of caffeic acid was recorded with LS3, revealing its esterification with tyrosine to yield rosmarinic acid may have favored its binding to LS3 active site. Only few studies on the glycosylation of rosmarinic acid have been reported. One example involves the use of a glycosyltransferase from *A. euchroma*.[25] Vanillic acid was the only phenolic compound tested that was transfructosylated to an insignificant level by all four LSs (below 4%). It is interesting to note that vanillic acid has only one hydroxyl group, while other selected phenolic compounds have more than one hydroxyl groups available for transfructosylation. In addition, it can also be hypothesized that the methoxy group of vanillic acid at position 3 might have contributed to steric hindrance, preventing the transfer of a fructosyl group to the adjacent hydroxyl group.

As far as the authors are aware, out of the nine investigated phenolic compounds, only catechin, rosmarinic acid, gallic acid, caffeic acid and coniferyl alcohol were previously tested as potential acceptor substrates of LS-catalyzed reactions.[28] Around 11%, 15%, 0%, 9% and 25% respectively of acceptor substrate conversion were reported when 0.1 U/mL LS from *Gluconacetobacter diazotrophicus* was used to fructosylate 25 mM phenolic compounds[28] Overall, LS from *V. natriegens* LS2 proved to be the most promising enzyme for the transfructosylation of phenolic compounds, successfully fructosylating all tested phenolic compounds besides vanillic acid. and achieving a maximum bioconversion yield of above 50% with catechol, catechin, epicatechin, gallic acid, caffeic acid and chlorogenic acid. *N. aromaticivorans* LS3 exhibited moderate transfructosylation activity towards catechol, catechin and epicatechin, while *P. graminis* LS4 fairly bioconverted catechol and catechin.

Then, LS from *G. oxydans* LS1, in general, did not favour the transfructosylation of phenolic compounds. In our previous study, substrate docking with homology models were performed for *G. oxydans* LS1, *V. natriegens* LS2 and *P. graminis* LS4 to examine their active site, compared to that of *B. subtilis* LS.[1b] The active site of LS1 had a lack of charged residues in its interior pocket compared to *B. subtilis* LS. This lack of charged residues, not seen with LS2 and LS4, might explain its lower affinity to phenolic compounds[1b].

Reaction Selectivity of Selected Levansucrases in the Presence of Phenolic Acceptors

The most promising bioconversion reactions of phenolic acids catalyzed by LSs (LS2- all acceptors besides vanillic acid; LS1- epicatechin; LS3- catechol and catechin; LS4- epicatechin) were investigated in terms of their transfructosylation and hydrolysis extent, as shown in Figure 2. The hydrolysis extent of sucrose is regarded as the transfer of the fructosyl group to water resulting in the release of fructose and glucose, while the transfructosylation extent refers to the transfer of the fructosyl group to phenolic compound acceptor and/or to the transfructosylation products (e.g. fructooligosaccharides, levan, phenolic fructosides).[1b,29] With catechol and catechin, higher transfructosylation extents of 6% and 17%, respectively, were recorded with *V. natriegens* LS2 than with *N. aromaticivorans* LS3 (2% and 5%), in line with the higher bioconversion yield of these phenolic compounds obtained with LS2 (Figure 1). Reactions with LS2 and LS3 generally recorded low bioconversion of sucrose used in excess at 0.9 M. The results also reveal that the presence of phenolic compounds favored the acceptor transfructosylation reaction catalysed by LS2 and LS3 than oligo/polymerisation reactions. The highest level of hydrolysis extent with LS2 was achieved with catechin and gallic acid as acceptors at 29%, while with LS3 a maximum of 31% hydrolysis extent was reached with acceptor catechin. Indeed, in our previous study,[1b] up to 60% of bioconversion of sucrose was reported with reactions carried out in the presence of 0.8 M sucrose and 5–7 U/mL *V. natriegens* LS2. Similarly, the final sucrose consumption by *N. aromaticivoran* LS3 was higher at a rate of 53%. Contrary to LS2 and LS3, *G. oxydans* LS1 and *P. graminis* LS4 did not show drastic differences in sucrose bioconversion in the presence of epicatechin acceptor, with previously reported yields of 78% and 81% respectively without acceptor.[1b] To the authors' knowledge, this is the first study that suggests the potential of phenolic compounds as inhibitors in some LS-catalyzed reactions, preventing the formation of other LS-catalyzed end-products.

LS can indeed catalyze the formation of levan and various FOSs when sucrose is used as the sole substrate. During polymerization, the growing fructan chain acts as the acceptor, synthesizing β-(2,6) linked oligofructans to form or elongate levan. If this processive reaction mechanism is not adopted, FOSs are instead formed via a non-processive/distributive reaction given the enzyme's lack of affinity for the synthesized product.[4b,30]

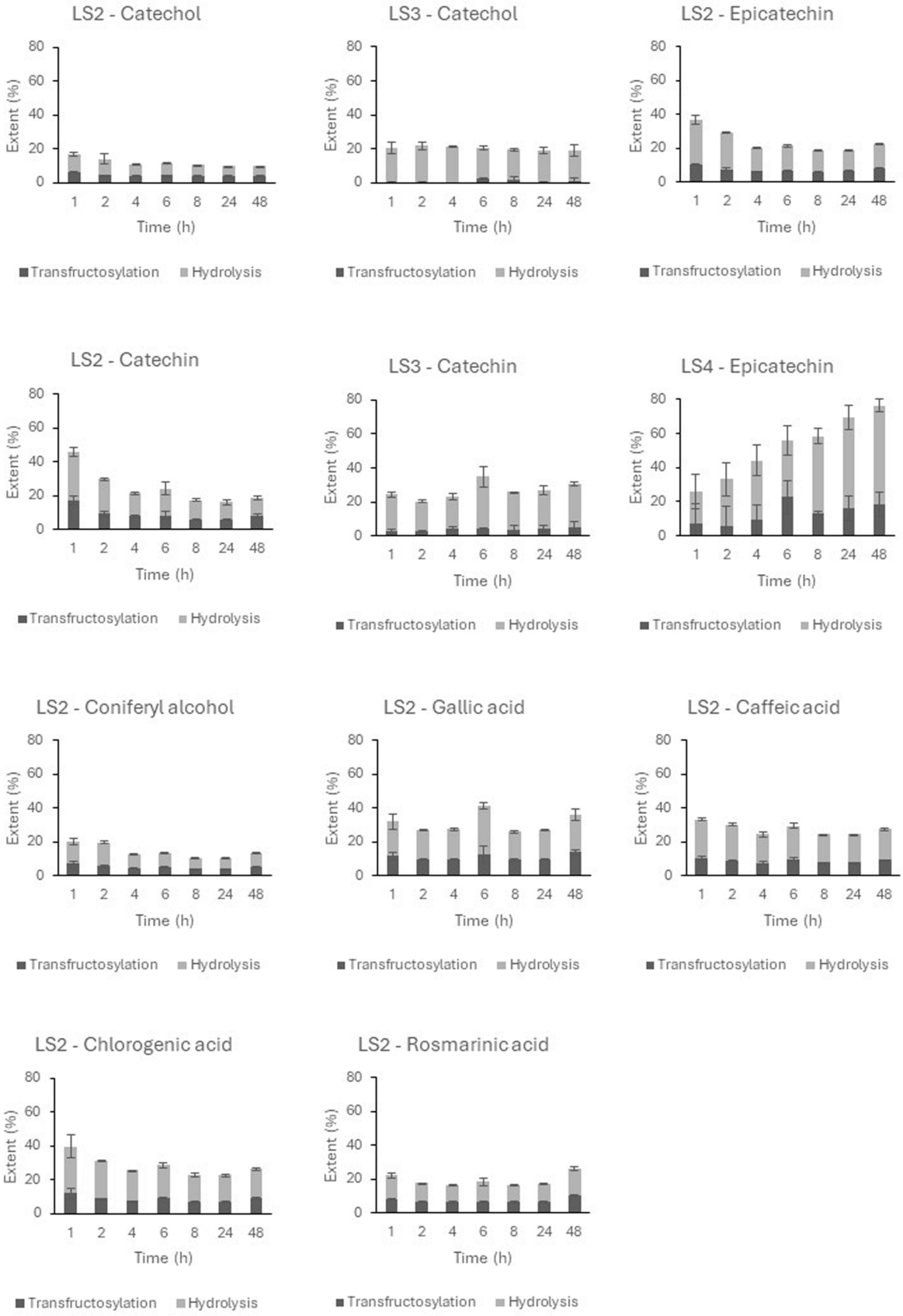

Figure 2. Time course for the reaction selectivity (transfructosylation/hydrolysis) of Levansucrase (LS)-catalyzed reactions (*G. oxydans* LS1, *V. natriegens* LS2, *N. aromaticivorans* LS3, and *P. graminis* LS4) in the presence of phenolic compound substrates.

LC–MS analysis was performed to analyze the FOSs formed. Supplementary Figure 4 illustrates the MS-MS fragmentation spectra of some FOSs produced. In general, reactions with *G. oxydans* LS1 led to the highest production of FOSs such as 1-kestose, 6-kestose, 6-nystose and 6-kestose-F–F. A notable level of 6-kestose was obtained with catechin at 30.6 g/L, and reactions with coniferyl alcohol resulted in the highest concentration of 6-nystose and 6-kestose-F–F at concentrations of 52.1 g/L and 32.9 g/L, respectively. With *V. natriegens* LS2, only 1-kestose was detected at low concentrations. As for reactions with *N. aromaticivorans* LS3, the presence of trace amounts of 6G-kestose, 1-kestose and nystose were obtained. Finally, with *P. graminis* LS4, 1-kestose, nystose and fructosyl nystose were formed, with vanillic acid producing 1-kestose at an important level of 19.7 g/L. In our previous study,[1b] the time courses for LS-catalyzed transfructosylation reaction with 0.8 M sucrose and 5–7 U/mL LS were carried out, and the resulting FOSs were characterized. Comparing the above results with our previous ones,[1b] it is evident that fewer FOSs are formed when phenolic compounds are successfully transfructosylated by LSs. For instance, with LS2 around 50 g/L of 1-kestose was produced after 50 h of reaction with sucrose as sole substrate, while in the presence of phenolic compounds, insignificant levels of 1-kestose were detected. Moreover, LS1 which showed the lowest affinity to phenolic compounds produced the highest levels of FOSs. This further suggests that phenolic compounds could potentially inhibit the production of FOSs by some LSs.

End-product Profiles of Phenolic Fructosides

The most promising reaction mixtures were also analyzed via LC–MS for the characterization of their produced phenolic fructosides. The end-product profiles characterization confirmed the successful transfructosylation of phenolic compounds catechol, catechin, epicatechin, gallic acid, chlorogenic acid, caffeic acid and coniferyl alcohol. Figure 3 displays the relative abundance of phenolic fructosides produced over time. The MS-MS fragmentation spectra of some fructosylated phenolic compounds obtained are shown in Figure 4 and Supplementary Figure 2 and their corresponding hypothesized structures are depicted in Supplementary Figure 3. The results demonstrate that the phenolic compounds could acquire more than one fructosyl group, with up to five fructosyl groups transferred to coniferyl alcohol and epicatechin using *V. natriegens* LS2 and *P. graminis* LS4, respectively. Referring to our previous study where the substrate docking with homology models were performed for *G. oxydans* LS1, *V. natriegens* LS2 and *P. graminis* LS4 to examine their active site, compared to that of *B. subtilis* LS, the growing phenolic fructosides can be explained from the LSs subsites structure.[1b] Indeed, for LS2, it was found that the sucrose orientation within its active site could direct the transfructosylation products toward a concentration of charged residues running along the left side of its active site. It can hence be hypothesized that the concentrated residues on the exterior of the active site can interact with the growing fructosylated phenolic compounds, resulting in a processive reaction.[1b] Núñez-López et al. (2019) also obtained mono-, di-, and tri-fructosides of puerarin, coniferyl alcohol and rosmarinic acid with reactions catalyzed by LS from *G. diazotrophicus*. With mangiferin, only mono-fructosides were formed. In another study, LS from *G. diazotrophicus* synthesized phlorizin mono- di- and tri-fructosides.[31] Our study further confirms the hypothesis that the acceptor specificity of LS towards phenolic compounds and their transfructosylation products depends greatly on the number of phenolic rings, the presence of sugar substituents and the reactivity of the hydroxyl groups.[28]

The number of fructosyl groups acquired not only depends on the phenolic substrates but also on the source of the enzyme. For instance, with epicatechin, LS2 could acquire three fructosyl groups, LS3 four fructosyl groups and LS4 up to five fructosyl groups. On the other hand, a maximum of three and four fructosyl groups were transferred to catechol with LS2 and LS3, respectively. Then with catechin, mono- and di-fructosides were formed with LS2 as compared to mono-, di-, tri- and tetra-fructosides with LS3. Previous studies indeed demonstrated how the source of the enzyme is a key determinant in fructosides formation with LS from *B. subtilis, L. mesenteroides* and *Zymomonas mobilis* producing mainly mono-fructosides while *G. diazotrophicus* resulting in mono-, di-, tri-, tetra-, and penta-fructosides with puerarin as acceptor.[32] The relative abundance of each fructosylated phenolic compound as well varies with reaction time. This suggests that the fructosides formed can act as both fructosyl donors and acceptors over time. The reaction time course of the transfructosylation of phlorizin by LS from *G. diazotrophicus* also showed varying fructosides production over time.[31]

Transfructosylation of Sugar Acceptors Catalysed by Selected Levansucrases

Reactions Selectivity of Levansucrases in the Presence of Sugar Acceptors

The time courses for the bioconversion reaction of sucrose in the presence of sugar acceptor substrates, including maltose, cellobiose and lactose, and sorbitol were investigated (See Supplementary Table 2). Total sucrose bioconversion was achieved after 48 h reaction in the presence of all acceptor substrates when *G. oxydans* LS1, *N. aromaticivorans* LS3 and *P. graminis* LS4 were used as biocatalysts. With *V. natriegens* LS2, a maximum bioconversion yield of 60% was reached after 24 h. Previously, up to 78%, 60%, 53% and 81% of sucrose bioconversion were reported at 50 h with reactions carried out in the presence of 0.8 M sucrose and 5–7 U/mL LS from *G. oxydans* LS1, *V. natriegens* LS2, *N. aromaticivoran* LS3 and *P. graminis* LS4.[1b] As for the bioconversion of acceptor substrates, LS1 successfully transfructosylated cellobiose and lactose; LS2 and LS4 catalysed the transfructosylation of maltose, cellobiose and lactose, and LS3 transferred a fructosyl group from sucrose to maltose and lactose. Sorbitol could not be transfructosylated by any of the selected LSs, revealing its low binding affinity to LS subsites. In general, the use of LS2 as a biocatalyst led to the

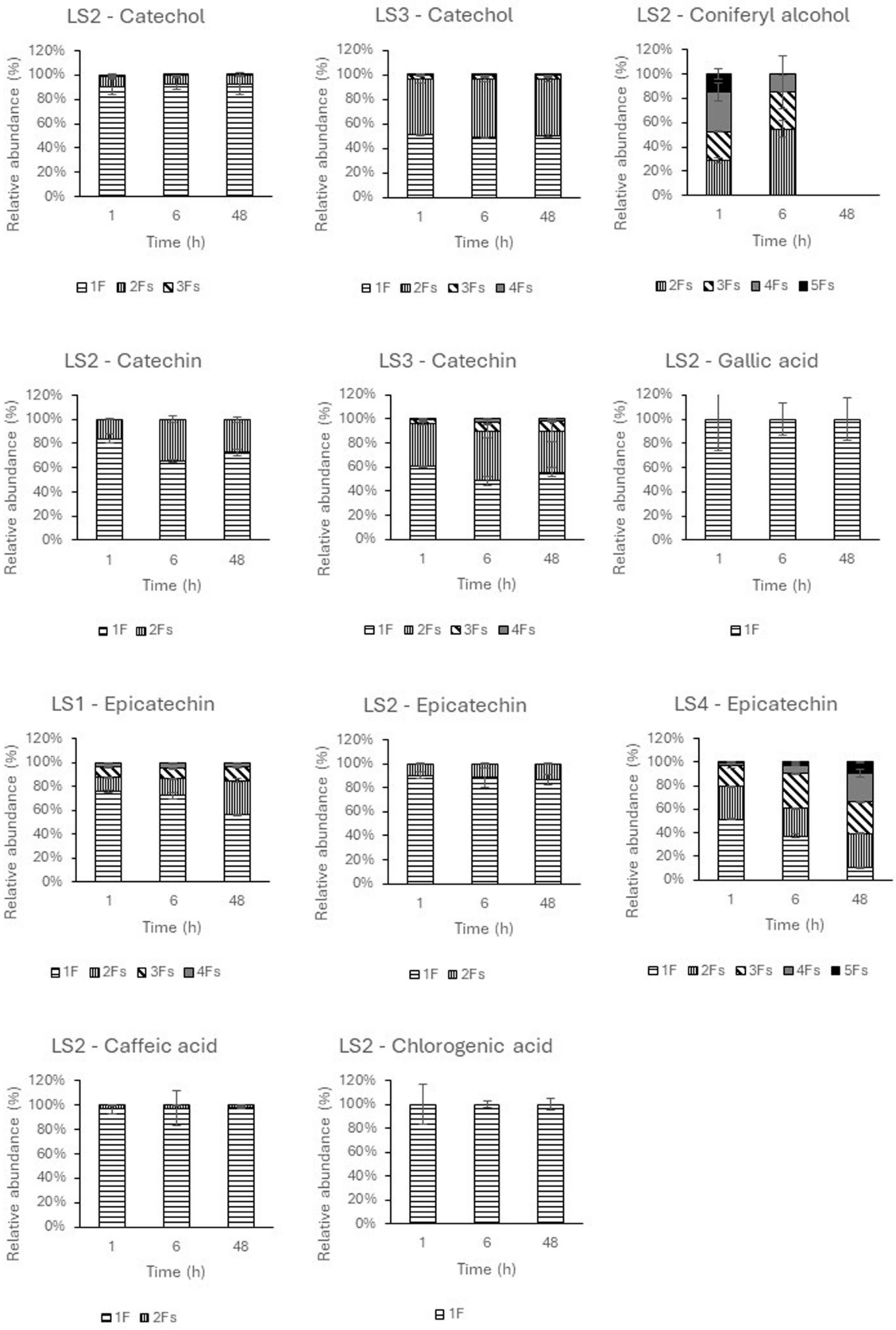

Figure 3. Relative abundance of phenolic fructosides* where 'F' represents the number of fructosyl groups acquired by phenolic acceptors (e.g., 1F = 1 fructosyl group transferred from sucrose to acceptor substrate). * *The relative abundance was calculated as the percentage of total phenolic fructosides with the same number of acquired fructosyl group compared to the total amount of phenolic fructosides detected.*

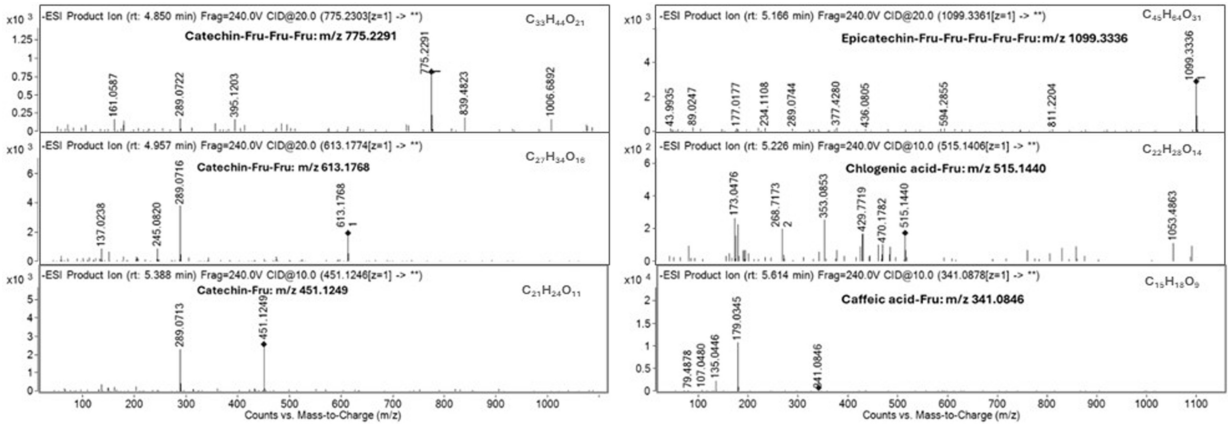

Figure 4. MS-MS fragmentation spectra of biotransformation end-products of LS-catalyzed transfructosylation reactions using catechin, chlorogenic acid, caffeic acid and epicatechin substrates. The major fragment for each of the end-product is its corresponding phenolic compound moiety (catechin and epicatechin moiety with m/z 289.0722, chlogenic acid moiety with m/z 353.0853 and caffeic acid moiety with m/z 179.0345). Diamond indicates parent ions.

highest bioconversion of acceptors with a maximum of 86%, 80% and 34% for maltose, cellobiose and lactose transfructosylation reaction, respectively. LS4 also converted a decent amount of acceptor substrates (54%, 76% and 17% with maltose, cellobiose and lactose, respectively), while LS1 and LS3 had rather low bioconversion yields (2–4%) overall.

The reaction selectivity of the most promising reactions were then determined by quantifying the free fructose (hydrolysis) and the transferred fructose (transfructosylation). Interestingly, although only *V. natriegens* LS2 and *P. graminis* LS4 were previously found to outstandingly convert the acceptor substrates, Figure 5 shows that both *G. oxydans* LS1 and *N. aromaticivorans* LS3 also resulted in noteworthy transfructosylation activities. This suggests the synthesis of levan and/or FOSs by LS1 and LS3 through the transfer of the fructosyl group to the fructosyl growing chains. With maltose, LS2 did not show a particular trend in its catalytic properties, with a rather constant transfructosylation extent of around 30%. LS3 resulted in a growing transfructosylation extent, as well as overall high hydrolytic activity. The transfructosylation extent of LS4-catalysed acceptor reaction of maltose showed a rising trend till 6 h, which then decreased at 48 h, revealing the shift of the reaction towards hydrolysis. Similar trends in transfructosylation extent were observed with LS2 and LS4 in the cellobiose acceptor reactions. Furthermore, an apparent decrease in hydrolysis was altogether observed at 48 h. It can be hypothesized that the release glucose may have been used as an acceptor, leading to the formation of sucrose analogues and to an apparent decrease in the hydrolysis and transfructosylation extents.

With LS1 and cellobiose acceptor, significant increase in the hydrolysis extent occurred with reaction time, while the transfructosylation extent intensified then declined at 48 h. Finally, when lactose was used as a substrate, LS1 and LS3 resulted in increasing hydrolytic and transfructosylation activities, with higher extent recorded with LS1. LS2 tranfructosylated lactose at a declining rate while LS4 did not show any particular transfructosylation trend. Increasing hydrolysis extent could however be evident with LS4. In our previous study, similar trends were observed, with LSs showing in general higher transfructosylation activity than hydrolytic one[33]

End-Product Profiles of Fructosylated Trisaccharides and Fructooligosaccharides

The end-product profiles of the sugar acceptor reactions were characterized via LC–MS. Overall, the fructosylated trisaccharides produced followed similar trends observed with their corresponding transfructosylation extent. In addition, the hypothesis that LS1 and LS3 produced FOSs was confirmed in Figure 6. Interestingly, comparing LS2 and LS4, LS2 showed a higher selectivity towards the transfructosylation of acceptor substrates maltose, cellobiose and lactose, producing only trance amounts of FOSs. Fructosylated sorbitol was not detected by LC–MS analysis, confirming that sorbitol could not act as acceptor substrates for the selected LSs. The production of FOSs in the presence of sorbitol shows that sorbitol did not have any inhibitory effect on LS-catalyzed reactions. The MS-MS fragmentation spectra of the resulting fructosylated trisaccharides and FOSs are shown in Figure 7 and Supplementary Figure 4.

When maltose was fructosylated, the non-reducing trisaccharide erlose (O-β-D-fructofuranosyl-(1,2)-O-α-D-glucopyranosyl (1,4)- α-D-glucopyranoside) was formed. Erlose is of high interest because of its sucrose-like taste and anticarious properties.[34] Erlose was previously enzymatically synthesized by various LSs such as those from *B. subtilis*,[35] *Bacillus amyloliquefaciens*,[29] *Beijerinckia indica subsp. Indica*, as well as our selected strains.[1b] Results for LS from *G. oxydans* LS1are however not presented in Figure 7 due to insignificant concentrations of erlose detected by LC–MS. Comparing LS2, LS3 and LS4, the highest erlose amount at 200 g/L after 1 h reaction was synthesized by *V. natriegens* LS2. Overall low concentrations were obtained with LS3, with the highest of 20 g/L after 4 h of reaction. High erlose concentrations were achieved in the LS4-

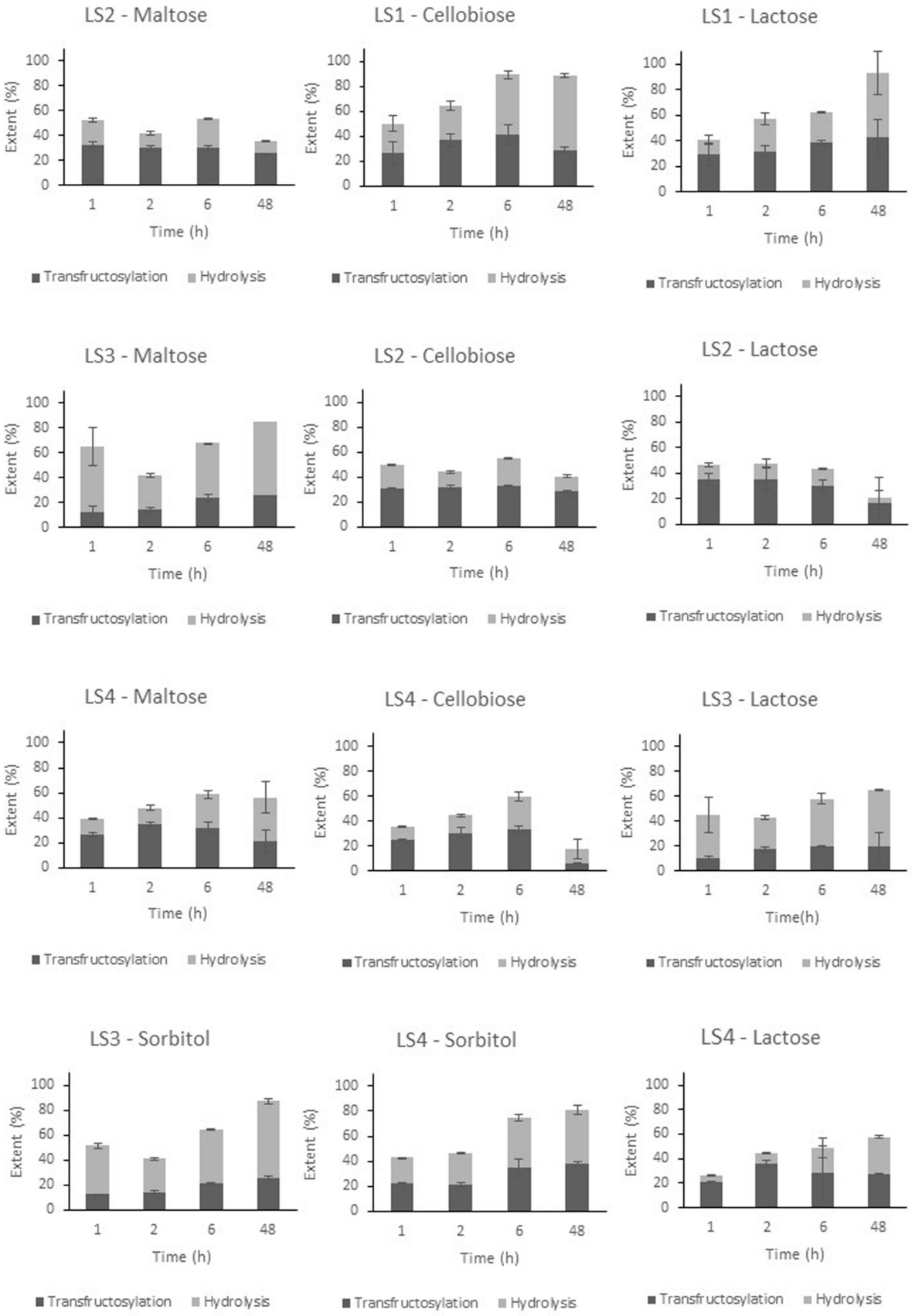

Figure 5. Time course for the reaction selectivity (transfructosylation/hydrolysis) of Levansucrase (LS)-catalysed reactions (*G. oxydans* LS1, *V. natriegens* LS2, *N. aromaticivorans* LS3, and *P. graminis* LS4) in the presence of sugar acceptor substrates.

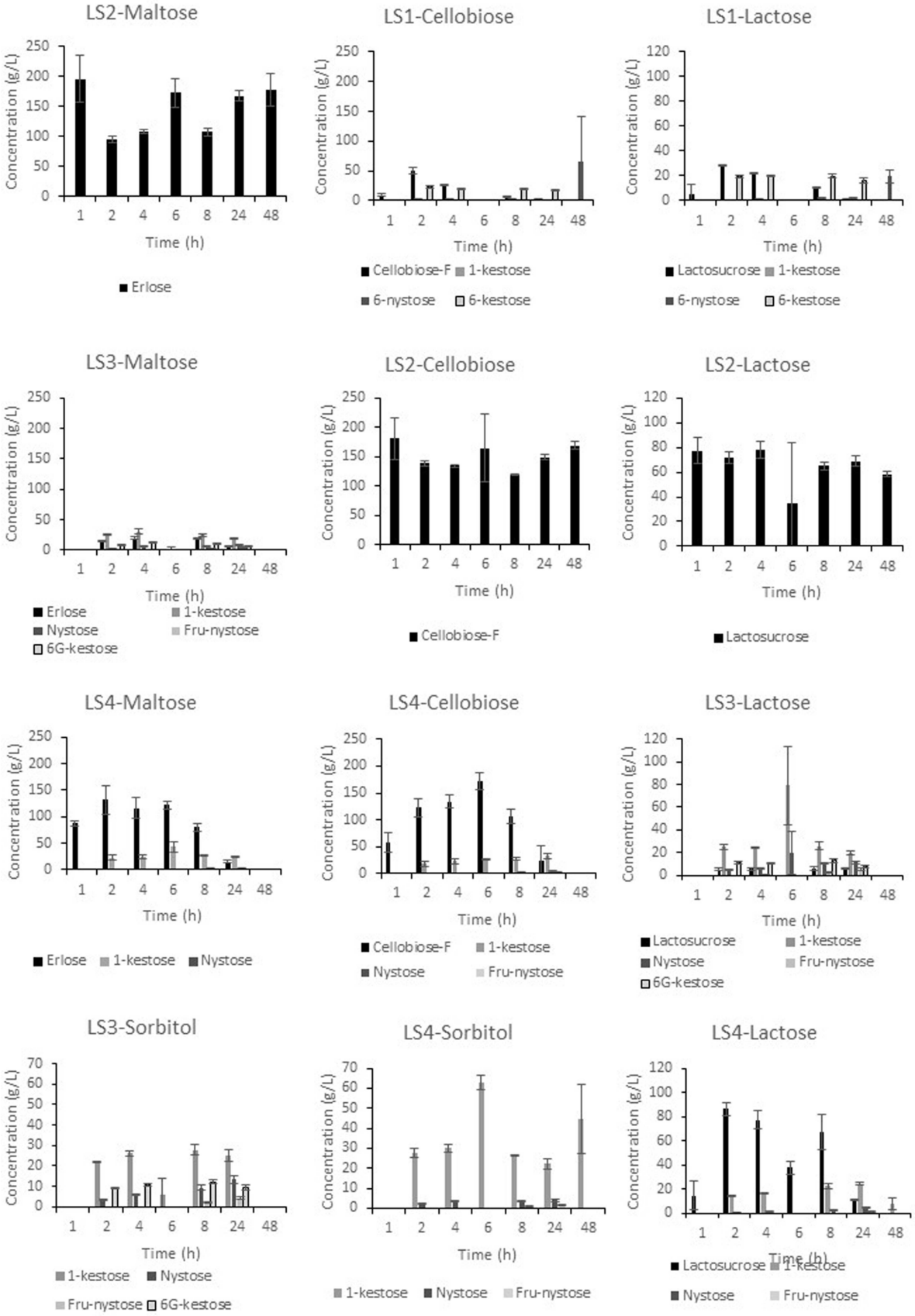

Figure 6. Biotransformation end-products in the presence of carbohydrate acceptors.

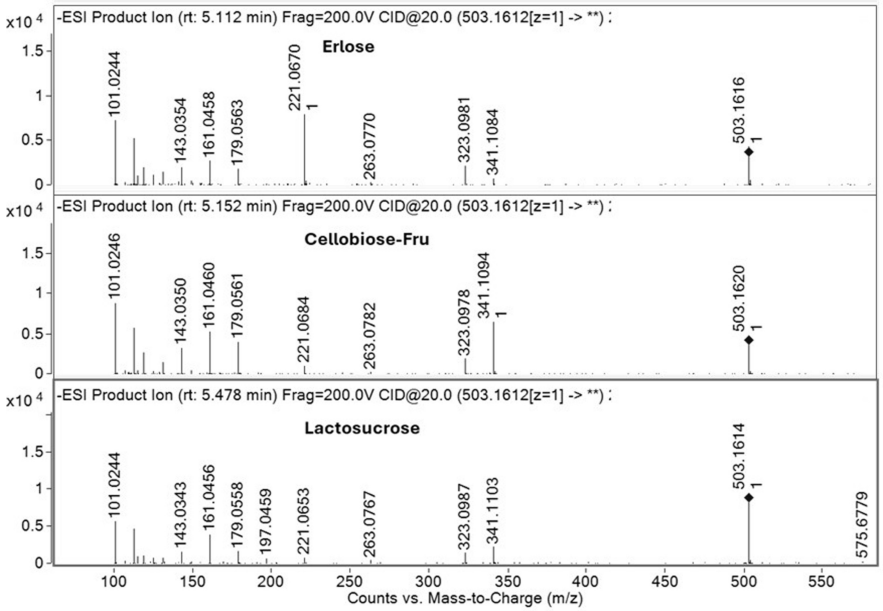

Figure 7. MS-MS fragmentation spectra of biotransformation oligosaccharide end-products with m/z 503.1612 (erlose, cellobiose-Fru, Lactosucrose) of LS-catalyzed transfructosylation reactions using maltose, cellobiose and lactose substrates, respectively. Diamond indicates parent ions.

catalysed maltose transfructosylation reaction within the first 8 h with a maximum of 130 g/L; however, this level reduced drastically at the later stage as a result of the shift of the reaction toward the hydrolysis as shown previously with the increase in the hydrolysis extent at 48 h (Figure 5).

Fructosylated cellobiose was obtained in the reactions catalyzed by LS1, LS2 and LS4. The highest concentration was achieved with LS2, reaching around 180 g/L after 1 h. With LS4, around 170 g/L of fructosylated cellobiose was formed after 6 h of reaction. With LS1 a maximum level of 50 g/L was reached. Besides resulting in the highest production yield, LS2 also has the advantage of being the least dependent on the reaction time. On the other hand, with LS4 or LS1, the fructosylated cellobiose concentration fluctuates over the 48 h reaction time course. Previously, cellobiose was also fructosylated by LSs from *Halomonas smyrnensis* AAD6^T,[36] *Microbacterium laevaniformans*,[37] *B. subtilis*[38] and *Bacillus licheniformis* 8-37-0-1 LS.[7] The physiological and physicochemical properties of fructosylated cellobiose allow its use as a low-calorie sugar substitute, to replace sucrose, in food and feed industries.[39]

Finally, the transfructosylation of lactose was reported by all four selected LSs. The transfructosylation of lactose produces lactosucrose (O-β-D-galactopyranosyl-(1,4)-O-α-D-glucopyranosyl-(1,2)-β-D-fructofuranoside). This trisaccharide has been valued for its potential prebiotic effects,[40] intestinal mineral absorption properties,[41] and ability to reduce body fat accumulation.[42] The water-holding capacity of lactosucrose also makes it an interesting ingredient for the food industry, particularly to produce fermented milk products like yogurts or cheese, to reduce syneresis or serum separation and act as a fat replacer.[43] Although the highest lactosucrose was reported with LS4 at around 85 g/L, the production declined over time. In general, LS2 seemed to be the most reliable LS, with high and rather constant lactosucrose yields. LS1 and LS3 resulted in significantly lower production at a maximum yield of around 30 g/L and 5 g/L, respectively. Similar trends were observed in our previous study with LS2 being the best catalyst in the transfructosylation of lactose and showing great potential in the use of dairy by-products as lactose substrates.[33]

A fluctuation in lactosucrose production was also observed. This was attributed to a possible shift of reaction towards lactosucrose hydrolysis and/or lactosucrose transfructosylation over the reaction course. In other words, lactosucrose could be utilized as a fructosyl donor and/or acceptor.[33] Lactosucrose was also previously found to be synthesized by LSs from *Aerobacter levanicum, Bacillus natto, B. subtilis, Brenneria goodwinii* and *L.* mesenteroides.[44]

Conclusions

The characterization of the acceptor specificity of selected LSs revealed that *V. natriegens* LS2 was the most efficient biocatalyst for the transfructosylation of phenolic compounds, including catechol, catechin, epicatechin, coniferyl alcohol, gallic acid, caffeic acid, chlorogenic acid, and rosmarinic acid, expect vanillic acid. Catechol, catechin and epicatechin were distinguishably the most versatile acceptors, being also significantly transfructosylated by other LSs (LS3- catechol, catechin, epicatechin; LS4- catechol and catechin). LC–MS analysis further proved that more than one fructosyl unit could be attached to the glycosylated phenolic compounds, with up to five fructosyl groups transferred to coniferyl alcohol and epicatechin using LS2 and LS4, respectively. The presence of phenolic compounds was also found to act as an inhibitor, preventing the formation of other LS-catalyzed end-products (FOSs). As for carbohydrate

substrates, maltose, cellobiose and lactose, they successfully acquired a fructosyl group from sucrose. No transfructosylation activity was reported with sorbitol. *V. natriegens* LS2 and *P. graminis* LS4 led to high yields of fructosylated trisaccharides. In addition, LS2 was more selective towards the fructosylation of disaccharides, while LS1, LS3 and LS4 simultaneously produced fructosylated trisaccharides and FOSs. This study highlights the potential of LS in the fructosylation of phenolic compounds and carbohydrates, resulting in the formation of functional compounds which can be of great interest to the pharmaceutical, cosmetics and food industries. Future works may include further characterization of the phenolic fructosides, determining their aqueous solubility, stability, and functional properties. The effects on levan production during the biotransformation reactions could also be investigated.

Experimental Section

Materials

Sucrose, D-(−)-fructose, D-(+)-glucose, (+)-catechin hydrate, gallic acid, vanillic acid, chlorogenic acid, rosmarinic acid, (−)-epicatechin, caffeic acid, D-(+)-Maltose monohydrate, D-Sorbitol, D-(+)-Cellobiose, α-Lactose monohydrat, myo-inositol, 3,5-dinitrosalicylic acid (DNS), potassium sodium tartrate (KNaC4H4O6), yeast extract, carbenicillin disodium salt, lysozyme from chicken egg white, DNase I, imidazole, C2H7NO2, NH4HCO3, NaOH solution were obtained from Sigma-Aldrich (Oakville, ON). Catechol, KH2PO4, K2HPO4, NaOH (Pellets/Certifies ACS), acetonitrile (ACN) HPLC grade, water optima LC–MS grade, bovine serum albumin (BSA), tryptone, NaCl, β-D-isothiogalactopyranoside (IPTG), PIPES, glycerol, tris-glycine-SDS 10x solution, acetone,and Pierce™ Coomassie Plus (Bradford) assay kit were provided by Fisher Scientific (Fair Lawn, NJ). Coniferyl alcohol was obtained from Thermo Fisher Scientific Inc. (Fair Lawn, NJ). 1-kestose, nystose, and 1F -fructofuranosylnystose were obtained from FUJIFILM Wako Chemicals U.S.A. Corporation (Richmond, VA). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) standards were purchased from Bio-Rad (Mississauga, ON). Terrific broth (TB) and lysogeny broth (LB) agar powder were acquired from Bio Basic (Markham, ON). *E. coli* BL21 (DE3) plysE strains were supplied by Invitrogen (Waltham, MA).

Production and Purification of Levansucrases

LSs from *G. oxydans* (strain 621H) (LS1), *V. natriegens* NBRC 15636 (LS2), *N. aromaticivorans* (LS3) and *P. graminis* C4D1 M (LS4) were produced and purified as described in our previous studies.[1a] *Escherichia coli* BL21(DE3) cells (Invitrogen) were first transformed with the LS genes of selected strains. The cells, plated on LB agar containing 100 μg/mL carbenicillin, were precultured in an LB media also containing 100 μg/mL carbenicillin for 8–10 h at 37 °C under 250 rpm. Terrific broth containing 2% v/v of the preculture and 100 μg/mL carbenicillin was then incubated at 37 °C under 250 rpm for around 4 h, until a bacterial growth turbidity of optical density of 1.2–1.6 at 600 nm was achieved. The enzyme expression was induced using 1 mM IPTG and the growth of the culture was resumed at room temperature for 18 h under 250 rpm. To collect the cells, centrifugation at 4 °C under 8000 rpm was carried out. The recovered pellets were resuspended in a sonication buffer (50 mM PIPES, 300 mM NaCl, and 10% glycerol; pH of 7.2; 4 ml/g). 4 mg/g lysozyme and 4 μL/g DNase were added to the suspensions which were then incubated at 18 °C under 50 rpm for 1 h. The cells were lysed by ultrasonication using a microtip (Misonix Ultrasonic Liquid Processor S-4000, Farmingdale, NY, USA) for 1 minute (10 s on, 60 s off, amplitude of 15) in an ice bath. The supernatants containing the enzymes were recovered after centrifugation at 4 °C under 14,000 rpm for 1 h, dialyzed against potassium phosphate buffer (5 mM; pH of 6) using a membrane with a molecular weight cut-off of 6–8 kDa, and then lyophilized. The LSs were purified via immobilized metal affinity chromatography on a HisTrap™ FF column (5 ml, GE Healthcare). After loading the resolubilized crude enzyme, the column was subsequently washed with sonication buffer, wash buffer (50 mM PIPES, 300 mM NaCl, and 10% glycerol; pH of 6.4), 5 mM imidazole, and 10 mM imidazole. LS enzyme was then eluted with 100 mM and 200 mM imidazole. SDS-PAGE electrophoresis analysis at 120 V using 15% SDS polyacrylamide gels and a 10x diluted Tris/Glycine/SDS buffer was performed to confirm the purity of the LSs.

The total specific activity of the purified enzyme was quantified as the total amount of reducing sugars produced per minute per mg of protein using a DNS test.[1a] The LS fractions with the highest purity and specific activity were pooled and stored at − 80 °C.

Enzymatic Biotrnasformation Reactions

Transfructosylation of Phenolic Compounds

For the LS catalyzed transfructosylation reaction of phenolic compounds, 5 U/mL of LS was incubated with 0.9 M sucrose and 0.03 M phenolic acceptor molecules in 10% DMSO at optimal temperature and pH of the selected LSs (LS1 and LS4- 50 mM ammonium acetate at pH 4/30 °C; LS2- 50 mM ammonium bicarbonate at pH 8/45 °C; LS3- 50 mM ammonium bicarbonate at pH 6/45 °C). The phenolic compounds studied include catechol, catechin, epicatechin, coniferyl alcohol, gallic acid, caffeic acid, chlorogenic acid, rosmarinic acid and vanillic acid. All reactions were performed in duplicates under 50 rpm. The biotransformation reactions were carried out over a time course of 48 h where aliquots were taken, placed in boiling water for 5 min to stop the reaction, and then stored at −20 °C until further analysis. The bioconversion yields of phenolic acceptors were quantified via HPLC. The separation was performed on an Agilent Zobrax SB–C18 reversed-phase column (250 mm ×4.6 mm, 5 μm), using a Beckman HPLC system equipped with an autosampler (Model 508), a UV/VIS DAD (Model 168) with computerized data handling and integration analysis (32 Karat, version 8). The samples were prepared by diluting them in 10:90 acetonitrile: water (v/v). They were analyzed using either of the two following gradient of water/formic acid 0.05% (v/v) and acetonitrile/formic acid 0.05% (v/v) (90/10 at 0 min, 50/50 at 20 min, 5/95 at 20.1 min and 90/10 at 35 min) or (90/10 at 0 min, 5/95 at 5 min, 90/10 at 25 min and 90/10 at 30 min) at a flow rate of 0.700 mL/min. The phenolic compounds were quantified using UV detection at 254 nm. The bioconversion yields were calculated as the difference between the initial and final concentrations of phenolic compounds as a percentage of the initial concentration of phenolic compounds.

Transfructosylation of Selected Carbohydrates

For the LS catalyzed transfructosylation reaction of carbohydrate and sugar alcohol acceptors, maltose, cellobiose, lactose and sorbitol, 5 U/mL of LS was incubated with 0.9 M sucrose and 0.45 M acceptor substrate. The biotransformation reactions were carried out at optimal temperature and pH of the selected LSs (LS1 and LS4- 50 mM ammonium acetate at pH 4/30 °C; LS2- 50 mM ammonium bicarbonate at pH 8/45 °C; LS3- 50 mM ammonium

bicarbonate at pH 6/45 °C). All reactions were performed in duplicates under 50 rpm over a time course of 48 h where aliquots were taken, placed in boiling water for 5 min to stop the reaction, and then stored at −20 °C until further analysis.

Reaction Selectivity (Hydrolysis vs Transfructosylation)

After the enzymatic biotransformation reactions, the remaining sucrose as well as the released glucose and fructose were quantified by high-pressure anion-exchange chromatography (HPAEC) using a Dionex ICS-3000 system equipped with a pulsed amperometric detector (PAD) and a CarboPac PA20 column (3×150 nm). The components of reaction mixtures were eluted with an isocratic mobile phase of 20 mM sodium hydroxide at a flow rate of 0.4 mL/min and 32 °C. The hydrolysis extent of sucrose was quantified as the concentration of released fructose taken as a percentage of the initial sucrose concentration, while the extent of sucrose transfructosylation was based on the difference between the concentrations of released fructose and glucose as a percentage of the initial sucrose concentration.

$$\textit{Transfructosylation yield } (\%) = \textit{Concentration of released glucose} - \frac{\textit{Concentration of released fructose}}{\textit{Initial sucrose concentration}} \times 100$$

$$\textit{Hydrolysis yield } (\%) = \frac{\textit{Concentration of released fructose}}{\textit{Initial sucrose concentration}} \times 100$$

End-Product Profiles Characterization

To characterize the phenolic fructosides produced, the reaction mixtures were analyzed by LC–MS using an Agilent 1290 Infinity II LC system coupled to the 6560-ion mobility Q-TOF -MS (Agilent Technologies, Santa Clara, USA). The samples were prepared by diluting them in 50:50 acetonitrile: water. The LC separation was conducted on a Poroshell120 EC–C18 analytical column (Agilent Technologies; 2.7 μm ×3 mm ×100 mm) connected with a Poroshell120 EC–C18 guard column (Agilent Technologies; 2.7 μm ×3 mm ×5 mm). The mobile phase A was HPLC water with 0.1 % formic acid and the mobile phase B was acetonitrile with 0.1 % formic acid. HPLC parameters were as follows: an injection volume of 4 μL, a flow rate of 0.3 mL/min and a column temperature set to 30 °C. The mobile phase profile used for the run-in negative ion mode was 2% B (0 to 1.0 min), 2%-20% B (1.0 to 4.0 min), 20%-100% B (4.0 to 8.0 min), 100% B (4.0 to 8.0 min), hold at 100% B (8.0–13.0 min), decrease to 2% B (13.0.0 to 13.5 min) and hold 2% B (13.5 to 14 min). The mass spectrometer was equipped with a Dual AJS ESI ion source operating in negative ionization mode. MS conditions were as follows: for ESI-, the drying gas temperature was 200 °C, the drying gas flow rate was 12 l/min, the sheath gas temperature was 250 °C, the sheath gas flow rate was 12 L/min, the pressure on the nebulizer was 35 psi, the capillary voltage was 4000 V, the fragmentor voltage was 240 V, and the nozzle voltage was 1000 V. Full scan MS data were recorded between mass-to-charge ratios (m/z) 100 and 1700 at a scan rate of 2 spectra/s, and were collected at both centroid and profile mode. Reference ions (m/z at 112.9856 and 1033.9881 for ESI−) were used for automatic mass recalibration of each acquired spectrum. Targeted MS/MS fragmentation (collision energy = 10, 20 V) were performed for selected reaction products and compared with fragmentation pattern of analytical standards of phenolic compounds. Data treatment was conducted using Quanlitative Analysis B.10.0 and Quantitative Analysis B.10.0 from Agilent MassHunter Workstation Software.

The sucrose concentration and end-product profiles of fructosylated trisaccharides and FOSs were characterized using an Agilent 1290 II liquid chromatography system coupled to an Agilent 6560-ion mobility Q-TOF –MS. The samples were prepared by diluting them in 50:50 acetonitrile: water (v/v) with the addition of myo-inositol (5 ppm) to serve as an internal standard. The analytes were separated with an InfinityLab Poroshell 120 HILIC–Z column (2.1×100 mm, 2.7 μm). Mobile phase A was LC–MS grade water with 0.3 % NH_4OH and mobile phase B was acetonitrile with 0.3 % NH_4OH. The flow rate was set at 0.4 mL/min with a column temperature of 35 °C. The constructed gradient started off with 85 % B (0.0 to 0.5 min) that had a linear decrease to 30 % B (0.5 to 9.0 min) where it was held (9.0 to 13.0 min) and then increased to 85 % B (13.0 to 15.0 min), followed by a 3 min post-run. The mass spectrometer was equipped with a Dual AJS ESI ion source operating in negative ionization mode. MS conditions for ESI was as follows: a drying gas temperature of 150 °C and a flow rate of 11 L/min, a sheath gas temperature of 350 °C and a flow rate of 12 L/min, a pressure on the nebulizer of 30 psig, a capillary voltage of 4000 V, a fragmentor voltage of 200 V, a skimmer voltage of 30 V, and a nozzle voltage of 2000 V. Full scan MS data were recorded at mass-to-charge ratios (m/z) from 80 to 1100 at a scan rate of 2 spectra/s and were collected at both centroid and profile mode. Reference ions (m/z at 112.985587 and 1033.988109 for ESI−) were used for automatic mass recalibration of each acquired spectrum. Targeted MS/MS fragmentation (collision energy = 10, 20 V) were performed for selected oligosaccharide products. Retention time and fragment pattern were compared with that of oligosaccharide analytical standards. Data treatment was conducted using Quanlitative Analysis B.10.0. The quantification was performed using Quantitative Analysis 10.0 from Agilent MassHunter Workstation Software.

Supporting Information

Supporting data associated with this article can be found online at https://doi.org/10.1002/cbic.202400107.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Levansucrase · Biocatalysis · Acceptor specificity · Phenolic fructosides · Fructosylated trisaccharides

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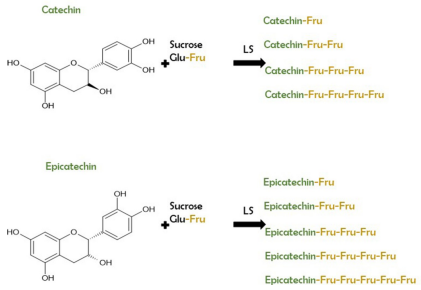
RESEARCH ARTICLE

Levansucrases from *Gluconobacter oxydans*, *Vibrio natriegens*, *Novosphingobium aromaticivorans*, and *Paraburkholderia graminis* were used to catalyze the transfructosylation of phenolic compounds and carbohydrates. The selected phenolic acceptor substrates were successfully glycosylated with up to five fructosyl groups transferred from the sucrose donor substrate. The transfructosylation of carbohydrates acceptors produced fructosylated trisaccharides that can act as low-calorie sugar substitutes and/or prebiotic ingredients.



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