Transglycosylation of steviol glycosides mediated by two novel thermophilic hydrolases, *Tt*bGal1 and *Mt*Bgl3a, and valorization of industrial byproducts as sugar donors.

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Stevia plant is likely to remain a major source of noncaloric high-potency sweeteners for the growing natural food market in the future. But, although the sales of products that contain Stevia compounds as the sole alternative sweetener source have more than doubled in the last 10 years, the bitter aftertaste of steviol glycosides, perceived by almost half of the human population, is still a troublesome feature. During the last 14 years, a great deal of attention has been paid to improve the quality of taste and simultaneously to increase the sweetness of steviol glycosides. Enzymatic glycosylation is a strategy to reduce stevioside bitterness, but reported glycosylation reactions suffer from low productivities. Although β-glycosidases and β-galactosidases are in vivo mainly hydrolytic enzymes, many of them can be used in vitro for synthetic purposes as transglycosidases. They display robustness, stability, and stereoselectivity. Nowadays, the industries generate high amounts of byproducts that may contain hight added value compounds with high functionality and/or bioactivity. In this study we used low-cost industrial by-products as the source of donor sugars for the transglycosylation of steviol glycosides. Regarding steviol glycoside transgalactosylation by β-Galactosidase galactosidase *Tt*bGal1, concentrated acid whey wastewater was used, derived from Greek strained yoghurt industry, which is rich in residual lactose. For β-glycosidase *Mt*bgl3a-mediated bioconversion of steviol glycosides, cellulose was chosen as a substrate, since it is the most abundant polymer on earth, and it is available as a by-product from most agro industrial activities, as part of lignocellulosic biomass.

The acid whey that was used for the enzymatic production of GOS was provided by Delta Foods SA and it was derived from production processes of Greek strained fat-free yoghurt. TtbGal1-mediated bioconversion, was routinely performed with 100 g L^{-1} lactose as the donor substrate, 5 g L^{-1} of either stevioside or rebA, 0.5 U mL^{-1} TtbGal1, in phosphate-citrate buffer pH 4.5, in 45 ^{o}C. *Mt*Bgl3a -mediated bioconversion was routinely performed with 50 g L^{-1} cellobiose as the donor substrate, 5 g L^{-1} of either stevioside or rebA, 64 μg mL^{-1} *Mt*Bgl3a, in phosphate-citrate buffer pH 5, in 45 ^{o}C. The acid whey preparation was also used for transgalactosylation reaction, *Tt*bGal1 was added to 1 mL of acid whey, with an activity of 0.5 U mL^{-1}, and 5 g L^{-1} of either stevioside or rebA. *Mt*Bgl3a (Karnaouri et al., 2013) was also used for the transglycosylation of stevioside and rebA, with hydrolysed microcrystalline cellulose (Avicel) as donor substrate. Avicel (100 g L^{-1}) was hydrolysed to cello-oligosaccharides by CBH1 (4.5 mg g^{-1}) and EG7 (2.7 mg g^{-1}) (Megazyme), at 1 mL reactions for 16 h in 45 ^{o}C under agitation (950 rpm). Then, *Mt*Bgl3a was added at a final concentration of 64 μg mL^{-1}, and stevioside or rebA at a final concentration of 5 g L^{-1}, and the reaction was incubated for a further 24 h. An LC-20AD HPLC system (Shimadzu) was employed for the analysis of the reaction products using a C18 CC 250/4.6 Nucleosil 100-5 column (Macherey-Nagel). The mobile phase consisted of 70% (v/v) phosphate buffer 10 mM pH 2.6 and 30 % (v/v) acetonitrile with 1 mL min^{-1} flow. Monitoring was performed at 210 nm using a UV–vis ProStar 335 Diode Array detector (Agilent Technologies). HILIC-ESI-QTOFMS technique was applied in negative ionization mode for the detection of transglycosylation products due to their high polarity.

The β-galactosidase *Tt*bGal1 was employed for the transglycosylation of stevioside and rebA, using lactose or low-cost industrial by-products as the glycoside donor. In case of lactose, the conversion of both stevioside and rebA started almost immediately, and the lowest concentration was measured after 4 hours of reaction in the case of stevioside, and after 24 hours of reaction for rebA. The maximum conversion of stevioside and rebA was 27.7 ± 1.4% and 31.8 ± 0.5 % respectively. When acid whey wastewater was used, which is rich in residual lactose, hydrolysis of the transglucosylation products was not observed, since the steviol glucosides concentration decreased up to 24 h. The results are shown Figure 1a where the maximum conversion observed for stevioside was 28.9 ± 7 %, while for rebA the maximum conversion was 19.5 ± 1.2 %.

The β- glycosidase *Mt*Bgl3a was also employed for the transglycosylation of stevioside and rebA, using cellobiose or hydrolysed microcrystalline cellulose as the donor. In the case of cellobiose the conversion of both stevioside and RebA started almost immediately, and the lowest concentration was measured after 24 hours. The maximum conversion of stevioside and rebA was 22.2 ± 2.4% and 22.7 ± 0.5 % respectively. When hydrolyzed

microcrystalline cellulose was used, lower conversion yields were obtained from cellulases. As shown in Figure 1b where the maximum conversion observed for stevioside was 13.8 ± 3.4 %, while for rebA the maximum conversion was 4.4 ± 0.5 %. The lower conversion yield obtained in the case of hydrolysed cellulose might be due to the low hydrolysis yield of cellulases, but also possibly to the lower affinity of *Mt*Bgl3a towards cello-oligosaccharides compared to cellobiose.

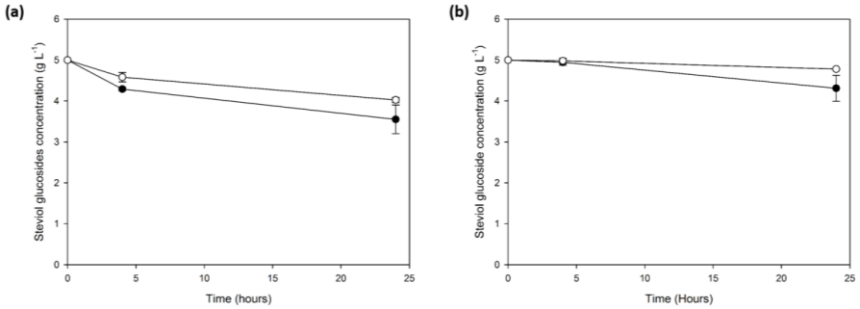

Figure 1. Transglycosylation of steviol glycosides by *Tt*bGal1 with concentrated acid whey as the donor (a), and *Mt*Bgl3a, with hydrolysed microcrystalline cellulose as the donor (b).

Regarding the HILIC-HRMS analysis of transglycosylation products, new transglycosylation products were detected and identified. For the m/z 1011.4279, two peaks were observed at Rt= 6.8 min and 7.3 min. For this peak, the suspect screening workflow was used, and first peak was identified as a mono-glycosylated product and later confirmed by reference standard at the level of identification confidence 1. The molecular formula, retention time and MS/MS (*in-silico*) were evaluated for the second peak and two most probable chemical structure were selected. Mono-glycosylated products of RebA ([M-glucose moiety +HCOO-]-) were tentatively identified at level of confidence 3 for this peak and more evidence was needed to prioritize these to candidates. For the m/z 1127.4764, two peaks were observed at Rt= 7.28 min and 7.46 min. Only one compound was matched to this m/z ([M-H]-) within mass accuracy of 8 mDa. The formate adduct for this mass was also detected ([C50H80O28+HCOO-]) reaching this the identification confidence of 4. To verify that the peak eluted at 7.28 min relates to a mono-glycosylated product of RebA, a clear MS/MS spectra is needed.

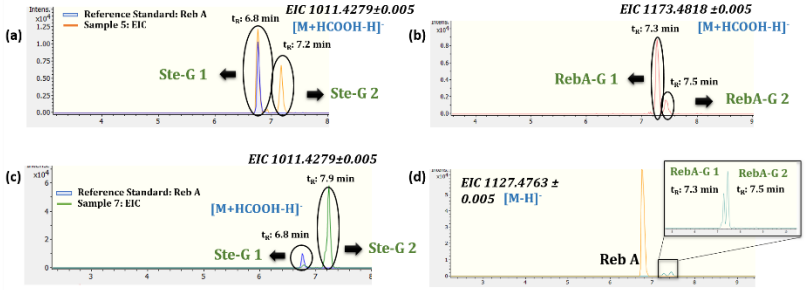

Figure 2. HILIC-HRMS analysis of transglycosylation products of stevioside (a and c) and RebA (b and d) from *Mt*Bgl3a and hydrolysed cellulose as donor (a and b) or *Tt*bgal1 and acid whey lactose as donor (c and d)

The main topic of this work was the enzymatic modification of steviol glycosides, performed with the aim of improving the organoleptic properties of steviol glycosides. In this respect, the modification of the carbohydrate moieties via bio catalysis *in vitro* is of special interest. In this context, the findings of the present work enhance and promote a different approach. The production of new transglycosylated products with new enzymatic systems is of critical importance in order to elucidate the correlation between taste and structure of steviol glycosides. There is also room for more explorative research, investigating the glycosylation specificity of new or engineered carbohydrate enzymes. This study demonstrated that acid whey wastewater and Avicel could be used as low-cost sugar donors for transglycosylated steviol glycoside production. These results could contribute to the design of a promising cost-effective process for the production of improved improved steviol glycosides from low-cost industrial by-products.

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