Exploring the glucosylation potential of glucansucrases

Devlamynck, Tim Nick

Chapter 6

Biocatalytic production of novel steviol glycosides with improved taste: scale-up, downstream processing and cost analysis

Tim Devlamynck[1,2], Evelien M. te Poele[2], Wim Soetaert[1], Lubbert Dijkhuizen[2]
Ghent University[1], University of Groningen[2]

Abstract

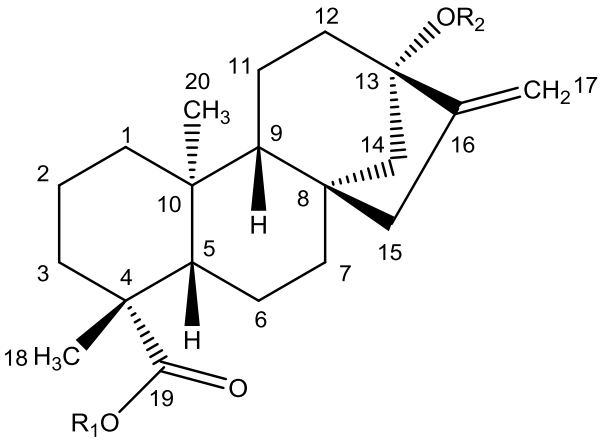
Previously we reported the efficient laboratory scale glucosylation of the *Stevia* components rebaudioside A (RebA) and stevioside with the Q1140E mutant of glucansucrase Gtf180-ΔN from *Lactobacillus reuteri* 180. Sensory analysis by a trained panel revealed that the glucosylated products possess a superior taste profile, displaying a significant reduction in bitterness compared to RebA and stevioside. As the developed technology holds excellent commercial potential, this chapter focused on the scale-up of the Gtf180-ΔN-ΔV-Q1140E catalyzed glucosylation of RebA and stevioside. An eco-friendly and efficient downstream processing of the glucosylated products was developed and demonstrated at 7.5 L scale, allowing the production of 250 g glucosylated RebA product. Estimates of the production costs indicated the economic feasibility of the overall process. The major factor in the total production cost was the acceptor substrate, i.e. RebA or stevioside; in contrast, the biocatalyst cost represented only a minor part. In an attempt to lower the overall production cost even further, the glucosylation of low-grade stevia extract (roughly a 50:50 mixture of stevioside and RebA) was demonstrated. The sensory properties of the resulting product mixture were perceived as excellent by a trained panel, mainly due to a significant reduction in bitterness.

1. Introduction

The steviol glycosides extracted from the plant *Stevia rebaudiana* (Figure 1), native in Paraguay and Brazil, were approved for use as high-intensity sweetener (HIS) in food products by the USA in 2009 and by the European Commission in 2011[43]. The share of HIS in the global sweetener market, estimated at $68 billion in 2014, is currently not significant[44], however, the world-wide increasing incidence of obesity and other diet-related diseases is expected to increase the consumer demand for low-calorie food products and beverages[46]. The HIS market is consequently predicted to grow at a fast compound annual growth rate (CAGR) of more than 5%. The natural character of stevia HIS gives it a competitive edge over synthetic HIS such as aspartame and saccharin, suffering from a bad reputation among consumers. As a result, stevia is currently the fastest growing HIS on the market, displaying a CAGR of roughly 8.5%[44]. The World Health Organization even projects that stevia will eventually replace 20% of the sugar segment, equaling a $10 billion industry[45], which is significantly greater than current stevia sales, estimated at $347.0 million in 2014[44].

The main impediment to accomplish these projections is the lingering bitterness displayed by most steviol glycosides, including stevioside (5-10% of leaf dry weight) and rebaudioside A (2-4% of leaf dry weight, RebA), the only steviol glycosides which can be economically extracted from the *Stevia* plant[190-192]. Not surprisingly, several companies have launched next-generation stevia products with improved taste. For example, USA-based MycoTechnology produces mushroom-derived enzymes (MycoZyme) which can be added to stevia products to remove their bitterness (http://www.mycotechcorp.com). In addition, a joint venture of Cargill and Evolva aims to develop a process for the fermentative production of rebaudioside D and M, two steviol glycosides perceived as less bitter than RebA, but difficult to extract from the *Stevia* leaves, in 2018 (http://www.evolva.com)[39,58,71]. However, the relatively high production costs, caused by inadequate strain characteristics and associated fermentation and downstream processing costs, have forced this joint venture to set back the

launching date several times since 2013. Dutch multinational DSM is developing a similar technology, applying a recombinant yeast (*Yarrowia lipolytica*) to produce steviol glycosides (http://www.dsm.com)[232]. However, no new announcements have followed the initial 2014 press release.



steviol (R_1 = R_2 = H)

Steviol glycoside	**R_1 (C-19)**	**R_2 (C-13)**
Stevioside	Glc(β1→	Glc(β1→2)Glc(β1→
Steviolbioside	H	Glc(β1→2)Glc(β1→
Rebaudioside A	Glc(β1→	Glc(β1→2)[Glc(β1→3)]Glc(β1→
Rebaudioside B	H	Glc(β1→2)[Glc(β1→3)]Glc(β1→
Rebaudioside C	Glc(β1→	Rha(α1→2)[Glc(β1→3)]Glc(β1→
Rebaudioside D	Glc(β1→2)Glc(β1→	Glc(β1→2)[Glc(β1→3)]Glc(β1→
Rebaudioside E	Glc(β1→2)Glc(β1→	Glc(β1→2)Glc(β1→
Rebaudioside F	Glc(β1→	Xyl(β1→2)[Glc(β1→3)]Glc(β1→
Rebaudioside M	Glc(β1→2)[Glc(β1→3)]Glc(β1→	Glc(β1→2)[Glc(β1→3)]Glc(β1→
Rubusoside	Glc(β1→	Glc(β1→
Dulcoside A	Glc(β1→	Rha(α1→2)Glc(β1→

Figure 1. Chemical structures of the most prevalent steviol glycosides found in the leaves of *Stevia rebaudiana*. Glucose (Glc), xylose (Xyl) and rhamnose (Rha) occur in the pyranose ring form. Glc and Xyl have the D configuration and Rha the L configuration.

Alternatively, enzymatic glucosylation has been proposed as a bitterness-eliminating process[8]. The main challenges faced here are to obtain an adequate product specificity, a complete RebA and stevioside conversion and a high space-time yield. The majority of described processes fail in fulfilling at least one of these three requirements. Glucosylations catalyzed by cyclodextrin glucanotransferases, α-amylases and β-amylases, are characterized by relatively high product yields, however, their lack of C-13/C-19-regiospecificity (Figure 1) renders them less useful as industrial biocatalyst: products with enhanced and reduced bitterness are obtained[50-56,60-62]. For example, (α1→4)-glucosylation of stevioside at the C-13 steviol position yielded products with improved intensity and quality of sweetness, whereas (α1→4)-glucosylation at the C-19 position resulted in an increased bitterness[52-54,196]. In contrast, the *in vitro* use of UDP-glycosyltransferases, catalyzing the conversion of RebA into rebaudioside D and M, yielded glycosides with improved sensory properties but suffered from very low productivities[57-59]. The application of β-glucosidases, introducing the naturally occurring β-linkages, resulted not only in the glucosylation of the steviol glycosides substrates but also in their hydrolysis, yielding products with an inferior taste profile[63-65].

As illustrated in chapters 4 and 5, the α-glucosylation of RebA and stevioside with the glucansucrase Gtf180-ΔN-Q1140E from *Lactobacillus reuteri* 180, using sucrose as donor substrate, offers a viable method for the production of next-generation stevia products[68-70]. The Q1140E mutation improved the conversion from roughly 50% to 95%, yielding 115 g/L and 50 g/L α-glucosylated product, for RebA and stevioside glucosylation, respectively. Structural analysis and sensory analysis by a trained panel revealed that introducing a single (α1→6) linked glucosyl moiety at their Glc(β1→C-19 residues yielded monoglucosylated products with a superior taste profile compared to their respective substrates. The multiglucosylated products displayed even better taste profiles (reduced bitterness and off-flavors), however, this was accompanied with an undesirable decrease in sweetness. Altogether, the mixtures of mono –and multiglucosylated RebA and Stev products showed most potential: an intensive sweetness combined with a very limited bitterness (see 3.4. Sensory analysis).

The RebA and stevioside glucosylation reactions were demonstrated only at laboratory scale, whereas chromatography was applied as purification method, a technology which is better avoided as it is too costly for industrial use. This chapter therefore aimed to develop this laboratory scale process with commercial potential into a cheap and straightforward pilot-plant process, allowing the synthesis of product samples for food safety analysis and for testing and tasting in several food products, such as chocolate, candy, etc. Furthermore, an accelerated shelf life study was performed to determine the stability of the introduced (α1→6) linkage in buffer solutions mimicking the acidic conditions in soft drinks. Finally, cost analysis of the glucosylation processes provided guidelines for future research and development activities in order to decrease the production costs.

2. Materials and methods

2.1. Stevioside, rebaudioside A and low-grade stevia extract

Stevioside was obtained from TCI Europe (> 85% pure, HPLC), rebaudioside A (RebA) from Tereos PureCircle Solutions (97% pure, HPLC). Low-grade stevia extract (Steviasol, 95% steviol glycosides) was mainly composed of RebA and stevioside (~ 50:50 mixture, as determined with HPLC).

2.2. Production and purification of recombinant Gtf180-ΔN-ΔV-Q1140E

Initial experiments with *Escherichia coli* BL21 (DE3) strains (Invitrogen, Carlsbad, USA), expressing either the N-terminally truncated Gtf180-ΔN-Q1140E[70,80,97] or the N- and V-terminally truncated[88] Gtf180-ΔN-ΔV-Q1140E[70,97] from *Lactobacillus reuteri* 180, were performed in shake flasks at 1 L scale, after which the biocatalyst production was performed at 7.5 L scale in a Labfors 5 bioreactor. The inoculum was routinely grown at 37 °C in shake flasks (200 rpm) containing LB medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.0) supplemented with 100 mg/L ampicillin. After overnight growth, 200 mL inoculum

was added to 5 L of LB medium supplemented with 30 g/L glucose and 100 mg/ L ampicillin. The temperature, agitation rate and aeration rate were set at 37 °C, 350 rpm and 1 vvm, respectively. The dissolved oxygen concentration was maintained above 30% by gradually and automatically increasing the agitation rate to 1200 rpm. The pH was maintained at 7.0 by automatic addition of 25 % NH_4OH and 1 M H_2SO_4. Anti-foam was added occasionally to prevent foaming. Optical density measurements were performed in a spectrophotometer at 600 nm (OD_{600}). At an OD_{600} of 5 the culture was induced with IPTG (final concentration of 0.1 mM). After 9 h of fermentation the glucose was depleted and the cells harvested by centrifugation (3000 g, 10 min). The cells were resuspended in lysis buffer and subsequently homogenized (800 bar, 3 cycles). Cell debris was removed by centrifugation (3000 g, 10 min) followed by microfiltration (0.5 µm). The crude enzyme was stable at 4 °C for several months and used accordingly for the production of glucosylated RebA (RebA-G).

2.3. Glucansucrase activity assay

One unit (U) of enzyme activity corresponds to the conversion of 1 µmole sucrose (used for hydrolysis and transglycosylation) in a solution of 100 mM sucrose, 25 mM sodium acetate (pH 4.7) and 1 mM $CaCl_2$ at 37 °C.

Enzyme activity assays, using approximately 50 mg/mL enzyme, were performed at 37°C with 100 mM sucrose in 25 mM sodium acetate (pH 4.7) and 1 mM $CaCl_2$. Samples of 150 µl were taken every min over a period of 8 min and immediately inactivated with 30 µl 1 M NaOH. The sucrose concentrations of the samples were subsequently quantified by means of HPLC analysis (see 2.4. HPLC analysis), allowing the calculation of the enzyme activity as defined above.

2.4. HPLC analysis

Two different types of HPLC analyses were performed. For the analysis of glucose, fructose and sucrose, an Agilent MetaCarb 67H column (300 mm × 6.5 mm) was used under isocratic conditions with 2.5 mM H_2SO_4 as the mobile

phase. The flow rate and temperature were set at 0.8 mL/min and 35°C, respectively. Detection was achieved with an RID detector. Calibration of the obtained peaks was accomplished using the corresponding standard curves.

For the analysis of the steviol glycosides an Agilent ZORBAX Eclipse Plus C18 column (100 mm × 4.6 mm, 3.5 µm) was used with water (solvent A) and acetonitrile (solvent B) as the mobile phase. The flow rate and temperature were set at 1.0 mL/min and 40 °C, respectively. The following gradient elution was used: 5-95% solvent B (0-25 min), 95% solvent B (25-27 min), 95-5% solvent B (27-30 min) and again 95% of solvent A (30-35 min). Detection was achieved with an evaporative light scattering detector (ELSD) (evaporation temperature: 90 °C, nebulization temperature: 70 °C, gas flow rate: 1.6 standard liter per min, SLM). Calibration of the obtained peaks was accomplished using the corresponding standard curves, obtained from previously purified glucosylated RebA (RebA-G) and stevioside (Chapters 4 and 5).

2.5. Yeast fermentation

RebA glucosylation reaction mixture (50 mM RebA, 125 mM sucrose) was incubated with 5, 10, 20 and 30 g/L (wet cell weight) fresh baker's yeast (*Saccharomyces cerevisiae*, AB Mauri) to remove sucrose, glucose, and fructose. Samples were taken at 0, 2, 4, 6 and 8 h, and subjected to HPLC analysis to determine the removal rate of both saccharides and RebA glucosides. The experiments were performed in duplicate.

2.6. Determination of adsorption and desorption characteristics

Adsorption experiments were carried out by adding a fixed amount of adsorbent (Lewatit® VP OC 1064 MD PH from LANXESS, 1 g) to six 50 ml Falcon tubes containing 10 mL dilutions of the RebA glucosylation mixture (with RebA-G concentrations ranging from 10 g/L to 60 g/L). The Falcon tubes were subsequently placed in a shaker at a temperature of 30 °C, 35 °C and 40 °C and

an agitation rate of 200 rpm for 60 min. The adsorption capacity q_e (g RebA-G/kg resin) and adsorption efficiency A (%) were calculated as follows:

$$q_e = \frac{(C_0 - C_e)V}{W}$$

$$A = \frac{C_0 - C_e}{C_0}$$

where C_0 is the initial RebA-G concentration (g/L), C_e is the RebA-G concentration at equilibrium (g/L), V is the volume of the solution (10 mL) and W is the mass of the adsorbent (1 g).

The obtained adsorption isotherms were analyzed using the Langmuir adsorption isotherm, which is represented by the following equation:

$$q_e = \frac{q_{max} K C_e}{1 + K C_e}$$

where q_{max} is the maximum adsorption capacity (g RebA-G/kg resin) and K is the Langmuir adsorption constant (L/g).

For the desorption experiments 10 mL of solvent was added to 4 g of adsorbent (equivalent to 1.5 bed volumes) loaded with a known amount of RebA-G. The 50 mL Falcon tubes were subsequently placed in a shaker at a room temperature and an agitation rate of 200 rpm for 60 min. The desorption capacity q_d (g RebA-G/kg resin) was calculated as follows:

$$q_d = \frac{C_d V}{W}$$

where C_d is the RebA-G concentration at equilibrium (g/L), V is the volume of the solution (10 mL) and W is the mass of the adsorbent (4 g). The experiments were performed in duplicate.

C_0, C_e and C_d were determined applying HPLC analysis as described before.

2.7. Continuous adsorption of glucosylated rebaudioside A

After optimization of the static adsorption of RebA-G onto Lewatit® VP OC 1064 MD PH resin, its continuous adsorption was evaluated at laboratory scale (120 g of resin, bed volume (BV) of 200 mL), and finally at pilot-plant scale (2 kg of resin, BV of 3.34 L). After supplying the RebA glucosylation reaction mixture, 3 BV of water were immediately supplied to remove any residual sugars and α-glucans. Subsequently, six BV of 70% isopropanol were added to elute the adsorbed RebA-G. Several flow rates (3, 4.5 and 6 BV/h) and adsorbate loadings (50-100 g RebA-G/kg resin) were tested at laboratory scale, after which the optimal continuous adsorption was performed at pilot-plant scale (see also 2.8.).

2.8. Production and purification of glucosylated rebaudioside A

The production of glucosylated RebA (RebA-G) was performed at 7.5 L scale in a Labfors 5 bioreactor. The temperature and agitation rate were set at 37 °C and 185 rpm, respectively. The pH was maintained at 4.7 by automatic addition of 1 M NH_4OH and 1 M H_2SO_4. No buffer agent was added. The medium contained the optimal substrate- and enzyme concentrations as determined in chapter 4 (84 mM RebA, 282 mM sucrose, 5 U/mL Gtf180-ΔN-ΔV-Q1140E) supplemented with 1 mM $CaCl_2$. RebA (97% purity, HPLC grade) was obtained from Tereos PureCircle Solutions.

After completion of the reaction, the protein was removed by briefly (10 min) incubating the reaction mixture at 95 °C, after which the precipitated protein was removed by filtration. RebA-G was isolated from the reaction mixture by adsorption onto Lewatit® VP OC 1064 MD PH macroporous resin. Washing thoroughly with water removed any residual sugars and α-glucans. RebA-G was desorbed with 70% isopropanol. The resulting mixture was concentrated by evaporation *in vacuo* and ultimately freeze-dried, yielding pure RebA-G.

2.9. Cost analysis

The base case used for the cost analysis was RebA-G production in batch mode. The production costs were estimated by using the process mass balances as obtained in chapter 4 (RebA glucosylation)[70] and the current chapter (enzyme production (see also 2.1.) and downstream processing (DSP) of RebA-G (see also 2.6. and 2.7.). Guidelines as presented by Tufvesson et al.[233] were followed for the cost estimation. Several assumptions were made in order to simplify the economic model (see supplementary information for detailed information). In short: The production scale of the enzyme fermentation and the enzymatic glucosylation were both fixed at 10 m^3 (75% working volume). To run the fermentation, including set-up, harvesting and cleaning, 96 man-hours were allocated. The cost to obtain crude enzyme (homogenization, centrifugation to remove cell debris, and finally microfiltration) was assigned a value of €200/kg enzyme[233]. For the execution of the enzymatic glucosylation, including product recovery, 120 man-hours was considered. The cost of direct labor was assigned a value of €30/h, based on data from Eurostat (http://ec.europa.eu/eurostat). Supervision costs and indirect operating costs such as quality control corresponded to 100% of the direct labor costs.

Tables SI and SII summarize the various raw materials needed to run one production cycle (for definition, see supplementary information). The prices of the raw materials were obtained from the respective suppliers. Tables SIII-VII summarize the equipment related costs, utilities costs, and finally labor costs.

2.10. Sensory analysis

The sensory analysis was performed in individual tasting booths at the UGent Sensolab (Belgium) by a trained panel (9 persons), as described in chapter 4[70]. In short, all solutions contained 588 mg/L of sweetener. Their taste (sweetness, liquorice, astringency and bitterness) was evaluated by swirling the sample in the mouth for 5 sec after which the sample was expectorated. Aftertaste was evaluated 10 sec after swallowing the solution. Lingering based on the maximum

taste intensity was rated 1 min later. Sucrose reference solutions (5%, 7.5% and 10% sucrose scoring 5, 7.5 and 10, respectively) were provided. Water (Spa Reine**)** and plain crackers were used as palate cleansers between sampling. All samples were evaluated in duplicate.

Statistical analyses were performed with SPSS 23 (SPSS Inc., Chicago, USA). All tests were done at a significance level of 0.05. One-Way ANOVA was used to investigate any significant difference between the solutions. Testing for equal variances was executed with the Modified Levene Test. When conditions for equal variance were fulfilled, the Tukey test was used to determine differences between samples[210]. In case variances were not equal, a Games-Howell post-hoc test was performed[211].

2.11. Stability analysis

The stability of RebA and RebA-G was determined by conducting an accelerated shelf life study at 80 °C in acidic buffer solutions (50 mM citric acid (pH 2.8 and 3.8) and 50 mM phosphate (pH 2.8 and 3.8)). Five mg of product was added to 10 ml buffer. All sample solutions were transferred to 1.5 mL Eppendorf tubes and incubated for 72 h in a thermoblock at 80 °C. Samples were taken at 0, 24, 48 and 72 h, and subjected to HPLC analysis.

3. Results and discussion

3.1. Biocatalyst production

The biocatalyst production generally forms an important factor of the total cost of a biocatalytic process at industrial scale[233]. A productive enzyme fermentation, yielding a highly active biocatalyst, are thus two essential features of any cost-effective enzymatic process.

The N-terminally truncated Gtf180-ΔN-Q1140E (117 kDa) and the N- and V-terminally truncated Gtf180-ΔN-ΔV-Q1140E (95 kDa) were both produced by fermenting the respective recombinant *Escherichia coli* strains in 1 L shake flasks, resulting in the production of 1306 U/mL and 1646 U/mL of enzyme, respectively. Incubation of 5 U/mL of the single enzymes in the optimal RebA glucosylation reaction mixture, as determined in chapter 4 (84 mM RebA, 282 mM sucrose)[70], resulted in identical RebA-G synthesis. Indeed, domain V is known to be crucial only for glucansucrase processivity; its deletion impairs polysaccharide synthesis, however, the acceptor reaction is not affected[88]. Nevertheless, deletion of domains N plus V resulted in a higher enzyme yield, which may reflect its lower molecular mass compared to the N-terminally truncated variant. In addition, the induction temperature is known to influence Gtf180 enzyme expression. Lower temperatures typically result in higher enzyme yields, due to improved enzyme folding and decreased aggregation of the protein into inclusion bodies. This was previously shown for the expression of GtfB[234] and Gtf180-ΔN[99] and was confirmed by studying the effect of the induction temperature on Gtf180-ΔN-ΔV-Q1140E expression (data not shown). However, as it was technically very complicated to lower the temperature below 37 °C at 7.5 L scale and, hence, at industrial scale, the fermentation was performed at 37 °C.

The production of Gtf180-ΔN-ΔV-Q1140E at 7.5 L scale yielded 38,320 U of enzyme or 815 mg of protein per L fermentation medium, which was obtained from roughly 45 g/L wet biomass (Figure S1). From the results obtained in chapter 4, it was calculated that 43,480 U of enzyme are needed to produce 1 kg RebA-G in batch mode. Consequently, the achieved enzyme production represented a theoretical production of 880 g RebA-G per L fermentation medium or about 1080 kg RebA-G per kg Gtf180-ΔN-ΔV-Q1140E. According to Tufvesson et al., a productivity of 670-1700 kg product/kg enzyme is required for the biocatalytic production of fine chemicals with a typical cost between €15-100/kg product[233,235,236]. The obtained productivity clearly meets this requirement. The effect of the Gtf180-ΔN-ΔV-Q1140E production cost on the RebA-G

production cost is discussed in more detail later in this chapter (3.3. Cost analysis).

3.2. Downstream processing of glucosylated RebA

Glucosylated RebA (RebA-G) was selected as model product for the development of the downstream processing (DSP). Glucosylated stevioside (Stev-G) was purified following the same principle as applied for RebA-G purification. The two products are consequently interchangeable in the context of DSP.

3.2.1. Yeast fermentation

In addition to the synthesis of RebA-G, two types of by-products are produced by the enzyme: fermentable sugars such as fructose and glucose (and some remaining sucrose substrate), and α-glucan oligo- and polysaccharides. A common strategy to remove fermentable sugars is *Saccharomyces cerevisiae* fermentation[237], a very cheap and sustainable DSP option, provided that the desired product (RebA-G) is not metabolized. As illustrated in Figure 2, *S. cerevisiae* was indeed able to ferment sucrose, fructose and glucose, while no loss of RebA-G was observed. Besides CO_2, ethanol and glycerol were produced by the yeast as side-products. A yellow color was observed after fermentation, suggesting the formation of other side-products.

3.2.2. Selective precipitation

As the α-glucans were not metabolized by the yeast, their removal required an additional step. In theory, the differences in solubility between α-glucans and RebA-G in organic solvents may result in their selective precipitation. The precipitate can then be easily separated from the supernatant. A patent of Cargill, describing the use of antisolvent crystallization (precipitation) to separate steviol glycosides, offered a useful protocol[238]. In short, the yeast-treated reaction mixture was evaporated until a sugar content of 30 °Bx (degrees Brix, the sugar

content of an aqueous solution, equaling 30% weight sugar), after which 3 volumes of ethanol, methanol or isopropanol were gradually added to provoke crystallization. Unfortunately, the majority of α-glucans precipitated simultaneously with RebA-G, preventing their complete separation. Hence, it was decided to change strategy and attempt separation by selective adsorption.

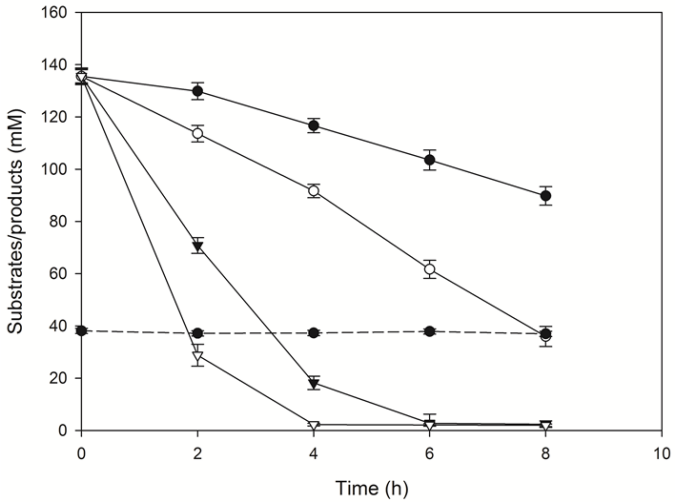


Figure 2. HPLC analysis of the utilization of fermentable sugars, present in RebA glucosylation reaction mixture (50 mM RebA, 125 mM sucrose), by *Saccharomyces cerevisiae* (● 5, ○ 10, ▼ 20, ▽ 30 g/L fresh yeast). RebA-G (dashed line) was not metabolized under all circumstances tested.

3.2.3. Selective adsorption

Selective adsorption of RebA-G onto several hydrophobic macroporous resins was evaluated. From the evaluated resins, Lewatit® VP OC 1064 MD PH displayed most potential and was consequently selected for further experimenting. The adsorption isotherms of RebA-G on this resin were determined at 30 °C, 35 °C and 40 °C and were described by the Langmuir model with good fit (R^2 of 0.92, 0.89 and 0.98 respectively, Figure 3). The highest adsorption capacity q_e was observed at 40 °C, suggesting an endothermic adsorption process, as previously described for the adsorption of RebA and stevioside onto mixed-mode macroporous resin[239]. Even at the lowest

temperature tested (30 °C), a sufficiently high adsorption efficiency (A) was obtained: as much as 92.0 g RebA-G could be adsorbed per kg resin with an A of 94%.

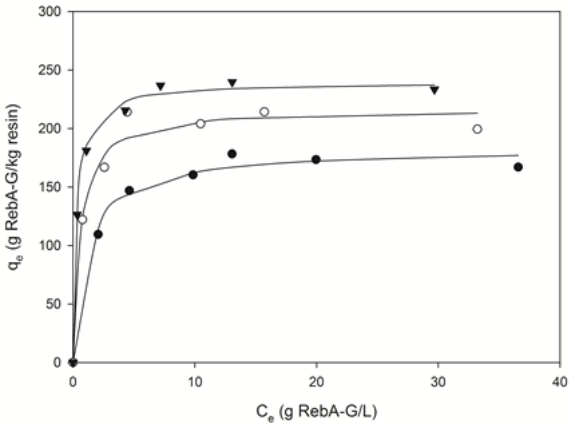


Figure 3. Adsorption isotherms of RebA-G on Lewatit VP OC 1064 MD PH resin at 30 °C (●), 35 °C (○) and 40 °C (▼). The obtained results were described by the Langmuir model with good fit (R^2 of 0.92, 0.89 and 0.98 respectively). $\mathbf{q_{max}}$ of 183, 217 and 240 g RebA-G/kg resin, respectively. **K** of 0.80, 1.73 and 3.1 L/g, respectively.

A compatible desorbing solvent was selected by evaluating the respective desorption capacities (q_d) at room temperature (Figure 4). Isopropanol (70 vol%) displayed most potential with a q_d of 91.3 g RebA-G/kg resin and was consequently selected as eluent for the production process.

Based on the previous results, an efficient continuous adsorption process was developed at room temperature, targeting complete adsorption with the highest dynamic adsorption capacity (DAC) possible. An important factor in the optimization of a continuous adsorption process is the flow rate. In general, low flow rates favor high adsorption efficiencies since the adsorbate has more time to interact with the adsorbent, preventing its breakthrough. The effect of the flow rate on the continuous adsorption of RebA-G onto Lewatit VP OC 1064 MD PH resin was therefore studied (Figure 5).

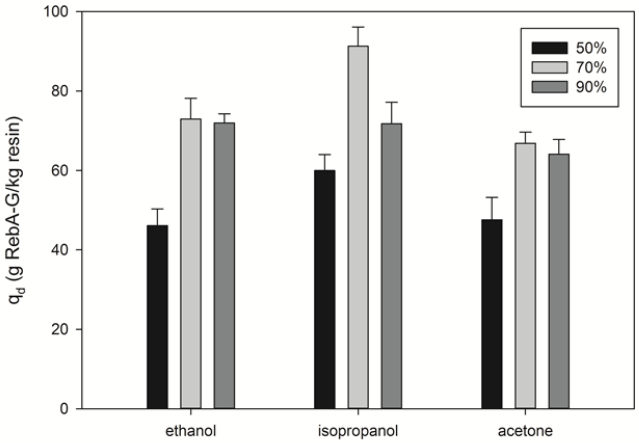


Figure 4. Desorption of RebA-G from Lewatit VP OC 1064 MD PH resin at room temperature, applying different percentages by volume of ethanol, isopropanol and acetone. Desorption capacities (**q_d**, defined in 2.5.) are given.

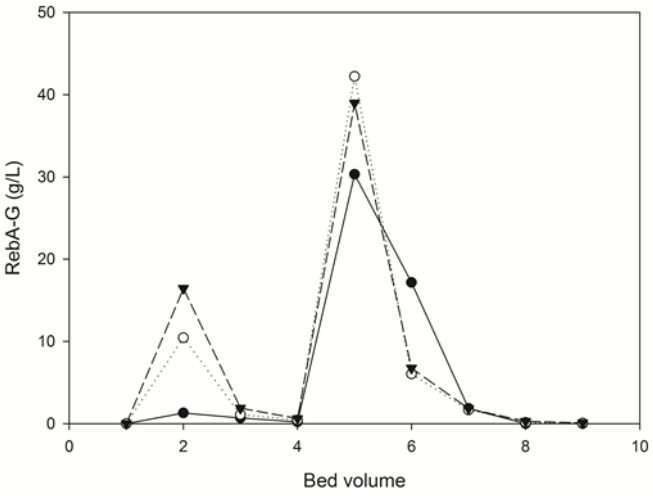


Figure 5. Continuous adsorption of the RebA glucosylation reaction mixture (at zero time: 84 mM RebA, 282 mM sucrose, 5 U/mL enzyme) onto Lewatit VP OC 1064 MD PH resin at a flow rate of 3 (●), 4.5 (○), and 6 (▼) BV/h. The amount of RebA-G (g/L) eluting from the resin is given.

Applying flow rates of 4.5 and 6 bed volumes per h (BV/h) caused considerable breakthrough of RebA-G, which resulted in suboptimal adsorption efficiencies of

79% and 68%, respectively. The application of a flow rate of 3 BV/h resulted in a near complete adsorption (96%) and a DAC of 95.7 g RebA-G/kg resin, similar to the optimal static adsorption capacity (92.0 g RebA-G/kg resin). Only 3 bed volumes of 70% isopropanol were subsequently needed for a near complete recovery of the product. The successful separation of RebA-G from the fermentable sugars and α-glucans is clearly shown by TLC analysis (Figure S2). The developed continuous adsorption was consequently used to treat 4 L RebA glucosylation reaction mixture (containing 60 g/L RebA-G), yielding 250 g of amorphous RebA-G, composed of (by mass) 4% RebA, 60% monoglucosylated RebA (RebA-G1) and 36% multiglucosylated RebA (RebA-G2+) (Figure S3). As the applied desorbing solvent (70% isopropanol) can be reused after evaporation, the developed DSP is not only efficient (almost no loss of RebA-G) but also eco-friendly.

3.3. Cost analysis of stevia glucosylation

3.3.1. Base case: RebA glucosylation

Any new production process must meet a number of criteria to be successfully implemented at industrial scale. Besides analyzing safety matters and environmental and legal issues, the process economics need to be evaluated[240]. Assessing the economic feasibility of a process is achieved by performing a production cost estimation, a powerful tool to guide research and development activities in order to turn lab-scale processes into commercially viable ones. Production costs can be divided into two categories: capital investment (CapEx) and operation costs (OpEx), both considered for the cost estimation of the base case (for detailed information, see supplementary information). A production scale of 10 m^3 for both the enzyme fermentation and the enzymatic glucosylation was assumed. It should be noted that production volume has a major impact on the production costs: increasing the volume from 1 m^3 to multiple cubic meters is accompanied by a cost reduction of several orders of magnitude[233]. The assignment of an adequate production scale is typically based on the expected sales, however, that was outside the scope of this chapter.

Tables I and II summarize the results obtained for the base case analysis. For the detailed results, see supplementary information (Tables SI-VII). Figure 6 illustrates the cost contribution of the different resources on the enzyme production (**A**) and RebA-G production, including DSP (**B**).

Table I. Cost analysis of Gtf180-ΔN-ΔV-Q1140E production in batch mode.

Resources	Cost (€/kg enzyme)	Relative cost (%)
Fermentation	2106	91
Raw materials	114	5
Utilities	34	1
Labor	950	41
Equipment	1008	44
Enzyme recovery	200	9
Total	2306	100

Table II. Cost analysis of RebA-G production and glucosylated steviol glycosides (GSG) production in batch mode, using RebA and low-grade stevia extract (steviol glycosides, SG) as acceptor substrate, respectively.

Resources	Cost (€/kg RebA-G)	Relative cost (%)	Cost (€/kg GSG)	Relative cost (%)
Biocatalysis	70.7	79	41.3	66
Enzyme	2.1	2	3.5	6
Sucrose	0.6	1	1.8	3
RebA/SG	56.5	63	17.1	27
Utilities	< 1	< 1	< 1	< 1
Labor	4.2	5	6.9	11
Equipment	7.1	8	11.6	19
DSP	18.5	21	21.3	34
Resin	12.7	14	12.7	20
IPA	1.1	1	1.1	2
Utilities	< 1	1	< 1	1
Labor	4.2	5	6.9	11
Total	89.2	100	62.6	100

A common drawback to implement an enzymatic process at industrial scale is the relatively high cost of the biocatalyst[233]. Although the enzyme production cost for the base case in itself is relatively high (€2306/kg enzyme), cost analysis revealed that the biocatalyst cost (€2.1/kg RebA-G) represents only 2% of the total RebA-G production cost. As rule of thumb, €1.5/kg product is an allowable cost contribution of the biocatalyst to the total production cost of fine chemicals, i.e. products with a typical cost between €15-100/kg product[233,235,236]. The estimated biocatalyst cost for the base case is roughly of the same order and consequently fulfills this requirement. The enzyme production consists mostly of labor- and equipment costs, which is typical for fermentation processes at 10 m^3 scale or lower (Figure 6).

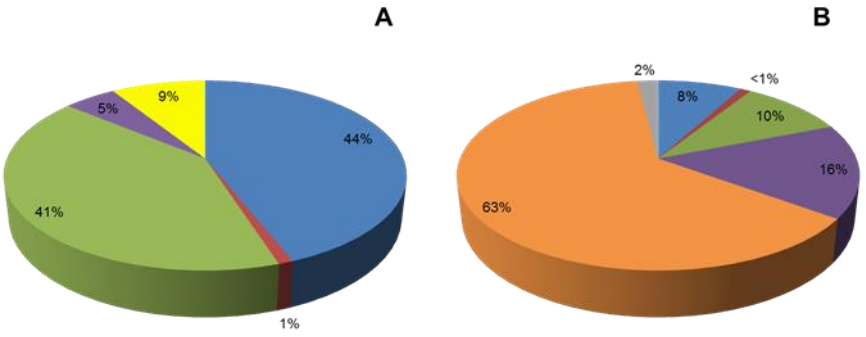


Figure 6. Cost contribution of various resources to the enzyme production cost (**A**) and the RebA-G production cost (**B**): ● Labor, ● Equipment, ● Raw materials, ● Utilities, ● Enzyme recovery, ● RebA, ● Enzyme (Gtf180-ΔN-ΔV-Q1140E).

The low cost contribution of Gtf180-ΔN-ΔV-Q1140E to the RebA-G production cost is explained by the high biocatalyst activity (k_{cat} for sucrose as donor substrate is 300/s)[98], allowing the addition of a relatively low amount of enzyme (5 U/mL) to the glucosylation reaction medium. As discussed previously, this results in a theoretical production of 880 g RebA-G per L fermentation medium. Nevertheless, there is still margin to reduce the fermentation cost. By performing the fermentation in fed-batch mode much higher cell densities (up to OD_{600} of 100) and consequently higher enzyme titers (up to 10 g/L) may be obtained[233]. As a result, labor- and equipment costs would decrease substantially.

The total RebA-G production cost equals €89.2/kg RebA-G, representing an extra cost of 12% compared to high-purity RebA, sold at €80/kg (Tereos PureCircle Solutions). Since the price of sucrose is roughly €0.70/kg and RebA-G is around 150 times sweeter than sucrose, a selling price of €105/kg RebA-G can still be considered as competitive. If RebA-G is sold at this price, a profit of €15/kg RebA-G is obtained.

As demonstrated in chapter 4, glucosylating RebA in fed-batch mode resulted in higher production yield compared to the batch production (270 vs 115 g/L RebA-G). However, the much lower productivity per kg enzyme (1080 vs 253 kg RebA-G/kg enzyme), due to the addition of 10 times more enzyme, was translated into an undesirable production cost increase of €11.1/kg RebA-G. From an economic perspective, the batch reaction is thus preferred. It has to be noted that further optimization of the fed-batch reaction, thereby decreasing the applied enzyme activity (U/mL), may allow further reduction of the production cost.

3.3.2. Alternative case: glucosylation of low-grade stevia extract

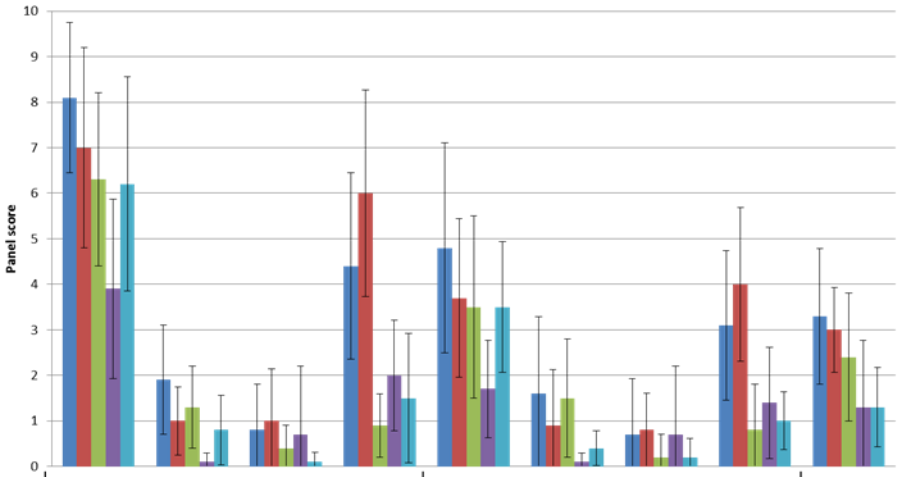
Remarkably, the base case analysis shows that RebA-G production cost is dominated by the cost of the acceptor substrate RebA (63%). A major cost reduction consequently may be achieved by changing the acceptor substrate: instead of high-purity RebA, low-grade stevia extract could be used as substrate, which is commonly marketed for roughly €40/kg (Tereos PureCircle Solutions, or half of the price for high-purity RebA (€80/kg, Tereos PureCircle Solutions). Moreover, glucosylating mixtures of steviol glycosides also offers the possibility to valorize stevioside, whose glucosylation by Gtf180-ΔN-Q1140E was already demonstrated in chapter 5. The glucosylation of different self-made stevioside/RebA mixtures and of low-grade stevia extract (Steviasol), basing the experimental design on the optimal reaction conditions for stevioside glucosylation, as determined in chapter 5 (31 mM steviol glycosides, 524 mM sucrose), was also successful (data not shown), yielding roughly 50 g/L glucosylated steviol glycosides (GSG). The economic model of the base case was changed accordingly, resulting in a production cost of €62.7/kg GSG, a

substantial reduction of almost 30% compared to RebA-G production (Table II). The glucosylation of a mixture of steviol glycosides consequently provides a substantial economic advantage over the glucosylation of high-purity RebA.

3.4. Sensory analysis of glucosylated stevioside/RebA mixture

The previous paragraph demonstrated the economic advantage of glucosylating a mixture of steviol glycosides. However, the sensory properties of the obtained product must at least be similar to those of RebA-G to justify this change of acceptor substrate. As proof of concept, a sensory analysis of a glucosylated mixture (obtained from glucosylating low-grade stevia extract), containing 50% Stev-G and 50% RebA-G (RebA-G/Stev-G), was performed by a trained panel, evaluating 9 different taste attributes. The mean scores of the attributes of the sweetened water solutions are shown in Figure 7 and were compared with the sensory properties of RebA-G and Stev-G.

The sensory properties of the RebA-G/Stev-G mixture, compared to RebA and stevioside, were clearly improved, mainly due to a significant reduction in bitterness. Interestingly, the relatively low sweetness of Stev-G was compensated by the high sweetness of RebA-G, yielding a product with excellent edulcorant properties. RebA-G was identified as the most promising high-intensity sweetener, combining a high sweetness with a very low off-flavor intensity. If taste has the highest priority, the glucosylation of high-purity RebA is thus preferred, as the presence of stevioside deteriorated the sensory properties of the RebA-G/Stev-G.



Sample	Taste				Aftertaste				Lingering
	Sweet	Liquorice	Astringent	Bitter	Sweet	Liquorice	Astringent	Bitter	
RebA	8.1^{A}	1.9^{A}	0.8^{A}	4.4^{AB}	4.8^{A}	1.6^{A}	0.7^{A}	3.1^{AB}	3.3^{A}
Stev	7.0^{AB}	1.0^{A}	1.0^{A}	6.0^{A}	3.7^{A}	0.9^{A}	0.8^{A}	4.0^{A}	3.0^{A}
RebA-G	6.3^{AB}	1.3^{A}	0.4^{A}	0.9^{B}	3.5^{A}	1.5^{A}	0.2^{A}	0.8^{B}	2.4^{A}
Stev-G	3.9^{B}	0.1^{B}	0.7^{A}	2.0^{B}	1.7^{B}	0.1^{A}	0.7^{A}	1.4^{B}	1.3^{A}
RebA-G/ Stev-G	6.2^{AB}	0.8^{AB}	0.1^{A}	1.5^{B}	3.5^{AB}	0.4^{A}	0.2^{A}	1.0^{B}	1.3^{A}

Figure 7. Sensory analysis of solutions containing 588 mg/L RebA, stevioside (Stev), RebA-G, Stev-G and a 50:50 RebA-G/Stev-G mixture. Sweetness, bitterness and off-flavors were rated on a scale of 10. Mean values are given in the table. ^{A,B}: different letters indicate significant differences ($p<0.05$) between solutions following one-way ANOVA and post-hoc test.

3.5. Stability analysis of glucosylated stevia products

In order to be used in beverages as high-intensity sweetener, the glucosylated stevia products need to be stable during storage over prolonged periods of time. Commercial soft drinks such as cola and lemonade typically have a low pH in the range of 2.8-3.8[241]. Several studies have indicated that the rate and extent of degradation of RebA and stevioside are dependent on pH, buffer type, buffer concentration and temperature[241-243]. Degradation typically involves the hydrolysis of the glycosidic linkages of the steviol glycosides, deteriorating their flavor. In case of RebA-G, it is particularly important that the enzymatically introduced (α1→6) linkage is stable so that the improved taste is maintained. The

stability of RebA and RebA-G was determined by conducting an accelerated shelf life study at 80 °C and pH 2.8 and 3.8 (50 mM citric acid and 50 mM phosphate), as shown in Figure 8.

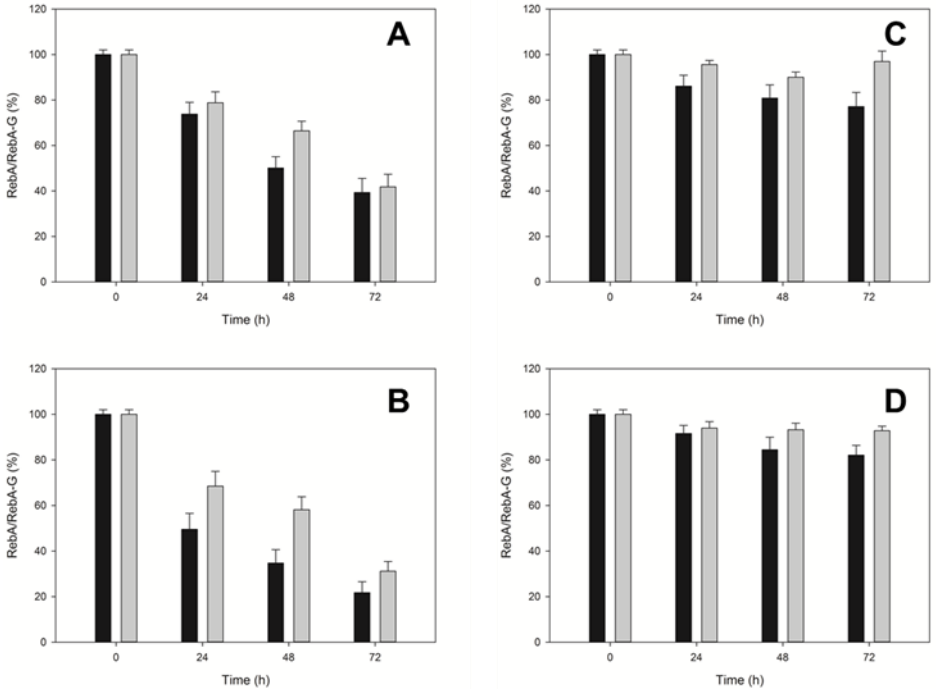


Figure 8. Stability of 500 mg/L RebA (*black*) and RebA-G (*grey*) in different acidic solutions. **A** 50 mM phosphate, pH 2.8; **B** 50 mM citric acid, pH 2.8; **C** 50 mM phosphate, pH 3.8; **D** 50 mM citric acid, pH 3.8.

RebA-G appears to be sufficiently stable to be applied in low pH soft drinks, it actually displayed a higher stability than RebA in all buffers tested. Buffer type and pH strongly influenced the degradation rate. In general, HPLC analysis revealed that incubations in citric acid buffer resulted in more degradation than in phosphate buffer. At pH 2.8 the half-life ($t_{1/2}$) of RebA-G in phosphate and citric acid buffer, was 63.9 and 52.0 h, respectively. In contrast, RebA-G was only slightly degraded at pH 3.8, even after 72 h ($t_{1/2}$ of 779.8 and 520.6 h, in phosphate and citric acid buffer, respectively). HPLC analysis suggested that RebA-G was degraded into RebA and subsequently into stevioside. Other degradation products could not be detected.

4. Conclusions

This chapter demonstrates the technical and economic feasibility of glucosylating steviol glycosides with the Gtf180-ΔN-ΔV-Q1140E enzyme. The strength of the technology lies in the high activity of this biocatalyst, suppressing the associated costs, as revealed by the cost analysis. Furthermore, selective adsorption of the glucosylated products onto macroporous resin offers a straightforward and efficient method for their purification. Based on the cost analysis, it was suggested to glucosylate mixtures of steviol glycosides rather than high-purity RebA or stevioside, as this results in a 30% cost reduction. From a taste quality perspective, the glucosylation of high-purity RebA is nevertheless preferred. Finally, the glucosylation reaction was successfully scaled up to 7.5 L scale, allowing the production of samples for food safety analysis and testing in several food products, such as chocolate, candy, etc. In future work demonstration of the complete process at pilot-plant scale will be an essential requirement to survive the so-called valley of death, which is faced by many novel technologies.

5. Supplementary information

5.1. Cost analysis: Assumptions and economic model

The base case used for the cost analysis is the glucosylation of high-purity RebA in batch mode at optimal reaction conditions (84 mM RebA, 282 mM sucrose, 5 U/mL Gtf180-ΔN-ΔV-Q1140E)[70].

General assumptions

- Plant is located in Western Europe.
- The facilities are part of a multi-purpose plant with shared utilities, services, offices, quality control, etc.
- Capital investment included equipment, instrumentation and piping needed for RebA-G production, excluding DSP.
- Scale: One bioreactor and one blending tank of 10 m^3 with 75% working volume, shared for fermentation and biocatalysis.
- Costs are given in €.

Production is as follows:

- One optimal fermentation at 10 m^3 yields enzyme to perform 8 enzymatic glucosylations at 10 m^3. As a result, 1 week of fermentation is followed by 8 weeks of biocatalysis, defined as 1 production cycle.
- Equipment is used to perform 5 production cycles per year, yielding 34.5 metric tons of RebA-G.
- The volume consumption of stevia is expected to reach 8,507 metric tons per year by the end of 2020[44]. The assumed production would consequently represent 0.4% of the total stevia market.

Raw materials

Table SI. Raw materials needed to run one fermentation batch at 10 m^3.

Raw materials	Quantity (kg)	Price (€/kg)
Glucose	225	0.5
Tryptone	75	3.0
Yeast extract	37.5	4.0
NaCl	37.5	2.1
Ampicillin	0.75	38.0
IPTG	0.18	154.0
H_2O	7125	0.01
Total cost		**694 €/batch**

It was assumed that the adsorption resin was reused during one year of production (40 times) and that the eluent (70% isopropanol) was recovered after evaporation and consequently reused during one year of production.

Table SII. Raw materials needed to run one enzymatic glucosylation reaction, including DSP, at 10 m^3.

Raw materials	Quantity (kg)	Price (€/kg)
Enzyme	0.8	2,306
Sucrose	723	0.7
RebA	609	80
$CaCl_2.2H_2O$	1	122
H_2O	6200	0.01
Resin	9,400	46.7
Isopropanol	25,875	1.5
H_2O	14,110	0.01

Calculation of the capital investment

To calculate the total installed cost (TIC), the total purchase cost was multiplied by the Lang factor $K = 5$[244].

To calculate the capital investment cost per production cycle, the total investment cost, equal to TIC, was converted into an equivalent annual cost by multiplying TIC with the annuity factor k:

$$k = \frac{i}{1 - (1 + i)^{-t}}$$

with the interest rate $i = 0.07$ and the equipment lifetime $t = 15$ yrs.

As one production cycle consists of 1 fermentation batch and 8 enzymatic glucosylations, the equipment cost per production cycle was allocated as follows: 11% to the fermentation, 89% to the glucosylation.

Table SIII. Equipment related costs.

Equipment	**Specifications**	**Price (€)**
Blending tank	10 m^3	51,500
Pump, gear	5 m^3/h	33,000
Heat exchanger	10 MW	50,000
Air compressor	10 m^3/min	54,000
Fermenter	10 m^3	175,000
Total purchase cost		**€363,500**
Total installed cost (TIC)		€1,817,500
Annuity (k = 0.14)		€199,500/year
Maintenance		€26,000/year
Other		€49,000/year
Annual cost		**€275,000/year**
Cost per production cycle		**€55,000**
Cost per fermentation batch		€6110
Cost per enzymatic glucosylation		€6110

Utilities

The price of electricity was fixed at €0.1/kWh.

Table SIV. Utilities needed to run one fermentation batch at 10 m^3.

Utilities	Specifications	kWh
Sterilization (20-140 °C)	4,187 J/kg.K	1047
Aeration (1 vvm)	5 kW/m^3	400
Agitation (500 rpm)	5 kW/m^3	400
Waste treatment	€2/m^3	
Total utilities cost		**€207/batch**

Table SV. Utilities needed to run one enzymatic glucosylation at 10 m^3.

Utilities	Specifications	kWh
Agitation (185 rpm)	1.9 kW/m^3	57
Heating (37 °C)	4,187 J/kg.K	148
Waste treatment	€2/m^3	
Total utilities cost		**€26/batch**

At kg scale, the last step of RebA-G production consisted of freeze-drying to remove the residual water. As freeze-drying is technically and economically not feasible at ton scale, spray drying was considered as last purification step for the cost analysis. Firstly, the mixture obtained after elution (2% solids) was evaporated until a solid content of 50% by means of a mechanical vapor recompression (MVR). Energy consumption of MVR and spray drying were based on Fox et al.[245].

Table SVI. Utilities needed to perform DSP of RebA-G.

Utilities	kWh
Evaporation	476
Spray drying	1917
Total utilities cost	**€249/batch**

Labor

Table SVII. Labor cost.

Utilities	Man-hours
Fermentation	96
Biocatalysis + DSP	120

5.2. Figures

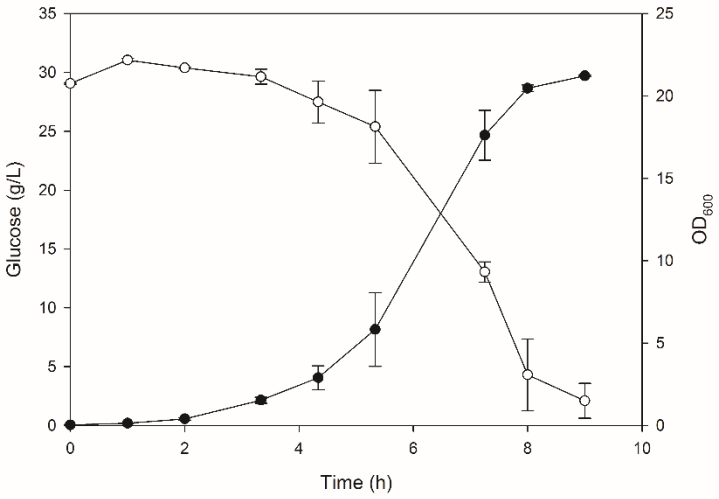


Figure S1. Biocatalyst production: growth of the recombinant *Escherichia coli* BL21 (DE3) strain, expressing Gtf180-ΔN-ΔV-Q1140E, in LB medium supplemented with 30 g/L glucose. Induction with IPTG after 4 h of incubation. ● OD_{600}, ○ glucose.

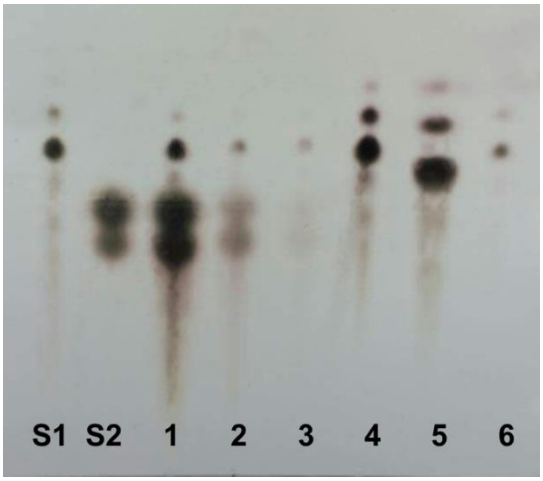


Figure S2. Continuous adsorption of RebA glucosylation reaction mixture (84 mM RebA, 282 mM sucrose, 5 U/mL enzyme) onto Lewatit VP OC 1064 MD PH resin at a flow rate of 4.5 BV/h. **S1** RebA-G; **S2** glucose, fructose and sucrose; **1-3** wash fractions; **4-6** elution fractions.

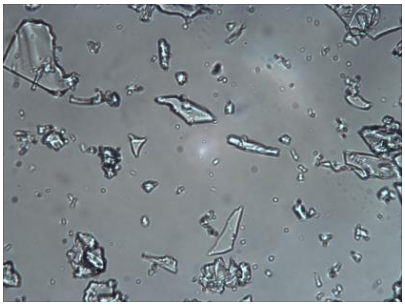


Figure S3. Glucosylated RebA (RebA-G), obtained after selective adsorption on macroporous resin, evaporation *in vacuo* and ultimately freeze-drying.