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ORIGINAL PAPER

Non-water miscible ionic liquid improves biocatalytic production of geranyl glucoside with *Escherichia coli* overexpressing a glucosyltransferase

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Abstract Whole cells of *Escherichia coli* overexpressing a glucosyltransferase from *Vitis vinifera* were used for the glucosylation of geraniol to geranyl glucoside. A high cell density cultivation process for the production of whole-cell biocatalysts was developed, gaining a dry cell mass concentration of up to 67.6 ± 1.2 g L^{-1} and a glucosyltransferase concentration of up to 2.7 ± 0.1 g protein L^{-1} within a process time of 48 h. Whole-cell batch biotransformations in milliliter-scale stirred-tank bioreactors showed highest conversion of geraniol at pH 7.0 although the pH optimum of the purified glucosyltransferase was at pH 8.5. The biocatalytic batch process performance was improved significantly by the addition of a water-immiscible ionic liquid (N-hexylpyridinium bis(trifluoromethylsulfonyl)imid) for in situ substrate supply. The so far highest final geranyl glucoside concentration (291 ± 9 mg L^{-1}) and conversion (71 ± 2 %) reported for whole-cell biotransformations of geraniol were achieved with 5 % (v/v) of the ionic liquid.

Keywords Whole-cell biotransformation · *Escherichia coli* · Geranyl glucoside · Glycosyltransferase · Ionic liquid

Introduction

The monoterpene alcohol geraniol represents one of the most important substances in the flavor and fragrance industry [1]. However, its high volatility and low water solubility require stabilization strategies to limit aroma degradation. One unique stabilization method to facilitate controlled and slow release of the fragrance is glycosylation by plant-derived glycosyltransferases, which can be expressed recombinantly in *Escherichia coli* (*E. coli*) [2–4]. The recombinant *E. coli* can be used in whole-cell biotransformations, which benefit from easy catalyst preparation, high enzyme stability, high product selectivity and intracellular cofactor regeneration [5]. Glycosyltransferases catalyze the transfer of a sugar moiety from an activated sugar donor, in this case UDP-glucose, to the substrate molecule. This reaction requires the continuous supply of the cofactor UDP-glucose, which can be regenerated intracellularly in whole-cell biotransformations. To address the low water solubility and toxic effects of the substrate, a biphasic system can be used [6]. In this respect, water-immiscible ionic liquids (IL's) are a promising alternative to common organic solvents, which often feature cell toxicity, negative environmental impacts and a volatile and flammable nature [7, 8]. IL's in contrast are easy-to-handle, stable solvents with no measureable vapor pressure, which can be adapted to particular processes by modifications of either the cation or the anion [9, 10]. Several studies have already demonstrated that the solvents show good biocompatibility and that a biphasic ionic liquid/water system can improve the process efficiency of whole-cell biocatalysis compared to pure aqueous systems or biphasic systems with the second phase being a common organic solvent [6, 7, 11, 12].

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Table 1 Product concentration and maximal conversion achieved in batch biotransformations for the production of geranyl glucoside at standard conditions (M9 mineral medium, pH 7.0, 0.2 g L^{-1} geraniol, 20 h, *E. coli* VvGT14a with 4.3 g L^{-1} dry cell mass) and at improved reaction conditions by the addition of the IL (HPYR)(NTF) as second phase (0.1 M KPi buffer, pH 7.0, 0.2 g L^{-1} geraniol, 5 % IL, 20 h, *E. coli* VvGT14a with 4.3 g L^{-1} dry cell mass) in comparison to the so far best reported values [4]

Biotransformation	Product concentration, mg L^{-1}	Maximal conversion, %
E. coli VvGT14a in buffer	118 ± 13	28 ± 3
E. coli VvGT14a in two-phase system (IL/buffer)	291 ± 9	71 ± 2
Literature [4]	165	41

In the present study, recombinant *E. coli* were grown in high cell density cultivations (HCDC) in stirred-tank bioreactors for the heterologous expression of the glucosyltransferase VvGT14a from *Vitis vinifera* [3]. The *E. coli* cells were subsequently used as whole-cell biocatalysts for the production of geranyl glucoside from geraniol and glucose. A biphasic system with the non-water miscible IL N-hexylpyridinium bis(trifluoromethylsulfonyl)imid [(HPYR)(NTF)] was used as substrate reservoir for the biocatalytic step. Reactions took place in a parallel reaction system developed at the Institute of Biochemical Engineering [13, 14]. This system allows for the parallel operation of 48 mL-scale gas-inducing stirred-tank bioreactors under controlled process conditions and is a convenient tool for process development. Dennewald et al. [14] proved the system's suitability for biphasic whole-cell biotransformations with non-water miscible IL's. Here, the parallel bioreactor system was applied to evaluate the influence of different media, pH and the addition of the IL (HPYR)(NTF) on the transformation of geraniol to geranyl glucoside. As the enormous multitude of possible anion/cation combinations of ILs results in up to 10^{18} possible compounds which leads to a large choice of ionic liquids, [15] and no clear structure–activity relationship is known to date, the choice of the applied IL (HPYR)(NTF) has been made on the basis of previous studies. (HPYR)(NTF) has been used successfully for process design of whole-cell biotransformations in the applied miniaturized bioreactors and the following transfer of the processes to liter-scale. Additionally, (HPYR)(NTF) has been proven to possess a high biocompatibility towards *E. coli* cells [14]. Moreover, pyridinium based ILs were reported to be catabolized by an activated sludge microbial community, whereby the pollution of water can be avoided [16, 17].

The aims of the study were to establish an efficient process for the production of the whole-cell biocatalysts, first. In a next step, the produced biocatalysts were applied for the improvement of the biotransformation of geraniol to geranyl glucoside with regard to product concentration and conversion to open new possibilities for further investigations on designing new industrial relevant processes including product recovery.

Materials and methods

Microorganism

An *E. coli* BL21(DE3) strain harboring a pET29a(+) plasmid with the gene for the glucosyltransferase VvGT14a from *Vitis vinifera* was used for the HCDC and the following biotransformation, which is henceforth abbreviated as *E. coli* VvGT14a.

Media, seed culture preparations and inoculation

Pre-cultures were grown in low salt LB medium (5 g L^{-1} yeast extract, 10 g L^{-1} peptone, 5 g L^{-1} NaCl, 30 mg L^{-1} Kanamycin), whereas seed cultures and all HCDC were carried out in defined medium according to Riesenberg et al. [18] with the following variations: No thiamin was needed for the used strain and another antifoam agent (Antifoam 204, Sigma Aldrich, Taufkirchen, Germany) was added manually, when foaming occurred. Glucose served as batch substrate at a concentration of 2 g L^{-1}. Kanamycin was used as selection marker at a concentration of 30 mg L^{-1}. For fed-batch cultivations, a feeding solution of 600 g L^{-1} glucose and 20 g L^{-1} $MgSO_4$ was prepared.

The biotransformations were carried out in either potassium phosphate buffer (0.1 M), defined medium according to Riesenberg et al. [18] or M9 mineral medium (1.0 g L^{-1} NH_4Cl, 100 µM $CaCl_2$, 6.0 g L^{-1} Na_2HPO_4, 3.0 g L^{-1} KH_2PO_4, 1 mM $MgSO_4$, 0.5 g L^{-1} NaCl) with 10 g $L^{-1}$of glucose as initial substrate concentration. H_3PO_4 and KOH were used to adjust the pH. Geraniol was added as substrate at concentrations of 0.2, 2.0, and 5.0 g L^{-1}. The ionic liquid (HPYR)(NTF) was used for biphasic biotransformations at concentrations of 5 % (v/v) or 20 % (v/v).

Pre-cultures were prepared in 15 mL test tubes with 5 mL LB medium. The tubes were inoculated with 100 µL of a frozen cell stock. The cells were grown to stationary phase at 200 rpm and 30 °C. Seed cultures were prepared in 500 mL Erlenmeyer flasks without baffles containing 95 mL defined medium. The flasks were inoculated with 5 mL of the pre-culture. The cells were grown to an optical density at 600 nm of $OD_{600} = 2$ at 250 rpm and 37 °C on a rotary shaker. All bioreactor cultivations were inoculated to $OD_{600} = 0.5$ after harvesting the seed culture by centrifugation (4.500 rpm, 5 min) and suspending the cells in the batch medium.

Cultivation systems, process monitoring, and control

The fed-batch cultivations on a liter-scale were carried out in a 7.5 L stirred-tank bioreactor equipped with three Rushton turbines (Labfors 2, Infors HT, Bottmingen, Switzerland), which was operated with an initial volume of 2 L. The pH was maintained at pH 6.8 with 25 % (w/v) H_3PO_4 and 12.5 % (v/v) NH_3. The dissolved oxygen concentration (DO) was maintained above 25 % air saturation by automatically increasing the stirrer speed up to 1200 rpm and the aeration up to 4.0 vvm.

The parallel biotransformations were performed in sterile single use stirred-tank bioreactors (provided by 2mag AG, Munich, Germany) with an initial reaction volume of 10 mL. The bioreactors were operated with gas-inducing stirrers in a parallel stirred-tank bioreactor system (bioREACTOR48, 2mag AG, Munich, Germany). The speed of the gas-inducing impellers was set to 2200 rpm. The headspace of each milliliter reactor was rinsed with 0.1 L min^{-1} sterile air. The sterile air was pre-saturated with water at room temperature (23 °C) by applying an L-sparger in a 1-L bottle. Losses in liquid volume by evaporation were prevented in the parallel stirred-tank bioreactors by setting the headspace cooling of each reactor to 20 °C.

HCDC and biotransformation protocols

For the HCDC of *E. coli* VvGT14a, a feeding profile was initiated immediately after inoculation with 0.35 g L h^{-1} glucose to control the growth rate to $\mu_{set} = 0.20\ h^{-1}$ at 37 °C. After 18 h, the maximal feeding rate of 8.75 g L^{-1} was achieved and was kept constant the following 2 h to avoid oxygen limitation. Afterwards, the temperature was decreased to 20 or 25 °C and 100 µM IPTG were added for the induction of the protein expression. The feeding rate was decreased to 3.25 g L^{-1} h^{-1} to avoid substrate accumulation during production phase (28 h).

For the biotransformation, the biocatalysts were harvested by centrifugation (4500 rpm, 5 min) and were suspended in the respective medium to $OD_{600} = 10$ (4.3 g L^{-1} dry cell mass). All biotransformations were carried out for 20 h at 20 °C. When biotransformations were performed in buffer, geraniol was added to the aqueous phase. For biotransformations with ionic liquids, geraniol was added to the non-water miscible ionic liquid phase to realize in situ substrate supply.

Monitoring of cell, glucose, acetate, and ammonium concentrations

Samples from HCDC were diluted manually with phosphate buffered saline (PBS; 8 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 1.44 g L^{-1} Na_2HPO_4, 0.24 g L^{-1} KH_2PO_4, pH 7.4). A GENESYS™ 10S UV–Vis Spectrophotometer (Thermo Electron Scientific Instruments LLC, Madison, USA) was used for the determination of OD_{600}. Dry cell weight (DCW) was measured gravimetrically by centrifuging (13,000 rpm, 3 min) and drying (80 °C, for at least 24 h) 2 mL of culture broth. Glucose, acetate and ammonium concentrations were measured enzymatically using a D-glucose kit, an acetic acid kit and an ammonia kit (R-Biopharm AG, Darmstadt, Germany).

Monitoring geraniol and geranyl glucoside concentration

Compounds were identified by their retention times, mass spectra, and product ion spectra in comparison with the data determined for authentic reference materials. The quantification was carried out by interpolation of the peak areas obtained by LC-UV-ESI-MS^n with standard curves. An Agilent LC/MSD Trap XCT ion trap mass spectrometer connected to a Jasco HPLC system (1500 series) equipped with a quaternary pump and a variable wavelength detector was utilized. Components were separated with a Phenomenex Synergi Max-RP column (50 mm long × 2.0 mm i.d., particle size 2 µM; Phenomenex, Aschaffenburg, Germany). HPLC was performed with the following binary gradient system: solvent A, water with 0.1 % formic acid and solvent B, 100 % Methanol with 0.1 % formic acid. The gradient program was as follows: 0–0.1 min, 30 % B, 0.1–6 min, 100 % B, 6.1–10 min, 30 % B. Injection volume was 5 µL and the flow rate was 0.4 mL min^{-1}. The detection wavelength was 210 nm. The ionization parameters were as follows: the voltage of the capillary was 4000 V and the end plate was set to −500 V. The capillary exit was 121 V and the Octopole RF amplitude 150 Vpp. The temperature of the dry gas (N_2) was 330 °C at a flow of 9 l min^{-1}. Tandem MS was carried out using helium as the collision gas (3.56×10^{-6} mbar) with 1 V collision voltage. Spectra were acquired in positive and negative ionization mode and target ions were fragmented in auto MS^2 mode.

Quantification of the glucosyltransferase VvGT14a

The cells were diluted in PBS to $OD_{600} = 2.5$ and were prepared for a SDS-PAGE analysis by incubation with Laemmli-buffer at 95 °C for 5 min. The probes were applied to SDS-PAGE gels containing 12.5 % acrylamide. A constant current of 35 mA was applied for a minimum of 60 min. The gels were stained according to Fairbanks et al. [19]. For the quantification of the protein concentration, a standard of bovine serum albumin was additionally applied to the gel. The gels were digitized by scanning (hp Scanjet 5470c, Hewlett-Packard GmbH, Berlin, Germany). The amount of dye adsorbed by the proteins was determined by densitometric analysis using the Win Image Studio Lite 4.0.21 software (LI-COR, Lincoln, USA).

Results and discussion

HCDC for the production of whole-cell biocatalysts

A HCDC protocol was established with *E. coli* VvGT14a in stirred-tank bioreactors on a 2 L-scale for the production of whole-cell biocatalysts for the biotransformation of geraniol to the corresponding glucoside. Preliminary studies showed that the growth rate had to be controlled to values below $\mu_{set} = 0.22\ h^{-1}$ during biomass production phase to avoid formation of inhibitory concentrations of acetate (data not shown). Two fed-batch processes were performed at a temperature of 20 °C after induction (see Fig. 1), gaining biomass concentrations of 67.6 ± 1.2 and 63.7 ± 1.2 g L^{-1} within a process time of 48.0 and 48.5 h. The dynamics of biomass production were identical during both cultivations. With an OD_{600} of 131.3 ± 3.2, the so far reported maximum final biocatalyst concentration could nearly be doubled for the production of *E. coli* whole-cell biocatalysts for the production of geranyl glucoside [4]. The growth rate during biomass production phase could be controlled to 0.21 ± 0.01 h^{-1} by implementing an exponential glucose feeding. Thereby, the glucose concentrations in the stirred-tank reactor were always kept below 0.15 g L^{-1} after depletion of the initial 2 g L^{-1} glucose. Maximal acetate concentrations of 0.50 g L^{-1} and 0.86 g L^{-1} were measured after biomass formation (20 h), which were significantly below inhibitory acetate concentrations of 5 g L^{-1} [20]. Ammonium limitation as well as inhibition could be avoided, as ammonium concentrations were kept between 1–2 g L^{-1}. The final glucosyltransferase concentrations of 2.1 ± 0.4 and 2.7 ± 0.1 g protein L^{-1} represent the lower limit of typical recombinant protein concentrations achievable with *E. coli* in HCDC processes (2–10 g L^{-1} [21]). The increase of the temperature during recombinant protein production phase to 25 or 30 °C delivered lower recombinant protein yields (data not shown).

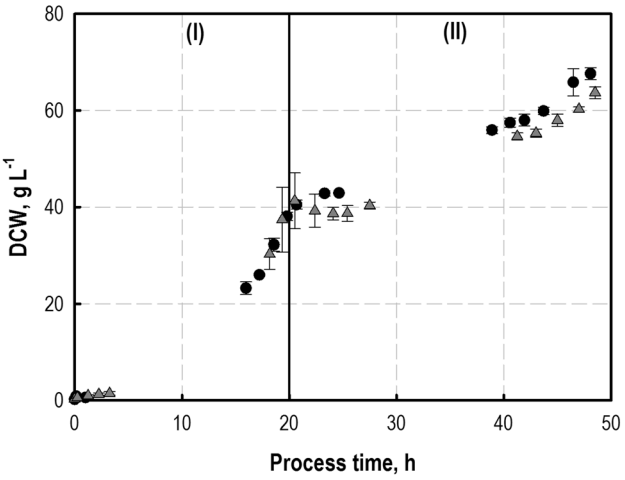

Fig. 1 Dry cell weight concentrations of two fed-batch processes (pH 6.8) with *E. coli* VvGT14a in stirred-tank reactors on a 2 L-scale. The *vertical line* separates the (*I*) biomass production phase ($T = 37$ °C, 2 g L^{-1} initial glucose and an exponential glucose feeding phase with $\mu_{set} = 0.2\ h^{-1}$ for 18 h followed by a constant feeding phase of 8.75 g L^{-1} h^{-1} glucose for 2 h) and (*II*) protein production phase (3.25 g L^{-1} h^{-1} glucose feeding, induction with 100 µM IPTG, T = 20 °C, 28 h)

Whole-cell biocatalysis for the production of geranyl glucoside

First, the influence of the initial pH on the biotransformation was evaluated in M9 mineral medium in batch processes using parallel operated milliliter-scale stirred-tank bioreactors (see Fig. 2). Whole-cell biocatalysts, which were produced at a temperature of 25 °C during protein expression phase, were applied for this study. The highest product concentrations could be observed at a pH between pH 6.0 and pH 7.5. A strong inhibition of the whole-cell biocatalysts at a pH above pH 7.5 and below pH 6.0 could be demonstrated. In contrast to the whole-cell biotransformations the purified glucosyltransferase from *Vitis vinifera* showed a maximal activity at pH 8.5 [3]. The necessity for stoichiometric regeneration of UDP-glucose in the *E. coli* cells as well as the difference between intracellular and extracellular pH may be an explanation for this difference in pH-optima.

After identification of the optimum initial pH, the influence of different medium compositions at pH 7.0 and the use of the non-water miscible IL (HPYR)(NTF) were characterized in batch whole-cell biotransformations using parallel operated milliliter-scale stirred-tank bioreactors. Whole-cell biocatalysts, which were produced at a temperature of 20 °C during protein expression phase, were used for this study, as they showed a significantly increased

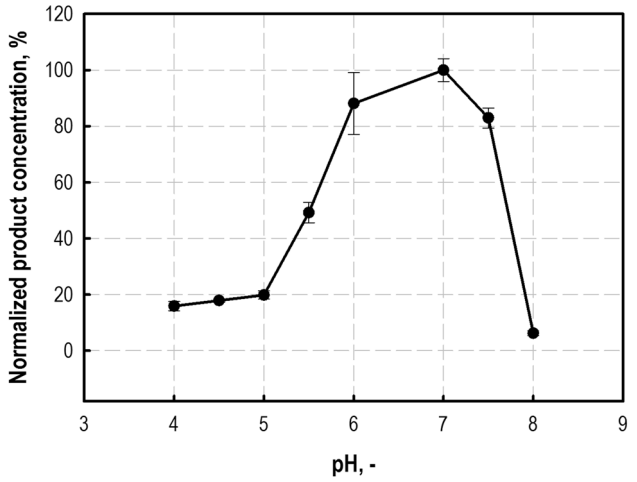

Fig. 2 Normalized geranyl glucoside concentration with standard deviations ($n = 3$) in parallel biotransformations as function of pH (stirred-tank reactors, $V = 10$ mL, $t = 20$ h, $T = 20$ °C, M9 mineral medium, 10 g L^{-1} glucose, 0.2 g L^{-1} geraniol, *E. coli* VvGT14a with 4.3 g L^{-1} dry cell mass). The product concentration was normalized to the geranyl glucoside concentration measured at pH 7.0 (16 mg L^{-1} geranyl glucoside)

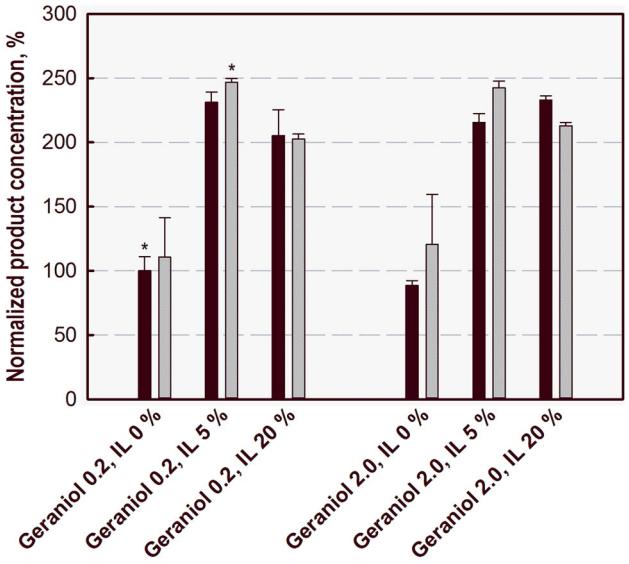

Fig. 3 Normalized geranyl glucoside concentration with standard deviations ($n = 3$) in parallel biotransformations [stirred-tank reactors, $V = 10$ mL, $t = 20$ h, $T = 20$ °C, 10 g L^{-1} glucose, pH 7.0, *E. coli* VvGT14a with 4.3 g L^{-1} dry cell mass) in M9 mineral medium (*black bars*) and 0.1 M KPi (*gray bars*) with initial geraniol concentrations of 0.2 and 2.0 g L^{-1} and ionic liquid (IL) additions of 0, 5, and 20 % (v/v)]. The biotransformations *highlighted* with *asterisk* are compared in Table 1. The product concentration was normalized to the geranyl glucoside concentration measured in M9 mineral medium at pH 7.0 (118 mg L^{-1} geranyl glucoside)

biocatalytic activity (about sevenfold increased final product titer) compared to biocatalysts produced at 25 °C. All biotransformations showed comparable performances in 0.1 M KPi buffer and M9 mineral medium (see Fig. 3), whereas the biotransformations in defined medium according to Riesenberg et al. [18], which was used for the HCDC process, showed a reduced final product titer of about 70 % (data not shown). For industrial applications, the KPi buffer is the preferential choice of medium in comparison to M9 mineral medium due to the lower salt content and thus easier separation of geranyl glucoside.

Increasing the initial concentration of geraniol in the aqueous media from 0.2 g L^{-1} by a factor of 10, 2.0 g L^{-1}, did not result in significant changes in the final product concentrations after a process time of 20 h. In situ supply of geraniol by adding (HPYR)(NTF) with the respective amounts of solved geraniol (0.2 and 2.0 g L^{-1}) as the second non-water miscible liquid phase resulted in an increase in final product concentrations by a factor of 2, 2.2, at the end of the biotransformations compared to the corresponding conversions in a single-phase system with 0.2 and 2.0 g L^{-1} geraniol. As the solubility of geraniol is about 20-fold higher in the ionic liquid phase compared to the aqueous buffer, it is most probable that the whole-cell biocatalysts in the aqueous phase were exposed to reduced geraniol concentrations during the whole two-phase batch processes. Higher geraniol conversions and geranyl glucoside concentrations may be possible in the two-phase batch processes by increasing the whole-cell biocatalyst concentrations and/or prolonging the process time.

Comparing the final geranyl glucoside concentrations and geraniol conversions of the batch processes with 0.2 g L^{-1} initial substrate concentrations and biocatalyst concentrations of $OD_{600} = 10$ with data from literature shows in the first instance that the single-phase whole-cell biotransformation resulted in lower conversion and product concentration compared to a production process of geranyl glucoside in a fed-batch process with continuous geraniol feeding and cell densities of about $OD_{600} = 70$–80 (see Table 1). Beyond that the beneficial effects of the two-phase system applied are clearly demonstrated. Conversion and final geranyl glucoside concentrations were significantly increased from 41 to 71 %, and from 165 to 291 mg L^{-1}, respectively.

The improved biotransformation applying a two-phase process can open new possibilities for further investigations on designing industrial relevant processes with in situ substrate supply. At this juncture, special focus will have to be placed on product recovery. As the solubility of geraniol is 20-fold higher in the aqueous phase compared to the ionic liquid phase whereas the solubility of geranyl glucoside is 35-fold higher in the ionic liquid phase compared to the aqueous phase, the substrate and product are already highly separated after the two-phase biotransformation. The remaining geranyl glucoside in the ionic liquid phase

could be retained by extraction with water. In general glucosides can be isolated by extraction, adsorption or distillation [22]. A common method for the isolation of glucosides is extraction with non-ionic resins (e.g., Amberlite XAD-2 [23]). With reported product recoveries of more than 90 % for the isolation of glucosides with non-ionic resins [23], the achievable isolated yield of the improved biotransformation can be assumed to be higher than 262 mg per liter reaction mixture.

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