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Research paper

Rational selection of biphasic reaction systems for geranyl glucoside production by *Escherichia coli* whole-cell biocatalysts

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ABSTRACT

Geranyl glucoside, the glucosylated, high-value derivative of the monoterpenoid geraniol, has various applications in the flavor and fragrance industry and can be produced through whole-cell biotransformation of geraniol with *Escherichia coli* whole-cell biocatalysts expressing the glucosyltransferase VvGT14a. However, the low water solubility and high cytotoxicity of geraniol require the design of a proper biphasic system where the second, non-aqueous phase functions as an *in-situ* substrate reservoir. In this work, a rational selection strategy was applied for choosing suitable sequestering phases for geranyl glucoside production by whole-cell biotransformation of geraniol. Hansen solubility parameters and octanol/water distribution coefficients were used as first principle methods in combination with extensive database research to preselect 12 liquid and 6 solid sequestering phases. Subsequently, experimental approaches were applied to determine physicochemical characteristics and the distribution of geraniol and geranyl glucoside between the phases. Moreover, the effects of the sequestering phases on the whole-cell biocatalysts and on the produced geranyl glucoside concentration were measured during parallel biotransformations in milliliter-scale stirred-tank bioreactors. The fatty acid ester isopropyl myristate emerged as the best choice due to its low viscosity, very poor water solubility, low price and compatibility with the whole-cell biocatalyst. The biphasic system containing 20 % (v/v) of this solvent boosted geranyl glucoside production (4.2-fold increase of geranyl glucoside concentration in comparison to aqueous system) and exhibits advantageous partitioning of geraniol into the organic phase (logD of 2.42 ± 0.03) and of geranyl glucoside into the water phase (logD of −2.08 ± 0.05). The systematic selection of a suitable biphasic system constitutes basic groundwork for the development of new bioprocesses involving geraniol. Moreover, this study can serve as a guideline for selecting sequestering phases for other whole-cell biotransformation processes.

1. Introduction

The rose-scented monoterpenoid geraniol has wide applications in the flavor and fragrance industry [1], but suffers from low water solubility and fast aroma degradation. To further increase the quality of respective consumables, aroma volatilization needs to be limited and controlled. One stabilization strategy depicts glucosylation of the monoterpenoid, resulting in highly water soluble and odorless geranyl glucoside. Cleavage of the glyosidic bond catalyzed by hydrolases of the skin microbiome or triggered by heat or change in pH results in slow release of the fragrance [2,3]. Besides classical chemical synthesis, which manifests several deficiencies [4], enzymatic glucosylation by plant-derived glycosyltransferases can be applied [5,6]. These glycosyltransferases can be expressed recombinantly in *Escherichia coli* (*E. coli*), which can be used in subsequent whole-cell biotransformations of geraniol [7]. Designing processes based on whole-cell catalysts instead of isolated enzymes is advantageous in order to guarantee the efficient regeneration of UDP-glucose. This nucleoside diphosphate sugar functions as a cosubstrate for glycosyltransferases, which catalyze the transfer of a sugar moiety from UDP-glucose to the substrate molecule [8].

The substrate geraniol is poorly water soluble (0.686 g L^{-1} at 20 °C) and cytotoxic [9]. A strategy to overcome these limitations is the application of a biphasic system where the non-aqueous phase serves as a sequestering phase for the substrate, resulting in low geraniol concentrations in the aqueous phase. The continuous transfer of the target compound from the non-aqueous into the aqueous phase is driven by the metabolic activity of the whole-cell biocatalysts and the retention of thermodynamic equilibrium in the biphasic system [10]. When designing a biphasic biocatalytic process, a systematic selection strategy is needed to decide on the variety of available non-aqueous phases as organic solvents, adsorbent and absorbent polymers or ionic liquids

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[11–14]. Whole-cell biocatalysis of geraniol for the production of geranyl glucoside has already been realized both in monophasic systems [7] and biphasic reaction systems using the ionic liquid N-hexylpyridinium bis(trifluoromethylsulfonyl)imid as a sequestering phase [15]. However, so far no systematic approach has been applied for selecting suitable non-aqueous phases for the biotransformation of geraniol.

Thus, the objective of this study was the identification of convenient sequestering phases for the whole-cell biotransformation of geraniol. *E. coli* expressing the glucosyltransferase VvGT14a from *Vitis vinifera* [5] was used as whole-cell biocatalyst and was produced by high cell density cultivation (HCDC). Biotransformations were performed at milliliter-scale in a parallel stirred-tank bioreactor system [15–17]. Sequestering phases were pre-selected based on theoretical considerations and compared based on the distribution of geraniol and geranyl glucoside between the phases, their physicochemical characteristics, their effect on the behavior of whole-cell biocatalysts and their influence on the biotransformation of geraniol. All in all, both liquid–liquid and solid-liquid reaction systems were analyzed and compared.

2. Materials and methods

2.1. Chemicals and microorganism

Geraniol (≥ 90 %) and isopropyl myristate (≥ 92 %) were obtained from Carl Roth (Karlsruhe, Germany). The organic solvents and the adsorbent resins were purchased from Sigma-Aldrich (Taufkirchen, Germany) and the polymers Elvax® 40W and Desmopan® 9370 AU were kindly provided by DuPont (Neu-Isenburg, Germany) and Covestro (Dormagen, Germany), respectively. The *E. coli* whole-cell biocatalyst BL21(DE3)pLysS carrying the expression vector pET29a(+) with the gene for the glucosyltransferase VvGT14a from *Vitis vinifera* was provided by the Associate Professorship of Biotechnology of Natural Products (Technical University of Munich, Freising, Germany).

2.2. High cell density cultivation and storage of whole-cell biocatalysts

E. coli whole-cell biocatalysts for subsequent biotransformations of geraniol were produced by HCDC, a method first depicted by Riesenberg et al. [18], in a 7.5 L stirred-tank bioreactor equipped with three Rushton turbines (Labfors 2, Infors HT, Bottmingen, Switzerland) as described by Schmideder et al. [15]. Only deviations from the original process will be delineated here. Immediately after inoculation of the medium in the bioreactor, exponential feeding (feeding solution: 600 g L^{-1} glucose and 20 g L^{-1} $MgSO_4$) was started with a growth rate of μ_{set} = 0.18 h^{-1}. After 18 h, the maximal feeding rate of 9 g L^{-1} h^{-1} glucose was reached and kept constant for 1.8 h. Subsequently, the temperature was lowered to 20 °C, protein expression was induced with 0.1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) and a linear feeding profile (starting point: 3.9 g L^{-1} h^{-1} glucose, increase: 0.15 g L^{-1} h^{-2} glucose) was applied for the following 28 h. After HCDC, the whole-cell biocatalysts were mixed with a cryoprotective sucrose solution (320 g L^{-1}) to a final concentration of 50% (v/v), stored at −80 °C for 4 h, lyophilized (Alpha 1–2 LD, Christ) and stored at 4 °C. Prior to further use, the freeze-dried cells were dissolved in 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, pH 7).

2.3. Selection criteria for sequestering phases

2.3.1. Hansen solubility parameters (HSP)

Hansen solubility parameters and Ra distances were determined with the software HSPiP (5th edition, version 5.0.05). The HSP method is based on the breakdown of solubility into three contributions: atomic dispersion interactions (δ_D), molecular dipole interactions (δ_P), and hydrogen bonding (δ_H). These parameters were used to calculate the HSP distance Ra:

$$Ra^2 = 4*(\delta_{D2} - \delta_{D1})^2 + (\delta_{P2} - \delta_{P1})^2 + (\delta_{H2} - \delta_{H1})^2$$

A low Ra distance indicates high affinity between solute and solvent or polymer, caused by similar solubility parameters. [19]

2.3.2. Distribution coefficients

Distribution coefficients of geraniol and geranyl glucoside between M9 mineral medium and different organic solvents were determined by adding 10 g L^{-1} geraniol and 2 g L^{-1} geranyl glucoside, respectively, to the sequestering phase, which made up 20 % (v/v) of the total reaction volume of 1 mL. After adding M9 mineral medium, the mixtures were incubated for 1 h at 25 s^{-1} in micro reaction tubes in a mixer mill (MM 200, Retsch Technology, Haan, Germany). Phases were separated (10 min, 16,249 g, benchtop centrifuge), if necessary diluted, and analyzed by HPLC.

2.3.3. Sorption isotherms

The sorption of geraniol and geranyl glucoside to different polymers was described by sorption isotherms. For geraniol, different amounts of the polymers (10, 25, 50, 100, 200, 250, 300 mg), which were washed with 2 mL M9 medium beforehand, were mixed with 10 mL of a geraniol solution (0.5 g L^{-1} in M9 medium) and incubated in sealed glass vials, which were agitated at 600 rpm by magnetic stirrers on a multi-stirrer plate at 30 °C for 8 h. Reactions were performed in triplicates. For geranyl glucoside, different amounts of XAD4 (10, 25, 50, 100, 250 mg) were mixed with 5 mL of a geranyl glucoside solution (0.5 g L^{-1} in M9 medium) and incubated at 30 °C and 600 rpm for 3 h. Reactions were performed in duplicates. The liquid phases were used for subsequent HPLC analysis.

2.3.4. Viscosity

Viscosity measurements of organic solvents were performed with a rotational viscometer (RheolabQC, Anton Paar) at 30 °C with a shear rate range of 100–2000 s^{-1}.

2.3.5. Biocompatibility, biofilm formation and bioavailability

The compatibility of organic solvents with the *E. coli* whole-cell biocatalyst was evaluated by counting the colonies formed on agar plates after incubation of the whole-cell biocatalysts with the respective solvent. A total reaction volume of 50 mL in 500 mL shake flasks consisting of 20 % (v/v) of the organic solvent in M9 mineral medium with 5 g L^{-1} glucose was inoculated with the biocatalyst to OD_{600} = 15. After incubating the dispersions at 30 °C and 200 rpm for 6 h, the aqueous phase was diluted by 10^7 in 1 x phosphate-buffered saline and 100 µL were plated on agar containing 0.1 g L^{-1} kanamycin. After incubation at 37 °C for 24 h, the colonies were counted. Incubation of the biocatalysts in pure M9 mineral medium served as a reference.

Adsorption of cells on polymers was evaluated indirectly as described by Morrish et Daugulis [14]. 300 mg polymers were added to 10 mL M9 medium containing 20 g L^{-1} glucose and biocatalysts with OD_{600} = 1. Preparations without polymers served as controls. The suspensions were incubated in sealed glass vials, which were agitated at 600 rpm by magnetic stirrers on a multi-stirrer plate at 30 °C. After 24 h, the OD_{600} was measured. A lower OD_{600} in suspensions containing polymers than in the controls was taken as an indicator for adsorption of cells on the polymer surfaces.

The assessment of bioavailability of polymers was performed with the same protocol, however without the addition of glucose. Mixtures without polymers served as controls. If the OD_{600} was higher in suspensions containing polymers than in the controls, bioavailability of the polymers was assumed.

2.4. Biotransformations

Whole-cell biotransformations of geraniol were performed in a parallel stirred-tank bioreactor system (bioREACTOR, 2mag, Munich,