Synthesis of the character impact compound raspberry ketone and additional flavoring phenylbutanoids of biotechnological interest with Corynebacterium glutamicum

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Abstract

Background: The phenylbutanoid 4-(4-hydroxyphenyl)butan-2-one, commonly known as raspberry ketone, is responsible for the typical scent and flavor of ripe raspberries. Chemical production of nature-identical raspberry ketone is well established as this compound is frequently used to flavor food, beverages and perfumes. However, high demand for natural raspberry ketone, but low natural abundance in raspberries, render raspberry ketone one of the most expensive natural flavoring components.

Results: In this study, Corynebacterium glutamicum was engineered for the microbial synthesis of the character impact compound raspberry ketone from supplemented p-coumaric acid. In this context, the NADPH-dependent curcumin/dihydrocurcumin reductase CurA from Escherichia coli was employed to catalyze the final step of raspberry ketone synthesis as it provides a hitherto unknown benzalacetone reductase activity. In combination with a 4-coumarate:CoA ligase from parsley (Petroselinum crispum) and a monofunctional benzalacetone synthase from Chinese rhubarb (Rheum palmatum), CurA constitutes the synthetic pathway for raspberry ketone synthesis in C. glutamicum. The resulting strain accumulated up to 99.8 mg/L (0.61 mM) raspberry ketone. In addition, supplementation of other phenylpropanoids allowed for the synthesis of two other naturally-occurring and flavoring phenylbutanoids, zingerone (70 mg/L, 0.36 mM) and benzylacetone (10.5 mg/L, 0.07 mM).

Conclusion: The aromatic product portfolio of C. glutamicum was extended towards the synthesis of the flavoring phenylbutanoids raspberry ketone, zingerone and benzylacetone. Key to success was the identification of CurA from E. coli having a benzalacetone reductase activity. We believe, that the constructed C. glutamicum strain represents a versatile platform for the production of natural flavoring phenylbutanoids at larger scale.

Keywords: NADPH-dependent curcumin reductase, Benzalacetone reductase, Corynebacterium glutamicum, Raspberry ketone, Metabolic engineering, Character impact compound

Introduction

The phenylbutanoid character impact compound raspberry ketone (4-(4-hydroxyphenyl)butan-2-one, RK) defines the typical scent and taste of raspberries. Thus, it is utilized by food and beverage industries to flavor beverages and foods, e.g. pudding, yogurt or sweets [1, 2]. In addition, its presumed activity as an anti-obesity or skin-whitening agent, drew consumers interest, although a
potential toxicity of this compound for humans has not yet been clarified [2–6].

Different strategies can be followed to obtain RK, e.g. extraction from natural plant material or chemical synthesis. Adversely, the natural concentration of RK in raspberries is not only very low (1–4 mg/kg), but also subject to seasonal and regional fluctuations, leading to high product costs of 3000–20,000 US$ per kg of natural, extracted RK [1, 7, 8]. Alternatively, RK can be chemically synthesized, but any RK produced by such processes is only considered as a nature-identical flavoring substance according to EU and US regulations, which no longer meets customers’ demands [9–11]. Contrary to this, RK obtained from microbial RK production is regarded as natural. Thus, microbial synthesis represents a promising approach for the sustainable production of natural RK. Prerequisite for establishing a microbial RK production process is the functional introduction of the natural biosynthesis pathway from the plant into a heterologous host.

In raspberry plants, RK synthesis starts from l-phenylalanine, which is provided by the shikimate pathway [12]. From there, l-phenylalanine is non-oxidatively deaminated by a phenylalanine ammonia lyase (PAL), yielding the phenylpropanoid cinnamic acid, which is subsequently hydroxylated towards p-coumaric acid (pCA). This compound in turn undergoes CoA-activation catalyzed by a 4-coumarate: CoA ligase (4CL, Fig. 1). The activated thioester is then condensed with one molecule of malonyl-CoA by a benzalacetone synthase (BAS), a type III polyketide synthase (PKS), yielding the diketide intermediate p-hydroxybenzalacetone (pHBA). Finally, a NADPH-dependent benzalacetone reductase (BAR) reduces pHBA to RK.

First studies on microbial RK production from supplemented pCA using BAS from Chinese rhubarb (Rheum palmatum) and CHS from raspberry (Rubus idaeus), respectively, reported product titers below 10 mg/L (0.06 mM) when using Escherichia coli or Saccharomyces cerevisiae as host strains [1, 13]. Interestingly, both studies relied on endogenous BAR activities by unknown endogenous reductase(s) in the respective host, rendering heterologous expression of a BAR-encoding gene unnecessary. Only recently, synthesis of up to 91 mg/L (0.55 mM) RK was demonstrated using E. coli BL21(DE3), which was developed for the expression of bas from R. palmatum and rzs1 from raspberry [8, 14]. The latter gene codes for the raspberry ketone/zingerone synthase RZS1 (RZS1Ri, UniProt ID: G1FCG0), which provides the required BAR activity.

Since various type III PKS-encoding genes of plant origin (encoding for stilbene synthases, chalcone synthases and a pentaketide chromone synthase) have been functionally expressed in Corynebacterium glutamicum previously, it is reasonable to assume that this is also true for a type III PKS gene providing BAS activity [15, 16].

In this context, C. glutamicum strains have been tailored towards increased malonyl-CoA supply for efficient synthesis of plant polyphenols and polyketides [16–18]. This was necessary, as typically only low intracellular concentrations of the unstable fatty acid precursor malonyl-CoA are maintained in microorganisms as its synthesis is strictly regulated, limiting overall product formation [19]. Although only one molecule of malonyl-CoA is required for the synthesis of one RK molecule, a C. glutamicum strain with increased malonyl-CoA availability is predestined for also establishing a heterologous pathway for the synthesis of RK.
In this study, we present the construction of a microbial *C. glutamicum* cell factory for the synthesis of the flavoring phenylbutanoids RK, zingerone and benzylacetone. Additionally, we identified a hitherto unknown BAR activity of the NADPH-dependent curcumin/dihydrocurcumin reductase CurA from *E. coli* allowing for the reduction of diketide intermediates.

**Results**

**Cytotoxicity of p-hydroxybenzalacetone and raspberry ketone**

In preparation of establishing a heterologous RK biosynthesis pathway from supplemented *p*CA, intermediate (*p*HBA) and product (RK) cytotoxicity on the designated host *C. glutamicum* was investigated. For this purpose, the strain *C. glutamicum* M-CoA, previously constructed for providing increased malonyl-CoA levels [16], was cultivated in CGXII medium with 4% glucose supplemented with different concentrations ranging from 0 to 1 g/L (6.17 mM) *p*HBA and RK (6.1 mM) using the BioLector microbioreactor system (Fig. 2). Concentrations ≥ 125 mg/L (0.77 mM) *p*HBA negatively affected microbial growth up to complete growth inhibition in the presence of 1 g/L (6.17 mM) *p*HBA. Bearing the designated supplementation of 5 mM *p*CA as precursor for RK synthesis in mind, resembling the standard production conditions for the synthesis of *p*CA-derived plant polyphenols using *C. glutamicum*, such toxic concentrations cannot be reached [18]. In contrast, no significant negative impact on growth could be observed upon supplementation of up to 1 g/L (6.1 mM) RK.

Previous studies utilizing either *E. coli* or *S. cerevisiae* for microbial RK synthesis demonstrated that both hosts provide an endogenous BAR activity [1, 13]. Hence, supernatants from the *C. glutamicum* microbioreactor cultivations performed in the context of the *p*HBA cytotoxicity experiments, were analyzed by HPLC for the presence of potentially accumulating RK. Indeed, RK was detected in all samples from cultivations supplemented with ≥ 125 mg/L (0.77 mM) *p*HBA reaching a maximum of 15.4 mg/L (0.094 mM) RK when 500 mg/L (3.09 mM) *p*HBA was present in the microbioreactor cultivations. Interestingly, even though the *C. glutamicum* cells did not grow in the presence of 1 g/L (6.17 mM) *p*HBA, up to 9.3 mg/L (0.057 mM) RK were formed. This particular experiment was repeated without cells to verify that *p*HBA reduction yielding RK is due to the presence of the *C. glutamicum* cells conferring an endogenous BAR activity and not the result of a spontaneous reduction under the selected cultivation conditions in CGXII medium. This control experiment showed that RK formation was only detectable in the presence of *C. glutamicum* cells. Therefore, a yet unknown endogenous BAR activity can also be ascribed to *C. glutamicum*.

**The curcumin reductase CurA from *E. coli* improves the BAR activity in *C. glutamicum***

With the aim to increase the endogenous BAR activity and to establish the full RK pathway in *C. glutamicum*, heterologous genes coding for BAS and BAR enzymes were episomally introduced into this bacterium. For this purpose, a codon-optimized gene variant encoding BAS

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**Fig. 2** Cytotoxic effects of a *p*-hydroxybenzalacetone and b raspberry ketone on growth of *C. glutamicum*. *C. glutamicum* M-CoA was cultivated in CGXII medium with 4% glucose supplemented with increasing concentrations of either *p*-hydroxybenzalacetone or raspberry ketone dissolved in DMSO using a BioLector microbioreactor. Biomass formation was followed by measuring the backscattered light intensity (gain 10) at a wavelength of 620 nm. The depicted data represent mean values from biological triplicates.
from *Rheum palmatum* (*basRpCg*, UniProt ID: Q94FV7) was combined with a gene for different BAR variants. A codon-optimized gene variant of RZS1R (*rzs1Ricg*, UniProt ID: G1FCG0) was used as this particular enzyme has already been successfully applied for the microbial synthesis of RK in *E. coli* [8]. Previously, cofactor specificity in a RZS1R-G191D mutant was reported to be relaxed resulting in the acceptance of NADH as reducing agent [14]. Based on this observation, the same amino acid substitution was also introduced into *rzslRicg* (*rzslRicg*-G191D). Additionally, available scientific data was analyzed to identify endogenous reductases involved in pHBA reduction in *E. coli* and *C. glutamicum*. In case of *E. coli*, the NADPH-dependent curcumin/dihydrocurcumin reductase CurA involved in the degradation of this polyphenol (*CurAEc*, UniProt ID: P76113) was identified as a promising candidate. Its natural substrate curcumin is a dimer of pHBA and thus the enzyme might also be active on the monomers (Additional file 1: Figure S1) [20]. Therefore, the native *curA* gene was amplified from the genome of *E. coli* MG1655 (*curA Ec*) but also ordered as codon-optimized variant (*curA ECG*) for a possible application in *C. glutamicum*. To enable IPTG-inducible heterologous gene expression from the strong T7 promoter, the plasmid pMKEx2 was selected [21]. The constructed plasmids were used for the transformation of *C. glutamicum* M-CoA. For evaluation of reductase activity, the generated strains were cultivated for 72 h in 50 mL defined CGXII medium with 4% glucose and 1 mM IPTG supplemented with 500 mg/L (3.09 mM) pHBA. Taken samples were extracted with ethyl acetate and analyzed for the synthesis of RK by HPLC (Fig. 3).

Surprisingly, both strains harboring an episomally encoded *rzslRicg* gene variant did not synthesize more RK from supplemented pHBA compared to *C. glutamicum* M-CoA harboring the empty vector (12.9 mg/L, 0.08 mM). This indicates that both *rzslRicg* variants are not functionally expressed in *C. glutamicum*. However, in presence of the *curA Ec*, 49.4 mg/L (0.3 mM) RK were synthesized, indicating not only its functional expression in *C. glutamicum* but also the capability of CurA to reduce pHBA. When utilizing the codon-optimized *curA ECG* gene, RK synthesis was increased further to 68.7 mg/L (0.42 mM). Hence, the pMKEx2-*basRpCg*-*curA ECG* plasmid was selected for all subsequent experiments. Noteworthy, at least one additional reductase conferring pHBA reducing abilities must be present in *E. coli*, since *E. coli* BL21 strains, previously also utilized for microbial RK synthesis, do not have the *curA* gene [1].

**Increased NADPH availability improves pHBA reduction**

The observed incomplete conversion of pHBA to RK suggested intracellular NADPH supply to be a limiting factor during phenylbutanoid synthesis. With the aim to improve pHBA reduction, previously described strategies for increasing NADPH availability were followed [22]. In particular, elimination of the endogenous lactate dehydrogenase activity, especially in combination with the heterologous expression of the transhydrogenase genes *pntAB* from *E. coli*, was shown to contribute to increased NADPH availability in *C. glutamicum*. The constructed strain *C. glutamicum* M-CoA ΔldhA was transformed using the plasmid pMKEx2-*basRpCg*-*curA Ec*. To evaluate, if the deletion of *ldhA* also increases NADPH availability and thus improves pHBA reduction of *C. glutamicum*, strains were cultivated both, absence or presence of pMKEx2-*basRpCg*-*curA Ec*. Strain cultivation and analysis of taken samples were
conducted as described above (Additional file 1: Figure S2). Under standard cultivation conditions, sole ldhA deletion did not affect growth, but did also not improve RK synthesis. However, the approach of deleting ldhA was not abandoned although no positive effect on absolute RK titers was observed at this stage. Obviously, limited effects on NADPH-dependent pHBA reduction generating RK are not surprising when taking into consideration that the abolished lactate-forming reaction is NADH-dependent, consequently increasing NADH availability. For increasing NADPH supply from NADH (NADH + NADP⁺ → NAD⁺ + NADPH), the membrane-bound transhydrogenase PntAB from *E. coli* (UniProt IDs: P07001 and P0AB67) described earlier was tested [22]. *E. coli* harbors two transhydrogenase isoforms. Whereas the energy-dependent PntAB enzyme catalyzes the transfer of a hydride ion from NADH to NADP⁺ under physiological conditions, the energy-independent cytoplasmatic variant UdhA (UniProt ID: P27306) operates in the reverse direction, when an excess of NADPH is present in the cell [23, 24]. However, in principal, both enzymes are capable of catalyzing both reactions.

The expression plasmids pEKEx3-pntABEc and pEKEx3-udhAEcCg either harboring the native pntAB genes from *E. coli* (pntABEc) or a codon-optimized *udhA* variant (udhAEcCg), each under control of the tac promoter, were constructed. Subsequently, these plasmids were used for transformation of *C. glutamicum* M-CoA ΔldhA harboring pMKEx2-basRPGC-curarAEcCg. The resulting strains were cultivated under the same conditions as described before. As heterologous expression of genes encoding for integral membrane proteins often cause growth defects, multiple IPTG concentrations ranging from 10 to 1000 µM were tested for the heterologous expression of pntABEc. These experiments showed that an increasing IPTG concentration was always associated with an increasing growth defect up to a complete arrest of growth (Additional file 1: Figure S3). HPLC analysis indicated a drastically impaired RK synthesis for all IPTG concentrations compared to the reference strain with 1 mM IPTG, rendering the heterologous expression of pntAB unsuitable for RK synthesis in *C. glutamicum*.

Contrary, episomal expression of udhAEcCg barely affected microbial growth but increased RK titers up to 25% (Additional file 1: Figure S4). This indicates that the functional expression of udhAEcCg in *C. glutamicum* allows for the hydride ion transfer from NADH to NADP⁺. Interestingly, functional expression of udhA from *E. coli* in *C. glutamicum* has already been demonstrated earlier, though utilized for the opposite hydride ion transfer [25]. Thus, *C. glutamicum* M-CoA ΔldhA harboring the two expression plasmids pMKEx2-basRPGC-curarAEcCg and pEKEx3-udhAEcCg provides the highest BAR activity, resembling a promising candidate for establishing (4CL and) BAS activity to complete the heterologous pathway for RK synthesis from supplemented pCA.

**Raspberry ketone synthesis from p-coumaric acid**

So far, RK synthesis in *C. glutamicum* was only achieved by supplementation of the diketide intermediate pHBA. As *C. glutamicum* M-CoA provides increased amounts of malonyl-CoA, synthesis of pHBA from pCA should be also possible. In addition to BAS from Chinese rhubarb (*R. palmatum*), which was shown to feature a novel catalytic mechanism allowing for the sole synthesis of pHBA, the bifunctional chalcone synthases PKS1 (UniProt ID: Q9AU11) and PKS4 (UniProt ID: B0LDU5) from raspberry (*R. idaeus*) were tested for pHBA synthesis from supplemented pCA in *C. glutamicum* [26–29]. Both enzymes were described to have a BAS side activity in addition to their CHS activity. Codon-optimized gene variants *pks1Ricg* and *pks4Ricg* were used to construct pMKEx2-pks1Ricg-curAEcCg and pMKEx2-pks4Ricg-curAEcCg. For evaluation of (4CL and) BAS activity, the constructed strains were cultivated and analyzed as described above with supplementation of 5 mM pCA instead of 3.09 mM pHBA. Stacked concentrations of pHBA and RK were used to assess the (4CL and) BAS activity (Fig. 4).

Utilization of the already applied *bas* gene from *R. palmatum* enabled RK synthesis from pCA in *C. glutamicum*. After 72 h of cultivation in the absence of udhAEcCg, 14 mg/L (0.09 mM) RK and 9.4 mg/L (0.06 mM) pHBA were detected by HPLC. In contrast to the previous experiments, udhAEcCg expression reduced pHBA- and RK synthesis. In addition, neither expression of *pks1* nor *pks4* in combination with *curA* enabled RK synthesis, suggesting that both genes were not functionally expressed in *C. glutamicum*. Since both enzymes are primarily chalcone synthases, it was also tested whether naringenin chalcone, or more precisely, naringenin was formed. However, also no detectable amounts of naringenin were synthesized, indicating that PKS1 and PKS4 might be incorrectly folded in *C. glutamicum*. Previously, N-terminal translational fusion with the maltose binding protein from *E. coli* (MαLEc) was demonstrated to efficiently increase functional expression of heterologous plant genes in *C. glutamicum* [30]. To test whether functional expression of *pks* genes could be achieved by mimicking this strategy, *C. glutamicum* strains harboring the plasmids pMKEx2-mαLEc-pks1Ricg-curAEcCg and pMKEx2-mαLEc-pks4Ricg-curAEcCg were constructed and cultivated. Although, general applicability of this strategy was indicated by the formation of RK when expressing
malEEc-pks1RiCg and malEEc-pks4RiCg significantly less RK was formed in comparison to BASRp.

Taken together, C. glutamicum M-CoA ΔldhA harboring the indicated expression plasmids was cultivated in 50 mL CGXII medium with 4% glucose and 5 mM p-coumaric acid in baffled flasks at 30 °C and 130 rpm for 72 h. a Growth and b p-hydroxybenzalacetone- and raspberry ketone synthesis was followed over time. Determined concentrations for p-hydroxybenzalacetone and raspberry ketone in cell extracts were stacked for a better visualization. The depicted data represent mean values with standard deviations from biological triplicates.

**Microbial synthesis of zingerone and benzylacetone**

Besides RK, other phenylbutanoids such as the ferulic acid-derived zingerone or the cinnamic acid-derived benzylacetone are of commercial interest as well. Zingerone is regarded as the molecule providing the characteristic flavor of cooked ginger, whereas benzylacetone is described to contribute to the characteristic taste of strawberries and jasmine [31–34]. The respective molecules differ from RK only in their hydroxylation/methoxylation pattern of the aromatic ring (Fig. 1). Thus, it is reasonable to assume that the enzymes of the RK pathway also accept ferulic acid and cinnamic acid (and their derivatives) as substrates, which would offer the opportunity for a combinatorial biosynthesis of zingerone or benzylacetone using the very same C. glutamicum strain (Fig. 1).

First, confirmation of reductase activity with the respective diketide intermediates of zingerone and benzylacetone synthesis was addressed as formation of the diketide pHBA from pCA by 4CLPc and BASRp was rather inefficient and might be even more challenging with alternative phenylpropanoids as substrates. To this end, CGXII medium was supplemented with the respective diketide precursors (3.09 mM) during cultivations of C. glutamicum M-CoA ΔldhA, optionally harboring pMKEx2-basRpCg-curAEcCg and pEKEx3-udhAEcCg. HPLC analysis of extracted samples demonstrated synthesis of 40.2 mg/L (0.21 mM) zingerone upon curAEcCg expression whereas the synthesis of benzylacetone was unaffected (0.6 mg/L, 0.01 mM) indicating that CurAEcCg cannot reduce benzalacetone. Therefore, benzalacetone appears to be solely reduced by the unknown endogenous reductase activity of C. glutamicum yielding benzylacetone. Interestingly, less zingerone (70 mg/L, 0.36 mM) compared to RK (99.8 mg/L, 0.61 mM) was produced from the respective diketide intermediate despite an even higher similarity to the curcumin structure (Fig. 5b).

Nevertheless, reductase activity of the constructed strain C. glutamicum M-CoA ΔldhA carrying pMKEx2-basRpCg-curAEcCg and pEKEx3-udhAEcCg was verified for all tested substitution patterns of the aromatic ring. To evaluate substrate promiscuity of the diketide forming enzymes 4CLPc and BASRp, C. glutamicum M-CoA ΔldhA harboring pMKEx2-basRpCg-curAEcCg and pEKEx3-udhAEcCg was cultivated using standard conditions with supplementation of the respective phenylpropanoids (5 mM). Extracted samples were analyzed by HPLC for the presence of respective diketides and ketones (Fig. 5d). After 72 h of cultivation, 7.9 mg/L (0.05 mM) pHBA and 4.7 mg/L (0.05 mM) RK.
were formed from pCA. When supplementing either ferulic acid or cinnamic acid, 0.8 mg/L (0.01 mM) vanillylidenacetone and 14.1 mg/L (0.07 mM) zingerone or 0.4 mg/L (0.01 mM) benzalacetone but no detectable benzylacetone was formed, respectively.

In principle, the precursors and intermediates of zingerone and benzylacetone synthesis can be converted also by the heterologous pathway for RK synthesis established in C. glutamicum. Nevertheless, benzylacetone could not be produced from cinnamic acid, probably due to the insufficient synthesis of the diketide intermediate benzylacetone. Contrary to previous results obtained from cultivations with supplemented diketide intermediates, the reduction of vanillylidenacetone appears to be more efficient compared to pHBA reduction, as almost all vanillylidenacetone synthesized was converted to zingerone.

**Discussion**

In this study, we constructed a C. glutamicum variant for the microbial synthesis of the flavoring phenylbutanoids RK, zingerone and benzylacetone. Initial cytotoxicity experiments of pHBA and RK suggested C. glutamicum to be more resistant to these compounds compared to E. coli and S. cerevisiae. For the latter two microorganisms, half maximal inhibitory concentrations (IC₅₀) have been calculated for both S. cerevisiae and E. coli [1]. Here, concentrations of 100 mg/L or 300 mg/L pHBA and 500 mg/L or 900 mg/L were determined for S. cerevisiae and E. coli to reduce biomass formation by 50%, respectively. Since the calculation of IC₅₀ values for C. glutamicum would be imprecise due to insufficient data for higher concentrations of both molecules, we cannot provide exact concentrations. Nevertheless, the cytotoxicity experiments allow to consider C. glutamicum to be more resistant to both pHBA and RK as the IC₅₀ concentrations have to be > 500 mg/L and > 1000 mg/L, respectively. More importantly, the constructed strain C. glutamicum M-CoA ΔldhA pMKEx2-basRpCg-curAEcCg pEKEx3-udhAEcCg accumulates up to 14 mg/L (0.09 mM) RK from supplemented pCA, which is comparable to the product titer determined for a S. cerevisiae strain (7.5 mg/L RK (0.05 mM)) [13].
However, synthesis of 91 mg/L (0.55 mM) RK from pCA using an engineered E. coli BL21(DE3) variant was recently reported [8].

Moreover, a yet unknown substrate promiscuity of the NADPH-dependent curcumin/dihydrocurcumin reductase CurA from E. coli MG1655 allowing for the efficient reduction of pHBA and vanillylideneacetone, respectively, was identified. Although E. coli BL21 has been previously reported to possess an endogenous BAR activity, this activity presumably cannot be traced back to CurA as this particular gene is not present in the utilized strain background [1]. Thus, it is likely that at least one additional reductase also features BAR activity in E. coli BL21. We presumed a possible enzymatic activity of CurA with pHBA due to structural similarities with its natural substrate curcumin (Additional file 1: Figure S1). As the additional methoxy group of vanillylideneacetone increases structural similarity to curcumin even more, a more efficient conversion to the corresponding ketone compared to the RK branch was expected prior to the conducted experiments. Indeed, when producing flavoring phenylbutanoids from supplemented phenylpropanoids, an almost complete conversion of vanillylideneacetone to zingerone was observed, whereas pHBA reduction was less efficient.

Furthermore, a hitherto unknown endogenous BAR activity must be also present in C. glutamicum as demonstrated by the reduction of the three tested diketide intermediates in the absence of curAEcCg. Apart from E. coli and C. glutamicum, such an activity has already been described for S. cerevisiae [13].

To increase NADPH supply for the efficient reduction of diketide intermediates, heterologous expression of genes encoding the membrane-bound as well as the cytoplasmatic transhydrogenases from E. coli was evaluated. Despite already being used to increase NADPH availability in an isobutanol producing C. glutamicum variant, the membrane-bound transhydrogenase PntAB turned out to be unsuitable for RK synthesis with the same bacterium [22]. With increasing induction strength, a severe growth defect in C. glutamicum was observed upon pntAB expression. This might indicate cytotoxic effects of the transhydrogenase itself, but also the absence of chaperones supporting folding or an altered membrane composition could be the cause [35]. Contrary, the cytoplasmatic transhydrogenase UdhA was beneficial for RK synthesis resembling a promising alternative to the membrane-bound PntAB. Despite being rather involved in the energy-independent hydride ion transfer from NADPH to NAD⁺ in vivo, UdhA can still catalyze the transfer in the reverse direction [23]. Nevertheless, the equilibrium of the transhydrogenation reaction could be shifted towards NADPH, when NADPH is constantly withdrawn by the reduction of pHBA to RK. Furthermore, deletion of ldhA increases NADH availability, which in turn also shifts the equilibrium of the transhydrogenase reaction towards NADPH. Further strategies for increased NADPH supply in C. glutamicum involve altering the coenzyme specificity of the NAD⁺-dependent glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to NADP⁺, which was done in the context of L-lysine production with C. glutamicum [36]. It should be noted, that an imbalanced NADH/NADPH distribution could perturb the cellular metabolism and might even inhibit cellular growth or glucose consumption [36].

Interestingly, expression of udhAEcCg appears to be disadvantageous for the synthesis of pHBA and RK from pCA. NADPH availability could not be limiting for the small amounts of pHBA produced from pCA, so that the described positive effect of additional NADPH supply only become significant at higher pHBA concentrations. The reduced cumulated titer of pHBA and RK might be due to the increased metabolic burden of the cell due to maintenance of the pEKEx3-udhAEcCg plasmid (requiring supplementation of a second antibiotic and expression of an additional antibiotic resistance gene) [37, 38].

Conclusion

In the present work, we extended the product portfolio of C. glutamicum towards flavoring phenylbutanoids. We identified an endogenous BAR activity of C. glutamicum and a yet unknown substrate promiscuity of CurA from E. coli that turned out to be a promising BAR. Moreover, the cytoplasmatic transhydrogenase UdhA from E. coli allowed for increased NADPH supply and ultimately improved RK synthesis. Taken together, the constructed strain C. glutamicum M-CoA ΔldhA harboring pMKEx2-basΔGcCg-curAEcCg and pEKEx3-udhAEcCg represents a versatile host for the synthesis of up to 99.8 mg/L (0.61 mM) RK, 70 mg/L (0.36 mM) zingerone and 10.5 mg/L (0.07 mM) benzylacetone.

Materials and methods

Bacterial strains, plasmids, media and growth conditions

All bacterial strains and plasmids with their respective characteristics used in this study are listed in Table 1. C. glutamicum strains were routinely cultivated aerobically at 30 °C in brain heart infusion (BHI) medium (Difco Laboratories, Detroit, USA) or defined CGXII medium with 4% (w/v) glucose as sole carbon and energy source [39]. E. coli DH5α, used solely for plasmid constructions, was cultivated in LB medium at 37 °C [40]. Where appropriate, kanamycin (E. coli 50 µg/mL, C. glutamicum 25 µg/mL) and/or spectinomycin (100 µg/mL for E. coli and C. glutamicum) was added to the respective medium.

Materials and methods
Table 1 Strains and plasmids used in this study

| Strain or plasmid | Characteristics | Source |
|-------------------|----------------|--------|
| C. glutamicum strains | C. glutamicum derivative with in-frame deletions of cg0344-47, cg0503 cg2625-40 and cg1126; harboring a chromosomally encoded codon-optimized 4clp, gene coding for 4-coumarate:CoA ligase from R. crispum under control of the T7 promoter and replacement of the native gilA promoter with the dapA promoter variant C5, mutated fadO binding sites upstream of accBC and accD1, two nucleotide exchanges in the ioT1 promoter and in-frame deletion of pyc | [16] |
| M-CoA ΔldhA | C. glutamicum M-CoA derivative with in-frame deletion of ldhA | This work |
| E. coli strains | | |
| DH5α | F-Φ80lacZΔM15 ΔlacZYA-argF]U169 recA1 endA1 hsdR17 (rKm2 mcrA) phoA supF44 λ- thi-1 gyrA96 relA1 | Invitrogen (Karlruhe, Germany) |

Plasmids

| Plasmid | Characteristics | Source |
|---------|----------------|--------|
| pK19mobsacB-ΔldhA | Vector for in-frame deletion of ldhA | [46] |
| pMKEx2 | kanR; E. coli-C. glutamicum shuttle vector (lacI, P_{T7}, lacO1, phM1519 or152, pACYC177 or152) | [21] |
| pMKEx2-basR_{Ec}--tzs1_{RiCg} | kanR; pMKEx2 derivative containing codon-optimized genes encoding benzalacetone synthase from R. palmatum (basR_{Ec}) and NADPH-dependent raspberry ketone/zingerone reductase from R. idaeus (tzs1_{RiCg}) | This work |
| pMKEx2-basR_{Ec}--tzs1_{RiCg}G191D | kanR; pMKEx2 derivative with mutations in the tzs1_{RiCg} nucleotide sequence leading to amino acid substitution G191D in R2S1_{RiCg} | This work |
| pMKEx2-basR_{Ec}--curA_{EcCg} | kanR; pMKEx2 derivative containing a codon-optimized gene encoding benzalacetone synthase from R. palmatum (basR_{Ec}) and the native gene encoding NADPH-dependent curcumin/dihydrocurcumin reductase CurA from E. coli (curA_{Ec}) | This work |
| pMKEx2-basR_{Ec}--curA_{EcCg}malEEc | kanR; pMKEx2 derivative containing codon-optimized genes encoding benzalacetone synthase from R. palmatum (basR_{Ec}) and NADPH-dependent curcumin/dihydrocurcumin reductase CurA from E. coli (curA_{Ec}) | This work |
| pMKEx2- pkS1_{EcCg}--curA_{EcCg} | kanR; pMKEx2 derivative containing codon-optimized genes encoding PKS1 from R. idaeus (pks1_{RiCg}) and NADPH-dependent curcumin/dihydrocurcumin reductase CurA from E. coli (curA_{Ec}) | This work |
| pMKEx2-pks4_{EcCg}--curA_{EcCg} | kanR; pMKEx2 derivative containing codon-optimized genes encoding PKS4 from R. idaeus (pks4_{RiCg}) and NADPH-dependent curcumin/dihydrocurcumin reductase CurA from E. coli (curA_{Ec}) | This work |
| pMKEx2-malEEc-pks1_{EcCg}--curA_{EcCg} | kanR; pMKEx2 derivative containing the native malE gene from E. coli fused to the codon-optimized gene encoding PKS1 from R. idaeus (malEEc-pks1_{RiCg}) and a codon-optimized gene encoding NADPH-dependent curcumin/dihydrocurcumin reductase CurA from E. coli (curA_{Ec}) | This work |
| pMKEx2-malEEc-pks4_{EcCg}--curA_{EcCg} | kanR; pMKEx2 derivative containing the native malE gene from E. coli fused to the codon-optimized gene encoding PKS4 from R. idaeus (malEEc-pks4_{RiCg}) and a codon-optimized gene encoding NADPH-dependent curcumin/dihydrocurcumin reductase CurA from E. coli (curA_{Ec}) | This work |
| pEKE3 | specE; E. coli-C. glutamicum shuttle vector (lacI, P_{lack}, lacO1, pBL1oriCg, pUCoriCg) | [47] |
| pEKE3-pntAB Ec | specE; pEKE3 derivative containing native pntAB genes from E. coli (pntAB_Ec) encoding a membrane-bound transhydrogenase | This work |
| pEKE3-udhA EcCg | specE; pEKE3 derivative containing udhA gene variant from E. coli (udhA_{EcCg}) encoding a cytoplasmic transhydrogenase | This work |
| pEKE3-malEEc-omtVv | specE; pEKE3 derivative containing malE gene from E. coli (malEEc) fused to the codon-optimized gene coding for resveratrol-di-O-methyltransferase from V. vinifera (omtVv) | [30] |

Bacterial growth was followed by measuring the optical density at 600 nm (OD_{600}). To cultivate C. glutamicum, a test tube with 5 mL BHI medium was inoculated with a single colony from an agar plate and grown for 6–8 h on a rotary shaker at 170 rpm (first preculture). This first preculture was used to inoculate 50 mL of defined CGXII medium with 4% (w/v) glucose in a 500 mL baffled Erlenmeyer flask (second preculture). The second preculture was cultivated overnight on a rotary shaker at 130 rpm. The main culture was subsequently inoculated from the second preculture to the indicated OD_{600} in defined CGXII medium with 4% (w/v) glucose. For microbial synthesis of phenylbutanoids, the main culture was inoculated to an OD_{600} of 5 in defined CGXII medium with 4% glucose and heterologous gene expression was induced 90 min after inoculation using 1 mM IPTG. 1 mL of the culture broth was sampled at
defined time points and stored at −20 °C until ethyl acetate extraction and HPLC analysis.

For evaluating cytotoxicity of pHBA and RK, C. glutamicum M-CoA was cultivated at 30 °C, 900 rpm and a humidity of 85% in 48-well Flowerplates containing 800 µL CGXII medium with 4% (w/v) glucose inoculated to an OD₆₀₀ of 1, using the BioLector microbioreactor (m2p-labs, Baesweiler, Germany). Increasing concentrations of both pHBA and RK (final concentrations 0, 15.625, 31.25, 62.5, 125, 250, 500 and 1000 mg/L), dissolved in 10 µL DMSO were added. Online measurement of the backscattered light intensity (620 nm, gain 10) was used for evaluation of cellular growth. To estimate IC₅₀ values, obtained backscattered light intensities after 72 h were plotted against the respective pHBA- and RK concentrations and subsequently analyzed using the GraphPad Prism 8.1.2 software (San Diego, CA, USA). The nonlinear regression [inhibitor] vs. response – Variable slope (four parameters) with the following specifications was used: top = 165, bottom = 15, as well as IC₅₀ = 0. The values for top and bottom correspond to the mean values for the determined backscatter values after 72 h in the absence of pHBA or RK, or the average value for the backscatter after 72 h in the presence of 1000 mg/L (6.17 mM) pHBA.

**Plasmid and strain construction**

Standard protocols of molecular cloning, such as PCR, restriction and ligation of DNA were carried out for recombinant DNA work [41]. All enzymes were obtained from Thermo Fisher Scientific (Schwert, Germany). Codon-optimized synthetic genes for C. glutamicum ATCC13032 were obtained from Thermo Fisher Scientific (formerly GeneArt, Darmstadt, Germany). Genes and chromosomal fragments were amplified by PCR from synthetic genes or genomic E. coli DNA as template using E. coli and chromosomal fragments were amplified by PCR from ATCC13032 were obtained from Thermo Fisher Scientific (Schwerte, Germany). Genes recombinant DNA work [41]. All enzymes were obtained from Thermo Fisher Scientific (Schwert, Germany). Codon-optimized synthetic genes for C. glutamicum ATCC13032 were obtained from Thermo Fisher Scientific (formerly GeneArt, Darmstadt, Germany). Genes and chromosomal fragments were amplified by PCR from synthetic genes or genomic E. coli DNA as template using E. coli and chromosomal fragments were amplified by PCR from ATCC13032 were obtained from Thermo Fisher Scientific (Schwerte, Germany). Genes recombinant DNA work [41]. All enzymes were obtained from Thermo Fisher Scientific (Schwert, Germany). Codon-optimized synthetic genes for C. glutamicum ATCC13032 were obtained from Thermo Fisher Scientific (formerly GeneArt, Darmstadt, Germany). Genes and chromosomal fragments were amplified by PCR from synthetic genes or genomic E. coli DNA as template using E. coli and chromosomal fragments were amplified by PCR from ATCC13032 were obtained from Thermo Fisher Scientific (Schwerte, Germany). Genes recombinant DNA work [41]. All enzymes were obtained from Thermo Fisher Scientific (Schwert, Germany). Codon-optimized synthetic genes for C. glutamicum ATCC13032 were obtained from Thermo Fisher Scientific (formerly GeneArt, Darmstadt, Germany). Genes and chromosomal fragments were amplified by PCR from synthetic genes or genomic E. coli DNA as template using E. coli and chromosomal fragments were amplified by PCR from ATCC13032 were obtained from Thermo Fisher Scientific (Schwerte, Germany). Genes recombinant DNA work [41]. All enzymes were obtained from Thermo Fisher Scientific (Schwert, Germany). Codon-optimized synthetic genes for C. glutamicum ATCC13032 were obtained from Thermo Fisher Scientific (formerly GeneArt, Darmstadt, Germany). Genes and chromosomal fragments were amplified by PCR from synthetic genes or genomic E. coli DNA as template using pair assembly [42]. In-frame gene deletions in the C. glutamicum genome were performed using the pK19mobscB system by a two-step homologous recombination method described previously [43, 44]. Integrity of all constructed plasmids was verified by colony PCR, restriction analysis, and DNA sequencing at Eurofins MWG Operon (Ebersberg, Germany) Techniques specific for C. glutamicum, e.g. electroporation of cells, were performed as described previously [45].

**Ethyl acetate extraction and HPLC quantification**

Phenylbutanoids and pathway intermediates were extracted from cultivation broth for subsequent HPLC analysis by mixing 1 mL of the culture broth with 1 mL ethyl acetate and vigorous shaking (1400 rpm, 10 min, 20 °C) in a Thermomixer (Eppendorf, Hamburg, Germany). The suspension was centrifuged for 5 min at 13,000 rpm and the upper ethyl acetate layer (800 µL) was transferred to an organic solvent resistant deep-well plate (Eppendorf, Hamburg, Germany). After evaporation to dryness, extracts were resuspended in the same volume of acetonitrile and subsequently used for HPLC analysis.

Metabolites were quantified using an Agilent high-performance liquid chromatography (HPLC) 1260 Infinity System equipped with a 1260 Infinity DAD (Agilent Technologies, Waldbronn, Germany). Authentic standards of benzalacetone, benzylacetone, cinnamic acid, ferulic acid, p-coumaric acid, vanillic acid and zingerone were purchased from Sigma-Aldrich (Taufkirchen, Germany), p-hydroxybenzalacetone was obtained from Alfa Aesar (Kandel, Germany) and raspberry ketone from Acros Organics (Geel, Belgium). LC separation was carried out with an InfinityLab Poroshell 120 2.7 µm EC-C₁₈ column (3.0 × 150 mm; Agilent Technologies, Waldbronn, Germany) at 50 °C. For elution, 0.1% acetic acid (solvent A) and acetonitrile supplemented with 0.1% acetic acid (solvent B) were applied as the mobile phases at a flow rate of 0.7 mL/min. Depending on the analyte, a different elution gradient was used, where the amount of solvent B was increased stepwise. Raspberry ketone: minute 0–10: 10%, minute 10–11: 10–90%, minute 11–13: 90%, minute 13–15: 90–10% and minute 15–17: 10%. Absorption was determined at 275 nm (raspberry ketone), 310 nm (p-coumaric acid) and 320 nm (p-hydroxybenzalacetone). Zingerone: minute 0–15: 10%, minute 15–16: 10–90%, minute 16–18: 90%, minute 18–20: 90–10% and minute 20–22: 10%. Absorption was determined at 275 nm (zingerone) and 320 nm (ferulic acid and vanillylidenacetone). Benzylacetone: minute 0–13: 10–50%, minute 13–15: 50%, minute 15–17: 50–10% and minute 17–19: 10%. Absorption was determined at 260 nm (benzylacetone) and 320 nm (cinnamic acid and benzalacetone). Area values of integrated signals were linear up to metabolite concentrations of at least 83.3 mg/L.
Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12934-020-01351-y.

Additional file 1. Additional information containing a list of oligonucleotides used in this study, the chemical structure of curcumin with highlighted pHBA structure and additional cultivation results.

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Authors’ contributions
LM and MM conceived the design of this study. LM and MM performed the experimental work. LM and JM wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article and its additional files.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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