Integration of computational and experimental synthetic biology approaches to enable innovative and sustainable biomanufacturing of cosmetic ingredients

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Abstract

Plants are a crucial source of bioactive molecules extensively utilized as cosmetic ingredients. However, traditional methods to produce these molecules, including plant extraction and chemical synthesis, are cost-ineffective and unsustainable. Synthetic biology heralds a transformative era in cosmetic material biomanufacturing, providing unparalleled customization, efficiency, and sustainability. The fusion of computational and experimental synthetic biology approaches holds the potential to revolutionize the cosmetics industry by generating innovative production processes while addressing environmental concerns.

In this study, we developed algorithms and software tools for scalable probabilistic model checking and pathway construction. Using these tools, we successfully established advanced computational models for the production of cosmetic ingredients, such as resveratrol and hydroxytyrosol. These models facilitated efficient in-silico testing of various parameters. Experimental implementation of the recommendations from modeling resulted in nearly a 3-fold increase in resveratrol yield, paving the way for large-scale production of this cosmetic ingredient. We also created a computational tool to predict natural product biosynthetic pathways and yielded an efficient pathway in *Escherichia coli* to produce high-quality hydroxytyrosol, a promising antioxidative and antiaging agent.

This research underscores how the integration of computational and experimental synthetic biology tools offers a novel and sustainable approach to cost-effective biomanufacturing of natural cosmetic materials.

Keywords: Synthetic biology; manufacturing; cosmetic ingredients; computational; experimental

Introduction

Natural products are produced by living organisms, such as animals, plants and microorganisms. These structurally and functionally diverse compounds have found a variety of applications in pharmaceutical, nutraceutical, agricultural and cosmetic industries. Plants are a well-known source of bioactive molecules extensively utilized as cosmetic ingredients, including the potent antioxidative and anti-aging agents such as resveratrol and hydroxytyrosol.

Resveratrol (INCI name: resveratrol), a natural polyphenolic compound found in grapes, berries, and peanuts, has garnered significant attention in the cosmetic industry due to its potent antioxidative and anti-aging properties. This compound helps protect skin cells from oxidative stress and environmental damage, thereby slowing down the aging process and reducing the appearance of fine lines and wrinkles. Additionally, resveratrol exhibits anti-inflammatory and antimicrobial activities, which can aid in calming irritated skin and preventing acne [1]. Its ability to enhance skin elasticity and hydration further contributes to a youthful complexion. Consequently, resveratrol is increasingly incorporated into a variety of skincare products, including serums, creams, and lotions, aimed at promoting healthy, vibrant skin [2, 3].

Hydroxytyrosol (INCI name: hydroxytyrosol), one of the most potent antioxidants in nature, has recently emerged as a valuable ingredient in the cosmetic industry due to its superior skin-protective and anti-aging properties. Renowned for its ability to neutralize free radicals, this olive-derived natural product helps prevent oxidative stress and the subsequent damage to skin cells, thereby reducing the appearance of wrinkles and fine lines. Its anti-inflammatory capabilities [4] make it an excellent choice for soothing irritated skin and reducing redness, while its antimicrobial properties help maintain a clear complexion by combating acne-causing bacteria [5]. Additionally, hydroxytyrosol enhances skin hydration and elasticity, promoting a smooth, youthful appearance. Consequently, this powerful compound is frequently incorporated into a variety of skincare products, such as serums, creams, and lotions, designed to promote healthy, radiant skin.

While plant extraction and chemical synthesis are widely used methods for isolating and producing plant molecules, they come with several disadvantages. Plant extraction often requires the use of large quantities of organic solvents, which can be hazardous to both human health and the environment due to their toxicity and flammability. Additionally, this method can be inefficient and costly, as it may necessitate multiple extraction and purification steps to achieve the desired purity. Furthermore, using farmlands for plant growth competes with food production. Chemical synthesis, on the other hand, involves complex procedures that use toxic chemical reagents and harsh reaction conditions and can produce harmful by-products and waste, posing environmental and disposal challenges. Moreover, synthetic routes often lack the specificity and selectivity of natural biosynthetic pathways, potentially leading to lower yields and the production of unwanted isomers or side products. Both methods also typically consume significant energy and resources, making them less sustainable compared to more environmentally friendly alternatives such as biotechnological production.

By contrast, biomanufacturing offers a promising alternative, enabling the sustainable production of plant cosmetic ingredients. Through the rational engineering of microbial cell factories, biosynthetic pathways for plant natural products can be constructed and optimized in microbial hosts, achieving high-yield production of desired molecules. Unlike plant extraction and chemical synthesis, biomanufacturing operates under mild conditions and in water. By precisely controlling production conditions in bioreactors, product quality remains consistent, and purity is high.

To enable biomanufacturing of cosmetic ingredients on an industrial scale, extensive optimization work is often required after the biosynthetic pathway is established in a microbial host to create a highly efficient production process. While testing various parameters in lab experiments is often time-consuming, labor-intensive and inefficient, computational tools can be incorporated into biomanufacturing to significantly enhance the overall efficiency of process development. This work aims to integrate computational and experimental approaches to achieve high-yield production of resveratrol and hydroxytyrosol. Computational tools are employed to construct efficient biosynthetic

pathways and simulate metabolic flux in *Escherichia coli* cells. The results are validated through wet lab experiments, and resulting data can be fed back into computational models for more accurate predictions.

Materials and Methods.

General equipment and experimental materials

Products were analyzed and purified using an Agilent 1200 HPLC instrument equipped with an Agilent Eclipse Plus-C18 column (5 μm, 250 mm × 4.6 mm). Samples were eluted with a methanol-water gradient (5:95 to 95:5, v/v, containing 0.1% formic acid) at a flow rate of 1 mL/min over 30 minutes. ESI-MS spectra were obtained on an Agilent 6130 single quadrupole LC-MS. ^{1}H NMR spectrum was collected on a Bruker AV III HD 500MHz spectrometer. Electroporation was performed using a MicroPulser™ apparatus from Bio-Rad, USA. Other major lab instruments include thermocycler, biosafety cabinet, autoclave, DNA electrophoresis system, incubator, bioreactor, centrifuge, microcentrifuge, rotavapor, and chemical hood.

Phusion® High-Fidelity DNA polymerase, T4 DNA ligase and various restriction enzymes were sourced from New England Biolabs or Thermo Fisher Scientific. Commercial standards of resveratrol and hydroxytyrosol were purchased from Aldrich-Sigma. Luria-Bertani (LB) medium, other chemicals and all solvents were obtained from Fisher Scientific (Rockford, IL, USA).

Strains, vectors, media, and culture condition

E. coli XL1-Blue and BL21(DE3) were purchased from Agilent for gene cloning and protein expression, respectively. *Pseudomonas putida* KT2440 (ATCC 47054) was acquired from the American Type Culture Collection. The pJET1.2 vector (Thermo Fisher Scientific, USA) was used for routine cloning. pET28a(+), pACYCDuet-1, and pCDFDuet-1 (EMD Millipore, USA) were used for enzyme expression. Genes were amplified through PCR or synthesized by Gene Universal. All primers were ordered from Thermo Fisher Scientific. Carbenicillin (50 μg/mL), kanamycin (50 μg/mL), streptomycin (50 μg/mL) and chloramphenicol (25 μg/mL) were supplemented into the culture media for selection of correct clones when appropriate. *E. coli* strains were routinely grown in LB medium (Fisher Scientific, USA) at 37 °C. Protein expression was induced by the addition of 0.2 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at 28 °C. 20 g/L glucose and 3 mM L-tyrosine were added after 5 hours of IPTG induction and the induced cultures were incubated with shaking at 250 rpm and 28 °C for an additional 48 hours to produce the desired products.

Construction of engineered *E. coli* strains for the production of resveratrol and hydroxytyrosol

The 4-coumarate:CoA ligase (4CL) from *Arabidopsis thaliana*, AhSTS (stilbene synthase or STS from *Arachis hypogaea*), VvSTS (STS from *Vitis vinifera*), and tyrosine ammonia lyase (TAL) from *Saccharothrix espanaensis* were selected for resveratrol biosynthesis. The corresponding genes were synthesized by Gene Universal. The 4CL gene was ligated into pACYCDuet-1 to yield pACYCDuet-1-4CL. Similarly, the *Ahsts* and *Vvsts* genes were ligated into pET28a to yield pET28a-Ahsts and pET28a-Vvsts, respectively. The TAL gene was ligated into pCDFDuet-1 to yield pCDFDuet-1-TAL. The *Ahsts* gene was then introduced into this plasmid to yield pCDFDuet-1-TAL-Ahsts.

These plasmids were introduced into *E. coli* BL21(DE3) using different combinations through electroporation for resveratrol production.

The HpaB, HpaC and ECAO genes were amplified via PCR from the genome of *E. coli*, and the DODC gene was cloned from the genome of *P. putida* KT2440. These genes were first ligated into pJET1.2 and subsequently into different expression vectors to create pET28a-DODC, pACYCDuet-1-HpaBC, and pCDFDUET-1-ECAO-ADH. These plasmids were introduced into *E. coli* BL21(DE3) through electroporation for hydroxytyrosol biosynthesis.

Product analysis

After fermentation, the bacterial cultures were centrifuged at 13,000 rpm for 10 minutes, and the supernatants were analyzed using HPLC to determine the product titers. Standard curves for resveratrol and hydroxytyrosol were established by injecting different amounts of commercial standards into the HPLC and plotting the correlation between the amounts and the peak areas of these compounds in Excel. The actual product titers were calculated based on the product peak areas from the samples.

Computational probabilistic model construction

The PRISM probabilistic modeling language was used to model the resveratrol biosynthetic pathway. The computational model includes two static species, L-tyrosine and pyruvate, and five dynamic species: malonyl-CoA, acetyl-CoA, *p*-coumaric acid, *p*-coumaroyl-CoA, and resveratrol. There are eight total transitions between them: three biosynthetic pathway transitions involving the TAL, 4CL, and STS enzymes, and five native metabolic reactions encompassing the intracellular production and uptake of acetyl-CoA and malonyl-CoA.

Integer variables represented the micromolar concentration of the five dynamic species, and guarded commands represented the eight transitions. Figure 1 shows a snippet of the PRISM model [6]. Two concurrently executing PRISM modules were created for the biosynthetic and native metabolic pathways. Within a pathway module, each reaction was modeled as a guarded command of the form [] *g* -> *r* : *u*. The empty brackets indicate no synchronization with another command is required, allowing independent evaluation and execution of each reaction. This empty synchronization label facilitates modeling concurrently occurring chemical reactions in a pathway module.

```
module native_pathway
// ---PDH: incoming acetyl-CoA---
 [] pyruvate > 0 -> rate_PDH : (acoa'=acoa+1);
// ---ACCOAC---
 [] acoa > 0 -> rate_ACCOAC : (mcoa'=mcoa+1)&(acoa'=acoa-1);
// ---PTA: cellular acetyl-CoA uptake---
 [] acetyl_CoA > 0 & rate_PTA > 0 -> rate_PTA : (acoa'=acoa-1);
// ---CS: cellular acetyl-CoA uptake---
 [] acetyl_CoA > 0 & rate_CS > 0 -> rate_CS : (acoa'=acoa-1);
// ---cellular malonyl-CoA uptake---
 [] malonyl_CoA > 0 & rate_mcuptk > 0 -> rate_mcoa_uptake : (mcoa'=mcoa-1);
endmodule

module synthetic_pathway
// ---TAL---
 [] L_tyrosine > 0 -> rate_TAL : (pca'=pca+1);
// ---4CL---
 [] pca > 0 -> rate_4CL : (pccoa'= pccoa+1)&(pca'=pca-1);
//---STS---
 [] pccoa > 0 & mcoa > 2 ->
   rate_STS : (resv'=resv+1)&(pccoa'=pccoa-1)&(mcoa'=mcoa-3);
endmodule
```

Figure 1. PRISM code snippet of modeled resveratrol biosynthetic pathway.

A reaction is enabled to occur if its guard expression *g* evaluates to true, typically ensuring that the substrate concentration is not zero. When enabled, the rate expression *r* determines the probability of the reaction occurring, based on the kinetic rate at the current model state. Finally, the update *u* modifies the model by increasing the product concentration and decreasing the dynamically changing substrate concentration.

Biosynthetic pathways prediction

To establish an efficient biosynthetic pathway for hydroxytyrosol in *E. coli*, we used computational tools to predict possible pathways and most efficient predicted pathways were selected for further

experimental validation.

One-step prediction

For one-step retrosynthetic prediction [7, 8], as illustrated in Figure 2, we adopted a Seq2Seq Transformer [9] to map product sequences to reactant sequences. This Transformer follows the encoder-decoder paradigm: the encoder processes the source SMILES sequence, transforming it into a latent representation, while the decoder combines this latent representation with its previous predictions to generate the target SMILES sequence. Trained end-to-end, the Transformer aims to minimize the discrepancy between the predicted and actual SMILES sequences, enabling the model to accurately infer the reactant precursors.

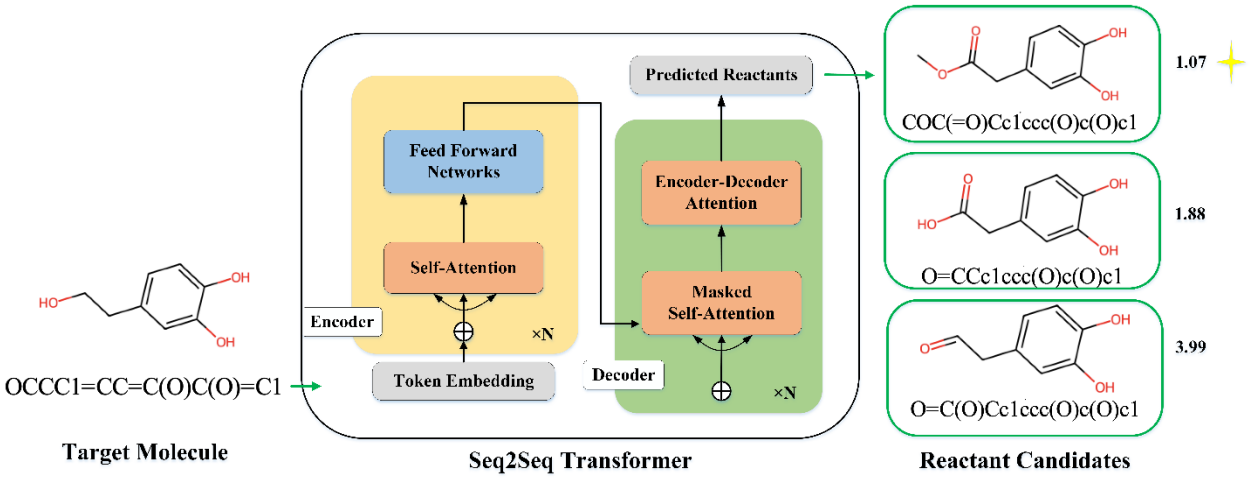

Figure 2. The Seq2Seq Transformer model for single-step prediction.

Multi-step planning

For multi-step planning [10, 11], as illustrated in Figure 3, we adopted Guided-Retro, which uses a best-first search algorithm to efficiently find high-quality synthetic routes. This approach translates the search into an AND-OR tree T, where molecule nodes are represented as 'OR' nodes and reaction nodes as 'AND' nodes. The search tree T begins with a single root compound node, representing the target compound n. At each iteration, a node d on the frontier of T is selected based on a value function. Node d is then expanded using the one-step prediction $p(d)$, extending T with one AND-OR stump. Subsequently, nodes with potential dependencies on d are updated.

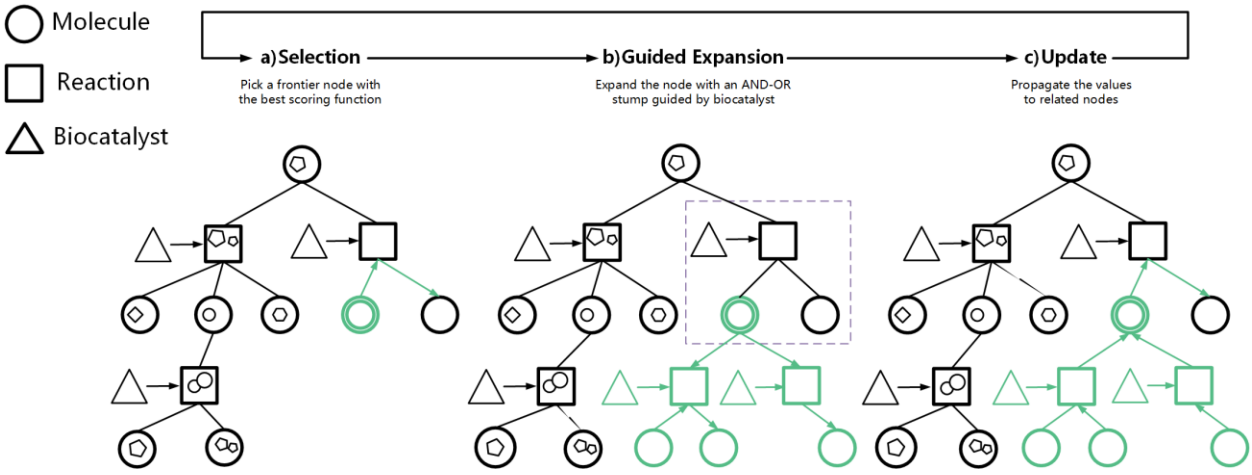

Figure 3. Multi-step biosynthesis planning with Guided-Retro.

The entire planning process consists of three steps: selection, guided expansion, and updating. In the selection step, the next node is chosen based on a carefully designed value function. Guided expansion involves using one-step prediction for the selected node to generate new molecule nodes and reaction nodes, along with their corresponding biocatalysts. During the updating step, the values of the relevant nodes are adjusted according to the difficulty of obtaining the biocatalysts and the cost of each reaction.

Results.

Construction of an artificial resveratrol biosynthetic pathway in *E. coli*

Tyrosine ammonia lyase (TAL) from *Saccharothrix espanaensis*, 4-coumarate:CoA ligase (4CL) from *Arabidopsis thaliana*, coumarate 3-hydroxylase (C3H) from *S. espanaensis*, and stilbene synthase (STS) from *Arachis hypogaea* were co-expressed in *E. coli* BL21(DE3) to create an artificial biosynthetic pathway of resveratrol in the bacterial host. As shown in Figure 4A, TAL catalyzes the deamination reaction of L-tyrosine to yield *p*-coumaric acid. 4CL ligates CoA to the carboxyl group of *p*-coumaric acid to generate *p*-coumaroyl-CoA. STS uses *p*-coumaroyl-CoA as the starter unit and malonyl-CoA as the extender unit to create resveratrol. HPLC analysis revealed that the engineered strain successfully produced resveratrol as the major product (Figure 4B).

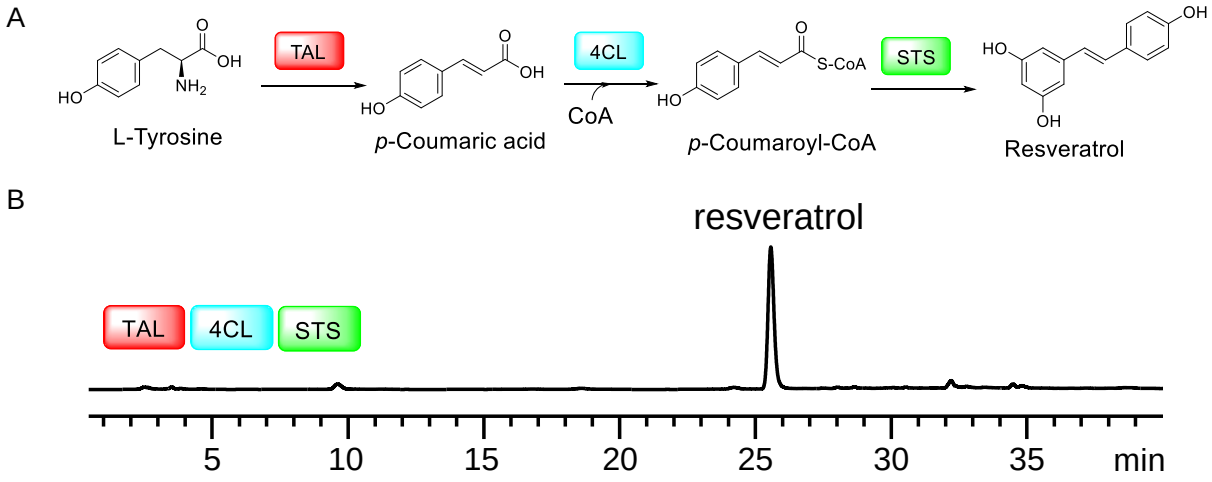

Figure 4. Reconstitution of resveratrol biosynthesis in *E. coli*. (A) An artificial biosynthetic pathway of resveratrol engineered into *E. coli*. (B) HPLC analysis of resveratrol production in engineered *E. coli* at 300 nm.

Probabilistic modeling and analysis of resveratrol production in *E. coli*

To circumvent the great time and material expense for testing a multitude of parameters, we applied an *in-silico* approach by constructing probabilistic models of the biosynthetic pathway for the production of resveratrol, and then performed stochastic simulation to analyze its interaction with native metabolism. The model consists of the reactions performed by the three biosynthetic enzymes including TAL, C3H and STS, along with a part of the cell's native central metabolism that feeds into the biosynthetic pathway. The constructed model successfully simulates the biological system in the expected manner with reasonable concentrations and outputs. The model runs concurrently, as each reaction can occur independently of all other reactions. The average of 100 random runs through the state space of the model produces an accurate approximation of the model's behavior, with an average confidence interval of around 1.5% of the original value, based on a 99% confidence level.

While many such metabolic models have been constructed in the past, most are deterministic, using either a system of differential equations or a flux balance analysis to solve for the product yield, disregarding kinetic parameters of each enzyme in the system.

Our work analyzed the enzyme's kinetic behavior by establishing a probabilistic model where the probability of each reaction occurring was determined by the relevant kinetic parameters and substrate concentrations, with a goal of understanding the intrinsic stochastic behavior observed in living systems [6]. The probability of choosing a particular reaction was determined by the corresponding enzyme's concentration-dependent kinetic rate at every state. A reaction whose enzyme has the fastest kinetic rate is most likely to occur; however, any reaction with a non-zero rate can also occur with lower probability. We measured the effects of varying concentration of enzymes in the biosynthetic pathway using stochastic simulation. Among the three biosynthetic enzymes, changing the concentrations of both TAL and 4CL showed a negligible increase in the predicted resveratrol yield, but the predicted yield varied proportionally to the concentration of STS. This behavior met our expectation because STS was identified as the rate-limiting enzyme by our stochastic analysis. This prediction was confirmed experimentally in the lab and improved the titer from 62.5 mg/L to 172.8 mg/L by increasing the expression level of STS in *E. coli* and replacing AhSTS with a more efficient STS - VvSTS, demonstrating the advantage of the computational modeling approach in analyzing a dynamic microbial production system.

Computational prediction of hydroxytyrosol biosynthetic pathways

We proposed a practical retro-biosynthesis model [8] and trained it on the MetaNetX [10] and REACT [12, 13] datasets. Unlike rule-based models, this model is fully data-driven, constructed based on elaborately curated biosynthetic and organic reaction data without the need for heuristic reaction template extraction. Additionally, during the pathway exploration, the model utilizes the promiscuity, fidelity, and diversity of enzyme catalysts [14] and incorporates advanced enzyme prediction tools [7, 10] as guides to rank the biological feasibility of potential pathways. This allows for timely discarding the pathways with low likelihood or high cost, thereby enhancing the efficiency of predictions. Using this approach, we predicted the retro-biosynthetic pathway for hydroxytyrosol, as shown in Figure 5.

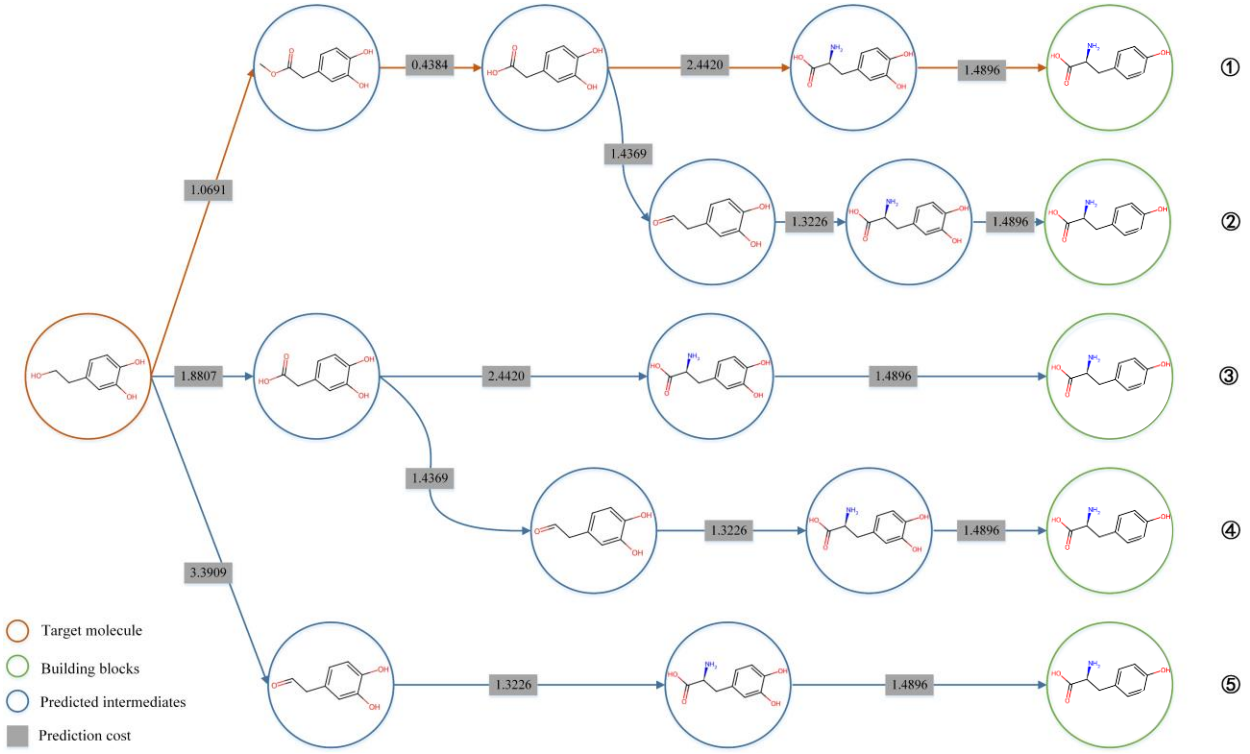

Figure 5. Predicted retro-biosynthesis pathways for hydroxytyrosol. Five representative candidate pathways are provided, with corresponding cost scores for each reaction.

From this figure, it can be seen that our model could generate various possible biosynthetic pathways, with predicted cost scores for initial evaluations. It is important to note that a few predicted pathways tend to take shortcuts through small cofactor-type molecules or by-products. To mitigate this issue, more meticulous data preprocessing and the integration of atom-mapping methods into the model can be employed. Among the five pathways shown in Figure 5, route (5) is more feasible with known enzymatic reactions and is further expanded in Figure 6 with the necessary enzymes.

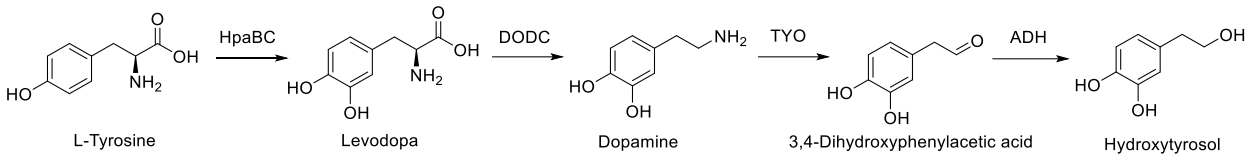

Figure 6. Artificial biosynthetic pathway of hydroxytyrosol established in *E. coli*. HpaB: 4-Hydroxyphenylacetate 3-hydroxylase; HpaC: Flavin reductase; DODC: Levodopa decarboxylase; TYO: tyramine oxidase; ADH: Alcohol dehydrogenase.

Engineered biosynthesis of hydroxytyrosol in *E. coli*

Based on the computational predictions, hydroxytyrosol can be synthesized from L-tyrosine (Figure 6). A total of five biosynthetic enzymes from various sources, including HpaB and HpaC from *E. coli*, DODC from *P. putida* KT2440, ECAO (TYO) from *E. coli* [15], and ADH from *E. coli* [16]. The corresponding genes were amplified through PCR from the genomes of *E. coli* and *P. putida*, respectively. They were inserted into pET28a, pACYCDuet-1 and pCDFDuet-1 to yield pET28a-DODC, pACYCDuet-1-HpaBC, and pCDFDUET-1-ECAO-ADH. These plasmids were co-transferred into *E. coli* BL21(DE3) and the resultant engineered strains were grown in LB medium in the presence of kanamycin, streptomycin, and chloramphenicol. Product formation was induced with IPTG. HPLC analysis of the culture of this strain showed that a major product was produced from L-tyrosine (Figure 7A). The UV spectrum of this product matched that of hydroxytyrosol (Figure 7B). Furthermore, ESI-MS of this product showed several ion peaks including $[M-H]^-$ at *m/z* 153.2, $[M+Cl]^-$ at *m/z* 189.2, and $[2M-H]^-$ at *m/z* 307.3 (Figure 7C), confirming that this product has a molecular weight of 154, further suggesting that this product is hydroxytyrosol. We then isolated this product (Figure 7D) and collected its ^1H NMR spectrum (Figure 7E). The signals were assigned, which are consistent with those reported for hydroxytyrosol [17], thus confirming that the product generated by the engineered *E. coli* strain is indeed hydroxytyrosol. This result indicated that an efficient hydroxytyrosol biosynthetic pathway has been successfully constructed in *E. coli*.

The engineered strain was cultured in a 5-L bioreactor. After the IPTG induction and addition of 25 g/L L-tyrosine in the fermentation broth, the titer of hydroxytyrosol reached 15.6 g/L in 48 hours, making it feasible for industrial scale production of this plant natural product through microbial fermentation.

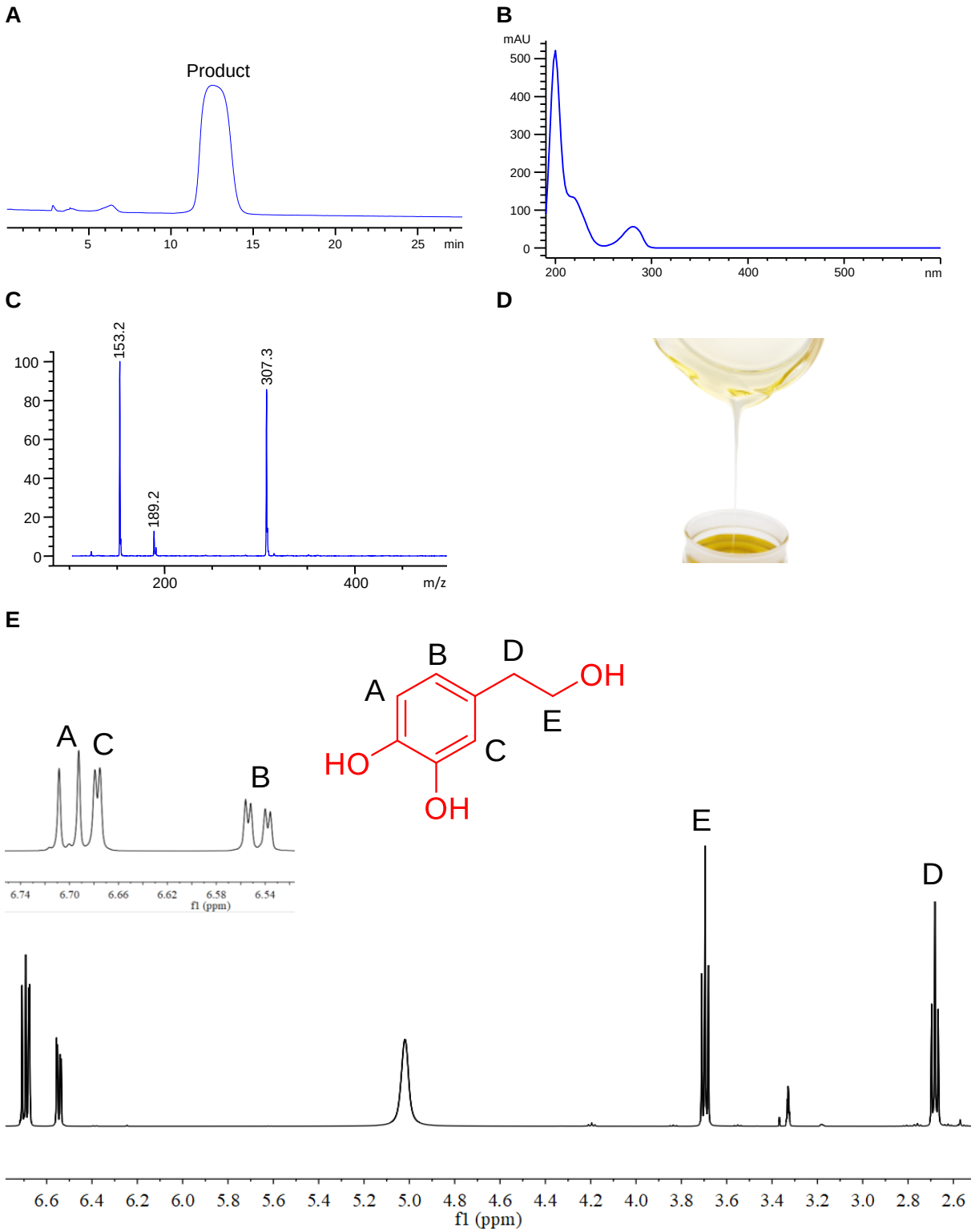

Figure 7. Engineered biosynthesis of hydroxytyrosol in *E. coli*. (A) HPLC analysis of hydroxytyrosol production in *E. coli* at 275 nm. (B) UV spectrum of the product. (C) Photo of purified hydroxytyrosol. (D) ESI-MS (-) spectrum of the product. (E) ^{1}H NMR spectrum of the product in CD_3OD.

Discussion.

Plant natural products serve as a rich source of bioactive compounds used in cosmetics. To address sustainability and cost-effectiveness, synthetic biomanufacturing offers a promising alternative to traditional methods like solvent-based plant extraction and chemical synthesis. Over the past two decades, the development of synthetic biology has enabled the construction of artificial biosynthetic pathways in microbial hosts such as *E. coli* and *Saccharomyces cerevisiae*. However, this type of work heavily relies on existing knowledge and individual expertise. Additionally, to move the technology from the laboratory to large-scale production, the process needs to be intensively optimized. Manual testing of each parameter can be time-consuming and inefficient.

In this work, we developed an integrated approach that combines both computational and experimental methods to innovate microbial production of plant-derived cosmetic ingredients. Two examples are provided in this paper.

First, we focused on resveratrol, a well-known antioxidative and anti-aging plant natural product. We constructed an artificial biosynthetic pathway in *E. coli* and successfully produced this cosmetic ingredient in the engineered bacterium. Furthermore, we established a computational model and performed in silico simulations of the biosynthetic pathway. By analyzing the kinetic parameters of the biosynthetic enzymes and manipulating enzyme concentrations within the computational model, we identified STS as the rate-limiting enzyme in this pathway. The catalytic efficiency and expression level of STS limited the overall metabolic flux towards resveratrol. This prediction was experimentally validated in the lab. By increasing the expression level of STS and utilizing a more efficient STS from another source, the resveratrol titer was enhanced by approximately 200%. Future work on enzyme evolution of STS to increase its efficiency may lead to further increases in resveratrol production.

The second example involves another plant-derived cosmetic ingredient, hydroxytyrosol. We constructed a computational platform capable of predicting possible biosynthetic pathways for selected products. This platform enables us to construct novel pathways for any product, even those with previously unknown pathways. The most efficient pathway for hydroxytyrosol, which uses L-tyrosine as the precursor, was selected and constructed in the *E. coli* host, resulting in a highly efficient producing strain. This strain is currently used for industrial production of this high-value product. The retro-biosynthesis model proposed in this work can enumerate diverse biosynthetic pathways, showing great potential in biogenesis analysis, biosynthetic pathway reconstruction, and rational design.

Conclusion.

A major challenge in efficient biomanufacturing of cosmetic ingredients lies in constructing an efficient biosynthetic pathway within microbial hosts. This research demonstrates that the integration of computational and experimental approaches can significantly improve the process, enabling the production of various cosmetic ingredients in a sustainable and cost-effective manner. Computational tools were created in this work, which not only predict feasible biosynthetic pathways for target compounds, but also identify metabolic flux bottlenecks. Experimental tools provide critical data for computational models to improve the accuracy of simulation and prediction. Furthermore, they can validate the computational results and result in highly efficient production processes for cosmetic ingredients. Therefore, this combined approach could yield innovative and sustainable biomanufacturing processes for biobased cosmetic ingredients.

Acknowledgments.

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Conflict of Interest Statement.

The hydroxytyrosol work was solely conducted by Viablife and this ingredient is currently produced commercially at the company's manufacturing site.

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