Biosynthesis and Biotechnological Synthesis of Hydroxytyrosol

Jiali Tang¹, Jiaying Wang¹, Pengfei Gong¹, Chengtao Wang¹, and Wei Chen¹

¹Beijing Technology and Business University School of Food and Health

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Abstract

Background: Hydroxytyrosol (HT), a phenolic compound derived from plants, exhibits robust antioxidant activity and possesses pharmacological properties, including anti-inflammatory, anti-cancer, anti-bacterial, and anti-viral effects. As a result, HT has gained significant attention as an excellent nutraceutical and food additive.

Purpose and scope: Currently HT is mainly produced by plant extraction and chemical synthesis. With the gradual development of biosynthesis technology and the increasing concern for food health, biosynthesis of HT has gained more and more attention from researchers.

Summary and conclusion: Biotechnological synthesis of HT offers distinct advantages over plant extraction and chemical synthesis, such as being environmentally friendly, safe, and cost-effective. This comprehensive review focuses specifically on the biosynthetic pathway of HT and recent advancements in biotechnological synthesis of HT. The review also discusses the potential applications of HT as a nutraceutical. Through a comprehensive analysis of the biosynthesis and biotechnological synthesis of HT, this review contributes to the growing body of knowledge in this field and paves the way for sustainable and scalable production of this versatile compound.

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Jiali Tang¹, Jiaying Wang¹, Pengfei Gong¹, Chengtao Wang¹,*, and Wei Chen¹,*

¹Key Laboratory of Geriatric Nutrition and Health, Ministry of Education, Beijing Advanced Innovation Center for Food Nutrition and Human Health, Beijing Engineering and Technology Research Center of Food Additives, School of Food and Health, Beijing Technology and Business University, Beijing 100048, PR China

*Correspondence to: Dr. Chengtao Wang, No.33 Fucheng Road, Haidian District, Beijing 100048, PR China
Tel.: +86-10-68984003
E-mail: wangchengtao@th.btbu.edu.cn

Dr. Wei Chen, No.33 Fucheng Road, Haidian District, Beijing 100048, PR China
Tel.: +86-10-68984003
mail: weichen@btbu.edu.cn

Abbreviations:

HT: Hydroxytyrosol; KSF: Key Success Factors; SGS: Societe Generale de Surveillance; SMRT: Single Molecule Real-Time; PPO: Polyphenol oxidase; DDC: Dopa decarboxylase; CuAO: Copperamine oxidase; ALDH: Acetaldehyde dehydrogenase; 3,4-DHPA: 3,4-dihydroxyphenylacetaldehyde; 4-HPA: 4-Hydroxyphenylacetic acid; L-DOPA: L-3,4-dihydroxyphenylalanine; GMOs: Genetic Modification Organisms

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Biotechnological synthesis of HT offers distinct advantages over plant extraction and chemical synthesis, such as being environmentally friendly, safe, and cost-effective. This comprehensive review focuses specifically on the biosynthetic pathway of HT and recent advancements in biotechnological synthesis of HT. The review also discusses the potential applications of HT as a nutraceutical. Through a comprehensive analysis of the biosynthesis and biotechnological synthesis of HT, this review contributes to the growing body of knowledge in this field and paves the way for sustainable and scalable production of this versatile compound.

Key points
- Introduction of Biosynthetic Routes and Biotechnological Pathways for Hydroxytyrosol Production.
- Overcoming the Rate-Limiting Phase in Hydroxytyrosol Biosynthesis and Creation of Novel Synthesis Routes.

Keywords
hydroxytyrosol; biosynthesis; biotechnological synthesis; nutraceutical; sustainable production

1 Introduction

Hydroxytyrosol (HT), recognized as the most potent and efficient antioxidant known\(^1\) is abundant in olives. In nature, HT is present in the olive plant as part of the secoiridoid compound oleuropein found in its leaves, fruit, oil, and oil production waste products. The "Mediterranean Diet", named after the countries around the Mediterranean Sea, Spain, Italy, France and Greece, is a healthy, light, simple and nutritious style of eating. As olive oil is a prime constituent of the health-promoting Mediterranean diet, HT has obtained recognition for its attributes, supported by a recent health claim of the European Food Safety Authority.

HT is currently used in cosmetics and food supplements, where it has anticancer, anti-inflammatory, anti-apoptotic and neuroprotective activities, and has been approved as a food additive by FDA GRAS. HT\(^2\), as a low-calorie and low-calorie alternative to sugar additives, is often added to beverages, candies, chocolates, pastries and other foods. (Figure 1).

At present, HT can be synthesized through physicochemical and biotechnological methods. Plant extraction involves the isolation of oleuropein from olive oil production waste or olive branches and leaves, followed by acid or catalytic hydrolysis to extract HT. Chemical synthesis is achieved under normal temperature conditions using solvents such as acetonitrile, tetrahydrofuran, dichloroethane, methylene chloride, or toluene. These solvents, along with 3,4-dimethoxyphenylethanol and aluminum triiodide, are mixed, followed by the addition of boron trifluoride tetrahydrofuran or boron trifluoride ether. Subsequently, the mixture is subjected to warming reflux, cooling, filtration, and activated carbon treatment to obtain white solid powdered HT. Biotechnological methods encompass enzymatic transformation, non-genetically engineered bacteria, and genetically engineered bacteria for HT production.

Fernandez-Bolanos et al.\(^3\) investigated the hydrothermal treatment of two-phase olive waste (alperujo) and its effect on HT solubility. They successfully extracted and purified HT from alperujo using an inexpensive chromatographic system, yielding approximately 4.5-5 kg of HT from 1000 kg of alperujo with a moisture content of 70%. Following purification, a minimum of 3 kg of HT with a purity of 90-95% was obtained. Neji
et al. \cite{1} catalyzed the generation of HT extracts from tyrosol through a wet hydrogen peroxide-catalyzed oxidation reaction using montmorillonite Key Success Factors (KSF) as a solid acid catalyst at room temperature. Papageorgiou et al. \cite{2} employed a solid-liquid extraction method to obtain HT from oleuropein, utilizing a water-ethanol mixture, hydrochloric acid hydrolysis, and optimized ethyl acetate extraction. They recovered 10-15 g of HT per kg of olive leaves. Squillaci et al. \cite{3} obtained acidic extracts rich in HT from olive oil dregs by incubating them at 37 degrees C for 1 h under pH 1.25 conditions. HT recovery of 92.50% was achieved using Amberlite XAD16N and XAD7HP resins with 25% ethanol as the optimal elution condition.

While plant extraction and chemical synthesis methods have certain drawbacks such as low recovery, lengthy cycle times, tedious steps, high costs, and environmental pollution due to the use of organic reagents, biotechnological synthesis of HT demonstrates advantages in terms of being environmentally friendly, safe, and cost-effective. The development of biological methods is rapidly gaining popularity and is expected to become mainstream in the future. Therefore, this review primarily focuses on the biosynthetic pathway of HT and the research pertaining to biological synthesis. The aim is to provide a theoretical foundation for further HT development, emphasizing the potential of biotechnological approaches \cite{1, 2, 3}.

### 2 Natural Biosynthetic Pathway of Hydroxytyrosol

The biosynthesis of HT, a potent antioxidant predominantly found in olive leaves, involves a complex network of enzymes that have not yet been fully elucidated. Guodong et al. \cite{4} employed a combined Societe Generale de Surveillance (SGS) and Single Molecule, Real-Time Sequencing (SMRT) approach to analyze the genes associated with the HT biosynthesis pathway, revealing that several enzymes, including polyphenol oxidase (PPO), Dopa decarboxylase (DDC), copperamine oxidase (CuAO), and acetaldehyde dehydrogenase (ALDH), play crucial roles. Mougiou et al. \cite{5} conducted transcriptome analysis of young olive fruits, demonstrating the presence of transcripts for all enzymes involved in the HT biosynthesis pathway. Enzymatic activities of tyrosine decarboxylase TDC, CuAO, ALDH, and PPO were identified from the proposed HT biosynthesis genes (tdc, mao, par, tyr, th, ddc, aldh). These findings revealed that two HT biosynthetic pathways can be formed using tyrosine as a precursor molecule. In one pathway, tyrosine is converted to L-DOPA by PPO, then to dopamine by TDC, and subsequently, CuAO generates 3,4-dihydroxyphenylacetaldehyde (3,4-DHPA), which ultimately leads to HT formation through ALDH. In the other pathway, tyrosine is transformed into tyramine by TDC, followed by CuAO-mediated conversion to 4-Hydroxyphenylacetic acid (4-HPA). ALDH then produces tyrosol, which is further converted to HT by PPO. Sanchez et al. \cite{6} identified two phenylethylaldehyde reductase genes, OePAR1.1 and OePAR1.2, involved in HT synthesis. The reaction catalyzed by OePAR is an important biochemical step in the formation of hydroxytyrosol from the amino acid L-3,4-dihydroxyphenylalanine (L-DOPA) in olives.

During alcoholic fermentation, various heteroalcohols are believed to be generated through amino acid metabolism pathways. These pathways include the deamination of amino acids to \(\alpha\)-keto acids, followed by decarboxylation to aldehydes and subsequent reduction to heteroalcohols (Ehrlich pathway). Additionally, \(\alpha\)-keto acids produced during amino acid biosynthesis from sugars can undergo degradation to form heteroalcohols. Gallardo-Fernandez et al. \cite{7} employed isotopic labeling analysis to investigate HT biosynthesis in \textit{Saccharomyces cerevisiae}. The study revealed the presence of unlabeled compounds alongside tyrosine, suggesting the existence of an alternative pathway for HT synthesis. Moreover, the concentration of unlabeled HT was approximately ten times higher than that of labeled HT, indicating that both the mangiferic acid pathway and the Ehrlich pathway contribute to HT formation, with the former playing a more prominent role in tyrosol synthesis. Consequently, sugar metabolism emerges as the primary precursor for the synthesis of aromatic higher alcohols.

In the context of low-temperature stress on olive trees and its relationship with genotype, Mougiou et al. \cite{8} investigated the physiological and biochemical effects. Their findings demonstrated that mRNA levels of PPO genes involved in HT biosynthesis and plant defense were upregulated after 24 hours of stress at 0 °C, with elevated expression persisting for an extended duration. Notably, three genes involved in HT biosynthesis exhibited increased expression levels following cold stress, suggesting that low-temperature conditions induce prolonged upregulation of mRNA and related genes associated with HT biosynthesis. Overall, understanding
the natural biosynthetic pathway of HT, including the involvement of specific enzymes and the impact of environmental factors, provides valuable insights for further research and the development of innovative strategies for HT production.

3 Biotransformation via enzyme

Enzymatic biotransformation has emerged as a promising strategy for the production of HT by utilizing structurally analogous substrates and specific enzymes. Several investigations have explored the utilization of diverse enzymes and substrates to achieve efficient HT synthesis. Hamza and Sayadi[12] employed Aspergillus niger-glucosidase generated by A. niger to catalyze the bioconversion of rape leaf extract and olive mill wastewater, resulting in the extraction of HT, with maximum concentrations of 1.1 and 0.5 g/L. They meticulously optimized the submerged culture conditions and devised parameters for the effective production of the enzyme. Chatzikonstantinou et al.[13] developed a potent biocatalyst by immobilizing β-glucosidase on chitosan-coated magnetic beads. This biocatalyst facilitated the modification of olive leaf extracts, attaining remarkable conversion of oleuropein (exceeding 90%) and a 2.5-fold enrichment of HT.

Briante et al.[14] immobilized partially purified hyperthermophilic β-glycosidase on chitosan support, thereby enabling the expedited biotransformation of oleaginous leaf extract into highly purified HT (91-94% in weight) within a short duration (14-16 h). Trincone et al.[15] synthetized novel HT mono- and disaccharide derivatives through the enzymatic conversion of tyrosol glycoside derivatives utilizing marine α-glucosidase from Aplysia fasciata and commercial tyrosinase from mushrooms. This methodology yielded HT products with final concentrations of 9.35 and 10.8 g/L, respectively.

Furthermore, engineered enzymes have been employed to enhance HT synthesis. Brouk et al.[16] harnessed protein engineering and statistical modeling to enhance the substrate specificity and oxidative activity of toluec monooxygenase. This approach enabled the efficient synthesis of HT from abundant and cost-effective 2-phenylethanol. Donadio et al.[17] investigated the biotransformation of unconventional substrates, including 2-phenoxyethanol, phthalate, and 2-indanol, utilizing recombinant toluene o-xylene monooxygenase expressed in Escherichia coli cells. Notably, they successfully generated six hydroxylated derivatives, including HT, from these substrates.

To surmount the limitations associated with the conversion of tyrosol to HT by tyrosinase, Deri-Zenaty et al.[18] developed a continuous dual enzyme reaction system utilizing sol-gel-immobilized tyrosinase from Bacillus megaterium and glucose dehydrogenase expressed in E. coli cell extract. Under optimized conditions, this system yielded HT with a final concentration of 7.68 g/L and a productivity of 2.30 mg HT/mg TyrBm bead.

Collectively, these investigations underscore the potential of enzymatic biotransformation as an efficient approach for HT production. By employing specific enzymes and substrates, researchers have demonstrated successful synthesis of HT from diverse sources. Further refinement and advancement of enzyme systems hold promise for enhancing HT production yields and productivity.

4 Production via Non-Genetic Modification Organisms (GMOs)

Researchers have explored non-GMO approaches for the production of HT using various microbial strains and immobilization techniques. These methods offer alternative and sustainable ways to produce HT without genetic modification (Figure 2). Allouche and Sayadi[19] conducted a screening of Serratia marcescens strain using p-tyrosol as the sole carbon source. They optimized the growth conditions and p-tyrosol concentration for the conversion of p-tyrosol to HT during bacterial growth. The best HT yield (80%) was obtained by Ser. marcescens growing cells at the end of the exponential phase with the addition of 2 g/L of p-tyrosol followed by 1 g/L of p-tyrosol after 7 hours of incubation. Bouallagui and Sayadi[20] employed immobilized resting cells of Pseudomonas aeruginosa in calcium alginate beads to enhance HT production. The biotransformation rate of cells immobilized in calcium alginate beads reached 86% in the presence of 5 g/L tyrosol during a single batch process. Carlozzi et al.[21] produced a sustainable and environmentally friendly product from olive oil mill effluent by using residual effluent as a feedstock and utilizing Rhodopseudomonas sp. S16-FVPT5 to
obtain a HT-rich mixture. Rebollo-Romero et al.\cite{22} investigated factors influencing HT yield during alcoholic fermentation, including yeast strain, initial tyrosine concentration as a precursor, and the effect of synthetic and sterilized natural grape juice. Commercially available yeast resulted in a higher final HT yield, reaching a maximum of 6.12 ng/mL. Anissi et al.\cite{23} focused on a Gram-positive bacterium capable of producing HT through the conversion of tyrosol or L-tyrosine. They identified the bacterium as *R. pyridinivorans* strain 3HYL DSM109178 based on phenotypic characteristics and 16S rDNA sequence. They also developed a plasmid-cured strain, *R. pyridinivorans* 3HYL-AO, through random chemical mutagenesis. The wild-type strain yielded 16.4 +/- 0.23 mmol/L of HT from tyrosol, while the mutant strain *R. pyridinivorans* 3HYL-AO produced 21.75 +/- 0.34 mmol/L of HT.

These non-GMO approaches highlight the potential of microbial strains and immobilization techniques in achieving efficient HT production. Further exploration of these strategies may contribute to the development of sustainable and scalable production methods for HT.

### 5 Production via GMOs

*E. coli* possesses several advantageous characteristics for the biosynthesis of HT, including a well-established genetic background, high expression levels of target genes, a robust expression system, ease of cultivation, rapid growth, strong resistance to contamination, and low cost. Furthermore, *E. coli* has been approved by the U.S. FDA as a safe genetically engineered host organism. As a result, it has found extensive use in HT biosynthesis.

HT biosynthesis can be hindered due to the through conversion of HT to ortho-quinone by tyrosinase or the lack of cofactors necessary for tyrosine hydroxylase. To address these challenges, researchers have explored various strategies. For instance, Satoh et al.\cite{24} demonstrated that the cofactor tetrahydroadenine (MH4) from *E. coli* could act as an alternative cofactor to TH in the presence of the BH4 regeneration pathway, preventing overoxidation. Additionally, the knockout of endogenous aromatic aldehyde oxidase was employed to prevent the formation of by-products, resulting in the nearly complete synthesis of HT in engineered *E. coli*. Other studies have utilized *E. coli* strains carrying specific hydroxylases to synthesize HT, resulting in notable yields of HT through strain optimization\cite{25-29}.

In the pursuit of efficient HT production, Chen et al.\cite{30} designed two biosynthetic pathways using engineered *E. coli* strains as biocatalysts. Each pathway contained a catalytic step that lacked an efficient enzyme. Through protein engineering, highly active tyrosol hydroxylases or tyramine hydroxylases were obtained to complete the pathways, resulting in elevated HT yields compared to previously reported routes. Furthermore, a monoxygenase mutant with hybrid function was developed through directed divergent evolution, which led to a significant increase in HT yield. Notably, the complex pathway achieved a much higher HT yield (93%) compared to pathway 1 (56%) and pathway 2 (54%) (Figure 3).

As shown in Figure 4, to overcome rate-limiting steps in HT biosynthesis, Yao et al.\cite{31} developed efficient engineered *E. coli* by addressing the limiting enzymatic step. By replacing mouse tyrosine hydroxylase with the HpaBC monoxygenase from *E. coli* BL21(DE3) strain and optimizing tyramine oxidase activity using an HT-responsive biosensor, the conversion from tyrosine to HT was achieved at a remarkable efficiency of 95%. Similarly, Zeng et al.\cite{32} established an enzymatic cascade pathway for HT synthesis using L-tyrosine as a substrate. This pathway involved the coordinated action of HpaBC, L-amino acid deaminase (LAAD), α-keto acid decarboxylase (ARO10), and phenol ammonia-lyase (PAL), resulting in high catalytic activity and efficient HT synthesis. Using this pathway, 24.27 mM of HT was obtained from 25 mM of L-tyrosine with a conversion rate of 97.1%.

Apart from *E. coli*, *S. cerevisiae* has emerged as a safe and promising host organism for HT production. Muniz-Calvo et al.\cite{33} improved HT production in *S. cerevisiae* by heterologously expressing the HpaBC enzyme complex from *E. coli*. Overexpression of *hpaB* and *hpaC* genes in *S. cerevisiae* led to enhanced HT production (3400 ± 500 μg/L), highlighting the potential of HpaBC overexpression as a tool to redirect central carbon flux towards tyrosine catabolism. Bisquert et al.\cite{34} integrated the HpaBC hydroxylase complex into the genome of *S. cerevisiae*, resulting in increased HT productivity without the need for exogenous tyrosol or
tyrosine supplementation (375 mg/L). Liu et al. [35] also achieved substantial improvements in HT production by constructing new synthetic pathways in \textit{S. cerevisiae} and optimizing key enzymatic steps (308.65 mg/L). These advancements resulted in the highest reported titer of HT biosynthesis in microorganisms to date (375 mg/L).

Co-culture studies involving engineered strains have shown promise for the production of HT with enhanced efficiency and yield. One example of such research is the study conducted by Liu et al. [36], where a coculture system consisting of \textit{S. cerevisiae} and \textit{E. coli} was developed to efficiently produce HT through \textit{de novo} biosynthesis. The metabolic capabilities of both microorganisms were leveraged, with \textit{S. cerevisiae} engineered to produce tyrosol using its endogenous Ehrlich pathway, and \textit{E. coli} dedicated to converting tyrosol to HT using HpaBC. To optimize HT production, intra- and intermodule engineering strategies were implemented in this microbial consortium, leading to the production of 435.32 mg/L of HT (Figure 5). In another study Gong et al. [26] developed a novel approach to improve the production of HT. The strategy involved designing a biosynthetic pathway using tyrosine as the substrate and selecting specific enzymes. Glutamate dehydrogenase GdhA was also overexpressed to facilitate cofactor cycling through coupled reactions catalyzed by transaminase and reductase. To optimize the process, the biosynthetic pathway was divided into two parts and utilized separate \textit{E. coli} strains. This approach successfully achieved a HT yield of 9.2 mM from 10 mM tyrosine through careful optimization.

These studies demonstrate the potential of co-culturing engineered strains for efficient HT production. By strategically designing and combining the metabolic pathways of different strains, co-culture systems can effectively convert precursor compounds into HT, leading to higher yields. The utilization of engineered strains in co-culture systems holds promise for the development of robust and sustainable processes for HT production.

In summary, metabolic engineering of microorganisms offers a promising approach for the sustainable production of HT. Both \textit{E. coli} and \textit{S. cerevisiae} have been extensively explored as host organisms, with genetic modifications and pathway optimizations leading to significant improvements in HT yields. These advancements demonstrate the potential for large-scale HT production using GMOs. Further research and optimization efforts in microbial biosynthesis hold great promise for meeting the increasing demand for HT in a sustainable manner.

6 CONCLUSION

In conclusion, the biosynthesis and production of HT have made significant progress, offering valuable insights for industrial development. HT possesses diverse properties and high value, making it an attractive compound with extensive potential applications. Biological production methods, particularly through genetic modification of microorganisms, are rapidly advancing and offer advantages such as scalability and sustainability (Figure 6).

However, there are still challenges to address in the production of HT. The limited availability of suitable cell factories is a current limitation that needs to be overcome. Research efforts should focus on expanding the range of organisms that can efficiently produce HT, exploring alternative host organisms, and optimizing existing ones. Cost is another important consideration. Although biological production is advantageous in terms of scalability, the cost of substrates, such as glucose, can still be significant. Future efforts should explore the use of cheaper raw materials or waste streams as feedstocks for HT production, aiming to reduce production costs and enhance economic viability. Furthermore, the extraction and stability of HT are areas that require attention. Developing efficient extraction methods and improving the stability of HT during processing, storage, and utilization are crucial for its commercial application.

Overall, future research and development should focus on expanding the repertoire of cell factories, optimizing production processes, reducing costs, and addressing extraction and stability issues. With continued efforts and advancements in these areas, the industrial production of HT can be further improved, enabling its widespread utilization in various industries.
Author Contributions

JT, CW and WC conceived the conceptualization. JT and JW designed the formal analysis, and methodology development. JT and PG conducted the data curation and the validation process. JT, CW and WC analyzed the data. JT and WC wrote the manuscript. JT, CW and WC contributed to the reviewing and editing. CW and WC provided the materials and agents and contributed to the project administration. All authors read and approved the manuscript.

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Data Availability Statement

Data will be made available on request.

Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Reference


Figure legends

Figure 1. Functions of hydroxytyrosol.

Figure 2. Production of hydroxytyrosol by non-genetically engineered bacteria.

Figure 3. Three synthetic pathways of hydroxytyrosol.

Figure 4. Pathway for oxidation of tyrosine to hydroxytyrosol. (a) L-tyrosine oxidation is catalyzed by tyrosine hydroxylase (TH) in the presence of the pterin cofactor. (b) Highly efficient mutant enzymes HpaBC and TYO were obtained by protein engineering, which sequentially lifted the two rate-limiting steps in the original hydroxytyrosol synthesis pathway and increased the hydroxytyrosol synthesis capacity of the original synthesis pathway.

Figure 5. De novo biosynthesis of hydroxytyrosol using a coculture system consisting of S. cerevisiae and E. coli.

Figure 6. Applications and perspectives of hydroxytyrosol.