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Review

Biotechnological Advances in Vanillin Production: From Natural Vanilla to Metabolic Engineering Platforms

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Abstract: Vanillin, an aromatic aldehyde, is one of the most popular flavors worldwide, extensively used in the food, cosmetics, pharmaceutical, and agrochemical industries. Despite its widespread use, less than 1% of the total vanillin production is natural, with the majority being synthesized chemically. While chemical synthesis can help to meet the growing demand for vanillin, a strong market trend has rapidly developed for products created from natural ingredients, including natural vanillin. Given the labor-intensive process of extracting vanillin from vanilla pods, there is a critical need for new metabolic engineering platforms to support the biotechnological production of nature-identical vanillin. This review highlights the significance of vanillin in various markets, its diverse applications, and the current state of bio-engineered production using both prokaryotic and eukaryotic biological systems. Although recent advancements have demonstrated successful vanillin production through biocatalytic approaches, our focus was to provide a current and innovative overview of vanillin bioengineering across various host systems with special consideration placed on microalgae, which are emerging as promising platforms for vanillin production through metabolic engineering. The use of these systems to support the biotechnological production of vanillin, while leveraging the photosynthetic capabilities of microalgae to capture CO_2 and convert it into biomass, can significantly reduce the overall carbon footprint.

Keywords: microalgae; vanilla; synthetic biology; biotechnological production; sustainability; photosynthetic microorganisms

Citation: Tazon, A.W.; Awwad, F.; Meddeb-Mouelhi, F.; Desgagné-Penix, I. Biotechnological Advances in Vanillin Production: From Natural Vanilla to Metabolic Engineering Platforms. *BioChem* **2024**, *4*, 323–349. https://doi.org/10.3390/biochem4040017

Academic Editors: Buyong Ma and Manuel Aureliano

Received: 9 October 2024
Revised: 5 November 2024
Accepted: 25 November 2024
Published: 27 November 2024

1. Introduction

In recent decades, the demand for flavor and fragrance compounds has surged, driven by the consumer preference for enhanced sensory experiences in food and other products [1]. Vanillin, in particular, has emerged as one of the most widely used flavoring agents worldwide, valued for its distinctive aroma and versatility [2,3]. Consequently, the aroma and fragrance industry has invested heavily in innovative production methods for vanillin to meet this growing demand sustainably and efficiently. Vanillin is the major organoleptic compound of vanilla aroma and is an aromatic aldehyde crystallized in the form of a white powder with a sweet taste and intense aroma [4]. The production of vanillin is possible through natural plant, biotechnological, and chemical processes [5]. However, only natural plant-extracted vanilla and vanillin produced using biotechnological platforms have been approved by the European Food Law (European Directive 88/388/EEC, OJ No. L184, 22 June 1988) and United States of America Food Law [6] as a "natural" food-grade spice [7]. Lately, a strong market trend has developed rapidly for products designed from natural ingredients [8], thus creating a strong demand for nature-identical vanillin, mainly for applications in beverages and foods with therapeutic properties. Currently, the global vanillin market is expected to grow from USD 12.73 billion in 2022 to USD 19.11 billion by 2030, with a Compound Annual Growth Rate (CAGR) of 5.2% over the forecast period

between 2022 and 2030 according to the market research company SNS Insider Strategy and Stats [9] (Figure 1).

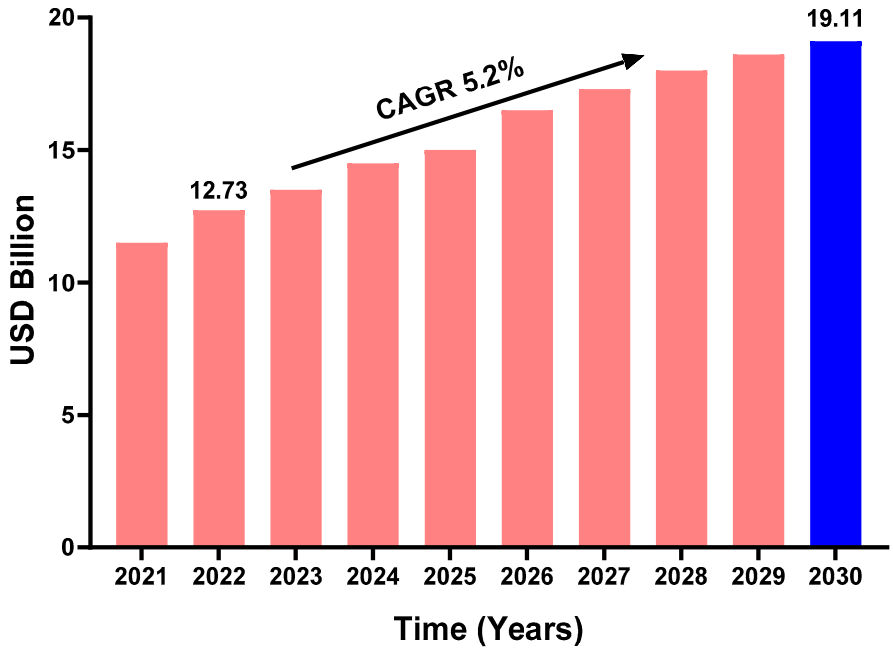

Figure 1. Vanillin market size reported by SNS Insider in 2022 [9].

Natural vanilla and vanillin, although often used interchangeably in everyday contexts, differ significantly in their source, composition, and production methods. Natural vanilla is derived from the beans of the vanilla orchid, involving a labor-intensive process of cultivation, hand-pollination, harvesting, and curing, resulting in a complex mixture of several hundred compounds that contribute to its rich and nuanced flavor profile. In contrast, vanillin is a single chemical compound, 4-hydroxy-3-methoxybenzaldehyde, which can be produced synthetically from precursors like guaiacol, or through biotechnological methods involving microbial fermentation. This simpler composition and more straightforward production process make vanillin more affordable and widely available, albeit with a less complex flavor compared to natural vanilla. Vanillin was isolated by Theodore Nicolas Gobley in 1858 [10]. Today, it is widely coveted by industries mainly for its applications in the areas of agri-food/beverages, perfumery/cosmetology, and medicine. Approximately 60% of industrial vanillin is used in the food/beverage industry, 33% in the perfumery/cosmetics industry, and 7% in the pharmaceutical industry [11].

The production of natural vanilla from vanilla pods is a costly and laborious process; to produce a single kilogram of vanilla, it requires approximately 500 kg of vanilla pods and a manual pollination of about 40,000 flowers [12]. This generates a high cost of natural vanilla on the market, between USD 1200 and 1400 per kg [13], compared to synthetic vanillin that costs less than USD 15 per kg [6,14]. For this reason, the biotechnological or bioengineering production of vanillin is of substantial interest for higher yields and more sustainable alternatives for spice production. As vanillin is derived from aromatic amino acid metabolism, screening *Vanilla planifolia* plants and other natural producers of vanillin such as *Glechoma hederacea*, *Oryza sativa*, and *Bruguiera gymnorhiza* [4,15,16] for the genes encoding the enzymes that are responsible for vanillin biosynthesis enables the heterologous production of vanillin in other organisms and its optimization into a sustainable high-yield system (Figure 2).

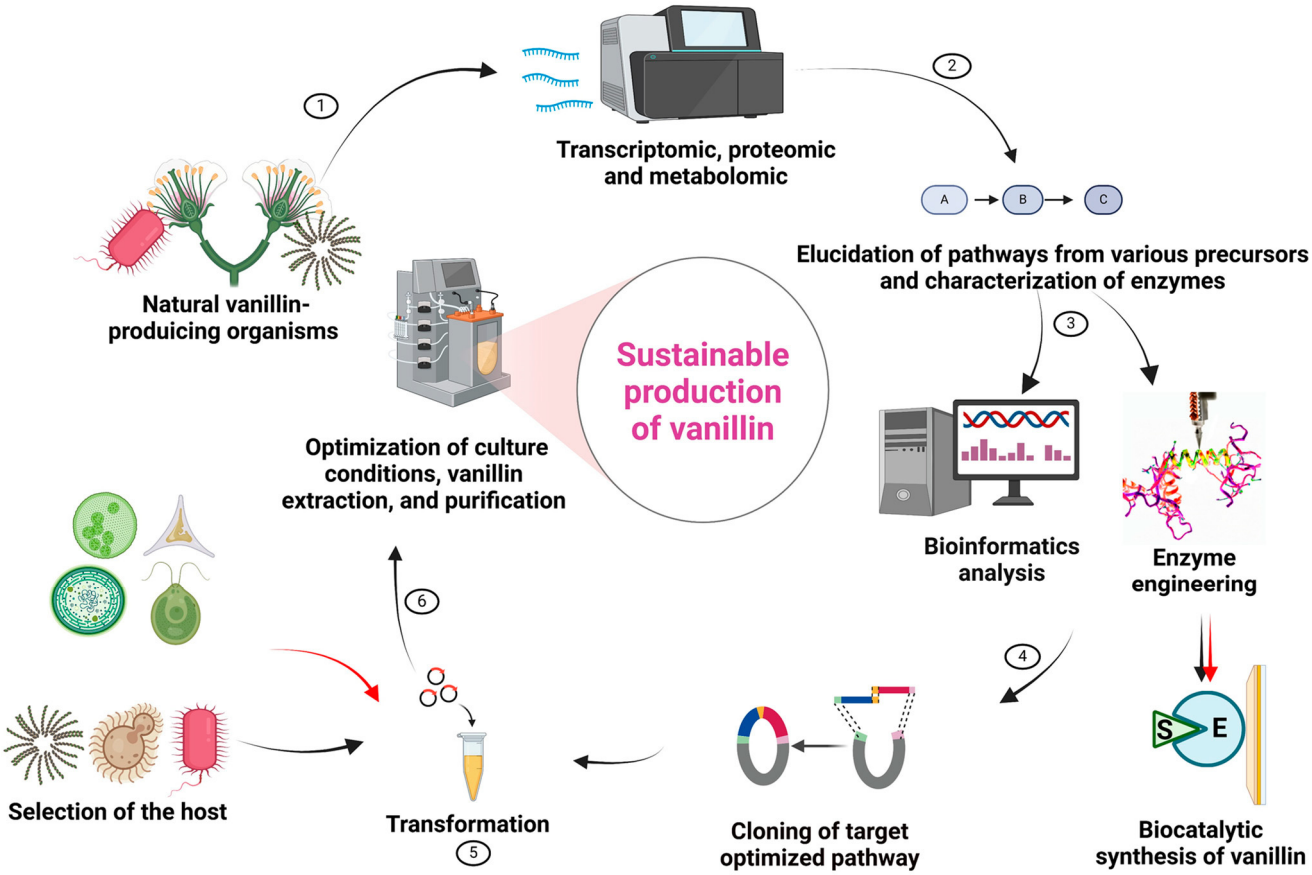

Figure 2. Current and future approaches for biologically engineered hosts for the production of vanillin. The black arrows indicate current approaches, while red arrows represent future approaches.

The biotechnological production of vanillin is carried out by the bioconversion of various natural substrates, such as ferulic acid, eugenol, isoeugenol, lignin, and glucose, using metabolically efficient biofactories, such as fungi, yeasts, or bacteria [17]. However, these current production systems often face several challenges, including inefficient metabolism in some cases, as well as cytotoxicity of the reaction products and/or intermediates in the culture media. Additionally, the formation of by-products due to the overexpression of endogenous vanillin dehydrogenase enzymes significantly impacts yield and quality, necessitating further purification steps. This is often viewed as a challenge for manufacturers and a bottleneck for environmental sustainability [7]. However, with the recent advances in genetic engineering, CRISPR-Cas9 technology, and purification techniques, the proportion of vanillin produced from these expression systems is increasingly expanding [3,18–20]. In order to support the biotechnological production of high-quality, sustainable, food-grade vanillin, photosynthetic organisms such as microalgae represent an ideal biomanufacturing system. Indeed, photosynthetic microorganisms can grow in the absence of a source of organic carbon. In addition, they can sequester carbon, allowing them to limit the industrial carbon dioxide (CO_2) emissions while reducing their impact in terms of greenhouse gases [21]. Several molecules of interest, including recombinant proteins such as pleiotropic antigens, and plant-specialized metabolites such as zeaxanthin, terpenoids, and growth hormones, have already been produced by this type of expression system [22–24]. Moreover, with the rapid advancement of metabolic engineering tools and techniques, there has been a surge in the algal production of high-value compounds. This review summarizes the importance of vanillin across various markets (food, perfumery/cosmetics, and pharmaceuticals/agrochemicals) and examines the current biological engineering production systems, including those based on bacteria, yeast, and plants. Special consideration is afforded to microalgae, which are emerging as promising platforms for supporting the biotechnological

production of vanillin through metabolic engineering. The manuscript also addresses the challenges associated with implementing microalgal-based vanillin production systems. This work aims to promote the use of these systems to enhance the biotechnological production of vanillin, leveraging the photosynthetic capabilities of microalgae to capture CO_2 and convert it into biomass, thereby reducing the overall carbon footprint.

2. Industrial Applications of Vanillin

2.1. Food and Beverage Industry Applications

Flavor is a sensation caused jointly by the taste and smell of a food. This is an organoleptic criterion that is regularly monitored in the agri-food industry in order to meet consumer demands [25]. The iconic and unmistakable flavor of vanillin, often described as sweet, is widely used in cold or baked confectionery, chocolate, pastries, and in the malting industry for its application to therapeutic drinks [11]. More interestingly, vanillin is often used in athletic beverage preparation to mask the bitter taste usually provided by some amino acids, such as arginine [25]. In addition to the human food applications, vanillin is applied as an additive to improve the feeding of chickens while protecting them against gastrointestinal infections [26]. In addition, the benefits of vanillin could be directly applied to the science of climate change. Ruminants (goats and dairy cows) have microorganisms (protozoa, fungi, and bacteria) in their rumen that are capable of fermenting complex carbohydrates to produce simple sugars and some atmospheric gases such as methane [27] which is known to be a potent greenhouse gas that is responsible for climate change [28]. Thus, the addition of vanillin as an ingredient in ruminant feed could improve its taste while inhibiting the growth of microorganisms, thereby limiting the release of methane into the atmosphere.

2.2. Applications in Perfume/Cosmetics

The tasty and pleasant scent of vanillin has applications in perfumery and cosmetology. Vanilla is used as a fragrance ingredient in perfumery formulations and preparations; it masks unpleasant odors and provides a very pleasant aroma [14]. Vanillin is used as a perfume fixative to preserve the appreciated fragrance in time and space. Due to its biological structure, vanillin stimulates the expression of the transcriptional factors (pOct-4, Oct-4, and Nanog) involved in the maintenance of pluripotent cells, the regeneration of epithelial stem cells in humans, and the expression of epithelial adhesive protein (E-cadherin), which helps to maintain the freshness of the body and increases its resistance against skin infections [29–31]. These regenerative and antimicrobial properties make vanillin a potential ingredient used in cosmetology and various applications in addition to its pleasant organoleptic properties.

2.3. Bioactivities/Pharmaceutical and Agrochemical Applications

Vanillin is used in the pharmaceutical industry as an excipient for several therapeutic drugs, such as antidepressants and antimicrobials [32,33]. This is the case for the antihypertensive drug methyl-dopamine (mixture consisting of 0.01 M of dopamine, vanillin, and N-methylacetamide, respectively) [34] used to treat hypertension, $_{L}$-dopamine used to treat Parkinson's disease, and a few anti-infectious agents such as trimethoprim [35]. In addition, numerous studies have demonstrated the pharmacological properties of vanillin, such as anticancer activity by mediating the antimutagenic potential and DNA damage [36], neuroprotective activity by attenuating biochemical impairments, learning and memory, and locomotor and motor coordination [37,38], antifungal activity through mitochondrial dysfunction [39], and, finally, antiviral and anti-inflammatory activities by neuraminidase inhibition and the modulation of the intracellular signaling pathways in THP-1 cells [40,41]. Moreover, it has recently been demonstrated that vanillin induces osteoblast-mediated bone-forming phenotypes through cell migration and F-actin polymerization [42]. Although vanillin is widely used in the food, pharmaceutical, and cosmetics industries, its application in agro-chemistry for the production of green pesticides has recently attracted

significant attention from the scientific community. Indeed, the chemical structure of vanillin, which contains a hydroxyl group and a highly modifiable aldehyde function, enables the addition of a series of bioactive motifs via an ether bridge, reductive amination, and thioacetalization to generate vanillin-derived pesticides [43]. Lou, et al. [44] recently provided a comprehensive summary of the advancements in vanillin-derived pesticides, highlighting how vanillin's unique properties are being harnessed to create more sustainable and environmentally friendly antiviral pest control solutions. These innovations contribute to the growing interest in green chemistry and sustainable agriculture, offering alternatives to the conventional synthetic pesticides that are often harmful to the environment and human health.

Due to the wide range of vanillin's applications, farmers, industrialists, and scientists are increasingly interested in the production systems of this highly valuable molecule to ensure its timely availability in large quantities.

3. Vanillin Biosynthesis in Vanilla Plants

Vanillin, or 3-methoxy-4-hydroxybenzaldehyde ($C_8H_8O_3$), is an aromatic aldehyde found in vanilla pods. Natural vanilla is a spice derived from orchids of the genus *Vanilla*, mainly obtained from the pods of *V. planifolia*, *V. pompona*, and *V. tahitensis* [7,45]. The de novo biosynthesis of vanillin through the phenylpropanoid pathway in *V. planifolia* was investigated by Gallage, Hansen, Kannangara, Olsen, Motawia, Jørgensen, Holme, Hebelstrup, Grisoni and Møller [4]. Vanillin biosynthesis involved several steps and enzymes, such as phenylalanine ammonia lyase (PAL; EC 4.3.1.25), cinnamate-4-hydroxylase (C4H; EC 1.14.13.11), 4-hydroxycinnamoyl-CoA ligase (4CL; EC 6.2.1.12), hydroxycinnamoyl transferase (HCT; EC 2.3.1.133), and 4-coumaroyl ester 3′-hydroxylase (C3′H; EC 1.14.13.36) [46,47]. The intermediates obtained from the catalytic reaction of these enzymes are coenzyme A derivatives as well as shikimate- and quinate esters of coumaric acid and caffeic acid [48]. Caffeic acid is a direct precursor of ferulic acid. It is methylated to ferulic acid by the enzyme caffeate *O*-methyltransferase (C*O*MT; EC 2.1.1.68). Using coupled transcription/translation assays and transient expression in tobacco and yeast, a gene *VpVAN* encoding an enzyme called vanillin synthase (*Vp*VAN; EC 4.1.2.41), which catalyzes the C2 side chain shortening of ferulic acid and its glucoside to vanillin or vanillin-b-D-glucoside, was identified in the chloroplasts and re-differentiated chloroplasts of *V. planifolia*. The discovery of this enzyme enabled Gallage, Hansen, Kannangara, Olsen, Motawia, Jørgensen, Holme, Hebelstrup, Grisoni and Møller [4] to elucidate the complete pathway of vanillin biosynthesis (Figure 3). A non-β-oxidative pathway for vanillin biosynthesis was also reported by Podstolski, et al. [49]. In this proposed pathway, 4-coumaric acid is first converted to 4-hydroxybenzaldehyde by 4-hydroxybenzaldehyde synthase (4-HBS; EC number not identified). The 3′-hydroxylation of 4-hydroxybenzaldehyde by the C3′H will then yield 3,4-dihydroxybenzaldehyde followed by the *O*-methylation of the 3′ hydroxyl group by an *O*-methyltransferase (*O*MT) to produce vanillin. Nevertheless, the yield of plant-based vanillin is relatively low, and the extraction and purification present several challenges [50]. In recent decades, the increased knowledge regarding the metabolic pathway and the corresponding enzymes involved in the conversion of various natural substrates, such as ferulic acid, eugenol, isoeugenol, lignin, and glucose, to bio-vanillin has provided new opportunities for bioengineering microorganisms for vanillin biosynthesis. Several authors have reported the biotechnological production of vanillin in plants, bacteria, and yeast, as summarized in Appendix A—Table A1. However, microalgae, as potential expression systems, may offer a promising alternative to support the biotechnological production of vanillin. This review will highlight the importance of these photosynthetic hosts for vanillin production, which remain underexplored compared to non-photosynthetic microorganisms despite their potential for more sustainable and efficient production.

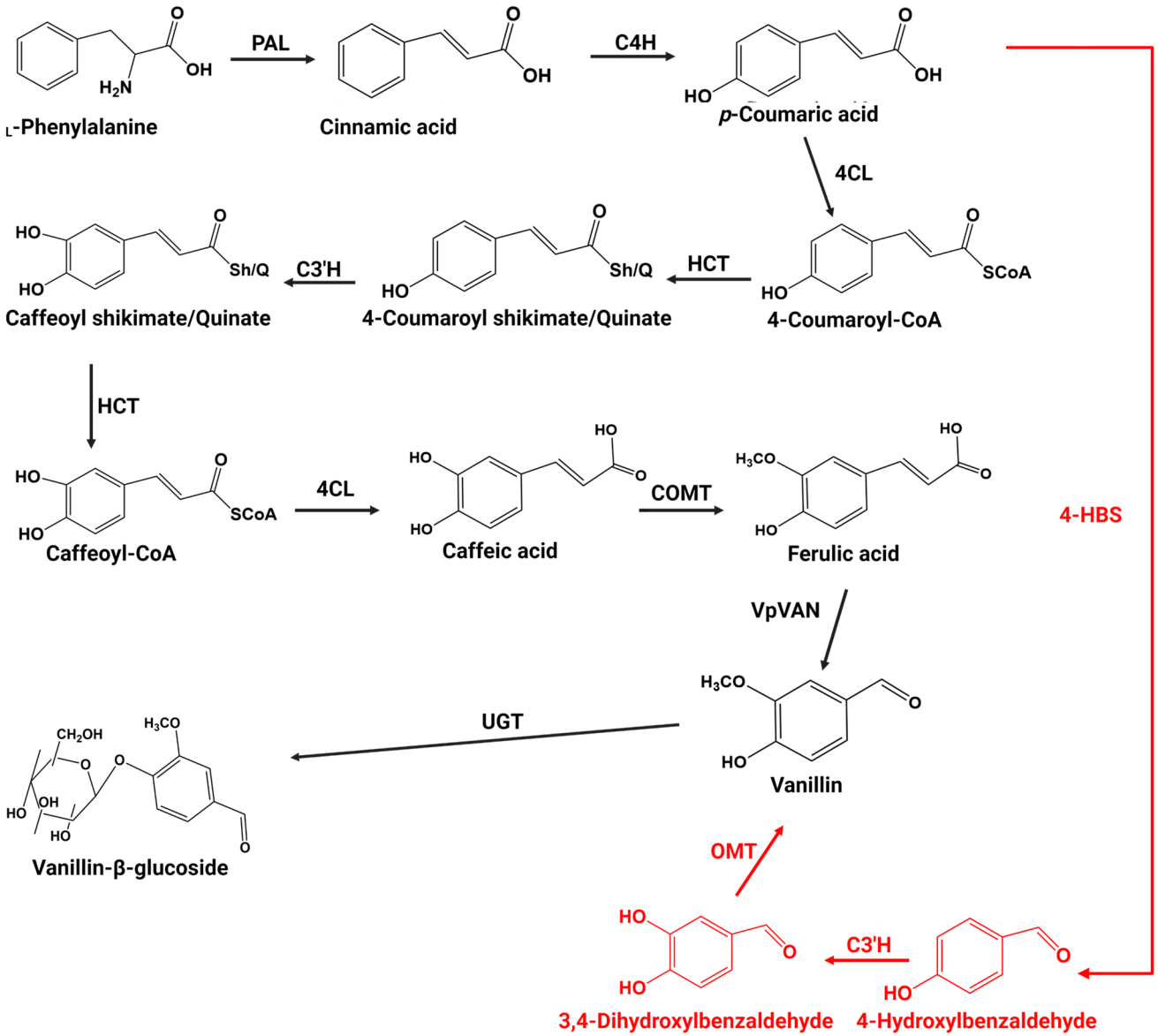

Figure 3. Vanillin and vanillin-β-D-glucoside biosynthesis pathways in *V. planifolia* pods. L-phenylalanine from the shikimate pathway will undergo a series of reactions to yield ferulic acid, which will subsequently be cleaved into vanillin by the enzyme vanillin synthetase according to Gallage, Hansen, Kannangara, Olsen, Motawia, Jørgensen, Holme, Hebelstrup, Grisoni and Møller [4]. As present in red according to Podstolski, Havkin-Frenkel, Malinowski, Blount, Kourteva and Dixon [49], 4-coumaric acid can be directly converted to 4-hydroxybenzaldehyde followed by 3′ hydroxylation and O-methylation to yield vanillin. Abbreviations: COMT: caffeate O-methyltransferase; PAL: phenylalanine ammonia lyase; OMT: O-methyltransferase; 4HBS: 4-hydroxybenzaldehyde synthase; C4H: cinnamate 4-hydroxylase; 4CL: 4-hydroxycinnamoyl-CoA ligase; HCT: hydroxycinnamoyl transferase; C3′H: coumaroyl ester 3′-hydroxylase; and VpVAN: *V. planifolia* vanillin synthase.

4. Engineering Prokaryotic and Eukaryotic Expression Systems for Sustainable Vanillin Production

Metabolic engineering aims to construct a metabolic pathway of interest in host organisms for the production of high-value-added compounds, thereby enhancing the yield and efficiency for industrial-scale production [51]. Microorganisms such as bacteria and yeast are widely used in metabolic engineering projects since they can be easily genetically modified with the availability of a wide range of molecular tools. This section summarizes the recent advancements and achievements in vanillin production using bacteria, yeast, and

plant tissues. It highlights the progress in optimizing the metabolic pathways, enhancing enzyme functionality, and applying innovative genetic engineering techniques to increase the vanillin yield across these biological systems. Additionally, the section addresses the current challenges in optimizing microbial-based vanillin production, such as low yield, metabolic bottlenecks, and strain stability. Potential strategies to overcome these challenges are also explored, including advanced metabolic engineering, adaptive laboratory evolution, and process optimization to improve scalability and commercial viability.

4.1. Engineering Prokaryotic Microorganisms for Vanillin Production

4.1.1. Engineering *Escherichia coli*

E. coli has been extensively used for the heterologous production of vanillin [52]. In particular, Torre, et al. [53] immobilized the recombinant strain M109/pBB1, which was mutated at the alcohol dehydrogenase (ADH; EC 1.1.1.1) locus and expressed the genes encoding feruloyl-CoA synthetase (FCS; EC 6.2.1.34) and enoyl-CoA hydratase/aldolase (ECH; EC 4.2.1.17) from *Pseudomonas fluorescens* BF13 in a reactor. This strategy resulted in the production of 0.080 g/L of vanillin from 0.200 g/L of ferulic acid in the immobilized-cell reactor. As a result, the combination of these two genes has been widely employed in *E. coli* expression systems. This is the case for Barghini, Di Gioia, Fava and Ruzzi [12], who were able to produce 0.53 g/L of vanillin from ferulic acid by co-expressing the same two genes in *E coli* strain JM109. Further progress in vanillin production using bacteria was realized eight years later by the research of Ni, et al. [54]. In fact, for the de novo production of vanillin in *E. coli*, this research group partially mimicked the vanillin biosynthetic pathway of the plant *V. planifolia* using the *E. coli* strain genetically engineered to overproduce $_{L}$-tyrosine (Figure 4B). The carbon flux from the primary metabolism of carbohydrates and simple organic compounds, such as glycerol and xyloses, was directed to vanillin via $_{L}$-tyrosine. This strategy produced 97.2, 19.3, 13.3, and 24.7 mg/L of vanillin from $_{L}$-tyrosine, glucose, glycerol, and xylose, respectively [54]. The conversion of protocatechuic acid to vanillic acid is a central step in the de novo biosynthesis of vanillin [55]. A central metabolism perturbation designed to increase the flux in the de novo biosynthetic pathway of vanillic acid increased the mean of the de novo vanillate yield from 132 to 205 mg/L [55]. However, protocatechuic acid, the precursor of vanillic acid, remained the dominant biosynthetic intermediate. To maximize this step of converting protocatechuic acid to vanillic acid, Kunjapur, Hyun and Prather [55] demonstrated that supplementation with methyl donor (methionine and homocysteine) improved the vanillin acid production from protocatechuic in *E. coli* strain DH10B. An artificial pathway for vanillic alcohol biosynthesis from simple carbon sources was proposed by Chen, et al. [56] using the *E. coli* BW25113 strain. This pathway required only four heterologous enzymes, namely *p*-hydroxybenzoate hydroxylase (PobA), carboxylic acid reductase (CAR), caffeate *O*-methyltransferase (COMT), and the CAR maturation factor phosphopantetheinyl transferase (Sfp) (Figure 4A), and led to 0.24 g/L of vanillyl alcohol released into the culture medium [56]. Vanillin can be converted from isoeugenol in one step via the oxidation of the side chains by the enzyme isoeugenol monooxygenase (IEM). The isoeugenol monooxygenase gene from the bacterium *Pseudomonas nitroreducens Jin1* was therefore cloned into *E. coli* [57]. This strain produced 38.34 g/L of vanillin from 49.2 g/L of isoeugenol. More recently, to optimize the yield of vanillyl alcohol production, Yang, et al. [58] mutated the shikimate dehydrogenase (*aroE*) gene in *E. coli* strain BW25113. In this study, gene *aroE* was knocked out to improve 3,4-DHBA biosynthesis of the upstream strain. Using the bioengineering *E. coli* BW25113 (YMC13/YMC14) co-culture strategy, this strain was able to produce 0.5 and 3.89 g/L of vanillic alcohol in shake-flask and fed-batch fermentations [58].

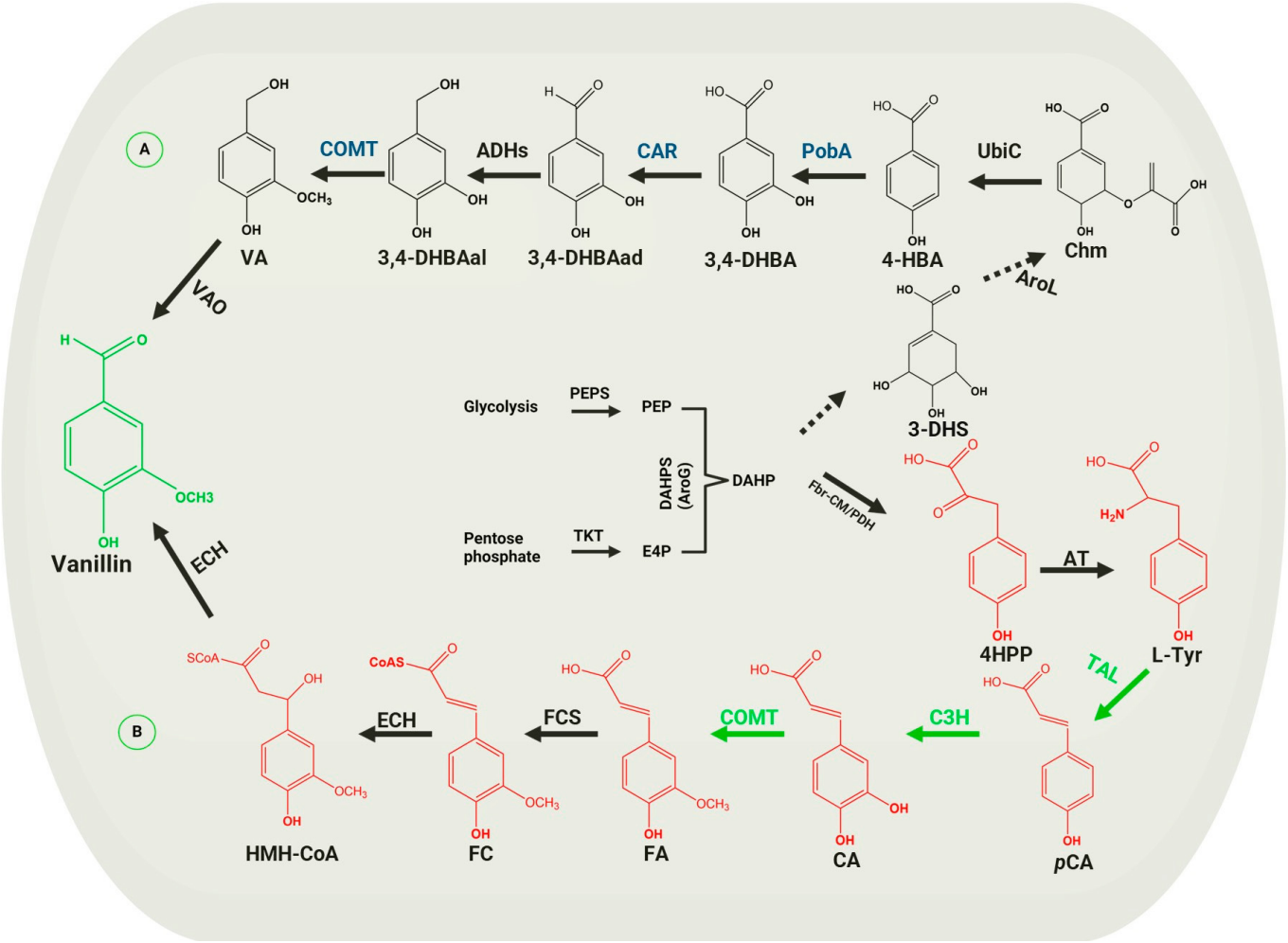

Figure 4. Engineered biosynthetic pathways for the de novo heterologous production of vanillin in *E. coli*. (**A**,**B**) represent the different metabolic pathways proposed by Chen, Shen, Wang, Wang, Zhang, Rey, Yuan and Yan [56] and Ni, Tao, Du and Xu [54]. (**A**) The arrows with black annotation in Chen's proposed pathway indicate the native pathway in *E. coli*, and the arrows blue annotation indicate the heterologous steps that have been added. (**B**) Pathway proposed by Ni, Tao, Du and Xu [54]; the green arrows contain three non-bacterial enzymes involved in the artificial pathway from tyrosine to ferulic acid, and the last black arrows of this pathway represent steps catalyzed by two bacterial enzymes involved in the conversion of ferulic acid to vanillin. Abbreviations. PEP: phosphoenolpyruvate; E4P: D-erythrose 4-phosphate; DAHP: 3-deoxy-D-arabinoheptulosonate 7-phosphate; 4-HPP: 4-hydroxyphenylpyruvate; $_{L}$-Tyr: $_{L}$-tyrosine; *p*CA: coumaric acid; CA: caffeic acid; FA: ferulic acid; FC: feruloyl-CoA; HMH-CoA: 4-hydroxy-3-methoxyphenyl-β-hydroxypropionyl-CoA; 3-DHS: 3-dehydroshikimic acid; Chim: chorismate; 4-HBA: 4-hydroxybenzoic acid; 3,4-DHBA: 3,4-dihydroxybenzoic acid; 3,4-DHBAad: 3,4-dihydroxybenzaldehyde; 3,4-DHBAal: 3,4-dihydroxybenzyl alcohol; VA: vanillyl alcohol; PEPS: phosphoenolpyruvate synthase; TKT: transketolase; AroG: 2-dehydro-3-deoxyphosphoheptonate aldolase; AroL: shikimate kinase II; UbiC: chorismate lyase; PobA: *p*-hydroxybenzoate hydroxylase; CAR: carboxylic acid reductase; ADH: alcohol dehydrogenases; COMT: caffeate O-methyltransferase; VAO: vanillyl alcohol oxidase; AT: aminotransferase; fbr-CM/PDH: fbr-chorismate mutase/prephenate dehydrogenase; TAL: tyrosine ammonia lyase; C3H: 4-coumarate 3-hydroxylase; FCS: *trans*-feruloyl-CoA synthetase; and ECH: enoyl-CoA hydratase/aldolase.

4.1.2. Engineering *Pseudomonas* sp.

Pseudomonas sp. has also been used for the production of various plants' specialized metabolites, including vanillin. To develop an effective strain of *Pseudomonas* that is capable of producing vanillin with high yields, research from Di Gioia, Luziatelli, Negroni, Ficca, Fava and Ruzzi [29] suppressed, by directed mutagenesis, the gene (*vdh*) encoding the vanillin dehydrogenase of the *Pseudomonas fluorescens* BF13 strain. The inactivation associated with the simultaneous expression of the structural genes encoding feruloyl-CoA synthetase and enoyl-CoA hydratase/aldolase from a low-copy-number plasmid enabled this strain to produce up to 1.27 g/L of vanillin from ferulic acid [29]. Later, the non-pathogenic strain *Pseudomonas putida* KT2440 was genetically optimized to convert ferulic acid into vanillin. This genetic modification strategy aimed to enhance the efficiency of the metabolic pathway responsible for converting ferulic acid into vanillin while minimizing the formation of undesirable by-products. This was achieved by downregulating the vanillin dehydrogenase (vdh) and trans-molybdate transporter genes while enhancing the expression of feruloyl-CoA synthetase and enoyl-CoA hydratase/aldolase under the tac promoter. As a result, a yield of 1.66 g/L of vanillin was obtained, indicating the success of the genetic modifications in increasing vanillin production while minimizing by-product formation [59].

4.1.3. Engineering *Corynebacterium glutamicum*

C. glutamicum is a promising host for metabolic engineering studies, mostly for the industrial production of amino acids [60]. However, some publications have demonstrated the capacity of the genetically modified strain to metabolize vanillin. Chen, et al. [61] investigated the ability of the strain in culture to resist high concentrations of vanillin produced from lignocellulosic biomass. A transcriptome analysis revealed that, in addition to stimulating vanillin degradation enzymes, high concentrations of vanillin also caused differential regulation of antioxidant enzymes, secretory proteins, and the cell envelope [61]. These insights may be useful for the engineering of vanillin tolerance strains for suitable production of biofuels from lignocellulosic biomass. Thus, the conversion of ferulic acid to protocatechuic acid was enabled in *C. glutamicum* ATCC 21420 by cloning the vanillic acid *O*-demethylase (*vanAB*) gene of *Corynebacterium efficiens* NBRC 100395, which converts vanillic acid into protocatechuic acid [62]. This transformed strain was able to produce 1.06 g/L of protocatechuic acid from 3.10 g/L of ferulic acid. Also, an artificial biosynthetic pathway expressing the *COMT* and *CAR* genes from *Rattus norvegicus* and *Nocardia iowensis*, respectively, has recently been cloned into *C. glutamicum* [63]. The carbon flux was directed towards vanillin by the deletion of the gene *NCgl0324* encoding an aromatic aldehyde reductase. The new strain PV-IYD0324 resulting in the culture produced 0.31 g/L of vanillin from endogenous 4-hydroxylbenzoic acid.

4.1.4. Engineering *Pediococcus acidilactici*

P. acidilactici is a Gram-positive coccus used as a host for the production of aromatic compounds such as vanillin. This bacterium has several advantages in metabolic engineering, particularly growth at high temperature, pH, and osmotic pressure values [64]. The *P. acidilactici* BD16 strain transformed by heat shock to express feruloyl-CoA synthetase and the enoyl-CoA hydratase/aldolase genes from *Amycolatopsis* sp. was cultured in the presence of ferulic acid [65]. When the culture conditions were optimized, 0.47 g/L of vanillin was recovered from 0.21 g/L of ferulic acid per mg of the recombinant cell biomass. Producing vanillin from expensive substrates such as ferulic acid has lower advantages over simple and available substrates such as glucose and industrial waste. Chakraborty, et al. [66] were able to recover 4.01 g/L of vanillin by culturing the recombinant *P. acidilactici* BD16 strain in the presence of rice bran.

In summary, prokaryotic microorganisms have been widely used for vanillin production by metabolic engineering. *E. coli* seems to be the best platform so far because few studies have mentioned the presence of vanillin degradation pathways in *E. coli*. Also, up to 38.3 g/L of vanillin could be produced in the bacteria in a single step using isoeugenol monooxygenase from *Pseudomonas nitroreducens Jin1*, as shown in Appendix A—Table A1 [57].

However, the expression of vanillin biosynthesis enzymes from plants in prokaryotic systems may, in some cases, result in poor enzyme stability or activity, requiring further optimization to improve the protein folding and expression levels. This issue can potentially be addressed by using eukaryotic expression systems, which typically possess more advanced protein-folding mechanisms and post-translational modifications (PTMs), thereby enhancing enzyme functionality and stability. Post-translational modifications such as glycosylation are essential for the optimal activity of some eukaryotic enzymes, affecting their folding, stability, and ability to interact with other molecules in the biosynthetic pathway [67,68]. When these modifications are absent or insufficient, it can lead to premature degradation of the enzymes, thereby reducing their activity and, consequently, the amount of vanillin produced.

4.2. Engineering Eukaryotic Organisms for Vanillin Production

Eukaryotic organisms, used as expression systems for vanillin production, are mainly yeasts and plant tissues.

4.2.1. Engineering *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*

S. cerevisiae is a yeast that is widely used in industry for the production of high-value-added compounds due to its alcohol tolerance, well-elucidated genome, and fermentation performance [69]. According to the Evolva project, Hansen, et al. [70] studied the ability of the yeast strains *S. cerevisiae* and *S. pombe* to produce vanillin from glucose. In this study, the vanillin biosynthetic pathway was constructed using the 3-dehydroshikimate dehydratase genes from the ascomycete *Podospora pauciseta*, the carboxylic acid reductase from the genus *Nocardia*, *O*-methyltransferase from *Homo sapiens*, and UDP-glycosyltransferase from *Arabidopsis thaliana* (red arrows in Figure 5A). The efficiency of this pathway was achieved by removing the alcohol dehydrogenase and the vanillin-β-D-glucosidase genes involved in vanillin and vanillin-β-glucoside degradation [71]. Therefore, 0.065 and 0.045g/L of vanillin were produced in *S. cerevisiae* and *S. pombe*, respectively, after 48 h of culture. Later, the glycosylated vanillin produced was optimized by Brochado, et al. [72] based on an in silico metabolic engineering algorithm. Indeed, this approach enables the identification of target reactions in a metabolic network whose inactivation would lead to an improvement in vanillin production. Bioinformatics analysis has thus identified pyruvate decarboxylase and glutamate dehydrogenase genes in *S. cerevisiae* whose suppression is thought to be associated with increased glycosyltransferase activity. The experimental analysis of four strains with two single gene deletions in these genes in the same study showed that three of the strains produced 1.5 times more glycosylated vanillin than the unmutated strains [72]. Plant tissues are mainly composed of lignin, which is a raw material residue obtained from various industrial bioprocesses. Due to its aliphatic and aromatic structures, it is a widely used substrate in the flavor industry [73]. Similarly, Shen, et al. [74] tested the ability of the EMV-8 strain of *S. cerevisiae* mutated with ethyl methane sulfonate to tolerate high concentrations of vanillin during the lignin biotransformation processes. The use of ethyl methane sulfonate is an engineering strategy that increases the oxidoreductase activity and antioxidant capacity of a microorganism. Such a mutation strategy enabled the EMV-8 strain to maintain a specific growth rate in the presence of 2 g/L of vanillin, in contrast to the reference strain NAN-27, which could not grow in the same conditions because of vanillin cytotoxicity [74]. To explain the redox mechanisms that allow the EMV-8 strain to tolerate high concentrations of vanillin, Liang, et al. [75] identified the different genes expressed and their implications in the mechanisms of vanillin tolerance in *S. cerevisiae*. Thus, the metabolism of the strain under investigation was characterized in a culture by the overexpression of the

mRNA-binding aldo-keto reductase (Gcy1), its Ypr1 paralog, the peroxisome membrane receptor (Pex5), and the multiprotein complex involved in specific growth (Mbf1). However, only the Gcy1 and Ypr1 proteins showed vanillin reductase activities that are dependent on NADPH and NADH [75]. To reproduce the de novo biosynthetic pathway of vanillin from tyrosine in *S. cerevisiae*, Qiu, et al. [76] developed a recombinant yeast strain by cloning five heterologous enzymes, namely tyrosine ammonia lyase (TAL) from the actinomycete *Saccharothrix espanaensis*, the combination of three hydroxylase enzymes [(4-coumarate 3-hydroxylase (C3H) from *Saccharothrix espanaensis*, 4-hydroxyphenylacetate 3-hydroxylase (4HPA3H) from *Pseudomonas aeruginosa*, and cytochrome P450 monooxygenase (CYP199A2) from *Rhodopseudomonas palustris*)], caffeate *O*-methyltransferase (COMT) from *Arabidopsis thaliana*, *trans*-feruloyl-CoA synthetase (FCS), and enoyl-CoA hydratase/aldolase (ECH) from *Streptomyces* sp. (dark blue arrows in Figure 5C). By mutating the *ARO4* and *ARO7* genes, which encode 3-deoxy-D-arabino heptulosonate-7-phosphate synthase (DAHPS) and chorismate mutase (CM), respectively, into others to limit the negative feedback of tyrosine and increase its flux to vanillin, then by substituting the rate-limiting enzyme tyrosine ammonia lyase (*sam8* gene) with 4-coumarate 3-hydroxylase (*sam5* gene) to increase the caffeic acid pool, these researchers were able to produce 0.008g/L of vanillin from 0.4 g/L of ferulic acid [76]. Most microorganisms lack the ability to accumulate aldehydes due to the presence of endogenous dehydrogenases and reductases, which facilitate their rapid metabolism and prevent their accumulation in the cells. To overcome this challenge in the case of vanillin production in yeast, Mo and Yuan [20] Mo and Yuan [20] recently developed a minimal aromatic aldehyde reduction (MARE) yeast platform for engineering vanillin production from glucose. They first used a combinatorial gene deletion approach, targeting the *adh6*, *adh7*, *gre3*, *gcy1*, *ydl124w*, *ypr1*, *ari1*, *ydr541c*, and *aad3* genes, which are critical for vanillin reduction and oxidation. Subsequently, they substituted the *hfd1* gene with *ubiC* to prevent vanillin oxidation and provide the precursor for ubiquinone synthesis, thus enhancing vanillin accumulation while limiting vanillyl alcohol production. Building upon this platform, they further optimized the co-factor supply (NADPH/SAM) and implemented dual precursor synthesis pathways along with metabolic reconfiguration using a phosphoketolase pathway (green arrows in Figure 5C). Through systematic engineering, they achieved a significant milestone of producing 0.36g/L of vanillin, demonstrating the potential of the MARE yeast platform for sustainable vanillin production. Similarly, Xin, et al. [77] meticulously engineered *Saccharomyces cerevisiae* BY4742, which carried deletions in twenty-two genes associated with vanillin reduction and oxidation [78], to facilitate the conversion of lignin-derived monomers into vanillin. They first reconstructed two pathways separately: the CoA-independent pathway, expressing *VpVAN* from *V. planifolia*, and the CoA-dependent non-β-oxidation pathway, expressing 4CL from *Petroselinum crispum* and Ech from *Pseudomonas putida* KT2440. In addition, they fused hpaC and hpaB enzymes to enable the simultaneous hydroxylation of *p*-coumaric acid and *p*-hydroxybenzaldehyde into caffeic acid and protocatechualdehyde, respectively. Furthermore, they regulated S-adenosylmethionine metabolism by expressing 3′-O-methyltransferase from *Arabidopsis thaliana* (AtCOMT), *Homo sapiens* (HsCOMT), and *Nicotiana tabacum* (NtCOMT), along with methylenetetrahydrofolate reductase from *Arabidopsis thaliana*. By also controlling the branch and shikimic acid pathways, the microbial cell factory, employing the CoA-dependent non-β-oxidation pathway, successfully produced 0.29g/L of vanillin from authentic lignocellulosic biomass hydrolysates [77].

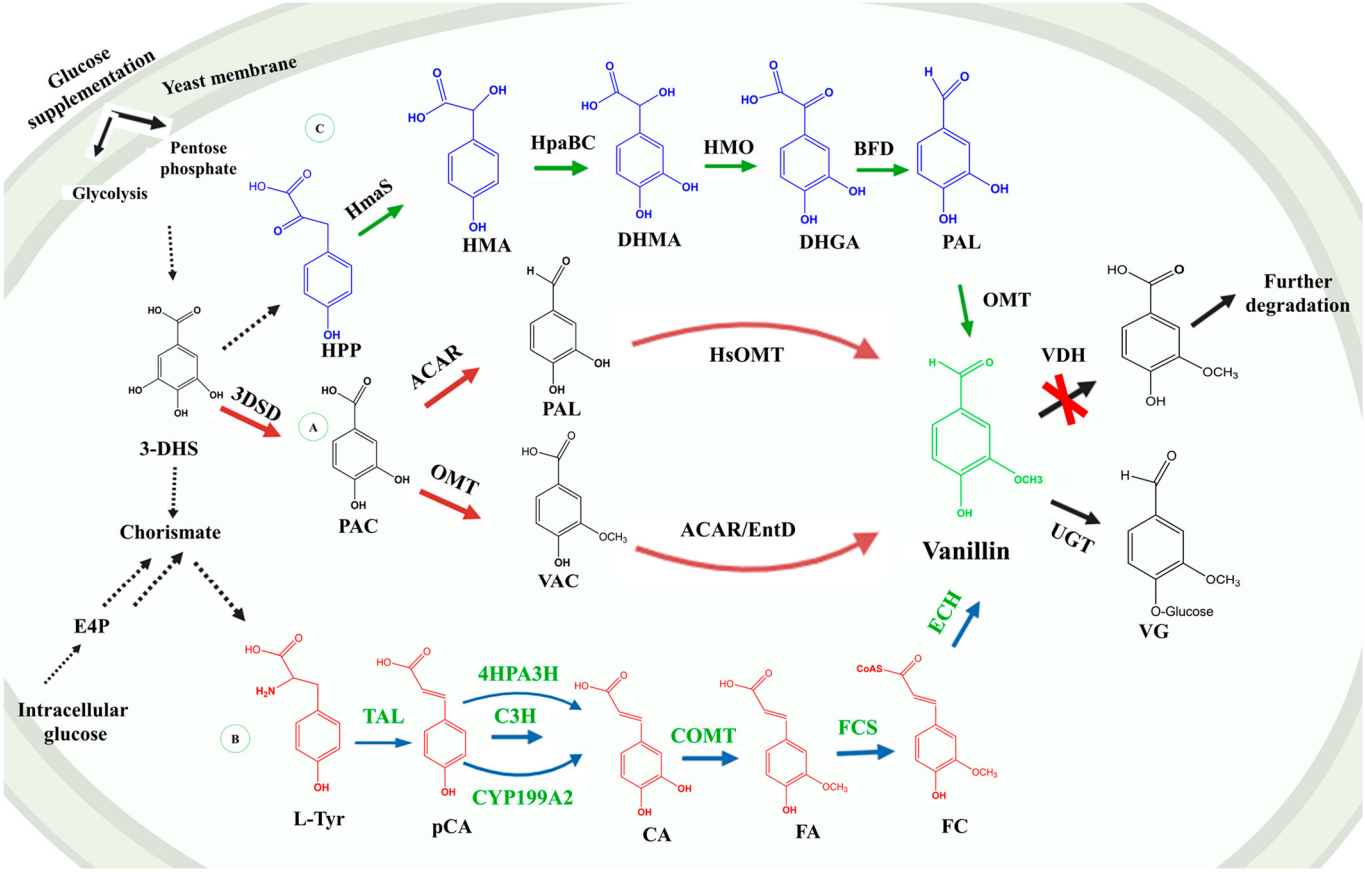

Figure 5. Biosynthesis pathways for vanillin and glycosylated vanillin production in *Saccharomyces cerevisiae* yeast [20,70,76,79]. (**A**) (Red arrows) represent the pathway proposed by Strucko, Magdenoska and Mortensen [79] and Hansen, Møller, Kock, Bünner, Kristensen, Jensen, Okkels, Olsen, Motawia and Hansen [70], while (**B**) (dark blue arrows) and (**C**) (green arrows) represent the pathways proposed by Qiu et al. (2022) and Mo and Yuan [20], respectively. Abbreviations. 3DHS: 3-dehydroshikimic acid; E4P: erythrose-4-phosphate; PCA: protocatechuic acid; L-Tyr: L-tyrosine; *p*-CA: *p*-coumaric acid; CA: caffeic acid; FA: ferulic acid; FC: feruloyl-CoA; 3,4-DHBAad: 3,4-dihydroxybenzaldehyde; PAL: protocatechuic aldehyde and VAC: vanillic acid; HPP: hydroxyphenylpyruvate; HMA: hydroxymandelate; DHMA: 3,4-dihydroxymandelate; DHGA: 3,4-dihydroxyphenylglyoxylate; 3-DSD: 3-dehydroshikimate dehydratase; ACAR: aromatic carboxylic acid reductase; EntD: phosphopantetheine transferase; HsOMT: O-methyltransferase (homo sapiens); UGT: UDP-glycosyltransferase; TAL: tyrosine ammonia-lyase; C3H: 4-coumarate 3-hydroxylase; 4HPA3H: 4-hydroxyphenylacetate-3-hydroxylase; COMT: caffeate O-methyltransferase; FCS: trans-feruloyl-CoA synthetase; ECH: enoyl-CoA hydratase/aldolase; HmaS: hydroxymandelate synthase; HpaBC: flavin-dependent monooxygenase; HMO: hydroxymandelate oxidase; BFD: benzoylformate decarboxylase; and VDH: vanillin dehydrogenase. The red cross materializes the suppression of genes involved in vanillin oxidation and reduction.

4.2.2. Engineering Plant Tissues for the Production of Vanillin

Although most of the studies are limited to the production of vanillin in microorganisms, a few have investigated plant tissues. Specifically, Chee, et al. [80] genetically transformed the pepper (*Capsicum frutescens)* cells with the vanillin synthetase gene from *Vanilla planifolia* (VpVAN) by microparticle bombardment. A high-performance liquid chromatography (HPLC) analysis of the extracts from the transformant cells in a culture led to the detection of 5.7×10^{-3} g/L of vanillin, which was sixteen times higher than the control [80]. An amount of 0.54 g of vanillin per gram of fresh callus culture was also produced from rice embryonic cells transformed by *Agrobacterium* to express the codon optimized

sequence of vanillin synthetase [81]. However, it is important to note that the enzymatic activity of this enzyme is subject to several controversies, and no kinetic parameters such as catalytic activity (Kcat) and Michaelis constant (Km) have yet been reported [82,83]. The progress of plant-tissue-culture-based vanillin synthesis has not been commercially viable and has been hindered by factors such as long culture duration, low yields, and increased by-product formation [3].

5. Current Challenges and Potential Strategies for Enhancing Microbial-Based Vanillin Production Systems

Despite the notable progress in microbial-based vanillin production, several challenges remain. One of the primary hurdles involves the low conversion efficiency and yield, largely due to the cytotoxicity of vanillin and its intermediates to host cells. This toxicity often impairs cell growth and enzyme functionality, limiting production [84]. Indeed, vanillin concentrations exceeding 0.5–1 g/L have been shown to significantly inhibit microbial growth, thereby reducing the final product [70]. Additionally, by-product formation during the biosynthesis process complicates the downstream purification, reducing the overall efficiency and increasing the production costs [50]. Another challenge is the metabolic burden on microorganisms engineered for vanillin production. Complex metabolic pathways, often involving multiple genetic modifications and CRISPR-Cas9 gene knockouts, can reduce cellular fitness, leading to lower productivity [85]. This strain on cellular resources often results in slower growth rates, decreased metabolic efficiency, and suboptimal vanillin yields, further complicating the scaling of biotechnological vanillin production for industrial applications. To address these challenges, potential strategies include metabolic pathway optimization by fine-tuning the pathways through adaptive laboratory evolution or optimizing the gene expression to enhance the flux toward vanillin biosynthesis, balancing precursor availability, co-factor regeneration, and improving enzyme activity [20,77,84,86]. Another approach is tolerance engineering, where microbial strains are engineered to increase their tolerance to vanillin and its intermediates, achieved through the overexpression of efflux pumps or modification of cellular stress response mechanisms to mitigate the cytotoxic effects of vanillin on microbial cells. Efflux pumps actively transport vanillin and its toxic intermediates out of the cell, reducing the intracellular concentrations and minimizing cellular damage [3]. Additionally, enhancing the stress response pathways can bolster the cell's ability to tolerate and recover from vanillin-induced stress, leading to improved growth and productivity [75]. These strategies are crucial for overcoming cytotoxicity, which is one of the major challenges in microbial-based vanillin production [3,72,87]. Enzyme engineering, using rational or directed evolution-based methods, can improve catalytic efficiency and reduce the formation of unwanted by-products, thereby enhancing yield and purity [86,88]. Diversifying host systems by exploring non-traditional microbial hosts, such as photosynthetic microalgae, presents a promising alternative for vanillin production, potentially lowering costs and integrating CO_2 fixation, which contributes to a more sustainable bioproduction platform.

6. Green Biology: Harnessing Microalgae as Natural Producers of Vanillin Precursors and Promising Hosts for Biotechnological Vanillin Production

Metabolically engineered photosynthetic microorganisms, such as microalgae, can offer several advantages as potential hosts for vanillin production. First, their ability to perform photosynthesis allows them to utilize CO_2 as a carbon source, reducing the reliance on external feedstocks and contributing to carbon sequestration [89,90]. This makes the process more sustainable compared to non-photosynthetic microorganisms. Additionally, microalgae can produce high-value precursors and metabolites relevant to vanillin biosynthesis, potentially enhancing the overall yield. Moreover, it has been documented that the cost associated with biotechnology production in microalgae is much cheaper compared to animal cells and even lower than that of microbes and plants [91].

6.1. Microalgae as Natural Producers of Vanillin Precursors

Microalgae, with their remarkable metabolic versatility, have developed intricate biochemical pathways that enable them to produce a wide range of specialized metabolites, including valuable phenolic compounds [92]. The biosynthesis of phenolic compounds in microalgae is a complex and highly regulated process, involving several metabolic pathways that enable the production of bioactive molecules that are essential for the survival and adaptation of microalgae to their environment [93,94]. This justifies the fact that *Phaeodactylum tricornutum* was able to produce various vanillin precursors and metabolites, such as coumaric acid, caffeic acid, ferulic acid, protocatechuic acid, gallic acid, and vanillic acid, from in vitro cultures grown in natural seawater containing elevated levels of copper (Cu) and iron (Fe) metals [93]. The presence of these metals likely enhanced the biosynthetic pathways involved in the production of phenolic compounds, including those that serve as precursors for vanillin synthesis. This demonstrates the potential of diatoms like *P. tricornutum* as biotechnological platforms for producing valuable aromatic compounds such as vanillin. Moreover, Bhuvana, et al. [95], using an HPLC method, detected peaks corresponding to caffeic acid derivatives in methanolic extracts of *Nannochloropsis oculata* and *Chlorella vulgaris.* Further, *Haematococcus pluvialis* was fed with vanillin precursors such as ferulic acid, coniferyl aldehyde, and *p*-coumaric acid by Tripathi, et al. [96], resulting in the production of vanillin (0.010 g/L), vanillyl alcohol (0.003 g/L), vanillic acid (0.005 g/L), protocatechuic acid (0.001 g/L), and *p*-hydroxybenzoic acid (0.001 g/L) in free and immobilized cell cultures. Together, these data provide compelling evidence for the presence of the putative enzymes involved in the synthesis of vanillin, its precursors, and metabolites in microalgae, as evaluated and outlined in the following Table 1. Indeed, we identified potential homologs of vanillin biosynthetic enzymes in microalgae by comparing the sequences to those already characterized in *V. planifolia* (VpVAN), *Streptomyces* sp. V-1 (FCS and ECH), and *Pseudomonas nitroreducens* Jin1 (IEM). An immature *Vp*VAN candidate displaying the cathepsin propeptide inhibitor (I29), peptidase_C1, and granulin domains was identified in the protein databases of various microalgae (Table 1). Additionally, a putative FCS similar to long-chain fatty acyl-CoA synthetases was identified in these microalgae species. This protein contains domains such as the fatty acyl-CoA synthetase domain, a dimer interface polypeptide, an acyl-activating enzyme (AAE) consensus motif, as well as AMP and CoA binding sites. Since the CoA-dependent non-β-oxidation pathway involves both FCS and ECH, a putative ECH with a crotonase-like domain, a substrate binding site, oxyanion hole (OAH)-forming residues, and a trimer interface polypeptide binding site was also found. However, for the isoeugenol monooxygenase pathway, only three of these microalgae species (*C. reinhardtii*, *H. pluvialis*, and *C. vulgaris*) present a putative IEM, characterized by a retinal pigment epithelial membrane protein domain. These putative proteins in microalgae could play roles similar to those in the vanillin biosynthesis pathway in *V. planifolia* or in bacteria. Indeed, phenolic compounds are primarily synthesized through the shikimic acid pathway, a biochemical route present in most plants and microorganisms [97–99]. This pathway, which leads to the formation of phenylpropanoid acid precursors like $_{L}$-phenylalanine, could serve as the upstream mechanism for the biosynthesis of various vanillin precursors. The key enzyme typically initiating phenolic compound biosynthesis is phenylalanine ammonia-lyase (PAL), which catalyzes the deamination of $_{L}$-phenylalanine to form cinnamic acid, a crucial precursor of vanillin. However, recent studies have shown that not all microalgal taxa possess the PAL enzyme [94]. This absence suggests that microalgae may utilize alternative pathways for phenolic compound biosynthesis, potentially opening up other routes for the production of vanillin precursors via different enzymatic mechanisms. Such alternative strategies highlight the metabolic diversity within microalgae, offering distinct advantages for engineering approaches aimed at optimizing vanillin production.

Table 1. Putative vanillin biosynthesis and degradation enzymes identified in microalgae.

Homologues of Vanillin biosynthesis Enzymes	Conserved Domains Database	Function	Component	Process	Target Biochemical Mechanisms
Putative Vanillin synthetase	214853, 425470, 239068, 197621.	Enables cysteine-type endopeptidase activity	Extracellular space and lysosome	Involved in protein catabolic process	Retro-aldol-type reaction [4]
Putative Feruloyl-CoA synthetase	341284;	Enables acyl-activating enzyme activity	NI	Catalyze the ATP-dependent acylation of fatty acids in a two-step reaction	CoA-dependent non-β-oxidation reaction [100]
Putative Enoyl-CoA Aldolase/hydratase	474030 119339	Enables enoyl-CoA hydratase activity	Mitochondrion	Involved in fatty acid beta-oxidation	CoA-dependent non-β-oxidation reaction [100]
Putative Isoeugenol Monooxygenase	442887	Enables carotenoid dioxygenase activity	NI	Involved in carotene catabolic process	Oxidative cleavage [101]

A BlastP was performed in NCBI using the vanillin synthase from *Vanilla planifolia* (GenBank: AKG47593.1); feruloyl-CoA synthetase from *Streptomyces* sp. *V-1* (GenBank: AGR34008.1); enoyl-CoA aldolase/hydratase from *Streptomyces* sp. *V-1* (GenBank: AGR34009.1), and isoeugenol monooxygenase from *Pseudomonas nitroreducens* Jin1 (GenBank: ACP17973.1) as queries in the protein databases of four microalgae species (*C. reinhardtii*, *P. tricornutum*, *H. pluvialis*, and *C. vulgaris*). Homologous enzymes sharing the same active domain were found in these microalgae species. CDD: Conserved Domains Database; NI: not identified.

6.2. Engineering and Modulation of Robust Microalgal-Based Vanillin Production Systems

The engineering of robust microalgal-based systems for vanillin production represents a promising frontier in biotechnology, aimed at utilizing the metabolic flexibility of microalgae for sustainable vanillin biosynthesis. This development involves the integration of advanced genetic engineering techniques to optimize the metabolic pathways responsible for producing vanillin and its precursors. The key strategies include introducing or enhancing the pathways involved in the synthesis of phenylpropanoid acids such as ferulic acid, *p*-coumaric acid, and caffeic acid, which serve as essential precursors for vanillin production [7]. Genetic engineering techniques, including the CRISPR-Cas9 and transgenic approaches, are widely employed to modify the key enzymes and regulatory elements in the microalgal genome [102,103]. These modifications may enable more precise control of the metabolic flux toward vanillin production, minimizing the formation of unwanted by-products while maximizing efficiency.

A significant challenge in engineering microalgae for vanillin production is the efficient expression of heterologous genes within their complex cellular environment. Research efforts have been undertaken to introduce vanillin biosynthesis pathways into microalgae like *P. tricornutum*. In that case, the researchers attempted to propagate the full vanillin biosynthesis pathway from *V. planifolia* into *P. tricornutum* through an episomal system, incorporating multiple key enzymes required for vanillin synthesis [104]. While the pathway introduction was successful at the mRNA level, issues such as episomal instability and difficulties in protein expression hindered the actual vanillin production [105]. Indeed, episomal plasmids are commonly used in genetic engineering to introduce new pathways into host cells without altering the cell's native genome [106–108]. However, these plasmids can be unstable, especially under prolonged culture conditions or selective pressures, leading to the gradual loss of the plasmid from the cell population. To overcome these challenges, researchers can explore strategies to improve the expression of heterologous genes, such as optimizing the gene integration into stable genomic locations, enhancing the promoter efficiency, and fine-tuning the expression systems [109,110]. Advances in algal synthetic bi-

ology tools, including modular plasmid systems and improved transformation techniques, are enabling more precise and efficient engineering of microalgae. Microalgae like *C. reinhardtii* offer a highly versatile platform for genetic manipulation, with extensive molecular tools and mutant libraries available [111,112]. Both the chloroplast and nuclear genomes of *C. reinhardtii* can be engineered to express vanillin biosynthesis enzymes, providing flexibility in targeting specific metabolic compartments for efficient vanillin production. Therefore, this approach enables the genetic modification of the nuclear genome (with the genes encoding enzymes that require N-glycosylation for enhanced activity) or the chloroplast genome (with the genes encoding enzymes that do not require N-glycosylation). This facilitates the reconstruction of partial vanillin biosynthesis pathways from *V. planifolia* or known bacterial pathways, ultimately leading to the development of industrial strains of microalgae with high vanillin yields.

Furthermore, the integration of efficient downstream processing techniques is critical to developing robust microalgal vanillin production systems. The extraction and purification of vanillin from microalgal biomass must be cost-effective and efficient to maximize the yield. Innovative approaches, such as the use of biocompatible solvents, microwave-assisted extraction, pulsed electric fields, supercritical fluids, crystallization, and membrane-based separation technologies, are currently being explored to streamline the recovery of vanillin from engineered microbial and plant-based vanillin production systems [50]. These techniques can also be adapted to microalgae, further enhancing their potential as a sustainable and scalable source of vanillin. By optimizing these extraction methods, the industrial viability of microalgal-based vanillin production could be significantly improved, ensuring high-purity vanillin at reduced processing costs and energy consumption. Ultimately, a vanillin-tolerant microalgae could be optimized for resistance to redox products, enabling the accumulation of vanillin without undergoing reduction or oxidation processes. Then, the optimized designed expression system carrying the vanillin biosynthesis pathway described in *V. planifolia* or the bacterial pathway can be used to engineer the nuclear and/or chloroplast genome of the tolerant microalgae. Finally, the optimized transformants are then cultivated on a large scale to produce vanillin for the cosmetics, food, and pharmaceutical industries.

7. Conclusions and Perspectives

This review has explored the potential of leveraging biotechnological advances to engineer microbial, plant, and algal systems for sustainable vanillin production. As the consumer demand for natural and eco-friendly ingredients grows, the biotechnological synthesis of vanillin offers a promising alternative to the traditional methods reliant on vanilla beans or petrochemical routes. We discussed and highlighted the production and industrial application of vanillin by deciphering the current knowledge regarding the key enzymes involved in vanillin biosynthesis. The insights gained from microbial and plant systems have already established a foundation for understanding vanillin biosynthesis. Advances in metabolic engineering have enabled the development of genetically modified microbial strains capable of efficiently producing vanillin and its precursors, although challenges such as yield optimization and pathway stability remain.

Microalgae have emerged as a promising platform for high-value molecule production owing to their metabolic diversity and capacity for large-scale cultivation. This unique biological system not only presents opportunities for vanillin biosynthesis but also offers added environmental benefits, such as carbon dioxide capture. While substantial progress has been achieved in the metabolic engineering of microalgae for the production of high-value molecules, the full potential of microalgae for vanillin production is yet to be realized. The complexity of heterologous gene expression in these systems remains a critical challenge. Researchers must continue to refine the gene integration strategies, enhance the promoter efficiency, and fine-tune the expression systems to ensure stable and high-yield production. The scaling-up of vanillin production also poses challenges. Although laboratory-level successes have demonstrated the feasibility of non-photosynthetic

microorganism systems for vanillin production, scaling these systems for commercial applications will require further optimization of the growth conditions, bioreactor designs, and downstream processing methods.

Looking ahead, several key areas will drive future research into biotechnological vanillin production using microalgae. First, a deeper understanding of microalgal metabolism, particularly in the context of vanillin biosynthesis, will enable more targeted and efficient metabolic engineering. Exploring the evolutionary diversity of microalgal species could uncover novel biosynthetic pathways and enzymes that further improve the efficiency and yield of vanillin production. Second, the integration of omics technologies, including genomics, transcriptomics, proteomics, and metabolomics, will provide valuable insights into the regulation of the vanillin biosynthesis pathways and the identification of bottlenecks that limit production. Collaboration between academia and industry will be crucial to accelerating the development of scalable, cost-effective production platforms. The growing interest in bio-based vanillin from the food, cosmetics, pharmaceutical, and agrochemical industries will drive investment in research and development. By addressing the challenges of gene expression, pathway optimization, and process scalability, it is likely that microbial and algal systems will become viable alternatives for sustainable vanillin production in the near future.

Author Contributions: Conceptualization, A.W.T. and I.D.-P.; validation, A.W.T., F.A., F.M.-M. and I.D.-P.; writing—original draft preparation, A.W.T.; writing—review and editing, A.W.T., F.A., F.M.-M. and I.D.-P.; visualization, A.W.T. and F.A.; supervision, I.D.-P.; project administration, F.M.-M. and I.D-P.; funding acquisition, I.D.-P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Université du Québec à Trois-Rivières—Annexe C program to I.D.-P.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the review and appendix; further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

Table A1. Metabolic engineering of prokaryotic and eukaryotic organisms for the production of vanillin.

Engineering Platform	Genes (Origin)	Bioengineering Strategies Transformation Method	Insertion Type	Mutations	Substrates (g/L)	Complexity	Product	Volume of Culture (mL)	Fermentation Time (h)	Yield (g/L)	Ref.
Escherichia coli BL21(DE3)	*IEM (Pseudomonas nitroreducens Jin1)*	Heat shock	Episomal	None	49.2 Isoeugenol	2	Vanillin	NI	16	38.3	[57]
Escherichia coli BL21(DE3)	*IEM (Pseudomonas putida IE27)*	NI	Genome integration	None	37.7 Isoeugenol	2	Vanillin	10	48	28.3	[113]
Escherichia coli BL21 (DE3)	*IEM (microbial metagenome)*	NI	NI	None	16.4 Isoeugenol	2	Vanillin	15	48	2.2	[114]
Escherichia coli JM109	*fcs and ech (Pseudomonas fluorescens BF13)*	NI	NI	None	0.64 Ferulic acid	4	Vanillin	NI	6	0.53	[12]
Escherichia coli FR13	*fcs and ech (Pseudomonas fluorescens BF13)*	NI	Genome integration	None	4.5 Ferulic acid	4	Vanillin	15	24	4.3	[115]
Escherichia coli BW25113	*PobA (Pseudomonas aeruginosa), CAR (Mycobacterium marinum), sfp (Bacillus subtilis), and comt (Arabidopsis thaliana)*	NI	NI	None	1 3,4-dihydroxy benzoyl alcohol.	4	Vanillyl alcohol	20	48	0.24	[56]
Escherichia coli K-12 MG1655	*CAR (Nocardia iowensis), sfp (Bacillus subtilis), comt (Homo sapiens), DSD (Bacillus thuringiensis)*	P1 bacteriophage transduction	Genome integration	Deletion of *yqhC-dkgA* genes	Protocatechuic acid	4	Vanillate	50	72	0.004	[55]
Pseudomonas fluorescens BF13	*fcs and ech (Pseudomonas fluorescens BF13)*	Heat shock	NI	Deletion of *vdh* gene	0.48 Ferulic acid	4	Vanillin	2000	24	1.27	[29]
Escherichia coli	*tal (Saccharothrix espanaensis), sam5 (actinomycete), comt (Arabidopsis thaliana), sam8 (Saccharothrix espanaensis), fcs, and ech (Streptomyces* sp.)	Co-transformation	Genome integration	Deletion of *TyrR* gene	L-Tyrosine	6	Vanillin	50	12	0.013–0.097	[54]

Table A1. *Cont.*

Engineering Platform	Genes (Origin)	Bioengineering Strategies Transformation Method	Insertion Type	Mutations	Substrates (g/L)	Complexity	Product	Volume of Culture (mL)	Fermentation Time (h)	Yield (g/L)	Ref.
Escherichia coli MG1655 RARE	*LCC (unknown prokaryote), TPADO (Comamonas* sp.*), DCDDH (Comamonas* sp.*), O-MT (Rattus norvegicus), and CAR (Nocardia iowensis).*	Heat shock	NI	None	Monomer terephthalic acid	6	Vanillin	10	24	0.11	[116]
Escherichia coli BW25113	*PobA (Pseudomonas aeruginosa), CAR (Mycobacterium marinum), sfp (Bacillus subtilis), and COMT (Arabidopsis thaliana)*	Heat shock	NI	Deletion of *aroE* gene	ND	6	Vanillyl alcohol	50 and 3000	2 and 24	3.89 and 0.55	[58]
Escherichia coli	*fcs and ech (Amycolatopsis* sp.*)*	NI	NI	None	Glucose	6	Vanillin	NI	48	0.50	[117]
Pediococcus acidilactici BD16	*fcs and ech (Amycolatopsis* sp.*)*	Heat shock	Episomal	None	0.21 Ferulic Acid per mg of recombinant cell biomass	4	Vanillin	100	0.33	0.47	[65]
Pediococcus acidilactici BD16	*fcs and ech (Pediococcus acidilactici BD16)*	Heat shock	Episomal	None	Rice bran	4	Vanillin	50	24	4.01	[66]
Pseudomonas putida KT2440	*Pp-SpuC and ATA (Chromobacterium), adh (Bacillus)*	Electroporation	Genome integration	None	Lignin-derived substrates	4	Vanillin and vanillylamine	50 and 100	24	0.10 and 0.13	[118]
Pseudomonas putida KT2440	*fcs and ech (Pseudomonas putida)*	Electroporation	Genome integration	Deletion of *vdh* and *modABC* genes	1.94 Ferulic acid	4	Vanillin	5	3	1.66	[59]
Corynebacterium glutamicum ATCC 21420	*vanAB (Corynebacterium efficiens NBRC 100395)*	Electroporation	Genome integration	None	3.1 Ferulic Acid	4	Protocatechuic acid	400	24	1.06	[62]
Corynebacterium glutamicum PV-IYΔ0324	*comt (Rattus norvegicus) and CAR (Nocardia iowensis)*	NI	Genome integration	Deletion of *NCgl0324* gene	Endogenous 4-Hydroxylbenzoic acid	6	Vanillin	25	48	0.31	[63]

Table A1. *Cont.*

Engineering Platform	Genes (Origin)	Bioengineering Strategies Transformation Method	Insertion Type	Mutations	Substrates (g/L)	Complexity	Product	Volume of Culture (mL)	Fermentation Time (h)	Yield (g/L)	Ref.
Amycolatopsis sp. *ATCC 39116*	*fcs and ech (Amycolatopsis* sp.*)*	Direct mycelium transformation	Genome integration	Deletion of *vdh* gene	5 Glucose	6	Vanillin	2000	20	22.3	[119]
Saccharomyces cerevisiae and Schizosaccharomyces pombe	*3DSD (Podospora pauciseta), ACAR (Nocardia* sp.*), O-MT (Homo sapiens), and UGT (Arabidopsis thaliana)*	Lithium acetate/polyethylene glycol/single carrier DNA	Genome integration	Deletion of *adh6* gene	Glucose	6	Vanillin	5	48	0.065 and 0.045	[70]
Saccharomyces cerevisiae VAN286	*UGT (Arabidopsis thaliana)*	NI	NI	Deletion of *pdc1* and *gdh1* genes	Glucose	6	Vanillin-β-D-glucoside	NI	90	0.50	[72]
Saccharomyces cerevisiae	*Sam8 (Actinomycete), sam5/hpaB/ CYP199A2(Saccharothrix espanaensis/Pseudomonas aeruginosa/Rhodopseudomonas palustris), comt (Arabidopsis thaliana), fcs, and ech (Streptomyces* sp.*).*	Lithium acetate/polyethylene glycol/single carrier DNA	Genome integration	Deletion of *Aro4* and *Aro7* genes	0.4 ferulic acid	6	Vanillin	50	96	0.008	[76]
Saccharomyces cerevisiae S288c and CEN.PK	*ACAR (Neurospora* sp.*), EntD (Escherichia coli), UGT (Arabidopsis thaliana), OMT (Homo sapiens), and 3DSD (Podospora pauciseta)*	Lithium acetate/polyethylene glycol/single carrier DNA	Genome integration	Deletion of *Bgl1* and *adh6* genes	20 Glucose	6	Vanillin-β-D-glucoside	50	45	2	[79]

Table A1. *Cont.*

Engineering Platform	Genes (Origin)	Bioengineering Strategies Transformation Method	Insertion Type	Mutations	Substrates (g/L)	Complexity	Product	Volume of Culture (mL)	Fermentation Time (h)	Yield (g/L)	Ref.
Saccharomyces cerevisiae BY4741	*AroZ (Podospora anserina), OMT (Homo sapiens), CAR (Segniliparus Rotundus), GapC (Clostridium acetobutylicum), Pos5c (S. cerevisiae), Sfp (Bacillus subtilis), EntD (E. coli), MetK (E. coli), hpaB (Pseudomonas aeruginosa), hpaC (Salmonella enterica), Xfpk (Bifidobacterium breve), CkPta (Clostridium kluyveri), HmaS (A. orientalis), HMO (S. coelicolor A3), and BFD (P. putida KT2440)*	Electroporation	Combinaison of both episomal expression and genome integration	Deletion of adh6, adh7, gre3, gcy1, ydl124w, ypr1, ari1, ydr541c, aad3, *hfd1, AKR, and AL DR family genes*	Glucose	4	Vanillin	10	120	0.36	[20]
Saccharomyces cerevisiae BY4742	*4CL (Petroselinum crispum), Ech (Pseudomonas putida KT2440), VpVAN (V. planifolia), hpaB(Pseudomonas aeruginosa), hpaC (Salmonella enterica), PobA (Pseudomonas putida KT2440), COMT (Arabidopsis thaliana), METHER (Arabidopsis thaliana), and PsVAO (Penicillium simplicissimum)*	NI	Genome integration	Deletion of *adh6, adh7, bdh2, bdh1, hfd1, pdc5, aro10, pha2, pgm1, trp2, pad1, ahfd1, AKR, and ALDR family* genes	lignocellulosic biomass	6	Vanillin	50	24	0.29	[77]

Table A1. *Cont.*

Engineering Platform	Genes (Origin)	Bioengineering Strategies Transformation Method	Insertion Type	Mutations	Substrates (g/L)	Complexity	Product	Volume of Culture (mL)	Fermentation Time (h)	Yield (g/L)	Ref.
Oryza sativa	*VpVAN (V. planifolia)*	Agrobacterium-mediated transformation	Genome integration	None	Endogenous ferulic Acid	4	Vanillin	NI	NI	0.00054	[81]
Capsicum frutescens	*VpVAN (V. planifolia)*	Microparticle bombardment	Genome integration	None	Endogenous ferulic Acid	4	Vanillin	NI	NI	0.00057	[80]

Abbreviations: TAL (*sam 8*): tyrosine ammonia lyase; C3H (sam5): 4-coumarate 3-hydroxylase; COMT: caffeate O-methyltransferase; fcs: feruloyl-CoA synthetase; ech: enoyl-CoA hydratase/aldolase; vdh: vanillin dehydrogenase; IEM: isoeugenol monooxygenase; PobA: 4-hydroxybenzoate hydroxylase; CAR: carboxyl acid reductase; Pp-SpuC: putrescine transaminase; ATA: amine transaminase; adh: alcohol dehydrogenase; sfp: phosphopantetheinyl transferase; COMT: catechol O-methyltransferase; 3DSD: 3-dehydroshikimate dehydratase; LCC: leaf–branch compost cutinase; TPADO: terephthalate 1,2-dioxygenase; DCDDH: 1,4-dicarboxylic acid dehydrogenase; OMT: O-methyltransferase; aroE: shikimate dehydrogenase; vanAB: vanillate O-demethylase; ACAR: aryl carboxylic acid reductase; UGT: UDP-glycosyltransferase; ADH6: alcohol dehydrogenase; Pdc1: pyruvate decarboxylase; gdh1: glutamate dehydrogenase; 4HPA3H (*hpaB* and *C*): 4- hydroxyphenylacetate 3-hydroxylase;CYP199A2: cytochrome P450 monooxygenase; DAHP: 3-deoxy-D-arabino heptulosonate-7-phosphate synthase; CHA: chorismate mutase; EntD: phosphopantetheine transferase; BGL1: beta-glucosidase; VpVAN: vanillin synthetase; NCgl0324: aromatic aldehyde reductase; 4CL: hydroxycinnamic acid CoA ligase; METHER: methylenetetrahydrofolate reductase; PsVAO: vanillyl alcohol oxidase; bdh1 and 2: 3-hydroxybutyrate dehydrogenase1 and 2; hfd1: fatty aldehyde deshydrogenase; pha2: prephenate dehydratase 2; pgm1: phosphoglucomutase 1; trp2: DOPAchrome tautomerase; pad1: phenylacrylic acid decarboxylase; AKR: aldo-keto reductases; ALDR: adrenoleukodystrophy-related; gcy1: glycerol 2-dehydrogenase; ari1: carbonyl reductase; ydr541c: aldehyde reductase; aad3: aryl-alcohol dehydrogenase; HpaBC: flavin-dependent monooxygenase; HMO: hydroxymandelate oxidase; BFD: benzoylformate decarboxylase. NI: not identified. The numbers 2, 4, and 6 represent the level of complexity of the heterologous pathway; 2 is less complex; 4 is moderately complex; and 6 is very complex. COMT in red is to differentiate the catechol O-methyltransferase from the caffeate O-methyltransferase.

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