UNIVERSITA' DEGLI STUDI DELL'INSUBRIA

DOTTORATO DI RICERCA IN SCIENZE DELLA VITA E BIOTECNOLOGIE

XXXVI CICLO

Systems Biocatalysis: production of high-value compounds from renewable by-products by a multienzymatic cascade process

Produzione di composti ad alto valore aggiunto da sottoprodotti rinnovabili mediante un sistema multienzimatico a cascata

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Anno accademico 2022-2023

Abstract

The efficient valorization of the lignocellulosic biomass components, and in particular the lignin fraction, could serve as a starting point for the establishment of a circular bioeconomy model aimed at the recycling and reutilization of industrial by-products over the exploitation of virgin feedstock. From lignocellulosic biomass both fermentable carbohydrate and aromatics can be obtained, which can be used for the production of biofuels and bioplastics thus reducing the dependence on petroleum-based feedstocks. The present Ph.D. thesis focuses on the development of novel biotechnological processes aimed at the extraction of aromatics from lignocellulosic by-products and their conversion to value-added products using whole-cell biocatalytic approaches.

Firstly, I developed an efficient and green process to produce ccMA from renewable feedstocks (i.e. kraft lignin and wheat bran) based on: a) the optimization of the extraction procedures of vanillin from lignin and of ferulic acid from wheat bran; b) the genetic engineering of an *E. coli* strain to modulate the expression of up to seven recombinant enzymes. In detail, vanillin was recovered from kraft lignin (4.5 mg vanillin/g kraft lignin) by an enzymatic treatment using the recombinant *Bacillus licheniformis* laccase, and ferulic acid from wheat bran (3.0 mg ferulic acid/g wheat bran) by a thermo-enzymatic method using the Ultraflo®XL commercial enzyme. The whole-cell biocatalyst used to convert vanillin into ccMA expresses the dehydrogenase LigV, the demethylase VanAB, the decarboxylase AroY and the dioxygenase C12O; meanwhile the whole-cell biocatalyst to convert ferulic acid to ccMA expresses all the above-mentioned enzymes plus the decarboxylase Fdc and the dioxygenase Ado. The engineered strains converted >95% of lignin-derived vanillin in 30 minutes, obtaining the production of 4.2 mg ccMA/g of kraft lignin. Starting from the wheat bran-derived ferulic acid, ccMA was produced with a >95% conversion yield in 10 hours, corresponding to 0.73 g ccMA/g ferulic acid, and 2.2 mg ccMA/g wheat bran biomass.

To further evaluate the capabilities of the whole-cell biocatlyst, the scaled-up production of ccMA from vanillin using the engineered *E. coli* growing cells was studied. The bioconversion reaction was carried out in a fermenter, providing improved control of the reaction conditions such as pH, dissolved oxygen and substrate pulse-feed rate, streamlining the biocatalytic process and enhancing scalability. The optimized growth medium composition (0.5 g/L glucose and 2 g/L lactose) and substrate addition strategy (1 mmol/h pulse-feed) enabled the engineered strain to produce 5.2 ± 0.36 g/L of ccMA in 48 hours, corresponding to 0.86 g ccMA/g vanillin. The purification of the produced ccMA from the fermentation broth was achieved through crystallization, yielding 2.58 ± 0.07 g per liter of broth, corresponding to a ≈50% purification yield.

Lastly, a preliminary analysis of a one-pot process for the production of 4-vinylguaiacol from wheat bran was conducted. The process involves the extraction of ferulic acid from the wheat bran using the three-step thermo-enzymatic protocol utilized previously and the simultaneous conversion of ferulic acid into 4-vinylguaicol using an engineered *E. coli* strain expressing the decarboxylase Fdc. The novelty of this process arises from the employment of the wheat bran crude extract as an auto-inducing growth medium, based on the presence of several fermentable carbohydrates and the utilization of a hybrid phenol-inducible promoter for the induction of Fdc expression, making the wheat bran-derived ferulic acid both the inducer and the substrate of the enzyme. The unoptimized process produced 1.8 mg 4-vinylguaiacol per gram of wheat bran, which correspond to the conversion of ≈75% of the ferulic acid extracted using the thermo-enzymatic method and ≈64% of the alkaline extractable ferulic acid present in the wheat bran.

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1. Introduction

1.1 Bioeconomy and Circular Economy

The huge impact human activities have on the environment can not be understated; indeed, Paul Crutzen suggested the establishment of a new geological epoch called Antropocene to underline the magnitude of our ecological footprint[1]. One striking data concerning this topic is the Earth Overshoot Day (Figure 1), which is the date that marks when all the biological resources that the Earth can renew during the entire year are finished. In 2023 was calculated to fall August 2nd. Due to human activities and related pollution, the Earth Overshoot Day has fallen earlier every new year; this clearly highlights the need to lower human ecological footprint and to find new industrial processes that do not require the over-exploitation of natural resources[2].

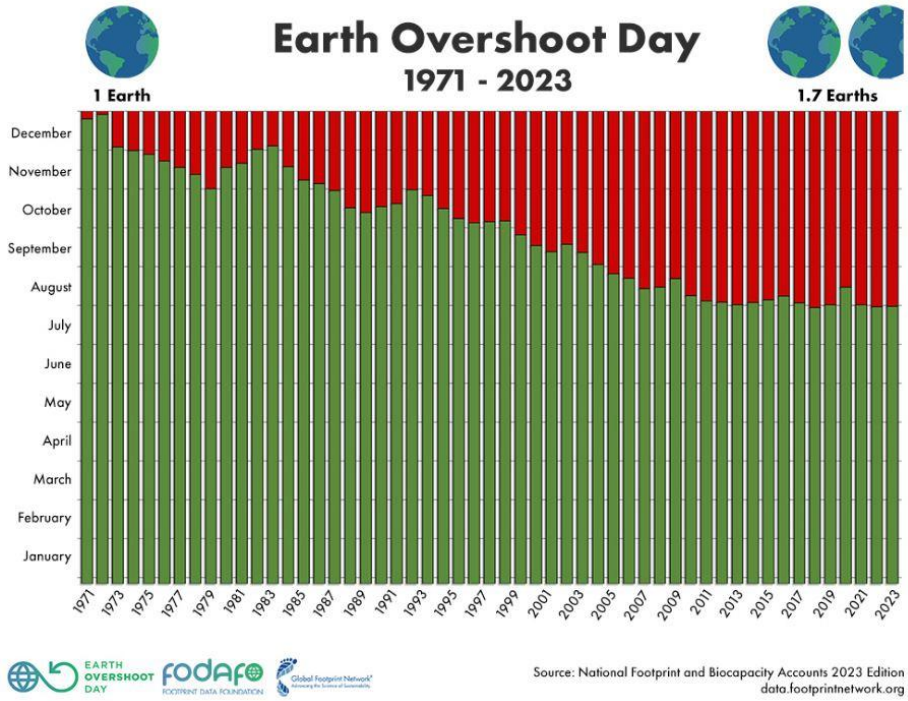

Figure 1. Earth overshoot day trend over the last 32 years. In this graph is clearly visible the unsustainability of our current economic model and related industrial processes which overexploit the natural resources of the Earth. In 2023 we consumed an amount of resources equal to 1.7 times the amount that are regenerated every year. Reprinted from the site www.overshootday.org.

This ecological crisis led the development of innovative economic models that seeks to combine economic profitability and strategic advancement with a commitment to both society and the preservation of our natural resources. In the late 1970s, a growing awareness emerged regarding the need for a more sustainable economic model to address the emerging social and environmental challenges. The ever-increasing global population, the repercussions of climate change, the unsustainable exploitation of land and ecosystems, and our heavy reliance on finite fossil resources,

prompted the introduction of the "Bioeconomy" concept[3]. During the First Global Bioeconomy Summit in Berlin in 2015, “bioeconomy” was defined for the first time as the "knowledge-based production and utilization of biological resources, biological processes and principles to sustainably provide goods and services across all economic sectors"[4]. In particular, the circular economy branch advocates an alternative to the conventional linear production model by discouraging the utilization of untouched fossil resources such as petroleum, coal, and natural gas, while instead aiming at establishing a closed-loop system designed to optimize the extraction of raw materials from waste products (Figure 2)[5–7]. While a single definition may not exist, the European Union encapsulates this approach through four pivotal terms, often referred to as the "4Rs": reuse, reduction, recycling, and recovery of waste[7].

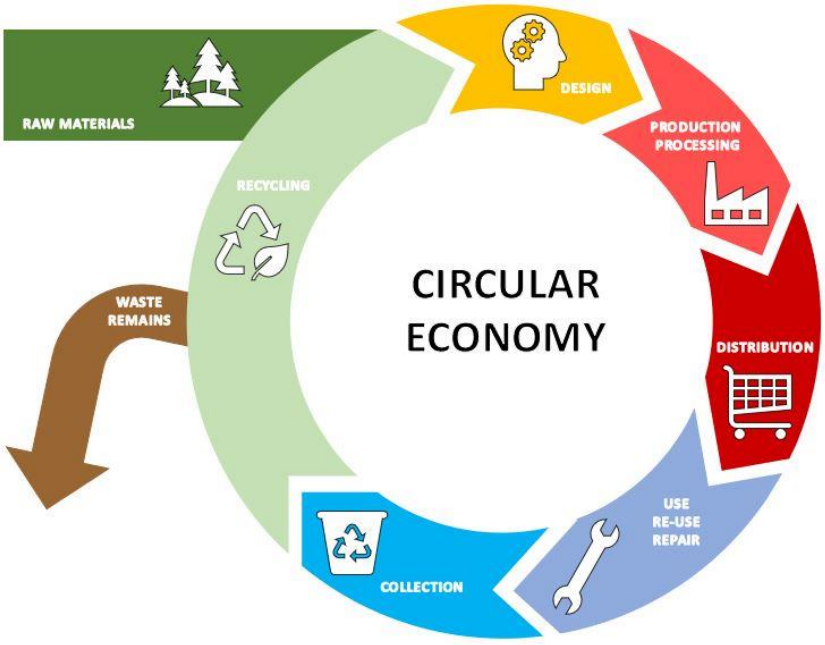

Figure 2. Schematic representation of the circular economy model. This economic model focuses particularly on recycling by-products and wastes generated during industrial processes, indeed the major goal of this model is the reduction of waste produced by human activities.

These circular processes are very similar to the metabolic pathways present in the biosphere, where by-products and waste materials generated by one organism can be utilized as substrate for the growth and survival by other organisms. In this view, “waste” materials can not be defined as “unwanted or unusable materials, substances, or by-products” anymore, but possess some inherent value that can (and should be) valorized. Therefore, the overarching goal of the circular economic system is to unlock even greater value from waste by establishing integrated facilities known as biorefineries that can rival their fossil-based counterparts[3,5,7].

The potential inherent in waste as a resource is staggering, with annual global quantities surpassing hundreds of megatonnes (i.e., $>10^8$ t). Of particular note, lignocellulosic biomass, which is estimated to exceed 2×10^{11} t/year in worldwide production[6,8], is considered the most suitable

renewable feedstock for replacing chemicals and fuels derived from fossil sources[5]. To address the mounting concern over global greenhouse gas emissions, the United States has funded numerous programs aimed at increasing the biomass percentage in commodities. This includes raising it from 5% in 2005 to 18% in 2020, with a target of reaching 25% by 2030[9]. Furthermore, harnessing biomass as a renewable resource not only carries significant environmental benefits but also offers to countries a means to achieve independence from fossil fuel reserves. These supplies, present in limited geographical regions on Earth, have sometimes been the subject of speculative practices and for international conflicts[9]. Due to this potential benefit, the circular economy has been identified as a potent approach that could assist reaching numerous Sustainable Development Goals (SDGs) outlined in the United Nations' 2015 document titled "Transforming our World: The 2030 Agenda for Sustainable Development"[10]. Moreover, the circular economy model has the potential to yield positive outcomes for both developing countries and developed nations: creating new jobs, allowing cost savings and fostering innovation[10]. Nevertheless, a significant impediment to transitioning from a linear economic system to a circular one lies in the workforce's skills gap and the technological innovations available. Addressing this challenge requires investments in innovation, infrastructure, and education, which are crucial for both promoting the circular economic model and improve the welfare[10,11]. Naturally, the viability of implementing the circular economic system must take into account the distinct attributes of each country, including the types of waste generated and the available infrastructure. In this regard, both the Life Cycle Assessment (LCA) and the Life Cycle Costing Analysis (LCCA) emerge as indispensable tools for conducting thorough environmental and economic assessments[3,7,9]. It's important to note that there won't be a singular all-encompassing bioeconomy; instead, there will be as many bioeconomies as ecosystems and socioeconomic models, making the whole system more flexible and robust[3]. Finally, an effective transition into a circular economic system would need a deep understanding of the underlying science and engineering required for the development of sustainable processes[3]. In this regard, **biocatalysis** is considered as one of the fundamental technologies helping this transition. Indeed, the use of enzymes as catalysts could overcome many of the disadvantages of traditional chemical synthesis. Enzymes are renewable, cost-effective, non-toxic, work under mild operational conditions (generally in water) and are highly selective, thus reducing the amount of by-products[12,13]. Some of these features, such as higher activity and/or selectivity, can even be achieved through protein engineering approaches[12]. Despite these advantages, such technologies often lack the efficiency needed to compete with traditional processes at an industrial scale and so there is still a long way to go before they can be fully implemented[14].

1.2 LignoCellulosic Biomass (LCB)

The utilization of plant biomass for both material and fuel purposes dates back to a time before the emergence of modern humans. This practice started with the development of primitive wooden tools and the use of firewood by the Neanderthals[15]. Despite the early utilization of lignocellulose as a raw material for various purposes such as textiles, fuels, and construction, it was not until the 1st century CE in China that people began employing primitive bleaching methods on lignocellulose to produce paper[16]. From such a moment, chemical processing techniques and innovative applications for plant biomass started to emerge; in particular, the introduction of chemically-assisted pulping made the mass production of paper from lignocellulose economically feasible[17]. Nowadays, we refer to plant biomass by using the term lignocellulosic biomass (LCB) due to being composed by two main biopolymers: lignin (10-20%) and holocellulose, which can be subdivided into cellulose (30-50%) and hemicellulose (15-35%), along with minimal quantities of fibrils and pectin (<0.1%)[5,18].

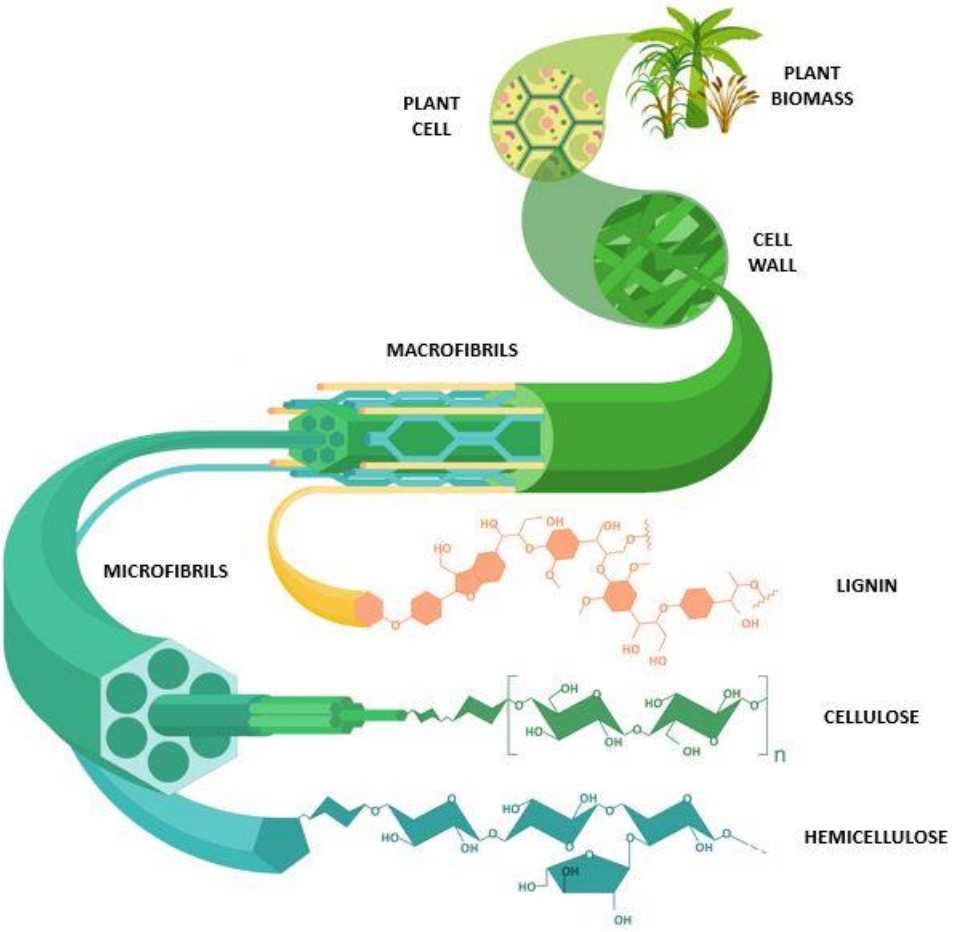

Figure 3. Structure of the lignocellulosic matrix: cellulose chains (green) are positioned at the core of the microfibrils, and are surrounded by hemicellulose (blue) and lignin (red) polymers cross-linked together through hydrogen and covalent bonds. Adapted by permission from [20], copyright 2019.

Lignocellulosic biomass (Figure 3) consists of primary and secondary plant cell walls. The primary cell wall primarily comprises cellulose and pectin, while the secondary wall is primarily composed of a complex polymeric matrix referred to as lignocellulose[19]. Cellulose $(C_6H_{10}O_5)n$ represents the main structural biopolymer in LCB: D-glucose monomers are interconnected through β-(1,4)-glycosidic bonds, forming polymeric chains of 10000 to 15000 monomers, further stabilized by numerous intra- and inter-molecular hydrogen bonds[21,22]. Hemicellulose is a branched hetero-polysaccharide consisting mainly of various pentoses and hexoses monomers linked by β-(1,4)-glycosidic bonds. Hemicellulose chain backbones are composed by 500 to 3000 sugar monomers with sidechains bound to the backbone through β-(1,2)-, β-(1,3)- and/or β-(1,6)- glycosidic bonds[23,24]. Unlike cellulose, the structure and sugar composition of hemicellulose vary depending on the plant species[25]. Lignin is a complex heterogeneous polymer composed by three aromatic units: syringyl (S), guaiacyl (G) and phydroxyphenyl (H) units. These units are bound together by ether linkages (α-O-4, β-O-4 and 4-O-5) and carbon–carbon linkages (β-β, β-5, 5–5 and β-1), with β-O-4 accounting for ≈50% of the total linkages present in lignin[26]. The highly irregular structure of lignin is cross-linked to holocellulose through covalent and hydrogen bonds, providing structural strength and depolymerization recalcitrance to LCB[17]. Just like hemicellulose, the aromatic composition of lignin can vary greatly between different plant species. The relative proportion of lignin, cellulose and hemicellulose in LCB can vary depending on the starting feedstock[27,28]; accordingly, it has been divided into woody feedstocks, agricultural residues, industrial byproducts, and municipal solid waste[5,27,29].

It is estimated that LCB production is $\approx 1.8*10^{11}$ tons/year[30], indeed holocellulose and lignin are the two most abundant biopolymer on the Earth[31]; it represents a valuable renewable resource to be exploited (Figure 4). However, LCB utilization is hampered by its heterogeneity and recalcitrance to depolymerization. Until now, LCB is processed using physical, chemical, physicochemical and/or biological pretreatments to break down the cross-links between lignin and the holocellulose. These pretreatments allow the separation of lignin from the holocellulose while increasing the material's porosity, making the polysaccharides more reactive to the following treatment[28]. After the pretreatment step, each LCB component (i.e. cellulose, hemicellulose and lignin) can be converted into a range of bioproducts through thermochemical processes (pyrolysis, gasification and torrefaction), enzymatic hydrolysis and microbial fermentation[8,32,33].

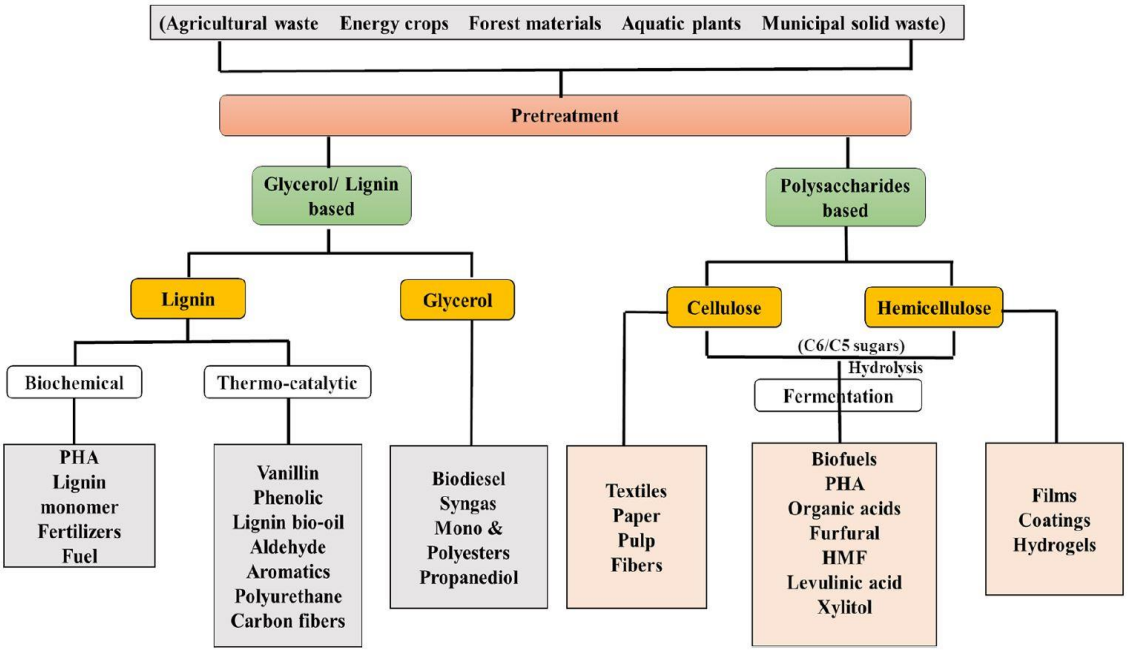

Figure 4. LCB valorization routes. Several LCB feedstocks can be pretreated to separate them into glycerol/lignin- and polysaccharides-based materials, thus allowing four different valorization streams (i.e. lignin, glycerol, cellulose and hemicellulose) resulting in the possible production of multiple added-value products. Reprinted by permission from [30], copyright 2022.

Traditionally, the pretreatment step aimed at the complete delignification of LCB in order to recover cellulose for applications in the paper industry (pulping techniques) or for the production of biofuels by extracting fermentable polysaccharides[34]. Despite the numerous different available approaches, there are no standardized methods for the valorization of lignocellulosic biomass due to the extensive variability in biomass composition stemming from its source, type, and recalcitrance level[30,35]. Thus, there is the need to develop taylor-made valorization processes based on the properties of the LCB feedstock.

In this view, this PhD project is focused at the valorization of two LCB by-products: Kraft lignin and wheat bran.

1.2.1 Kraft lignin

The LCB pretreatment processes lead to the separation of cellulose, polysaccharides and the so called technical lignins. Technical lignins differ substantially from their native lignin counterparts due to the various reactions that occur during processing, including lignin depolymerization, condensation of lignin fragments, and the formation of new functional groups[32,36,37]. The prevalent LCB pretreatment method is the Kraft pulping process, which is utilized during the pulping processes to produce paper, generating ≈85% of total technical lignin world-wide[35]. During this process, wood chips react with an aqueous solution of sodium hydroxide and sodium sulfide, called white liquor, at a temperature ranging from 155 to 175 °C[33,36]. The strongly alkaline environment

during the cooking process leads to the cleavage of aromatic ether bonds within lignin, resulting in the formation of soluble thio-lignin fragments often referred to as black liquor; these fragments can be precipitated in a condensed form upon acidification[38,39]. The recovered Kraft lignin typically exhibits a molecular mass falling within the range of 1,500 to 5,000 g/mol. It is characterized by a low presence of β-O-4 ether linkages, a sulfur content of 0.5 - 3.0 wt% and a residual ash content of 1 to 5 wt% following the cooking and washing steps involving diluted sulfuric acid[35].

Kraft lignin is primarily commercialized by companies like MeadWestvaco and Metso Corporation, the developer of LignoBoost technology for lignin recovery[32,33], at a price of 0.1-0.5 $/kg[9]. Regrettably, more than 80% of the lignin produced annually (≈63 × 10^4 tons) is utilized as a low value fuel to produce energy and only ≈10^5 tons are valorized through the production of carbon fibers, binders, ion-exchange resins, carriers for fertilizers and pesticides, as well as low molecular mass aromatics[35,40,41]. Despite its potential, the industrial valorization of lignin is hampered by its inherent heterogeneity and chemical stability. Lignin depolymerization technique aims at bypass these drawbacks by breaking down the polymer into its components, thus obtaining the enrichment of desired lignin-derived substrates that can be converted into valuable products[42]. At present, the depolymerization techniques can be divided in i) thermal, ii) chemical, iii) thermo-chemical, iv) microwave-assisted, and v) biological processes[42]. Depending on the technique used, it is possible to obtain different aromatic compounds from lignin depolymerization such as guaiacol, syringol, catechol, vanillin and its derivatives which have garnered significant attention due to their potential as key components in the production of flavors, fragrances, and polymers[8,43].

In summary, due to its low price and the great amount produced annually, Kraft lignin could represent a great source of renewable aromatics allowing the sustainable production of value-added products.

1.2.2 Wheat bran

Wheat, along with maize and rice, constitutes roughly 90% of the world's cereal production[44]. The annual wheat production is ≈770 million tons[45], with ≈70% of it earmarked for food consumption[44]. The majority of wheat is milled to produce white wheat flour: during this process, wheat bran is generated alongside other valuable components like wheat germ and portions of the endosperm. Approximately, one-fifth of the total weight of cultivated wheat being transformed into bran, amounting to 100-150 million tons annually[46]. Currently, wheat bran is considered an agricultural by-product and is mainly used as livestock feed, with only limited quantities marketed for human consumption[47]. The composition of wheat bran comprises roughly 8-12% moisture, 13-18% protein, 36-57% carbohydrates (typically 20-40% dietary fibers and 10-25% starch), 5-6% ash,

4-5.5% fat and 1% phenolic acids[46,47]. The relative abundances can vary between species and/or different climate conditions.

The most abundant phenolic acid in wheat bran is ferulic acid, which is generated through the phenylpropanoid pathway[48]. Ferulic acid plays important roles in plant cell walls, such as the protection against pathogen invasion[48] and as a cross-linker of polysaccharides chain[49]. In wheat, ferulic acid accumulates mainly in the bran where ≈90% is bound to arabinofuranosyl residues of arabinoxylans and other cell wall structures, while the remaining ≈10% exists as either a soluble, unattached compound or a conjugated moiety esterified to sugars[50]. In details, inside the cell wall there are short chains composed of xylose units connected by β-(1–4) glycosidic linkages, called arabinoxylan oligosaccharides. Ferulic acid can be found esterified to α-arabinose substituent bound to the arabinoxylan oligosaccharides in O-2 and/or O-3 positions[51] (Figure 5). These structural organization and its low price (0.05-1 €/kg)[52,53] make wheat bran an ideal source material for ferulic acid production.

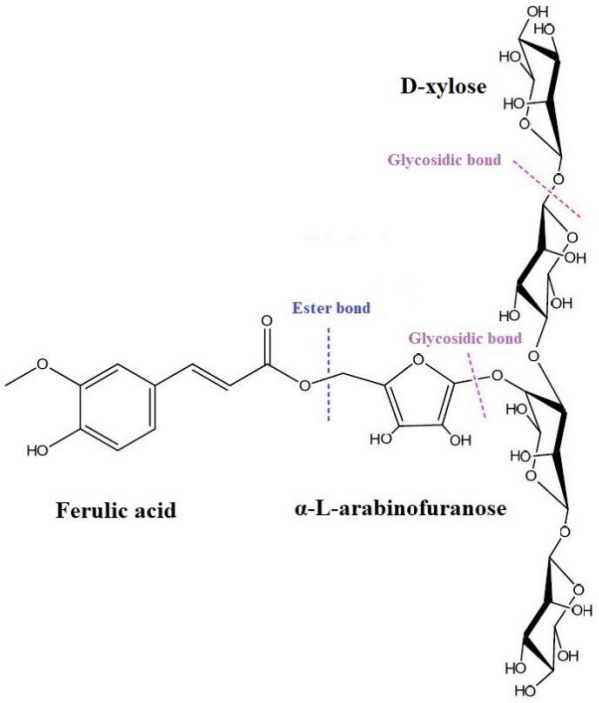

Figure 5. Ferulic acid bound to wheat bran hemicellulose chain. Hemicellulose in wheat bran is mainly composed by linear chain of D-xylose units; ferulic acid can be found esterified (blue) to α-L-arabinofuranose bound to the linear chain D-xylose in O-2 and/or O-3 positions through a glycosidic bond (purple). Adapted by permission from [30], copyright 2020.

The extraction of ferulic acid from wheat bran can be achieved through chemical or biotechnological (using enzymes or microorganisms) methods. Chemical approaches often involve alkaline or acid hydrolysis procedures aimed at breaking down the lignin/phenolic-carbohydrate complex. However, after these treatments, a mixture containing different compounds such as

additional phenols, proteins and carbohydrates, is obtained, thus requiring additional downstream purification steps[47,54]. Biotechnological approaches are generally considered more environmentally friendly compared to chemical methods because they are more energy-efficient and selective, typically involving digestion with esterases[48]. These biotechnological treatments are gentler, selectively releasing ferulic acid without causing damage to other valuable chemicals, which can occur during alkaline extraction, thus requiring a lower number of purification steps[46]. Once extracted, ferulic acid can be used in the food, healthcare, and cosmetic industries thanks to its antioxidant and anti-cancer effect[44,47]. Additionally, ferulic acid can be converted into bio-vanillin[55] or can serve as a building block for polymerization, enabling the production of bioplastics[56,57]. Wheat bran is not only a promising feedstock for ferulic acid extraction, in fact it can be utilized as a direct source of starch, arabinoxylans and proteins[47]; furthermore, wheat bran carbohydrates can be utilized for the fermentative production of succinic acid, acetone, butanol, ethanol, amino acids and gamma-aminobutyric acid or for the chemical synthesis of furfural and 5-hydroxymethylfurfural[47].

Due to its many applications and the large amount produced worldwide, wheat bran is an agricultural by-product that need to be valorized more.

1.3 Biocatalysis

Biocatalysis can be defined as the use of a biological entity, an enzyme or a whole cell, for the conversion of a substrate into a product of interest. Humans have unknowingly utilized biocatalytic processes since 6000 BC during fermentation of several types of foods and beverages[58]. From the early 20th century we started to utilize it more consciously, employing specific microorganisms and developing complex biocatalytic processes to produce valuable industrial products[59]. From such a date, the number of biotechnological methods has steadily increased due to ongoing advancements in the field and their applications in various transformation processes. In contemporary industrial applications, the use of biocatalytic processes continues to expand, encompassing a wide range of products, from specialty chemicals to bulk chemicals[59,60].

Another efficient technology widely used at industrial level, is the chemocatalysis, where the catalyst is an inorganic compound instead of a biological entity[61]. In some instances, such as polymer synthesis[62] and petroleum cracking/refining[63], the reaction conditions are not compatible with the use of enzymes making the chemocatalytic approach mandatory. Nonetheless, if an enzyme can catalyze the same reaction of chemocatalyst, these two strategies can compete and the choice between the two depends on the specific application. However, the utilization of biocatalysts instead of an inorganic catalysts offers several technological and environmental benefits:

1. Specificity: enzymes are highly specific, catalyzing regio-, stereo- and/or enantiospecific reactions with minimal or no side-reactions. Their specificity can lead to higher yields and purer products compared to chemocatalysis, which may produce unwanted byproducts, thus requiring a higher number of downstream purification steps[12,60,64,65].
2. Mild reaction conditions: biocatalytic reactions typically occur in aqueous buffers under mild temperature and pH operational conditions. This can reduce the need for harsh chemicals (such as organic solvents and metal catalysts) and high temperature values, making the process more environmentally friendly and energy-efficient. Moreover, this diminishes the generation of toxic byproducts and wastes[12,60,64,65].
3. Sustainable catalyst: biocatalysts are biocompatible, derived from biological renewable sources, and biodegradable, which simplifies disposal and reduces the environmental impact of the process[12,60,64,65].
4. Cost-effectiveness: while the initial development of biocatalysts may be expensive, once established, they can be more step-efficient and cost-effective for large-scale production compared to chemocatalysis[12,60,64,65].
5. Discovery: new enzymes/organisms can be discovered through environmental sampling and genome mining broadening the biocatalytic's toolbox[12,60,64,65].
6. Protein engineering: enzymes selectivity and activity can be modified and/or enhanced through protein engineering, either by rational design or directed evolution approaches. Moreover, biocatalytic organism characteristics can be modified through Adaptive Laboratory Evolution (ALE) and/or metabolic engineering[12,60,64,65].
7. Multi-step catalysis: multi-step enzymatic cascades mimic natural metabolic pathways, enabling one-pot multi-step conversion of compounds that are challenging to produce through traditional chemical methods[12,60,64,65]. This approach combines several enzymes in vitro/in vivo to build biochemical pathways allowing cascades modification of substrates. Multi-enzymatic cascades are a promising approach that try to overcome some flaws of single-enzyme reactions. Indeed, this approach could eliminate the need to isolate intermediates, reducing waste generation, improve the reaction yield and productivity by circumventing possible intermediate inhibition and help streamlining the whole process, resulting in lowered operational costs and reduced energy consumption[66,67]. Noteworthy, multi-enzymatic cascades are particularly cost-efficient when applied to whole-cell biocatalysis, where there is no need to extract and purify every single enzyme of the pathway[64].

Despite these advantages, it is important to note that biocatalysis shows limitations. Generally, biocatalytic processes are less efficient than chemocatalytic ones, have lower productivity and

generate products at lower concentrations. This means that obtaining the same amount of product is more time-consuming and/or bigger reactors are needed, thus using a higher amount of buffer (i.e. aqueous buffer) per amount of product. This is partially due to "obligated" mild reaction conditions making some substrates less reactive and some products less soluble in water; this could be partially solved by biocatalyst engineering. Another disadvantage of using aqueous buffer is the downstream purification of the product. Finally, the cost-effectiveness of biocatalysis is a contentious argument since the cost of developing and optimizing an industrial biocatalytic process is very high. If there are no chemocatalytic alternatives, it could be worth the investment, otherwise their use can be more economically viable for companies[12,60].

Nonetheless, when focusing on the environmental aspect it is clear that biocatalytic processes have the potential to reduce the wastes and toxic/hazardous materials generation. Sheldon and Woodly in their review[12] effectively explained how biocatalysis conforms almost perfectly with the 12 principles of the green chemistry formulated by Anastas and Warner in 1998 in their book "Green Chemistry: Theory and Practice" [68]. Their aim was to outline a framework for making chemocatalysis more sustainable, from the substrates used, the reaction conditions and the products (and by-products) generated. The summarized 12 concept of green chemistry and their connection to biocatalysis are shown in Table 1.

Table 1. Connections between the principles of green chemistry and the biocatalytic approach

	Green chemistry principles	Biocatalysis
1	**WASTE PREVENTION**: It is better to prevent waste than to treat or clean up waste after it has been created	Biocatalytic process produce less amount of dangerous waste
2	**ATOM ECONOMY:** Synthetic methods should be designed to maximize incorporation of all materials used in the process into the final product	Due to the specificity of enzymes, biocatalytic processes are generally more atom-economical
3	**LESS HAZARDOUS CHEMICAL SYNTHESIS:** Wherever practicable, synthetic methods should be designed to use and generate substances that possess little or no toxicity to human health and the environment	Enzymes and biocatalytic organisms generally have low toxicity
4	**DESIGN SAFER CHEMICALS:** Chemical products should be designed to preserve efficacy of function while reducing toxicity	Not relevant
5	**SAFER SOLVENTS AND AUXILIARIES:** The use of auxiliary substances (e.g., solvents, separation agents, etc.) should be made unnecessary wherever possible, and innocuous when used	Biocatalytic reactions are usually performed in aqueous solutions
6	**DESIGN FOR ENERGY EFFICIENCY:** Energy requirements should be recognized for their environmental and economic impacts and should be minimized. Synthetic methods should be conducted at ambient temperature and pressure	The use of milder conditions usually results in higher energy efficiency

7	**USE OF RENEWABLE FEEDSTOCK:** A raw material or feedstock should be renewable rather than depleting whenever technically and economically practicable	Enzymes and biocatalytic organisms are produced from renewable feedstocks
8	**REDUCE DERIVATIZATION:** Unnecessary derivatization (use of blocking groups, protection/deprotection, temporary modification of physical/chemical processes) should be minimized or avoided if possible, because such steps require additional reagents and can generate waste	Biocatalytic process completely avoids derivatization steps
9	**CATALYSIS:** Catalytic reagents (as selective as possible) are superior to stoichiometric reagents	Enzymes and biocatalytic organisms are catalysts
10	**DESIGN FOR DEGRADATION:** Chemical products should be designed so that at the end of their function they break down into innocuous degradation products and do not persist in the environment	Not relevant
11	**REAL-TIME ANALYSIS FOR POLLUTION PREVENTION:** Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances	Easily applicable to biocatalytic processes
12	**INHERENTLY SAFER CHEMISTRY FOR ACCIDENT PREVENTION:** Substances and the form of a substance used in a chemical process should be chosen to minimize the potential for chemical accidents, including releases, explosions, and fires	The use of milder conditions and the low toxicity of biocatalyst results in safer processes

It should be clear that, despite some drawbacks, biocatalysis represents one of the key technologies allowing the transition from a linear economy, focused on production and efficiency, to a circular bio-economy more thoughtful about the environment[12,60]. In this regard, several parameters to evaluate the environmental friendliness of a process have been formulated[12,69]. The most utilized is the **E-factor** defined as the kg waste/kg product: for an ideal process that produces negligible amount of waste per amounts of products the E-factor is equal to zero[12,69]. This parameter has been further refined by adding an **environmental quotient** (EQ) that considers the toxicity and biodegradability of the waste produced[12,69]. Another important tool to evaluate the ecological footprint of a process is the **Life Cycle Assessment** (LCA). This analysis evaluates the environmental effects of a product throughout its entire lifecycle, encompassing stages from the extraction of raw materials to materials processing, distribution, usage, and possible disposal or recycling[12,69]. Despite these parameters and evaluation are useful to have a better understanding of the ecological footprint of a process, these are not sufficient to assess the economical sustainability of a process[12]. Noteworthy, biocatalysis is not only an approach opposed to chemocatalysis, but biocatalysis is a complex field offering several different strategies. In particular, it can be subdivided into 3 main branches: i) isolated enzymes (Figure 6A), ii) resting cells (figure 6B) and iii) fermentation (Figure 6C).

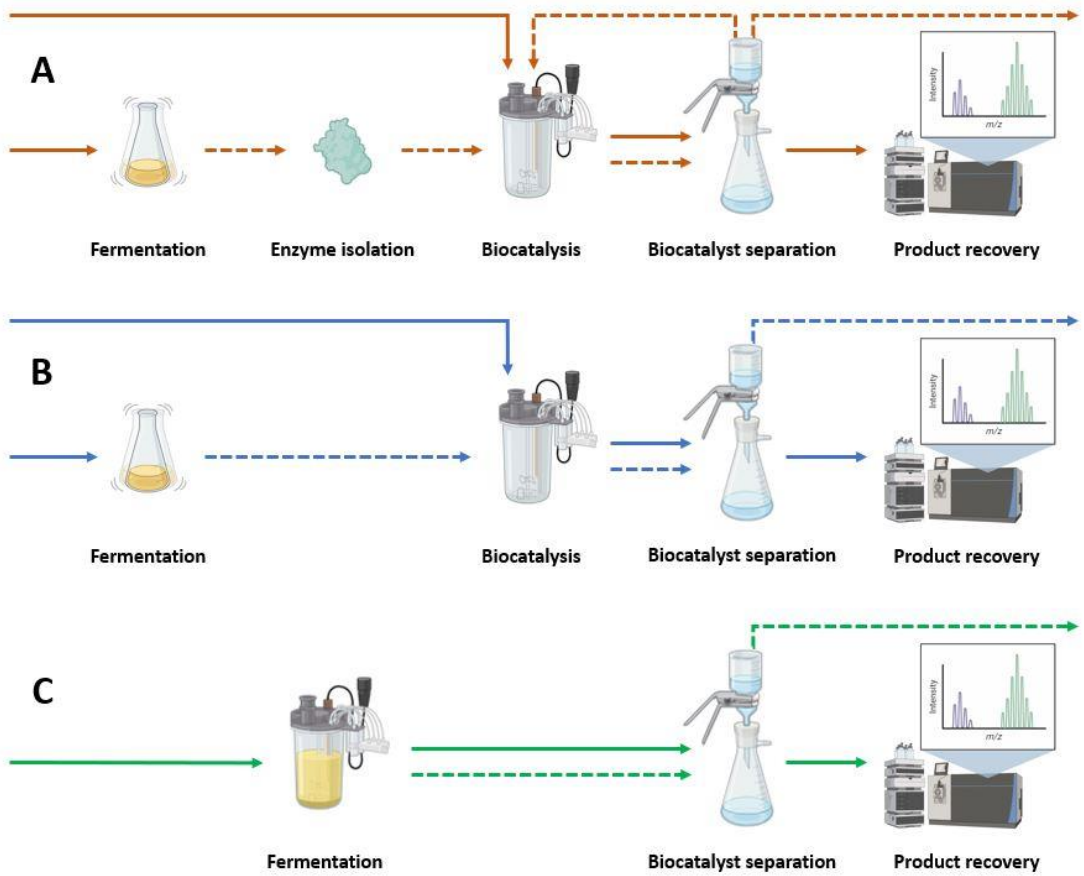

Figure 6. Biocatalytic branches flowsheets. A) Isolated enzyme biocatalytic approach, B) resting cells biocatalytic approach, and C) fermentative biocatalytic approach. Solid lines indicate the streams of substrate/product. Dotted lines indicate biocatalyst streams.

These branches can be then grouped into cell-free approaches (i.e. isolated enzymes) and whole-cell approaches (i.e. resting cells and fermentation).

In the cell-free biocatalysis, the cellular components, particularly the enzymes, are extracted from the cells and utilized outside the cells. After the extraction, the crude cell lysate can be utilized for the conversion, or the enzymes can be further purified from the other cellular components before being used[70]. The cell-free approach shows advantages over the use of a whole-cell system:

1. Cell-free systems are less complex and more controllable, making them suitable for well-defined reactions and research applications. They are less likely to have unwanted side reactions or interference from cellular components. Moreover, due to the simplicity of the reaction system, the promiscuous activity of certain enzymes can be exploited to utilize non-natural substrates[12,70].
2. Cell-free systems can be customized by selecting specific enzymes or cofactors for a particular reaction, allowing for greater control over the reaction conditions and product formation. In some cases, even non-natural operational conditions can be utilized to improve the activity on certain substrates (e.g., higher temperature, harsh pH, use of solvents)[12,70].

3. Cell-free systems are amenable to optimization through adjustments to reaction conditions and the addition of specific enzymes, making them more suitable for fine-tuning reaction parameters. The lower number of components and the absence of side reactions allow an easier parameterization of the reaction system, making it more suitable for in silico optimization[12,70,71].
4. In cell-free systems, it is possible to work in presence of enzyme and substrate concentrations not obtainable *in vivo*, thus increasing the yield, titer and productivity of the whole system[12,70].
5. Lastly, some substrate can not be internalized in cells, due to steric hindrance and/or not having a specific transporter. Use of isolated enzymes allows the bioconversion of these substrates without the need of time-consuming engineering of a microbial strain[12,70].

In whole-cell biocatalysis, the microbial host (bacteria, yeast, etc.) is used as the catalyst. The cell contains all the necessary machinery, including enzymes and cofactors, to perform the desired biochemical reactions[64]. These whole-cell biocatalysts can be natural (i.e. isolated form the environment) or genetically engineered to produce certain compounds[64]. The whole-cell approach shows advantages over a cell-free system:

1. Thanks to cellular mechanisms, whole-cell systems are more robust and stable against perturbations, providing a protective environment for enzymes. This enzyme's compartmentalization can potentially prolongs their activity and lifespan, obtaining a more durable biocatalyst[12,60,64].
2. Whole-cell systems are cheaper to produce and maintain, avoiding the expensive and time-consuming step needed for the extraction and purification of enzymes. Moreover, the endogenous metabolism of the cells can be exploited to recycle the cofactor needed in the biocatalytic reaction without additional costs[12,60,64].
3. Whole-cell systems often have membrane transport systems that can facilitate the uptake of substrates, increasing the local concentration inside the cells, and excretion of products, reducing issues related to product inhibition[12,60,64].
4. Whole-cell systems are more easily separated from the products of the biocatalysis, either by precipitation or filtration, thus reducing the cost of downstream processing[12,60,64].
5. Multi-enzymatic cascades are cheaper to set up in whole cell systems, allowing to perform complex multistep reactions or build metabolic pathways without the need to express and purify every single enzyme of the pathway. This makes them well-suited for applications requiring the conversion of substrates through various intermediates to produce a desired product without the need of intermediate steps of purification[12,60,64].

The choice between a whole-cell and a cell-free biocatalytic approach depends on the specific application, the complexity of the desired reaction, and the need for control and customization. Whole-cell systems are often preferred for robustness and versatility, while cell-free systems offer more precision and customization[12,60].

There is another possible subdivision that can be made between the three aforementioned branches of biocatalysis: the growth-associated biocatalysis (fermentation) and the growth-separated biocatalysis (isolated enzymes and resting cells)[12]. As the name suggests, in growth-associated biocatalysis, the growth of the biocatalyst and the bioconversion are simultaneous. The conversion is carried out in the same medium used for growth and the substrate can be some form of nutrients for the organism. In this condition, the metabolism of the whole biocatalyst is highly active[72], allowing the efficient recycling of cofactors, and the cost of the process is minimized by reducing the required steps[64,73]. Meanwhile, in the growth-separated biocatalysis, the preparation and the utilization of the biocatalyst are separated steps. The major advantages of this approach is the degree of freedom in the choice of operational reaction conditions (e.g., temperature, pH , reaction media, organic solvent addition) and in the concentration of the biocatalyst, thus productivity (space-time yield) can be substantially enhanced compared to that of a fermentation[12]. Moreover, the use of less complex reaction media allow a cheaper and less time-consuming downstream purification[12,64,70].

The purification step can be simplified further by immobilizing the biocatalyst on a solid support. The **immobilization** is typically achieved on a solid sphere or, more commonly, within a porous support, making it readily removable from the reaction system through filtration. The immobilization also makes biocatalyst reutilization easier and enhances its stability by blocking it into a more robust conformation[74], thus making the biocatalyst more productive (kg product/kg enzyme)[74]. The increased rigidity of the enzymes' structures generally causes a decrease in their catalytic properties, but, in some cases, the structural distortions caused by the immobilization can be exploited to alter the catalytic features of an enzyme[75]. In the case of a cofactor-dependent enzyme, an interesting approach is the co-immobilization of the biocatalytic enzyme and the respective cofactor or of a cofactor regenerating enzymatic system. The proximity between the enzyme and the cofactor can increase its catalytic efficiency and remove the need for cofactor addition, making it a self-sufficient heterologous biocatalyst[76]. As an example, the covalent binding of α-chymotrypsin to nanoporous silica improved the half-life of the enzyme at 40 °C up to 1000-fold and increase its activity in organic solvent up to 100-fold: in particular, it retains 35% of its activity after 2 h of incubation in methanol[77]. Furthermore, biocatalyst immobilization allowed their use in continuous-mode reactors, the so-called flow biocatalysis approach. By following this

approach it is possible to increase the lifespan of the biocatalyst, improve productivity and reduce waste[78]. Futhermore, flow biocatalysis offers two peculiar advantages over the batch counterpart: i) telescoped reactions and ii) automation. In telescoped reactions, the different steps of a multi-enzymatic cascade can be carried out in different bioreactors sequentially; in this way a substrate can be converted by the corresponding enzyme in a reactor, the product can be then funneled in a second reactor to be converted by a second enzyme and so on. This approach reduces inhibition problems, and the needs of intermediates' purification and allows to work under the optimized operational conditions of every enzyme involved in the biosynthetic pathway[78]. Finally, due to the modular nature of a flow reactor, it is easier to set up automated procedures that allow for cheaper and faster biocatalytic processes[79]; the two advantages can even be combined for the development of an automated multistep synthesis in flow[80]. A flow biocatalytic approach has been implemented using an immobilized amine transaminase from *Halomonas elongata* achieving a reduction of reaction time of up to 2-fold and an increase in productivity compared to the batch approach[81].

In conclusion, biocatalysis has the potential to be one of the key technologies enabling the establishment of circular bioeconomy.

1.4 Strain improvement

Enzymes are the primary component of any biocatalytic system. In the isolated enzymes approach, enzymes are the sole component of the system; in contrast, in the whole-cell biocatalysis approach they are employed within microbial chassis. This chassis possesses its own metabolic pathways, that compete with the biocatalytic enzyme for the same cellular machinery and cofactors. This competition for resources can influence the bioconversion capabilities of the biocatalyst, and vice versa, the biocatalytic reaction/pathway can have an effect on the homeostasis of the cell[82–84]. In fact, exogenous proteins overexpression is known to be a stress to the cellular environment, resulting in a reduced fitness of the microbial strain[85]. Furthermore, even an accumulation of the substrate, intermediate(s) or final product can generate stresses for the microbial chassis, reducing the overall productivity of the biocatalytic system[86]. Thus, to improve the production of the target compound, a balance between the endogenous metabolism and the expression of biocatalytic enzymes needs to be found; in theory, this balance is obtained by finding the catalytic amount of enzyme needed to convert the substrate into the desired product at a sufficient rate[85]. This field is called metabolic pathway balancing or metabolic engineering[84,87] and try to find the aforementioned balance through different approaches (Figure 7).

The most straightforward method is to control enzyme's expression at DNA-level, transcription-level and/or translation-level. Starting from DNA-level control, the easier and most utilized

method adopted in this field, the enzyme's expression can be modulated by changing the gene copy number[84]. If the gene of interest is chromosomally integrated, decreasing the copies of the gene of interest in the genome will lead to a reduced expression. Instead, when using plasmid vectors it possible to minimize the metabolic burden on the microbial chassis by diminishing the expression of the gene of interest using a low copy number plasmid[85]. Low copy number plasmids not only help establishing the balance between biocatalysis and endogenous metabolism but can also ensure the segregational stability and a consistent copy number throughout the microbial culture[85]. Transcription-level control is naturally imposed by promoters, that can have different strength and can be constitutive or inducible[84,85]. Despite numerous promoters are found in Nature, the discovery and characterization of new promoters is difficult[88] and the ones already utilized and studied have high strength, meaning they induce the overexpression of the target gene generating stress to the microbial host[85]. To solve this issue, several techniques have been utilized to engineer known promoter sequences in order to produce libraries of promoters with variable strength, by: i) error-prone PCR[88,89]; ii) saturation mutagenesis of the spacer sequence (regions surrounding the consensus regions, i.e. -35 and -10 for bacteria)[88,89]; iii) hybrid promoter engineering to modify promoter regulation and strength by combining consensus and spacer regions of different promoters[88,89]; iv) site-directed mutagenesis of the transcription factor binding site, to modulate the strength and/or regulation of the promoter[88,89]. Another interesting technique that could help in balancing the expression of the biocatalytic enzymes is the so-called "dynamic balancing", where the expression of the pathway enzymes can be up-regulated or down-regulated under the control of a genetic circuit sensible to one or more cellular metabolite(s)[84]; as an example, by creating a fatty acid-sensitive sensor regulator system utilizing the transcription regulator fadR, the production of fatty acid ethyl ester in *E. coli* was improved by almost 3-fold, from 9.4% to 28% theoretical maximum[90]. Finally, transcription-level control can be imposed by modifying the ribosome binding site (RBS) sequences. In this case, the correlation between RBS sequence and RBS strength is well understood: a digital RBS calculator tool was developed and optimized, thus it is now possible to build libraries of RBS with various strength[84]. RBS engineering has been utilized in order to optimize pathway's enzymes expression for the production of fatty acids in *E. coli*: the better expression balance allowed to reach a 46% fatty acids production increase[91].

In some cases, particularly when using xenobiotics, the limiting steps of a biocatalytic pathway can be represented by the import of the substrate and/or the export of the product. If the substrate accumulates in the extracellular medium due to low intracellular transport, it can be degraded or subjected to side-reactions; similarly, if the product accumulates in the cytosol, it can have cytotoxic effects (e.g., osmotic stress) and or inhibit biocatalytic enzymes activity, reducing the overall

product's yield[92]. The transporter active on the substrate/product can be identified through a transcriptomic and gene knock-out analysis, so that the transporter expression could then be enhanced by genome editing or recombinant expression. Moreover, if the selected microbial chassis of choice does not possess an efficient transport mechanism, it is possible to improve it by protein engineering methods[92,93]. To improve the production of succinate in *E. coli*, the expression of the succinate transporter genes *dcuC* and *dcuD* was modulated using RBS engineering, obtaining 34% improved succinate production compared to the parental strain[94].

One of the more grueling tasks in metabolic engineering is identifying the bottlenecks of a bioconversion system, since there are several factors that can hinder the optimal metabolic flux. This task can be handled by -omics approaches, such as transcriptomics[95] and metabolomics[96], that enable extensive studies of cellular regulation and metabolic networks. From these data it is possible to build a Genome-scale Metabolic Model (GEM)[87,97] of a particular organism, which can then be used to perform **Flux Balance Analysis** (FBA)[87,98] to identify possible bottlenecks.

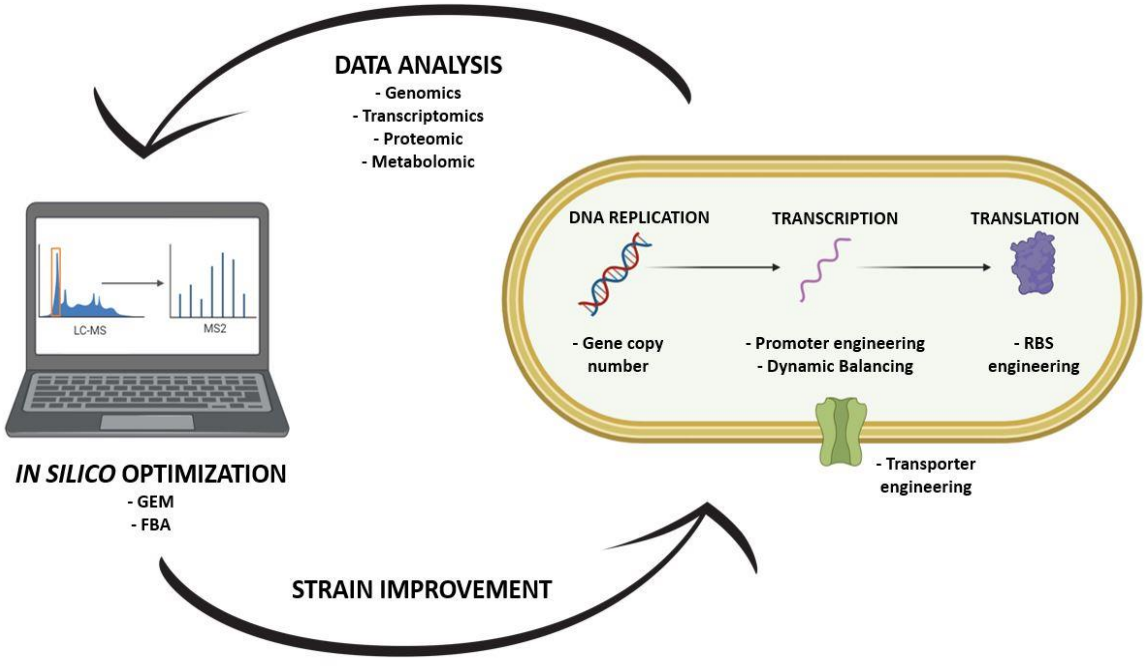

Figure 7. Metabolic engineering process scheme. Metabolic engineering is an iterative process composed of 3 major stages: i) data analysis, ii) *in silico* optimization and iii) strain improvement. After the first iteration of this process, data obtained from the improved strain starting a new cycle to further improve strain characteristics.

In details, FBA is a computational technique used to analyze and predict the flow of metabolites through a metabolic network, it operates on the principles of mass conservation and aims to find a set of metabolic fluxes (the rates of chemical reactions) that achieve specific objectives while satisfying constraints on the overall system. FBA has been instrumental in metabolic engineering, the study of microbial physiology, and the design of bioengineering processes. It is particularly

useful for modeling and optimizing the metabolic processes in microorganisms, such as bacteria and yeasts, as well as in other biological systems[98]. Thereafter, the limiting steps can be optimized by gene knock-out or knock-in, or by recombinant expression of heterologous proteins[99]. The major drawback of in silico simulation of biological systems is the great degree of uncertainty due to their innate complexity and adaptiveness[85]. The development and optimization of a biocatalytic strain require a deep knowledge of the physiology, genetics and biochemistry of the host microbe. Despite the recent advancements in this field, there is still a lot of work to be done in order to fully understand and develop the optimal biocatalytic strain.

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catalysts

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Review

Lignin Valorization: Production of High Value-Added Compounds by Engineered Microorganisms

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Abstract: Lignin is the second most abundant polymer in nature, which is also widely generated during biomass fractionation in lignocellulose biorefineries. At present, most of technical lignin is simply burnt for energy supply although it represents the richest natural source of aromatics, and thus it is a promising feedstock for generation of value-added compounds. Lignin is heterogeneous in composition and recalcitrant to degradation, with this substantially hampering its use. Notably, microbes have evolved particular enzymes and specialized metabolic pathways to degrade this polymer and metabolize its various aromatic components. In recent years, novel pathways have been designed allowing to establish engineered microbial cell factories able to efficiently funnel the lignin degradation products into few metabolic intermediates, representing suitable starting points for the synthesis of a variety of valuable molecules. This review focuses on recent success cases (at the laboratory/pilot scale) based on systems metabolic engineering studies aimed at generating value-added and specialty chemicals, with much emphasis on the production of *cis*,*cis*-muconic acid, a building block of recognized industrial value for the synthesis of plastic materials. The upgrade of this global waste stream promises a sustainable product portfolio, which will become an industrial reality when economic issues related to process scale up will be tackled.

Keywords: lignin valorization; biocatalysis; *cis*,*cis*-muconic acid production; aromatic compounds; value-added compounds; metabolic engineering; platform chemical; microbial cell factory

Citation: Rosini, E.; Molinari, F.; Miani, D.; Pollegioni, L. Lignin Valorization: Production of High Value-Added Compounds by Engineered Microorganisms. *Catalysts* **2023**, *13*, 555. https://doi.org/10.3390/catal13030555

Academic Editor: Roberto Fernandez-Lafuente

Received: 15 February 2023
Revised: 3 March 2023
Accepted: 7 March 2023
Published: 9 March 2023

1. Introduction

Lignin, the second most abundant polymer on Earth, is a main component of lignocellulose in plants. Lignin is a complex polymer assembled from aromatic building blocks, which represents the most underutilized renewable. About 300 billion tons of lignin are available in the biosphere, thus providing a huge resource for sustainable processes [1]. Lignin is also largely produced by various production systems: in biorefinery plants, when one liter of cellulosic ethanol is produced, about one kilogram of lignin is also generated. Lignin is a main by-product of the paper and pulp industry. For example, Kraft pulping generates about 130 million tons of lignin per year [2,3]. At present, most lignin is burnt for energy supply or discarded into the environment: partial valorization of lignin to target chemicals represents a main environmental benefit [4].

Lignin is mainly composed of three phenylpropanoid units: the monolignols coniferyl alcohol (G), sinapyl alcohol (S) and *p*-coumaryl alcohol (H). About 50–80% of all interunit bonds are β-O-4 ether bonds and α-O-4, β-5, β-β and 5–5 linkages and biphenyl and diaryl ether structures are also present (Figure 1) [5]. Lignin content and composition strongly depend on the plant source [6]. Because of its heterogeneous character, lignin depolymerization is a difficult task that generates a wide range of aromatic compounds.

Several excellent reviews reported about catalytic and biological methods for lignin depolymerization [5,7–10]. As a general rule, biological systems seem better suited to handling heterogeneous mixtures than physico-chemical methods: the lignin-degrading

microbes utilize funneling pathways to channel multiple monolignols into key intermediates [11], thus allowing the production of a platform of chemicals. These processes guarantee sustainable green alternatives to fossil routes and also enhance the efficiency of the existing second generation biorefineries. For a comparison of chemical and biological methods for lignin depolymerization, see Table 1.

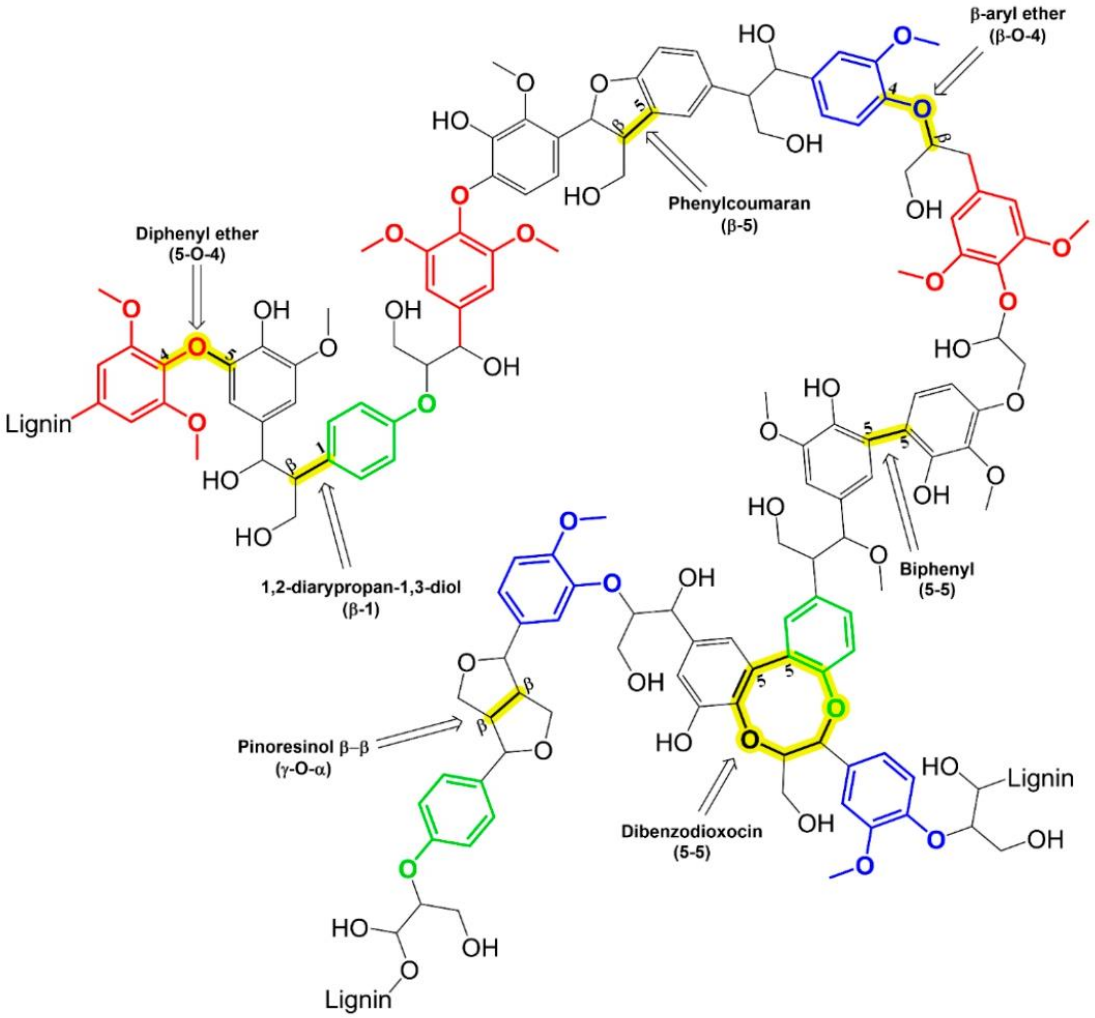

Figure 1. Schematic representation of the lignin structure. The figure shows the three main moieties: *p*-hydrophenyl (H) (green), guaiacyl (G) (blue) and syringyl (S) (red). The most frequent bonds are highlighted in yellow.

White-rot and brown-rot fungi have long been considered the only lignin-metabolizing organisms [12,13]. However, a number of investigations reported on bacteria as lignin-degrading organisms [13], especially the ones belonging to phyla *Proteobacteria*, *Actinobacteria*, and *Firmicutes*, as well as members of the archaeal phylum *Bathyarchaeota* [13–16]. Biological lignin depolymerization is mainly due to extracellular oxidases, such as lignin peroxidase, manganese peroxidase, versatile peroxidase, dye-decolorizing peroxidase, and

laccase [17–20]. Once simple aromatics are released from lignin breakdown, additional microbes are allowed to participate. Notably, aromatics from G-type and H-type lignin are funneled only into protocatechuate and catechol, whereas S-type lignin-derived aromatics degradation occurs via a non-interacting branch (Figure 2) [21–23]. Following lignin depolymerization, aromatics catabolism and ring cleavage, the carbon of these compounds ultimately integrates into tricarboxylic acid cycle.

Table 1. A brief overview of chemical and biological lignin depolymerization methods.

Methods	Advantages	Limitations
Chemical treatments		
Acid catalysts Base catalysts Metallic catalysts Ionic liquids assisted catalysis Supercritical fluids assisted catalysis Oxidative catalysis	Effective lignin degradation High stability Low substrate/product inhibition effects	Low selectivity Environmental concern Costly Difficult recovery of products from the mixture Harsh operational conditions
Biological treatments		
Bacteria Fungi In vitro enzymatic depolymerization	Mild operational conditions Environmentally friendly Optimized by metabolic engineering Optimized by protein engineering Funneling pathways to key intermediates High selectivity and catalytic efficiency	Low conversion yield Long culture/biotransformation time Substrate/product inhibition Difficult to scale up

Biological conversion of lignin is still hampered by the lack of details about the related catabolism pathways and the low conversion ability of microbes. Furthermore, the crude mixture of recovered and pretreated lignin could contain toxic aromatics and inhibiting substances (e.g., sulphite), also showing extreme pH values. Multiple approaches can be relevant in generating a versatile system able to employ a variety of aromatics. Among these approaches are: (i) the search for additional suitable microbial strains; (ii) the determination and recognition of additional lignin degradation pathways; (iii) the synergy between lignin-degrading microbes and novel enzymes: this approach strongly depends on the design/engineering of enzymes with selected properties, e.g., enzymes with improved tolerance, enhanced substrate and cofactor specificity [24,25]; (iv) the use of systems biology to shed light on the metabolic pathways and degradation mechanisms of lignin/aromatics in microorganisms; (v) the systems biology-guided engineering of lignin-degrading microbial strains to build useful strains for utilizing and converting lignin into specific chemicals. The technology platform for targeted and customized strain engineering is quickly growing by incorporating additional elements from synthetic biology, such as libraries of synthetic promoters, ribosomal binding sites and bicistronic elements to allow tunable expression. Furthermore, novel molecular biology toolboxes for DNA synthesis and assembly, as well as targeted and multiplex genome editing allow fast and throughput genetic modifications.

Since the processing of lignin strongly depends on the plant biomass source and the isolation method used, a flexible lignin-degrading strain/approach needs to be generated. Beyond the cellular engineering of existing living organisms, the number of reports focusing on the design, creation and application of synthetic cells are rapidly increasing [26]: such systems aim at the modular programming of genetic circuits and/or synthetic genomes towards a new era of biotechnology.

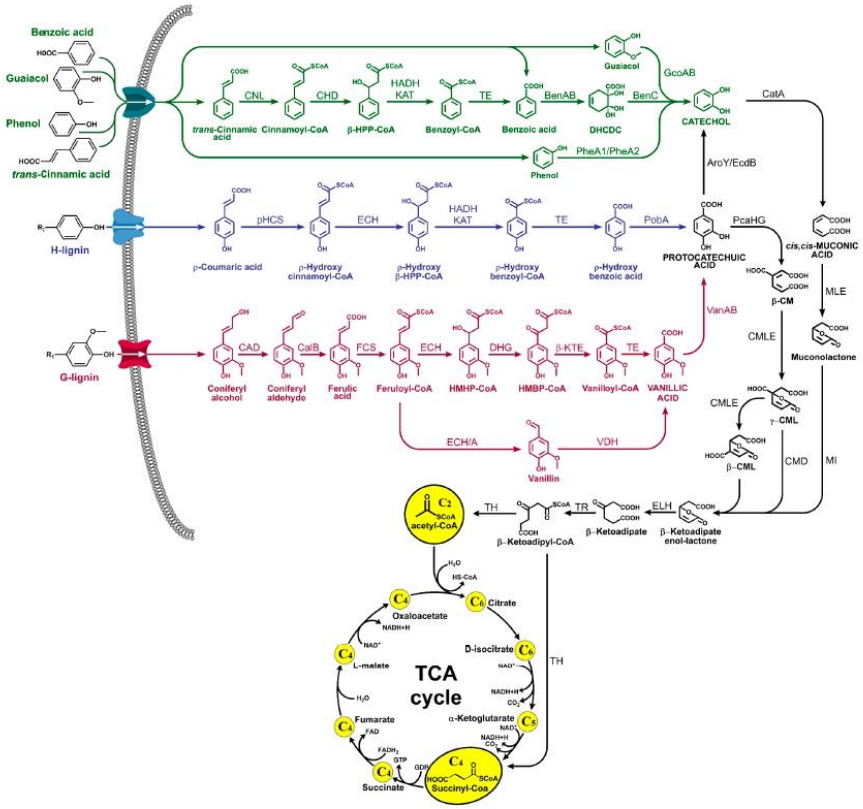

Figure 2. Scheme of the main metabolic pathways involved in the degradation of the aromatic monomers derived from lignin depolymerization. Enzymes involved in the degradation of *trans*-cinnamate, phenol, guaiacol and benzoate (green branch): CNL, cinnamoyl-CoA ligase; CHD, cinnamoyl-CoA hydratase/dehydrogenase; BenA, benzoate 1,2-dioxygenase subunit A; BenB, benzoate 1,2-dioxygenase subunit B; BenC, benzoate 1,2-dioxygenase ferredoxin reductase; BenD, 3,5-cyclohexadiene-1,2-diol-1-carboxylate dehydrogenase; PheA1, large subunit phenol hydroxylase; PheA2, small subunit phenol hydroxylase; GcoA, cytochrome P450; GcoB, cytochrome P450 reductase. Enzymes involved in the conversion of *cis,cis*-muconic acid (black branch): CatA, catechol 1,2-dioxygenase; MLE, *cis,cis*-muconate lactonase; MI, muconolactone isomerase; PcaHG, protocatechuate 3,4-dioxygenase; CMLE, β-carboxy-*cis,cis*-muconate lactonase; CMD, γ-carboxy-muconolactone decarboxylase; ELH, β-ketoadipate enol-lactone hydrolase; TR, β-ketoadipate succinyl-CoA transferase; TH, β-ketoadipyl-CoA thiolase. Enzymes involved in the degradation of H-lignin monomers (blue branch): pHCS, *p*-hydroxycinnamoyl-CoA synthetase; HADH, 3-hydroxyacyl-CoA dehydrogenase; KAT, 3-ketoacyl-CoA thiolase; PobA, *p*-hydroxybenzoate hydroxylase. Enzymes involved in the degradation of G-lignin monomers (red branch): CAD, coniferyl alcohol dehydrogenase; CalB, conyferyl aldehyde dehydrogenase; FCS, feruloyl-CoA synthetase; ECH, enoyl-CoA hydratase; DHG, dehydrogenase; β-KTE, β-keto thiolase; TE, thiolase; ECH/A, enoyl-CoA-hydratase/aldolase; VDH, vanillin dehydrogenase; VanAB, vanillate demethylase A + vanillate *O*-demethylase oxidoreductase; AroY, protocatechuate decarboxylase; EcdB, Ubix family prenyltransferase; HMHP-CoA, 3-(4-hydroxy-3-methoxyphenyl)-3-hydroxypropanoic acid; HMBP-CoA, 4-hydroxy-3-methoxyphenyl-β-ketopropionyl-CoA; β-HPP-CoA, β-hydroxy propionyl-CoA; DHCDC, 1,2-dihydroxy-cyclohexa-3,5-diene-1-carboxylate; β-CM, β-carboxymuconate; β-CML, β-carboxymuconolactone; γ-CML, γ-carboxymuconolactone.

This review highlights recent advances in the field of systems metabolic engineering aimed at exploring the possible conversion of lignin-derived monomers into value-added products, placing much emphasis on the potential practical production of the plastics precursor *cis*,*cis*-muconic acid and of pharmaceutical precursors. The critical review of recent developments of novel biological conversion processes (focusing on their advantages and drawbacks) represents a comprehensive proof of concepts for great potentiality of renewable biomasses-derived aromatics.

Making the whole process of lignin conversion into high-value compounds cost-effective at industrial scale requires the improvement of productivity/yield of the target products. Therefore, the studies discussed in this review, concerning the use of metabolic engineering and systems biology, emphasize that the biological valorization of lignin components into value-added bioproducts is expected to generate considerable market growth and to make the lignin valorization process profitable and sustainable in the near future.

2. Microbial Lignin Depolymerization

Besides its complexity and recalcitrance to degradation, the main problem in industrial lignin valorization is that it is still considered more a problem than a valuable and unexploited resource. All current industrial applications are experimental, pioneering and mainly focused on lignin elimination from the main product mixture. Different strains of white-rot, brown-rot and soft-rot fungi were tested and experimentally used in the bio-pulping process to prepare different vegetal fibrous materials for subsequent chemical and physical treatments in the paper industry. The main drawback is that existing processes still do not match the strict industrial requirements: in most cases the overall process takes days or even months to achieve a suitable degree of delignification [27]. However, in the last decade, several projects not related to bio-pulping were aimed at obtaining valuable chemicals or surfactants directly from lignin [28,29] or lignin-related material, such as advanced biopolymers [30,31].

The first step in lignin microbial degradation is its rough depolymerization performed by extracellular enzymes like peroxidases and laccases. These enzymes cleave unselectively different bonds in lignin, yielding different dimers and monomers. These molecules are small enough to be internalized and funneled through different metabolic pathways (Figure 2).

Both free enzymes and cell fermentation-driven lignin depolymerization suffer the same drawbacks. First of all, enzymatic production is often quantitatively inadequate for industrial purposes: these enzymes are naturally mildly active and expressed at low levels, thus providing limited substrate amounts to sustain the microorganism metabolism. In addition, under ideal operating conditions, wild-type ligninolytic enzymes reach just a fraction of the 10,000 turnovers (moles of product/moles of enzyme) conventionally required as the minimum to economically sustain an industrial bioprocess [32]. Therefore, under harsh pH and temperature conditions and in presence of solvents, the activity of these enzymes decreases very quickly. Moreover, many of these enzymes are subjected to self-deactivation due to heme destruction, suicide inactivation (excess of H_2O_2) and formation of amino acid-based free radicals, yielding a decrease in enzyme reduction potential.

2.1. Microbial Consortia

In nature, lignin depolymerization is often performed by a concert of different organisms, rather than a single one. This usually allows each organism to overcome the deficiency of the others, yielding a quite efficient degradation process. Therefore, one practical strategy to mimic what happens in nature is based on assembling microorganism consortia which may provide a better and faster process. The main problem of this approach is that many microorganisms are still not easily culturable in the laboratory and must be studied using culture-independent strategies, such as metagenomics analyses.

A green fluorescence protein (GFP)-based biosensor was used to detect vanillin and syringaldehyde, which are typical products of lignin metabolism, in high-throughput screening [33]. A total of 147 clones from coal beds were identified for their ability to grow on hardwood and Kraft lignin-enriched media, mostly coming from uncultivable archaea and bacteria. Later on, the same method was used from the aforementioned coal bed sites to identify aromatic transaminases that were able to use lignin-derived monoaromatics producing value-added feedstocks for pharmaceutical synthesis. A novel transaminase, able to aminate as many as 14 monoaromatic lignin-related aldehydes and ketones, was found and characterized [34].

The microbial communities involved in lignin degradation from moderately thermophilic environments such as hot spring sediment (52 °C) and woody "hog fuel" piles (53–62 °C) were studied by a mixed strategy using ^{13}C-ring-labeled synthetic lignin as substrate and monitoring both released $^{13}CO_2$, as a degree of lignin mineralization, and isotopic enrichment of DNA, to identify the genome(s) belonging to lignin-degrading organism(s) [35]. A total of 14 out of 125 draft genomes was enriched in ^{13}C and contained several novel putative laccase-like multi-copper oxidase (LMCO) encoding genes.

Consortia can also be modified and "optimized". To obtain a minimal and effective lignocellulolytic microbial consortium (MELMC), a dilution-to-stimulation/extinction method was implemented using samples from the Andean Forest soil cultured with a mixture of three agricultural wastes. This "dilution-to-stimulation" enriched and stabilized approximately 50 bacterial strains ascribable to the lignocellulolytic component of the original microbial population. Then, this enriched media was inoculated in serial dilutions of the substrate until 10^{-11}, where only the *Pseudomonas* sp. and *Paenibacillus* sp. bacterial species were highly abundant (>99%) [36].

One of the most tricky approaches to consortia is the creation of an artificial one. Consortia can be made up in two ways: (i) top-down, starting from natural consortia and through their characterization and optimization; (ii) bottom-up, trying to establish synergic relationships between different microorganisms not necessarily already involved in a consortium. A valuable example is the method provided by Hu et al. where possible microbial interactions were tested by screening as many microorganisms as possible [37]: several lignocellulosic strains of fungi were tested on a minimal media and the best synergic ligninolytic activities were identified and measured. Then, a screened community enriched in lignin-degrading bacteria was introduced to develop synergic activities with the already-formed fungal consortia. As a result, two fungal (*Trichoderma* and *Aspergillus*) and 16 bacterial strains (from *Bacillus*, *Enterococcus*, *Lactococcus*, *Acinetobacter*, and *Pseudomonas* species) were identified as members of a synergistic microbial consortium showing an improved activity up to 197% [38].

2.2. Extremophilic Microorganisms

The enzymes from extremophiles could represent a suitable alternative in lignin depolymerization to mesophilic bacteria because they already operate in harsh conditions. *Thermobifida fusca* (growing at 45–60 °C) [39] and *Clostridium thermocellum* (growing at 60 °C) [40] are mildly thermophilic bacteria which show or express thermophilic enzymes with detectable lignin depolymerization activity. At higher temperatures, fewer bacteria are able to use lignin. As an example, *Archaeoglobus fulgidus* (growing at 80 °C) is suspected to use lignin-derived substrates based on a recently discovered methoxydotrophic metabolism, which acts on methoxylated aromatic molecules [41] quite common in lignin structure. Similarly, a laccase from the hyperthermophilic *Thermus thermophilus* strain showed an optimum of temperature at 90 °C [42].

On the other hand, several microorganisms show a remarkable lignin degradation activity at low temperatures, this offering advantages for biotechnological processes and for low-energy treatments. Several psychrophilic microorganisms were identified from different environments, both fungi such as *Rhodosporidiobolus colostri* from the alpine forest (1–25 °C) [43] and various Antarctic filamentous fungi from King George Island in

Antarctica (growing at 1–15 °C) [44], and bacteria, such as *Arthrobacter* sp. from Chinese Manchuria (growing at 15 °C) [45] and *Burkholderia* sp. from Alaskan tundra soil (growing at 15 °C) [46]. Recently, from the marine *Halomonas* sp. strain M68 isolated from marine biofilm and water samples collected in Terra Nova Bay (Antarctica), an intracellular thermo- and halo-tolerant laccase-like activity, with an optimal temperature in the 40–50 °C range, was isolated [47].

Halophiles bacteria are quite interesting due to the uncommon resistance to high saline levels, a feature usually linked to remarkable tolerance to UV light radiations, temperature, and extreme pH values. Actually, *Penicillium chrysogenum*, isolated in saline soil, has the capability to depolymerize lignin directly on olive milled substrate from oil production [48]; meanwhile, several halophilic ligninolytic enzymes were isolated and tested on raw substrates such as sugar beet pulp [49], almond shells [50] or peanut shells [51]. All these microorganisms, and related enzymes, were tested successfully on such substrates with minimal or no pre-treatment.

Significantly, several laccases were identified as solvent-resistant [19,52]. Besides only a few of them that were tested and characterized, both fungi [53] and bacteria [54] proved to be a promising source of solvent-tolerant enzymes, particularly useful to treat high-molecular weight lignin polymers, usually scarcely soluble in aqueous media [13].

3. Lignin Valorization: The Biological Routes towards Value-Added and Specialty Chemicals

Microorganisms show the ability to convert lignin, as well as lignin-derived compounds, into several value-added molecules, see Table 2 [55–62]. Regardless, due to the mentioned heterogeneity of lignin, its depolymerization results in the production of a number of monomeric aromatic compounds such as vanillin, guaiacol, catechol and protocatechuic acid (PCA) (Figure 2): their funneling into targeted chemicals of industrial value (e.g., bio-based plastic precursors as *cis*,*cis*-muconic acid, ccMA), is crucial for an efficient biomass valorization. The biological route approach to lignin valorization is very promising, mainly due to the abundant biochemical pathways present in nature [63,64], or assembled in the laboratory [65,66].

Table 2. Value-added compounds obtained from lignin or lignin-derived aromatics by microbial degradation pathways.

Product	Host	Starting Material	Yield	Reference
Coniferyl alcohol	*Amycolatopsis* sp. HR167 pRLE6SK*vaom*	Eugenol	4.7 g/L	[55]
Ferulate	*E. coli* XL-1 Blue pSK*vaomPcalAmcalB*	Eugenol	14.7 g/L	[56]
Lactate	*P. putida* KT2440-CJ127 *P. putida* KT2440-CJ124	Benzoate *p*-Coumarate	0.5 g/L 0.5 g/L	[57] [57]
PCA	*P. putida* KT2440	Ethanol-assisted depolymerized lignin	6.7 mg/L	[58]
Pyruvate	*P. putida* KT2440-CJ112 *P. putida* KT2440-CJ116	Benzoate *p*-Coumarate	0.7 g/L 2 g/L	[57] [57]
Succinate	*Phanerochaete chrysosporium*	Lignin	20 mg/L	[59]
Vanillin	*R. jostii* RHA045 *Shewanella putrefaciens* *Pseudomonas* sp. HR199 Δ*vdh* *E. coli* pSK*echE*/*Hfcs* *Streptomyces setonii*	Wheat straw lignocellulose Lignin from wheat straw Eugenol Ferulate Ferulate	96 mg/L 275 mg/L 400 mg/L 300 mg/L 6.4 g/L	[60] [61] [62] [56] [67]

The most investigated metabolic routes employ upper pathways to drive aromatic compounds into the β-ketoadipate pathway via the catechol node, a central hub of aromatic catabolism ("biological funneling approach"): many aromatic-catabolizing microorganisms employ a catechol-1,2-dioxygenase (CatA) enzyme to open the catechol ring to produce

ccMA [23]. Moreover, different aromatics from guaiacyl- and *p*-hydroxyphenyl-units of lignin are catabolized into PCA and further metabolized into ccMA (see Figure 2).

3.1. Cis,cis-Muconic Acid

ccMA is a dicarboxylic acid of recognized industrial value [68,69]: it represents a potential platform chemical for the synthesis of a variety of polymers (polyamides, polyethylenes, and polyurethanes) [70–72]. ccMA can be easily hydrogenated into adipic acid, a building block of commercial nylons and polyurethanes, or converted into terephtalic acid (TPA), one of the two main monomeric components of the high-demand polyethylene terephthalate (PET) [70,73]. It has been estimated that the global market for ccMA derivatives is USD 22 billion [74], with a market for ccMA alone at more than USD 100 million [72].

Traditionally, ccMA has been chemically produced using petroleum-based feedstocks and high concentrations of heavy metal catalysts, thus generating toxic intermediates with heavy environmental pollution. Moreover, the chemical process produces a mixture of muconic acid enantiomers (i.e., ccMA and *cis,trans*-muconic acid), thus requiring downstream purification processes and additional costs [74]. Therefore, the production of ccMA from renewable sources such as lignocellulosic biomass represents a suitable and appealing green alternative.

The current biological production of ccMA is mainly focused on the introduction in the selected microorganisms of synthetic pathways to convert the intermediates metabolites of the natural shikimate pathway into ccMA starting from glucose [73,75,76]. Significantly, while sugars derived from biomass pre-treatment are in high demand for food, lignin-derived aromatics represent a feedstock not competing with the food chain also available at a lower cost than sugars. Since the 1970s, the biobased production of ccMA from benzoic acid and toluene has been reported, reaching a >93% yield (moles of ccMA per moles of benzoic acid) [77], but environmental concerns pushed towards renewable feedstocks [78,79]. The engineered strains such as *Amycolatopsis* sp. [80], *Corynebacterium glutamicum* [81,82], *Rhodococcus opacus* [83,84], *Escherichia coli* [74,85–87], and *Pseudomonas putida* KT2440 [23,88–94] have been reported to produce high ccMA titers from lignin-related aromatics. As a general rule, different systems biocatalysis approaches rely on the blockage of ccMA degradation pathways to favor its accumulation and on the overexpression of heterologous enzymes involved in the conversion of lignin-derived aromatics into ccMA. A summary of engineering strategies adopted for constructing optimized ccMA-producing strains is reported in Table 3.

3.1.1. *Amycolatopsis* sp. ATCC 39166

Amycolatopsis sp. ATCC 39166 has been reported as one of the few microorganisms able to utilize guaiacol in a metabolic pathway involving its demethylation into catechol, a precursor of ccMA [95]. A tailored genomic modification of the actinomycete *Amycolatopsis* sp. ATCC 39166 has been successfully developed by homologous recombination to produce ccMA from guaiacol. By deletion of two genes (AATC3_020100018510, 1104 bp and AATC3_020100009302, 1278 bp) encoding for putative muconate cycloisomerases (CatB) involved in the β-ketoadipate pathway, the MA-2 mutant strain was generated (Figure 3A): the optimized engineered strain produces 3.1 g/L ccMA from guaiacol after 24 h incubation, with a 96% yield, when cultivated at 37 °C in a fed-batch process using a stirred tank bioreactor initially supplemented with 5 mM guaiacol and 6 g/L glucose, followed by a pulse-wise guaiacol addition (Table 3).

Interestingly, when the hydrothermal depolymerization of an industrial softwood Kraft lignin from pine (Indulin AT) was conducted for 20 min at 330 °C, four most abundant aromatics were generated with a 12% conversion yield containing guaiacol (44%), catechol (30%), phenol (14%), and *o*-cresol (12%). The additional distillation step enriched the lignin hydrolysate of guaiacol: the total process provided an aqueous solution containing 7 g/L guaiacol, 3 g/L *o*-cresol, and 0.2 g/L phenol [80]. Significantly, starting from this lignin hydrolysate, the optimized MA-2 strain consumed all aromatics (2.5 mM) within about

10 h generating 1.8 mM ccMA and improving up to 6-fold the production efficiency in comparison to the wild-type strain (Table 3).

Table 3. Metabolic engineering strategies for constructing optimized ccMA-producing strains.

Host	Starting Material	Deleted Genes	Overexpressed Genes	Productivity (g/L/h)	Reference
Amycolatopsis sp. ATCC 39166	Guaiacol Hydrothermal depolymerized Kraft lignin	*G10GW-3575, G10GW-1735* (putative cycloisomerases)	-	0.13 0.26	[80]
C. glutamicum	Catechol Hydrothermal depolymerized Kraft lignin	*catB*	*catA*	2.4 0.07	[82]
R. opacus	PCA Lignin from corn stover	*catB, pcaHG*	*aroY, ecdB*	0.56 0.02	[83]
	Vanillin	*LPD03722, LPD04406* (vanillin reductases) *pcaHG*	*vdh, aroY, ecdB*	0.22	[84]
E. coli	Vanillin	-	*vdh, vanAB, catA, aroY, kpdB*	0.04	[85]
			ligV, ligM, aroY, catA	0.01	[74]
			ligV, vanAB, aroY, catA	0.7	[86]
	Vanillin from lignin	-	*ligV, vanAB, aroY, catA*	0.3	
	Isoeugenol	-	*ado, hfd1, vanAB, gdc, catA*	0.14	[87]
	Ferulic acid Ferulic acid from wheat bran	-	*fdc, ado, ligV, vanAB, aroY, catA*	0.14 0.04	[86]
P. putida	*p*-Coumarate Alkaline pretreated liquor	*pcaHG, catBC, dmpKMLNOP*	*aroY*	0.17 0.03	[88]
	p-Coumarate Ferulic acid	*pcaHG, catRBCA, crc*	*catA, aroy*	0.08 0.01	[90]
	p-Coumarate	*pobA, catRBCA, crc*	*praI, ecdBD, vanAB*	0.4	[91]
	Catechol Hydrothermal depolymerized Kraft lignin	*catBC, endA-1, endA-2*	*catA* *catA, dmpKLMOP*	4.3 0.24	[92]
	Vanillic acid	*pcaHG, catB*	*pdc*	0.03	[94]
	Vanillic acid Sugar cane bagasse alkaline extract	*catB*	*pcaHG, aroY, catA. vanAB*	0.21 0.01	[93]

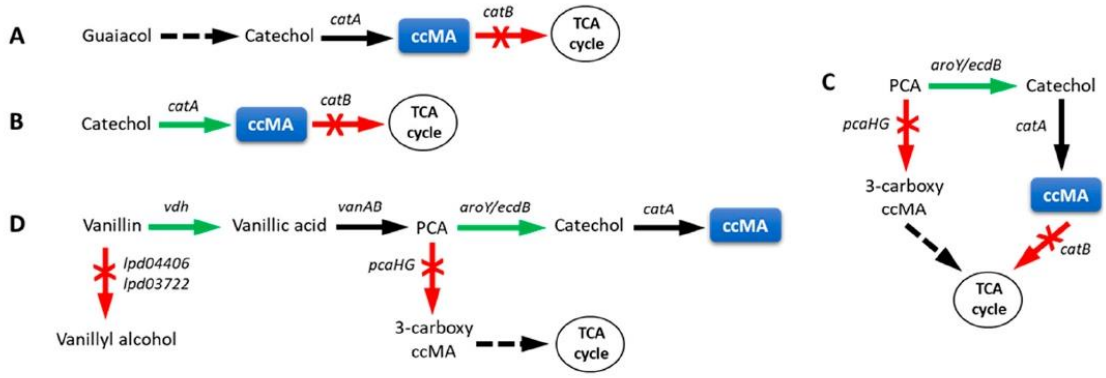

Figure 3. Scheme of ccMA production from engineered (**A**) *Amycolatopsis* sp. ATCC 39166 [80], (**B**) *Corynebacterium glutamicum* [82], and (**C**,**D**) *Rhodococcus opacus* [83,84]. Encoding genes: *aroY*, protocatechuate decarboxylase; *catA*, catechol 1,2-dioxygenase; *catB*, muconate cycloisomerase; *ecdB*, flavin prenyltransferase; *lpd03722* and *lpd04406*, putative vanillin reductases; *pcaHG*, protocatechuate 3,4-dioxygenase; *vdh*, vanillin dehydrogenase; *vanAB*, vanillate demethylase. Black arrows show the endogenous pathway; green arrows show overexpressed pathways; red crossed arrows identify deleted pathways; dashed arrows show additional multi-step pathways.

3.1.2. *Corynebacterium glutamicum*

Similar to the genetic modification reported above for *Amycolatopsis* sp ATCC 39166, a metabolic engineering strategy to upgrade the ccMA production has been recently carried out for the soil bacterium *C. glutamicum* ATCC 13032 [82]. This microorganism is able to degrade a wide spectrum of aromatic compounds via the β-ketoadipate pathway, representing the main assimilation pathway of lignin-derived compounds [22].

A stable genome manipulation by using the integrative, non-replicating plasmid pClik in sacB [81] was carried out at the level of the catechol branch of the β-ketoadipate pathway: an engineered strain deleted of the gene *catB* (NCgl2318) encoding the muconate cycloisomerase enzyme (to block the catabolism of ccMA) was established (*C. glutamicum* MA-1). When cultivated in shake flasks at 30 °C, 5 mM phenol, 10 mM catechol, and 20 mM benzoic acid have been fully converted into ccMA in 24 h. In addition, to enable faster ccMA production, the targeted overexpression of catechol-1,2-dioxygenase (CatA), catalyzing the intradiolic opening of the aromatic ring of catechol to give ccMA was established by using the strong constitutive P_{tuf} promoter (NCgl0480): for the obtained strain (*C. glutamicum* MA-2), a 10-fold higher CatA expression level was achieved in comparison to the single mutant strain *C. glutamicum* MA-1 [82] (Figure 3B). The constitutive expression of the *catA* gene (NCgl2319) reached a ccMA production rate of 5.2 mmol g^{-1} h^{-1} starting from catechol, a value 25-fold higher than that observed for the non-induced strain, with a full molar yield. Moreover, in the fed-batch process using pulse-wise feeding of catechol (to avoid toxic levels) and of glucose (to maintain its level in the 5–15 g/L range), the optimized *C. glutamicum* MA-2 strain accumulated up to 85 g/L ccMA after 60 h of incubation, with a maximum volumetric productivity of 2.4 g L^{-1} h^{-1} (Table 3). The performance of the established strain was also evaluated on real lignin samples: the hydrothermal treatment of the softwood Kraft lignin from pine (Indulin AT) was carried out in supercritical water, resulting in the enrichment of the liquid phase in catechol (101 mM) and phenol (25 mM). The lignin hydrolysate, supplemented with glucose, was added as pulse to the *C. glutamicum* MA-2 culture grown in minimal medium in baffled shake flasks: a production of 1.8 g of ccMA per liter of broth after 27 h of incubation was achieved (corresponding to 0.07 g L^{-1} h^{-1}, Table 3).

3.1.3. *Rhodococcus opacus*

R. opacus PD630 is a Gram-positive bacterium well suited for lignin valorization mainly because of its high tolerance to lignin-derived breakdown compounds coupled to the ability to catabolize a number of aromatics [87–89]. Recently, *R. opacus* has been engineered for the first time to produce ccMA from lignin-derived monomeric aromatic compounds and real lignin samples, towards the catechol and PCA funneling nodes (Figure 2), blocking the related degradation pathways [74]. A number of *R. opacus* mutants has been successfully established by using a gene deletion/insertion strategy based on a mutant phenylalanyl-tRNA synthase gene (pheS*) as a counter-selection marker in the second homologous recombination, and with the *p*-Cl-phenylalanine as a screening factor. Briefly, when the pheS* expression cassette is integrated into the *R. opacus* PD630 genome, the recombinant strain will be sensitive to *p*-Cl-Phe, whereas mutants loosing the pheS* expression cassette will recover *p*-Cl-Phe resistance [90]. Based on the genome analysis of *R. opacus* PD630, at first the gene encoding for the muconate cycloisomerase CatB (Pd630_LPD06567) has been deleted (to block the ccMA degradation in the β-ketoadipate pathway), by using the suicide plasmid pK18mob-pheS * yielding the *R. opacus* PD630-MA1 strain. When 10 mM catechol was supplied, the engineered *R. opacus* PD630-MA1 strain generated 9.7 mM ccMA (Table 3). Analogously, to block the PCA degradation pathway, the genes encoding the α- (Pd630_LPD05451) and β-subunit (Pd630_LPD05450) of protocatechuate 3,4-dioxygenase PcaHG in the *R. opacus* PD630-MA2 mutant have been deleted generating the *R. opacus* PD630-MA4 mutant. Simultaneously, to push protocatechuate into the catechol degradation pathway, the genes encoding protocatechuate decarboxylase AroY (GenBank: ADF61496) and UbiX-like prenyltransferase EcdB (GenBank: ADF63617) from *Enterobacter cloacae* have

been inserted in the endogenous plasmid of the PD630-MA4 strain, thus generating the PD630-MA6 mutant (Figure 3C). The established strain accumulated ccMA from PCA with a yield of 0.91 mol/mol (Table 3) [74]. Significantly, when the *R. opacus* PD630-MA6 mutant was incubated with a commercial alkali lignin pretreated with 1% (*w*/*w*) H_2SO_4 and 1% (*w*/*w*) NaOH at 120 °C for 30 min, and added to the commercial laccase from *Aspergillus*, 1.63 g/L ccMA was produced by a fed-batch fermentation process after 72 h incubation (Table 3) [74].

Interestingly, the engineered strain converted several lignin-derived aromatics (*p*-coumarate, vanillin, vanillyl alcohol, vanillate, coniferyl alcohol, ferulate, *p*-hydroxybenzoate, *p*-hydroxybenzaldehyde, and *p*-hydroxybenzyl alcohol) into ccMA: after incubation with 10 mM of each substrate, their concentration decreased up to 2–5 mM. In particular, the observation that only 10% vanillin and 4% *p*-hydroxybenzaldehyde (mol/mol) were converted into ccMA indicates that alternative metabolic pathways for these aromatic aldehydes are active, e.g., vanillin was mainly converted into vanillyl alcohol [75]. A total of 23 potential vanillin reductases were predicted by BLASTp analysis, using the sequence of the verified enzyme from *Pseudomonas* sp. strain 9.1 (FEZ21_09870) [91], and their transcriptional level assessed by qRT-PCR [75]. The 11 selected putative vanillin reductases have been expressed in *E. coli* BL21 cells to evaluate their ability to convert vanillin into vanillyl alcohol: the enzymes encoded by the genes *Pd630_LPD03722* and *Pd630_LPD04406* showed the highest conversion yield. Accordingly, a "reducing expenditure and broadening sources" strategy has been adopted to engineer the *R. opacus* PD630-MA6 strain, see above. The mentioned genes encoding putative vanillin reductases were deleted from the *R. opacus* PD630-MA6 mutant, and the gene encoding vanillin dehydrogenase Vdh from *Sphingobium* sp. SYK-6 (BAK65381.1) was overexpressed, to draw the vanillin metabolic flux towards ccMA [75] (Figure 3D). Notably, the vanillyl alcohol production decreased from 85.6% to 1.7%, thus enhancing the ccMA production up to a 97.8% yield after 24 h of incubation, in comparison to the starting 10% (mol/mol) bioconversion yield (Table 3).

3.1.4. *Escherichia coli*

E. coli represents a promising cell factory for ccMA production, mainly due to its incomparable fast growth and established gene manipulations tools available for its metabolic engineering. The first report on the ccMA production from lignin-derived aromatics via a biosynthetic pathway established in *E. coli* relies on the conversion of vanillin into ccMA by the mixture of *E. coli* XL-1blue/pHM002 and pTS052 recombinant strains [85]. The whole cell system has been co-transformed with the pHM002 plasmid encoding vanillin dehydrogenase (Vdh), the α- and β-subunits of vanillate demethylase (VanAB), and catechol dioxygenase (CatA) from *P. putida* NBRC100650, and the plasmid pTS052 encoding protocatechuate decarboxylase (AroY) from *Klebsiella pneumoniae* A170-10 and the β-subunit of the 4-hydroxybenzoate decarboxylase (KpdB) from *K. pneumoniae* NBRC14940, to improve the PCA decarboxylase activity (reaching a 14-fold increase compared to the strain expressing the AroY enzyme alone) [85]. The resting cells have been incubated at 30 °C under shaking in 1 mL of M9 medium containing 1 mM vanillin: a ≈ 80% bioconversion yield was achieved after 3 h of incubation with vanillic acid accumulation, probably due to the ccMA feedback inhibition of the demethylase activity (Table 3).

Later on, the valorization of lignin-derived vanillin has been demonstrated by a hybrid biochemical route combining alkali lignin depolymerization and the reconstruction of the heterologous synthetic pathway in the *E. coli* DH1 strain [74]. Vanillin has been obtained by oxidation of Kraft lignin added to hydrogen peroxide as a catalyst, with a yield in the range of 3–5% (*w*/*w*), and used as substrate for the bioconversion reaction. To accomplish the bioconversion of vanillin into ccMA, the genes encoding vanillin dehydrogenase (LigV), 3-*O*-methylgallate-*O*-demethylase (LigM) from *Sphyngobium* sp. SYK-6 [66,96], and protocatechuate decarboxylase (AroY) [66] from *K. pneumoniae* were stacked in a single operon in the pBbE1a plasmid under the control of the P_{trc} promoter, while the gene encoding catechol 1,2-dioxygenase (CatApmt2) from *P. putida* mt-2 was cloned in the pBbE1 plasmid under

the control of the T7 promoter. All genes were codon optimized for expression in *E. coli*. The whole cells harboring both plasmids were harvested, resuspended in M9 medium added of 10 g/L glucose and 0.5 g/L vanillin in a 5% of the original volume, and incubated under shaking at 30 °C: a bioconversion yield of 341 mg/L ccMA was achieved, corresponding to 0.69 g of ccMA produced from 1 g vanillin, without accumulation of intermediate metabolites in the reaction mixture [74] (Table 3). The whole cell platform expressing the synthetic pathway under the isopropyl-β-D-1-thiogalactopyranoside (IPTG)-inducer promoter has been further optimized by the successful integration of an autoregulatory vanillin-inducible system and the co-expression of an active aromatic transporter [97]. In detail, the biocatalytic pathway is under induction of the vanillin self-inducible promoter ADH7 from *Saccharomyces cerevisiae* [98], and at the same time, vanillin is efficiently transported across cell membranes by co-expressing the CouP transporter of the ABC system from *Rhodopseudomonas palustris* (under the control of the ADH7 promoter), showing a dissociation constant for phenylpropanoid ligands in the nanomolar range [99]. This strain showed a 40% increase in catechol production compared to the one not expressing CouP.

The engineered *E. coli* system represents an appealing route for lignin valorization notwithstanding the cost (and often the toxicity) of inducers [100], such as IPTG. Moreover, different lignin-derived aromatics present in lignin depolymerization mixtures have been reported to inhibit the enzyme activity and cell growth [101,102]: a self-regulation system could reduce the "toxicity" of aromatics such as vanillin, which can be therefore used as both inducer and substrate.

A useful strategy to improve the bioconversion yield, and in particular to reduce the bottlenecks represented by alternative biosynthetic routes, is represented by the engineered *E. coli* K-12 MG1655 RARE [103], a strain showing low aromatic aldehyde reduction activities and thus aimed at preventing the conversion of vanillin into the by-product vanillyl alcohol [66]. A whole cell biocatalyst to convert isoeugenol into ccMA has been developed using the engineered strain expressing aromatic dioxygenase (Ado) from *Thermothelomyces thermophila* [104], aldehyde dehydrogenase (Hfd1) from *S. cerevisiae* [105], vanillic acid *O*-demethylase (VanAB) from *P. putida* KT2440 [94], gallate decarboxylase (Gdc) from *Talaromyces atroroseus*, and catechol dioxygenase (CatA) from *Acinetobacter radioresistens* [106] (Figure 4A). The whole cell biocatalyst expressing Ado and Hfd1 enzymes almost completely converted 10 mM isoeugenol into vanillic acid [87]. When the engineered strain expressing the overall synthetic pathway was fed with 10 mM isoeugenol, 2.1 mM ccMA only was produced, with accumulation of vanillic acid, thus suggesting inhibition of VanAB activity by isoeugenol. This bottleneck was overcome by a two-step approach combined with a vanillic acid pulse-feeding strategy (i.e., by adding 10 mM vanillic acid every 90 min) (Figure 4A): 25 mM isoeugenol-derived vanillic acid has been converted into 23.3 mM ccMA after 24 h of incubation, with a 93.1% conversion yield (Table 3) [87]. Significantly, a low conversion rate was observed when the vanillic acid concentration was increased up to 50 mM because of the drop in the pH value of the reaction mixture due to the ccMA accumulation: pH control at 8.0 resulted in the complete consumption of vanillic acid and a 87.6% conversion yield into ccMA.

Very recently, the *E. coli* K-12 MG1655 RARE strain has been employed to convert in one-pot both vanillin arising from lignin and ferulic acid isolated from wheat bran into ccMA [86]. The whole cell biocatalyst has been engineered with three plasmids differing in copy numbers to modulate the expression of seven recombinant enzymes to convert vanillin into ccMA: the phenolic acid decarboxylase Fdc from *Bacillus pumilus* and the aromatic dioxygenase Ado from *T. thermophila* to convert ferulic acid into vanillin, and the vanillin dehydrogenase LigV from *Sphingobium* sp. SYK-6, the vanillate *O*-demethylase oxygenase VanAB, the protocatechuate decarboxylase AroY from *Klebsiella pneumonia* subsp., and the catechol dioxygenase from *Acinetobacter radioresistens* S13 (Figure 4B). The optimization of the extraction procedures for vanillin from lignin and of ferulic acid from wheat bran, combined with selected reaction conditions of the whole cell biocatalyst, allowed the production of ccMA from lignin-derived vanillin in one-pot with a >95% conversion

yield in 30 min and a productivity of 4.2 mg of ccMA per g of Kraft lignin. Moreover, the optimized whole-cell system expressing all the above-mentioned enzymes produced 2.2 mg of ccMA/g of wheat bran in 10 h, with a >95% conversion yield (Table 3) [86].

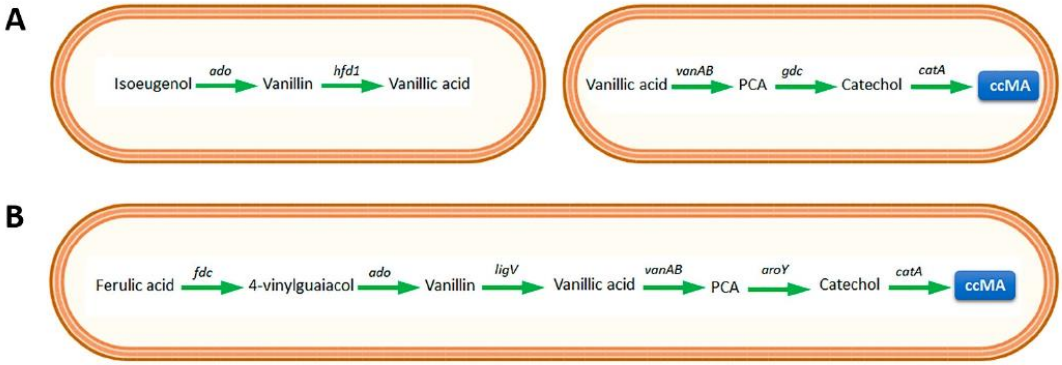

Figure 4. Synthesis of ccMA from (**A**) isoeugenol in a two-step approach [87], and from (**B**) ferulic acid in one-pot [86] using engineered *Escherichia coli* strains. Encoding genes: *ado*, aromatic dioxygenase; *aroY*, protocatechuate decarboxylase; *catA*, catechol 1,2-dioxygenase; *fdc*, phenolic acid decarboxylase; *gdc*, gallate decarboxylase; *hfd1*, aldehyde dehydrogenase; *ligV*, vanillin dehydrogenase; *vanAB*, vanillate demethylase. Green arrows show overexpressed pathways.

3.1.5. *Pseudomonas putida*

P. putida KT2440 is able to catabolize a number of lignin-derived aromatics, thus representing a native ccMA producer. The known genetic manipulation strategies, combined with the ability to tolerate a variety of physical and chemical stresses, made this microorganism the most used cell factory system to produce ccMA from many aromatics. *P. putida* KT2440 has been metabolically engineered to convert a number of lignin-derived aromatics such as ferulic acid, vanillin, coniferyl alcohol and *p*-coumarate into ccMA through both the catechol and PCA branches of the β-ketoadipate pathway (Figure 2) [88]. In detail, the *pcaHG* gene encoding the protocatechuate 3,4-dioxygenase was replaced by the *aroY* gene for the PCA decarboxylase from *Enterobacter cloacae*, in order to funnel the PCA metabolism toward the production of catechol and block the conversion of PCA into β-ketoadipate. To enable the constitutive expression of the catechol 1,2-dioxygenase CatA enzyme, to avoid further ccMA conversion and to expand substrate utilization (e.g., including phenol, a commonly-derived lignin degradation intermediate), the genome region corresponding to genes *catR*, *catBC*, and to promoter for *catBCA* were replaced with the P_{tac} promoter, and the genes *dmpKLMNOP* encoding the phenol monooxygenase from *Pseudomonas* sp. CF600 [107] were integrated downstream the *catA* gene under the tac promoter (Figure 5). The optimized engineered *P. putida* KT2440-CJ103 strain successfully converted 10 mM catechol, phenol and benzoate (via the catechol node) and 10 mM PCA, coniferyl alcohol, ferulic acid, vanillin, caffeic acid, *p*-coumarate and 4-hydroxybenzoic acid (4-HBA) (via the PCA node) into ccMA, using acetate as carbon and energy source, with a yield in the 14–93% range. A significant vanillic acid accumulation resulted starting from vanillin, ferulic acid and coniferyl alcohol, probably ascribable to the mentioned transcriptional regulation strategies. The engineered strain was used in a fed-batch bioreactor with aeration and pH control: a ccMA titer of 13.5 g/L was achieved after 78.5 h of incubation using *p*-coumarate as substrate (2 mM), a value 15-fold higher than the bioconversion yield obtained in shake flasks cultivation [88]. Interestingly, the *P. putida* KT2440-CJ103 strain produced 0.7 g/L of ccMA after 24 h of incubation from an alkaline liquor stream obtained by the corn stover pre-treatment with NaOH and antraquinone (Table 3). Considering that ferulic acid and *p*-coumarate were initially present as the two major aromatics (0.34

and 0.92 g/L, respectively), a 67% molar yield was achieved. Significantly, following the addition of activated carbon (12.5% wt/vol) to the culture media under stirring for 1 h to remove oxygenated aromatics, ccMA was precipitated by reducing the pH and temperature values and crystals recovered by vacuum filtration: a 74% recovery yield was obtained, with a >97% degree of purity.

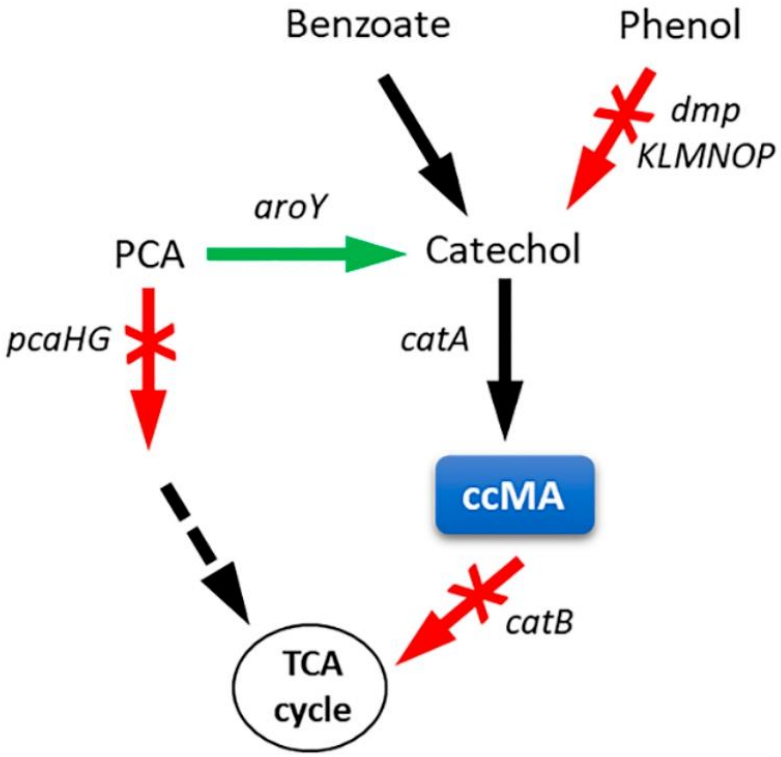

Figure 5. Metabolic engineering of *Pseudomonas putida* KT2440 to convert protocatechuic acid (PCA) and catechol derived from benzoate and phenol into ccMA [88]. Encoding genes: *aroY*, protocatechuate decarboxylase; *catA*, catechol 1,2-dioxygenase; *catB*, muconate cycloisomerase; *dmpKLMNOP*, phenol monooxygenase; *pcaHG*, protocatechuate 3,4-dioxygenase. Black arrows show the endogenous pathway; green arrows show overexpressed pathways; red crossed arrows identify deleted pathways; dashed arrows show additional multi-step pathways.

It is well known that many aromatic degradation pathways involved in the ccMA production are controlled by transcriptional and translational regulators, resulting in the accumulation of intermediates such as vanillate, PCA and 4-HBA, thus reducing the productivity of the overall process [88,108]. In *Pseudomonas*, the most important of these mechanisms is controlled by the Crc (catabolite repression control) protein, a translational regulator that inhibits both the transcription of mRNA encoding transcriptional regulators of catabolic enzymes and transporters of substrates inside cells [89]. By a mass spectrometry-based proteomics investigation, the 4-HBA hydroxylase PobA enzyme and the vanillic acid demethylase VanAB enzymes (involved in the bioconversion of 4-HBA and vanillic acid into PCA) have been identified as targets of the Crc protein [90]: the deletion of the *crc* gene led to enhanced ccMA production from ferulic acid and *p*-coumarate when glucose or acetate were used as carbon and energy sources [90]. The resulting *P. putida* KT2440-CJ238 engineered strain was grown on 10 mM glucose or 30 mM sodium acetate (with

additional feeding every 12 h to provide energy and carbon for growth) in the presence of 20 mM ferulic acid or *p*-coumarate as substrates. In cultures grown on glucose, the ccMA was obtained with a yield of 94.6 ± 0.6% (mol/mol) after 36 h of incubation in comparison to a 56.0 ± 0.3% (mol/mol) figure observed for the parental strain (Table 3). Similarly, a faster metabolism of the vanillate intermediate was apparent using ferulic acid as substrate: after 72 h of incubation, ccMA was produced by the engineered KT2440-CJ238 strain with a 28.3 ± 3.3% (mol/mol) yield, compared to 12.0 ± 2.3% (mol/mol) for the KT2440-CJ102 parental strain. Less pronounced effects were observed when acetate was used as a cost-effective alternative substrate, with a 47.7 ± 0.6% (mol/mol) yield after 24 h from *p*-coumarate and a 16.9 ± 1.4% (mol/mol) yield after 72 h from ferulic acid [90].

The bottleneck represented by the rate-limiting activity of the 4-HBA hydroxylase has been also alleviated by the engineering of the *P. putida* KT2440 strain expressing an alternative hydroxylase enzyme: the endogenous gene encoding PobA (a NADPH obligate enzyme) has been replaced by the *praI* gene encoding the enzyme from *Paenibacillus* sp. JJ-1b, which utilizes either NADH and NADPH as cofactor [91]. The engineered CJ475 strain with enhanced activity of the protocatechuate decarboxylase AroY through the co-expression of EcdBD, deleted of a global regulator of carbon catabolite repression and of the *catRBCA* gene, overexpressing the native vanillate *O*-demethylase VanAB [109], has been further engineered by integrating the *praI* gene into the bacterial genome and deleting the *pobA* gene (CJ781 strain) [91]. In a bioreactor cultivation system, with a feed of 6 mmol of *p*-coumarate per hour, a ccMA titer of 43 g/L was achieved with a 96 mol% yield, and a productivity of 0.4 g L^{-1} h^{-1} (Table 3).

A useful metabolic strategy to increase the catechol tolerance has been reported by [92]. The engineered *P. putida* KT2440-M6 strain was constructed by deleting the *catBC* gene, as reported before for the KT2440-CJ103 strain [88], and two endonuclease encoding genes *endA-1* and *endA-2*, and by boosting the expression of the catechol 1,2-dioxygenase CatA by the insertion of a second genomic copy of the *catA2* gene downstream of the *catA* gene under the control of the native P_{cat} promoter [92]. The engineered strain showed a 20% improvement in catechol tolerance, thus allowing the production of 64.2 g/L ccMA from catechol after 15 h of incubation in a fed batch process, with a productivity of 4.3 g L^{-1} h^{-1} (Table 3).

As reported before for the engineered *P. putida* KT2440-CJ103 strain [88], and with the aim to expand the substrate spectrum to cresols, the gene *dmpKLMOP* encoding the phenol hydroxylase from *P. putida* CF600 was inserted into the genome of the KT2440-M6 strain, designated as M9 strain. The engineered biocatalyst successfully converted the hydrolysate obtained by the hydrothermal treatment of softwood lignin in supercritical water (which mainly contained catechol, phenol and small amounts of *p*-cresol and *o*-cresol) into ccMA with a titer of 13 g/L after 54 h of incubation (Table 3). After two treatments of the reaction mixture with active carbon, followed by crystallization and lyophilization, a dry powder containing 96.3% ccMA was obtained. The established process has been successfully scaled up to a 50-L pilot scale reactor: maintaining the concentration of catechol below 2 mM by a pulse-feeding strategy, a total of 1.5 kg of ccMA was recovered as a white powder [92].

A *P. putida* KT2440 mutant strain able to perform a sugar-free ccMA production has been also developed by [94]. As also reported previously for the *P. putida* KT2440 strain, the genes encoding the ccMA cycloisomerase (CatB) and the PCA 3,4 dioxygenase (PcaHG) were deleted, and the gene encoding the PCA decarboxylase enzyme (PDC) from *K. pneumoniae* was introduced in the engineered IDPC/pTS110 strain. When 25 mM 4-HBA was supplied as a carbon source, 25 mM vanillic acid was converted into ccMA with a 19.0% yield after 48 h of incubation, evaluated as mol (ccMA)/mol (vanillic acid + 4-HBA) (Table 3): both 4-HBA and vanillic acid were metabolized to PCA, then used to produce biomass and energy via the PCA 3,4-dioxygenase pathway, and ccMA via the PCA decarboxylation pathway ("PCA shunt", Figure 2). The engineered strain generated 0.11 mM ccMA by growing on Japanese cedar lignin pretreated via the alkaline nitrobenzene oxidation process [94].

To bypass the constrain for the application of the *P. putida* KT2440 strain in the ccMA production, and to employ reaction mixtures containing syringyl nucleus compounds as sources for cell growth, the novel *Pseudomonas* sp. NGC7 strain, isolated as syringate-assimilating bacterium [110], was used [93]. The plasmid pTS119 carrying the *pcaHG*, *aroY*, *catA*, and *vanAB* genes was introduced in a NGC7 mutant strain not expressing the PcaHG and CatB enzymes (NGC703/pTS119). In an oxygen-dissolved (DO)-stat fed-batch culture and lowering the DO up to 2.5%, ccMA accumulated up to 35.4 mol% yield (15 g/L) from a mixture of vanillic acid and 4-HBA when the initial feeding rate of the substrates (0.2 mL/h) was increased twice and three times 42 h and 50 h after the inoculation, respectively (Table 3). The engineered strain successfully grew on the sugar cane bagasse alkaline extract prepared via a mild alkaline treatment and accumulated ccMA with a 18.7 mol% yield without sugar feeding (Table 3) [93].

3.2. Bio-Based Plastics Precursors

Besides ccMA, other valuable bio-based plastics precursors can be obtained from the bioconversion of lignin monomers. Moreover, these precursors could be utilized as copolymers to improve the biodegradability of plastic materials [111] or to produce novel biodegradable plastic polymers [112].

Adipic acid is an aliphatic dicarboxylic acid used for the production of various types of plastics, mostly used to produce Nylon 6,6. Due to its many applications, it has a market value of around USD 6 billion, with an annual production of nearly three million tons [113]. Almost all of the adipic acid present on the market is produced from petroleum-based feedstocks, through an energy-intensive process that generates ≈ 10% of anthropogenic N_2O emissions worldwide [114]. Different techno-economic analyses investigated several adipic acid production routes: it was estimated that adipic acid obtained from glucose using a fully biological route would be 20% cheaper (USD 1.36/kg) than the one from chemical route. Another techno-economic analysis demonstrated that using lignin-derived feedstocks instead of glucose as a starting material would reduce the production cost by 50% [115].

To obtain the full biological production of adipic acid from catechol, Kruyer et al. engineered an *E. coli* ΔiscR strain to co-express the catechol 1,2-dioxygenase (CatA) from *Rhodococcus* sp. AN22 and muconic acid reductase (MAR) from *Bacillus coagulans* (Figure 6A) [115]. The engineered strain was cultivated at 37 °C and fed with 1 g/L catechol, carrying out the bioconversion in a two-stage fermentative process: the first 2 h under aerobic conditions and the last 22 h in anaerobic conditions, to have a reducing environment useful to improve ccMA reduction. The bioconversion produced 1.6 mg/L of adipic acid (0.24% yield) from catechol in 24 h (Table 4) [115]. In another work, adipic acid was produced from guaiacol using a BL21(DE3) *E. coli* strain transformed with the pQLinkN plasmid harboring the genes encoding cytochrome P450 GcoAB from *Amycolatopsis* sp. ATCC 39116, catechol 1,2-dioxygenase CatA from *P. putida* and enoate reductase BcER from *B. coagulans* (Figure 6B) [115]. The strain was co-transformed with pGro7 to improve the BcER solubility through chaperonine co-expression. Adipic acid production was performed using resting cells ($OD_{600\ nm}$ = 20) in M9 medium containing 5 mM guaiacol at 37 °C (61% yield in 24 h, Table 4) [114]. Indeed, *P. putida* KT2440 strain was engineered to obtain adipic acid from *p*-coumaric acid and ferulic acid. The gene *pcaF* was knocked-out to funnel the carbon flux towards adipic acid production while the genes encoding 3-ketoadipoyl-CoA reductase PaaH from *E. coli*, 3-hydroxyadipoyl-CoA dehydratase PaaF from *E. coli* and 2,3-dehydroadipoyl-CoA reductase from *Treponema denticola* were integrated into the chromosome under the control of the P_{tac} promoter (Figure 6C) [116]. The fermentation process was optimized by lowering aeration after 24 h to promote the formation of a reducing environment (helpful for adipic acid production) and pulse-feeding glucose to a final concentration of 2.5 g/L: this would allow growth, cofactor recycling and repression of endogenous aromatics metabolism. The fermentation was carried out at 30 °C for 96

h and resulted in 0.76 g/L (18.4% molar yield) of adipic acid from *p*-coumaric acid and ferulic acid (Table 4) [116].

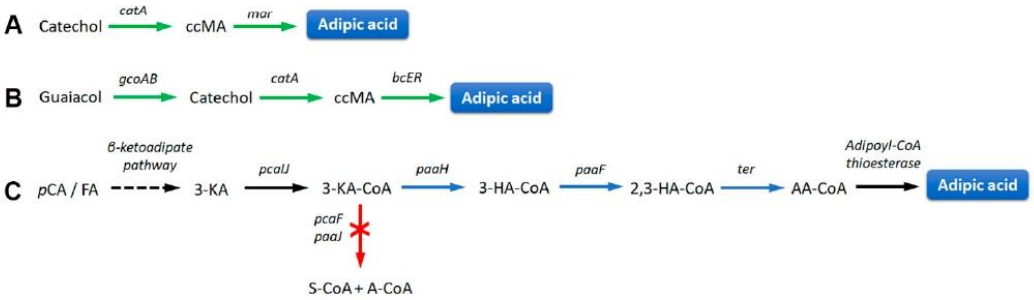

Figure 6. Scheme of adipic acid production from engineered (**A**) *E. coli* ΔiscR [115], (**B**) *E. coli* RARE [114] and (**C**) *P. putida* KT2440 [116]. Encoding genes: *catA*, catechol 1,2-dioxygenase; *mar*, muconic acid reductase; *gcoAB*, cytochrome P450 GcoAB; *bcER*, enoate reductase; *paaH*, 3-ketoadipoyl-CoA reductase; *paaF*, 3-hydroxyadipoyl-CoA dehydratase; *ter*, 2,3-dehydroadipoyl-CoA reductase; pCA, *p*-coumaric acid; 3-KA, 3-ketoadipic acid; 3-KA-CoA, 3-ketoadipoyl-CoA; 3-HA-CoA, 3-hydroxyadipoyl-CoA; 2,3-HA-CoA, 2,3-dehydroadipoyl-CoA; AA-CoA, adipoyl-CoA; S-CoA, succinyl-CoA; A-CoA, acetyl-CoA. Black arrows show the endogenous pathway; green arrows show overexpressed pathways; blue arrows show chromosomal inserted pathways; red crossed arrows identify deleted pathways; dashed arrows show additional multi-step pathways.

Table 4. Metabolic engineering strategies for the production of bio-based plastics and pharmaceuticals precursors.

Product	Host	Starting Material	Deleted Genes	Chromosome Insertion	Overexpressed Genes	Productivity (g/L/h)	Reference
Adipic acid	*E. coli*	Catechol	-	-	*catA, mar*	0.6×10^{-4}	[115]
		Guaiacol	-	-	*gcoAB, catA, bcER*	0.02	[114]
	P. putida KT2440	*p*-Coumarate Ferulic acid	*pcaF*	*paaH, paaF, 2,3-dehydroadipoyl-CoA reductase*	-	0.01	[116]
PHA	*P. putida* KT2440	Ethanol-assisted depolymerized lignin	-	-	*hps, phi, ada*	0.3×10^{-2}	[58]
		p-Coumarate from lignin	*fadAB*	*phaG, alkK, phaC1, phaC2*	-	1.2	[58]
PDC	*P. putida* KT2440	Syringic acid Ferulic acid *p*-Coumarate	*pcaHG, vanAB*	*vanABHR199, ligAB, ligC*	-	0.02	[117]
		p-Coumarate	*pcaHG*	-	*ligAB, ligC*	0.19	[118]
	P. putida PDH	Depolymerized lignin	-	-	*vanAB, ligV, ligAb, ligC, desZ*	0.03	[111]
	N. aromaticivorans DSM12444	Vanillin Vanillic acid	*desC, desD, ligI*	-	-	0.1	[119]
		Depolymerized lignin				0.1×10^{-2}	[119]
2,4-PDCA	*R. jostii* RHA1	Wheat straw Kraft lignin	-	-	*praA*	0.5×10^{-6}	[112]
		Wheat straw Protobind lignin	*pcaHG*	*praA*	*dyp2*	0.007	[120]
2,5-PDCA	*R. jostii* RHA1	Wheat straw	-	-	*ligAB*	0.5×10^{-3}	[112]

Table 4. *Cont.*

Product	Host	Starting Material	Deleted Genes	Chromosome Insertion	Overexpressed Genes	Productivity (g/L/h)	Reference
Gallic acid	*E. coli* MG1655 RARE	*p*-Coumarate	-	-	*hpaBC, fcs, ech, hfd1, pobA*	0.1	[121]
		Ferulic acid	-	-	*fcs, ech, hfd1, vanAB, pobA*	0.1	[121]
	R. opacus PD630	Base-depolymerized AFEX lignin	*protocatechuate 3,4-dioxygenase, putative catechol 2,3-dioxygenase*	*pobA, desV, desA, ligM, metF, ligH*	-	0.6×10^{-2}	[122]
Pyrogallol	*E. coli* DH1	Base-depolymerized Kraft lignin	-	-	*ligM, desA, lpdc*	0.3×10^{-3}	[74]
L-veratrylglycine	*E. coli* BL21(DE3)	Ferulic acid	*metJ*	-	*ejomt, nmtN, luxS, metk, AL-11*	0.01	[123]

Polyhydroxyalkanoate (PHA) is a naturally produced biodegradable polymer with promising physical properties [124]. However, extensive biological PHA production is hampered by the cost of the carbon source (i.e., sugars) which accounts for approximately 50% of the process cost. Therefore, using lignin as starting material for PHA production could be an effective strategy to improve the economic and environmental sustainability of the process [58]. Nguien et al. constructed a whole-cell biocatalyst able to convert ethanol-assisted depolymerized lignin into PHA. The *P. putida* KT2440 strain was engineered by introducing the pAWP89 plasmid that allows the expression of hexulose-6-phosphate synthase HPS and 6-phospho-3-hexulose isomerase PHI from *Methylomicrobium alcaliphilum* 20Z, and acetaldehyde dehydrogenase ADA from *Dickeya zeae* under the control of P_{tac} promoter. HPS and PHI allow the use of formaldehyde generated from vanillic acid demethylation by the endogenous enzyme VanAB as a carbon source, and ADA would improve the efficient utilization of ethanol present in the ethanol-assisted depolymerized lignin solution. This strain was cultivated in M9 medium supplemented with depolymerized lignin solution at 30 °C: after 96 h, the fermentation resulted in ≈300 mg/L of PHA (21.3% dcw, Table 4) [58]. Another study utilized a more extensive engineering approach of *P. putida* KT2440 to achieve the conversion of lignin-derived *p*-coumaric acid to PHA. In this latter work, the genes encoding enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase FadB and 3-ketoacyl-CoA thiolase FadA, involved in the PHA degradation pathway, were knocked-out; meanwhile, the genes encoding hydroxyacyl-ACP thiolase PhaG, hydroxyacyl-CoA synthase AlkK and two PHA polymerases PhaC1 and PhaC2 from *P. putida* were integrated into the bacterial genome and overexpressed using the constitutive P_{tac} promoter. The engineered strain was grown in M9 medium supplemented with *p*-coumaric acid at 30 °C and fed with additional *p*-coumaric acid: a titer of ≈950 mg/mL of PHA (≈54% g/g yield) was achieved after 96 h of incubation (Table 4). The same strain produced ≈115 mg/mL of PHA when incubated in modified M9 medium supplemented with 75% sterile soluble lignin stream at 30 °C [125].

2-Pyrone-4,6-dicarboxylic acid (PDC) is a stable chemical intermediate of microbial lignin depolymerization. PDC polyester showed remarkable biodegradability and physical properties [126], making it a promising substitute for TPA in polyester production [118]. A tailored *P. putida* KT2440 strain was engineered to funnel compounds derived from S-, G- and H-lignin monomers (syringic acid, ferulic acid and *p*-coumaric acid, respectively) into PDC production. To achieve this biological funneling, the gene encoding PCA 3,4-dioxygenase PcaHG was knocked-out (to prevent PCA use as a carbon source) and the native *vanAB* gene was substituted with the *HR199 vanAB* from *P. putida* to prevent 3-*O*-methylgallate demethylation while maintaining demethylation activity on syringic acid and PCA. Meanwhile, the genes encoding PCA 4,5-dioxygenase LigAB and CHMS dehydrogenase LigC from *Sphingobium* sp. SYK-6 were integrated into the chromosome under P_{tac} promoter control (Figure 7A) [117]. The fermentation, performed at 30 °C in M9

medium supplemented with 40 mM glucose and 1.5 mM of each lignin monomer, produced 4 mM PDC (93% mol/mol yield) in 36 h (Table 4) [117].

Another study developed and optimized the conversion of *p*-coumaric acid into PDC using an engineered *P. putida* KT2440 strain. Here, the gene encoding PCA 3,4-dioxygenase PcaHG was knocked-out and the genes encoding for PCA 4,5-dioxygenase LigAB and CHMS dehydrogenase LigC from *Sphingobium* sp. SYK-6 were cloned into the pSEVA631 plasmid under the control of P_{tac} promoter (Figure 7B) [118]. Due to the accumulation of 4-HBA in the fermentation broth, the oxygen concentration was raised to 30% to improve the activity of the endogenous enzyme PobA which converts PCA into 4-HBA. The strain was cultivated in M9 medium supplemented with 2.5 g/L glucose and 10 g/L glycerol, and the reaction system was fed with a *p*-coumaric acid solution to maintain a 30 mM concentration. The optimized bioconversion system produced PDC from 120 mM *p*-coumaric acid with a molar yield of ≈100% in 115 h (Table 4) [118].

Quian et al. engineered a *P. putida* PDH strain to convert vanillin and syringaldehyde present in the lignin depolymerization extract [111]. Two different plasmids were built and transferred into the bacterium to improve lignin conversion: (i) pKT230 plasmid harboring the genes encoding vanillate demethylase VanAB from *P. putida* KT2440, vanillin aldehyde dehydrogenase LigV, protocatechuate dioxygenase LigAB and hydroxymuconate-6-semialdehyde dehydrogenase LigC from *Sphingobium* sp. SYK-6; (ii) pJB866 plasmid carrying the gene encoding 3-*O*-methylgallate dioxygenase DesZ from *Sphingomonas paucimobilis* (Figure 7C) [111]. The fermentation in a minimal medium containing 7 g/L glucose and 1.15 mg/mL of Birch extract or Japanese cedar extract at 28 °C produced 0.48 mg/mL and 0.65 mg/mL of PDC, respectively (Table 4).

The *Novosphingobium aromaticivorans* DSM12444 strain was also used to produce PDC from depolymerized lignin, exploiting its innate ability to utilize lignin monomers as a carbon source. The genes *desC*, *desD* and *ligI* were knocked-out from the bacterial genome to funnel lignin monomers metabolism towards PDC production and prevent its degradation [119]. Despite the strain optimization, when cultured in SISnc-V0 media supplemented with 20 mM glucose and a solution of depolymerized lignin containing different lignin monomers at 30 °C, all the aromatic compounds were consumed and 0.5 mM PDC (59% molar yield) was produced only after 77 h. When the engineered strain was cultured in a fed-batch reactor and fed with a solution containing 226 mM vanillic acid, 34 mM vanillin, 550 mM glucose, 15 g/L ammonium sulfate, and 5% (*v*/*v*) DMSO, a total of 26.7 mM (4.9 g/L) PDC was produced in 48 h (Table 4) [119].

Pyridine 2,4-dicarboxylic acid (2,4-PDCA) and **pyridine 2,5-dicarboxylic acid (2,5-PDCA)** are two interesting lignin-derived chemicals that could replace petroleum-derived TPA in polybutyrate adipate terephthalate (PBAT) polymer, thus producing a new plastic polymer [112]. Furthermore, due to soil bacteria's capability to degrade pyridine derivatives [127], the new polymer should be biodegradable. The first attempt to produce these pyridine derivatives was made by engineering *R. jostii* RHA1 to obtain the meta-ring cleavage compound which is then subjected to ammonia cyclization, catalyzed by NH_4Cl, to generate the final product. To produce 2,5-PDCA, the plasmid pTipQ2 harboring the gene encoding protocatechuate 4,5-dioxygenase LigAB from *S. paucimobilis* was transferred into *R. jostii* RHA1; meanwhile, to produce 2,4-PDCA, the plasmid pTipQ2 carrying the gene encoding protocatechuate 2,3-dioxygenase PraA from *Paenibacillus* sp. JJ-1b was employed [112]. The engineered strains were cultivated in M9 medium (containing NH_4Cl) supplemented with either 1% wheat straw or 0.5% Kraft lignin at 30 °C. The fermentation of LigAB-expressing strain produced 125 mg/L of 2,4-PDCA from wheat straw (after 9 days) and 53 mg/L of 2,4-PDCA from Kraft lignin (after 4 days) (Table 4). The fermentation of PraA-expressing strain produced 106 mg/L of 2,5-PDCA from wheat straw (after 9 days) while no product formation was observed when fed with Kraft lignin (Table 4) [112]. The same research group optimized the 2,4-PDCA production through further strain engineering: in detail, the gene encoding LigAB was integrated into the chromosome under control of the engineered promoter P_{tpc5}, the gene *pcaHG* was knocked-out to block the

competing β-ketoadipate pathway and the strain was transformed with the expression plasmid pTipQC2 harboring the genes encoding peroxidase Dyp2 from *Amycolatopsis* sp. 75iv2, to improve lignin oxidation rate [120]. The improved whole-cell biocatalyst was cultivated in M9 medium (containing NH_4Cl) supplemented with either 1% wheat straw or 1% Green Value Protobind lignin at 30 °C. The fermentation resulted in 330 mg/L of 2,4-PDCA produced from wheat straw and 240 mg/L of 2,4-PDCA produced from Green Value Protobind lignin in 40 h (Table 4) [120].

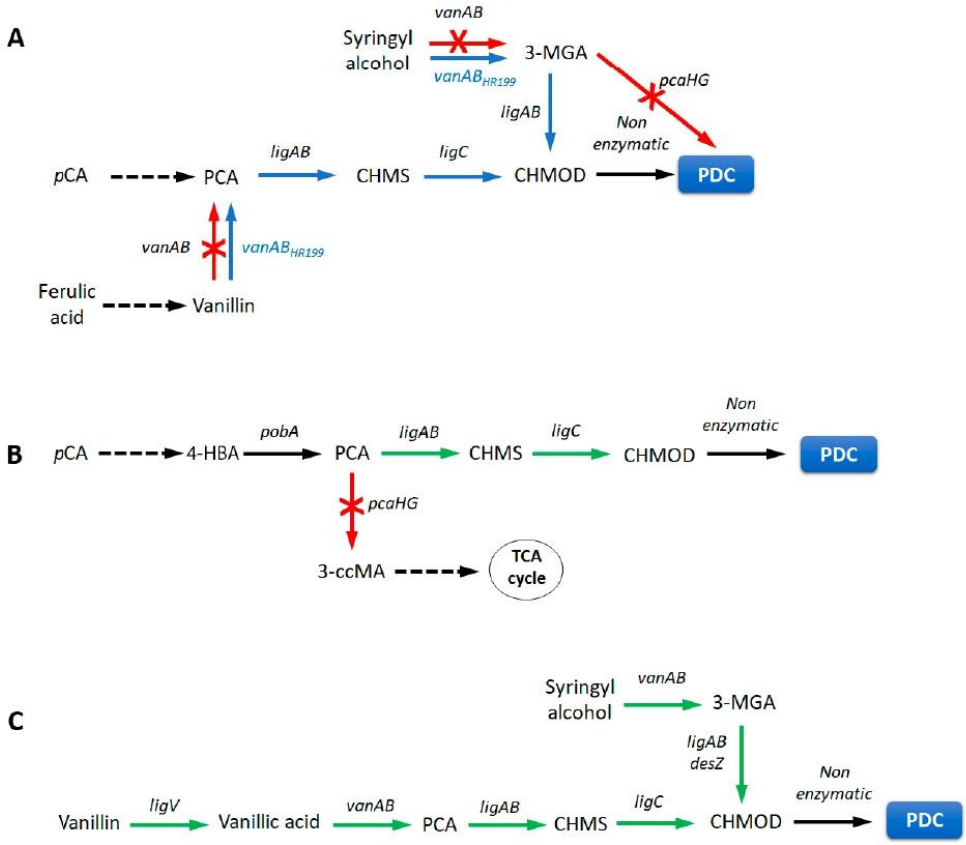

Figure 7. Scheme of PDC production from engineered (**A**,**B**) *P. putida* KT2440 [117,118] and (**C**) *P. putida* PDH [111]. Encoding genes: *pcaHG*, PCA 3,4-dioxygenase; *vanAB*, vanillate demethylase; *ligAB*, PCA 4,5-dioxygenase; *ligC*, CHMS dehydrogenase; *pobA*, *p*-hydroxybenzoate hydroxylase; *ligV*, vanillin dehydrogenase; *desZ*, 3-*O*-methylgallate dioxygenase; pCA, *p*-coumaric acid; PCA, protocatechuic acid; CHMS, 4-carboxy-2-hydroxy-cis,cis-muconate 6-semialdehyde; CHMOD, 4-carboxy-2-hydroxy-6-methyoxy-6-oxohexa-2,4-dienoate; 3-MGA, 3-methoxygallate; 4-HBA, 4-hydroxybenzoic acid; 3ccMA, 3-carboxy-ccMA. Black arrows show the endogenous pathway; green arrows show overexpressed pathways; blue arrows show chromosomal inserted pathways; red crossed arrows identify deleted pathways; dashed arrows show additional multi-step pathways.

3.3. Pharmaceuticals

Interestingly, lignin could also represent an interesting source of bioactive molecules and pharmaceutical precursors [64]. The development of biotechnological processes to produce these high-value compounds could represent the driving force to establish competitive lignin biorefineries [128].

Gallic acid is a natural phenolic compound occurring in many different plant species and showing several interesting biological activities, such as anti-cancer, anti-oxidant and anti-inflammatory effects among others [129]. In addition, this compound is utilized in the food and cosmetic industries as preservative, and is a precursor for the production of various value-added products [130]. Nowadays, gallic acid is mainly obtained from chemical or enzymatic hydrolysis of tannins, making its production a high-cost process dependent on raw material quality and availability [122]. Fu et al. developed two whole-cell biocatalysts to produce gallic acid lignin-derived aromatics from *p*-coumaric acid and from ferulic acid [121]. The strain of choice for this bioconversion was *E. coli* MG1655 RARE [103]. To construct the biocatalyst using *p*-coumaric acid as substrate, three different expression plasmids were introduced in *E. coli*: pETDuet-1 harboring genes encoding feruloyl-CoA synthetase FCS and enoyl-CoA hydratase/aldolase ECH from *P. putida*, pRSFDuet-1 carrying genes encoding aldehyde dehydrogenase HFD1 from *S. cerevisiae* and pACYCDuet-1 plasmid to express vanillic acid *O*-demethylase VanAB from *P. putida* and the *p*-hydroxybenzoate hydroxylase PobA-Y385F variant from *P. putida* (Figure 8A, top) [121]. The biocatalyst to convert ferulic acid into gallic acid was built using the same plasmids, but with the plasmid pACYCDuet-1 carrying the genes encoding the two-component flavin-dependent monooxygenase HpaBC from *E. coli* instead of *vanAB* gene (Figure 8B, bottom). The bioconversion was performed with resting cells in 200 mM phosphate buffer at pH 8.0 and 30 °C, at 10 mM initial substrate concentration and feeding with additional 10 mM of substrate after 6 h. Starting from *p*-coumaric acid, ≈19.5 mM gallic acid (≈98% yield) was produced in 36 h; meanwhile, using ferulic acid, ≈19.9 mM gallic acid (≈99% yield) was produced in 36 h (Table 4) [121]. Gallic acid was also generated from base-depolymerized lignin using an engineered *R. opacus* PD630 strain, capable of utilizing S-, G- and H-lignin monomers as substrates. The Y385F and T294A substitutions were introduced in the endogenous enzyme PobA to improve the hydroxylation activity, and the genes encoding protocatechuate 3,4-dioxygenase and a putative catechol 2,3-dioxygenase were deleted to block gallate and PCA biodegradation pathways. Then, to improve S-lignin monomers utilization, the genes encoding aldehyde hydrogenase DesV, THF-dependent *O*-demethylases DesA and LigM, and the tetrahydrofolate recycling system MetF and LigH from *Sphingobium* sp. SYK-6 were integrated in the genome (Figure 8B) [122]. The engineered strain was cultivated in M9 medium supplemented with 10 mM glucose and 0.5 g/L of soluble base-depolymerized AFEX lignin as substrate at 30 °C: after 48 h of incubation, 1.8 mM gallic acid was produced (Table 4) [122].

Pyrogallol is a phenolic compound used as a substrate for the chemical synthesis of different biologically active molecules [131] that could have potential use as anti-proliferative agent on cancer cells [74]. Pyrogallol is produced through the thermal decarboxylation of gallic acid [131]: accordingly, its current industrial production shows the same drawbacks as the gallic acid one. Wu et al. attempted the production of pyrogallol from syringic acid obtained from base-catalyzed oxidation of Kraft lignin using an engineered *E. coli* DH1 strain [74]. The biochemical pathway was constructed by inserting into the pBbE1a plasmid the genes encoding the enzymes tetrahydrofolate-dependent *O*-demethylases LigM and DesA from *Sphingomonas* sp. SYK-6, and the decarboxylase Lpdc from *Lactobacillus plantarum* WCFS1 (Figure 8C) [74]. The bioconversion of lignin-derived syringate to pyrogallol was assayed using both growing cells and resting cells. The fermentation was performed in LB medium supplemented with 20 g/L glucose and 1 g/L of lignin-derived syringate at 37 °C and produced 7.3 mg/L pyrogallol and 18 mg/L of gallic acid. The bioconversion using resting cells was carried out in M9 medium supplemented with 10 g/L glucose and 0.5 g/L of lignin-derived syringate at 30 °C: this approach improved the gallate production (59.6 mg/L) but the pyrogallol production was similar to the fermentation system (6.2 mg/mL) (Table 4) [74].

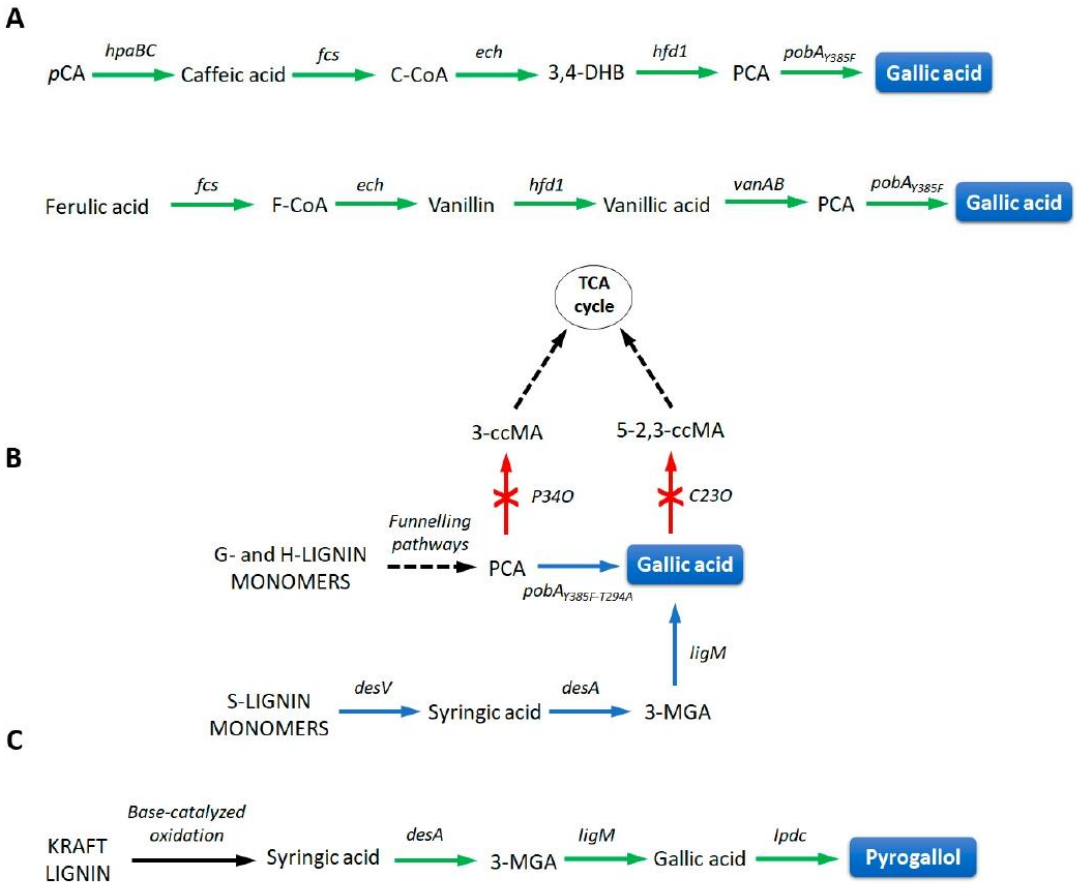

Figure 8. Scheme of gallic acid production from (**A**) engineered *E. coli* RARE [121] and (**B**) *R. opacus* PD630 [122], and of pyrogallol production from (**C**) engineered *E. coli* DH1 [74]. Encoding genes: *hpaBC*, two-component flavin-dependent monooxygenase; *fcs*, feruloyl-CoA synthetase; *ech*, enoyl-CoA hydratase/aldolase; *hfd1*, aldehyde dehydrogenase; *pobA*, *p*-hydroxybenzoate hydroxylase; *vanAB*, vanillate demethylase; *desV*, aldehyde hydrogenase; *desA* and *ligM*, THF-dependent *O*-demethylases; *P34O*, protocatechuate 3,4-dioxygenase; *P23O*, putative catechol 2,3-dioxygenase; *lpdc*, decarboxylase. pCA, *p*-coumaric acid; C-CoA, caffeoyl-CoA; 3,4-DHB, 3,4-dihydroxybenzoic acid; F-CoA, feruoyl-CoA; 5-2,3-ccMA, 5-carboxy-2,3-dihydroxymuconate 6-semialdehyde; 3ccMA, 3-carboxy-ccMA; 3-MGA, 3-methoxygallate. Black arrows show the endogenous pathway; green arrows show overexpressed pathways; blue arrows show chromosomal inserted pathways; red crossed arrows identify deleted pathways; dashed arrows show additional multi-step pathways.

3,4-Dihydroxyphenyl-L-alanine (L-DOPA) is the precursor of dopamine and one of the most effective drugs used to treat Parkinson's disease. Due to its role, the global market demand is approximately 250 tons per year with a market value of approximately USD 101 billion [132]. Currently, L-DOPA is produced via chemical synthesis and extraction from plants; however, these production methods are not sustainable [133]. Galman et al. developed a two-stage one-pot process to produce biobased L-veratrylglycine, a precursor of L-DOPA [123]. In the first step, ferulic acid is methylated to 3,4-dimethoxycinnamic acid: the plasmid pET28 harboring the gene encoding a variant of *O*-methyltransferase EjOMT enzyme (I133S/L138V/L342V) from *Eisenia japonica* and the plasmid p-ACYCDuet-1 carry-

ing the gene encoding S-adenosylhomocysteine nucleosidase MntN, S-ribosylhomocysteine lyase LuxS and a variant of SAM synthetase MetK enzyme (I303V) from *E. coli* were transferred into the *E. coli* BL21(DE3) strain. To increase the methionine availability, the endogenous transcriptional repressor MetJ was deleted from the E. coli's genome. The engineered strain (at 0.7% *w*/*v*), when incubated in M9 medium supplemented with 5 mM D,L-methionine and 3 mM ferulic acid at 30 °C, produced 0.6 g/L of 3,4-dimethoxycinnamic acid (99% conversion) in 48 h. In the second step, an engineered *E. coli* BL21(DE3) strain overexpressing a variant of the ammonia lyase AL-11 enzyme (Q84V) was added to the reaction mixture (0.8% *w*/*v*) together with 4 M ammonium carbamate: after an additional 18 h of incubation at 30 °C, the complete conversion in L-veratrylglycine into L-DOPA (>99% ee) was obtained (Table 4) [123].

4. Perspectives

The cost-effectiveness of biological lignin valorization largely depends on converting the lignin-derived aromatics to value-added compounds. To improve the economic viability of the overall lignin valorization process, especially at the industrial scale, and to be cost-competitive with petroleum routes of chemical synthesis, several concepts/tools/strategies seem particularly relevant to fill the gap:

- the screening/use of engineered promoters with varying strength that could lead to tunable systems aimed at maximization of protein expression of selected enzymes of the relevant pathways;
- the use of metabolic engineering and protein engineering approaches to remove any bottleneck of the relevant pathways;
- the optimization of fermentation conditions;
- the efficient supply of large quantities of lignin in a relatively uniform, purified and biocompatible form;
- the optimization of lignin depolymerization to yield a substrate with high monomer concentrations;
- the limitation of toxicity of lignin degradation products;
- the optimization of the products' separation process that has been estimated to account for over 60% of production costs [134].

These points are expected to guide future research in the field.

5. Conclusions

The future economic success of lignocellulosic-based biorefineries is tied to the valorization of lignin. Lignin is an excellent material for producing a number of value-added bioproducts. Microorganisms and enzymes are well suited to handle the inherent recalcitrant properties of lignin, its heterogeneity and its toxicity. As illustrated, metabolic engineering (also supported by protein engineering to optimize selected enzymatic steps) resulted in microbial cell factories for the production of relevant chemicals from lignin at the laboratory scale and at the pilot scale.

Biological lignin valorization has not yet been conducted at an industrial/commercial scale, so techno-economic analyses have not been fully evaluated. A recent study highlighted that the biological conversion of lignin into bioplastics had significant potential to improve the profitability of lignin valorization [135]. A "plug-in processes of lignin' with the integration of five fractionation pretreatments has been set up: the optimized process could enable a minimum polyhydroxyalkanoate selling price at as low as USD 6.18/kg, with operating costs accounting for about 47–58% of total production [135]. Overall, low costs of lignin fractionation and of downstream separation processes are essential to the economic success of lignin valorization. The development of innovative approaches, such as synthetic biology and metabolic engineering, could be helpful to increase the production yields and to design new bioconversion pathways thus reducing the price of the final products and further increasing and diversifying the portfolio of products from lignin.

Although at present the bacterial lignin valorization is still a "field of dreams" [64], growing research and innovation will make it a reality in the short period and a central pillar in sustainability.

Author Contributions: Conceptualization, E.R. and L.P.; writing—original draft preparation, E.R., F.M., D.M. and L.P.; writing—review and editing, E.R. and L.P.; visualization, E.R., F.M. and D.M.; supervision, E.R. and L.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: L.P. and E.R. thank the support of Fondo di Ateneo per la Ricerca and CIB, Consorzio Interuniversitario per le Biotecnologie. F.M. and D.M. are students of the Ph.D. course "Life Sciences and Biotechnology", University of Insubria.

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

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2. Aim of the work

The current linear production model relies primarily on the exploitation of virgin resources, like fossil fuels, and generates large amount of waste materials, making it completely unstainable in the long run. Consequently, the use of economic models focusing on the environmental sustainability of the process, such as the bioeconomy and circular economy model is mandatory, in order to reduce the overexploitation and pollution of the natural resources. Plastic materials have several excellent properties that made them indispensable in many fields of everyday life; nonetheless, their production is almost completely reliant on dwindling petroleum-based feedstocks, thus demanding innovative production processes rom non-toxic renewable materials.

In this view, the lignocellulosic biomass has a great potential to become the renewable alternative to oil, thanks to its great abundance and its heterogeneity, allowing its use for several production processes. Cellulose and hemicellulose are already industrially utilized for the production of paper and biofuels, meanwhile lignin, despite being a natural and renewable source of aromatics, is underutilized due to the high chemical and physical recalcitrance of its structure. Therefore, the biotechnological valorization of lignin could not only help coping with the diminishing supply of petroleum but also induce the transition to more sustainable industrial processes to produce plastic precursors and other valuable chemicals.

This PhD project is aimed at the development of a biotechnological process for the valorization of lignocellulosic by-products (i.e. wheat bran and kraft lignin), focusing on the use of whole-cell biocatalytic approaches for the conversion of lignocellulose-derived aromatics into value-added compounds. For this purpose, at first two thermo-enzymatic treatments for the extraction of ferulic acid and vanillin, from wheat bran and kraft lignin, respectively, will be assayed and optimized. Ferulic acid can be converted into vanillin by a two-step biochemical pathway, and vanillin can be further converted into *cis,cis*-muconic acid, a precursor of several plastic materials, through a four-step biochemical pathway. Thus, to convert the lignocellulose-derived aromatics into added-value compounds, an engineered *E. coli* strain expressing up to 7 recombinant enzymes will be designed and built up. The engineered strain will be used following a resting cells approach, making the setup of the recombinant pathways easier compared to isolated enzymes, while allowing the adjustment of several reaction parameters. To further assay the biocatalytic capabilities of the engineered strain converting vanillin into *cis,cis*-muconic acid, the bioconversion will be conducted in a bioreactor using the growing cells approach. The combination of using a growing cells approach combined with an auto-inducing growth medium, will make the process more straightforward, cheaper and overall more scalable. Lastly, a one-pot process for the production of the plastic precursor 4-

vinylguaicol directly from wheat bran will be evaluated. An engineered *E. coli* strain expressing a decarboxylase enzyme, that converts ferulic acid into 4-vinylguaiacol, under the control of a phenol-inducible promoter, will allow the use the extracted ferulic acid as an inducer, meanwhile the *E. coli* strain will grow in the carbohydrate-rich wheat bran crude extract. This one-pot strategy is aimed to reach an almost complete utilization of the starting material wheat bran, eliminating the need for commercial growth ingredients and external inducers, hence making the process more sustainable.

3. Results

3.1 Whole-Cell Bioconversion of Renewable Biomass-Related Aromatics to cis,cis-Muconic Acid

Molinari, F.; Pollegioni, L.; Rosini, E. Whole-Cell Bioconversion of Renewable Biomasses-Related Aromatics to *cis,cis*-Muconic Acid. *ACS Sustain. Chem. Eng.* **2023**, *11* (6), 2476–2485.

3.2 Bio-based *cis,cis*-muconic acid production from vanillin using a growing cells approach

Unpublished data

3.3 One-pot biotechnological valorization of wheat bran into 4-vinylguaiacol

Unpublished data

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Research Article

Whole-Cell Bioconversion of Renewable Biomasses-Related Aromatics to *cis,cis*-Muconic Acid

Filippo Molinari, Loredano Pollegioni,* and Elena Rosini*

Cite This: *ACS Sustainable Chem. Eng.* 2023, 11, 2476−2485

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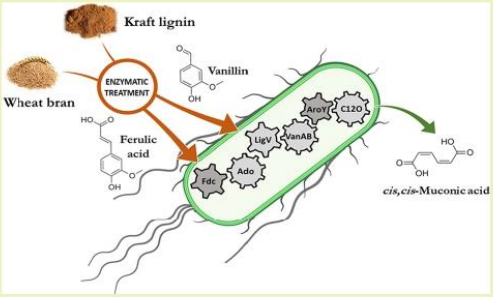
ABSTRACT: Lignin and wheat bran represent renewable feedstocks for generation of useful and value-added compounds such as vanillin (a popular flavoring agent) and *cis,cis*-muconic acid (ccMA, a building block for the synthesis of plastic materials). In the present work, we report on the setup of an efficient and green process for producing such valuable compounds based on (a) the optimization of the extraction procedures for vanillin from lignin and ferulic acid from wheat bran and (b) the genetic engineering of an *Escherichia coli* strain with up to three plasmids differing in copy numbers to modulate the expression of up to seven recombinant enzymes. In detail, we used two sequential reactions catalyzed by the decarboxylase Fdc and the dioxygenase Ado to convert wheat bran-derived ferulic acid into vanillin: nature-identical vanillin was produced in one pot with a >85% yield in 20 h. Next, the dehydrogenase LigV, the demethylase VanAB, the decarboxylase AroY, and the dioxygenase C12O converted lignin-derived vanillin into ccMA with a >95% conversion yield and a productivity of 4.2 mg of ccMA/g of Kraft lignin in 30 min. Finally, when the optimized *E. coli* strain expressing all the abovementioned enzymes was used, ccMA was produced with a >95% conversion yield starting from ferulic acid in 10 h following product isolation, corresponding to 0.73 g of ccMA/g of ferulic acid, 1.4 g of ccMA/L, and 2.2 g of ccMA/g of wheat bran biomass. The optimized whole-cell system represents a sustainable and cost-competitive process for producing high value-added products from renewable resources.



KEYWORDS: *biotransformation, cascade reaction, renewable biomasses, wheat bran, lignin valorization, vanillin, system biocatalysis, engineered E. coli*

INTRODUCTION

Lignin accounts for 15−25% of plant cell wall lignocellulose and represents the largest renewable source of aromatic compounds in the biosphere.[1,2] Massive amounts of technical lignins are generated industrially during biomass fractionation in lignocellulose processing (e.g., 50 million metric tons of lignin is generated from the pulp and paper industry every year).[3] At present, lignin is largely burnt for energy instead to be valorized into useful chemical products.[4−6] Furthermore, the wheat grain milling process generates wheat bran, usually used as livestock feed or as a fiber-rich ingredient in food products.[7] Wheat bran is rich in phytochemicals; among them, ferulic acid represents the most abundant phenolic compound.

Lignin and wheat bran represent renewable feedstocks for generation of useful and value-added compounds. On this side, vanillin (4-hydroxy-3-methoxybenzaldehyde), the second most popular flavoring agent after saffron used in the food and cosmetics industry, is of main industrial interest. Three types of vanillin—namely, natural, nature-identical (biotechnologically produced from ferulic acid), and synthetic—are available on the market: only natural and nature-identical vanillins are considered as food-grade additives. Due to the high cost and small amount produced (≈6500 tons/year), the worldwide production of natural vanillin does not fulfill the market demands.[8] Vanillin is also widely used as a building block in pharmaceutical synthesis and for the production of biopolymers.[9] Furthermore, a well-suited starting building block for the synthesis of relevant chemicals (adipic acid and terephthalic acid) and precursors of commercial plastics (such as nylon 6,6-polytrimethylene terephthalate) is represented by *cis,cis*-muconic acid (*cis,cis*-2,4-hexadienedioic acid, ccMA).

One strategy for biocatalytic valorization of lignin and wheat bran is to funnel a heterogeneous mixture of lignin-derived compounds into few, selected value-added compounds, also

Received: November 2, 2022
Revised: January 18, 2023
Published: February 1, 2023

https://doi.org/10.1021/acssuschemeng.2c06534
ACS Sustainable Chem. Eng. 2023, 11, 2476−2485

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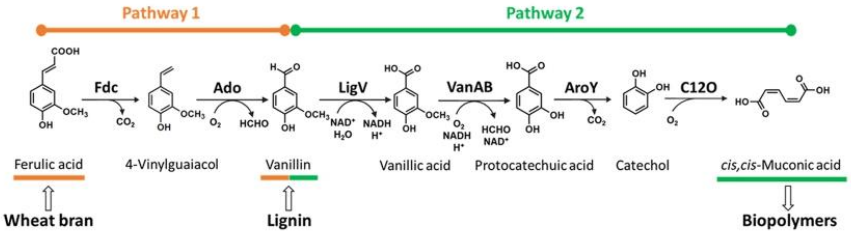
Scheme 1. Artificial Biosynthetic Pathway to Produce *cis,cis*-Muconic Acid from Ferulic Acid[a]



[a]Pathway 1: production of vanillin from ferulic acid by a cofactor-independent decarboxylase/oxygenase two-step process catalyzed by Fdc and Ado. Pathway 2: bioconversion of vanillin to *cis,cis*-muconic acid catalyzed by the multi-enzymatic system comprising the vanillin dehydrogenase LigV, the vanillic acid *O*-demethylase VanAB, the protocatechuate decarboxylase AroY, and the catechol 1,2-dioxygenase C12O. The alternative oxygenase enzyme Cso2 was also evaluated for the whole-cell biotransformation.

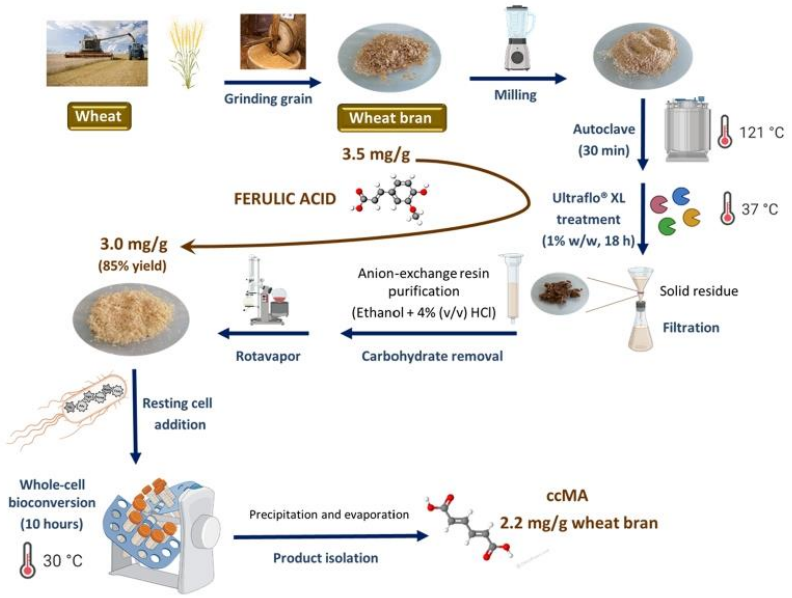


Figure 1. Schematic representation of the optimized protocol for recovery and purification of ferulic acid from wheat bran (see treatment iv; Table 2) followed by its bioconversion into ccMA.

taking advantage of engineered microorganisms, in the so-called "biological funneling" approach.[6]

Whole-cell biocatalysts offer several advantages, such as multi-step reactions with cofactor regeneration, high catalytic efficiency, and mild conditions, but require tailoring by means of protein and metabolic engineering approaches[10] and a critical evaluation of the results in comparison to cell-free systems.[11] Recently, we used a system biocatalysis approach to develop the first multi-enzymatic, one-pot bioconversion process of vanillin into ccMA based on four sequential reactions catalyzed by the commercial enzyme xanthine oxidase, the recombinant enzyme *O*-demethylase LigM in the presence of tetrahydrofolate (THF) and of the cofactor-regeneration enzyme methyl transferase MetE, and *Escherichia coli* cells expressing the decarboxylase AroY and the recombinant enzyme catechol 1,2-dioxygenase C12O.[12] The optimized lab-scale procedure converted 5 mM vanillin into ccMA in ≈30 h with a 95% yield.

Here, we focused on the generation of an engineered *E. coli* strain able to convert in one-pot both vanillin arising from lignin and ferulic acid isolated from wheat bran into ccMA (Scheme 1). The previous aromatic compound was isolated from Kraft lignin using the enzymatic procedure described by Vignali et al.,[13] the latter from wheat bran using an optimized thermo-enzymatic process. The engineered cell system efficiently generated ccMA, a useful metabolite of main industrial relevance: the global market of the ccMA derivatives is estimated at $22 billion,[14] with a market for ccMA alone at more than $100 million.[15]

MATERIALS AND METHODS

Reagents. Methanol (ACS Grade, ≥99%), formic acid (ACS Grade, ≥98%), softwood Kraft lignin, 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO), ethyl acetate, and analytical grade standards of ferulic acid (*trans*-hydroxy-3-methoxycinnamic acid), 4-vinylguaiacol (2-methoxy-4-vinylphenol), vanillin (4-hydroxy-3-methoxybenzalde-

https://doi.org/10.1021/acssuschemeng.2c06534
ACS Sustainable Chem. Eng. 2023, 11, 2476−2485

Table 1. Synthetic Genes Used in this Study

gene	accession number	enzyme	source	encoded protein (amino acids)	reference
Fdc	CAC18719.1	phenolic acid decarboxylase	*Bacillus pumilus*	161	18
Cso2 (A49P-Q390A)	ADG10219.1	9-*cis*-epoxycarotenoid dioxygenase	*Caulobacter segnis*	491	18
Ado	XP_003665585	aromatic dioxygenase	*Thermothelomyces thermophila*	603	19
LigV	BAK65381.1	vanillin dehydrogenase	*Sphingobium* sp. *SYK-6*	480	14
VanA	AAN69332.1	vanillate *O*-demethylase oxygenase	*Pseudomonas putida KT2440*	355	19
VanB	AAN69333.1	NAD(*P*)H oxidoreductase	*P. putida KT2440*	316	19
AroY	BAH20873	protocatechuate decarboxylase	*Klebsiella pneumonia* subsp.	502	19
C12O	AF182166.3	catechol 1,2-dioxygenase	*Acinetobacter radioresistens S13*	294	19

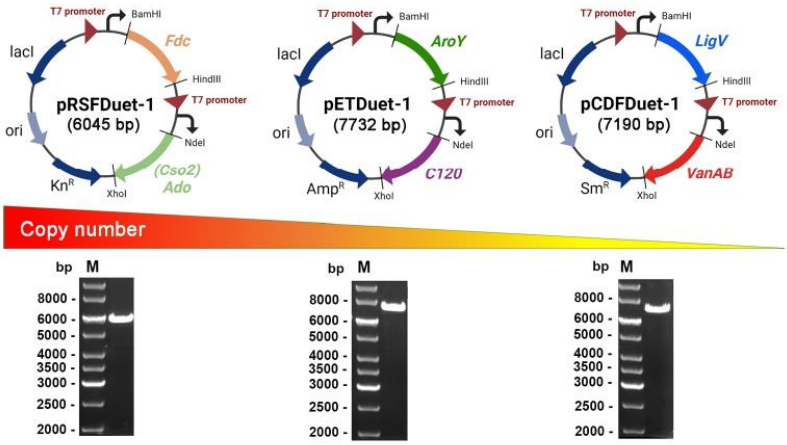


Figure 2. (Top) Physical map of plasmids pRSFDuet-1 (high copy number), pETDuet-1 (medium copy number), and pCDFDuet-1 (low copy number) carrying the genes encoding Fdc (orange) and Cso2/Ado (light green), AroY (dark green) and C12O (purple), and LigV (blue) and VanAB (red) enzymes, respectively. The main functional elements of the plasmids include two multiple cloning sites, both preceded by a T7*lac* promoter and a ribosome binding site as well as a sequence encoding a 6-His tag at the N-terminal end and an S-tag at the C-terminal end. The plasmids also carry the kanamycin (Kn^R), ampicillin (Amp^R), and streptomycin (Sm^R) resistance genes. (Bottom) For each construct, the nucleic acid gel electrophoresis analysis of the DNA plasmid linearized by the *BamHI* restriction enzyme is shown. M = GeneRuler 1 kb DNA ladder (Thermo Fisher Scientific).

hyde), vanillic acid (4-hydroxy-3-methoxybenzoic acid), protocatechuic acid (3,4-dihydroxybenzoic acid), catechol (1,2-dihydroxybenzene), and *cis,cis*-muconic acid were purchased by Merck/Carlo Erba (Merck KGaA, Darmstadt, Germany). The commercial food-grade enzyme UltrafloXL was kindly supplied by Novozymes (Bagsværd, Copenhagen, Denmark), and wheat bran was a generous gift of Molino Dallagiovanna (Gragnano Trebbiense, Piacenza, Italy).

Ferulic Acid Recovery from Wheat Bran. The recovery was carried out using a thermo-enzymatic method similar to the one reported by Bautista-Expósito et al.[7] To improve the release of ferulic acid, wheat bran was pre-treated by milling it to a fine powder (3 min of total time by alternating 30 s of milling to a 30 s pause) before the thermo-enzymatic extraction. Wheat bran powder was suspended at a 1:20 solid to solvent ratio (g/mL) in 100 mM potassium phosphate pH 6.0 and autoclaved at 121 °C, 1 bar, for 30 min. Then, the suspension was cooled to room temperature and 1% UltrafloXL (enzyme to wheat bran powder dry weight ratio, w/w) was added. The enzymatic step was carried out at 37 °C, under shaking (130 rpm), for 24 h. The amount of alkaline-extractable ferulic acid, the reference value used to calculate the thermo-enzymatic recovery yield, was determined as stated by Di Gioia et al.[16] In detail, 0.5 g of wheat bran was suspended in 30 mL of 2 M NaOH and incubated at 30 °C on a rotatory shaker for 2 h; 25 μL of the reaction mixture was added to 50 μL of solvent A (see the HPLC Analytical Method section) and centrifuged for 5 min at 11,000*g*, 4 °C, and 20 μL of the supernatant was withdrawn for HPLC analyses.

The amount of soluble carbohydrates in the supernatant (diluted 1:250) was quantified using the phenol-sulfuric acid method. Briefly, the concentrated sulfuric acid was able to break down all polysaccharides into monosaccharides that react with phenol to form stable yellow-gold compounds that can be measured spectrophotometrically; the absorbance value at 480 nm was measured using a Jasco V-580 spectrophotometer (Cremella, Italy). The calibration curve was built using standard solutions of xylose in the 100−300 μg/mL concentration range.

Ferulic Acid Purification. To selectively recover ferulic acid from the crude extract after the thermo-enzymatic treatment, a method adapted from Di Gioia et al. was used.[16] The solid residue was removed from the crude extract by paper filtration, and then, the strong anion exchange resin Amberlite IRA 401 Cl^- (DuPont, Wilmington, USA) was added (1 g of resin to 40 mL of solution) to the enzymatic hydrolysate. The solution was incubated at room temperature on a rotatory shaker for 3−4 h. The suspension was dried, added to ethanol with 4% (v/v) HCl (15 mL per g of resin), and then incubated at room temperature on a rotatory shaker for 1 h to elute the bound ferulic acid. The alcoholic solution containing ferulic acid was separated from the resin, neutralized by adding 2 M NaOH, and then concentrated by a rotavapor. The resulting powder was solubilized in 100 mM Tris−HCl buffer, pH 8.0 and used for HPLC analyses (to evaluate the recovery yield) and as the substrate for the whole-cell biotransformation. A schematic representation of the recovery and purification of ferulic acid from the wheat bran is depicted in Figure 1.

https://doi.org/10.1021/acssuschemeng.2c06534
ACS Sustainable Chem. Eng. 2023, 11, 2476−2485

Vanillin Recovery from Kraft Lignin. Vanillin was isolated from Kraft lignin following the procedure described by Vignali et al.[13] In detail, a 100 mL mixture containing 0.5 g of Kraft lignin and 2 mM TEMPO was added to the recombinant laccase from *Bacillus licheniformis* (BALL) (100 U, 0.2 U enzyme per mg of lignin).[13,15,17] After 2 h of incubation at 30 °C under shaking (100 rpm) in 0.5 L baffled flasks, 1 N HCl was added until a final pH of 1.0 was reached. The sample was extracted using 3:1 (v/v) ethyl acetate as the organic solvent; the dewatered organic phase was then filtered using Whatman filter paper, dried in a rotary evaporator, and resuspended in 200 mM Tris−HCl buffer, pH 8.0 for HPLC analyses (to evaluate the recovery yield) and as the substrate for the whole-cell biotransformation. Following this procedure, vanillin was obtained with a recovery yield of ≈4.5 mg per gram of lignin, as evaluated by GC−MS analyses.[13]

Construction of Plasmids and Whole-Cell Biocatalysts. The synthetic genes encoding the enzymes involved in the biosynthetic pathway (Scheme 1) were designed by *in silico* back translation of the amino acid sequence reported in the GenBank database (Table 1), and the codon usage was optimized for expression in *E. coli*.

To facilitate subcloning into pRSFDuet-1, pETDuet-1, and pCDFDuet-1 (Novagen, Darmstadt, Germany), the sequences corresponding to *BamHI* (GGATCC) and *HindIII* (AAGCTT) restriction sites were added at the 5′- and 3′-ends of *Fdc*, *AroY*, and *LigV* genes, while the sequences corresponding to *NdeI* (CATATG) and *XhoI* (CTCGAG) restriction sites were added at the 5′- and 3′-ends of *Cso2*, *Ado*, *C12O*, and *VanAB* genes (Figure 2). The synthetic genes were produced by Twist Bioscience HQ (San Francisco, USA). The synthetic genes and the three plasmids were digested with the corresponding restriction enzymes (Fast Digest, Thermo Fisher Scientific, Monza, Italy) and ligated with T4 DNA ligase (Thermo Fisher Scientific), see Figure 2.

The ligation mixtures were used to transform NEB10-β (pRSFDuet-1 and pETDuet-1 plasmids) or JM109 (pCDFDuet-1 plasmid) *E. coli* chemically competent cells (the latter strain was selected since the NEB10-β cells are streptomycin-resistant). The presence of the insert was verified by digestion with the corresponding restriction enzymes. The *Fdc* and *Cso2*/*Ado* genes were inserted into the pRSFDuet-1 plasmid obtaining pRSFD:Fdc-Cso2 and pRSFD:Fdc-Ado, *VanAB* and *LigV* genes were inserted into the pCDFDuet-1 plasmid obtaining pCDFD:VanAB-LigV, and *AroY* and *C12O* genes were inserted into the pETDuet-1 plasmid obtaining pETD:AroY-C12O (Figure 2). Finally, different combinations of plasmids were introduced into the *E. coli* MG1655 RARE[20] strain through chemical transformation to construct the whole-cell biocatalysts. The cell biocatalyst containing pRSFD:Fdc-Cso2 was also co-transformed with the pGro7 (Takara Bio Europe, Saint-Germain-en-Laye, France) plasmid encoding the GroEL and GroES chaperone proteins.[8]

Cultivation of the Whole-Cell Biocatalysts. For the starting culture, the engineered strains used as the biocatalyst were inoculated in the LB medium containing the appropriate antibiotic (30 μg/mL kanamycin, 100 μg/mL ampicillin, or 50 μg/mL streptomycin) and grown at 37 °C, 130 rpm, for 18 h. For the strain containing the pGro7 plasmid, 25 μg/mL chloramphenicol was also added. The next day, 100 mL of Terrific broth containing the appropriate antibiotic was inoculated with an amount of starting culture to have an initial OD_{600nm} = 0.1 and the culture was incubated at 37 °C, 130 rpm, until OD_{600nm} ≈ 0.6. Then, the incubation temperature was lowered to 20 °C and the proteins' expression was induced by adding 0.5 mM IPTG. The cells were further grown at 20 °C, 130 rpm, for 18 h. When required, 1 mM $FeSO_4$ was also added together with IPTG. The cells expressing the Fdc and Cso2 enzymes were prepared following the protocol reported by Furuya et al.:[18] 100 mL of inoculated LB broth containing 100 μg/mL ampicillin (adding 25 μg/mL chloramphenicol and 4 mg/mL arabinose for recombinant cells carrying pGro7) was incubated at 37 °C, 130 rpm, until OD_{600nm} ≈ 0.8−1.0; the cell culture was then added to 0.1 mM IPTG and 1 mM $FeCl_2$ and incubated at 25 °C, 130 rpm, for 18 h. The cells were harvested by centrifugation (8000*g*, 10 min, 4 °C), washed once in reaction buffer (see the Whole-Cell Biotransformation section), and resuspended in the same buffer to have a final concentration of 350 mg/mL. Notably, the same expression protocol, followed by the harvesting and resuspension steps, was performed for untransformed *E. coli* MG1655 RARE cells, used as control during the bioconversion reactions.

Whole-Cell Biotransformation. The whole-cell biotransformation reactions were set up using the analytical grade substrates and the recombinant *E. coli* cells (70 mg cww/mL), in either 100−200 mM Tris−HCl pH 8.0, 100 mM NaOH-glycine pH 9.5, or M9 medium. The reaction was carried out in a 1 mL final volume, into a 4 mL glass vial. To test the effect of different additives on the biotransformation yield, glucose (10 g/L), glycerol (10% v/v), and DMSO (10% v/v) were added to the reaction mixture. The biotransformation reactions using wheat bran-derived ferulic acid or lignin-derived vanillin were set up similarly using wheat bran or lignin extracts instead of the analytical grade standards, adjusting the extract volume based on the substrate concentration. All biotransformation reactions were performed at 30 on a rotatory shaker. The biocatalytic processes were monitored by withdrawing at different times 50 μL of the reaction mixture, centrifuged for 5 min at 16,000*g*, 4 °C, to remove the cells. Then, 5 μL of the supernatant was added to 145 μL of solvent A (see the HPLC Analytical Method section) and centrifuged for 5 min at 16,000*g*, 4 °C, and 20 μL of the supernatant was analyzed by HPLC (see below).

Product Isolation and Characterization. The reaction mixture from the bioconversion of 10 mM ferulic acid (100 mL) was treated with 100 μL of methanol, centrifuged to eliminate *E. coli* cells, evaporated, and then dissolved in 500 μL of methanol. The product ccMA was characterized by mass spectrometry analysis using Waters ZQ-2000 instrument single quadruple in ESI mode,[12] and ^{1}H-NMR spectra were recorded in deuterated DMSO-d_6 (δ = 2.50 ppm) on a spectrometer operating at 400.16 MHz.

HPLC Analytical Method. HPLC analyses were performed on a Jasco apparatus equipped with a Kromaphase C8 column 100 Å, 5 μm, and 4.6 × 250 mm (Scharlab, Barcelona, Spain) and with a UV detector set at 276 nm. The flow rate was 1 mL/min, and the column oven was set at 25 °C. A binary system of solvent A (2.5% v/v formic acid) and solvent B (methanol) was used with the following gradient: 0 min, 90% solvent A + 10% solvent B; 0−20 min, ramping up to 25% solvent A + 75% solvent B; 20−21 min, ramping up to 100% solvent B. Calibration curves were obtained by solubilizing standards of ferulic acid, 4-vinylguaiacol, vanillin, vanillic acid, protocatechuic acid (PCA), catechol, and ccMA in 100 mM Tris−HCl, pH 8.0, at a final concentration of 40 mM. Subsequently, after dilution in the 0.04−2 mM range, 25 μL of each sample was added to 50 μL of solvent A and centrifuged for 2 min at 11,000*g*, 4 °C: 20 μL of the supernatant were injected for HPLC analyses. Retention times for standards of PCA, ccMA, catechol, vanillic acid, vanillin, ferulic acid, and 4-vinylguaiacol were 8.6, 10.4, 11.0, 12.8, 14.0, 15.1, and 21.1 min, respectively. The calibration curves are reported in the Supporting Information, Figure S1.

RESULTS AND DISCUSSION

Ferulic Acid Recovery from Wheat Bran. The wheat bran predominantly contains non-starch polysaccharides (38%), starch (19%), proteins (18%), and lignin (6%), which is mainly a (G-S)-type (i.e., a ratio S:G:H of 0.9:1.0:0.19).[21,22] Noteworthy, ferulic acid is the most abundant phenolic compound in wheat bran lignin followed by *p*-coumaric acid: figures of 9.7 ± 0.4 and 2.6 ± 0.5 mg/g wheat bran have been reported.[22] Ferulic acid is linked to arabinoxylans through ester links:[7] in the context, to develop a sustainable process for its release, the enzymatic hydrolysis of ester bonds is particularly attractive because of its environmentally friendliness and energy efficiency.[23] On the basis of its high efficiency for the release of free ferulic acid from wheat bran,[9] the UltrafloXL (containing cellulase and xylanase activities) was selected. To evaluate different pre-treatments,

https://doi.org/10.1021/acssuschemeng.2c06534
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reactions were set up starting from 2 g of wheat bran by (i) autoclaving at 121 °C, 1 bar, for 30 min; (ii) enzymatic treatment alone; (iii) autoclaving followed by the enzymatic treatment; and (iv) milling, autoclaving, and enzymatic treatment. The optimization of the "green" recovery process, representing the best choice to produce nature-identical vanillin compared to a chemical approach,[7,16] was set up based on the ferulic acid recovery yields using the value achieved employing an alkaline extraction treatment as reference. The latter method was used to determine the maximal amount of alkaline-extractable ferulic acid, under experimental conditions widely reported in literature:[24,25] a maximal value of 3.4 mg of ferulic acid per gram of wheat bran was achieved.[7,16] As shown in Table 2, the autoclaving and the enzymatic treatment alone reached an 7 and 22% recovery yield in comparison to the amount of ferulic acid obtained by the alkaline treatment, respectively, while a synergistic cooperative effect was observed for the combination of these two treatments: a 70.1 ± 3.2% yield was achieved. The obtained results suggest that the hydrothermal pretreatment allows easier access of hydrolytic enzymes by opening the cell wall structure, thus enhancing the breakdown of phenolic cross-links, as reported by Merali et al.[22] Noteworthy, compared with other pretreatment strategies, the hydrothermal treatment shows a significantly lower environmental impact, lower capital investment, the lack of chemicals, and a low byproduct generation.[26] The highest recovery yield (≈85% of the alkaline recovered ferulic acid) was obtained by milling the wheat bran to a fine powder before the combined (autoclaving and enzymatic hydrolysis) treatment: starting from 1 g of wheat bran, 3 mg of ferulic acid was recovered. Based on these results, the full treatment (milling and autoclaving pretreatments followed by the enzymatic hydrolysis; Table 2 and Figure 1) was selected for the ferulic acid isolation.

Table 2. Wheat Bran-Derived Ferulic Acid Recovery Yield

treatment	mg ferulic acid/g wheat bran	yield (%)
alkaline hydrolysis[a]	3.4	100
(i) autoclaving	0.3 ± 0.1	7.4 ± 1.5
(ii) enzymatic hydrolysis	0.8 ± 0.1	22 ± 3
(iii) autoclaving + enzymatic hydrolysis	2.3 ± 0.2	70 ± 3
(iv) milling + autoclaving + enzymatic hydrolysis	3.0 ± 0.2	85 ± 6

[a]Treatment used to calculate the maximal amount of achievable ferulic acid, taken as 100% recovery yield.

Ferulic acid was then purified through selective binding to an anion exchange resin: after the incubation with ethanol +4% HCl, more than 70% of the extracted ferulic acid was eluted. The solution was concentrated by a rotavapor obtaining a fine powder that could then be solubilized in the bioconversion buffer to be used as the substrate for the whole-cell biotransformation reactions (Figure 1). Notably, the water phase following the combined treatment contained 0.65 ± 0.06 g of carbohydrates per gram of wheat bran, allowing a valorization of such a component too.

Vanillin Production from Ferulic Acid. To explore the vanillin production from ferulic acid, a cofactor-independent enzymatic reaction system was set up and optimized: ferulic acid is converted to 4-vinylguaiacol by the ferulic acid decarboxylase Fdc from *B. pumilus*, and the carotenoid cleavage oxygenase Cso2 from *C. segnis* converts 4-vinylguaiacol to vanillin (pathway 1; Scheme 1).[18] The synthetic genes encoding Fdc and Cso2 (Table 1) were cloned in the high copy number pRSFDuet-1 plasmid, designed for the co-expression of two target genes (Figure 2). In particular, it was previously reported that the low catalytic efficiency of Cso2 and its thermal instability could affect the vanillin production.[27] In our study, the amino acidic substitutions A49P and Q390A were introduced in Cso2 (Table 1), as suggested by the site-directed mutagenesis study performed by Yao et al.,[28] and the high-copy number plasmid was used for its recombinant production.

The whole-cell biocatalyst was established in the engineered *E. coli* K-12 MG1655 RARE,[20] a strain showing reduced aromatic aldehyde reduction activities and aimed at preventing the conversion of vanillin into the byproduct vanillyl alcohol,[12] see below. Biotransformation reactions using resting cells were carried out using the engineered *E. coli* cells (35, 70, or 140 mg cww/mL), harvested and suspended in an M9 minimal medium or 100−200 mM Tris−HCl buffer, pH 8.0. As shown in Figure 3A, the whole-cell biocatalyst expressing Fdc and Cso2 (70 mg/mL cww incubated at 30 °C in 100 mM TrisHCl buffer, pH 8.0, hereinafter defined as "standard conditions") rapidly decarboxylated 10 mM ferulic acid into 4-vinylguaiacol while a limited amount of the final product vanillin was generated (reaching a 25% bioconversion yield only). The addition of 10 g/L glucose, 10% (v/v) glycerol, or 10% (v/v) DMSO to the reaction mixture significantly inhibited vanillin production: in all cases, the almost complete conversion of ferulic acid into 4-vinylguaiacol was achieved with no vanillin production (see Figure S2A). The co-expression of the GroEL and GroES chaperone proteins increased the vanillin yield up to a 50% value after 20 h of

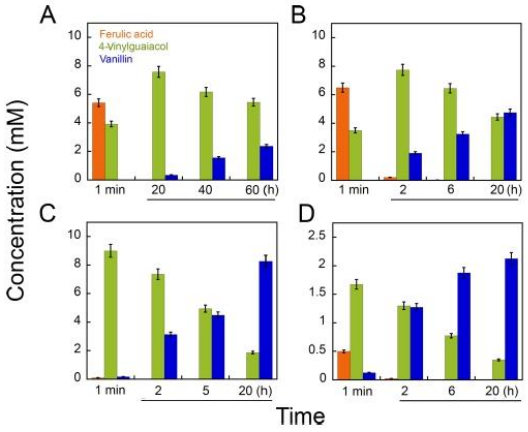

Figure 3. Time course of the one-pot bioconversion of 10 mM ferulic acid into vanillin (pH 8.0, 30 °C) by the *E. coli* whole-cell biocatalyst (100 mg/mL) expressing the enzymes (A) Fdc and Cso2; (B) Fdc, Cso2, and the chaperone proteins GroES and GroEL; and (C) Fdc and Ado. (D) Time course of the one-pot bioconversion of 2.5 mM ferulic acid extracted from wheat bran by the *E. coli* cell system expressing Fdc and Ado (strain *E. coli* II, 1 mL final reaction volume). The low level of ferulic acid at 1 min of reaction highlights the quick import and conversion (by the enzyme Fdc) of this compound. The values are reported as mean ± standard deviation (n = 3).

https://doi.org/10.1021/acssuschemeng.2c06534
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incubation under standard conditions (see Figure 3B and Figure S2B), indicating that the efficient folding of Cso2 protein is largely responsible of vanillin production. On the other hand, the addition of compounds able to favor protein folding, such as glycerol (10% v/v) and/or DMSO (10% v/v), as well as the incubation in 100 mM glycine-NaOH buffer, pH 9.5, did not improve the bioconversion yield.

To bypass the rate-limiting step catalyzed by the oxygenase Cso2 in the production of vanillin, the use of the alternative enzyme aromatic dioxygenase Ado from the thermophilic fungus *T. thermophila* (showing a 44% amino acid identity with Cso2) has been evaluated (Scheme 1).[19] The purified Ado enzyme shows a catalytic efficiency 78,500-fold higher than that of wild-type Cso2.[19] The *E. coli* biocatalyst expressing both Fdc and Ado enzymes, and named *E. coli* II, converted 10 mM ferulic acid in one-pot into the final product vanillin in a total of 20 h under standard conditions with a 90% yield (Figure 3C and Figure S2C). The incubation of 10 mM ferulic acid without the whole-cell biocatalyst or using untransformed *E. coli* cells did not generate any detectable reaction products. Taking into account that the Ado enzyme belongs to the carotenoid cleavage oxygenase family containing iron as a prosthetic group,[29] the effect of Fe^{2+} on the oxygenase reaction was investigated: adding 1 mM $FeSO_4$ in the medium (see the Materials and Methods section) did not affect the reaction rate and the bioconversion yield of the overall biotransformation of ferulic acid into vanillin. Moreover, the simultaneous addition of IPTG and 10 mM benzyl alcohol, to induce chaperone co-expression,[30] did not improve the bioconversion yield, and the supplement of 10 g/L glucose in the reaction mixture, to enhance the NAD(*P*)H cofactor regeneration,[31] significantly inhibited the vanillin production: a 30.2 ± 1.4% bioconversion yield was observed after 24 h of incubation.

As shown in Figure 3C, the oxidation of 4-vinylguaiacol to vanillin is the rate-limiting step of pathway 1 (Scheme 1). The bottleneck cannot be ascribed to a difference in the expression levels of Fdc and Ado enzymes (3 mg/g cells and 9.5 mg/g cells, respectively, see Figure S3). Although at present not supported by evidence, a chemical reaction between 4-vinylguaiacol and the formaldehyde produced by the oxygenase enzyme could explain the experimental observation, as reported for the same bioconversion pathway by Saito et al.[32]

The fast bioconversion of ferulic acid into 4-vinylguaiacol suggests that *E. coli* II cells can support high rates of substrate uptake, making this system useful for industrial applications. Moreover, the almost complete transformation of ferulic acid into vanillin demonstrates that the selected *E. coli* strain has no endogenous enzymes metabolizing these compounds. Noteworthy, the *E. coli* II whole-cell biocatalyst converted 85.4 ± 3.2% wheat bran-derived ferulic acid (2.5 mM) into vanillin in 20 h (Figure 3D), highlighting the possibility to obtain a fine specialty chemical such as vanillin at significant yield from an agricultural byproduct, a topic of high interest in wheat-producing countries.[33]

Bioconversion of Vanillin into *cis,cis*-Muconic Acid. Starting from the enzymatic cascade process set up to convert vanillin into ccMA,[12] now, we evaluated the assembling of this pathway in a single-engineered *E. coli* K-12 MG1655 RARE strain to promote the diffusion of intermediates in the confined intracellular space, thus reducing the mass transfer problem.[10] Compared to the cell-free bioconversion system,[12] the commercial enzyme xanthine oxidase catalyzing the oxidation of vanillin into vanillic acid and the THF cofactor-dependent demethylase LigM catalyzing the demethylation of vanillic acid into protocatechuic acid (PCA) have been substituted by the enzymes vanillin dehydrogenase LigV from *Sphingomonas paucimobilis* SYK-6 and vanillic acid *O*-demethylase VanAB from *P. putida* KT2440 (see Scheme 1; this strain encoding five recombinant enzymes was named *E. coli* V).

Recent synthetic biology studies highlighted an enhanced yield and productivity by balancing the expression of genes encoding enzymes involved in whole-cell biotransformation.[34,35] Accordingly, the synthetic genes encoding LigV and VanAB (Table 1) were cloned in the low copy number pCDFDuet-1 plasmid and the genes encoding AroY and C12O were cloned in the medium copy number pETDuet-1 plasmid (see Figure 2). The pETDuet-1 plasmid was selected for AroY expression since it harbors the same replication origin of pET24 previously used to express this decarboxylase.[12]

The cells expressing the vanillin dehydrogenase LigV completely converted 10 mM vanillin into vanillic acid in 20 h (Figure 4A). The co-expression of the demethylase activity consisting of the two-component enzymatic system VanA, an iron-dependent monooxygenase, and its partner electron transfer protein VanB, accepting both NADH and NADPH as the cofactor, pushed the vanillin oxidation and the oxidative demethylation reaction, thus resulting in the PCA production in a total of 2 h of incubation with a 95.2 ± 3.5% yield (Figure

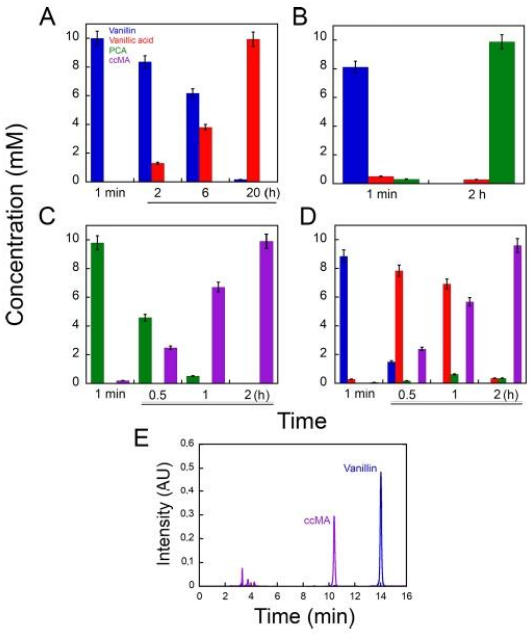

Figure 4. Time course of the one-pot bioconversion of (A) vanillin into vanillic acid, (B) vanillin into PCA, (C) PCA into ccMA, and (D) vanillin into ccMA catalyzed by the *E. coli* whole-cell biocatalyst expressing the enzymes LigV (A), LigV and VanAB (B), AroY and C12O (C), and LigV, VanAB, AroY, and C12O (strain *E. coli* V) (D). All the reactions were carried out on a 10 mM substrate in 200 mM Tris−HCl, pH 8.0, 30 °C (1 mL final volume). The values are reported as mean ± standard deviation (n = 3). (E) HPLC chromatograms of the mixture composition at the end of the biotransformation reaction reported in panel (D).

https://doi.org/10.1021/acssuschemeng.2c06534
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4B). Concerning the last two steps of the biocatalytic pathway, the whole-cell biocatalyst expressing AroY and C12O rapidly decarboxylated PCA into catechol and completely converted 10 mM of this substrate into ccMA (Figure 4C).

Noteworthy, the whole-cell biocatalyst was implemented by assembling the overall synthetic pathway (made of five enzymes) in a single strain and completely converted in one-pot 10 mM vanillin into ccMA in a total of 2 h (Figure 4D,E). The co-expression of all enzymes in the same biocatalyst shifted the reaction equilibria toward the production of the final, desired product ccMA and sped up the reaction rate, with no accumulation of intermediates (Figure 4E): a 95.2 ± 3.8% bioconversion yield was obtained in 2 h. Interestingly, the *E. coli* V whole-cell biocatalyst achieved the almost complete conversion of 1 mM lignin-derived vanillin in 30 min of incubation, thus highlighting the possibility to produce ccMA from a renewable and inexpensive biomass: under optimized conditions, 4.2 mg of ccMA has been produced from 1 g of Kraft lignin.

Production of *cis,cis*-Muconic Acid from Ferulic Acid. The whole-cell biocatalysis has been further implemented by assembling both pathways (see Scheme 1) in a single strain, thus offering the potential for production in a single pot: the *E. coli* VII whole-cell biocatalyst harbors three plasmids encoding all enzymes catalyzing the conversion of ferulic acid into ccMA (pathways 1 and 2 in Scheme 1). Under optimized conditions, 10 mM substrate was converted into ccMA with a 40.0 ± 2.1% yield in a total of 22 h of incubation: at such a time, a peak corresponding to ≈6 mM vanillic acid was still present (see Figure S4A). This could be due to the inhibition of the enzymatic activity of VanAB by 4-vinylguaiacol. Actually, an inhibition of the demethylase enzyme has been observed at increasing concentrations of isoeugenol (>5 mM), an aromatic compound structurally resembling 4-vinylguaiacol.[36] In agreement with this hypothesis, when the whole-cell biocatalyst expressing the enzymes LigV, VanAB, AroY, and C12O (*E. coli* V) was incubated with 10 mM vanillic acid in the presence of 5 mM 4-vinylguaiacol, the final product ccMA was obtained with a 30.1 ± 1.7% bioconversion yield after 2 h of incubation: two peaks corresponding to ≈5 mM vanillic acid and ≈3 mM PCA were still present at the end of the incubation (see Figure S4B). Noteworthy, in the absence of 4-vinylguaiacol, the almost complete conversion of vanillic acid into the final product was apparent (Figure S4B). Accordingly, the supplementation of an additional amount of the *E. coli* VII whole-cell biocatalyst (100 mg cww/mL final concentration) after 6 h of incubation (i.e., when the peak corresponding to 4-vinylguaiacol disappeared) allowed the almost complete conversion of ferulic acid into the final product ccMA in a total of 10 h of incubation (see Figure 5A).

Interestingly, the optimized reaction conditions were also used for the bioconversion of wheat bran-derived ferulic acid (2.5 mM): based on the intensity of the peak corresponding to ccMA, a ferulic acid bioconversion yield of 95.1 ± 4.1% after 10 h of incubation was apparent (see Figure 5B). The established bioconversion process produced 2.2 mg of ccMA from 1 g of the wheat bran biomass (see Figure 1).

The scale up of the bioconversion was performed at 10 mM ferulic acid in a 100 mL final reaction volume: the substrate was converted into the final product ccMA with a 96.1 ± 3.8% yield in a total of 10 h of incubation (see Figure 6). The product was recovered from the reaction mixture by methanol precipitation (15 mg) and identified by NMR and ESI-MS (see Figure S5): the ESI-full scan mass spectrum shows an (M-1)- m/z 141.27 and an intense product ion at m/z 97.05 that resulted from a decarboxylation process. An identical fragmentation pattern was observed for the commercial ccMA dissolved in methanol.[12]

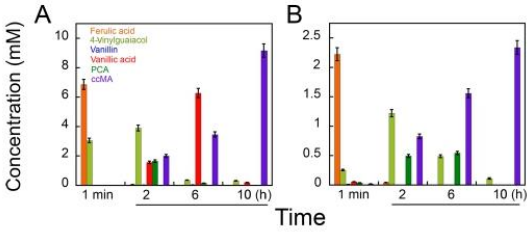

Figure 5. Time course of the one-pot bioconversion of (A) 10 mM standard ferulic acid and (B) 2.5 mM ferulic acid extracted from wheat bran into ccMA by the *E. coli* VII cell system, in 200 mM Tris–HCl pH 8.0, 30 °C (1 mL final volume). In both cases, after 6 h of incubation, an additional amount of cells (100 mg ccw/mL final concentration) was added. The low level of ferulic acid at 1 min of reaction highlights the quick import and conversion (by the enzyme Fdc) of this compound. The values are reported as mean ± standard deviation (n = 3).

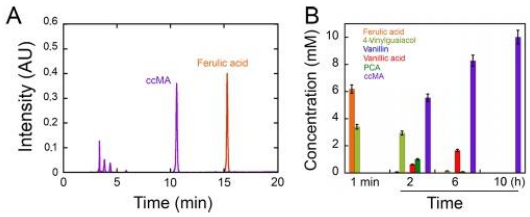

Figure 6. Bioconversion of 10 mM ferulic acid into ccMA, in 200 mM Tris–HCl, pH 8.0, 30 °C (100 mL final reaction volume). (A) HPLC chromatograms of ferulic acid (t0) and of ccMA produced after 10 h. (B) Time course of the reaction: the column bars report the relative amount of ferulic acid, 4-vinylguaiacol, vanillin, vanillic acid, PCA, and ccMA. The low level of ferulic acid at 1 min of reaction highlights the quick import and conversion (by the enzyme Fdc) of this compound. The values are reported as mean ± standard deviation (n = 3).

CONCLUSIONS

The global market potential of ccMA and its derivatives was estimated to exceed $22 billion per year,[14] compared to a global bio-vanillin market expected to reach $400 million by the end of 2025.[37] Accordingly, the production of ccMA from low-cost, renewable feedstocks using an engineered *E. coli* strain (and through the generation of ferulic acid and vanillin) represents a significant improvement toward the generation of relevant bioproducts from biomasses (such as adipic acid, a useful building block for the production of nylon 6,6-polyamide). Bacterial production of ccMA from lignin or lignin-model compounds has been recently revised.[36,38] The engineered strains such as *P. putida*, *Amycolatopsis* sp., *Rhodococcus opacus*, *E. coli*, and *Corynebacterium glutamicum* have been reported to produce high ccMA yields from lignin-based aromatics. The metabolic engineering of synthetic pathways, combined with the optimization of fermentation conditions in a fed-batch process, achieved high production levels of ccMA starting from different lignin-derived aromatics.

https://doi.org/10.1021/acssuschemeng.2c06534
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Table 3. Production of ccMA by Engineered Bacterial Strains

organism	production (g/L)	productivity (g/L·h)	fermentation process	reference
		p-Coumaric acid → ccMA		
P. putida KT2440 CJ103	13.5	0.17	fed-batch	42
P. putida KT2440 CJ238	55.4	0.77	fed-batch	39
P. putida KT2440 CJ242	49.7	0.50	fed-batch	47
P. putida KT2440 CJ781	43.0	0.40	fed-batch	48
		Guaiacol → ccMA		
Amycolatopsis sp. ATCC 39166 MA-2	3.1	0.13	fed-batch	49
		Catechol → ccMA		
C. glutamicum MA-2	85.0	2.4	fed-batch	40
P. putida KT2440 MA-9	36.1	4.4	fed-batch	41
		Pre-treated lignin → ccMA		
P. putida KT2440 CJ103	0.7	0.03	batch	42
P. putida KT2440 TMBHV002	0.3	0.01	batch	43
P. putida KT2440 MA-9	13.0	0.24	fed-batch	41
Amycolatopsis sp. ATCC 39166 MA-2	0.2	0.02	fed-batch	49
C. glutamicum MA-2	1.8	0.07	fed-batch	40
R. opacus PD630-MA6	1.6	0.02	fed-batch	50

Table 4. Comparison of Whole-Cell Production of ccMA from Vanillin and Ferulic Acid

organism	volume (mL)	substrate (mM)	time (h)	production (g/L)	productivity (g/L·h)	g ccMA/g Van or FA	reference
			vanillin (Van) → ccMA				
engineered *E. coli*	5	3.3	24	0.341	0.014	0.690	14
engineered *R. opacus* PD63	50	0.5	24	0.711	0.030	0.934	51
engineered *E. coli*	1	1	3	0.120	0.040	0.805	52
engineered *E. coli* (strain V)	1	10	2	1.421	0.710	0.934	this paper
engineered *E. coli* (strain V) starting from lignin	1	1	0.5	0.142	0.284	0.934	this paper
			ferulic acid (FA) → ccMA				
engineered *P. putida* KT2440	25	20	72	0.796	0.011	0.204	53
engineered *E. coli* (strain VII)	100	10	10	1.421	0.142	0.731	this paper
engineered *E. coli* (strain VII) starting from wheat bran	2.5	2.5	10	0.355	0.035	0.731	this paper

Recent state-of-the-art whole-cell conversion studies are reported in Table 3. The production of ccMA from *p*-coumaric acid reached 55 g/L using the engineered *P. putida* KT2440 CJ238, in which a global regulator of carbon catabolite repression was eliminated.[39] Becker et al.[40] engineered the *C. glutamicum* MA-2 strain with the elimination of muconate cycloisomerase enzyme (CatB) and the overexpression of the catechol-1 and 2-dioxygenase (CatA): final titers of 85 and 1.8 g/L ccMA were produced from catechol and hydrothermal pre-treated softwood lignin, respectively (Table 3). Noteworthy, a titer of 13 g/L ccMA was reached starting from a hydrothermal treated-softwood lignin in supercritical water (mainly containing catechol) using the engineered strain *P. putida* KT2440 MA-9 after 54 h of incubation.[41] Concerning batch processes in a shake flask, a titer of 0.3−0.7 g/L of ccMA was obtained by two different engineered *P. putida* KT2440 strains starting from an alkaline pre-treated liquor stream and the acid-soluble residue was obtained by the corn stover.[42,43]

The success of our proposed process using resting cells in a batch process is due to the optimization of the extraction procedures for selected starting molecules (i.e., vanillin from lignin and ferulic acid from wheat bran, the latter procedure generating a sugar-rich water fraction of further usefulness) and to the genetic engineering of an *E. coli* strain (with low aromatic aldehyde reduction activities) based on the use of plasmid combinations differing in copy numbers for the expression of the various recombinant enzymes. Actually, using the *E. coli* II strain, vanillin was produced at 85.4 ± 3.2% yield from 10 mM ferulic acid (Figure 3C), with a productivity of 1.3 g/L, in agreement with data reported in literature using an engineered *P. putida* KT2440 strain.[44] Noteworthy, the *E. coli* II strain produced nature-identical vanillin from 2.5 mM wheat bran-derived ferulic acid in 20 h (Figure 3D): vanillin produced from renewable resources can be labeled as "natural" by the European and U.S. legislation,[45] thus increasing its value. A similar process based on Fdc and Cso2 enzymes was previously reported:[18] we substituted the rate-limiting Cso2 with the most active aromatic dioxygenase Ado and employed a high-copy number plasmid for expression. Indeed, the analysis of the conversion of 10 mM ferulic acid by the *E. coli* VII strain (expressing all the enzymes of the novel biosynthetic pathway reported in Scheme 1) did not show any intermediate accumulation and highlighted the efficient transport of ferulic acid by the bacterial inner membrane transporters. The process generated 0.73 g of ccMA/g of ferulic acid following the isolation of the final product, corresponding to 1.4 g of ccMA/L in 10 h. Table 4 reports a comparison with previous whole-cell bioconversion processes of vanillin and ferulic acid into ccMA in a batch process: a faster ccMA production and a higher conversion of ferulic acid were obtained using the *E. coli* VII cell system.

Usually, a newly established biosynthetic process does not immediately fulfill the economic requirements for commercial operation. Biological lignin valorization has not yet been

https://doi.org/10.1021/acssuschemeng.2c06534
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conducted at an industrial/commercial scale; thus, a technoeconomic analysis (TEA) has not yet been reported.[38] From an economic point of view, the cost of a product = A + B/yield + C/Pv, where A is the capital cost, B is the raw material cost, C is the operating cost, and Pv is the volumetric productivity.[10] The use of a cheap raw material, such as wheat bran and lignin, an efficient bioconversion yield (0.73 g of ccMA/g of ferulic acid), a good volumetric productivity (1.4 g/L), and an easy product recovery, allows us to greatly reduce the production cost and indicate that our proof-of-concept study not only is technically feasible as demonstrated but also possesses significant potential to improve the profitability of renewable biomass valorization. Moreover, compared to a biocatalytic process, the use of whole cells circumvents the need for cell lysis and enzyme purification, thus reducing the catalyst cost. Taking into account the commercial cost of ferulic acid (1 €/g) and the low operational cost (40 €/L cell culture), our optimized whole-cell biocatalyst produces 1 g of ccMA from 1.4 g of ferulic acid starting from 20 mL of fermentation broth at approximately 5% of the commercial cost of the final product ccMA (40 €/g) and the use of ferulic acid recovered from an inexpensive renewable biomass such as wheat bran (at a commercial cost of 0.2 €/kg) leads to a 300-fold increase in economic value.

The ability of an engineered *E. coli* strain to generate ccMA from different natural and renewable feedstocks (instead of petroleum) makes this system well suited to set up a number of innovative processes aimed at generating bioproducts and bioplastic monomers: bacterial lignin valorization seems no more a "field of dreams".[46]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.2c06534.

HPLC calibration curves for ferulic acid, 4-vinylguaiacol, vanillin, vanillic acid, PCA, catechol, and ccMA; HPLC chromatograms of the bioconversion of ferulic acid into vanillin; SDS-PAGE analysis of cell pellets of the strain expressing Fdc and Ado enzymes; HPLC chromatograms of the bioconversion of ferulic acid and vanillic acid into ccMA; and identification of the final product ccMA by ESI-MS and ^{1}H-NMR analyses (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

L.P. and E.R. thank the support of Fondo di Ateneo per la Ricerca and CIB, Consorzio Interuniversitario per le Biotecnologie. We are grateful to Silvia Gazzola for the mass spectrometry and NMR analyses. F.M. is a student of the PhD course "Life Sciences and Biotechnology", University of Insubria.

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https://doi.org/10.1021/acssuschemeng.2c06534
ACS Sustainable Chem. Eng. 2023, 11, 2476−2485

3.2 Bio-based *cis,cis*-muconic acid production from vanillin using a growing cells approach

INTRODUCTION

Muconic acid (2,4-hexadienedioic acid) is a six-carbon di-unsaturated dicarboxylic acid which occurs in three isomeric forms: *cis,cis*-muconic acid (ccMA), *trans,trans*-muconic acis, and *cis,trans*-muconic acid. Muconic acid can be produced by chemical synthesis (from non-renewable oil-derived chemicals), bioconversion of lignin (from biotransformation of lignin-based aromatic compounds), and microbial fermentation of sugar; for a recent review see[1]. Muconic acid is a molecule of recognized industrial value[2] (estimated market in 2024 of US$ 119.4 million)[3]: it can be hydrogenated into adipic acid, a widely applied building block of commercial nylons and polyurethanes, and can be used as the starting material for making terephthalic acid in the synthesis of the plastic polymer polyethylene terephthalate (PET)[4]. Both adipic acid and terephthalic acid are also used in cosmetic, pharmaceutical, textile and food sectors[2]. Based on the increasing prices and diminishing oil availability, plastics production through environmentally friendly fermentation from renewables explain the increasing interest in bio-based muconic acid.

Notably, strains which produce ccMA from aromatics can employ lignin hydrolysates as starting material[5–7]. Lignin represents the second most abundant polymer on Earth; it can be treated to generate mixtures of aromatics through thermochemical[8,9] and biological de-polymerization[10–12]. Lignin is strongly underutilized: 98% is simply burned for energy supply, so that it represents a considerable source of renewable carbon. In past years, a number of efforts focused on the improvement of productivity and yield of high-value added compounds from lignin conversion by metabolic engineering of various bacteria. The biological production of ccMA (and related compounds) from lignin has been recently reviewed, see[1,13] and references therein.

Recently, our group setup of a bioprocess for producing ccMA based on the optimization of the extraction procedures for ferulic acid from wheat bran and vanillin from lignin, and the engineering of an *Escherichia coli* strain expressing up to seven recombinant enzymes[14]. In detail, the decarboxylase Fdc and the dioxygenase Ado converted wheat bran-derived ferulic acid into vanillin: vanillin was produced in one pot with a >85% yield in 20 h. Next, the dehydrogenase LigV, the demethylase VanAB, the decarboxylase AroY, and the dioxygenase C12O converted lignin-derived vanillin into ccMA with a >95% conversion yield (4.2 mg of ccMA/g of Kraft lignin in 30 min). When the optimized *E. coli* strain expressing all the seven enzymes was used on ferulic acid, ccMA was produced with a >95% conversion yield in 10 h: following product isolation, 0.73 g of ccMA/g of ferulic acid, and 2.2 g of ccMA/g of wheat bran biomass was produced.

In this study, we evaluated the ccMA production from vanillin by the abovementioned engineered *E. coli* strain using a growing cells approach by optimizing the medium composition and vanillin supply, followed by ccMA recovery and purification. We established an integrated process for ccMA production by bacterial fermentation.

MATERIALS AND METHODS

Strain, Growth Medium and Reagents

The *E. coli* MG1655 RARE strain[15] carrying the plasmids pETDuet-1:AroY-C12O and pCDFDuet-1:VanAB-LigV was constructed in a previous study[14]. Luria Bertani broth (Lennox), Terrific broth (modified), glycerol, D-(+)-glucose, α-lactose monohydrate and Antifoam 204 were purchased from Merck KGaA (Darmstadt, Germany). Methanol (ACS Grade, ≥99%), formic acid (ACS Grade, ≥98%), sulfuric acid (ACS Reagent, 95-98%), activated charcoal (DARCO®, 100 mesh particle size) and analytical grade standards of vanillin (4- hydroxy-3-methoxybenzaldehyde), vanillic acid (4-hydroxy-3-methoxybenzoic acid), protocatechuic acid (3,4-dihydroxybenzoic acid), catechol (1,2-dihydroxybenzene) and *cis,cis*-muconic acid were purchased by Merck KGaA (Darmstadt, Germany).

Bioconversion: Flask Cultivation

For the starting culture, the engineered strain was inoculated in Luria Bertani broth (LB) containing the appropriate antibiotics (100 μg/mL ampicillin and 50 μg/mL streptomycin) and grown at 30 °C, 180 rpm, for 18 h. The next day, 100 mL of Terrific broth (TB) containing the appropriate antibiotic, 10 g/L glycerol, 0.5 g/L glucose and lactose (2, 4 or 10 g/L) were inoculated with an amount of starting culture to have an initial OD_{600nm} = 0.1 and the culture was incubated at 30 °C, 130 rpm. When the OD_{600nm} value reached 3.0 – 3.5 (≈ 6 h), vanillin was added to the flask up to a final concentration of 10 mM. The bioconversion was monitored by withdrawing at different times aliquots of fermentation broth (2 mL) and processed for HPLC analysis (see "HPLC analytical method" section).

Bioconversion: Resting Cell Assay

Samples were withdrawn from the bioreactor at different times of cultivations, the cells were harvested by centrifugation (4000 rpm, 20 min, 4 °C), washed once in 200 mM Tris-HCl pH 8.0 and resuspended in the same buffer to have a final concentration of 350 mg cww/mL. The whole-cell biotransformation reactions were carried out in 1 mL final volume, into a 2 mL plastic tube.

The reactions were set up in 200 mM Tris-HCl pH 8.0 containing 70 mg cww/mL recombinant *E. coli* cells and 10 mM vanillin. All biotransformation reactions were performed at 37 °C on a rotatory shaker. The biocatalytic processes were monitored by withdrawing at different times 100 µL of the reaction mixture and processed for HPLC anlysis (see "HPLC analytical method" section).

Bioreactor Cultivations

Cultivations were performed in 2.5 L bioreactor vessels (BioBook compact – Kbiotech, Switzerland), autoclaved at 121 °C for 20 min before usage. Inoculum cultures of the engineered strain were grown in 50 mL of LB containing the appropriate antibiotics in 250 mL flasks at 30 °C and shaking at 180 rpm for 20 h. The composition of the media was: 50 g/L TB powder, 10 g/L glycerol, 0.5 g/L glucose, 2 g/L of lactose, 0.005% (v/v) Antifoam 204 and the appropriate antibiotics (unless stated otherwise). Culture aliquots (about 25 mL) were centrifuged at 4000 g at 4 °C for 10 min and the obtained bacterial pellets were resuspended using 20 mL of sterile medium collected from each bioreactor vessel. Cultivations were initiated by inoculating the cell suspension into the bioreactors through a sterile rubber septum at an initial $OD_{600nm} \approx 0.1$. Each batch cultivation was carried out in 1 L medium at 30 °C at a stirring speed of 200 rpm for 24-48 h. Dissolved oxygen (DO) in the medium was kept at 1% by increasing the stirring speed (up to 400 rpm) and by sparging compressed air and O_2, and pH was kept at 7.5 by adding 3 M NaOH. Vanillin was added by pulse-feeding a solution of 1 M vanillin in ethanol at a rate of 1 mL/h (unless stated otherwise). Samples were withdrawn at different times to evaluate the amount of biomass, carbon sources, acetate, ethanol and intermediates and product of the recombinant pathway in the medium (see "Growth Analysis" and "HPLC Analytical Methods").

Growth Analysis

Optical density of the samples was spectrophotometrically measured at 600 nm using MSE PRO Single Beam UV/VIS spectrophotometer apparatus: the experimental data points obtained were analyzed by the Gompertz equation[16] in order to build growth curves and calculate the maximum specific growth rates (μmax). Furthermore, the weight-optical density ratio (g/OD_{600}) for the cells was calculated. After the spectrophotometric analysis to evaluate the optical density of the culture, 10 mL of the sample were filtered by dead-end filtration using a 0.22 µm PTFE filter (previously weighted) to entrap *E. coli* cells on the filter. The filter was washed with MilliQ water and was weighted to measure the cells wet weight (cww). Then, the filter was dried in oven at 70 °C for 1 day and kept in a desiccator jar at room temperature for 1 day before being weighted again to

measure the cells dry weight (cdw). The weight-optical density ratio was estimated by linear regression over the experimental points plotted as total optical density versus weight (cww or cdw).

cis,cis-Muconic Acid Purification

ccMA was purified from the cultivation medium following the procedure described by Wang et al.[17] In detail, the broth was centrifuged at 8000 rpm, 4 °C for 20 min and the pellet was discarded. The supernatant was incubated with 10% (v/v) of activated charcoal at 37 °C, 180 rpm for 1 h; the activated charcoal was separated from the supernatant by centrifugation at 8000 rpm, 25 °C for 20 min and the remaining particulate in the supernatant was removed by dead-end vacuum filtration using a 0.45 μM PTFE filter. The filtrate was cooled down to 4 °C before adjusting the pH below 2.0 through H_2SO_4 (98-99%) addition. The resulting solution was incubated at 4 °C for 1 h to improve the ccMA crystals precipitation. ccMA crystals were recovered by dead-end vacuum filtration using a 0.45 μM PTFE filter, then the filter was dried at 37 °C for 48 h. The activated charcoal was incubated in MilliQ water at 25 °C for 10 min, the resulting suspension was then processed to recover ccMA crystal, as already stated. After the drying step was completed, ccMA crystals were weighted and the purity was assayed by HPLC analysis.

HPLC Analytical Method

Samples from growing and resting cell were centrifuged at 4000 rpm for 10 min and supernatants recovered for HPLC analysis. The quantification of glucose, lactose, glycerol, ethanol and acetate was performed on a Jasco apparatus equipped with a RezexTM ROA-Organic Acid H + (8%) column (300 × 7.8 mm; Phenomenex, Castel Maggiore, Italy) and a refractive index (IR) detector. The analysis was carried out using 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.8 mL/min, the oven was set at 80 °C. Medium sample supernatants were diluted 1:5 (v/v) in the mobile phase and passed through a 0.22 μm filter, then 100 μL were injected for HPLC analyses. The quantification of vanillin, vanillic acid, protocatechuic acid, catechol and ccMA was performed using a Kromaphase C8 column (100 A, 5 μm, and 4.6 × 250 mm; Scharlab, Barcelona, Spain) and with a UV detector set at 276 nm. The flow rate was 1 mL/min, and the column oven was set at 25 °C. A binary system of solvent A (2.5% v/v formic acid) and solvent B (methanol) was used with the following gradient: 0 min, 90% solvent A + 10% solvent B; 0−20 min, ramping up to 25% solvent A + 75% solvent B; 20−21 min, ramping up to 100% solvent B. Fermentation and resting cell sample supernatants were diluted 1:30 (v/v) in the solvent A and passed through 0.22 μm filter, then 100 μL were injected for HPLC analyses.

RESULTS

Optimization of Medium Formulation and Growth Analysis

The medium composition was set up to maintain similar operational conditions utilized for the whole-cell bioconversion system optimized in our previous study[14]: 50 g/L TB powder and 10 g/L (or 0.008% v/v) glycerol were used. To improve the economic sustainability of the overall process, the recombinant proteins expression was induced using lactose instead of isopropyl β-D-1-thiogalactopyranoside (IPTG), which is cheaper[18] and is safer on *E. coli* cells [19,20]. Lactose has been utilized successfully at different concentration to achieve high levels of expression of different recombinant proteins[18,21–24]. Since in our previous work[14] the expression of pathway's recombinant enzymes was induced using 0.5 mM IPTG, 2 g/L lactose was added to the fermentation medium to achieve a similar level of expression, as reported in[18]. Noteworthy, an additional advantage derived from the use of lactose as inducer, is that glucose can be added to the fermentation medium to prevent lactose uptake and metabolism while allowing rapid growth of the recombinant strain to the desired amount of biomass before protein induction starts, in the so-called "auto-inducing medium" expression[24]. Hence, to make the fermentation step more straightforward, lactose and glucose has been added to the growth medium to make it auto-inducing. The amount of glucose needed to build a new *E. coli* cell during growth under aerobic condition is ≈2.6×10^{9} molecules, a figure obtained by the sum of glucose molecules needed for carbon atoms (≈2×10^{9}; BNID 101859) and energy during aerobic growth (≈3-6×10^{8}; BNID 101778, 114702) needed to make one *E. coli* cell[25]. Since the concentration of *E. coli* K-12 MG1655 cells at 1 OD/mL is ≈7×10^{8} cell/mL (BNID 104831)[26], the amount of glucose needed for *E. coli* growth based to a certain generation number (G) was calculated according to Eq. (1):

$$Glucose\ consumption\ \left(\frac{g}{L}\right) = K\ \left(\frac{mg}{OD}\right) * N_i\ \left(\frac{OD}{mL}\right) *\ 2^G\ , \qquad (1)$$

where K (0.54 mg/OD) represents the amount of glucose utilized to build ≈7×10^{8} *E. coli* cells and N_i is the initial optical density of the culture. Since it is reported that starting lactose auto-induction during the log phase could inhibit bacterial growth resulting in low saturation density cultures[18], the culture should reach the late-log phase before the start of the proteins expression. In order to reach a high amount of biomass before recombinant proteins induction starts, it was estimated using Eq. (1), that an *E. coli* culture with an initial OD/mL of 0.1 could grow to 6.4 OD/mL (6^{th} generation) with 3.45 g/L of glucose. Accordingly, 3 g/L of glucose were added to the medium.

The pH value of the growth medium was maintained at 7.5 with the addition of 3 M NaOH, thus allowing either the engineered strain growth and an optimal pH for recombinant enzymes' activity.

Based on these assumptions, the final composition of the fermentation medium was: 50 g/L TB powder, 10 g/L glycerol, 3 g/L glucose, 2 g/L lactose, pH 7.5. Glycerol has been kept in the medium since it can be used as a carbon source without causing the inhibition of lactose metabolism, hence allowing the expression of recombinant proteins[27]. Lastly, in order to have a compromise between high conversion rate of the pathway's recombinant enzymes and the rapid growth of *E. coli,* the growth temperature was set at 30 °C.

The addition of vanillin to the fermentation medium prior to the inoculum could result in the growth inhibition[14,28], therefore growth analysis of the engineered strain to understand when the pathway's enzymes are expressed. The bioconversion yields of the engineered strain *E. coli* MG1655 RARE pCDFDuet-1:LigV-VanAB pETDuet-1:AroY-C12O were assayed by harvesting cells from the bioreactor at different times during growth and setting up bioconversion reactions with resting cells, in order to identify the optimal growth phase to add vanillin. Moreover, the metabolic profile was analyzed to understand when carbon sources were depleted in the growth medium, with particular attention to glucose consumption. The bioreactor cultures were started by inoculating an amount of pre-inoculum culture to have an initial OD/mL of ≈0.1.

In the aforementioned conditions, the engineered strain had a specific growth rate (μ) equal to 1.02 ± 0.04 h^{-1} (Figure 1A) and a saturation density of 6.0 ± 0.2 OD/mL (Figure 1B). HPLC analysis of the medium showed the complete consumption of glucose after 7 h of growth while lactose and glycerol remained in the medium at the concentration of 1.6 g/L and 5.1 g/L, respectively, up to 28 h of growth (Figure 1B). The ccMA production yield of the engineered strain was assayed by assembling resting cells bioconversion of vanillin into ccMA using cells harvested after 8 h (i.e. one hour after glucose was completely consumed) and 24 h of growth to verify if the longest induction time of expression of the pathway's enzymes could increase the bioconversion yield. Despite the complete conversion of vanillin into vanillic acid, only a small fraction of vanillin was converted into ccMA: ≈0.1% and ≈5.4% bioconversion yield, by the cells collected at 8 h and at 24 h, respectively (Table 1). The inducer of the lactose operon is the metabolite allolactose, generated from the transglycosylation of lactose catalyzed by the enzyme β-galactosidase[29]. The fact that lactose was still present in the medium after 24 h (i.e. 1.5 g/L) could indicate that the enzyme β-galactosidase was not highly expressed under these growing conditions, and therefore the concentration of allolactose was not enough for the efficient expression of the pathway's recombinant enzymes. This could be due to the persistence of glucose in the fermentation medium until the culture has already reached the initial stationary phase (Figure 1B), causing a weak induction of the lactose operon.

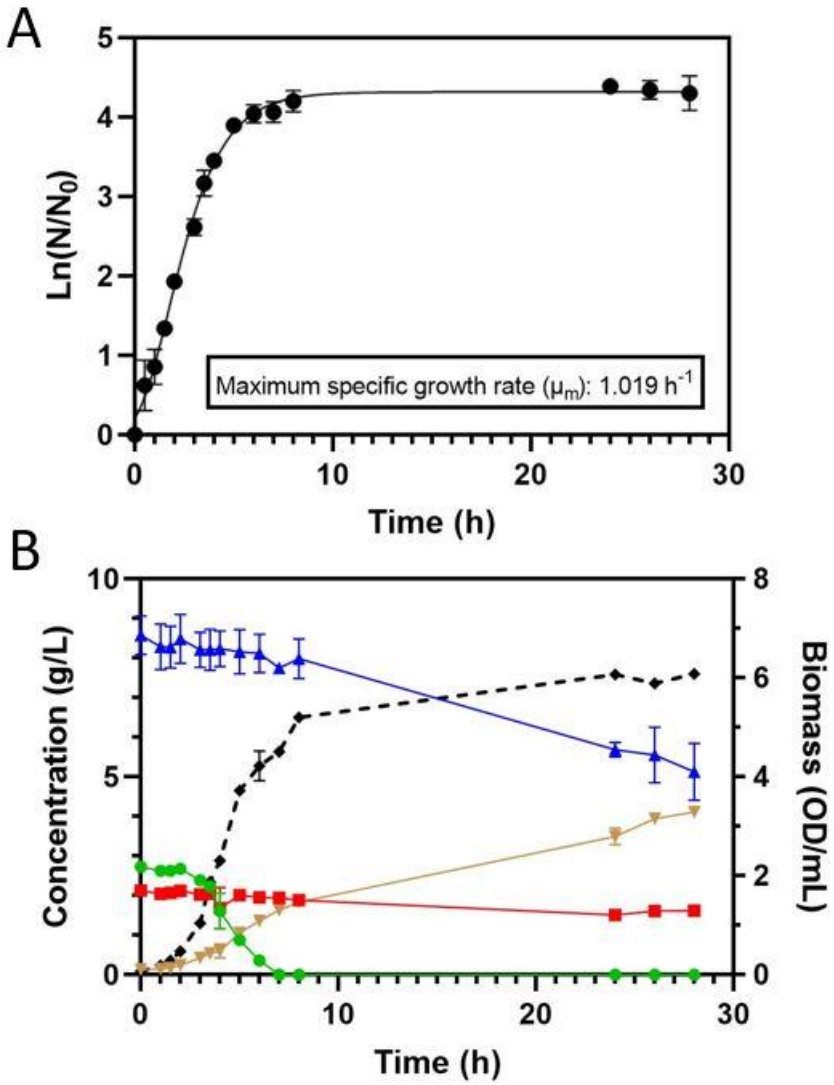

Figure 1. Growth curve, metabolic profile and biomass concentration of the engineered strain grown in fermentation medium containing 3 g/L glucose and 2 g/L lactose, without the addition of vanillin. A) Growth curve of the engineered strain calculated using Modified Gompertz equation[16]. B) Metabolic profile (continuous line) and biomass concentration (dotted line) of the engineered strain: glucose (green) is completely consumed after 7 h while ≈80% lactose (red; ≈1.6 g/L) remains in the medium after 28 h of growth. Glycerol and acetate labels are in blue and yellow, respectively. Data were collected from two independent biological replicates.

To solve this drawback, the amount of glucose added to the fermentation medium was decreased to start using lactose during the exponential phase. According to Eq. (1) the amount of glucose needed to allow the growth to early exponential phase (≈1.0 OD/mL) is 0.5 g/L. Besides using a lower concentration of glucose, the induction using 2 and 4 g/L of lactose was evaluated. When the growth medium contained 2 g/L lactose the engineered strain showed a specific growth rate equal to 1.22 ± 0.08 h^{-1} (Figure 2A) and a saturation density of 5.6 OD/mL (Figure 2B). HPLC analysis of the fermentation medium showed the complete consumption of glucose after 5 h of growth while lactose and glycerol remained in the medium at the concentration of 1.1 g/L and 5.1 g/L, respectively, up to 24 h of growth (Figure 2B). As expected, *E. coli* starts metabolising lactose after glucose has been depleted, between the 3rd and 5th hour of growth (Figure 2B). In this

condition, resting cells bioconversions were set up using cells harvested one hour after glucose was completely consumed (i.e. after 6 h fermentation), as well as at 8 and 24 h, to see if increasing the induction time could improve the bioconversion yield. The best conversion yield (≈32%) was obtained by the cells harvested at 6 h (Table 1), highlighthing that the engineered strain is able to convert vanillin into ccMA as early as 1 h after the glucose is completely consumed.

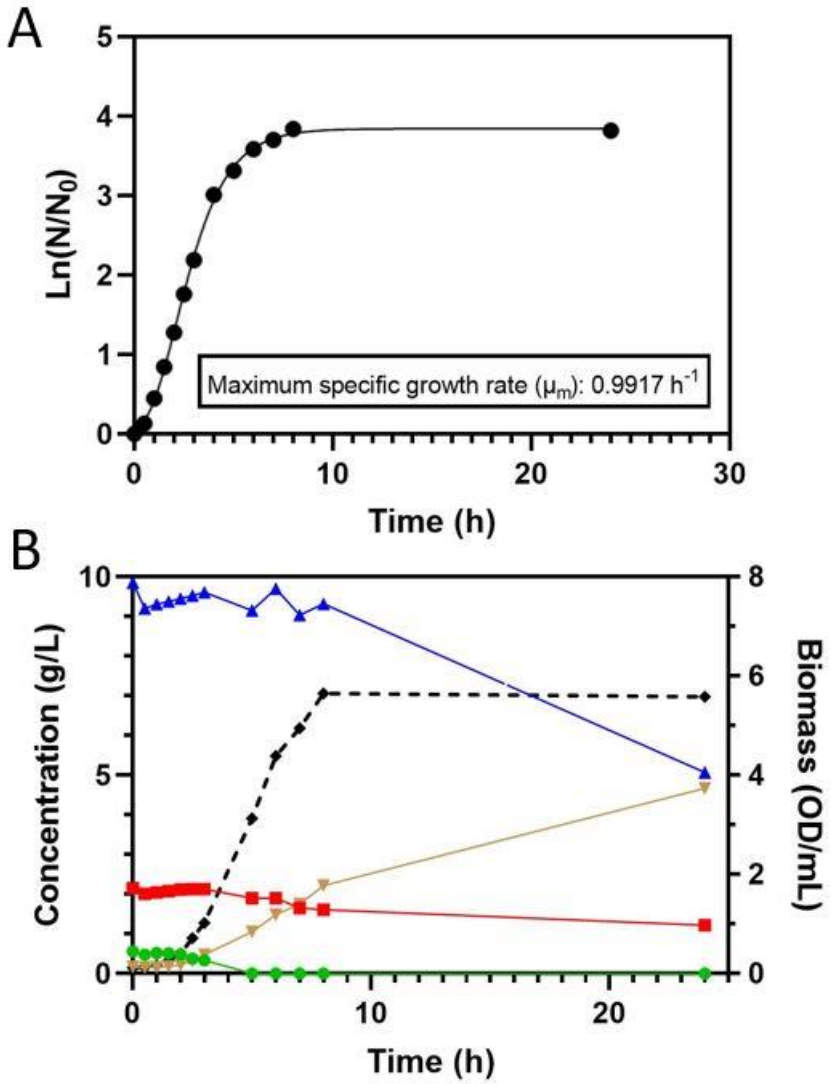

Figure 2. Growth curve, metabolic profile and biomass concentration of the engineered strain grown in fermentation medium containing 0.5 g/L glucose and 2 g/L lactose, without the addition of vanillin. A) Growth curve of the engineered strain calculated using Modified Gompertz equation[16]. B) Metabolic profile (continuous line) and biomass concentration (dotted line) of the engineered strain: glucose (green) is completely consumed after 5 h while ≈55% lactose (red; ≈1.1 g/L) remains in the medium after 24 h fermentation. Glycerol and acetate labels are in blue and yellow, respectively. Data were collected from a single biological replicate.

The increase of the amount of lactose in the medium could improve pathway's enzymes expression and, therefore, the bioconversion yield. Accordingly, the engineered strain was growth in a medium with double the amount of lactose (4 g/L). Despite the growth with 4 g/L lactose of the engineered strain showed a course similar with the one obtained using 2 g/L of lactose, with a μ of 1.12 ± 0.08 h^{-1} (Figure S1A) and a saturation density of 5.1 OD/mL (Figure S1B), the best conversion yield

for resting cells was only ≈21% and was obtained with cells harvested after 8 h of growth. Based on these evidences the medium formulation containing 0.5 g/L glucose and 2 g/L lactose, with the addition of vanillin after 6 h from the start of the culture was used. During these cultivations the build-up of foam was observed in the bioreactor, therefore 0.005% (v/v) Antifoam 204 was added. The presence of the antifoam does not alter the specific growth rate (Figure 3A), metabolic profile and saturation density (Figure 3B), so it was used for the following bioreactor cultivations.

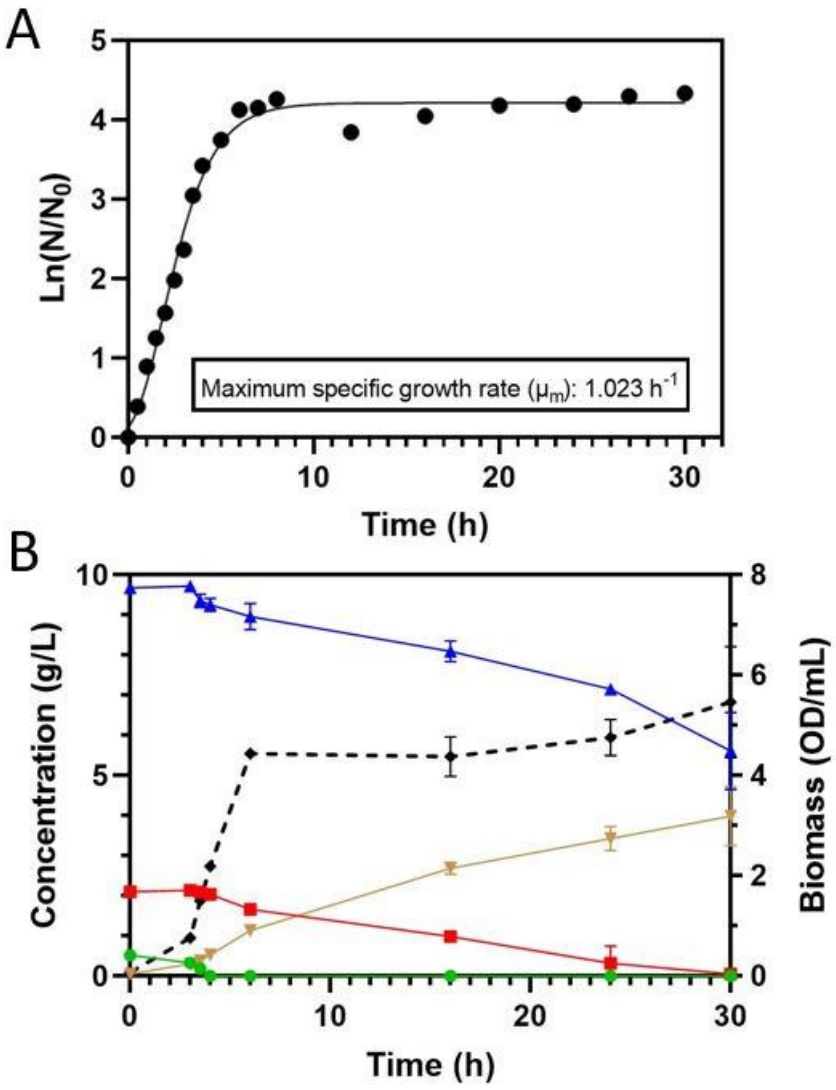

Figure 3. Growth curve, metabolic profile and biomass concentration of the engineered strain grown in fermentation medium containing 0.5 g/L glucose, 2 g/L lactose and 0.005% (v/v) Antifoam 204, without the addition of vanillin. A) Growth curve of the engineered strain calculated using Modified Gompertz equation[16]. B) Metabolic profile (continuous line) and biomass concentration (dotted line) of the engineered strain: glucose (green) is completely consumed after 4 h and lactose (red) after 24 h fermentation. Glycerol and acetate labels are in blue and yellow, respectively. Data were collected from two independent biological replicates.

Table 1. ccMA production yield obtained by bioconversion reactions of 10 mM vanillin using resting cells harvested from the cultivation using different media formulation.

Glucose (g/L)	Lactose (g/L)	Harvest time (h)	ccMA production yield (%)
3	2	8	0.11
		24	5.41
0.5	2	6	31.98
		8	22.62
		24	27.57
0.5	4	6	9.81
		8	21.34

Bioreactor Fermentation Optimization

Since it has been reported that VanA activity is inhibited by high amount of vanillic acid in the reaction system, it has been supposed that the modality of vanillin addition could have a significant impact on ccMA production[30]. Thus, two different strategies of vanillin addition were evaluated: i) spike and ii) pulse-feeding mode. In details, in the spike approach 10 mmol of vanillin (10 mM final concentration) were added all at once 6 h after the inoculum of the biocatalyst strain *E. coli* MG1655 RARE pCFD:LigV-VanAB pETD:AroY-C12O whereas in the pulse-feeding method vanillin was added at a rate of 1 mmol/h starting from 6 h. In the spike approach, vanillin was almost completely converted into ccMA in 30 h and an accumulation of vanillic acid was observed from 8 to 24 hours (Figure 4A). Using the pulse-feed approach, the almost complete conversion was reached after 16 h of incubation (10 h after the start of the pulse-feed), this representing almost half of the time required to reach the same result using the spike approach (Figure 4B).

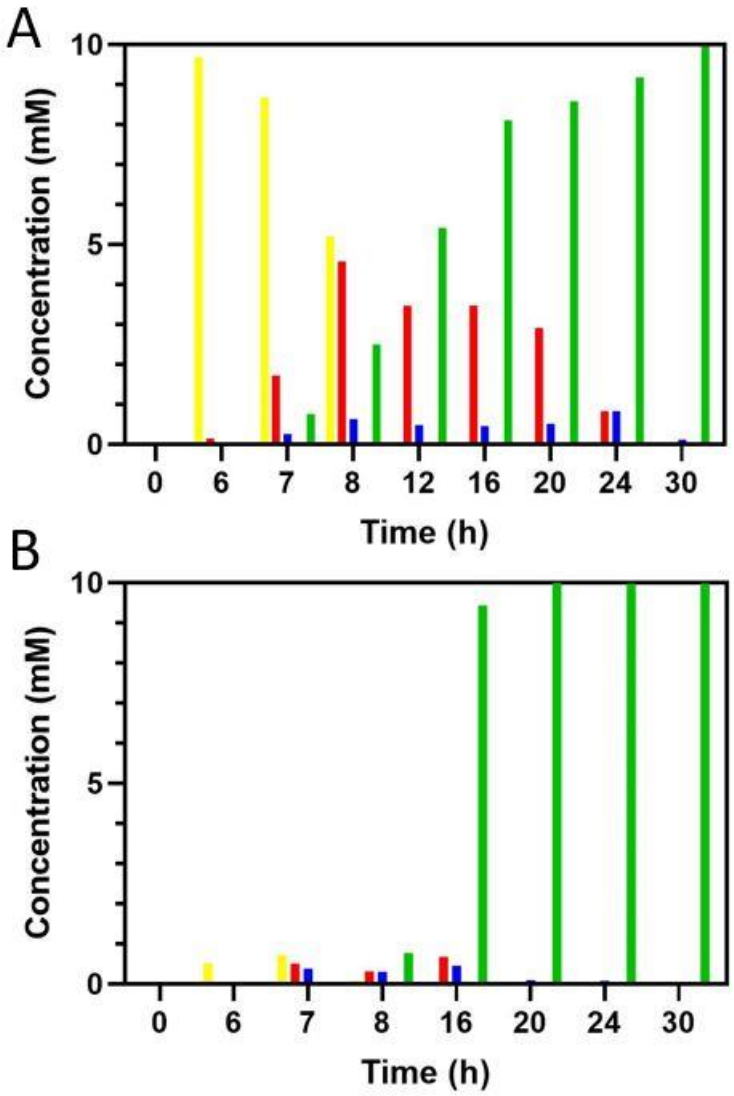

Figure 4. Time course of the bioconversion of 10 mM vanillin, added to growing cells using two different strategies. A) Spike mode: 10 mmol of vanillin were added in the bioreactor 6 h after the growth started. B) Pulse-feed mode: 6 h after the growth started 10 mmol of vanillin were added in the bioreactor through a 1 mmol/h pulse-feed that lasted for 10 h. The metabolites label are as follow: vanillin is yellow, vanillic acid is red, protocatechuic acid is blue, catechol is brown and *cis,cis*-muconic acid is green. Data were collected from a single biological replicate.

Subsequently, two different growths were set up both at a final vanillin concentration of 40 mM: i) 2 mmol/h vanillin pulse-feeding for 20 h, to verify if the engineered strain is able to maintain a high conversion yield at a higher vanillin addition rate, and ii) 1 mmol/h vanillin pulse-feeding for 40 h, to test whether the engineered strain maintain its catalytic prowess at a higher amount of vanillin and when used for longer periods of time. As shown in Figure 5, an accumulation of vanillic acid (≈25 mM at 30 h) in the 2 mmol/h pulse-feeding cultivation was observed, showing that the engineered strain is not able to fully convert vanillin into ccMA at this addition rate. Notably, the engineered strain's growth seems to be impaired by this vanillin addition rate: the saturation density observed was only ≈3.4 OD/mL (Figure 5B) vs. 5.0 - 6.0 OD/mL (Figure 3). Accordingly, the low

biomass concentration in the bioreactor could be involved in the accumulation of vanillin acid and the consequently low ccMA production yield (Figure 5A).

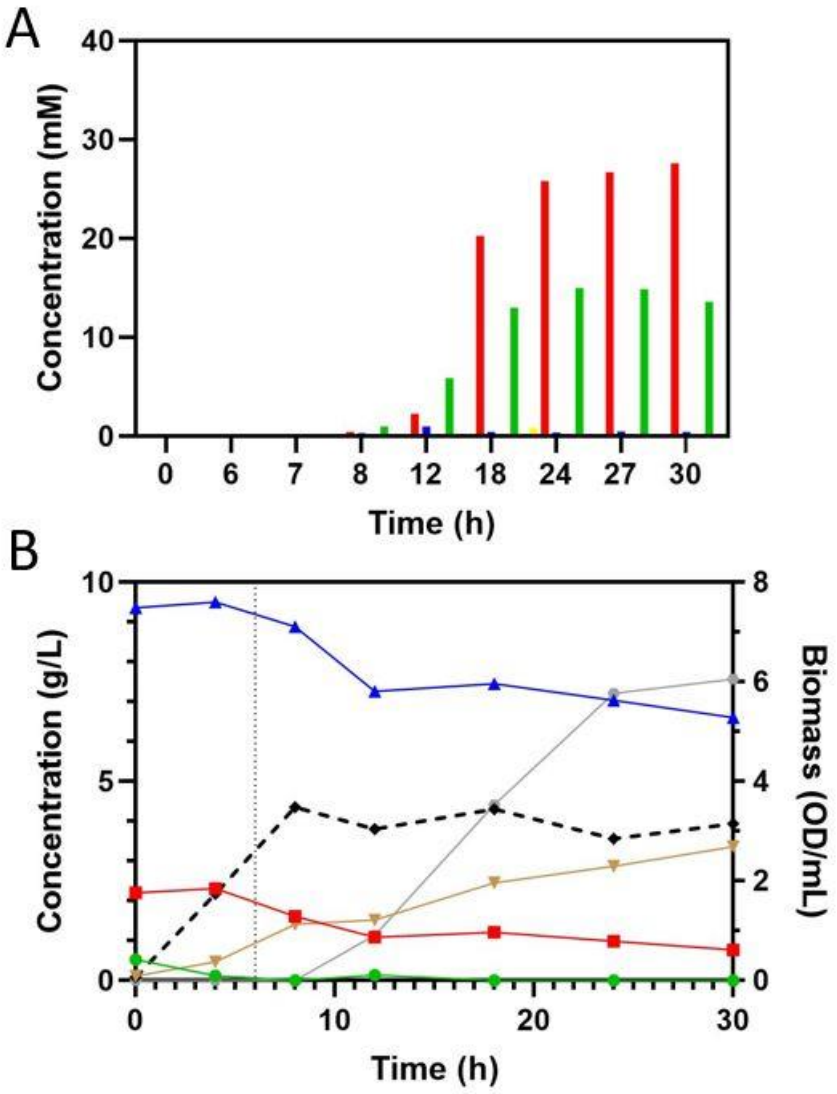

Figure 5. Time course, metabolic profile and biomass concentration of the bioconversion of 40 mM vanillin added through a 2 mmol/h pulse-feed. A) Time course of the bioconversion. The bioconversion labels are as follow: vanillin is yellow, vanillic acid is red, protocatechuic acid is blue, catechol is brown and *cis,cis*-muconic acid is green. B) Metabolic profile (continuous line) and biomass concentration (dotted line) of the bioconversion: glucose (green) is completely consumed after 4 h, ≈40% lactose (red; ≈0.8 g/L) and ≈65% glycerol (blue; ≈6.5 g/L) remained in the bioreactor after 30 h fermentation. The labels for acetate and ethanol are in yellow and grey, respectively. The vertical dotted line indicates when the vanillin pulse-feed started. Data were collected from a single biological replicate.

Interestingly, in the cultivation at 1 mmol/h vanillin pulse-feeding addition with a duration of 40 h, showed a saturation density of the culture comparable with the biomass concentration (≈5.0 OD/mL) obtained during the growth analysis (Figure 3) and the lactose and glycerol completely depleted after 24 h and 42 h, respectively (Figure 6B). Under these conditions, the engineered strain produced ≈36 mM (≈5.1 g/L) ccMA from 40 mM vanillin over 48 h of incubation (Figure 6A), thus reaching a ≈90% bioconversion yield of the vanillin added in the bioreactor. Notably, all the carbon sources were completely depleted during the fermentation, meaning that the engineered

strain remained in active growth over the 48 h, and that the conversion of vanillin into ccMA continued even after the lactose was completely consumed. The bioconversion of 40 mmol vanillin into ccMA using 1 mmol/h pulse-feeding approach was carried out in triplicate: an overall 91.7 ± 0.06 conversion yield of vanillin into ccMA with a final ccMA titer of 36.7 ± 2.5 mM after 48 h was achieved, with a similar metabolic profile (Figure S2).

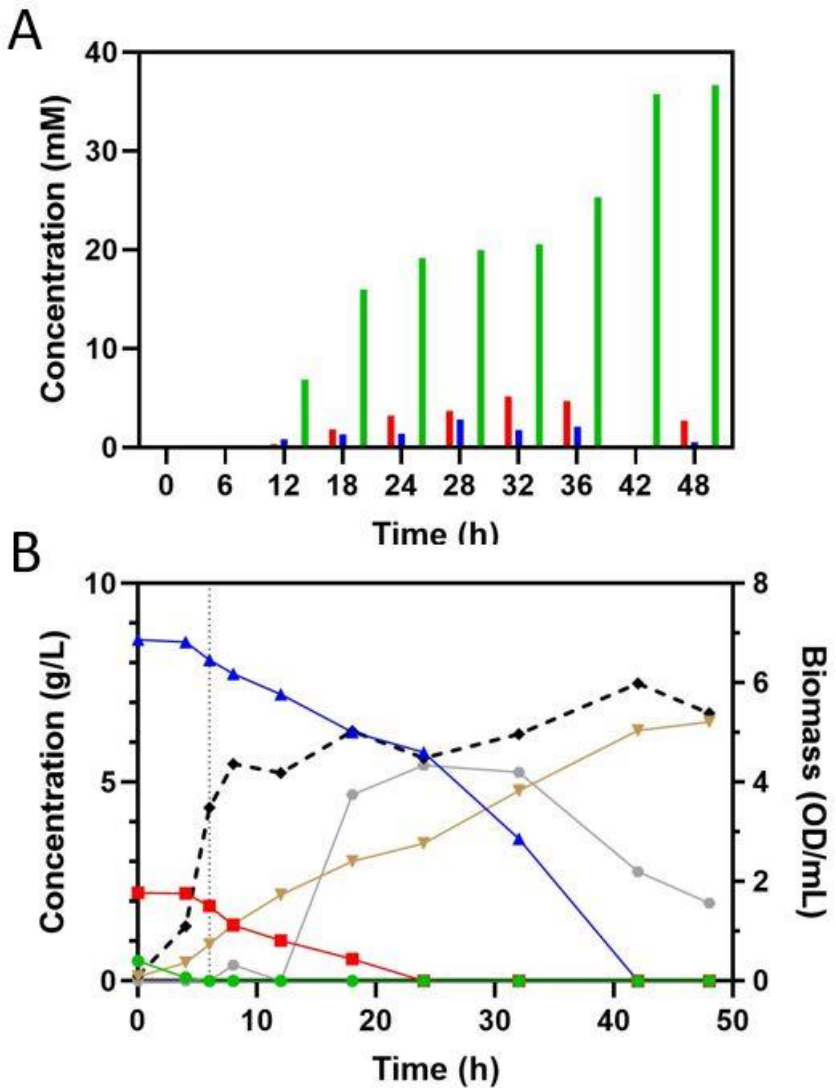

Figure 6. Time course, metabolic profile and biomass concentration of the bioconversion of 40 mM vanillin added thorugh a 1 mmol/h pulse-feed. A) Time course of the bioconversion. The bioconversion labels are as follow: vanillin is yellow, vanillic acid is red, protocatechuic acid is blue, catechol is brown and *cis,cis*-muconic acid is green. B) Metabolic profile (continuous line) and biomass concentration (dotted line) of the bioconversion: glucose (green) is completely consumed after 4 h, lactose (red) after 24 h and glycerol (blue) after 42 h. The labels for acetate and ethanol are in yellow and grey, respectively. The vertical dotted line indicates when the vanillin pulse-feed started. Data were collected from a single biological replicate.

cis,cis-Muconic Acid Purification

The supernatants were separated from the biomass obtained under the optimized condition (see above) through centrifugation and ccMA was purified, a total of 2.58 ± 0.07 g ccMA per liter of

supernatant was obtained corresponding to a recovery yield of 49.5 ± 0.07%, with a >95% purity of ccMA crystals. The recovery yield and the purity of ccMA crystals were comparable to the ones reported by Wang et al.[17] In order to increase the recovery yield, the used activated carbon was resuspended in water and the purification protocol was restarted from the step involving the incubation at 37 °C for 1 h in agitation[17]. Despite following the same protocol, no additional ccMA was recovered. The lower overall recovery yield could be due to the difference between the ccMA concentrations in the supernatants, 5.11 g/L (this work) and 20.55 g/L in [17], that combined with ccMA losses due to activated charcoal interactions and the minimal solubility of protonated ccMA in water (≈ 1 g/L[31]) do not allow the separation of the remaining ccMA by crystallization.

DISCUSSION

ccMA is a valuable precursor for the production of polyamides' and polyesters' monomers, such as adipic acid, caprolactam and terephthalic acid[1,13,32,33]. Indeed, the global market for ccMA estimated to have an annual growth of 7% reaching a value of 110 million USD in 2024[33]. Moreover, the projected annual global market potential ccMA derivatives is estimated to surpass $22 billion[32]. Consequently, the development of a biotechnological process for the production of bio-based ccMA is of great interest.

ccMA production using a biocatalytic approach from renewable feedstocks could be a more cost-effective and sustainable alternative to the chemocatalytic approach using petroleum-based feedstock, diminishing oil reliance for the production of plastics materials[1,33,34]. The microbial ccMA production has been attempted using either fermentable substrates (i.e. glucose and glycerol) and/or aromatic compounds obtainable from lignin hydrolysis[1]. ccMA bioconversion from fermentable substrates has been carried out mainly using engineered *E. coli* and *S. cerevisiae* strains: the highest ccMA yields reported are 64.5 g/L in 120 h with an engineered *E. coli* strain[35] and 22.5 g/L in 118 h with an engineered *S. cerevisiae* strain[17]. On the other hand, for the production of ccMA from lignin-derived aromatics, the use of engineered lignin-utilizing microorganisms such as *C. glutamicum*, *P. putida*, *Amicolatopsis* sp. and *R. opacus*, is generally preferred, due to their resistance to high concentrations of phenolic compounds and their innate ability to metabolize lignin-derived substrates[1,13,34,36]. The bioconversion yields vary widely depending on several different lignin-derived aromatics used as substrate and the microbial species. Two of the most notable results are the production of 85 g/L of ccMA from catechol in 36 h using an engineered *C. glutamicum* strain[37] and the production of 55.4 g/L of ccMA from *p*-coumaric acid in 72 h using an engineered *P. putida* strain[38]. Nevertheless, for the polysaccharides fraction and the glycerol used for the lignocellulose

pretreatment there are already established valorization processes mostly aimed at biofuels production[39]. Meanwhile, there is no established process for the valorization of the lignin fraction, thus, there is a greater interest in developing new efficient and scalable lignin valorization processes. In this view, an engineered *E. coli* strain harboring genes encoding four different recombinant enzymes (LigV, VanAB, AroY and C12O) to convert vanillin into ccMA was developed. This engineered strain, when utilized in a biocatalytic process using resting cells (70 g cww/L) converted 10 mM vanillin into ccMA with a >95% yield, in 2 h[14]. In this work, the aforementioned engineered *E. coli* strain was used to develop and optimize a more efficient and economic biocatalytic process to convert vanillin into ccMA. The bioconversion reaction was performed through a cultivation in bioreactor suing growing cells, which not only make easier to control reactions conditions, such as pH and substrate pulse-feed rate, but reduce the number of steps in the biocatalytic process and make it more scalable. Moreover, to reduce process' cost, recombinant enzyme's expression has been induced using lactose instead of IPTG[18]. The best conditions for the vanillin bioconversion were investigated by modulating the amount of glucose and lactose in the fermentation medium[40]. The best result was obtained using 2 g/L lactose, as already reported in literature[40]; further addition of lactose (4 g/L) resulted in a lower specific growth rate and saturation density of the culture. In addition, vanillin was added using a pulse-feed strategy (1 mmol/h) in order to reduce the inhibition of VanA activity that could arise from vanillic acid accumulation in the reaction system[30]. The optimized growth medium composition and the substrate's addition strategy used, allowed the engineered strain to produce 5.2 ± 0.36 g/L of ccMA in 48 h, corresponding to 0.86 g ccMA/g vanillin. The produced ccMA was purified from the fermentation broth by crystallization, obtaining ≈2.6 g per liter of broth with a purity of ≈50%.

As shown in Table 2, green metrics has been calculated for the bioconversion of vanillin into ccMA with resting cells, the cultivation process alone and the cultivation process plus the downstream ccMA purification step.

Table 2. Comparison between the green metrics of bioconversion processes using resting cells, cultivation with growing cells alone, and the cultivation process plus the downstream ccMA purification step.

	Stoichiometric Factor (SF)[41]	**Carbon Efficiency (CE)**[42]	**Atom Efficiency (AE)**[43]	**Actual AE (AAE)**[43]	**E-value**[44]	**Process Mass Intensity (PMI)**[45]
Resting cells	1.0	67.5	93.4	93.4	30.2	1417.1
Growing cells	1.0	67.5	93.4	84.1	0.4	195.2
+ ccMA purification				42.0	39.2	428.9

For a better comparison of two different strategies of biocatalysis, metrics have been evaluated by calculating the amount of biomass, substrate and solutions needed to produce the amount of ccMA obtained by the fermentative process (i.e. 5.2 g/L). The AE, CE and SF are the same for all bioconversion processes due to using the same biocatalytic pathway, since these parameters evaluate the amount of substrate mass retained in the product[41–43]. The bioconversion using resting cells showed a higher AAE value compared to the process performed in fermentation due to the higher conversion rate (this parameter is calculated by multiplying AE with product's relative yield[43]). The E-value is the ratio of the amount of waste produced to the amount product obtained[44] and the PMI is the ratio of the amount of materials used for the process to the amount product obtained[45]. In both cases, the approach using growing cells is more sustainable than the resting cells one and the PMI results lower even when the purification step is taken into consideration. Comparing the E-factor of our processes with the ones designated by Sheldon[44], the process based on growing cells alone has an E-value suitable for the production of bulk chemicals (<1-5 kg product/kg waste) while when the purification step is considered the E-value arose to a figure acceptable for the production of fine chemicals (5-50 kg product/kg waste).

The values reported in Table 2, highlight that the approach based on growing cells is more sustainable compared to the bioconversion with resting cells, despite the lower AAE, even when is combined with the product purification step. This is due to the lower number of steps, thus removing the use of buffer salts and additional water, and the lower amount of biocatalyst needed (2 g cdw vs. 36 g cdw) for the fermentative process. The ccMA purification step has not been considered for the resting cells process because of the lower ccMA titer (1.41 g/L) that does not allow an efficient crystallization. A comparison of the biocatalytic parameters for bioconversions performed with resting cells and with the fermentative process is shown in Table 3. The resting cells process was evaluated also considering the biocatalyst preparation step (requiring a total of 26 h) and the 2 h bioconversion reaction.

Table 3. Comparison of biocatalytic parameters for the bioconversion process performed using resting cells and growing cells.

	Production (g/L)	**Catalyst production (g/L×g cdw cells)**	**Productivity (g/L×h)**	**Catalyst productivity (g/L×h×g cdw cells)**	**g ccMA / g Vanillin**
Resting cells	1.42	0.14	0.71	0.07	0.93
+ preparation			0.05	0.005	0.93
Growing cells	5.2	2.6	0.11	0.05	0.86

The process with growing cells allowed to reach a 4-fold higher ccMA production compared to resting cells, 5.2 g/L and 1.42 g/L respectively. ccMA production difference are most notable when normalized on the amount of cell utilized in the bioconversion: 2 g cdw/L and 10 g cdw/L in the growing and resting cells approach, respectively, which is ≈17-fold higher in the fermentation (2.6 g/L×g cdw cells vs. 0.14 g/L×g cdw cells). On the other hand, the resting cells approach showed a better productivity (0.7 g/L×h) and catalyst productivity (0.07 g/L×h×g cdw cells) in comparison to the growing cells process, with 0.11 g/L×h and 0.05 g/L×h×g cdw cells, respectively. Nonetheless, when the preparation step of the resting cells process is taken into consideration, the productivity of the process based on growing cells is ≈2-fold higher than the one with resting cells (0.11 g/L×h vs. 0.05 g/L×h) which became ≈10-fold higher (0.05 g/L×h×g cdw cells vs. 0.005 g/L×h×g cdw cells) when normalized on the amount of cells utilized.

To the best of our knowledge, this is the first report of a biocatalytic process producing ccMA from vanillin using an engineered *E. coli* strain grown in a bioreactor. Despite the improvements of using growing cells over the resting cells approach, this is a pilot-study on the possible production of plastic materials from lignin-derived feedstocks by microbial fermentation. The proposed process could be further optimized by increasing the biomass concentration in the bioreactor, which could lead to a higher achievable ccMA titer allowing for more effective purification step and making the process industrially viable and better comparable with alternative bacterial strains[37,38].

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Supplementary materials

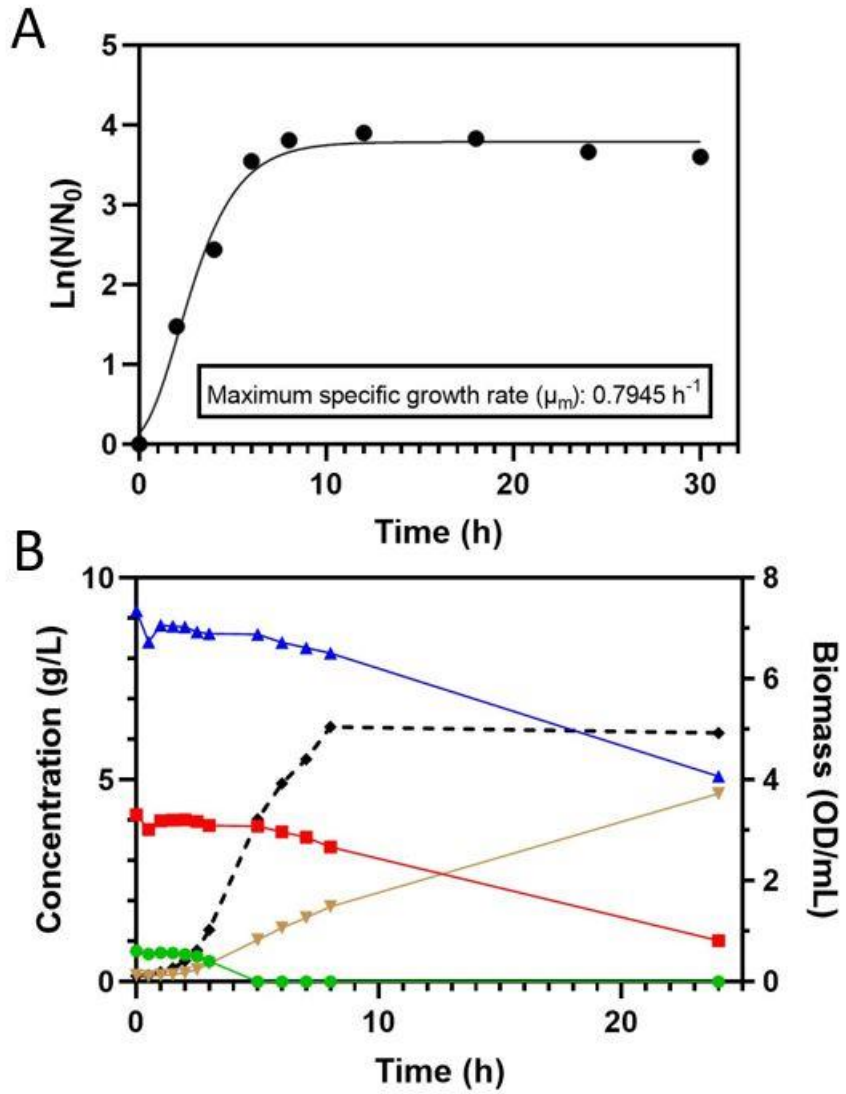

Figure S1. Growth curve, metabolic profile and biomass concentration of the engineered strain grown in fermentation medium containing 0.5 g/L glucose and 4 g/L lactose, without the addition of vanillin. A) Growth curve of the engineered strain calculated using Modified Gompertz equation[3]. B) Metabolic profile (continuous line) and biomass concentration (dotted line) of the engineered strain. The metabolites labels are as follow: glucose is green, lactose is red, glycerol is blue and acetate is yellow. Data were collected from a single biological replicate.

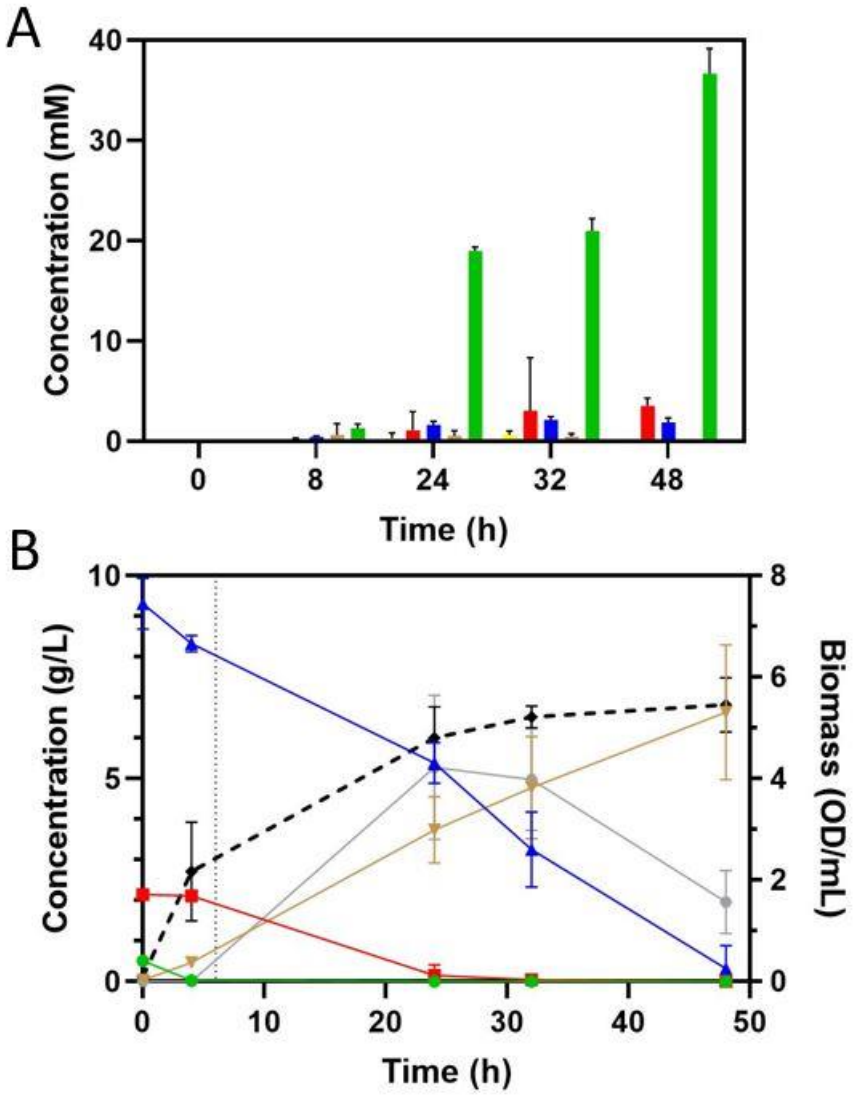

Figure S2. Time course, metabolic profile and biomass concentration of the bioconversion of 40 mM vanillin added thorugh a 1 mmol/h pulse-feed. A) Time course of the bioconversion. The bioconversion labels are as follow: vanillin is yellow, vanillic acid is red, protocatechuic acid is blue, catechol is brown and *cis,cis*-muconic acid is green. B) Metabolic profile (continuous line) and biomass concentration (dotted line) of the bioconversion. The metabolites labels are as follow: glucose is green, lactose is red, glycerol is blue, acetate is yellow and ethanol is grey. The vertical dotted line indicates when the vanillin pulse-feed started. Data were collected from three indipendent biological replicate.

3.3 One-pot biotechnological valorization of wheat bran into 4-vinylguaiacol

INTRODUCTION

4-vinylguiacol (2-methoxy-4-vinylphenol, 4VG) is a lignin-derived aromatic compound, which is mainly used as a flavoring agent in the beverages, perfumery and food industry[1]. Recently, 4VG has been successfully utilized as a precursor for the production of several polymeric materials[2–4]. The three different chemical moieties on the molecule (i.e. vinyl, methoxy and hydroxyl group) allow the polymerization of 4VG monomers, thus allowing the subsequent functionalization of the polymer. In an our previous work, vanillin and *cis,cis*-muconic acid were produced through microbial conversion (based on resting cells approach) starting from wheat-bran derived ferulic acid using an engineered *E. coli* strain[5]. One intermediate of this previous recombinant pathway is 4VG, which was obtained from the decarboxylation of ferulic acid by the Fdc decarboxylase from *B. pumilus*[6]. Ferulic acid was extracted from wheat bran (WB) following a three-step thermo-enzymatic treatment using the commercial enzyme Ultraflo®XL, which breaks down WB polysaccharides releasing ferulic acid (and monosaccharides). The extraction step was followed by the purification of the ferulic acid from the WB crude extract using an anion exchange resin and the carbohydrates-rich supernatant was discarded (anyway it can be used as a fermentable substrate for microbial growth)[7]. The presence of WB-derived carbohydrates in the crude extract and the fact that the conditions of the enzymatic step of the extraction (37 °C, pH 6.0) are well suited for Fdc activity[6], open the possibility to produce 4VG directly from WB in a one-pot fermentative process using an engineered *E. coli* strain expressing Fdc. This one-pot process was inspired by the Simultaneous Saccharification and Co-Fermentation (SSCF) approach used for the microbial conversion of lignocellulosic biomass[8]. In the SSCF, while the hydrolysis of the holocellulose (cellulose and hemicellulose) is carried out, the released carbohydrates are simultaneously used by a microbial strain for growth and production of the compound of interest. In the proposed process (Figure 1), the carbohydrates are used for the growth of the engineered strain while the ferulic acid is converted to 4VG by the decarboxylase Fdc, thus allowing for a complete and straightforward valorization of WB. Moreover, to make the process more sustainable, *Fdc* gene can be under the control of phenol inducible promoter to induce the expression of the enzyme by the ferulic acid released from the WB.

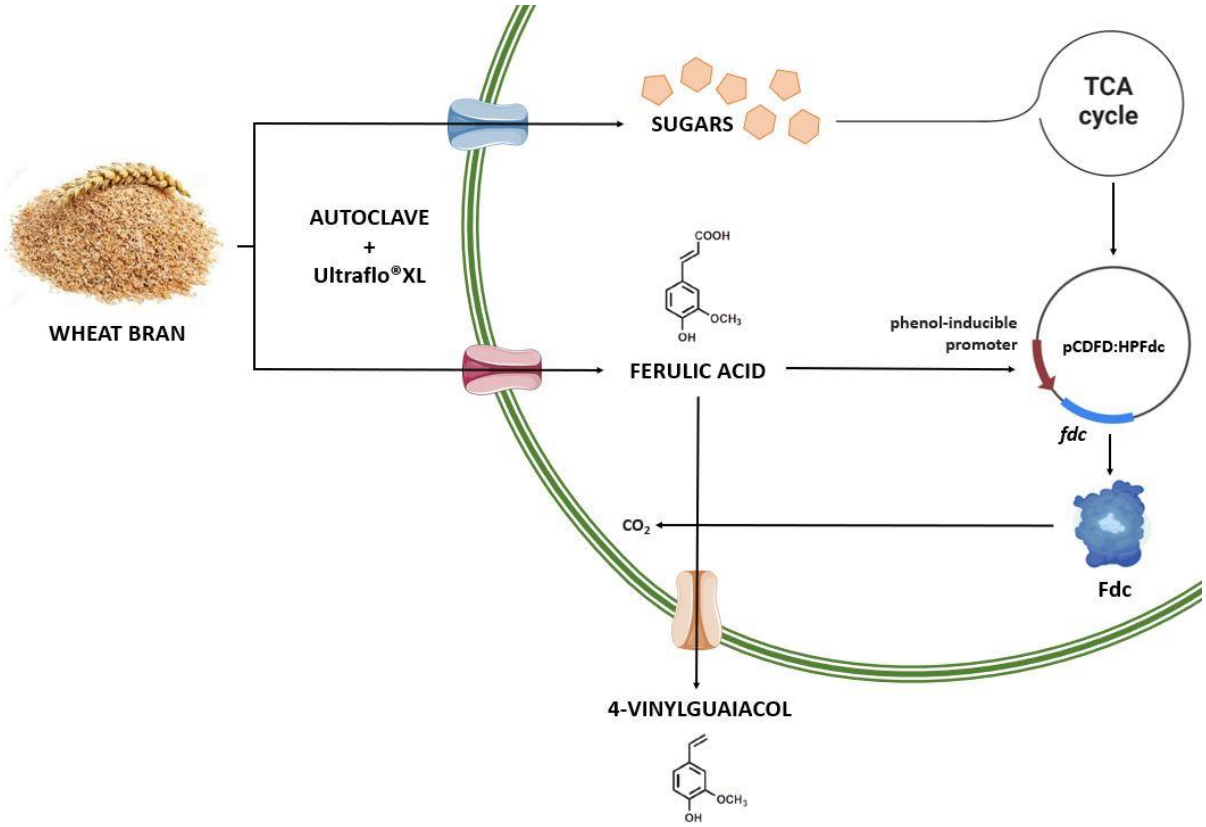

Figure 1. Schematic representation of the one-pot SSCF-like process aimed at the production of 4-vinylguaiacol from wheat bran-derived ferulic acid.

Varman et al.[9] developed a hybrid phenol-inducible promoter for *E. coli* by combining the up and down regulatory sequences from the endogenous P_{emrR} promoter, an *E. coli* promoter involved in the phenol detoxification response, and the spacer region between the -10 and -35 sequences of the high strength P_{tac} promoter, thus creating a hybrid phenol-inducible promoter (P_{vtac}) with higher strength than the natural one. The transcription factor emrR of *E. coli* binds to P_{emrR} promoter repressing the transcription of target gene[10]; in the presence of lignin-derived phenolic compounds, such as vanillin and ferulic acid, these phenolics can bind to emrR causing the release from the P_{emrR} promoter and the consequent activation of the transcription of the target gene[11]. Hence, by using this hybrid promoter, the WB crude extract could become an auto-inducing medium that allows the expression of recombinant enzymes without the need for external inducers. Finally, the WB-derived 4VG, could be purified from the crude extract using the reverse phase adsorbent resin Amberlite XAD4[1] and used as a precursor for the production of novel polymeric materials with enhanced functionality.

MATERIALS AND METHODS

Materials

Methanol (ACS Grade, ≥99%), formic acid (ACS Grade, ≥98%) and analytical grade standards of ferulic acid (*trans*-hydroxy-3-methoxycinnamic acid) and 4-vinylguaiacol (2- methoxy-4-vinylphenol, 4VG) were purchased by Merck/Carlo Erba (Merck KGaA, Darmstadt, Germany). The commercial food-grade enzyme UltrafloXL was kindly provided by Novozymes (Bagsvard, Copenhagen, Denmark), and wheat bran was a generous gift of Molino Dallagiovanna (Gragnano Trebbiense, Piacenza, Italy).

Promoter design, cloning and *E. coli* transformation

The synthetic nucleotide sequence of the hybrid phenol promoter was designed as reported in the paper[9]. To facilitate subcloning of the hybrid phenol promoter into pCDFDuet-1 (Novagen, Darmstadt, Germany), the sequences corresponding to *NcoI* (CCATGG) and *BamHI* (GGATCC) restriction sites were added at the 5'- and 3'-ends of the hybrid phenol promoter, while the sequence corresponding to *EcoRI* (GAATCC) restriction site was added in the spacer sequence to allow the screening of the ligation products. The synthetic promoter was produced by Twist Bioscience HQ (San Francisco, USA). The gene coding for the decarboxylase Fdc from *B. pumilus*[6], available in our laboratory[5], the sequence was flanked by the sequences corresponding to *BamHI* (GGATCC) and *HindIII* (AAGCTT) restriction sites at the 5'- and 3'-ends, respectively, allowing subcloning into the pCDFDuet-1 plasmid. The synthetic *Fdc* gene, the sequence of the hybrid phenol promoter and the plasmid were digested with the corresponding restriction enzymes (Fast Digest, Thermo Fisher Scientific, Monza, Italy) and ligated with T4 DNA ligase (Thermo Fisher Scientific). The ligation mixtures were used to transform JM109 *E. coli* chemically competent cells. The presence of the *Fdc* gene was verified by digestion with the corresponding restriction enzymes and the presence of the hybrid phenol promoter was verified by *EcoRI* restriction analysis. The *Fdc* gene was inserted into the pCDFDuet-1 plasmid obtaining the pCDFD:Fdc plasmid. Next, the hybrid phenol promoter was inserted upstream the *Fdc* gene in the pCDFD:Fdc plamid, obtaining the pCDFD:HPFdc plasmid. Finally, the two plasmids (pCDFD:Fdc and pCDFD:HPFdc) were introduced into the *E. coli* MG1655 RARE[12] strain through chemical transformation to construct the whole-cell biocatalyst.

WB Hydrolysis

The ferulic acid recovery was carried out using a thermo-enzymatic method similar to the one reported by Bautista-Expósito et al.[13], and already utilized in our previous work[5]. Wheat bran was pre-treated by milling it to a fine powder (3 min of total time by alternating 30 s of milling to a 30 s pause) before the thermo-enzymatic extraction. Wheat bran powder was suspended at a 1:20 solid to solvent ratio (g/mL) in an acqueous solution and autoclaved at 121 °C, 1 bar, for 30 min. The acqueous solution tested were MilliQ water, 100 mM potassium phosphate pH 6.0, and 100 mM potassium phosphate, pH 8.0. Then, the suspension was cooled to room temperature and 1% UltrafloXL (enzyme to wheat bran powder dry weight ratio, w/w) was added. The enzymatic step was carried out at 37 °C, under shaking (130 rpm), for 16-24 h. The inoculum of the engineered strain in the crude extract have been carried out both simultaneously or delayed (≈16 h) to the addition of UltrafloXL.

Culture and bioconversion

For the starting culture, the engineered strain (used as the biocatalyst) was inoculated in the LB medium containing 50 μg/mL streptomycin and grown at 37 °C, 130 rpm, for 10-18 h. Then, the wheat bran crude extract, added of 50 μg/mL streptomycin, was inoculated with an amount of starting culture to reach an initial $OD_{600\ nm}$ = 0.05 and the culture was incubated at 37 °C, 130 rpm. To find the best conditions for 4VG conversion, the inoculum of the wheat bran crude extract has been performed both together with UltrafloXL addition or 15 h after the addition of UltrafloXL. The growth of the engineered strain on WB crude extract was assayed by counting colony forming units (CFU) on LB agar plates added of streptomycin. To allow the sedimentation of the solid matter present in the crude extract, cultures were decanted for 1-2 minutes before withdrawing the samples. Then, withdrawn samples were serially diluted with 1% w/v NaCl to achieve an overall million-fold diluition before spreading 100 μL of each sample on selective LB plates in duplicate. The plates were incubated at 37 °C for ≈18 h before manual colony count.

HPLC analysis

HPLC analyses were performed on a Jasco apparatus equipped with a Kromaphase C8 column 100 A, 5 μm, and 4.6 × 250 mm (Scharlab, Barcelona, Spain) and with a UV detector set at 276 nm. The flow rate was 1 mL/min, and the column oven was set at 25 °C. A binary system of solvent A (2.5% v/v formic acid) and solvent B (methanol) was used with the following gradient: 0 min, 60% solvent A + 40% solvent B; 0−15 min, ramping up to 10% solvent A + 90% solvent B; 15−17 min, maintaining 10% solvent A + 90% solvent B. Calibration curves were obtained by solubilizing

standards of ferulic acid in 100 mM Tris−HCl pH 8.0, and 4-vinylguaiacol in DMSO, at a final concentration of 40 mM. Subsequently, after dilution in the 0.04−2 mM range, 25 µL of each sample were added to 50 µL of solvent A and centrifuged for 2 min at 11,000 *g*, 4 °C: 20 µL of the supernatant were injected for HPLC analysis. Retention times for ferulic acid and 4VG standards were 6.9 and 12.0 min, respectively.

RESULTS

Cell growth assay

The engineered *E. coli* strain for the production of 4VG was built by subcloning the synthetic gene encoding the ferulic acid decarboxylase Fdc from *B. pumilus*[6] in a pCDFDuet-1 plasmid. The sequence of the hybrid phenol-inducible promoter P_{vtac} was then subcloned upstream to the Fdc sequence, allowing the phenol-induced expression of the recombinant enzyme (See Figure 2).

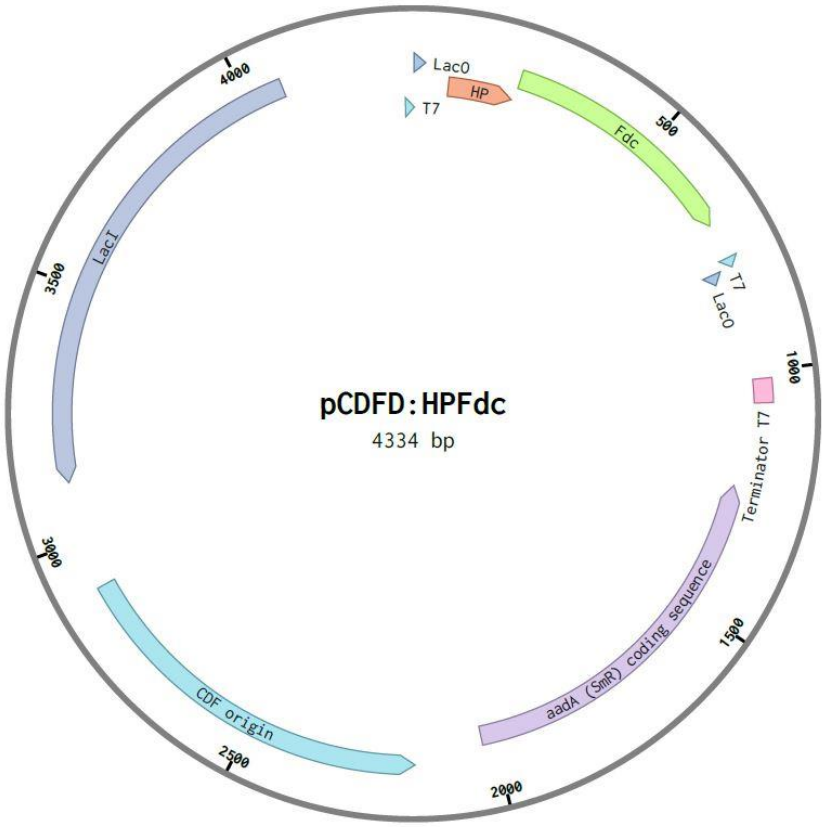

Figure 2. Physical map of plasmids pCDFDuet-1 carrying the genes encoding Fdc (green) under the control of the hybrid phenol-inducible promoter (HP, orange). The main functional elements of the plasmids include two multiple cloning sites, both preceded by a T7*lac* promoter and a ribosome binding site as well as a sequence encoding a 6-His tag at the N-terminal end and an S-tag at the C-terminal end. The plasmids also carry the kanamycin (KnR), ampicillin (AmpR), and streptomycin (SmR) resistance genes.

The final plasmid pCDFD:HPFdc was then transformed in *E. coli* MG1655 RARE[12] cells to generate the biocatalytic strain. In an our previous work[5], we established that 10 mM ferulic acid

has little to no inhibitory effect on *E. coli* viability when grown in LB medium at 37 °C. To test whether the enzymatic cocktail UltrafloXL affects the growth of *E. coli*, the optical density of an *E. coli* culture in LB added of streptomycin and UltrafloXL (at the same concentration used for the WB hydrolysis, 0.0005 % v/v) was monitored. The engineered strain RARE pCDFD:HPFdc grown in presence of UltrafloXL showed no significant differences, both in growth rate and saturation density, when compared to a growth under the same condition without UltrafloXL (data not shown); thus, it was concluded that the addition of the UltrafloXL enzymatic cocktail to the fermentation broth does not have any inhibitory effect on *E. coli* growth under the tested conditions. Lastly, *E. coli* was grown in the presence of 1 mM 4VG, since it is reported that generally vinyl phenol derivatives obtained by the decarboxylation of phenolic acids are more toxic for *E. coli*[14]. After an incubation for 18 h at 37 °C, 130 rpm, the engineered strain *E. coli* showed a similar saturation density to control culture, thus excluding 4VG as inhibitor of *E. coli* growth. Unexpectedly, after the incubation, only 81.1 ± 2.7% of 4VG remained in the culture broth, meaning that the 4VG is partially converted to an unknown compound, probably by some endogenous enzymatic activities of *E. coli*. Furthermore, simultaneously to the area decrease of the peak corresponding to 4VG in HPLC analysis, the appearance of a new peak in the chromatogram was observed (Figure 3) which could correspond to the product of 4VG conversion during the incubation with *E. coli* cells. Hence, this sample will be identified by HPLC-MS analysis to understand the reaction (chemical or enzymatic) causing the disappearance of 4VG.

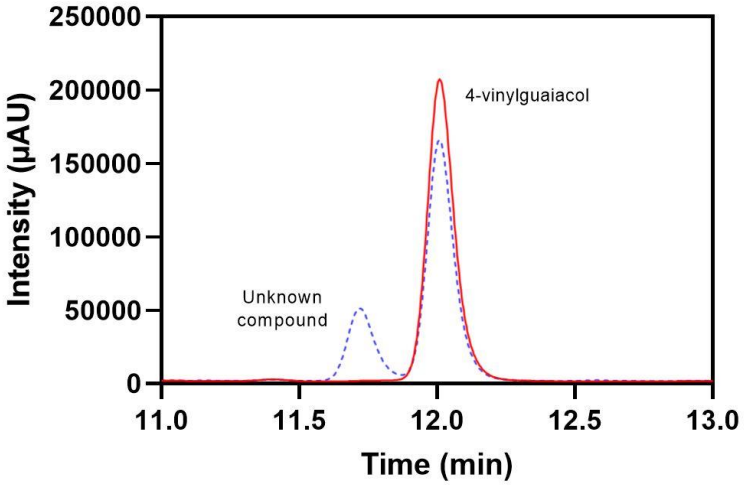

Figure 3. Chromatogram profile of the 4VG stability assay. The sample taken at the start of the incubation with *E. coli* MG1655 RARE pCDFD:HPFdc cells is represented by the red continuous line; the sample taken after 18 h of incubation is represented by the dotted blue line.

SSCF-like bioconversion

The production of 4VG from WB was assayed using different aqueous solutions and by the time of the engineered strain RARE pCDFD:HPFdc inoculum. The aqueous solutions tested were: i) MilliQ water, ii) 100 mM phosphate buffer, pH 6.0, and iii) 100 mM phosphate buffer, pH 8.0. The inoculum was performed both simultaneously (co-ultra) and 16 h after (post-ultra) the addition of UltrafloXL. Moreover, two control reactions were set up: in the first only the UltrafloXL cocktail was added, and in the second the UltrafloXL was added together with an *E. coli* strain not expressing Fdc; in this way, the effect of *E. coli* cells on the UltrafloXL activity could be evaluated. In our previous work[5], the three-step extraction method allowed to obtain a supernatant in which the ferulic acid concentration was 0.7 - 0.8 mM; therefore this was set as the maximal concentration of 4VG in the fermentation broth following the conversion of ferulic acid. Table 1 shows the concentration pf 4VG obtained under different reaction conditions.

Table 1. 4VG production from WB using a one-pot reaction with UltrafloXL and *E. coli* cells expressing the Fdc enzyme (a maximal amount of ferulic acid corresponding to 0.75 mM is estimated after WB extraction[5]).

Reaction conditions	**Time (h)**	**Ferulic acid (mM)**	**4-Vinylguaiacol (mM)**	**Conversion (%)**
pH 6.0 co-ultra	16	0.01	0.49	65.7
	20	0.04	0.51	68.0
	24	0.03	0.51	68.0
H_2O co-ultra	16	0.01	0.56	74.8
	20	0.04	0.55	73.4
	24	0.04	0.58	77.1
pH 8.0 co-ultra	16	0.03	0.47	63.3
	20	0.03	0.47	62.2
	24	0.05	0.49	65.4
pH 6.0 post-ultra	16	0.73	0.01	1.3
	18	0.37	0.32	43.0
	20	0.07	0.48	64.0
	24	0.03	0.53	71.0
H_2O post-ultra	16	0.80	0.02	2.0
	18	0.33	0.39	51.4
	20	0.08	0.59	78.4
	24	0.03	0.58	77.9
pH 8.0 post-ultra	16	0.76	0	0
	18	0.50	0.21	28.0
	20	0.15	0.48	63.4
	24	0.02	0.50	66.3

CTRL ultra	16	0.74	0.01	1.7
	24	0.79	0	0
CTRL ultra + E. coli	16	0.72	0.01	1.8
	24	0.75	0.01	1.4

The engineered strain was catalytically active in all the conditions tested, indicating that the hybrid phenol-inducible promoter allows to induce the expression of the Fdc enzyme in a sufficient amount. Despite the ferulic acid is completely depleted during the reaction, only 65 - 78% of the expected 4VG is produced, suggesting that 4VG is probably converted to other compound(s). No significant difference has been observed by performing the bioconversion in the phosphate buffer at pH 6.0 (68 – 71%) or pH 8.0 (65 – 65%), meanwhile performing the reaction in MilliQ water (77 - 78%) seems to optimal condition for 4VG production. Interestingly, the time of the inoculum does not seem to exert any effect on the production of 4VG: the highest productivity was observed in water, at 16 h of the co-ultra condition (0.035 mM/h). Actually, the presence of *E. coli* in crude extract does not have any inhibitory effect on UltrafloXL activity. The *E. coli* concentration in the crude extract was evaluated by diluting the supernatant and plating the resulting dilution on a selective LB plate containing streptomycin. Despite the time of the inoculum does not affect the production of 4VG, the cell concentration in the co-ultra reactions (0.71 ± 0.05 OD/mL) was slightly lower than the post-ultra reactions (0.86 ± 0.06 OD/mL).

Furthermore, the addition of nitrogen sources to the crude extract was also evaluated since it could be scarce in the crude extract, making the expression of endogenous and recombinant enzymes suboptimal. Accordingly, ammonium chloride (1 g/L) or yeast extract (0.5 g/L) were added before the autoclave step of the extraction process: no effect on the final titer of 4VG was observed (data not shown).

DISCUSSION AND FUTURE PERSPECTIVES

These results show the feasibility of the one-pot production of 4VG from WB using the enzymatic cocktail UltrafloXL and the engineered *E. coli* strain expressing Fdc under the control of phenol-inducible promoter. Despite the encouraging results, there is still space to optimize and better understand the whole process. For instance, the cause of the conversion of 4VG needs to be further investigated. Moreover, the strength of the hybrid phenol-inducible promoter should be compared to the T7 promoter; this will be done by analyzing the intensity of the fluorescence generated by a Green Fluorescent Protein under the control of both promoters. The purification of 4VG from the crude extract using Amberlite XAD4 resin have still to be carried out. Finally, the levels of carbohydrates released by the three-step extraction protocol and the ones consumed

during the engineered strain growth will also be assayed by HPLC coupled with a refractive index detector.

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4. Discussion

Nowadays plastic materials play an ubiquitous role in various facets of our everyday lives, encompassing transportation, telecommunications, clothing, footwear, and serving as packaging materials essential for transporting a diverse array of food, beverages, and goods[1]. Plastic polymers are broadly utilized in several applications thanks to their favorable properties: mechanical strength, malleability, chemical and physical durability, inexpensiveness, and high electrical and thermal insulation capabilities. The potential for innovative applications of plastics is vast, promising future benefits such as novel medical uses and the reduction of energy consumption in transportation[1]. Despite these advantages, plastics production heavily relies on fossil sources for the generation of precursors and monomers[2]. The dwindling reserve of petroleum and the environmental risks linked with its extraction and exploitation makes the current process of plastic production unsustainable, therefore there is a great interest developing innovative technologies that allow the production of plastics from renewable and non-toxic sources[2]. One of the most interesting raw materials is the lignocellulosic biomass (LCB); the abundance ($\approx 1.8\times10^{11}$ tons/year)[3] and the heterogeneity composition (hexoses, pentoses and aromatic compounds)[4,5] of this renewable feedstock make it a promising alternative to fossil sources to produce several value-added compounds. This realization has led to the development of the biorefinery concept, where the lignocellulosic biomasses are separated into carbohydrate- and lignin-rich streams and valorized according to their chemical and physical characteristics. Currently, the carbohydrate-rich fraction is utilized mainly for the production of paper and biofuels, conversely, the recalcitrant lignin-rich fraction is still underutilized despite being a great renewable source of aromatics[3,6].

This PhD project aimed at the development of a novel biotechnological process for the valorization of kraft lignin and wheat bran, two LCB by-products produced in large amounts worldwide[7–9]. In particular, it focused on the design and optimization of bioconversion processes using engineered *E. coli* strains expressing recombinant biochemical pathways to convert LCB-derived compounds to value-added chemicals and plastic precursors (i.e. *cis,cis*-muconic acid, vanillin and 4-vinylguaicol). A whole-cell biocatalytic strategy was employed aiming at the development of a cheap and straightforward process which could be scaled-up to an industrial scale, and most importantly, to make it safe and environmentally sustainable. It was reported that ≈60% of the reported industrial biocatalytic reactions utilize whole-cells over isolated enzymes in virtue of these advantages[10]. The production cost of whole-cell biocatalysts (35-100 €/kg) is estimated to be much lower compared to the cost of isolated enzymes (250-1000 €/kg), making this strategy more appealing for the production of bulk and specialty chemicals[11]. This cost difference could be even

higher for multi-step enzymatic processes, requiring the separate production and purification of every employed enzyme.

Firstly, a biotechnological process for the production of vanillin and *cis,cis*-muconic acid (ccMA) from kraft lignin and/or wheat bran was developed. This process involves two major stages: i) the thermo-enzymatic extraction of vanillin from kraft lignin and ferulic acid from wheat bran; and ii) the microbial conversion of the extracted compound using an engineered *E. coli* strain expressing up to seven recombinant enzymes. In the first stage, vanillin was extracted form kraft lignin by an enzymatic treatment using recombinant *Bacillus licheniformis* laccase following a procedure already established by our laboratory[12]: this allowed the recovery of 4.5 mg vanillin/g kraft lignin. The efficient extraction of ferulic acid from wheat bran was obtained by optimizing a three-step thermo-enzymatic treatment using Ultraflo®XL, a commercial enzymatic cocktail. The three-step treatment followed by a purification step using an anion exchange resin resulted in the recovery of 3.0 ± 0.2 mg ferulic acid/g wheat bran, corresponding to 85 ± 6% of the total alkaline extractable ferulic acid. Concerning the second stage, three different biocatalysts were setup starting from the engineered *E. coli* MG1655 RARE strain, which is capable of accumulating aromatic aldehydes without reducing them to alcohols[13]. The *E. coli* strain was transformed with the pRSFDuet-1 plasmid harboring the genes coding for decarboxylase Fdc[14] and the dioxygenase Ado[15] (strain II), which allowed the conversion of ferulic acid into vanillin. *E. coli* strain was co-transformed with the pCDFDuet-1 plasmid harboring the genes coding for dehydrogenase LigV[16], demethylase VanA and reductase VanB[17] together with the pETDuet-1 plasmid harboring the genes coding for decarboxylase AroY[16] and cathecol-1,2-dioxygenase (C12O)[18] (strain V): the expression of these latter enzymes allowed the conversion of vanillin into ccMA. Noteworthy, the final optimized biocatalyst (strain VII) has been obtained by the co-transformation with all the aforementioned plasmids, making it capable of producing ccMA from ferulic acid in one-pot.

The whole-cell bioconversions were all conducted using the resting cells approach, to make the process cheaper compared to the use of purified recombinant enzymes and to allow the cofactors regeneration during the biocatalytic reaction[19]. The whole-cell biocatalytic reactions were optimized to work at pH 8.0 and at 30 °C, which are optimal operational conditions for most of the enzymes of the pathways, and to use a low amount of biocatalyst (≈70 g cww/L). The *E. coli* strain II converted ≈90% of 10 mM ferulic acid and 85.4 ± 3.2% of 2.5 mM wheat bran-derived ferulic acid into vanillin in 20 h, showing the feasibility of producing bio-vanillin from an agricultural by-product. The *E. coli* strain V proved to be very efficient in the conversion of vanillin into ccMA, indeed it was capable of converting 95.2 ± 3.8% of 10 mM vanillin into ccMA in just 2 h and to completely convert 1 mM of kraft lignin-derived vanillin into ccMA in 30 min. When the two

pathways were assembled into the final *E. coli* strain VII, the resulting whole-cell biocatalyst converted 96.1 ± 3.8% of 10 mM ferulic acid and 95.1 ± 4.1% of 2.5 mM wheat bran-derived ferulic acid into ccMA in 10 h. The optimization of the extraction methods and the development of a novel whole-cell biocatalyst allowed the production of 4.2 mg ccMA/g kraft lignin and 2.2 mg ccMA/g wheat bran. The conversion of vanillin into ccMA has been performed by using an *in vitro* multi-enzymatic system developed in our laboratory[12]: noteworthy, the engineered strain converted a double amount (10 mM vs. 5 mM) of vanillin into ccMA with a higher yield (≈95% vs. ≈90%) and in a fifteenth of the time (2 h vs. 30 h). Interestingly, the use of the whole-cells approach proved to be not only cheaper than isolated enzymes approach, but even more performing. Moreover, to the best of our knowledge, the microbial conversion of ferulic acid into ccMA has been attempted only once using an engineered *P. putida*, strain which was chosen due its innate ability of utilizing monolignols as a carbon source. The knock-out of crc repressor, pcaGH and catA/A2 allowed the fermentative conversion of ≈28% of 20 mM ferulic acid into ccMA in 72 h, obtaining a ccMA titer of ≈5.6 mM[20]. Our proposed biocatalytic process using the *E. coli* strain VII achieved the almost complete conversion of 10 mM ferulic acid into ccMA in a seventh of the time (10 h vs. 72 h). Interestingly, scientific works on the direct conversion of wheat bran-derived ferulic acid into ccMA were not found in literature, making our biocatalytic process the first reported one-pot bioconversion.

The capability of an engineered *E. coli* strain to produce ccMA from renewable feedstocks, rather than relying on petroleum, identifies this system as a suitable alternative for the production of bioproducts and bioplastic monomers.

Subsequently to the encouraging results obtained, the scaled-up production of ccMA from vanillin using engineered *E. coli* (i.e. *E. coli* strain V) growing cells was studied. The bioconversion reaction was carried out in a fermenter, providing improved control of the reaction conditions such as pH, dissolved oxygen and substrate pulse-feed rate, streamlining the biocatalytic process, and enhancing scalability. By using this strategy, it could be possible to enhance the efficiency and cost-effectiveness of the biocatalytic process; indeed, the use of growing cells instead of resting cells reduces the number of steps needed, and the improved reaction control could help increase the biocatalyst productivity and obtain a higher product titer in the reaction system. The first step has been the formulation of an auto-inducing medium, allowing initial rapid growth of the engineered strain and the subsequent induction of the recombinant enzymes production, without the need to use the expensive inducer IPTG[21]. Using the data reported in literature, an equation to correlate the amount of glucose present in a media and the *E. coli* growth was formulated; accordingly, 0.5 g/L of glucose was calculated to achieve a biomass of ≈1 OD/mL, a value confirmed by

experimental data and scientific literature[22]. The efficient induction of pathway's enzymes expression was achieved by the combined use of 0.5 g/L glucose and 2 g/L lactose, allowing the engineered strain to convert vanillin into ccMA already after 6 h from the initial inoculum. Further lactose addition (4 g/L) resulted in a diminished specific growth rate and saturation density of the culture. Additionally, to mitigate potential inhibition of VanA activity caused by vanillic acid accumulation[17], vanillin was added to the fermentation broth using a pulse-feed strategy (1 mmol/h). The optimized growth medium composition and substrate addition strategy enabled the engineered strain to produce 5.20 ± 0.36 g/L of ccMA in 48 hours, corresponding to 0.86 g ccMA/g vanillin. The purification of the produced ccMA from the fermentation broth was achieved through crystallization, yielding 2.58 ± 0.07 g per liter of broth corresponding to a purification yield of 49.50 ± 0.07%.

A similar ccMA yield was obtained by Chen et al.[17] using an engineered *E. coli* strain expressing VanAB, GDC and CatA, and by utilizing a pulse-feed approach for the addition of the substrate. In this work, the resting cells of the engineered strain converted 87.6% of 50 mM vanillic acid into ccMA in 30 h, achieving a final ccMA titer of ≈6.2 g/L. Compared to the proposed process, a higher productivity (0.109 g/L×h vs. 0.124 g/L×h) has been achieved, but with a 10-fold decrease in catalyst productivity (0.054 g/L×h×g cdw cells vs. 0.0083 g/L×h×g); despite this, the use of a resting cells approach and the required addition of 20 g/L glucose to the bioconversion reaction, make this process more expensive and difficult to scale-up. An interesting result was obtained using an engineered *Pseudomonas* sp. strain NGC7, which is able to convert vanillate into ccMA while using syringate as the sole carbon source[23]. The engineered strain was built by deleting protocatechuic acid and ccMA cycloisomerase genes and inserting an expression plasmid carrying the gene encoding a protocatechuic acid decarboxylase, flavin prenyltransferase, vanillate *O*-demethylase and catechol 1,2-dioxygenase. This engineered strain was able to convert ≈75% of 30 mM vanillic acid into ccMA in 72 h, obtaining a final ccMA titer of ≈3.2 g/L. Despite this process generates a lower ccMA yield (3.2 g/L vs. 5.2 g/L) in a longer time (72 h vs. 48 h) compared to our proposed process, the engineered strain was able to produce ccMA (≈0.15 g/L) from birch lignin-derived phenols. Moreover, it allowed the glucose-free production of ccMA, thanks to its ability to use S-lignin monomers as carbon source, thus reducing the overall cost of the process.

As shown in paragraph 3.2, the bioconversion with growing cells, beyond achieving higher ccMA titer than resting cells, is even more environmentally friendly and so more prone to be used for the development of a sustainable industrial-scale process. Noteworthy, the biocatalytic parameters of the resting cells were obtained from a batch reaction without the same control of several parameters of the reaction system and without the option of a pulse-feed addition of the substrate. Therefore,

the use of resting cells (of the engineered strain) in a controlled bioreactor could lead to a further increase in productivity and a higher product titer, making a more viable option to produce bio-based ccMA. To the best of our knowledge, the production of ccMA from vanillin using an engineered *E. coli* strain by a controlled bioreactor was not reported in literature yet.

Lastly, a preliminary analysis of a one-pot process for the production of 4-vinylguaiacol from wheat bran was conducted. The process involves the extraction of ferulic acid from the wheat bran using the three-step thermo-enzymatic protocol already established[24] and the simultaneous conversion of wheat bran-derived ferulic acid into 4-vinylguaicol using an engineered *E. coli* RARE strain[13] expressing the decarboxylase Fdc[14]. The novelty of this process arises from the employment of the wheat bran crude extract as an auto-inducing growth medium, based on the presence of several fermentable carbohydrates[25] and the utilization of a hybrid phenol-inducible promoter[26] for the induction of Fdc expression, making the wheat bran-derived ferulic acid both the inducer and the substrate of the enzyme. This strategy could help in increasing the economic sustainability of the process by removing the needs of commercially available growth media and external inducers (such as IPTG and lactose), and by reducing the number of needed steps. Moreover, the wheat bran-derived 4-vinylguaicol will be purified using a reverse phase resin and used for the production of novel plastic polymers. The preliminary results of this project proved that the engineered strain is capable of growing and expressing the recombinant enzyme in the crude extract of wheat bran, and that the simultaneous extraction and conversion strategy is feasible. The unoptimized process produced 1.8 mg 4-vinylguaiacol per gram of wheat bran, which corresponds to the conversion of ≈75% of the ferulic acid extracted using the thermo-enzymatic method and ≈64% of the alkaline extractable ferulic acid present in the wheat bran. The suboptimal yield is probably due to the partial conversion of 4-vinylguaicol by competitive *E. coli* endogenous enzymatic activities: further analysis will allow to understand and solve this issue.

In conclusion, the overall results obtained during this PhD project should contribute to the ever-growing biotechnological toolbox for the valorization of LCB. The optimization of LCB valorization could help in the transition to a fossil sources-free economy, allowing the development of a more sustainable and more environmentally careful society. The production of the plastic precursor ccMA from lignocellulose feedstocks using a whole-cell biocatalyst was explored using both resting cells and growing cells approach. The growing cells approach proved to be more productive and more environmentally sustainable than the resting cells approach, mainly due to the use of a bioreactor that allowed for a better control of the reaction parameters. Furthermore, a novel one-pot process for the production of 4-vinylguaiacol from wheat bran by combining a thermo-enzymatic extraction to a microbial fermentation using an engineered *E. coli* strain was

studied. Despite the early stage of this project, it could represent a streamlined and sustainable alternative to 4-vinylguaicol production; when optimized, it could allow the purification of the bio-based 4-vinylguaicol and its utilization as a precursor for novel plastic polymers.

This PhD project highlights the possible benefits of utilizing microbial biocatalytic approaches for the valorization of industrial lignocellulosic by-products. The values of this approach are best described by Davide Pearlman's laws of applied microbiology[27]:

1. the microorganism is always right, your friend, and a sensitive partner;
2. there are no stupid microorganisms;
3. microorganisms can and will do anything;
4. microorganisms are smarter, wiser, more energetic than chemists, engineers, and others;
5. if you take care of your microbial friends, they will take care of your future.

Indeed, the use of whole microorganisms allows to design tailor-made processes, from the design of an engineered strain to the selection of the cultivation/conversion protocols, making them exploitable in several different production processes. Moreover, whole microorganisms enable the development of more stepwise processes, allowing for a better scalability and the reduction of waste produced. Therefore, microbial biocatalysis could be one of the key enabling technologies that will facilitate the transition to more sustainable industrial processes, creating favorable conditions for the establishment of the circular bioeconomy model.

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