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From wheat bran to 4-vinylguaiacol: A green bioprocess featuring *in situ* product recovery

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ABSTRACT

Valorizing agro-industrial byproducts such as wheat bran (WB) through microbial and enzymatic processing offers a sustainable route to high-value aromatic compounds like 4-vinylguaiacol (4VG). This study reports a novel one-pot biocatalytic system that couples the enzymatic hydrolysis of WB (UltrafloXL) - releasing ferulic acid (FA) and sugars – with the microbial conversion of FA into 4VG by an engineered *E. coli* strain expressing ferulic acid decarboxylase (Fdc) under a phenol-inducible promoter. The process is self-sustaining: FA acts both as the enzymatic substrate and as the inducer for Fdc expression, while glucose released from WB sustains microbial growth. Integrated *in situ* product recovery (ISPR) using a tea-bag resin system enhances yields while streamlining purification. The system achieved a 4VG yield of approximately 2.0 mg/g of WB, corresponding to an overall conversion efficiency of ~88 %. Proof-of-concept experiments at 0.5 L scale showed efficient extraction (96 ± 1.5 %) and purification (76 ± 1.2 %) yields, together with a low materials-based cost, supporting the techno-economic viability of 4VG production from renewable feedstocks. To our knowledge, this is the first report of a WB-based 4VG biosynthesis using an engineered *E. coli* in a system where FA acts both as substrate and activator. This integrated, autoinductive platform represents a green alternative for aromatic compounds production in alignment with bioeconomy principles.

1. Introduction

The biotechnological production of 4-vinylguaiacol (4VG) from wheat bran (WB) has emerged as a promising strategy to valorize agricultural residues into high-value aromatic compounds. Fundamental to this process is the enzymatic release of ferulic acid (FA), a phenolic acid naturally bound within the WB lignocellulosic matrix, followed by microbial or enzymatic decarboxylation to 4VG. This molecule is industrially mainly used as a flavoring agent in the beverages, perfumery and food industry [1]. Additionally, 4VG is a key intermediate for the synthesis of biobased polymers and fine chemicals, contributing to the transition toward a more sustainable and circular bioeconomy [2–5]: the three different chemical moieties on the molecule (i.e. vinyl, methoxy and hydroxyl group) allow the polymerization of 4VG monomers and the functionalization of the polymer. The commercial relevance of 4VG lies not only in its versatility but also in its economic value. Bio-based aromatic compounds like 4VG can value several hundred USD/kg, especially when produced at food- or pharma-grade purity. Its use in producing vanillin, thermoplastic monomers, and antioxidant-rich materials increases its market potential [1].

Conventional chemical decarboxylation of FA requires high temperatures (>180 °C) and metal catalysts (e.g., CuO, ZnO), leading to energy-intensive processes with low selectivity, while also raising safety and environmental concerns [6]. As a result, more sustainable enzymatic and microbial strategies for the production of 4VG from renewable feedstocks have been investigated. Although some yeasts and fungi have been tested, bacteria remain the most extensively studied - including *Streptomyces setonii, Cupriavidus, Enterobacter, Lactobacillus farciminis, and Bacillus spp*. However, wild-type strains generally exhibit low activity, producing modest amounts of 4VG (e.g., 720–885 mg/L) [7]. To enhance productivity, recombinant phenolic acid decarboxylases from a range of microorganisms have been expressed in heterologous hosts, including genes from *Enterobacter* spp., *Bacillus* spp., and *Aspergillus luchuensis*, enabling higher enzyme expression and better control

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over reaction conditions. Nonetheless, the inherent toxicity of both FA and 4VG limits the maximum achievable titers, which often remain below the thresholds required for efficient downstream processing [8]. Additionally, several bacterial recombinant whole-cell systems have been reported - primarily exploiting phenolic acid decarboxylases from *Bacillus pumilus, Bacillus atrophaeus,* and *Bacillus licheniformis* - achieving 4VG titers of up to 237 g/L [3,9,10]. Notably, these approaches rely on purified FA as the starting substrate and require external induction of decarboxylase expression (e.g., IPTG-based systems).

In a previous work, we produced vanillin and *cis,cis*-muconic acid starting from WB-derived FA through microbial conversion (based on a resting cell approach) by an engineered *E. coli* strain [11]. 4VG is an intermediate of this recombinant pathway, generated from the FA decarboxylation by the *B. pumilus* Fdc decarboxylase [12]. FA was extracted from WB following a three-step thermo-enzymatic treatment which breaks down WB polysaccharides releasing FA (and monosaccharides). The extraction step was followed by the purification of FA from the WB crude extract using an anion exchange resin while the carbohydrates-rich supernatant was discarded (notably, it can be further used as a fermentable substrate for microbial growth) [13].

The presence of WB-derived carbohydrates in the crude extract and the use of conditions for the enzymatic extraction step well suited for Fdc activity [12], 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 [14]. 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 (Fig. 1), the carbohydrates are used for the growth of the engineered strain and the FA is converted into 4VG by the Fdc decarboxylase, thus allowing for a straightforward valorization of WB. Moreover, to make the process more sustainable, *Fdc* gene can be under the control of a phenol inducible promoter to induce the expression of the enzyme by the FA released from the WB, avoiding the use of expensive inducers. Varman et al. [15] developed a hybrid phenol-inducible promoter for *E. coli* by combining the up and down regulatory sequences from the endogenous P_{emrR} 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 *E. coli* emrR transcription factor binds to P_{emrR} promoter repressing the transcription of the target gene; in the presence of lignin-derived phenolic compounds, such as vanillin and FA, these phenolics bind emrR causing its release from the P_{emrR} promoter and the consequent transcription of the target gene. 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. In addition, the Integrating in Situ Product Recovery (ISPR) approach [16–18], based on the WB-derived 4VG adsorption on the Amberlite XAD4 resin [2] in the tea bag system, may represent a low-cost, and high reproducible recovery strategy.

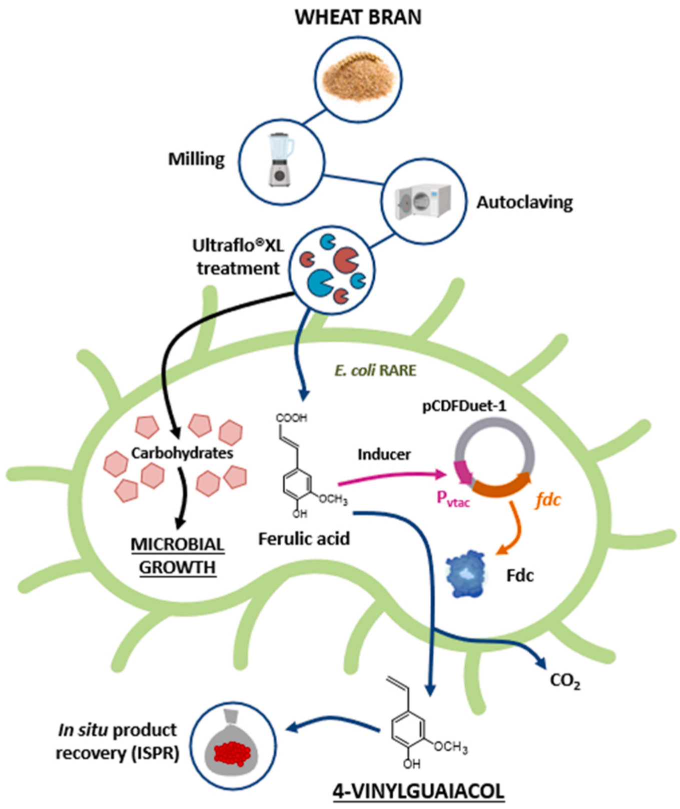

Fig. 1. Schematic representation of the one-pot simultaneous saccharification and co-fermentation (SSCF)-like process aimed at producing 4-vinylguaiacol from wheat bran-derived ferulic acid, used as both substrate and Fdc-expression inducer.

While our previous work demonstrated the feasibility of converting WB-derived FA into 4VG, it relied on resting-cell biotransformations, purified FA, and external induction of enzyme expression [12]. In this study, we advanced that approach by developing a fully self-sustaining, one-pot, autoinductive system. This configuration enables the direct bioconversion of FA released from WB without prior purification, integrating enzyme-assisted hydrolysis, microbial FA decarboxylation, and in situ product recovery within a single streamlined process. Such an autoinductive one-pot strategy represents a substantial step toward scalable, green biotechnological production of 4VG and related aromatic compounds.

2. Materials and methods

2.1. Materials

Methanol (ACS Grade, ≥99 %), formic acid (ACS Grade, ≥98 %), Amberlite™XAD4 and analytical grade standards of ferulic acid (*trans*-4-Hydroxy-3-methoxycinnamic acid, FA) and 4-vinylguaiacol (2-methoxy-4-vinylphenol, 4VG) were purchased from Merck (Merck KGaA, Darmstadt, Germany). The commercial food-grade enzymatic cocktail Ultraflo®XL was kindly provided by Novozymes (Bagsvard, Copenhagen, Denmark), and wheat bran was a generous gift of Molino Dallagiovanna (Gragnano Trebbiense, Piacenza, Italy).

2.2. Promoter design, cloning and E. coli transformation

The synthetic nucleotide sequence of the hybrid phenol promoter P_{vtac} was designed as reported in Varman et al., 2018 [15]. To facilitate the subcloning 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 P_{vtac} promoter, while the sequence corresponding to *EcoRI* (GAATTC) restriction site was added in the spacer sequence to allow the screening of the ligation products (see Table S1). The gene sequence coding for the decarboxylase Fdc from *B. pumilus* [12] 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 P_{vtac} promoter (produced by Twist Bioscience HQ, San Francisco, USA) and the plasmid were digested with the corresponding restriction enzymes (FastDigest, Thermo Fisher Scientific, Monza, Italy) and ligated using T4 DNA ligase (Thermo Fisher Scientific). The ligation mixtures were used to transform JM109 *E. coli* chemically competent cells. The *Fdc* gene was inserted into the pCDFDuet-1 plasmid obtaining the pCDFD:T7Fdc plasmid. Next, the P_{vtac} promoter was inserted upstream the *Fdc* gene in the pCDFD:T7Fdc plasmid, obtaining the pCDFD:HPFdc plasmid (Figure S1). The presence of the *Fdc* gene was verified by digestion with the corresponding restriction enzymes; the presence of the hybrid phenol promoter was verified by *EcoRI* restriction analysis. Finally, the two plasmids (pCDFD:T7Fdc and pCDFD:HPFdc, Table S1) were introduced into the *E. coli* MG1655 RARE [11] (reduced aromatic aldehyde reduction, bacterial strain #61440, Addgene, Watertown, USA) strain through chemical transformation to generate the whole-cell biocatalysts T7Fdc and HPFdc, respectively.

2.3. *Wheat bran hydrolysis*

The FA recovery from WB was carried out using a thermo-enzymatic method similar to the one reported by Bautista-Expósito et al. [19], and already utilized in our previous work [12]. In detail, WB 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). WB powder was suspended at a 1:20 solid to solution ratio (g/mL) and autoclaved at 121 °C, 1 bar, for 30 min. Different aqueous solutions were used: MilliQ water, 50–100–200 mM potassium phosphate pH 6.0, 100 mM citrate pH 5.0, 100 mM Tris-HCl pH 8.0, and 100 mM potassium phosphate pH 8.0. Then, the suspension was cooled to room temperature and 1 % UltrafloXL (enzyme to WB dry powder weight ratio, w/w) was added. The enzymatic hydrolysis was carried out at 37 °C, under shaking (130 rpm), for 16–24 h (WB crude extract).

2.4. *Growth analysis*

For the starting culture, the *E. coli* RARE strain was inoculated in LB medium and grown at 37 °C, 130 rpm, for 18 h. The next day, 50 mL of LB medium eventually added with 0.0005 % (v/v) UltrafloXL or 1 mM 4VG were inoculated with an amount of starting culture to an initial $OD_{600\ nm} = 0.1$, and the culture was incubated at 37 °C, 130 rpm. The optical density of the bacterial culture was spectrophotometrically recorded at 600 nm at different times and the experimental data points were analyzed by the Gompertz equation [20] to build growth curves and calculate the maximum specific growth rate (μ_{max}).

2.5. *RT-qPCR*

The *E. coli* T7Fdc and HPFdc biocatalysts were grown, and the Fdc expression induced, under the same conditions used for the specific activity measurement (see “Bioconversion: whole-cell Fdc specific activity assay” paragraph). After 3 h, an amount of culture volume equivalent to an $OD_{600\ nm} = 1$ was collected. Cells were treated with the RNAprotect® reagent (Qiagen), lysed by sonication (6 cycles of 20 s impulse at 20 % amplitude, and 60 s of pause in ice) and total RNA was purified by RNeasy mini kit® (Qiagen) according to manufacturer’s instructions. cDNAs were synthetized with the iScript™ kit (Bio-Rad) using 750 ng RNA. qRT-PCR reactions (15 µL volumes, in triplicate for each sample) were performed in a CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using the SensiFAST™ SYBR® No-ROX reagent kit (Meridian Bioscience). RT-qPCR amplification conditions were: 2 min initial denaturation at 95 °C, 40 cycles at 95 °C for 5 s, 58 °C for 10 s and 72 °C for 10 s. Relative expression level of Fdc was calculated by the 2-ΔΔCt method using *idnT* gene as internal reference gene [21]. The primers used for the qPCR quantification of *Fdc* and *idnT* genes are reported in Table S2.

2.6. *Bioconversion: whole-cell Fdc specific activity assay*

For the starting culture, the biocatalysts T7Fdc and HPFdc *E. coli* strains were inoculated in M9 medium [22] containing 50 µg/mL streptomycin and grown at 37 °C, 130 rpm for 18 h. The next day, 50 mL of M9 medium containing 50 µg/mL streptomycin were inoculated with an amount of starting culture to an initial $OD_{600\ nm} = 0.1$, and the culture was incubated at 37 °C, 130 rpm, until the $OD_{600\ nm} \approx 0.6–0.8$. Then, 0.1 mM IPTG or 1 mM FA was added to the cultures to induce the expression of Fdc, and the cells were further grown at 37 °C, 130 rpm, for 3 h. The cells were harvested by centrifugation (8000 g, 10 min, 4 °C), washed once in 100 mM Tris-HCl pH 8.0, and resuspended in the same buffer to reach a final concentration of 350 mg_{cww}/mL. The same expression protocol was also performed without adding the inducers (i.e. IPTG or FA), to be used as control. The whole-cell Fdc specific activity assays were set up using 2 mM FA as substrate and the recombinant *E. coli* cells (0, 5, 10 and 20 mg_{cww}/mL) in 100 mM Tris-HCl pH 8.0. All biotransformations were performed at room temperature (25 °C) in 1 mL final volume; after 10 min of incubation, a 50 µL aliquot was sampled and diluted 1:3 in 2.5 % formic acid to stop the enzymatic reaction. The diluted sample was centrifuged (10 min, 10000 g, 25 °C) and 40 µL of the supernatant were analysed by HPLC (see “HPLC analyses” paragraph).

2.7. *Bioconversion: whole-cell growth on the WB crude extract*

For the starting culture, the engineered HPFdc *E. coli* strain was inoculated in the LB medium containing 50 µg/mL streptomycin and grown at 37 °C, 130 rpm, for 10–18 h. Then, the WB crude extract, supplemented with 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 for 16 h. The WB crude extract was added simultaneously or 16 h after the addition of UltrafloXL. The growth of the engineered strain on the WB crude extract was assayed by counting colony forming units (CFU) on LB agar plates added of streptomycin: the withdrawn samples were serially diluted in 1 % (w/v) NaCl to achieve an overall million-fold dilution 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.

2.8. *4VG recovery*

The 4VG was recovered from the growth medium using the reverse phase adsorbent resin Amberlite XAD4 [2]. Before use, the resin was activated by incubation in 96 % ethanol, overnight at 4 °C. The activated resin was packed in a 10 mL propylene column with porous filter to remove the ethanol and washed with 10–20 volumes of MilliQ water. After the removal of the water, the resin was weighed and used for the purification of 4VG. To assay the binding capability of the resin, 10 mg resin were added to 1 mL of 0.3–1–3–9 mM 4VG in MilliQ water or to the WB crude extract, at 25 or 37 °C. At different incubation times, the supernatant was sampled and analyzed by HPLC (see “HPLC analyses” paragraph) to evaluate the residual amount of 4VG in the supernatant. After the binding step, the resin was washed in MilliQ water to remove WB particles. The bound 4VG was released by two sequential elution steps: the resin was incubated twice in 96 % ethanol (20 mL per g of resin) at 37 °C, 130 rpm for 1 h. The alcoholic solutions were analyzed by HPLC (see “HPLC analyses” paragraph).

2.9. *One-pot 4VG production*

The “teabags” for 4VG recovery were built by thermosealing a nylon filter (100 mesh) containing 1 g of Amberlite XAD4 resin, from now on named XADbags. The activation and elution of the XADbags were performed following the same protocol used for the free resin. The one-pot 4VG production was carried out in a 250 mL flask (5 g WB and 100 mL MilliQ water) or in a 2 L flask (25 g WB and 500 mL MilliQ water). The WB-containing flasks were autoclaved (121 °C, 30 min) and after cooling at room temperature, 50 µg/mL streptomycin, 1 % UltrafloXL (enzyme to WB dry powder weight ratio, w/w), 1 % (v/v) starting culture (see “Bioconversion: whole-cell growth on the WB crude extract” paragraph) and 1 or 5 XADbags were added. The flasks were incubated at 37 °C, 130 rpm for 16 h. At this point, the XADbags were recovered, washed with MilliQ water and the bound 4VG eluted following the same procedure reported in "4VG recovery" section. A schematic representation of the 4VG production and recovery process is depicted in Fig. 1.

2.10. HPLC analyses

HPLC analyses of aromatics 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 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 produced by solubilizing commercial standards of FA in 100 mM Tris-HCl, pH 8.0, and 4VG in DMSO, at a final concentration of 40 mM. 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 used for HPLC analysis. Retention times for FA and 4VG standards were 6.9 and 12.0 min, respectively.

The quantification of glucose, lactose, fructose, xylose, arabinose, galactose, maltose and mannitol was also performed by HPLC using a PRONTOGEL H column (300 × 8.0 mm; Bischoff, Leonberg, Germany) and a refractive index 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 50 °C. Calibration curves were generated by solubilizing standard carbohydrates in MilliQ water at a final concentration of 40 g/L; after dilution in the 0.15 –20 g/L range, the samples were diluted 1:5 in the mobile phase, centrifuged for 2 min at 11,000 g, 4 °C before injecting 50 µL in the HPLC system. Retention times for glucose, lactose, fructose, xylose, arabinose, galactose, maltose and mannitol were 7.15, 6.25, 7.75, 7.6, 8.25, 7.5, 6.15 and 7.9 min, respectively.

2.11. GC-MS analysis

GC-MS analysis was performed on the alcoholic eluates from the XAD bags. Gas chromatographic separation was achieved using a Trace 1600 GC (Thermo Fisher Scientific) equipped with an Autosampler 1610. A TraceGOLD TG-5MG column (30 m × 0.25 mm, 0.25 µm film thickness; Thermo Fisher Scientific) served as the stationary phase, while helium (UHP grade) was used as the carrier gas. Mass spectrometric detection was carried out with an ISQ7610 single quadrupole MS operating at 70 eV ionization energy. The system was configured with an initial pressure of 100 kPa, an injection temperature of 280 °C, and an interface temperature of 200 °C. The GC oven temperature program was set as follows: initial temperature of 50 °C held for 1 min, ramped at 10 °C min^{-1} to 280 °C, and held for 15 min. Instrument control and data acquisition were performed using Chromeleon 7.3.2 software. Compound identification was accomplished by comparison with the NIST20 mass spectral library.

2.12. Statistical analysis

Statistical analysis was performed using GraphPad Prism software (version 9.0.0) applying multiple Student's *t*-tests and two-way ANOVA with a 95 % confidence interval. Significance was assessed at $p < 0.05$. Results were expressed as the mean ± standard error.

3. Results and discussion

3.1. Microbial growth in WB crude extract

The engineered HPFdc strain was generated by subcloning the synthetic gene encoding the *B. pumilus* Fdc decarboxylase [23] in pCDFDuet-1 plasmid. The DNA fragment containing 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 S1).

The final pCDFD:HPFdc plasmid was used to transform *E. coli* MG1655 RARE [11] cells to obtain the HPFdc strain. To test whether the addition of the enzymatic UltrafloXL cocktail (containing cellulase and xylanase activities) [12] affects the growth of *E. coli*, the μ_{max} of the *E. coli* RARE cells culture in LB medium added of UltrafloXL (0.0005 % v/v, i.e. the same concentration used for the following WB hydrolysis) was determined: no inhibitory effect on *E. coli* growth was observed under the tested conditions ($\mu_{max} = 2.3 \pm 0.3\ h^{-1}$ compared to $2.0 \pm 0.5\ h^{-1}$ in the control growth conditions) (see Figure S2). Then, the *E. coli* RARE strain was grown in the presence of 1 mM 4VG, since it is reported that vinylphenol compounds could be toxic for *E. coli* strains compared to the respective acid derivative [24]. As expected, the growth rate of the strain incubated with 4VG was lower ($1.8 \pm 0.3\ h^{-1}$) compared to the control conditions, reaching a lower final saturation density value after 24 h of incubation ($3.9 \pm 0.1\ OD_{600\ nm}$/mL vs $5.1 \pm 0.1\ OD_{600\ nm}$/mL). Unexpectedly, after the incubation, 81.1 ± 2.7 % of 4VG remained in the culture broth, suggesting that 4VG was partially degraded by some endogenous *E. coli* enzymatic activities (4VG is stable under these conditions in the absence of *E. coli* cells). Finally, the addition of 10 mM FA to cell culture has little to no inhibitory effect on the *E. coli* growth, as also demonstrated in our previous work [12].

The thermo-enzymatic treatment of WB enables the release of FA and carbohydrates, the latter useful to sustain *E. coli* growth. In detail, the UltrafloXL enzyme cocktail containing cellulase and xylanase activities, effectively hydrolyzes WB's complex polysaccharides—mainly arabinoxylans and cellulose—thereby releasing FA and increasing the availability of monosaccharides such as arabinose, xylose, and glucose [12, 25]. In this view, the carbohydrate concentration has been assayed after: i) the autoclave step, ii) the following enzymatic incubation with UltrafloXL, and iii) after the growth of the engineered strain in the WB crude extract. As shown in Fig. 2, the highest amount of carbohydrates was released following the enzymatic step (blue bars). Interestingly,

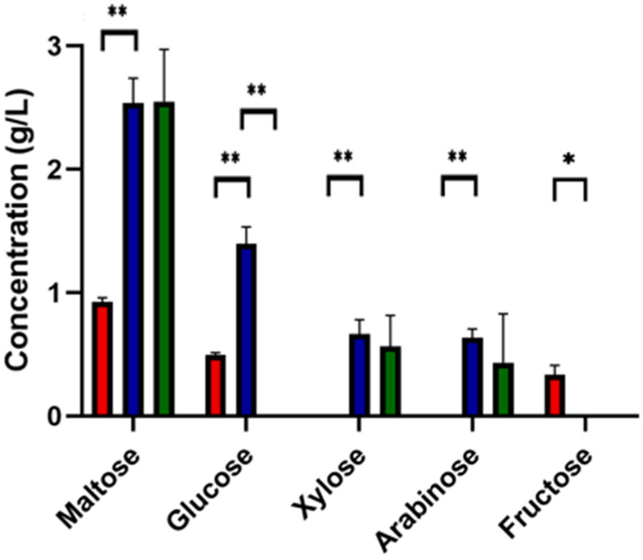

Fig. 2. Carbohydrates released by the thermo-enzymatic treatment of WB. Carbohydrates have been quantified following the autoclave step (red), the autoclave and enzymatic step (blue), and at the end of the engineered strain growth in the WB crude extract (green). The values are reported as mean ± standard deviation (n = 3). Statistical analysis was performed using two-way ANOVA followed by a Tukey's multiple comparison test. * = $p < 0.05$; ** = $p < 0.0001$.

when the engineered strain was inoculated in the WB crude extract and incubated at 37 °C for 18 h, glucose was completely consumed, and a limited consumption of arabinose and xylose was also observed (see Fig. 2, green bars).

Notably, since glucose is already released by the autoclave step, the engineered strain should grow on the WB crude extract even before the enzymatic treatment by UltrafloXL. To clarify this point, in the "post-ultra" condition, WB was autoclaved and treated with UltrafloXL before inoculating *E. coli*, and in the "co-ultra" condition *E. coli* was added to the WB crude extract together with UltrafloXL; afterward, both samples were incubated at 37 °C, 130 rpm for 16 h before being plated. The cell density obtained in the co-ultra condition was slightly lower (0.71 ± 0.05 OD/mL) compared to the value obtained in the post-ultra (i.e. 0.86 ± 0.06 OD/mL). This result demonstrates that the engineered strain is able to grow on the WB crude extract even without the UltrafloXL treatment, opening to the option to perform in one-pot the extraction of FA from WB by UltrafloXL and its conversion to 4VG.

3.2. P_{vtac} promoter strength assessment

The strength of the P_{vtac} promoter was compared to the strong T7 promoter by measuring the transcription efficiency by RT-qPCR of *Fdc* gene and the specific activity of Fdc in the T7Fdc and HPFdc strains. As shown in Fig. 3A, both strains showed a ≈ 20-fold increase of the relative *Fdc* expression level when induced with 0.1 mM IPTG, while using FA as inducer, the transcription of the *Fdc* gene was observed only for the HPFdc strain (≈ 3.3-fold increase in comparison to the control), highlighting that the presence of the P_{vtac} promoter is fundamental to allow FA-induced transcription. The strength of the hybrid promoter was ≈ 6-fold lower compared to the T7 one.

In view of a practical application, the promoter strength was evaluated assessing the Fdc enzymatic specific activity to verify whether the observed increased transcription results in increased enzyme activity, and therefore 4VG production. As shown in Fig. 3B, the induction by 0.1 mM IPTG resulted in a ≈ 5.5-fold increase of the Fdc specific activity in both strains, since the T7 promoter is present in both plasmids. Furthermore, the addition of 5 mM FA resulted in a significant effect only for the HPFdc strain carrying the P_{vtac} promoter (while the T7Fdc strain showed a specific activity similar to the control): a ≈ 3-fold increase of Fdc specific activity was observed, a figure 2-fold lower compared to the maximal value obtained using IPTG (2.0 ± 0.2 vs. 3.9 ± 0.2 μmol min^{-1} mg$_{cww}^{-1}$).

3.3. SSCF-like bioconversion

The ability of the HPFdc strain to convert the substrate FA (also acting as inducer of Fdc expression) into 4VG, was evaluated at different incubation times in two buffered solutions (100 mM phosphate buffer, pH 6.0 or pH 8.0) to evaluate the effect of pH on 4VG production, as well as in water, which use could reduce the cost of the overall process (Table S3). The HPFdc strain was inoculated into the WB extract generated by autoclaving both simultaneously (co-ultra) or 16 h after (post-ultra) the addition of UltrafloXL. Moreover, three control reactions were set up for the co-ultra setting by adding: i) UltrafloXL (ultra), ii) UltrafloXL and an *E. coli* strain not expressing the Fdc enzyme (*E. coli*), iii) UltrafloXL and the T7Fdc strain (T7Fdc).

Considering that the previously optimized three-step FA extraction method from WB resulted in 3.0 ± 0.2 mg$_{FA}$/g$_{WB}$ (15.5 ± 1.3 μmol/g$_{WB}$) [12], the maximal achievable production of 4VG should correspond to 2.3 mg$_{4VG}$/g$_{WB}$. Interestingly, the presence of *E. coli* cells in the WB crude extract did not exert any inhibitory effect on the UltrafloXL activity: the FA extraction yield (2.85 ± 0.06 mg/g$_{WB}$, 14.68 ± 0.01 μmol/g$_{WB}$) was similar with or without the *E. coli* cells (Fig. 4 A). The HPFdc strain fully converted FA into 4VG in all tested conditions, while a partial conversion (17.0 ± 1.1 %) of the WB-derived FA was obtained for the T7Fdc strain, indicating that the Fdc expression under the P_{vtac} promoter is fundamental to efficiently convert FA to 4VG. This result may be related to the basal expression of Fdc since even the non-induced strains catalyze the partial decarboxylation of FA (CTRL in Fig. 3B).

Although FA was completely depleted during the reaction, only 65–78 % of the expected 4VG was produced, suggesting that 4VG may be further converted into other compound(s), either spontaneously or through endogenous *E. coli* enzymatic activities. On this side, 1 mM 4VG remained unaltered when incubated at 37 °C for 16 h in MilliQ water or LB medium, while in the presence of *E. coli* cells a 22–35 % decrease was observed. These results confirm that the loss of 4VG in the SSCF-like process is mainly attributable to cellular uptake or metabolic conversion by *E. coli* cells, rather than to spontaneous degradation. As shown in Fig. 4 A, FA remained stable under the tested conditions. Interestingly, comparable 4VG yield were obtained when the bioconversion was carried out in phosphate buffer at pH 6.0 (1.51 ± 0.04 mg$_{4VG}$/g$_{WB}$, 10.05 ± 0.01 μmol/g$_{WB}$) or pH 8.0 (1.47 ± 0.03 mg$_{4VG}$/g$_{WB}$, 9.79 ± 0.01 μmol/g$_{WB}$), meanwhile a higher yield was achieved in MilliQ water (1.72 ± 0.06 mg$_{4VG}$/g$_{WB}$, 11.45 ± 0.01 μmol/g$_{WB}$). This difference may be related to the final pH value of the cultures: while the two buffered

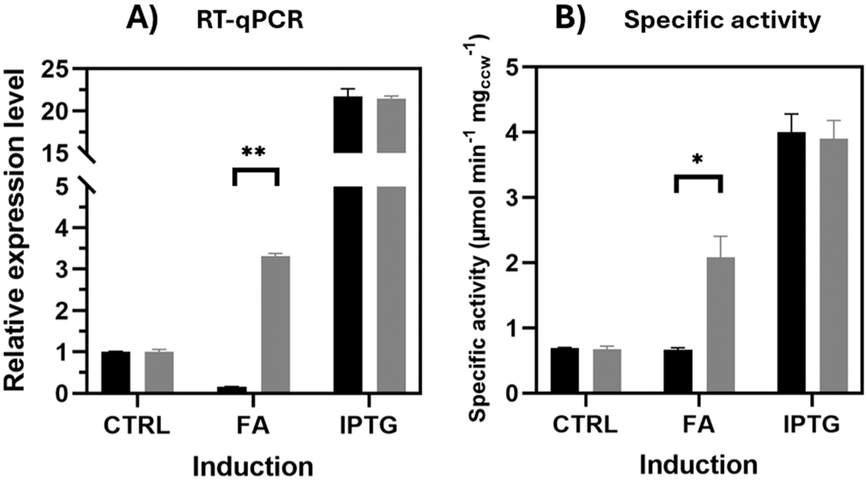

Fig. 3. Assessment of the promoters strength. The expression of the Fdc enzyme in T7Fdc (black) and HPFdc (gray) strains was induced adding 5 mM FA (FA) or 0.1 mM IPTG (IPTG). A) RT-qPCR analysis reporting the relative expression level of *Fdc* vs. *idnT* (an endogenous *E. coli* gene) as internal reference. B) Whole-cell Fdc specific activity level: the enzymatic activity was determined by measuring by HPLC the 4VG produced in a resting cell approach. The values are reported as mean ± standard deviation (n = 3). Statistical analysis was performed using two-way ANOVA followed by a Tukey's multiple comparison test. * = $p < 0.05$; ** = $p < 0.0001$.

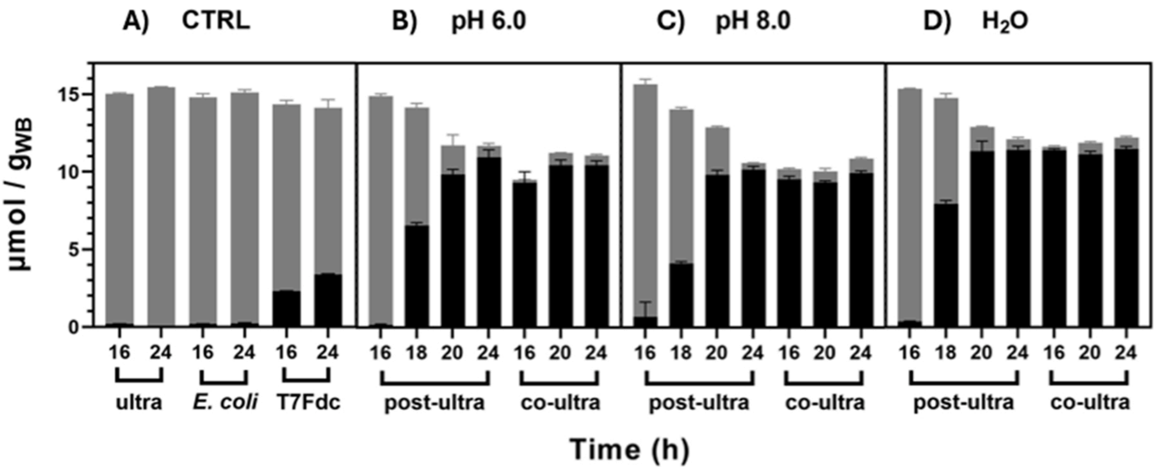

Fig. 4. 4VG production from WB in a SSCF-like approach. A) Control reactions (CTRL): ultra, containing only UltrafloXL; *E. coli*, containing UltrafloXL and *E. coli* RARE strain; T7Fdc, containing UltrafloXL and T7Fdc strain. B, C) Bioconversion reactions carried out in 100 mM potassium phosphate buffer, pH 6.0 or pH 8.0. D) Bioconversion reactions carried out in MilliQ water. 4VG: black bars; FA: gray bars.

solutions maintained pH$\geq$ 6.0, the MilliQ culture reached a final pH $\approx$ 5.0, which appears to favour 4VG (Fig. 4B-D). To further verify whether the differences in 4VG yield were primarily driven by the pH of the reaction medium rather than by the presence or composition of the buffer, additional bioconversion experiments were performed using different buffering systems (50 and 200 mM phosphate, pH 6.0; 100 mM citrate, pH 5.0; and 100 mM Tris-HCl, pH 8.0) (Figure S3). Comparable 4VG titres were obtained under all conditions, with a slightly higher production observed in 100 mM citrate at pH 5.0 — the only condition showing a statistically significant increase compared to the other buffers. These findings indicate that the enhanced 4VG accumulation previously observed in MilliQ water is primarily attributable to the lower final pH of the culture, rather than to any inhibitory or compositional effect of the buffer. Notably, pH 5.0 corresponds to the reported optimum for Fdc enzyme activity [23], further supporting this value as the most favorable for both enzymatic decarboxylation and product stability.

Furthermore, when ammonium chloride (1 g/L) or yeast extract (0.5 g/L) were added before the autoclave step of the extraction process as additional nitrogen source for *E. coli* growth, the amount of produced 4VG was unaffected (data not shown).

The highest 4VG productivity was obtained after 16 h in MilliQ water using the co-ultra inoculum (5.3 ± 0.1 mg/L•h, 35.3 ± 0.7 μmol/L•h); accordingly, this condition was used for the following one-pot 4VG production. Notably, the time-course profile of 4VG production under the selected conditions (co-ultra in MilliQ water) shows that maximum productivity is reached within the first 8 h of incubation (Figure S4, dotted line). However, when the reaction is carried out in the presence of a tea bag containing XAD4 resin, a longer incubation period ($>$8 h) is required to achieve $>$ 90 % adsorption of the produced 4VG (Figure S4, solid line). Accordingly, a 16 h incubation time was selected to ensure efficient product recovery and compatibility with the subsequent one-pot processing (Section 3.4), underscoring the need to balance conversion kinetics with effective in situ product capture.

3.4. One-pot 4VG production and recovery

The recovery of 4VG from the reaction mixture was performed using the reverse phase adsorbent resin Amberlite XAD4 [2]: in particular, the "tea-bag" approach based on XADbag could allow the simultaneous production and recovery of the 4VG product (Fig. 5).

At first, the binding capability of the XAD4 resin was evaluated using the commercial 4VG dissolved in MilliQ water, at 37 °C in view of using the resin during the bioconversion step. The adsorption performance of XAD4 resin was investigated by varying the 4VG-to-resin ratio (i.e. 5, 15, 45, and 135 mg$_{4VG}$ per gram of resin), by monitoring residual 4VG present in the supernatant after 1 h of incubation: the highest adsorption efficiency (96.1 ± 0.5 %) was obtained at 15 mg$_{4VG}$/g resin (see Table S4). This ratio was used in all following experiments and in the one-pot bioconversion assays. Notably, using the WB-derived 4VG present in the WB crude extract after the thermo-enzymatic treatment and HPFdc bioconversion, a recovery yield similar to the value obtained for commercial 4VG was obtained after 1 h of incubation (i.e. 95.1 ± 1.1 %). No FA adsorption to the XAD4 resin was observed under the same conditions.

The simultaneous FA extraction, conversion and 4VG recovery was then evaluated using the XAD4 resin particles in the reaction mixture: the resin was added to the WB crude extract incubated with UltrafloXL

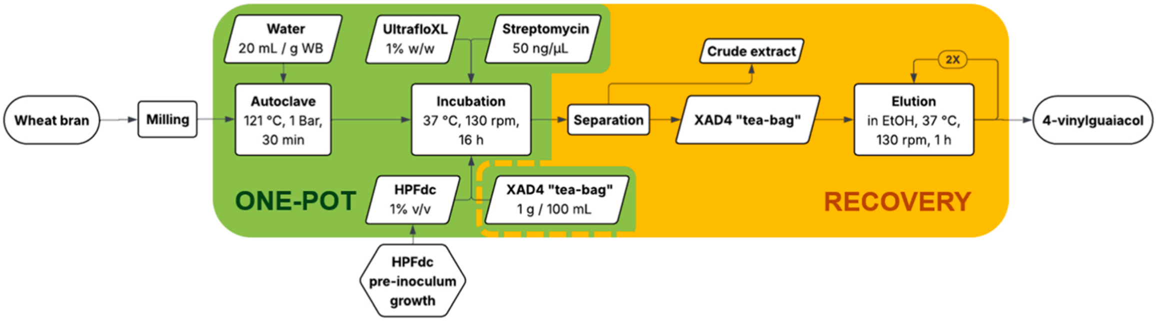

Fig. 5. Workflow of the proposed biocatalytic process for the 4VG production from WB. The steps highlighted in green and orange represent the one-pot bioconverison and the recovery approach, respectively.

and HPFdc strain. After the incubation at 37 °C for 16 h, the resin was removed from the WB crude extract by filtration. In the first elution step, 84.5 ± 1.5 % of the bound 4VG was released, corresponding to 1.51 ± 0.03 mg_{4VG}/g_{WB}, and 0.29 ± 0.02 mg_{4VG}/g_{WB} were recovered in the following elution step.

Finally, the overall process was evaluated using XADbags: after the autoclave treatment, 100 mL of WB crude extract containing 15 mg 4VG was added of one XADbag (1 g resin, to maintain the optimal 15 $mg_{4VG}/$g resin ratio), UltrafloXL and the HPFdc strain. After 16 h of incubation at 37 °C, the XADbag was retrieved from the bioconversion mixture. Interestingly, an increase in the overall recovery yield was achieved: the total amount of recovered aromatics (FA + 4VG = 17.1 ± 0.1 μmol/g_{WB}) was similar to the amount of FA extracted using the NaOH method (17.5 ± 0.2 μmol/g_{WB}, 3.4 ± 0.1 mg/g_{WB}), and 9.5 ± 0.6 % higher than FA extraction yield using the UltrafloXL only (15.45 ± 1.3 μmol/g_{WB}, 3.0 ± 0.2 mg/g_{WB}). Most importantly, the consumption of 4VG by *E. coli* cells observed during the bioconversion assay (see "SSCF-like bioconversion" paragraph) was not apparent using XADbags, leading to a ≈ 23.5 ± 1.6 % increase of the total extraction and bioconversion yield. The amount of 4VG recovered from the XADbag was 2.17 ± 0.13 mg_{4VG}/g_{WB} (15.1 ± 0.2 μmol/g_{WB}) corresponding to a 88.3 ± 2.5 % recovery yield vs. the 84.5 ± 1.9 % figure obtained for the free resin. These results confirm that the XADbags efficiently captures 4VG during the process, preventing its microbial degradation while maintaining a recovery yield consistent with the theoretical maximum expected from WB.

The identity of 4VG was confirmed by GC-MS analysis of the ethanol eluates from the XAD4 resin bags (see Figure S5). The chromatogram revealed that 4VG accounted for ≥ 80 % of the detected compounds, while a minor peak (2.5 %) corresponded to 4-vinylphenol, likely originating from *p*-coumaric acid naturally present in WB.

This one-pot process was performed on a larger sample (25 g of WB) using a 2 L flasks containing 500 mL of MilliQ water added with 5 XADbags, thus maintaining the same resin-to-biomass ratio. Noteworthy, a 96.6 ± 2.2 % (mol/mol) aromatics (FA + 4VG) extraction yield was achieved: the amount of 4VG eluted from the XADbags was 1.86 ± 0.13 mg/g_{WB}, corresponding to a 75.9 ± 3.9 % recovery yield, confirming good performance under higher substrate load. Notably, the XAD resin could be reused up to three times without any loss of 4VG recovery efficiency.

4. Conclusion

Valorizing agro-industrial byproducts such as WB through microbial or enzymatic processes represents a promising strategy for the sustainable synthesis of aromatic compounds such as 4VG. This approach not only supports the transition toward bio-based alternatives to petrochemical products, but also aligns with bioeconomy principles by converting low-cost residues into value-added biochemicals. Technically, the main challenges lie in efficiently releasing FA from the lignocellulosic matrix of WB and subsequently converting it into 4VG. The limited FA content of WB (0.1–1.0 mg FA g^{-1} WB) inherently constrains the maximum theoretical yield [26]. Nevertheless, advances in enzyme engineering and the use of engineered microorganisms have markedly improved conversion efficiencies and product titers [14,27]. The use of whole-cell biocatalysts enables mild reaction conditions and reusability, which can lower the operational costs; when coupled to the tea-bag-based product recovery system, this approach also enhances reproducibility and reduces downstream processing costs.

Starting from a WB sample containing 3.5 mg of FA per gram of WB [12] - theoretically corresponding to a maximum of 2.3 mg of 4VG per gram of WB - the proposed one-pot biocatalytic process achieved an overall 76 % recovery yield, resulting in 2.0 mg of 4VG per gram of WB. This outcome was attained using a fully green technology that integrates enzymatic hydrolysis via the UltrafloXL cocktail with the microbial bioconversion by an engineered *E. coli* strain expressing Fdc under the control of a phenol-inducible promoter.

A key innovation of the proposed process lies in its autoinductive and self-sustaining nature: the same FA extracted from WB acts as both the substrate and the inducer for the expression of the decarboxylase enzyme Fdc, since the engineered *E. coli* strain carries a phenol-inducible promoter (P_{vtac}). Simultaneously, the glucose released from WB supports microbial growth, enabling the entire system to function using only components derived from the starting biomass. As highlighted in Fig. 1, this design allows the valorization of WB within a one-pot, SSCF-like configuration, without requiring external inducers or carbon sources.

The carbohydrates released from the WB by our previous three-step method (based on milling, autoclaving and UltrafloXL enzymatic hydrolysis) [12], and those obtained in this study, are compared in Table 1 to the ones reported by Di Gioia et al. in their work [28] normalized by the amount of WB used. Differences in terms of total amount of released carbohydrates and mixture composition are apparent that can be ascribed to the different starting materials (i.e. the origin of WB) and to the different commercial enzymes used in the thermo-enzymatic treatment. Actually, the enzymatic activities reported for UltrafloXL are β-endoglucanase and xylanase, while the two commercial enzymes used by Di Gioia et al. [28] were Fungamyl® SUPER AX and Celluclast®BG containing α-amylase and cellulase activities, respectively.

The proposed bioconversion system simultaneously valorizes FA and glucose released from WB; however, the other soluble sugars (see Table 1), as well residual solid biomass, remain largely unexploited under the present conditions, and could be further valorised through complementary routes. Growth experiments confirmed that *E. coli* selectively consumed glucose, while the other released mono- and disaccharides were not metabolized. Nevertheless, these sugars represent attractive feedstocks for integration into a broader biorefinery framework. Xylose and arabinose, for instance, can be directed toward microbial production of xylitol, arabinitol, lactic acid, or succinic acid using specialized fermentative strains [29–31], whereas maltose may be used for the synthesis of value-added oligosaccharides or fermented to ethanol by yeasts such as *Saccharomyces cerevisiae* and *Rhodotorula* sp. [32,33], Moreover, hemicellulosic fractions rich in arabinoxylans could be further converted into arabinoxylo-oligosaccharides or dietary fibres for food and feed applications [34,35]. Importantly, in our process the residual soluble carbohydrates do not interfere with 4VG recovery or purity, as they remain confined to the aqueous phase and are not co-eluted with the product during ethanol-based extraction.

Given that WB typically contains 0.1–1 mg_{FA}/g_{WB} [26], the maximal production level of 4VG from WB remains moderate, typically in the range of milligrams per gram of raw substrate. In contrast, studies using purified FA [3] and a solvent-tolerant phenolic acid decarboxylase from *Bacillus atrophaeus,* reported very high product titers, up to 237 g/L. Similarly, phototrophic cyanobacteria (*Synechococcus sp.* PCC 11901) have been engineered to reach gram-scale 4VG production from FA feedstocks [5]. Recently, a novel bioprocess using *Rhizopus oryzae*, an aromatic compound-degrading fungus, was developed for the co-production of 4VG and fumaric acid: when cultured on a glucose-xylose mixture, the strain yielded 4.6 g/L of 4VG and 11.3 g/L of fumaric acid [4]. For comparison, the maximum 4VG concentration obtained in our system (2.0 mg g^{-1} WB, corresponding to roughly

Table 1
Comparison of the carbohydrates released from WB in this work and in Di Gioia et al. [28].

	Released sugars (mg/g WB)	
Sugar	This study	Di Gioia et al. [28]
Maltose	50.7	149.7
Glucose	27.9	29.8
Xylose	13.3	46.6
Arabinose	12.7	20.1
Total carbohydrates	650*	508

*Molinari et al. [12]

100 mg L^{-1} in the reaction mixture) is comparable to the values reported by Williamson et al. [36], who achieved 15–62 mg L^{-1} 4VG in engineered *Pseudomonas putida* Δech::padC cultures grown on lignocellulosic substrates such as Green Value Protobind lignin. In their system, padC expression was placed under control of the ferR operon - inducible by several hydroxycinnamic acids - and gene deletions (*fcs, ech*) were required to prevent FA catabolism. Despite the lower FA content of WB relative to lignin-rich feedstocks [36], our platform reached comparable 4VG titres, underscoring the efficiency of this simplified, heterologous bioconversion platform.

To the best of our knowledge, our process is the first report of a biocatalytic process producing 4VG from WB-derived FA using an engineered *E. coli* strain, especially in a self-sufficient process where FA is used as inducer and substrate. Moreover, in the proposed process, the combination of enzyme-assisted hydrolysis, microbial bioconversion, and ISPR via tea-bag resin systems not only simplifies downstream processing, but also enhances the overall yields by limiting product loss. This system reached a 4VG production of approximately 2.0 mg per gram of WB, corresponding to an overall conversion efficiency of ~88 %. Moreover, the robustness of the process is confirmed by successful operation in 2 L flasks with 25 g of WB, maintaining a high FA extraction yield (~97 %) and 4VG purification efficiency (~76 %). These results provide a proof-of-concept for ISPR via a resin-based adsorption strategy, which could be further adapted to more advanced configurations for process intensification. Although the “tea-bag” setup is not directly scalable, the underlying principle is fully compatible with industrial adsorption systems such as packed-bed, expanded-bed, or circulating fluidized-bed columns, which are well established as effective solutions for continuous downstream bioprocessing [37]. Future developments should prioritize resin regeneration and reuse, fouling mitigation, and integration with membrane-assisted or hybrid systems to enhance productivity and more efficiently manage dilute process streams.

A preliminary cost estimation of 4VG production was conducted to provide an initial indication of the potential economic relevance of the proposed system. The analysis considered only the cost of raw materials and reagents used at laboratory-scale — specifically WB, UltrafloXL, culture medium components, and Amberlite XAD4 resin — resulting in an estimated cost of approximately 0.15 € per liter of reaction mixture (≈1.5 € per g of 4VG). This estimate excludes utilities, energy, labor, and equipment-related expenses, which would substantially raise the overall production cost at larger scales. For reference, the commercial price of ≥ 98 % purity 4VG from Merck KGaA is around 25 € per gram. Although this comparison underscores the economic promise of renewable feedstock-based 4VG production, a comprehensive techno-economic assessment will only be meaningfully once the process is implemented at pilot or industrial scale.

Overall, the auto-inductive system developed in this study demonstrates the feasibility of integrating enzymatic biomass deconstruction, microbial catalysis, and ISPR into a single, self-sustaining process. While similar integrated bioprocesses have been explored for lignocellulosic valorization, their application to the biosynthesis of aromatic compounds remains comparatively limited [38,39]. The achieved conversion efficiency and volumetric productivity are promising, although still below industrially relevant targets. Further optimization of the hybrid phenol-inducible promoter — for example by fine-tuning operator spacing or modulating transcription factor binding affinity — could broaden its dynamic range and responsiveness to FA levels [40,41].

The ISPR strategy adopted here — including the use of a tea-bag resin system — effectively mitigated product volatilization and potential inhibition effects. However, future studies should evaluate long-term resin performance, including saturation kinetics, regeneration efficiency, and mechanical stability under continuous or fed-batch operation [17].

Microbial robustness also remains a critical factor for process intensification. Continuous exposure to phenolic intermediates can induce oxidative stress and compromise membrane integrity, ultimately reducing cell viability during extended biotransformations. In parallel, monitoring the formation of potential by-products will be essential to avoid pathway diversion and unwanted degradation of 4VG. Looking ahead, the integration of auto-inductive platforms with modular cascade systems could enable fully self-regulated microbial factories powered by renewable feedstocks. Such developments would represent a significant step toward sustainable, low-input biotechnological production of aromatic building blocks.

CRediT authorship contribution statement

Luca Brambilla: Supervision, Methodology, Investigation. **Loredano Pollegioni:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Conceptualization. **Stefano Busti:** Methodology, Investigation. **Marco Vanoni:** Supervision, Methodology, Investigation. **Filippo Molinari:** Writing – original draft, Visualization, Methodology, Investigation. **Alex Pessina:** Writing – original draft, Methodology, Investigation. **Elena Rosini:** Writing – original draft, Validation, Supervision, Methodology, Investigation, Conceptualization.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2025.11.003.

Data availability

Data will be made available on request.

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