Metabolic and Process Engineering for Synthesis of Monolignols Ligneering

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This project aims to produce phenolics using a biotechnological approach. Such compounds are found e.g. in plants, where they are important precursors to biopolymers or contribute to taste, color, flavour and nutraceutical properties of crops. Due to their often limited availability from plants source, this project is focused on the production of plant phenolics by microbial fermentation. Advantageously, glucose as a renewable feedstock will be used. The produced monolignols are then to be polymerized to produce lignin-like structures, the so-called dehydrogenation polymers (DHP), which could potentially be used as a substitute for petroleum-based chemicals such as epoxide-based resins and glues.
LIGNEERING is a consortium of two academic partners, the Martin-Luther-Universität Halle-Wittenberg and the Leibniz Institute of Plant Biochemistry. The combined expertise of the partners facilitates the creation of microbial producer strains for selected phenolics (e.g. monolignols), which includes the development of suitable biocatalysts, their integration into the microbial hosts and the technical implementation of production processes by fermentation. Thus, the aim is to establish a “green”, biosynthetic and high-yield production platform, which will help to substitute similar products made from fossil carbon and to significantly reduce CO_2 in the future and to provide substitute materials for petrol-based commodities.

I. Topic, open questions and purpose

Microbial processes for production of the monolignols *p*-coumaryl alcohol (Cm-OH), coniferyl alcohol (C-OH), sinapyl alcohol (S-OH) developed previously (Monomer project, FKZ 031A551) showed two main limitations regarding efficiency and achievable product concentrations. Due to unwanted basal expression of the enzymes for the intended biotransformation, the growth of the *E. coli* cells in minimal medium was hampered, caused presumably by the presence of a cryptic promoter sequence in the used plasmid (Dillmann 2020). Cell growth and biomass production was only possible by supplementation of the medium with complex components like yeast extract or peptone, which could increase the cost for purification of the product after the biotransformation in large scale production. Furthermore, it was observed that the

accumulation of the monolignol products in the medium had toxic effects on the production strain which prevented further conversion of the fed substrates and limited the overall yield. Previous experiments achieved product concentrations of up to 11 mM of coniferyl alcohol (C-OH) in a small scale bioreactor (0.4 L) by using a two-step process with initial production of biocatalysts by *E. coli* followed by cell harvest, washing of cells and transfer in fresh medium for conversion of ferulic acid (F-COOH) to C-OH (Dillmann 2020). The main focus of the Ligneering project was the optimization of the microbial production process of C-OH by overcoming aforementioned limitations. This included investigations on the enzyme of the reaction cascade, suitable media composition and efficient strategies for in situ product removal in order to develop a one-pot production process of C-OH in gram scale.

The formation of lignin, a second valuable product in focus of the current project, from monolignols occurs in nature in a so-called end-wise polymerization in which oxidized monolignol radicals undergo cross-coupling reactions with radicals formed on the free-phenolic ends of growing lignin polymers (Tobimatsu and Schuetz 2019). The coupling itself is a nonenzymatic process but the radicals are formed by peroxidases and/or laccases. In most studies the commercially available horseradish peroxidase (HRP) is used to synthesize DHPs, which has only a low affinity towards S-OH and is not able to directly oxidize oligomers or even polymers, so the radical has to be transferred from the oxidized monomers to oligomers and polymers which in the end hinders the polymer growth (Sasaki, Nishida et al. 2004). Therefore, other oxidizing enzymes (mainly peroxidases) were tested to more suitable biocatalysts for the generation of lignin-like compounds.

II. Theory and methods

The investigations on strain and process development for the production of monolignols were based on *E. coli* production strains that were developed in a previous project (FKZ 031A551). The base strain *E. coli* BL21 ΔtyrR:T7 carries an insertion in transcription regulator *tyrR*. The knock out of tyrR leads to deregulation and overexpression of several enzymes in tyrosine synthesis pathway and transporter for aromatic amino acids (Pittard and Davidson 1991). The insertion carries the expression cassette for an inducible T7 polymerase.

The base strain was transformed with plasmids carrying either construct TCCN or CCN containing the genes of enzymes Tyrosine ammonia-lyase (TAL), Carboxylic acid reductase (CAR), Cinnamyl alcohol dehydrogenase (CAD) and Norcadia phosphopantetheine transferase (NPT; needed for activation of CAR). Since the TAL enzyme is not necessary for the intended conversion of F-COOH, the CCN construct was mainly used in the experiments regarding strain and process development (figure 1).

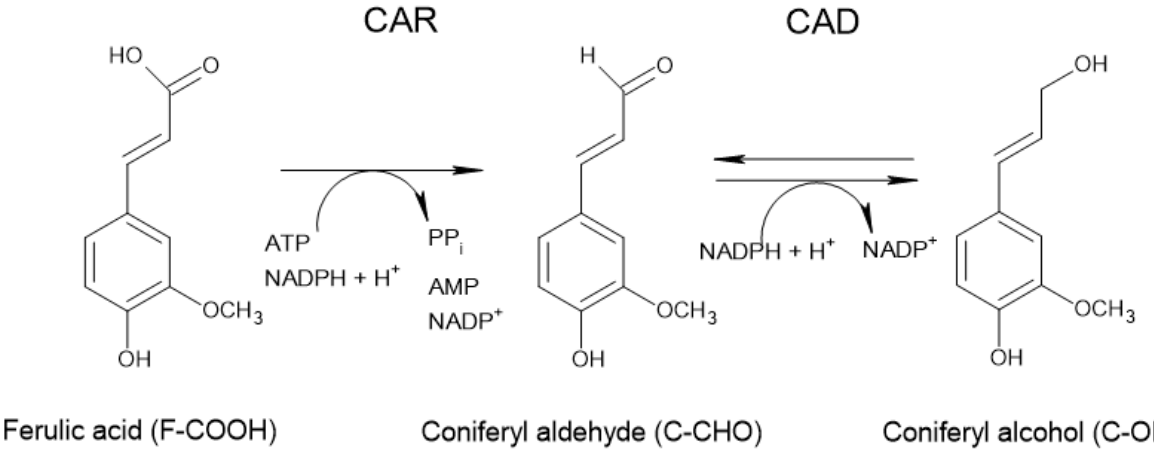

Figure 1: Reaction scheme for conversion of ferulic acid to coniferyl aldehyde by carboxylic acid reductase (CAR) and further conversion to coniferyl alcohol by cinnamyl alcohol deyhdrogenase (CAD).

In contrast to the monolignol production, the subsequent polymerization will be performed *in vitro* with purified enzymes. Therefore, the genes of selected enzymes had to be cloned and transferred into suitable expression hosts. After purification a spectrophotometric assay was used to determine the activity against C-OH. Active enzymes were then used in the polymerization process, were syringe pumps are used to slowly add the reaction compounds to the enzyme solution (Nakamura, Matsushita et al. 2006). This process is used to mimic the natural polymerization of lignin. After acetylation, the polymers are to be first analyzed by gel permeation chromatography (GPC) to determine their molecular weight distribution. Later 2D-NMR methods and GC-MS will be used to get detailed informations about the type and amount of the different linkages between monomer units within the polymer.

III. Results and perspectives

Initial investigations were carried out regarding suitable media compositions for establishing a one-pot C-OH production process without the need of a cell harvest / medium exchange step. This included shake flask cultivations with peptone derived from different feedstocks and variations in final concentrations in order to reduce the overall amount of complex compounds in the medium. With addition of only 0.1 % of peptone to the medium the production strain carrying the plasmid showed the same maximum specific growth rate of 0.66 h^{-1} compared to the reference strain without plasmid.

In the next step, bioreactor cultivations in parallel fermenter setup in 0.5 L scale were executed in order to optimize inducer conditions (lactose vs. IPTG) for recombinant enzyme production. Unexpectedly, adding no inducer at all (vs. 0.5 mM IPTG, 0.35 % lactose, 0.75 % lactose) led to highest conversion of 8.0 mM F-COOH (82 %) and highest C-OH concentration of 6.7 mM.

Furthermore, adjustments of the enzyme cascade were carried out, where reductases from different organism were tested in order to replace the unspecific CAR from *Nocardia iowensis* (NiCCN) in the initial construct. Constructs with reductases from

Segniliparus rotundus (SrCCN), *Neurospora crassa* (NcCCN) and *Mycolicibacterium neoaurum* (MnCCN) were generated and screened regarding the ability for efficient conversion of F-COOH. In addition, a change of plasmid backbone was performed (pACYC_duett to pET28) to avoid the putative cryptic promoter sequence causing basal expression of recombinant genes. After plasmid change higher cell growth on minimal medium without supplementation of peptone (up to 0.5 h^{-1}) and no basal expression in absence of inducer could be observed in 100 mL shake flask scale. It was found that SrCAR and MnCAR are suitable alternatives to NiCAR by showing higher conversion rates after addition of F-COOH (figure 2). Due to the fact that the strain carrying the MnCCN construct showed slower growth in shake flask cultivations, SrCCN was chosen for all subsequent experiments.

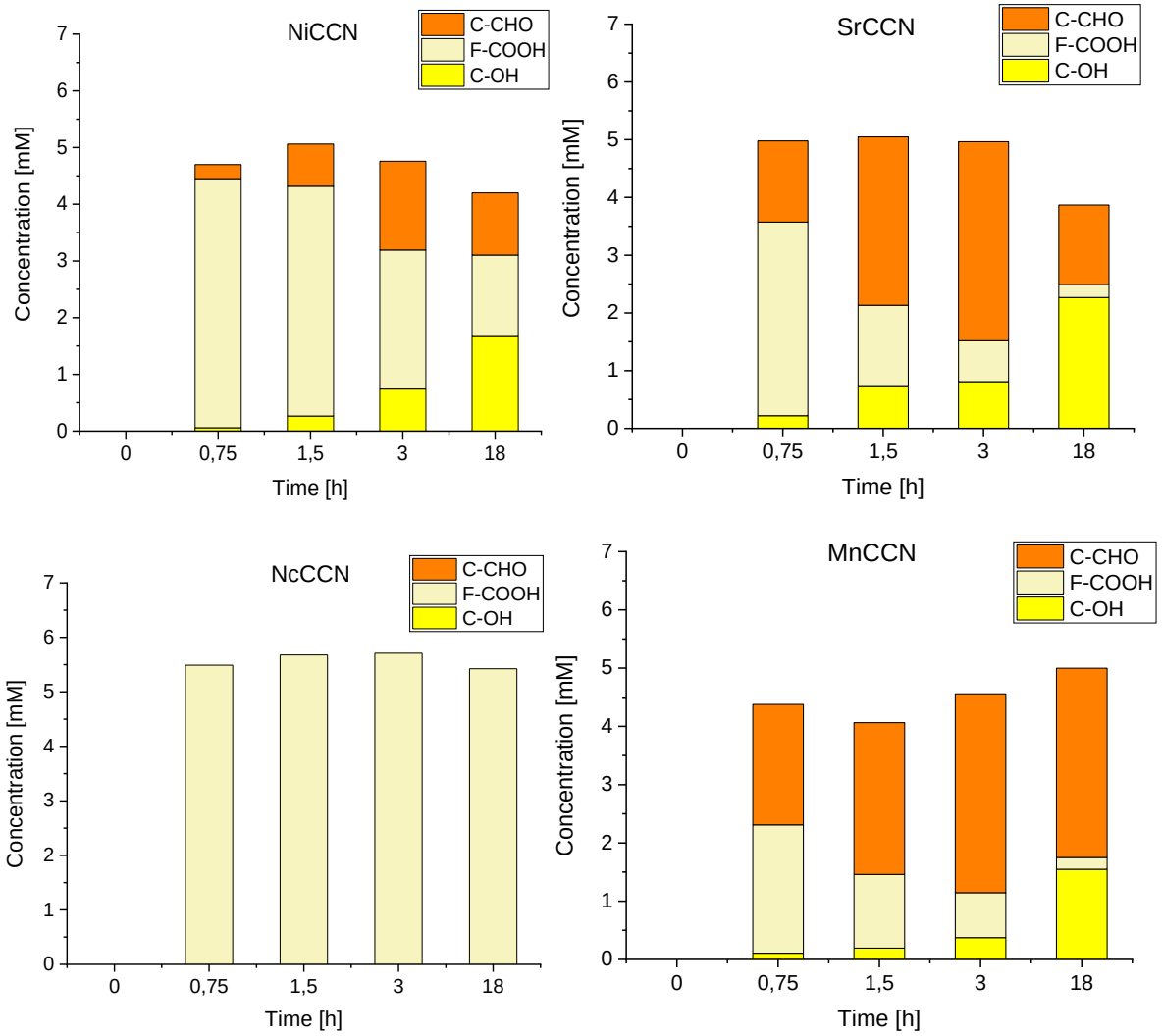

Figure 2: Influence of carboxylic acid reductase variant on production of coniferyl alcohol from ferulic acid by *E. coli* BL21 ΔtyrR:T7 XCCN in 100 mL shake flask scale with minimal medium at 37 °C and 110 rpm. Product formation was started by addition of 5 mM ferulic acid.

Increasing the rate of the CAR reaction lead to the accumulation of high amounts of C-CHO in the medium, meaning the subsequent CAD reaction became the rate limiting

step. Several experiments regarding variations in induction conditions (e.g. time for induction) and addition of medium supplements like Zn^{2+} (Choi, Chung et al. 2015) were carried out. Almost full conversion of F-COOH to C-OH in less than 3 h without significant amounts of C-CHO being present in the medium could be reached by increasing time for induction to > 13 h, whereas the supplementation of Zn^{2+} showed no positive effect.

In addition to modifications to the enzyme cascade in order to overcome productivity limitations (mentioned above), different strategies for *in-situ* product removal were tested. These included microfiltration, two-phase cultivation and adsorption techniques.

Initial test runs in 3 L bioreactor scale were carried out using a bypass with 0.2 µm microfiltration module for continuous removal of product-containing medium. The media volume removed by filtration was replenished by feeding fresh medium containing glucose and F-COOH. This setup showed feasibility regarding the avoidance of C-OH concentrations above 1.3 mM. However, only low substrate concentrations and high dilution rates in the feed could be used, which led to high volumes of permeate and an overall reduction in space-time yield compared to batch process.

As second option the *in-situ* separation of C-OH was investigated using two phase cultivation setups, where an organic phase is added to the culture, which is able to selectively extract the product, is immiscible with the medium and does not inhibit the activity of the biocatalyst. It was shown that phenol and phenolic compounds could be efficiently extracted from culture media by adding the biocompatible solvent tributyrin (Miao, Li et al. 2015, Luo, Cho et al. 2019, Tharmasothirajan, Wellfonder et al. 2021). Applying the tributyrin to the production of C-OH in shake flask scale led to complete conversion of 5 mM F-COOH in 18 h and extraction of 98 % of produced C-OH to the organic phase. Unfortunately, separation of the organic phase from cells and culture medium after centrifugation was not possible without significant loss of product due to instable sediments and formation of several interphases containing oil-in-water- and water-in-oil-emulsions. Additionally, back extraction of C-OH from tributyrin by using 0.1 M NaOH caused degradation of product and increased the overall loss.

Therefore, investigations on adsorption of C-OH from culture medium were carried out. Different adsorber materials were screened regarding the ability of selective adsorption and elution of C-OH. Sepabeads SP850, a polystyrene / divinylbenzene matrix, showed best results and was used in shake flask experiments for *in-situ* removal of C-OH from cultivation broth. Based on these findings fed-batch process was designed in 3 L scale, consisting of an initial batch phase at 37°C, an induction phase at 24 °C for 17 h and a production phase at 37 °C, which was started by adding 200 g of adsorber and feeding F-COOH (48 mmol) to the reactor (figure 3). In the process a complete conversion of fed F-COOH was observed. After elution of C-OH from the matrix, 44.5 mmol could be recovered corresponding to a yield of 93 %. In subsequent steps, the eluted product was concentrated by rotary evaporation. 5 g of 12.4 g concentrate was crystallized in dichlormethane / hexane mixture once yielding 1.2 g with a purity of 34

%. This corresponds to a theoretical, isolated yield of 1 g C-OH that was produced in the cultivation.

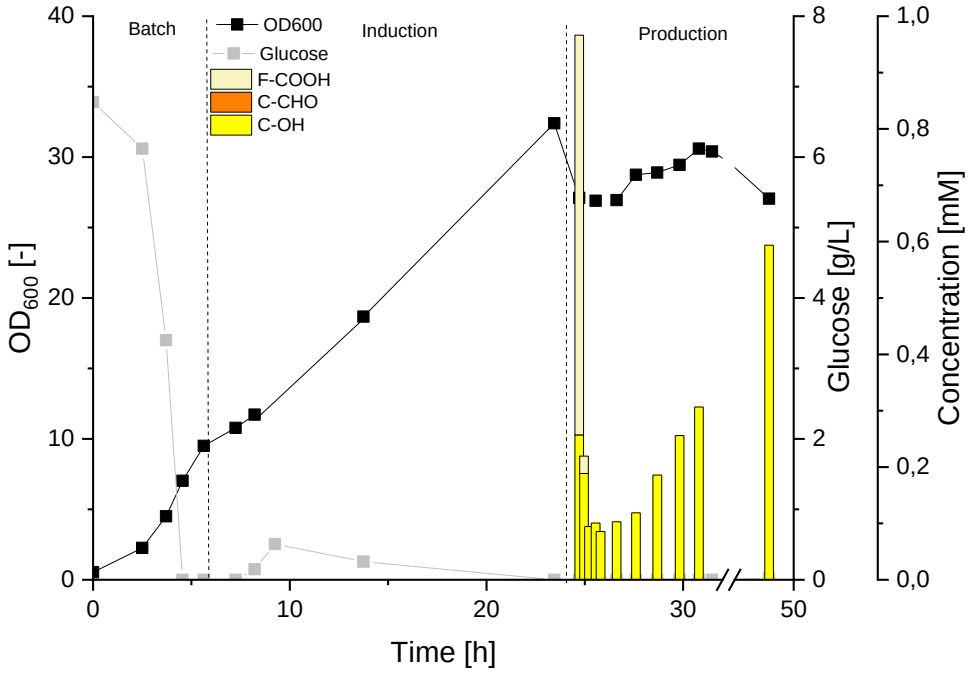

Figure 3: Production of C-OH by *E. coli* BL21 (DE3) SrCCN in 3 L minimal medium with addition of 48 mmol F-COOH and glucose at constant rates. Induction was carried out at 24 °C with 0.5 mM IPTG after 6 h. After 23 h, temperature was increased to 37 °C again and 200 g of adsorber Sepabeads SP850 as well as F-COOH was added. Concentration of F-COOH, C-CHO and C-OH was determined in the supernatant of the medium.

Future investigations should focus on improving the production yield. This could be done by increasing the amount of biocatalyst/cell mass in the bioreactor, further modifications of the enzyme cascade (more active CAD variants) and optimization of the induction conditions. By adjusting the ferulic acid feeding strategy based on the amount of active biocatalyst in the reactor, it could be possible to avoid accumulation of C-CHO completely.
Further optimization of product yield could be achieved by increasing the amount of adsorber in the process and aiming for higher loading of C-OH on the matrix by feeding more substrate. Installation of an external loop containing the adsorber could facilitate a scale up of the adsorber capacity, without being limited by the maximum working volume of bioreactor. Combining a cell retention module and adsorption module in an external loop could enable the development of a continuous process, with benefits of less change-over times and higher productivity. In this setup, two adsorption columns can be operated in rotation in adsorption mode and elution (regeneration) mode, respectively.
In regard of costs of substrates, less expensive and more sustainable feedstocks like glucose from beech wood hydrolysate and F-COOH from microbial production (Ferubase project) should be tested.

The first enzymes of interest for the polymerization experiments were peroxidases found in cell cultures of *Picea abies*. These extracellular peroxidases showed the ability to bind lignin and they were used to form DHPs that are more similar to natural lignin (i.e. with a higher amount of β-O-4 bonds) than those formed by HRP (Warinowski, Koutaniemi et al. 2016). *Pichia pastoris* was selected as the expression host and a modular secretion system based on Golden Gate Cloning (Püllmann, Knorrscheidt et al. 2021) was used to test different promotor-signal peptide combinations to obtain soluble, secreted peroxidases. Unfortunately, no peroxidases could be detected in the supernatant. Because of the difficulties concerning the recombinant expression of plant peroxidases, we focused on Dye-decolorizing peroxidases (DyPs) - bacterial peroxidases which can be easily expressed in *E. coli* and also show activity on lignin and related substrates. The synthetic genes of the two selected DyPs from *Saccharomonospora viridis* (*Svi*DyP) and *Thermomonospora fusca* (*Tfu*DyP) were cloned in pET28a and expressed in *E. coli* BL21 in good yields as hexahistidine-tagged fusion proteins. Adding 5-aminolevulinic acid and $FeCl_3$ to the expression medium increased the amount of heme loaded protein and therefore also increased the activity against ABTS, guaiacol and C-OH. Nevertheless, the activity of the DyPs against C-OH is much lower compared with HRP. Therefore, the polymerization method from Nakamura et al. had to be adjusted to keep the polymerization conditions comparable. Additionally, the buffer substance and pH were changed to avoid protein precipitation and improve peroxidase activity. The obtained products were chemically acetylated and analyzed by GPC. Although polymerization products were found, only a low average molecular weight corresponding to dimers and trimers was observed (table 1).

Table 1: Average molar mass distribution of the acetylated products from peroxidase-catalyzed polymerization of C-OH.

peroxidase	yield[1] [%]	M_n	M_w	M_w/M_n
HRP	13.8 ± 1.6	228 ± 1	826 ± 206	3.62
SviDyP	3.9 ± 1.5	286 ± 14.5	1367 ± 206	4.78
TfuDyP	41.5 ± 0.1	273 ± 30.5	1033 ± 542	3.78

[1] The yield is calculated using the initial mass of C-OH and the mass of the isolated non-acetylated product.

Possible reasons for the low molecular weight of the polymerization products are the relatively low activity of DyPs against C-OH, low enzyme stability under the used polymerization conditions or a too high concentration of H_2O_2 added as a co-substrate. Therefore, future investigations should focus on the improvement of peroxidase activity and stability, either by screening/mining of peroxidases from other organisms that are involved in lignin synthesis or degradation, or by engineering a suitable DyP that already shows basal activity against C-OH and/or the other monolignols. Laccases could also be used for polymerization with the advantage that they use oxygen instead

of H_2O_2 as reducing agent which simplifies the polymerization setup and avoids protein denaturation through H_2O_2.

IV. Literature

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