Metabolic and process engineering for microbial production of protocatechuate with Corynebacterium glutamicum

Mohamed Labib1,2 | Jonas Görtz2,3 | Christian Brüsseler1,2 | Nicolai Kallscheuer1,2 | Jochem Gärtgens1,2 | Andreas Jupke2,3 | Jan Marienhagen1,2,4 | Stephan Noack1,2

1Institute of Bio- and Geosciences (IBG-1): Biotechnology, Forschungszentrum Jülich GmbH, Jülich, Germany
2Bioeconomy Science Center (BioSC), Forschungszentrum Jülich GmbH, Jülich, Germany
3Aachener Verfahrenstechnik – Fluid Process Engineering (AVT.FVT), RWTH Aachen University, Aachen, Germany
4Institute of Biotechnology, RWTH Aachen University, Aachen, Germany

Correspondence
Stephan Noack, Institute of Bio- and Geosciences (IBG-1): Biotechnology, Forschungszentrum Jülich GmbH, D-52425 Jülich, Germany.
Email: s.noack@fz-juelich.de

Funding information
NRW Strategieprojekt BioSC, Grant/Award Number: 313/323-400-002 13

Abstract
3,4-Dihydroxybenzoate (protocatechuate, PCA) is a phenolic compound naturally found in edible vegetables and medicinal herbs. PCA is of high interest in the chemical industry and has wide potential for pharmaceutical applications. We designed and constructed a novel Corynebacterium glutamicum strain to enable the efficient utilization of D-xylose for microbial production of PCA. Shake flask cultivation of the engineered strain showed a maximum PCA titer of 62.1 ± 12.1 mM (9.6 ± 1.9 g L⁻¹) from D-xylose as the primary carbon and energy source. The corresponding yield was 0.33 C-mol PCA per C-mol D-xylose, which corresponds to 38% of the maximum theoretical yield. Under growth-decoupled bioreactor conditions, a comparable PCA titer and a total amount of 16.5 ± 1.1 g PCA could be achieved when D-glucose and D-xylose were combined as orthogonal carbon substrates for biocatalyst provision and product synthesis, respectively. Downstream processing of PCA was realized via electrochemically induced crystallization by taking advantage of the pH-dependent properties of PCA. This resulted in a maximum final purity of 95.4%. The established PCA production process represents a highly sustainable approach, which will serve as a blueprint for the bio-based production of other hydroxybenzoic acids from alternative sugar feedstocks.

KEYWORDS
Corynebacterium glutamicum, electrochemically induced crystallization, isomerase pathway, protocatechuate, xylose

1 | INTRODUCTION

Due to the increasing environmental concerns and the desire for a sustainable bioeconomy there is a growing interest in the production of high-value compounds from renewable feedstocks. Improving the native properties of microorganisms or designing new microbial production hosts is a promising way into that direction. Applications for aromatic compounds, such as the phenolic acid 3,4-dihydroxybenzoate (protocatechuate, PCA), are steadily increasing due to their use in the pharmaceutical and polymer industries.
PCA has a wide range of pharmaceutical applications as an antibacterial, antiviral, antiangiogenic, or antifibrotic agent (Kakkar & Baïs, 2014). Furthermore, its anticancer activity has been reported in terms of an induction of apoptosis of human leukemia cells (Lin et al., 2007). Additionally, the antioxidative activity of PCA is based on the counteraction against free radical formation by upregulation of genes encoding enzymes with neutralizing activities. Industrially, the copolymer of PCA and aniline serves as an electrode with high electrochemical potential rendering PCA a precursor of polymers and plastics (Sun et al., 1998).

PCA naturally occurs as a secondary metabolite in various plant species. For example, it is present in Acai oil, obtained from the fruit of the Acai palm (Euterpe oleracea) (Hassan et al., 2009). It is also found as an antifungal agent in the pigmented onion scales of Allium cepa, enabling them to resist onion smudge (Vitaglione et al., 2007). Recently, a selective PCA extraction method for plant material using molecularly imprinted polymers was presented (Li & Row, 2018). It is application to the leaves of Ilex chinensis Simd yielded 8.46 µg g⁻¹. PCA was also purified from the bark of Terminalia nigrowenulosa using methanol extraction, followed by fractionation with different solvents (Nguyen et al., 2013). The freeze-dried ethyl acetate fraction resulted in the detection of 1.0 mg mL⁻¹ PCA, which is still far too low for an industrial production scale.

Various microorganisms have also shown their potential for PCA biosynthesis. For example, the ubiquitous soil bacterium Bacillus thuringiensis excretes PCA into the medium under iron-limiting conditions (Garner et al., 2004). Furthermore, Azotobacter paspalli accumulated PCA upon its cultivation in a defined medium containing acetate and D-glucose or sucrose as carbon sources (Collinson et al., 1987). However, the resulting natural product titers in both cases are very low.

The Gram-positive, nonsporulating bacterium Corynebacterium glutamicum is widely used in industrial biotechnology for the large scale production of various amino acids such as L-lysine (>1.4 million t/a) and L-glutamate (>2 million t/a) (Eggeling & Bott, 2015). Furthermore, the production of biobased organic acids such as pyruvate, lactate, and succinate has been reported using genetically engineered C. glutamicum (Wieschalka et al., 2013). The broad spectrum of carbon utilization and plasticity of its metabolism are physical properties that render C. glutamicum accessible to manipulation and robust for cultivation under industrial conditions (Baumgärt et al., 2018).

In a first approach towards PCA production, the D-phenylalanine-producing strain C. glutamicum ATCC 21420 was further modified to express the gene ubiC coding for chomisomer pyruvate lyase from Escherichia coli, which enabled the formation of 7.4 mM PCA from D-glucose after 96 h of fed-batch cultivation (Okai et al., 2016). Recombinant expression of the gene vanAB encoding the heterodimeric vanillate O-demethylase from Corynebacterium efficiens NBRC 100395 in the same parental strain enabled the bioconversion of 16.0 mM ferulic acid to 6.91 mM PCA after 12 h of fed-batch cultivation (Okai et al., 2017). Noteworthy, C. glutamicum is capable of utilizing PCA as the sole carbon and energy source (Shen & Liu, 2005; Unthan et al., 2014) and in both approaches followed by Okai et al. (2017), the natural PCA catabolism of C. glutamicum was not inactivated.

A respective platform strain, in which the peripheral and central degradation pathways for aromatic compounds have been abolished, was constructed recently (Kallschüer & Marienhagen, 2018). Additional engineering work focused on improved D-glucose import through deregulation of the gene coding for the glucose/myo-inositol permease IolT1. Further modifications were introduced to improve the carbon flux into the shikimate pathway. These included a reduced flux towards the tricarboxylic acid (TCA) cycle by reduction of native citrate synthase activity and expression of a codon-optimized and truncated version of the gene aroF from E. coli (designated aroF*). This gene codes for an L-tyrosine feedback-resistant 3-deoxy-D-arabinoheptulosonate-7-phosphate (DAHP) synthase catalyzing the initial and committed step of the shikimate pathway. In combination with overexpression of native genes coding for the 3-dehydroshikimate dehydratase (QsuB) and transketolase (Tkt), the constructed strain C. glutamicum DelAro5 C7 PDelE[io1T1 pMKE2x_arOF*QsuB pEKE3x_tkt (abbreviated as PCAglc) produced 13 mM PCA in shake flask cultures starting from 4% (w v⁻¹) D-glucose as sole carbon and energy source in defined CGXII medium (Kallschüer & Marienhagen, 2018).

Most recently, a high-titer production process for PCA from D-glucose was presented (Kogure et al., 2020). For reaching the reported titer of 82.7 g L⁻¹ PCA a two-step fermentation approach was applied. Cells were first grown to high densities using a complex medium, followed by a manual centrifugation step to harvest the cells for subsequent biotransformation on D-glucose.

Refined D-glucose is the major substrate for glycolytic pathways and is thus preferably used as a feedstock for biotechnological production using engineered microorganisms (Cueto-Rojas et al., 2015; Monod, 1949; Straathof, 2014). Plants such as bananas or pears are an important source of D-glucose and also grow on farmland suitable for food production. Therefore, the use of pure D-glucose for bioprocesses is considered competitive with human food and may increase commodity prices (Ekman et al., 2013; Vilkari et al., 2012). By contrast, the C₅ sugar D-xyllose is the second most abundant fraction of lignocellulosic biomass generated as waste from agricultural, pulp, and paper industry (Buschke et al., 2013; Kawaguchi et al., 2006). Consequently, it is a cost-effective renewable carbon source compared with typically used hexoses.

When targeting bio-based PCA through microbial production from renewable resources, a suitable protocol for product extraction from the cultivation broth is required. Here, ultrafiltration (Galaniakis et al., 2010) and adsorption (Sarma & Mahiuddin, 2014) are energy- and time-consuming options (Khin et al., 2012). Recently, tri-n-butyl phosphate was used as a reactive extraction agent to form a complex with protonated PCA in the aqueous phase that can be extracted using an organic phase (Antony & Wasewar, 2018; Antony & Wasewar, 2019; De et al., 2018, 2019). Moreover, an in situ product removal concept using reactive extraction was suggested to prevent product inhibition during the fermentation process (De et al., 2019). Nevertheless, this concept is limited for fermentation processes with
a pH below the respective pK_a,COOH value of 4.48 (Lax & Synowietz, 1967). Additionally, the solubility of organic solvents in the aqueous phase can negatively affect the fermentation process (Kreyenschulte et al., 2018). Most recently, a promising, electrochemical downstream processing strategy for succinic acid was developed (Kocks et al., 2020) that can be adopted for the separation of PCA (Figure S5). The modeling and production process (Gausmann et al., 2020; Kocks et al., 2020). Adjusted using water electrolysis. Thereby, neutral salt emission can be significantly reduced to enhance the feasibility of the whole production process (Gausmann et al., 2020; Kocks et al., 2020).

In this study, we established a one-pot production process for bio-based PCA that enables biomass and product formation utilizing D-glucose and D-xylose as complementary carbon substrates. By combining in silico strain design with targeted metabolic engineering and process development including downstream processing, a sustainable and industrially relevant production process for PCA could be developed.

2 MATERIALS AND METHODS

2.1 Model-based strain design and performance characterization

Flux balance analysis (FBA) was performed using a core model of the central metabolism in C. glutamicum, which was additionally extended for the biosynthetic route of PCA (Figure S5). The modeling and visualization tool Omix was used for model definition and FBA was carried out using the available plug-in (Droste et al., 2011). Maximization of PCA formation was used as objective function.

For the determination of key performance indicators (including maximum titers, rates, and yields) of the different batch and fed-batch experiments conducted in this study, bioprocess modeling was performed using the pyFOOMB package (Hemmerich et al., 2020). The general bioprocess model and corresponding parameter estimates after fitting the model to the different experimental data sets can be found in the Online Supplementary Information (Figures S6-S10 and Table S1).

2.2 Bacterial strains, media, and growth conditions

All bacterial strains including their characteristics and sources are listed in Table 1. E. coli DH5α was used for cloning purposes and routinely cultivated aerobically at 37°C either in reaction tubes containing 5 mL lysogeny broth (LB) (Bertani, 1951) medium within a rotatory shaker (170 rpm) or on LB agar plates (with 1.8% (w v⁻¹) agar). All C. glutamicum strains are derived from C. glutamicum ATCC 13032 (Abe et al., 1967) and were grown aerobically at 30°C, either on brain heart infusion (BHI) (Difco Laboratories) agar plates (with 1.8% (w v⁻¹) agar) or in reaction tubes filled with 5 mL BHI medium on a rotatory shaker at 170 rpm. Kanamycin was added to a final concentration of 25 µg mL⁻¹ and 50 µg mL⁻¹ for C. glutamicum and E. coli strains harboring the cloning and construction vector pK19mobsacB, respectively. Spectinomycin (100 µg mL⁻¹) and

| Strain | Relevant characteristics | Source or reference |
|--------|-------------------------|---------------------|
| Escherichia coli DH5α | F− ΔfhoA lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17 (rK−, mK +) phoA supE44 λ-thi−1 gryA96 relA1 | Invitrogen (Karlsruhe, Germany) |
| Corynebacterium glutamicum MB001(DE3) | Prophage-free derivative of ATCC 13032 with chromosomal expression of the T7 RNA polymerase gene under control of P lacUV5 (IPTG-inducible) | Kortmann et al., 2015 |
| DelAro5 C7 P O6=ioT1 | MB001(DE3) derivative with in-frame deletions of cg0344-cg0347, cg2625-cg2640, cg1226, cg0502, and cg3349-cg3354 combined with an exchange of the native promoter of the citrate synthase gene gltA by the dapA promoter variant C7 as well as two point mutations in the promoter of the inositol transporter gene iot1 that abolishes repression of iot1 by the regulator protein IolR | Kallscheuer & Marienhagen, 2018 |
| DelAro5 C7 P O6=ioT1 Δpyk | derivative of DelAro5 C7 P O6=ioT1 with in-frame deletion of pyk (cg2291) coding for the pyruvate kinase | This study |
| PCA GLC | DelAro5 C7 P O6=ioT1 derivative harboring the plasmids pMKEx2_aroF−qsuB and pEKEx3_tkt | Kallscheuer & Marienhagen, 2018 |
| PCA GLC Δpyk | PCA GLC derivative with in-frame deletion of pyk (cg2291) coding for the pyruvate kinase | This study |
| PCA XYL | DelAro5 C7 P O6=ioT1 derivative harboring the plasmids pMKEx2_aroF−qsuB and pEKEx3-xyIAx−xyIBx | This study |
| PCA XYL Δpyk | PCA XYL derivative with in-frame deletion of pyk (cg2291) coding for the pyruvate kinase | This study |
kanamycin (30 μg mL\(^{-1}\)) were supplemented for the cultivation of \(C.\ glutamicum\) strains harboring the expression vectors pEKEx3 and pMKEx2, respectively. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM for induction of regulated gene expression.

### 2.3 Plasmid and strain construction

All enzymes were purchased from Thermo Fisher Scientific. Standard protocols of molecular cloning, such as polymerase chain reaction (PCR) and Gibson assembly were used (Gibson et al., 2009; Sambrook & Russel, 2001). \(E.\ coli\) DH5α was transformed via heat shock at 42°C for 90 s with chemically competent cells prepared using the RbCl method. \(C.\ glutamicum\) was transformed by electroporation followed by a heat shock at 46°C for 6 min in BHIS medium (BHIS medium supplemented with 90 g L\(^{-1}\) sorbitol). Regeneration of cells took place on a rotary shaker at 170 rpm (37°C and 60 min for \(E.\ coli\); 30°C and 120 min for \(C.\ glutamicum\)) (Eggeling & Bott, 2005; Hanahan, 1983). The in-frame deletion of the pyk gene (cg2291) coding for pyruvate kinase in \(C.\ glutamicum\) was performed by two-step homologous recombination using the plasmid pK19mobsacB-Δpyk according to a previously described protocol (Niebisch & Bott, 2001). Verification of the constructed plasmid for gene deletion was performed by restriction analysis and deletion of pyk was verified by colony-PCR. The used plasmids and oligonucleotides are listed in Tables S2 and S3.

### 2.4 Shake flask cultivations

Pre-cultures in 100 mL baffled shake flasks filled with 15 mL BHI were inoculated with single colonies from a fresh BHI agar plate and incubated for 8 h at 30°C on a rotatory shaker at 250 rpm. These cultures were then used to inoculate a second pre-culture in 500 mL baffled shake flasks with 50 mL of defined CGXII medium (Keilhauer et al., 1993) containing 4% (222 mM) δ-glucose as carbon and energy source. The incubation was performed for 15 h at 30°C on a rotatory shaker at 250 rpm. Finally, the main culture was inoculated to an optical density at 600 nm (OD\(_{600}\)) of 5.0 in 50 mL defined CGXII medium containing either 4% (222 mM) δ-glucose or 4% (266 mM) δ-xylose. Incubation was performed for 98 h at 30°C on a rotatory shaker at 250 rpm. During cultivations, samples were taken for biomass and supernatant analysis at the indicated time points.

### 2.5 Lab-scale bioreactor cultivations

Bioreactor cultivations were performed according to a previously described method (Tenhaef et al., 2018). Lab-scale cultivations were performed as biological duplicates using a parallel bioreactor system (Eppendorf/DASGIP) and the cultivations were started with an initial working volume of 1.2 L. During the cultivation, the pH was measured using a pH electrode (405-DPAS-SC-K80/225), and was maintained at 7.0 by addition of 5 M H\(_3\)PO\(_4\) and 5 M NH\(_4\)OH on demand while the temperature was kept at 30°C. To achieve aerobic process conditions with a dissolved oxygen concentration (DO) of at least 30%, the airflow was set to 0.5 vvm while stirring at 400–1200 rpm. DO electrode (VisiFermDO 225) and exhaust gas composition (GA4, DASGIP AG) were used for online measurements. The cultivation started with 10 g L\(^{-1}\) δ-glucose and an initial OD\(_{600}\) of 0.5 was achieved from an exponentially growing pre-culture containing defined CGXII medium (40 g L\(^{-1}\) δ-glucose).

After complete consumption of δ-glucose (indicated by a sudden increase in the DO signal), IPTG was added to a final concentration of 1 mM and δ-xylose pulse-feeding was started (fed-batch condition A). A solution of 450 g L\(^{-1}\) δ-xylose in deionized water was used for δ-xylose feeding and a total feeding volume of 360 mL δ-xylose solution was distributed into pulses to maintain the δ-xylose concentration above 70 mM until the end of the cultivation. The δ-xylose pulses of 71, 49, 60, 60, and 60 mL were injected after 13, 23, 35, 41, 54, and 68 h of cultivation, respectively. For fed-batch condition B, slow δ-glucose feeding was started in addition to the δ-xylose pulses. The δ-glucose feed solution consisted of 500 g L\(^{-1}\) δ-glucose in deionized water and the feed rate was set to 1.5 mL h\(^{-1}\). Feeding was performed for 94 h and was stopped after δ-glucose started to accumulate.

### 2.6 Biomass and supernatant analysis

Cell densities were assessed as OD\(_{600}\) measured using an UV-1800 spectrophotometer (Shimadzu). 1 mL cultivation broth was collected through a septum and diluted in 0.9% (w v\(^{-1}\)) NaCl to an OD\(_{600}\) value between 0.1 and 0.3. However, at a wavelength of 600 nm, PCA shows interference with the absorbance spectrum (Figure S4). Therefore, all OD\(_{600}\) measurements were corrected by subtracting the OD\(_{600}\) value of the supernatant (obtained by sample centrifugation at 13,000 rpm for 10 min) from the OD\(_{600}\) value of the culture broth. Cell dry weight (CDW) was determined gravimetrically as previously described (Limberg et al., 2017). In a weighted reaction tube, 2 mL cultivation broth was centrifuged (13,000 rpm, 10 min) and the resulting pellet was resuspended in 0.9% (w v\(^{-1}\)) NaCl. After a second round of centrifugation, the supernatant was removed by decantation and the cell pellet was dried (80°C, 24 h) for gravimetric CDW determination.

A previously described high performance liquid chromatography (HPLC) method for quantification of sugars and acids was adopted (Tenhaef et al., 2018). For quantification of substrate and product, additional culture samples were centrifuged (13,000 rpm, 4°C, 10 min) and the resulting supernatants were filtered through a cellulose-acetate syringe filter (0.2 μm, DIA-Nielsen). δ-glucose, δ-xylose, and PCA were measured using an HPLC system (Agilent 1100 Infinity, Agilent Technologies). The separation method used the Organic Acid Resin HPLC Column 300×8 mm (CS Chromatography) as stationary phase and the mobile phase consisted of 0.1 M H\(_2\)SO\(_4\) with a flow rate of 0.6 mL min\(^{-1}\). The column temperature and
injection volume were set to 55°C and 20 µL, respectively. A Refractive Index Detector at 35°C was used for the detection of D-glucose and D-xylose while PCA was detected using UV light absorption at 215 nm with a Diode Array Detector. An external standard in a weighted linear regression approach was used for determination of concentrations.

Untargeted metabolome analysis for the culture supernatants was performed via an Agilent 6890N gas chromatograph coupled to a Waters Micromass GCT Premier high resolution time of flight mass spectrometer. Sample preparation and peak identification as well as details of mass spectrometer operation are described in a previous study (Paczia et al., 2012).

2.7 Downstream processing of PCA

The pH-dependent solid-liquid equilibrium for PCA at 30°C was taken from a previous study (Holtz et al., 2020) and the solubility at 5°C was determined using the shake-flask method (Alsenz & Kansy, 2007). The solubility of PCA was calculated for different pH values using solubility data, the pKₐ,COOH value of PCA, and the Henderson–Hasselbalch equation (Avdeef et al., 2000; Hasselbalch, 1916).

Two samples from the replicate bioreactor cultivations applying condition B were centrifuged (8000 rpm, 45 min, 4°C) and the supernatants were filtered (0.2 µm, Filtroz) and concentrated (from 0.67 L and 0.84 L to 0.25 L and 0.28 L, respectively) at 101°C at atmospheric pressure using a temperature-controlled magnetic hotplate stirrer with a ceramic plate (VWR). The PCA concentration was measured using the HPLC analytics protocol described above. The pH was measured with the pH-electrode Phenomenal® 110 (VWR).

Afterwards, the pH of the concentrated samples was electrochemically shifted in a three-chamber electrolysis setup. In addition to the previously published setup (Gaumann et al., 2020; Kocks et al., 2020), the anode chamber was divided by inserting the anode into a cage consisting of polyethylene terephthalate glycol and a cation exchange membrane (Fumapem 14.100). This step was included to avoid degradation of PCA at the anode, a phenomenon that was observed in preliminary experiments and previously published work (Poulos et al., 1999). A ruthenium oxide-coated titanium electrode (MAGNETO special Anodes B.V.) and a nickel sheet (99% purity) were used as anode and cathode, respectively. The temperature of all chambers was controlled at 30°C and a magnetic stirrer was used for stirring the liquid in the anode chamber.

At the start of the experiment, the concentrated fermentation broth was filled into the anode chamber. 150 and 40 mL aqueous electrolyte, containing 0.5 M sodium dihydrogen phosphate dihydrate (NaH₂PO₄·2H₂O, purity >98%, Carl Roth), were added to the cathode chamber and the cage around the anode, respectively. During the experiment, the voltage was limited to 40 V and the current to 0.2 A. The pH was measured hourly and a sample was taken every 2 h.

After the electrochemical pH shift, both liquids of the anode chamber were mixed and the pH and PCA concentration were measured. Finally, 100 mL medium were cooled to 5°C and in seeded experiments, 0.32 g of sieved PCA seeds (d₅₀ = 395 µm, d₁₀ = 240 µm, d₀ = 600 µm) were added to 350 mL medium and cooled to 5°C. The crystallization efficiency was defined as the difference between start and end concentration divided by the maximal achievable crystal mass:

\[ \xi = \frac{c_{\text{start}} - c_{\text{end}}}{c_{\text{start}} - c_{\text{equilibrium}}} \]

The identity and purity of the obtained crystals were analyzed using the described GC-ToF-MS and HPLC methods.

3 RESULTS AND DISCUSSION

3.1 In silico-guided improvement of PCA-producing C. glutamicum strains

Flux balance analyses were carried out to identify optimal PCA production routes and to derive further promising genetic engineering targets ultimately leading to improved PCA production in C. glutamicum. Starting from D-glucose as sole carbon and energy source a maximum PCA yield of 0.84 C·mol⁻¹ PCA C·mol⁻¹ GLC was predicted, which could theoretically be realized under aerobic, growth-decoupled conditions (Figure 1a). The inactivation of pyruvate kinase is predicted as target to avoid loss of the PCA precursor phosphoenolpyruvate (PEP), which is mostly converted to pyruvate and subsequently to the TCA cycle-fueling substrate acetyl-CoA. In fact, deletion of the corresponding pyk gene has already been tested for the production of 4-hydroxybenzoate from D-glucose with C. glutamicum (Kitade et al., 2018). In this case, however, the resulting increase in product yield was only 1%. This is likely due to the fact that the active (i.e., non-abolished) phosphotransferase system (PTS)-dependent uptake of D-glucose results in the formation of one mole of pyruvate per mol of imported D-glucose, which would have to be recycled to recover the equivalent amount of PEP for PCA synthesis. In C. glutamicum, this could theoretically be achieved by a reaction sequence involving pyruvate carboxylase and phosphoenolpyruvate carboxykinase (PEPCK) (cf. Figure 1a). Although PEPCK is reported to be also active under glycolytic conditions (Petersen et al., 2001), its high net reverse operation under in vivo conditions is thermodynamically unfavorable.

Alternatively, a non-PTS route for D-glucose phosphorylation via ATP-dependent hexokinase/glucokinase is conceivable, which would circumvent the loss of PEP and even enable the maximum theoretical yield of 0.86 C·mol⁻¹ PCA C·mol⁻¹ GLC. Kogure et al. (2016) demonstrated high-yield production of shikimate with a PTS-deficient C. glutamicum strain and combined expression of ppgK (encoding polyphosphate glucokinase) and iolT1 (encoding the permease iolT1). The authors also tested the additional deletion of the pyk gene to...
FIGURE 1  In silico strain design and resulting metabolic engineering of Corynebacterium glutamicum for PCA production. (a) Optimal PCA production route from D-glucose following PTS-coupled uptake. (b) Optimal PCA production route from D-xylose by utilizing the isomerase pathway. In both cases, the colored reactions represent active steps and the thickness of each arrow corresponds to its flux value in relation to the uptake rate. The fully annotated network model is shown in Figure S5. (c) Selected engineering targets for C. glutamicum enabling production of PCA from D-glucose and D-xylose as primary carbon source for growth and production, respectively. Highlighted arrows represent reactions steps that are enforced through plasmid-based (over)expression or targeted inactivation of native gene regulation. Red crosses represent gene deletions while the blue cross represents a targeted downregulation of gene expression (to 10% residual activity compared to the wild-type strain). Cs, citrate synthase; EMP, Embden–Meyerhof–Parnas pathway (most common glycolytic pathway); ISO, isomerase pathway; PCA, protocatechuate; Pk, pyruvate kinase; PPP, pentose phosphate pathway; PTS, phosphotransferase system; TCA, tricarboxylic acid cycle (citrate cycle); Tkt, transketolase
minimize loss of PEP, but the resulting strain was impaired in growth. To circumvent this problem, a tunable downregulation of the pyruvate kinase activity was proposed that should allow a first growth phase on d-glucose, followed by a growth-decoupled production phase (Kogure et al., 2016). However, such an inducible mechanism for the inhibition of the activity of already synthesized proteins is not yet known.

Noteworthy, when using d-glucose as a carbon source, the combined operation of the oxidative and reductive part of the pentose phosphate pathway is not the preferred route for supply of erythrose-4-phosphate (E4P, the second substrate of DAHP synthase besides PEP), since this would result in a loss of carbon in the form of CO₂. Instead, operation of the transketolase 2 enzyme in the direction of E4P formation is required (cf. Figure 1a), which could be supported by additional overexpression of the tkt gene (cf. Table 1).

Taking all these aspects into consideration, we decided to follow a different production strategy for PCA that relies on the utilization of one primary carbon substrate (i.e., d-glucose) for biomass production and another one (i.e., d-xylose) for product formation. By introducing the bacterial isomerase pathway into the native metabolic network of C. glutamicum, the non-PTS substrate d-xylose could be converted to PCA with the same maximum theoretical yield of 0.86 C-molPCA C-mol⁻¹XYL (Figure 1b). While growth-decoupling and PEP accumulation could be realized through pyk deletion (without interference of growth on d-glucose), the increased supply of E4P is also guaranteed by using the isomerase pathway for utilization of d-xylose.

Following the predictions of our initial in silico study aiming to avoid flux of PEP towards the TCA cycle, we deleted the gene coding for the pyruvate kinase (pyk) in our parental producer strain PCA.GLCL yielding C. glutamicum DelAro5 C7 P₆₈-iolT1 Δpyk pMKEx2-arocqusB pEKE3x-tkt (referred to as PCA.GLCL Δpyk). For establishing the d-xylose-based production of PCA, we implemented the isomerase pathway for the degradation of d-xylose in both strains by expressing the heterologous genes coding for xylose isomerase (xylA) from Xanthomonas campestris and overexpression of the endogenous xylose kinase gene (xylB) instead of expressing the transketolase gene (tkt). The resulting strain C. glutamicum DelAro5 C7 P₆₈-iolT1 pMKEx2-arocqusB pEKE3x-xylAx-xylBc₈ and its derivative with the additional pyk deletion are abbreviated in the following as PCA.XYL and PCA.XYL Δpyk, respectively (cf. Figure 1c).

### 3.2 Comparative phenotyping of engineered PCA producers

To study the impact of d-xylose assimilation and pyk deletion on PCA production, all four strains were cultivated in shake flasks in defined CGXII medium, supplemented with either 222 mM d-glucose or 266 mM d-xylose as sole carbon and energy source. Induction of episomal gene expression was achieved by supplementation of the inducer IPTG directly after inoculation of the cultures. All four strains take up d-glucose by the native PTS-system for glucose as well as by the transporter IolT1. The gene coding for the latter is normally not expressed under the chosen cultivation conditions, however, an engineered constitutive expression of the iolT1 gene in all strains described in this study is ensured by modification of the respective operator/promoter sequence (indicated by P₆₈-iolT1 in the strain designations). The transporter IolT1 is also responsible for PTS-independent uptake of d-xylose (Brüsseler et al., 2018).

The cultivation of strains PCA.GLCL and PCA.GLCL Δpyk on d-glucose showed comparable growth rates of 0.21 h⁻¹ and 0.20 h⁻¹.
TABLE 2  Performance indicators of engineered *Corynebacterium glutamicum* protocatechuate (PCA) producer strains obtained from batch and fed-batch cultivation experiments

| Strain/condition                                      | Batch shake flask (n = 3) | Fed-batch bioreactor (n = 2) |
|-------------------------------------------------------|---------------------------|------------------------------|
|                                                        | PCA<sub>GLC</sub>         | PCA<sub>GLC</sub> Δpyk      | PCA<sub>XYL</sub>          | PCA<sub>XYL</sub> Δpyk |
|                                                        | Final OD<sub>600</sub> [-] | Final PCA titer [mM]        | Maximum growth rate [h<sup>-1</sup>] | Maximum D<sub>6</sub>-glucose uptake rate [C·mmol<sub>gCDW</sub> h<sup>-1</sup>] |
|                                                        | 74.2 ± 8.5                | 3.5 ± 1.2                   | 0.21 [0.205, 0.208]         | 15.6 [15.57, 16.00]  |
|                                                        | 67.7 ± 6.9                | 7.8 ± 1.6                   | 0.20 [0.198, 0.202]         | 17.4 [17.38, 17.82]  |
|                                                        | 41.0 ± 4.0                | 12.0 ± 1.1                  | 0.11 [0.105, 0.108]         | 13.0 [12.84, 13.61]  |
|                                                        | 24.7 ± 6.5                | 62.1 ± 12.1                 | 0.04 [0.035, 0.042]         | 6.5 [5.95, 7.17]     |
|                                                        | 11.6 ± 7.5                | 18.0 ± 12.4                 | 0.40 [0.404, 0.422]         | 3.5 [3.43, 3.79]     |
|                                                        | 50.8 ± 9.5                | 61.7 ± 4.0                  | -                            | -                  |
|                                                        |                           |                               | a Following the additional D<sub>6</sub>-glucose feed the resulting dynamics of measured process quantities were too complex for fitting a valid process model for rate estimation. |
|                                                        |                           |                               | b Calculated from the total amount of formed PCA divided by the total amount of consumed D<sub>6</sub>-xylose. |
respectively, as well as comparable substrate uptake rates of 15.6 C·mmol\textsubscript{GLC} g\textsubscript{CDW} \textsuperscript{-1} h\textsuperscript{-1} and 17.4 C·mmol\textsubscript{GLC} g\textsubscript{CDW} \textsuperscript{-1} h\textsuperscript{-1}, respectively (Figure 2 and Table 2). However, the PCA production rate (1.22 C·mmol\textsubscript{PCA} g\textsubscript{CDW} \textsuperscript{-1} h\textsuperscript{-1}), the final PCA titer (7.8 ± 1.6 mM), and PCA yield (0.04 C·mol\textsubscript{PCA} C·mol\textsubscript{GLC}) of strain PCA\textsubscript{GLC} \Delta\text{pyk} were significantly increased compared to its predecessor strain PCA\textsubscript{GLC}. Nevertheless, the PCA yield is still more than one order of magnitude lower than the maximum theoretical yield mentioned above. Cultivation of strain PCA\textsubscript{XYL} on D-xylene resulted in a significantly reduced growth rate (0.11 h\textsuperscript{-1}) and final biomass concentration. While the D-xylene uptake rate (13.0 C·mmol\textsubscript{XYL} g\textsubscript{CDW} \textsuperscript{-1} h\textsuperscript{-1}) was slightly lower, the overall PCA production performance was further improved in comparison to the PCA\textsubscript{GLC} \Delta\text{pyk} strain. Remarkably, cultivation of strain PCA\textsubscript{XYL} \Delta\text{pyk} on D-xylene showed the lowest growth rate (0.04 h\textsuperscript{-1}), the lowest substrate uptake rate (6.5 C·mmol\textsubscript{XYL} g\textsubscript{CDW} \textsuperscript{-1} h\textsuperscript{-1}), but the highest PCA production rate (1.47 C·mmol\textsubscript{PCA} g\textsubscript{CDW} \textsuperscript{-1} h\textsuperscript{-1}), the highest final PCA titer (62.1 ± 12.1 mM) and the highest PCA yield (0.33 C·mol\textsubscript{PCA} C·mol\textsubscript{XYL}) of all four strains (cf. Figure 2 and Table 2). By contrast, cultivation of strain PCA\textsubscript{XYL} \Delta\text{pyk} on D-glucose showed again significantly higher biomass production and lower PCA formation (Figure S1).

Overall, strain PCA\textsubscript{XYL} \Delta\text{pyk} demonstrated a strong increase in the production-related performance indicators related to D-xylene as carbon source in comparison to the previously published PCA producer strain PCA\textsubscript{GLC} related to D-glucose (Kallscheuer & Marienhagen, 2018). In particular, the PCA yield of strain PCA\textsubscript{XYL} \Delta\text{pyk} corresponds to 38% of the maximum theoretical yield from D-xylene, which is quite acceptable for a de novo produced benzoic acid at laboratory scale.

As expected, the inactivation of pyruvate kinase has no effect on growth of the D-glucose-based PCA producer strain. For \textit{C. glutamicum} wild type and the corresponding pyk-deletion mutant it was already demonstrated that growth was not affected in defined CGXII medium with D-glucose as sole carbon and energy source (Sawada et al., 2015). The high demand for pyruvate and further pyruvate-derived precursors for biomass production might be completely fulfilled by the PTS-coupled D-glucose uptake. Alternatively, pyruvate could be formed via the concerted action of PEP carboxylase, malate dehydrogenase, and malic enzyme, as shown previously for a pyruvate kinase-deficient wild-type strain of \textit{E. coli} cultivated under D-glucose-limiting conditions (Emmerling et al., 2002).

By contrast, the additional inactivation of pyruvate kinase in the strain PCA\textsubscript{XYL} resulted in a strong reduction of the specific growth rate (cf. Table 2). The observed slow, but steady growth of strain PCA\textsubscript{XYL} \Delta\text{pyk} up to an OD<sub>600</sub> = 25 during shake flask cultivation might be explained by an alternative flux mode for pyruvate supply involving the operation of malic enzyme. In fact, a similar role of malic enzyme was shown for \textit{C. glutamicum} \Delta\text{pyk} when grown on different gluconeogenic substrates (Netzer et al., 2004). From our GC-ToF-MS analysis for endpoint samples, we found extracellular accumulation of malate exclusively with strain PCA\textsubscript{XYL} \Delta\text{pyk} (Figures S2 and S3). This could point to malic enzyme as limiting step for a sufficient supply of pyruvate for biomass synthesis in this strain.

![Figure 3](https://example.com/figure3.png) Fed-batch bioreactor cultivations of \textit{Corynebacterium glutamicum} PCA\textsubscript{XYL} \Delta\text{pyk} strain in CGXII medium containing 10 g L\textsuperscript{-1} D-glucose for initial batch growth. In condition A, starting at \(t = 13\) h, a total of 162 g D-xylene was pulsed over the whole production phase. In condition B an additional feed of 0.75 g h\textsuperscript{-1} D-glucose was introduced. D-Glucose feeding was stopped after 107 h of cultivation to avoid its further accumulation. Mean values (dashed lines) and standard deviations (shaded areas) were derived from the two depicted independent cultures. PCA, protocatechuate
Most importantly, the combined inactivation of pyruvate kinase and introduction of the non-PTS substrate d-xylose in strain PCAXYL Δpyk led to the observed superior PCA production performance. This is mainly due to the higher availability of both PCA precursors, E4P and PEP, which also resulted in a significantly higher accumulation of the direct condensation product DAHP compared to all other strains (Figures S2 and S3).

### 3.3 Development of a one-pot PCA production process

Process development for PCA production was initiated by cultivating strain PCAXYL Δpyk in a parallel bioreactor system (1.2 L) in defined CGXII medium containing 1% (w v⁻¹) d-glucose as sole carbon and energy source for biomass growth, followed by repeated pulse-feeding of d-xylose to foster PCA formation (Figure 3, cond A).

Within the first 13 h of cultivation, d-glucose was completely consumed and the cell dry weight increased to 5.5 ± 0.1 g L⁻¹. Subsequently, both plasmids (i.e., for d-xylose assimilation and PCA formation) were induced by IPTG and d-xylose feeding was initiated for PCA production. During the cultivation, the d-xylose conversion and PCA formation rate significantly slowed down, resulting in final titers of 9.2 mM and 26.7 mM in both replicates, respectively.

The decrease of the PCA formation rate towards the end of the cultivation could be attributed to the reduced activity of 3-dehydroshikimate dehydratase (QsuB). Previous in vitro analysis of the substrate-saturated QsuB enzyme from C. glutamicum showed non-competitive inhibition (Kᵢ ~0.96 mM) at a concentration of 0.6 mM PCA (Shmonova et al., 2020). Further in vitro analysis of this enzyme showed its highest activity in presence of 1 mM Co²⁺ and at a pH of 8.0–8.4. Although both factors are considered not physiologically optimal for the cultivation of C. glutamicum, this bacterium is still able to grow at extracellular concentrations of 2 mM Co²⁺ (Fanous et al., 2010) and establish pH homeostasis over a pH range from 6.5 to 8.0 (Michel et al., 2015). Therefore, strategies such as in situ product removal and media optimization could presumably maintain the enzymatic stability required for efficient PCA production over longer cultivation periods. However, no clear distinction could be made from our data regarding the physiological reasons for the decreased activity of 3-dehydroshikimate dehydratase. Therefore, the total loss of enzyme activity is modeled by assuming constant enzyme degradation and this approach resulted in a good description of the observed PCA dynamics (Figure S10) and derived rate estimates (cf. Table 2).

To achieve a higher PCA productivity with strain PCAXYL Δpyk, an additional d-glucose feed of 0.75 gC₆H₁₂O₆ h⁻¹ after the initial batch phase was included (Figure 3, cond B). As expected, this resulted in an additional and substantial increase in biomass production up to 17.3 ± 0.41 g L⁻¹ within 40 h of cultivation before another medium component essential for growth became limiting. During the production phase, a total amount of 162 g d-xylose was fed from which 119.8 g was consumed and partly converted into PCA. A final titer of 61.7 ± 4.0 mM (9.5 ± 0.6 g L⁻¹) and yield of 0.19 C molPCA C molΔpyk was achieved. For the yield calculation, a final bioreactor volume of 1.73 L was taken into account. The observed higher PCA production was likely due to the increased biomass that remained metabolically active during assimilation of d-glucose.

The microbial production of PCA from d-glucose has been previously reported in various bioreactor fermentations using engineered C. glutamicum strains. A production titer of 1.1 g L⁻¹ PCA was reported after 96 h of fed-batch cultivation using a mini-jar fermenter containing a complex medium (Okai et al., 2016). Most recently, by following a two-step fed-batch approach, a much higher titer of 82.7 g L⁻¹ PCA was realized (Kogure et al., 2020). In this approach, the engineered strain was first grown to high cell density using complex medium, and then the biotransformation step was started from 10% (w v⁻¹) of the harvested biocatalyst. Nevertheless, the potential process inefficiencies in terms of high substrate costs and technically demanding cell separation and PCA purification could be offset by the high PCA titer obtained.

In our study, a one-pot fermentation process based on comparably cheap defined media was developed for reaching 1.7% (w v⁻¹) cell density and production of 9.51 g L⁻¹ PCA. When considering lignocellulosic hydrolysate as a potential source of d-glucose and d-xylose our process could be cost-effective and not competing with human food.

### 3.4 Separation and purification of PCA

Following the final one-pot production process, PCA was separated from the cell-free supernatants of two independent cultures R1 and R2. The applied process concept for the purification of PCA was adapted from a very recent study (Kocks et al., 2020) and consists of a concentration step, an electrochemical pH shift, and a cooling crystallization step (Figure 4a). The latter two unit operations were split to ensure sufficient conductivity during the pH shift (Brinkmann et al., 2014). The used solid-liquid equilibria of PCA in the fermentation medium for different pH values are shown in Figure 4b. The course of the solubility over the pH of recent experimental data for 30°C fits well with the results from the fitted Henderson–Hasselbalch equation (Hassellbalch, 1916; Holtz et al., 2020). Based on the measured solubility of 5.0 g L⁻¹ at 5°C and a pH of 2.99, the equation was then used to estimate the solubility of PCA over the pH at a temperature of 5°C.

Initially, the concentration of supernatants from the two replicates (R1 and R2) amounted 9.0 and 9.9 g L⁻¹, respectively. After the evaporation step, the concentration in both samples was increased to 26.4 and 30.1 g L⁻¹, and the pH was measured as 5.11 and 4.83, respectively. Next, the electrochemical shift was induced and in both cases, the pH decreased linearly, while the concentration of PCA remained almost constant (Figure 4c). At the end of the shift the concentration in R1 slightly decreased, which may indicate leakage through the electrode cage. Since the initial pH of sample R1 was higher than that of R2, the required electrical charge was enlarged.
FIGURE 4  Separation of biotechnologically produced protocatechuate (PCA). (a) Process concept adapted from Kocks et al., 2020. The supernatant is concentrated (1), the pH is electrochemical shifted (2), afterwards cooled to 5°C (3) and filtered (4). The dashed line represents a possible recycle of the mother liquid to the fermentation. (b) Solubility of PCA in fermentation medium from Holtz et al., 2020 (diamond) for 30°C and experimental result for 5°C (circle). The Henderson–Hasselbalch equation (Avdeef et al., 2000; Hasselbalch, 1916) was used to correlate pH-dependent solubility for 30°C (black line) and 5°C (gray line). (c) Course of pH (black) and concentration of PCA (gray) during the electrochemical pH shift of fermentation medium from fed-batch replicates R1 (circles) and R2 (squares) following condition B (cf. Figure 3). The average current in both experiments was \( I = 0.2 \) A. (d) Scanning Electron Microscope pictures of the crystalline product of seeded (left) and non-seeded (right) crystallization captured with amplifications of 800 and 500, respectively.
Though, the linear slope is similar due to the nearly equal concentrations. The final pH for R1 and R2 was measured as 3.58 and 3.80, respectively.

The seeded and non-seeded crystallization experiments exhibited slow crystallization kinetics (Table 3). Even though the liquid was supersaturated roughly fivefold, the final concentration of the seeded crystallization still amounted 13.8 g L\(^{-1}\) after 14 days. Crystal nucleation in the non-seeded experiment was observed between 7 and 14 days of experimental runtime. Such slow kinetics could be a consequence of the sample matrix, which may have contained sufficiently concentrated fermentation by-products to affect crystallization.

The crystal surfaces of the seeded and non-seeded crystallization exhibited defects and showed the integration of small agglomerates (Figure 4d). This suggests that crystal growth was hindered and small PCA crystals formed agglomerates instead. Since the crystallization of PCA from the fermentation broth has not been studied before, this effect has to be investigated further. Sarma and Mahiuddin (2014) detected different morphologies of PCA depending on the present temperature. At temperatures below 10°C, PCA forms needle-shaped crystals that have a tendency to break, agglomerate and decelerate crystal growth (Beckmann, 2013). Therefore, the crystallization of PCA at higher temperatures could be beneficial for the process.

Finally, the purity of the gained crystals was determined by HPLC for the seeded and non-seeded crystallization as 95.4 and 91.8 wt %, respectively. The overall recovery ratio of the downstream process amounted 51% with seeds and 77% without the addition of seeds.

### TABLE 3 Results from the seeded and nonseeded crystallization experiments

| Parameter/condition | Seeded | Non-seeded |
|---------------------|--------|------------|
| Initial concentration [g L\(^{-1}\)] | 27.4 | 27.4 |
| End concentration [g L\(^{-1}\)] | 13.75 | 6.4 |
| Crystallization efficiency ξ [%] | 60.9 | 93.7 |
| Recovery ratio [-] | 0.51 | 0.77 |
| Purity [wt %] | 95.4 | 91.8 |

### 4 | CONCLUSIONS

In this study, a highly sustainable bioprocess is presented for the microbial production and downstream processing of PCA. Combining in silico strain design with targeted metabolic engineering enabled the use of d-glucose and d-xylene as complementary carbon sources for cell growth and product synthesis. The inactivation of pyruvate kinase and introduction of the non-PTS substrate d-xylene have significantly improved the PCA production performance in the engineered C. glutamicum strains. Purification of PCA was achieved by following a salt-free processing concept and yielded high-grade pure PCA crystals. With the established production and downstream processes, the sustainable biosynthesis of other hydroxybenzoic acids from alternative sugar feedstocks and their purification is within reach.

### ACKNOWLEDGMENTS

The authors acknowledge the financial support of the Bioeconomy Science Center as part of the projects HyImPAct ("Hybrid processes for Important Precursor and Active pharmaceutical ingredients") and R2HPBio ("Renewables to high-performance bioplastics by sustainable production ways"). The scientific activities of the Bioeconomy Science Center were financially supported by the Ministry of Innovation, Science and Research within the framework of the NRW Strategieprojekt BioSC (no. 313/323-400-002 13). Open Access funding enabled and organized by Projekt DEAL.

### CONFLICT OF INTERESTS

Mohamed Labib, Christian Brüsseler, Jan Marienhagen and Stephan Noack are involved in a patent application concerning aspects of the manuscript.

### DATA AVAILABILITY STATEMENT

The data used in this study can be made available upon reasonable request to the corresponding author.

### ORCID

Mohamed Labib https://orcid.org/0000-0003-2616-9608
Christian Brüsseler https://orcid.org/0000-0003-0128-7827
Nicolai Kallscheuer https://orcid.org/0000-0003-4925-6923
Andreas Jupke https://orcid.org/0000-0001-6551-5695
Jan Marienhagen https://orcid.org/0000-0001-5513-3730
Stephan Noack https://orcid.org/0000-0001-9784-3626

### REFERENCES

Abe, S., Takayama, K.-I., & Kinoshita, S. (1967). Taxonomical studies on glutamic acid-producing bacteria. The Journal of General and Applied Microbiology, 13(3), 279–301.
Alsén, J., & Kansy, M. (2007). High throughput solubility measurement in drug discovery and development. Advanced Drug Delivery Reviews, 59(7), 546–567.
Antony, F. M., & Wasewar, K. (2019). Reactive extraction: a promising approach to separate protocatechuic acid. Environmental Science and Pollution Research, 27, 1–13.
Antony, F. M., & Wasewar, K. L. (2018). Reactive separation of protocatechuic acid using tri-n-octyl amine and di(2-ethylhexyl) phosphoric acid in methyl isobutyl ketone. Separation and Purification Technology, 207, 99–107.
Avdeef, A., Berger, C. M., & Brownell, C. (2000). pH-metric solubility. 2: Correlation between the acid-base titration and the saturation–shake-flask solubility-pH methods. Pharmaceutical Research, 17(1), 85–89.
Baumgart, M., Unthan, S., Kloss, R., Radek, A., Polen, T., Tenhaef, N., & Noack, S. (2018). Corynebacterium glutamicum Chassis C1*: Building and testing a novel platform host for synthetic biology and industrial biotechnology. ACS Synthetic Biology, 7(1), 132–144. https://doi.org/10.1021/acssynbio.7b00261
Beckmann, W. (2013). Crystallization: Basic concepts and industrial applications. John Wiley & Sons.
Lax, E., & Synowitz, C. (1967). D'Ans-Lax: Handbuch für chemiker und physiker (Vol. I, 3rd ed.). Springer.

Li, G., & Row, K. H. (2018). Selective extraction of 3,4-dihydroxybenzoic acid in *L. chinenisis* Sims by meticulous mini-solid-phase microextraction using ternary deep eutectic solvent-based molecularly imprinted polymers. *Analytical and Bioanalytical Chemistry*, 410(30), 7849–7858. https://doi.org/10.1007/s00216-018-1406-y

Limberg, M. H., Schulte, J., Aryani, T., Mahr, R., Baumgart, M., Bott, M., & Oldiges, M. (2017). Metabolic profile of 1,5-diaminopentane producing *Corynebacterium glutamicum* under scale-down conditions: Blueprint for robustness to bioreactor inhomogeneities. *Biotechnology and Bioengineering*, 114(3), 560–575. https://doi.org/10.1002/bit.26184

Lin, H. H., Chen, J. H., Huang, C. C., & Wang, C. J. (2007). Apoptotic effect of 3,4-dihydroxybenzoic acid on human gastric carcinoma cells involving JNK/p38 MAPK signaling activation. *International Journal of Cancer*, 120(11), 2306–2316. https://doi.org/10.1002/ijc.22571

Michel, A., Koch-Koerges, A., Krumbach, K., Brocker, M., & Bott, M. (2015). Anaerobic growth of *Corynebacterium glutamicum* via mixed-acid fermentation. *Applied and Environmental Microbiology*, 81(21), 7496–7505. https://doi.org/10.1128/AEM.02413-15

Monod, J. (1949). The growth of bacterial cultures. *Annual Review of Microbiology*, 3(1), 371–394. https://doi.org/10.1146/annurev.mi.03.100149.021020

Netzer, R., Krause, M., Rittmann, D., Peters-Wendisch, P. G., Eggeling, L., Wendisch, V. F., & Sahm, H. (2004). Roles of pyruvate kinase and malic enzyme in *Corynebacterium glutamicum* for growth on carbon sources requiring gluconeogenesis. *Archives of Microbiology*, 182(5), 354–363. https://doi.org/10.1007/s00203-004-0710-4

Nguyen, D.-M.-C., Seo, D.-J., Kim, K.-Y., Park, R.-D., Kim, D.-H., Han, Y.-S., & Jung, W.-J. (2013). Nematicidal activity of 3,4-dihydroxybenzoic acid purified from *Terminalia nigrerulessa* bark against Meliodygynne incognita. *Microbial Pathogenesis*, 59–60, 52–59. https://doi.org/10.1016/j.micpat.2013.04.005

Niebisch, A., & Bott, M. (2001). Molecular analysis of the cytochrome bc1-aa3 branch of the *Corynebacterium glutamicum* respiratory chain containing an unusual diheme cytochrome c1. *Archives of Microbiology*, 175(4), 282–294. https://doi.org/10.1007/s0020300262

Okai, N., Masuda, T., Takeshima, Y., Tanaka, K., Yoshida, K. I., Miyamoto, M., & Kondo, A. (2017). Biotransformation of fenolic acid to protocatechuic acid by *Corynebacterium glutamicum* ATCC 21420 engineered to express vanillate O-demethylase. *AMB Express*, 7(1), 130. https://doi.org/10.1186/s13568-017-0427-9

Okai, N., Miyoshi, T., Takeshima, Y., Kuwahara, H., Ogino, C., & Kondo, A. (2016). Production of protocatechuic acid by *Corynebacterium glutamicum* expressing chorismate-pyruvate lyase from *Escherichia coli*. *Applied Microbiology and Biotechnology*, 100(1), 135–145. https://doi.org/10.1007/s00216-015-6976-4

Paczia, N., Nilgen, A., Lehrmann, T., Gätgens, J., Wiechert, W., & Noack, S. (2012). Extensive exometabolome analysis reveals extended overflow metabolism in various microorganisms. *Microbial Cell Factories*, 11(1), 122. https://doi.org/10.1186/1475-2859-11-122

Petersen, S., Mack, C., de Graaf, A. A., Riedel, C., Elkmann, B. J., & Sahm, H. (2003). Metabolic consequences of altered phosphoenolpyruvate carboxykinase activity in *Corynebacterium glutamicum* reveal anaplerotic regulation mechanisms in vivo. *Metabolic Engineering*, 3(4), 344–361. https://doi.org/10.1016/j.men.2001.0198

Poulos, L., Makri, D., & Prohaska, X. (1999). Photocatalytic treatment of olive millling waste water: Oxidation of protocatechue acid. *Global Nest*, 1(1), 55–62.

Sambrook, J., & Russel, D. W. (2001). *Molecular cloning: A laboratory manual* (Vol. 1, 3rd ed.). Cold Spring Harbor Laboratory Press.

Sarma, J., & Mahiuddin, S. (2014). Specific ion effect on the point of zero charge of α-alumina and on the adsorption of 3, 4-dihydroxybenzoic acid onto α-alumina surface. *Colloids and Surfaces, A: Physicochemical and Engineering Aspects*, 457, 419–424.

Sawada, K., Wada, M., Hagiwara, T., Zen-in, S., Imai, K., & Yokota, A. (2015). Effect of pyruvate kinase gene deletion on the physiology of *Corynebacterium glutamicum* ATCC13032 under biotin-sufficient non-glutamate-producing conditions: Enhanced biomass production. *Metabolic Engineering Communications*, 2, 67–75. https://doi.org/10.1016/j.jmeteco.2015.07.001

Shen, X., & Liu, S. (2005). Key enzymes of the protocatechuate branch of the beta-ketoaipate pathway for aromatic degradation in *Corynebacterium glutamicum*. *Science in China, Series C: Life Sciences*, 48(3), 241–249. https://doi.org/10.1007/BF03183617

Shmonova, E. A., Voloshina, O. V., Ovsienko, M. V., Smirnov, S. V., Nolde, D. E., & Doroshenko, V. G. (2020). Characterization of the *Corynebacterium glutamicum* dehydroshikimate dehydratase QsuB and its potential for microbial production of protocatechuic acid. *PLoS One*, 15(8), e0231560. https://doi.org/10.1371/journal.pone.0231560

Strathof, A. J. J. (2014). Transformation of biomass into commodity chemicals using enzymes or cells. *Chemical Reviews*, 114(3), 1871–1908. https://doi.org/10.1021/cr400309c

Sun, J.-J., Zhou, D.-M., Fang, H.-Q., & Chen, H.-Y. (1998). The electrochemical copolymerization of 3,4-dihydroxybenzoic acid and aniline at microdisk gold electrode and its amperometric determination for ascorbic acid. *Talanta*, 45(5), 851–856. https://doi.org/10.1016/S0039-9194(97)00183-5

Tenhaef, N., Brüsseler, C., Radek, A., Hilmes, R., Unrean, P., Marienhagen, J., & Noack, S. (2018). Production of D-xylonic acid using a non-recombinant *Corynebacterium glutamicum* strain. *Bioresources Technology*, 268, 332–339. https://doi.org/10.1016/j.biortech.2018.07.127

Unthan, S., Grünberger, A., van Ooyen, J., Gätgens, J., Heinrich, J., Paczia, N., & Noack, S. (2014). Beyond growth rate 0.6: What drives *Corynebacterium glutamicum* to higher growth rates in defined medium. *Biotechnology and Bioengineering*, 111(2), 359–371. https://doi.org/10.1002/bit.25103

Vilkar, L., Vehmaanperä, J., & Koivula, A. (2012). Lignocellulosic ethanol: From science to industry. *Biomass and Bioenergy*, 46, 13–24. https://doi.org/10.1016/j.biombioe.2012.05.008

Vitaglione, P., Donnarumma, G., Napolitano, A., Galvano, F., Gallo, A., Scaffi, L., & Fogliano, V. (2007). Protocatechuc acid is the major human metabolite of cyanidin-glucosides. *The Journal of Nutrition*, 137(9), 2043–2048.

Wieschalka, S., Blomback, B., Bott, M., & Eikmanns, B. J. (2013). Bio-based production of organic acids with *Corynebacterium glutamicum*. *Microbial Biotechnology*, 6(2), 87–102. https://doi.org/10.1111/1751-7915.12013

**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the supporting information tab for this article.

To cite this article: Labib, M., Görtz, J., Brüsseler, C., Kallscheuer, N., Gätgens, J., Jupke, A., Marienhagen, J., & Noack, S. (2021). Metabolic and process engineering for microbial production of protocatechuate with *Corynebacterium glutamicum*. *Biotechnology and Bioengineering*, 118, 4414–4427. https://doi.org/10.1002/bit.27909