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Preparation of 4-Vinylphenol Using pHCA Decarboxylase in a Two-Solvent Medium

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Abstract:

An efficient synthesis of 4-vinylphenol (4-VP) from *para*-hydroxycinnamic acid (pHCA) that uses the enzyme *para*-hydroxycinnamic acid decarboxylase (PDC) in a two-phase aqueous−organic solvent has been developed. The 4-VP titer and catalyst productivity (17 g L^{-1}; 1010 g 4-VP isolated g^{-1} catalyst) greatly exceed those for intact cells expressing PDC or calcium alginate bead-immobilized PDC-expressing cells in an aqueous buffer (5 g L^{-1}; 4.4 to 64 g of isolated 4-VP per gram of catalyst). In this two-phase reaction design, the organic solvent (e.g., toluene) enabled continuous extraction of 4-VP into the organic phase and either its recovery as a crystalline solid with low color or its direct conversion to 4-acetoxystyrene.

Introduction

4-Vinylphenol (CAS 2628-17-3; 4-hydroxystyrene; hydroxystyrene monomer; HSM) is a polymer precursor and may be reacted either as-is or as a protected (acetoxy or butoxycarbonyl) derivative to produce branched or linear polymers, respectively. The inherent reactivity of 4-VP is a factor in the maximum solution concentrations available from commercial vendors: alcoholic solutions (propylene glycol) do not exceed 10 wt % of the vinyl monomer. The linear homopolymer, poly(4-hydroxystyrene), has good UV transparency and solubility properties for use as a negative photoresist for 248 nm photolithography.[1] Reported synthetic routes to 4-VP include a two-step sequence involving Knoevenagel condensation of malonic acid and hydroxybenzaldehyde[2] and a single-stage Grignard reaction using 4-bromophenol and vinylmagnesium bromide.[3] Preparations of 4-VP by decarboxylation of *para*-hydroxycinnamic acid (pHCA) have also been described. Representative chemical decarboxylation reactions have been demonstrated using thermal, high-pressure conditions,[4] aqueous basic conditions,[5] and microwave heating with base catalysis[6] to induce pHCA decarboxylation to 4-VP. Biotransformations to 4-VP employing whole cells with pHCA decarboxylase activity suffer from long reaction times, low yields, and product concentrations that are far below levels that would be attractive for commercial use.[7−9] The low productivity of biocatalytic methods is partly due to the toxicity of 4-VP[10] and enzymatic inactivation.[11]

We sought biocatalytic routes to 4-VP that met two objectives: first, the route should use a renewable feedstock; second, the route should be feasible for scale-up and competitive with existing chemical syntheses to 4-VP. Recently, we described the construction of a novel recombinant *Escherichia coli* strain that fermented glucose to 4-VP.[11] In this fermentation scheme, glucose was converted to tyrosine via an augmented aromatic amino acid pathway; the tyrosine was subsequently converted to pHCA and 4-VP by the heterologously expressed yeast *Rhodotorula glutinis* phenylalanine/tyrosine ammonia lyase (PAL/TAL, encoded by *PAL* gene) and the bacterial *Lactobacillus plantarum* pHCA decarboxylase (PDC, encoded by *pdc* gene).[11] This recombinant *E. coli* strain expressed only modest levels of PAL/TAL and PDC and produced only 0.4−0.6 g L^{-1} 4-VP under optimized glucose fermentation conditions. The toxicity of 4-VP precluded achieving higher titers in the fermentation route and undoubtedly contributed to the low titers of 4-VP biotransformations reported by others.[7−9] We designed an alternative production strategy in which the decarboxylase gene was removed from the recombinant fermentation strain and pHCA, the end product of glucose fermentation, was converted into 4-VP in a stand-alone biotransformation using pHCA decarboxylase.[12]

The nonoxidative aromatic decarboxylases encoded by the *padC* and *pdc* gene families are particularly suitable as biotransformation catalysts as they are generally stable, require no exogenous cofactor, and contain no internally

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bound cofactor.[13−21] The absolute requirement for a 4-hydroxy aromatic substrate and the absence of exogenous cofactors suggest a mechanism that proceeds by acid−base chemistry via a *para*-quinone methide intermediate.[21,22] The enzyme-catalyzed decarboxylation and thermal decarboxylation mediated by base catalysis may both proceed by the quinone methide intermediate.[4] Except for the use of isotopically labeled substrates to determine the reaction stereoselectivity,[22] there have been few mechanistic studies of phenolic decarboxylase enzymes, and no protein structure has been solved to date.

Herein, we describe optimization of the decarboxylase biotransformation on fermentation-derived pHCA (bio-pHCA). The approach is similar to that used by Lee et al. in which vinylguaiacol (3-methoxy 4-vinylphenol) was produced at 9.6 g L^{-1} from ferulic acid in a water/hexane mixture with ferulic decarboxylase.[23] We used *E. coli* recombinants for stable overexpression of PDC activity and adopted the two-solvent, two-phase strategy in which the product 4-VP is selectively removed from the aqueous medium. The decarboxylation can be performed using crude fermentation preparations of pHCA with little effect on product yield, an advantage of enzymatic catalysis over chemical syntheses, which exhibit less tolerance for reactant impurities. Since the desired monomer for low branching polymerization is acetoxystyrene (ASM), we demonstrated its convenient preparation by a one-pot, two-stage process where 4-VP is immediately acetylated after the aqueous medium is drained from the two-solvent reactor.

Experimental Section

General. All chemicals were reagent-grade and used as received from either the manufacturer or distributor. Unless otherwise noted, the biochemicals were obtained from Sigma-Aldrich Chemical (St. Louis, MO); various dehydrated culture media were obtained from Difco (Detroit, MI). Authentic 4-VP was purchased from Lancaster Synthesis (Windham, NH). Bottled, spectroscopic grade water was obtained from either EMD Chemicals (Gibbstown, NJ) or Aldrich (Milwaukee, WI). *Lactobacillus plantarum* (ATCC14917) and *Bacillus subtilis* (ATCC6633) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The pHCA-producing *E. coli* strain used to prepare bio-pHCA in these studies was a tyrosine overproducing strain expressing the *Rhodotorula glutinis* phenylalanine/tyrosine ammonia lyase gene (*pal*).[12] SDS PAGE analyses were performed by diluting the samples in 2X sample loading buffer (LDS, Invitrogen) to achieve a final protein concentration of 0.5 mg mL^{-1}. Samples were placed in capped microfuge tubes, heat denatured at 70 °C for 10 min and then loaded (10 μg protein/lane) onto NuPage 4−12% Bis-Tris gels (Invitrogen) using the MES buffer system for enhanced resolution of lower molecular weight proteins. The Mark 12 molecular weight standards (Invitrogen) were used. Gels were run at constant voltage (200 V) for 40 min. When SDS-PAGE gels were used for Western blots for expression level measurements, purified *L. plantarum* decarboxylase protein (1.0 to 25 ng protein per lane)[11] was loaded as the standard. When protein levels in cell-free extracts were very high, the decarboxylase concentrations on the gels were quantified directly by staining in Simply Blue Safe Stain (Invitrogen) for 1.0 h. After 3 × 1.0 min washes with distilled, deionized water, the destained gels were photographed with a FluorChem 8000 system (Alpha Innotech, San Leandro, CA) and protein expression was quantified by following the manufacturer's protocols. Decarboxylation reactions were followed using thin layer chromatography (TLC): solid support, Merck Silica gel $60F_{254}$; mobile phase, ethyl acetate, 100%; typical R_f values for pHCA and 4-VP were 0.4 and 0.7, respectively. High performance liquid chromatography (HPLC) analysis was performed on an Agilent 1100 series liquid chromatograph (Agilent Technologies, Palo Alto, CA) with a photodiode array detector using a Zorbax SB-C18 column (4.6 mm × 250 mm or 4.6 mm × 150 mm rapid resolution); UV detector wavelengths, 258 and 312 nm; temperature, 40 °C; mobile phase, a gradient combining solvent A, 0.1% trifluoroacetic acid in water, and solvent B, 0.1% trifluoroacetic acid in acetonitrile, with 95% solvent A/5% solvent B at start; linear gradient to 20% solvent A and 80% solvent B over 8 min; holding at 20% solvent A/80% solvent B for 2 min and then a linear gradient to 95% solvent A/5% solvent B over 1 min; flow rate, 1.0 mL min^{-1}. Typical retention times for tyrosine, pHCA, and 4-VP were 3.5, 5.3, and 7.4 min, respectively. UV/vis spectrometry was performed on a Cary 100 Bio UV/vis spectrophotometer (Palo Alto, CA). Extracted or purified 4-VP samples were analyzed by proton NMR (^{1}H NMR) spectroscopy at 500 MHz (Bruker-BioSpin, Billerica, MA). Chemical shifts are reported in ppm (δ). The morphology of the cells was determined using transmission electron microscopy (TEM) (Tecnai F-20, FEI, Hillsboro, OR). The cells were sedimented by centrifugation (10 000 × *g*, 20 min, 4 °C), and the paste was applied to copper planchets and cryofixed in a Leica EM Pact (Leica, Deerfield, IL) high-pressure freezing instrument. Sample-containing planchets were held in liquid nitrogen and transferred to a freezer at −85 °C for freeze substitution in 2% w/v osmium tetroxide in anhydrous acetone for 60 h. They were then placed in a Leica AFS automatic freeze substitution unit at −85 °C for further processing. Cells were then ramped from −85 °C to −30 °C (10 °C h^{-1}), held at −30 °C for 13 h, after which

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they were ramped again from −30 °C to 0 °C (10 °C h^{-1}) and held at 0 °C for 30 min. The vials holding the samples were transferred to the hood and allowed to come to room temperature over 1.0 h. After two changes of fresh acetone, the samples were infiltrated and embedded in Epon resin and cured at 60 °C for 60 h. Ultrathin sections were cut on an Ultracut E ultramicrotome (Leica, Deerfield, IL), stained for contrast with aqueous uranyl acetate (4%) followed by lead citrate, and examined using TEM operating at 200 kV.

Construction of Recombinant *E. coli* Strains Containing the pHCA Decarboxylase Genes from *L. plantarum* (*pdc*) or *B. subtilis* (*padC*). To construct *E. coli* strains DPD5004 (expressing *pdc*) and DPD5005 (expressing *padC*), the genomic DNA from either *L. plantarum* grown in the MRS medium or *B. subtilis* grown in the Luria−Bertani (LB) medium was isolated using a DNeasy kit (Qiagen, Valencia, CA). The oligonucleotide primers used for the *pdc* (designated *pdc*1 herein) gene from *L. plantarum* (GenBank Accession no. U63827) were 5′-GGTAATT*CATATG*ACAAA-3′ and 5′-TCACGTGAAACATTACTTATT-3′ which included the *Nde*I site (underlined nucleotides). The oligonucleotide primers used for the *padC* (designated *pdc*2 herein) gene (GenBank Accession no. AF-17117) from *B. subtilis* were 5′-GTGTGT*CATATG*GAAAACT-3′ and 5′-TCGCGGGAATTGTGATGGT-3′, which also included the *NdeI* site (underlined nucleotides). The oligotide primers used for the *padC* gene were 5′-GTGTGT*CATATG*GAAAACT-3′ and 5′-TCGCGGGAATTGTGATGGT-3′, which also included the *Nde*I site (underlined nucleotides). The predicted 550-bp DNA fragments for both *pdc*1 and *pdc*2 genes were purified by using a Qiagen PCR clean up kit and ligated into the pCRII-TOPO cloning vector using the TA Cloning Kit (Invitrogen). Subsequently, these plasmids were digested with *BamH*I and *Xba*I, and fragments containing the *pdc* genes were ligated into the expression vector pKSM715, which had been previously digested with *BamH*I and *Xba*I, to form plasmids pKSM-*pdc*1 and pKSM-*pdc*2, respectively. Transformants were selected following overnight growth on LB agar plates containing kanamycin (50 μg mL^{-1}) at 37 °C. Plasmids pKSM-*pdc*1 and pKSM-*pdc*2 were digested with *Nde*I and *EcoR*I and ligated into the expression vector pET17b (Novagen, Madison, MI) which had already been digested with *Nde*I and *EcoR*I. The resultant plasmids were designated pET17b.*pdc*1 and pET17b.*pdc*2, respectively,[11] and each was transformed into *E. coli* strain BL21(DE3) [genotype: recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1 f80lacZ dM15 d(lacZYA-argF)U169 (DE3)] for expression of high concentrations of PDC1 and PDC2. The final strains were designated as DPD5004 (expressing PDC1 enzyme) and DPD5005 (expressing PDC2 enzyme).

Growth of DPD5004 and DPD5005 Strains. Both strains were grown overnight (37 °C, 250 rpm) in the LB broth containing ampicillin (150 μg $mL^{-1,}$ LB-amp), and an aliquot (2.0 mL) was used to inoculate each of four 0.5-L flasks containing 100 mL of the LB-amp medium. The cultures were grown to an OD_{600} of 0.5A before they were induced by isopropyl β-D-thiogalactopyranoside (IPTG, final concn 0.5 mM). The induced cultures were grown for 60 min before centrifugation (20 min, 10 000 × *g*, 4 °C) and the cell paste (ca. 1.1 g) was stored at 4 °C until use in 4-VP biotransformations. The PDC activity and expression levels in DPD5004 and DPD5005 were determined from their corresponding cell-free extracts as described below.

Preparation of Cell-Free Extracts and Determination of PDC Activity. Cell-free extracts were prepared by resuspending either DPD5004 or DPD5005 cells in 25 mM sodium phosphate buffer (pH 6.0) to an OD_{600} of 35 or 70A mL^{-1}. The cell suspension was lysed by passage through a French Pressure Cell (1200 psi), and supernatant was obtained following centrifugation (10 000 × *g*, 4 °C, 20 min). The percentages of PDC in the total protein of the extracts were determined by measuring the density of protein bands on a 10−20% SDS-PAGE gel stained with Coomassie blue. The pHCA decarboxylase activity was measured as previously described by Cavin et al.:[14,15] the conversion of pHCA to 4-VP was followed at 30 °C by measuring the disappearance of pHCA with the decrease in absorbance at 315 nm. The assay buffer was 25 mM sodium phosphate (pH 6.0) containing 0.2 mM pHCA. The enzyme extract was added to initiate the reaction, and the initial rate was recorded at 315 nm. The enzyme activity in units mL^{-1} (micromoles of 4-VP formed min^{-1} mL^{-1}) was calculated using the molar absorptivity coefficient (ϵ) of 10 000 cm^{-1} M^{-1} for pHCA, and the protein concentration was determined using the BioRad protein kit (Richmond, CA) and 1.0 mg mL^{-1} bovine serum albumin (BSA) as the standard. Cell-free extracts prepared for use in 4-VP biotransformations were stored at −20 °C until use.

Immobilization of DPD5005 Cells Using Calcium Alginate. A 25 mL Pyrex screw-top bottle, equipped with a Teflon stir bar, was charged with 6.0 mL of distilled, deionized water and placed in a water bath at room temperature. Protonal LF 1060 alginate (0.33 g, FMC BioPolymers, Mechanicsburg, PA) was slowly added; the bottle was covered, and the water bath was heated to 80 °C. The suspension was mixed until the alginate was completely dissolved (e.g., 5.24 wt % alginate) prior to allowing it to cool to ambient temperature. DPD5005 cells (37 mg dry cell weight, dcw) in 0.25 mL of distilled, deionized water were added to 0.25 mL of the alginate mixture with stirring at 25 °C. Bead-making was conducted at 25 °C in a 30 mL beaker equipped with a stir bar and containing 16 mL of 0.2 M calcium acetate. The magnetic stirrer speed was adjusted to a rate high enough to keep the beads suspended in solution. A 1.0 mL capacity syringe, fitted with a blunt end 17-gauge needle (1.5 cm length), was filled with the decarboxylase extract/alginate solution while care was taken to avoid bubbles. The solution was slowly added dropwise to the calcium acetate solution at 25 °C with stirring. When addition of the decarboxylase/alginate solution was completed, the formed beads were stirred in the 0.2 M calcium acetate for 2 h at 25 °C. The beads were stored in 5 mM calcium acetate buffer (pH 6.0) at 4 °C until use. Enzyme activity was determined using a single bead in a 4.5 mL cuvette containing a stir bar and 4.0 mL of a mixture containing 25 mM MOPS buffer (pH 6.0), 5.0 mM calcium acetate, and 0.2 mM pHCA.

Decarboxylation of pHCA to 4-VP by DPD 5005 in a Single Phase Aqueous Reaction. (a) *Recycling whole cells*. A 22-mL glass vial containing a small stir bar was charged with 10 mL of a mixture containing 30 mM pHCA in 0.2 M sodium phosphate buffer (pH 6.0) and cells (OD_{600} of 20A mL^{-1} reaction) and stirred at room temperature while the reaction progress was followed using TLC. HPLC analysis was used at the end of the reaction to confirm complete consumption of pHCA. The cells were then removed by centrifugation, washed in 25 mM sodium phosphate buffer (pH 6.0), and resuspended in 0.2 M sodium phosphate buffer (pH 6.0) containing 30 mM pHCA for another round of catalysis. These steps were repeated until the rate and yield of pHCA conversion were greatly reduced (after four reaction cycles). (b) *Recycling cell-free extracts.* A 22-mL glass vial containing a small stir bar was charged with 10 mL of 0.2 M sodium phosphate buffer (pH 6.0) containing 30 mM pHCA and 1.2 mg mL^{-1} BSA. An aliquot (15 μL, 152 μg protein; 15 units PDC activity) of cell-free extract was added to initiate the reaction. The vial was capped, placed on a magnetic stir plate, and vigorously stirred, and the reaction progress was monitored by TLC until it either was completed or did not proceed further. The protein in the cell-free extracts was recovered by ultrafiltration (Amicon YM10 membrane, Millipore, Billerica, MA) and washed several times to remove any residual pHCA or 4-VP before it was restored to its initial reaction volume. The steps outlined above were repeated until no decarboxylase activity was detected.

Decarboxylation of pHCA to 4-VP Using Recycled PDC Cell-Free Extracts in a Biphasic, Two-Solvent Reaction System. A 22-mL glass vial containing a small stir bar was charged with 5.0 mL of a water-immiscible organic solvent (e.g., toluene, dichloromethane, methyl decanoate, or 2-undecanone) to which 10 mL of 0.2 M sodium phosphate buffer (pH 6.0) containing 30 mM pHCA and 1.2 mg mL^{-1} BSA and an aliquot of cell-free extract of either DPD5004 (500 μL, 2.15 mg protein; 15 Units PDC activity) or DPD5005 (15 μL, 152 μg protein; 15 Units PDC activity) was added. The vial was capped, placed on a magnetic stir plate, and vigorously stirred while the reaction progress was monitored by TLC. When the reaction was completed, the immiscible layers were allowed to separate in a separatory funnel and the aqueous layer was filtered by ultrafiltration (YM-10 membrane) to recover the protein catalyst. Depletion of pHCA in the aqueous filtrate was analyzed by HPLC. The aqueous decarboxylase-containing retentate was washed twice with 0.2 M sodium phosphate buffer (pH 6.0) and brought up to the original reaction volume, and residual enzyme activity was determined as described above. The sample was then filtered, and the retained protein was resuspended in a volume of the pHCA-containing buffer solution equivalent to the aqueous layer volume at the end of the previous reaction cycle. The restored aqueous catalyst solution was added to fresh organic solvent, the two phases were stirred sufficiently to achieve intimate mixing, and the above steps were repeated until the reaction time for complete conversion of pHCA to 4-VP had doubled relative to the initial reaction time. The organic layer from nine toluene reactions were pooled and poured through a funnel containing silicone-treated filter paper (1PS Phase Separators, Whatman, Maidstone, U.K.). The organic layer was filtered and dried over anhydrous magnesium sulfate, and a minute quantity of polymerization inhibitor, 2,6-di-*tert*-butyl-4-methyl-phenol (CAS 98-29-3; Aldrich), was added. An ivory-white crystalline solid was recovered after the sample was taken to dryness under a vacuum (200 mmHg, at 45 °C). Further purification of the product was achieved by recrystallization from hexane yielding white crystalline solids (237 mg, 73% yield). The product was characterized by ^{1}H NMR analysis in d_4-methanol (4 mg dissolved in 0.8 mL of solvent). Purity of the product by HPLC was >98%; ^{1}H NMR (500 MHz, d_4-MeOH), δ (ppm): 7.3 (doublet, 2 H, 5, 6- protons of **Ar**omatic ring); 6.78 (doublet, 2 H, 2, 3- protons of aromatic ring); 6.65 (multiplet, 1 H (Ar—**H**C═CH_2); 5.6 (doublet, 1 H, (Ar—C═C**H**, cis); 5.05 (doublet, 1 H (Ar—C═C**H**, trans).

Preparative Scale Decarboxylation of pHCA to 4-VP by PDC Cell-Free Extracts in a Biphasic, Two-Solvent Reaction. A 500-mL jacketed Wheaton CelStir reactor was charged with 280 mL of 0.2 M sodium phosphate buffer (pH 6.0) containing 5 g L^{-1} pHCA and 300 mg of BSA. The cell-free extract of DPD5004 (2.5 mL, 13 mg protein, 69.5 Units PDC) was added along with 0.15 L of toluene, and the reactor was capped, placed on a magnetic stir plate, and stirred at moderate speeds at 33 °C. The reaction appeared to be complete, as judged by TLC, after 30 min and was worked up as described above. The ivory-colored residue (1.1 g) was recrystallized from hexane, collected into a vial, dried under a vacuum (60 mmHg) to constant weight, and characterized by ^{1}H NMR analyses. The recrystallized 4-VP purity was >98% as determined by HPLC.

Conversion of "Bio-pHCA" to 4-VP Using PDC Cell-Free Extracts in a Biphasic, Two-Solvent Reaction System. (a) *Preparation of "bio-pHCA"*: "Bio-pHCA" was prepared by glucose fermentation using a recombinant *E. coli* strain as previously described.[12] The "bio-pHCA-rich" supernatant was prepared by centrifugation (10 000 × *g*, 20 min, 4 °C) of the fermentation medium to remove cells and other insoluble products. (b) *Biphasic, two-solvent reaction*: A 500-mL jacketed Wheaton CelStir reactor was charged with 100 mL of crude "bio-pHCA" supernatant, which, in addition to "bio-pHCA", contained cinnamic acid (CA), phenylalanine, tyrosine, and other growth medium components at pH 6.8. The buffering capacity against a base was increased by adding 200 mL of 0.2 M sodium phosphate buffer (pH 6.0), and commercial pHCA was added to increase the total [pHCA] in the aqueous medium to 17.7 g/L. Cell-free extract (0.5 mL, 1.1 mg protein, 12 Units PDC) was added along with 150 mL of toluene, and the reactor was capped, placed on a magnetic stir plate, and stirred at a moderate speed at 33 °C for 15 h before terminating the reaction. The emulsified organic phase was separated from the aqueous layer and was filtered through a glass-fritted funnel of medium porosity to break the emulsion. The combined organic layers were worked up as described above. The crude solid (ca. 2 g, 78% yield) was recrystallized from

Table 1. **pHCA decarboxylase activity in *E. coli* strains expressing *pdc* and *padC***

pHCA decarboxylase *gene* (enzyme)	recombinant *E. coli* strain	plasmid	percent decarboxylase (% total protein)	activity (U/mL)	specific activity (U/mg)
pdc (PDC1)	DPD5004	pET17b.*pdc1*	13	15 ± 6	4 ± 2
padC (PDC2)	DPD5005	pET17b.*pdc2*	31	133 ± 51	19 ± 4

hexane, collected, and dried under a vacuum (60 mmHg) to constant weight (1.1 g, 43% yield). The overall catalyst productivity was 1010 g of 4-VP produced g^{-1} of cell-free catalyst. A similar reaction sequence was carried out using cell-free extracts of DPD5005 and yielded a catalyst productivity of 1420 g of 4-VP g^{-1} of cell-free catalyst. The ^{1}H NMR spectra of both biocatalytically produced products were identical to an authentic sample of 4-VP.

Preparation of "Bio-4-Acetoxystyrene" Using PDC Cell-Free Extracts. For a convenient synthesis of "bio-4-acetoxystyrene" (bio-ASM), "bio-pHCA" was isolated by an acid precipitation method that involved acidifying the fermentation broth to pH 6.5 with sulfuric acid; filtering to remove the solids; adding sulfuric acid to further lower pH to ~2.2; filtering to recover the bio-pHCA solids; extracting with methanol; recrystallizing from water; and drying under a vacuum. HPLC analysis provided an estimated purity for the solid "bio-pHCA" of 92 ± 4%. The "bio-pHCA" (83 g) was mixed in water and titrated with sodium hydroxide to prepare an aqueous solution of 0.46 M pHCA at pH 6.1. Four successive enzyme-catalyzed decarboxylation reactions were performed in a 1.0-L CelStir reactor that contained 250 mL of 0.46 M pHCA and 10 mL of 2.0 M phosphate buffer (pH 6.0) to which cell-free extract (5 mL, 48 mg protein, 438 Units PDC) was added followed by 500 mL of toluene and 125 mg of Prostab 5415 inhibitor (Ciba Specialty Chemicals, Tarrytown, NY). The reaction was stirred at ambient temperature for ~8 h before the biphasic mixture was separated into an organic layer and an opaque bottom aqueous layer. The 4-VP-rich toluene layers (~1.7 L) were combined and transferred into a four-necked 3-L round-bottomed flask equipped with a stir bar. Acetic anhydride (41 g, 30% molar excess) and 4-*N*,*N*-dimethylaminopyridine (37 mg, 0.1 mol %) were added to the flask, and the contents were stirred at 50 °C until only a trace of 4-VP remained (5 h). The reaction was monitored by TLC (ethyl acetate/hexane, 2:1; R_f for ASM = 0.9; R_f 4-VP = 0.6) until near complete. Toluene was removed under a vacuum to yield a crude brown oil (65 g, 80% overall yield from pHCA) of ASM. The crude product was distilled under a vacuum to yield 51.5 g (~64% overall yield) of a clear, colorless liquid identified as ASM by ^{1}H NMR and by comparison against an authentic sample.

Results and Discussion

pHCA Decarboxylase Activities of *L. plantarum Pdc* and *B. subtilis PadC* Expressed in *E. coli*. The pHCA decarboxylase activities of *L. plantarum*, *B. subtilis*, and other microorganisms have previously been evaluated in our laboratories.[11] In this communication we concentrated on using these enzymes, heterologously expressed in *E. coli*, for development of convenient biocatalytic routes for decarboxylation of pHCA. We observed the highest decarboxylase activity in both DPD5004 and DP5005 strains 60 min after IPTG induction as outlined in Table 1.

Table 2. **Comparison of protein content and pHCA decarboxylase activity in supernatant of DPD5005 cells in control versus 4-VP producing cells**

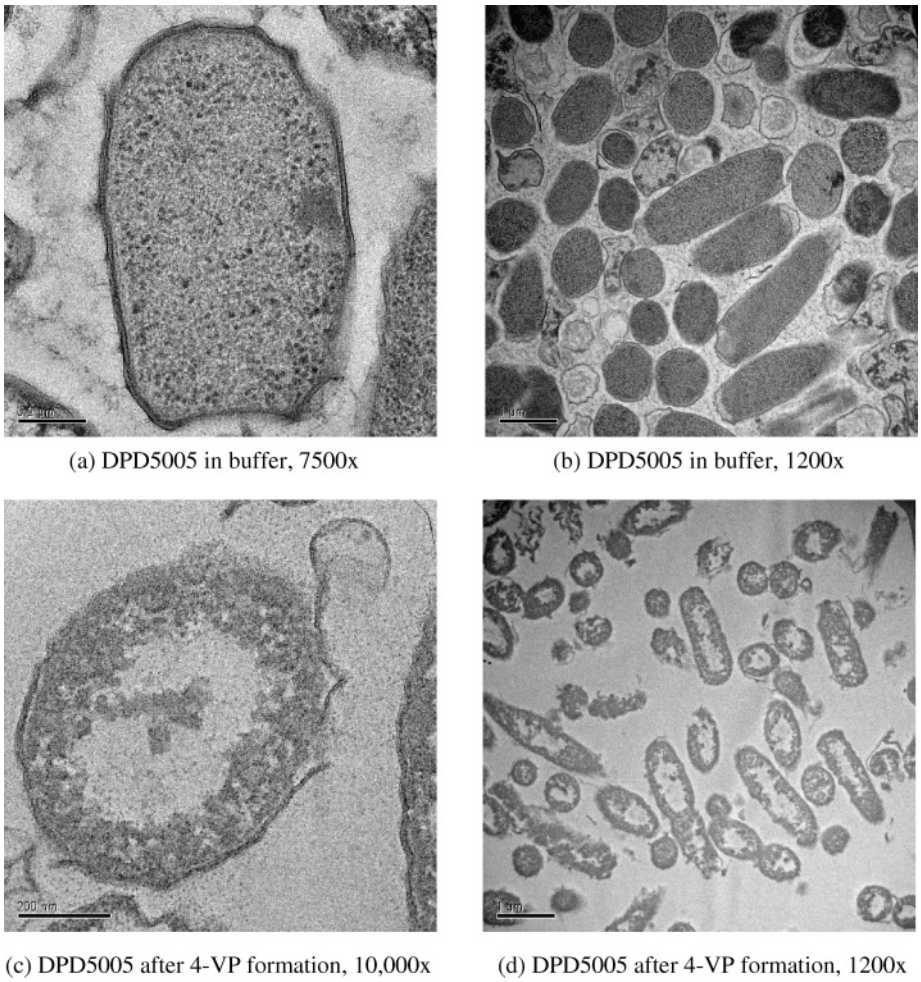
reaction conditions	[protein] mg/mL	activity (U/mL)
0.2 M sodium phosphate buffer (pH 6.0) (control)	1.1	2.0
0.2 M sodium phosphate buffer (pH 6.0) plus 30 mM pHCA (4-VP producing conditions)	0.5	0.1

Biotransformations of pHCA to 4-VP Using Whole Cells. A high concentration (e.g., 0.2 M) of sodium phosphate buffer was required in biocatalytic reactions to counter the pH rise associated with the decarboxylation reaction. The cells were recovered by centrifugation and reused until no further decarboxylation of pHCA occurred. Decarboxylase activity ceased after three cycles, and the cumulative 4-VP produced by DPD5004 and DPD5005 were 40.3 and 64 g g^{-1} of dcw, respectively. While the catalyst productivities observed with these two recombinant strains were considerably higher than the productivity we had previously observed in a fermentation scheme to 4-VP (11), both fell short of our target of 1000 g of 4-VP g^{-1} of dcw.

Freeze/thaw cycles and washes with detergents or organic solvents are commonly used to permeabilize cells and to facilitate substrate and product movement across their membranes. We tested the effect of both freeze/thaw cycles and washing with either 0.01−1.0% cetyltrimethylammonium bromide, 0.1−1.0% toluene in water or 0.1−1.0% chloroform in water on DPD5005 and found that none of these treatments improved the biocatalyst's performance.[25]

Effect of 4-VP on DPD5005 Cells and TEM Studies. We observed that cells used in a pHCA to 4-VP biotransformation reaction displayed unusual resistance to breakage. They aggregated, were difficult to suspend in solution, and were not easily disrupted by either passage through the French Pressure Cell or sonication. Table 2 summarizes the protein content and the decarboxylase activity in supernatants of cells passed through a French Pressure Cell compared to the control. Furthermore, after a single 4-VP production cycle, the decarboxylase-containing cells were not viable when streaked on agar plates. The possible toxicity of pHCA

(25) Experiments performed by ACS Project SEED intern, Saman Uppal. Washing of the cells with 1% chloroform in water was deleterious to the cell integrity, and very little of the decarboxylase activity was retained inside the cell biocatalyst.



(a) DPD5005 in buffer, 7500x (b) DPD5005 in buffer, 1200x

(c) DPD5005 after 4-VP formation, 10,000x (d) DPD5005 after 4-VP formation, 1200x

Figure 1. **Transmission Electron Micrographs of *E. coli* DPD5005 in (a, b) phosphate buffer and (c, d) after formation of 4-VP.**

was ruled out when cells lacking pHCA decarboxylase activity and exposed to a pHCA-containing medium remained viable.

TEM studies were performed to investigate the effect of 4-VP on cells. The micrographs revealed dramatic changes in the cell membrane/wall and the interior of the *E. coli* host. Figure 1a−d are pairs of photos that contrast DPD5005 exposed to 0.2 M sodium phosphate buffer (pH 6.0) with the same strain after 4-VP production. After 4-VP production, the cell walls and membranes were damaged, and cytoplasm components were coagulated and compacted around the cell's periphery. The deleterious effects of 4-VP are not limited to *E. coli* cells since we observed a similar loss of viability, resistance to membrane breakage, and lack of protein release when two *Pseudomonas* strains (KT1440 and S12) devoid of pHCA decarboxylating activity were exposed to 1.0 g L^{-1} of 4-VP. It is therefore reasonable to assume that these 4-VP-induced changes may be a general phenolic toxicity effect.[26]

Calcium Alginate Bead Immobilized Cells as Decarboxylase Catalysts. We immobilized DPD5005 cells in calcium alginate beads in an attempt to improve the PDC enzyme stability. The cells were immobilized as described by DiCosimo et al.[27] Neither calcium alginate nor calcium alginate/polyethyleneimine/glutaraldehyde entrapped cells were effective catalysts. After four reaction cycles, the productivity of the calcium alginate immobilized cells was only 16 g of 4-VP g^{-1} of dcw, which was far less than the 64 g of 4-VP g^{-1} of dcw attained with nonimmobilized cells after only three reaction cycles. Furthermore, the alginate beads were embrittled and encrusted with a white deposit following reaction with pHCA, presumably due to polymerization of 4-VP on the beads. However, further detailed studies are required to confirm this assumption.

Stability of PDC Activity in Cell-Free Extracts. We studied the loss of activity in crude cell-free extracts of both DPD5004 and DPD5005 stored in 25 mM phosphate buffer (pH 6.0) at ambient (23 °C), 4 °C, −20 °C, and −80 °C temperatures. Both cell-free preparations were stable for at least 1 year when stored at temperatures equal to or below 4 °C. At ambient temperature, DPD5004 preparations consistently lost significant activity after 60 h. By contrast, DPD5005 extracts lost no activity when stored at room temperature for over 5 months.

Biotransformations of pHCA to 4-VP Using PDC Cell-Free Extracts in Various Solvent Systems. The possibility that an organic solvent might prolong the pHCA decarboxylase activity was tested with cell-free extracts. We first tested the decarboxylase activity of cell-free extracts of both DPD5004 and DPD5005 in the presence of 19 separate solvents (Table 3). Under fixed reaction conditions (1:1 0.2

(26) S. Haynie observations on cells. Data not shown.
(27) DiCosimo, R.; Ben-Bassat, A.; Fallon, R. D. U.S. 6,251,646. 2002.

Table 3. **Decarboxylase activities from cell-free extracts of DPD5004 and DPD5005 during pHCA to 4-VP biotransformation in biphasic reactions**

solvent in second phase	log *P*	DPD5004 % PDC activity[a]	DPD5005 % PDC activity[a]
none (control)		100	100
ethyl acetate	0.86	0	15
methylene chloride	1.34	73	65
2-heptanone	1.8	0	2.5
2-octanone	2.22	0	2.5
2-heptanol	2.24	0	36
toluene	2.54	100	82
2-nonanone	2.71	20	9
3-nonanone	2.71	28	18
1-octanol	2.81	0	9.5
2-decanone	3.2	20	8
3-decanone	3.2	43	36
4-decanone	3.2	42	44
2-nonanol	3.22	23	42
hexane	3.29	43	42
1-nonanol	3.3	25	35
2-decanol	3.71	38	51
4-decanol	3.71	58	42
2-undecanone	4.09	55	40
methyldecanoate	4.41	77	75

[a] Activity values are expressed as a percent of the control.

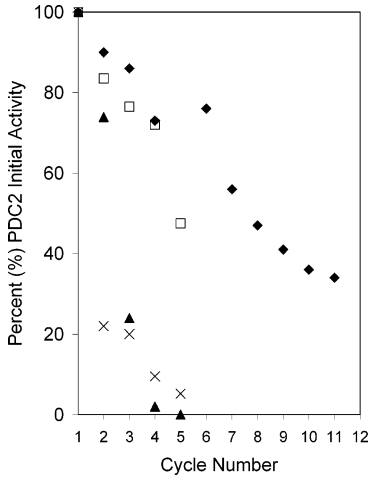


Figure 2. **Stability of PDC2 activity in DPD5005 cell-free extracts in four solvent mixtures containing 5 g L^{-1} pHCA in sodium phosphate buffer (pH 6.0) and (a) ×, no cosolvent; (b) ◆, toluene; (c) □, dichloromethane; and (d) ▲, methyldecanoate.**

M sodium phosphate buffer (pH 6.0)/organic solvent, 30 mM pHCA, either DPD5005 (70 μg of protein, 1.8 Units PDC) or DPD5004 (270 μg of protein, 1.5 Units PDC), and BSA to bring total protein to 1.0 mg mL^{-1}), we could readily discriminate among solvents (Table 3). The decarboxylase activity during a single pHCA to 4-VP cycle had no correlation with the log *P* of the organic solvent. Although DPD5004 was more sensitive than DPD5005 to deactivation by organic solvents, toluene, methyldecanoate, and methylene dichloride were the least harmful solvents to both enzyme preparations. Since it was difficult to distinguish among the three solvents, they were further evaluated in an enzyme stability study using a cell-free extract of DPD5005. Figure 2 depicts the activity versus cycle profiles of the DPD5005 decarboxylase activity in aqueous buffer and in a biphasic

Table 4. **PDC activity following pHCA to 4-VP conversion using PDC catalyst in various conditions**

catalyst system	% PDC activity after one reaction cycle
whole cell	2
calcium alginate immobilized cell	2
cell-free extract	22
cell-free extract/toluene	90

reaction pairing with toluene, dichloromethane, or methyldecanoate. The order of stability is toluene > dichloromethane ≫ methyldecanoate; after a single cycle all have higher activities than those from buffer alone where no organic solvent is present.

Each of these organic solvents is partially soluble in water and could thus influence the enzyme stability. The different solvent dependencies of PDC1 and PDC2 and the absence of a relationship with log *P* suggest that the observed solvent effect is due to specific influences of the organic solvent on the decarboxylase enzyme, not differences in the 4-VP extraction efficiency by the different solvents.

Based on observations outlined above, it is clear that the overall enzyme stability depends on the nature of the catalyst system used for decarboxylation of pHCA to 4-VP. Table 4 shows that the biphasic system is far superior to other catalyst systems in retaining high PDC activity after 4-VP formation. From these data we conclude that 4-VP is detrimental to the enzyme's stability and that removal of 4-VP from the aqueous phase can effectively prolong the enzyme's stability. Cosolvents in the presence of whole cells did not accomplish this extension of PDC activity, and we ascribe the more rapid loss of activity in both free and immobilized whole cells as due to the complications of hardening of the cell membrane and the encrusting of the beads which retard 4-VP removal from the enzyme.

Preparation of 4-VP Monomer Using Biphasic Reactions with PDC Cell-Free Extracts. Results outlined above demonstrated that toluene was the preferred cosolvent with which to perform larger scale reactions to evaluate the influence of temperature, substrate concentration, and crude fermentation components on 4-VP productivity. In addition to prolonging enzymatic activity, toluene affords several operational advantages. Its differential ability to dissolve pHCA and 4-VP allows efficient extraction of 4-VP while maintaining high substrate concentration of pHCA in the aqueous phase. Toluene can be removed from the product mixture by modest heating under a vacuum, or the toluene solution of 4VP can be used directly in a subsequent acetylation reaction to form ASM. Reactions were run at 0.09, 0.3, and 0.8 L scales to examine the catalyst productivity and ease of isolating 4-VP. The ratio of aqueous buffer to toluene ranged from 2:1 to 5:1 in the 0.8 L reaction. We were successful in attaining high conversions when using very low quantities of PDC1 or PDC2 biocatalyst to achieve high catalyst productivity in a single reaction cycle (Table 5). The single operational complication of the two-phase system is the formation of stable emulsions at the organic/aqueous interface. The emulsions trapped the product and

Table 5. **4-VP syntheses with pHCA decarboxylase cell-free extracts in aqueous buffer/toluene**

catalyst system	reaction conditions	reaction time (min)	isolated 4-VP (productivity)
DPD5004: 13 mg of cell-free extract	[pHCA] = 5 g/L; 0.28 L (8.5 mmol) 33 °C	<30	1.0 g (77)
DPD5004: 1.1 mg of cell-free extract	[pHCA] = 18 g/L; 0.3 L (32 mmol) 33 °C	900	1.1 g (1010)
DPD5005: 0.15 mg of cell-free extract	[pHCA] = 5 g/L; 0.090 L (9 recycle reactions, 2.7 mmol) 23 °C	90 (per cycle)	0.24 g (1560)
DPD5005: 2.0 mg of cell-free extract	[pHCA] = 14 g/L; 0.8 L (68 mmol) 45 °C	900	2.9 g (1420)

complicated the separation of the two phases and, thus, lowered 4-VP yields. We observed that a higher proportion of organic solvent to water (e.g., 1:1) significantly diminished emulsion formation and improved the recovery of 4-VP from the organic phase. We have neither optimized the biphasic conditions to mitigate emulsion formation nor explored the upper limits for productivity of this two-solvent design. However, our reported productivity values are conservative, reflecting *isolated* 4-VP product, and are probably lower than what was actually attained in these reactions.

Conclusions

Phenols are known to have biocidal activity against microorganisms,[28] and we observed that 4-VP caused aggregation of bacterial cell contents. Perhaps for this reason, a single-stage fermentation of glucose to 4-VP in which 4-VP is allowed to accumulate may never achieve high product titers.[11] Such aggregation may also limit the efficacy of a pHCA decarboxylase whole-cell biotransformation of pHCA to 4-VP. Although we were unable to successfully adapt immobilization technology for the intact cells, we do not rule out the possibility of identifying an immobilization support suitable for enriched cell-free extracts of either the tetramer PDC1 or the dimer PDC2. An immobilized pHCA decarboxylase would lend ease and convenience and enhanced stability for catalyst reuse.

(28) Srivastava, R. B.; Thompson, R. E. M. *Br. J. Exp. Pathol.* **1966**, *97*, 315−323.

Placing an extractive organic solvent in the reaction medium was a straightforward approach that improved the biocatalyst stability and simplified product isolation. On the basis of the initial productivity values achieved in this study, it is practical to contemplate a one-time use of an enriched decarboxylase cell-free extract. This study has demonstrated a practical biotransformation of pHCA to 4-VP resulting in formation of a product that meets specifications for applications requiring the highest purity of product.

This two-phase biocatalyst route to 4-VP may be competitive with one of the better chemical syntheses to 4-VP;[4] a simple comparison suggests that there is a tradeoff of disadvantages and the process burden associated with pHCA isolation and purification (chemical route) may be equivalent to the burden associated with larger reaction vessels that may be required to accommodate less concentrated reaction media (biocatalyzed route). The thermal, base-catalyzed chemical route[4] has demonstrated high yields of 4-VP (or the corresponding ASM derivative) from purified pHCA solutions up to a 2 M concentration, while the two-solvent PDC system has been limited to pHCA levels up to 0.2 M, above which we observed a precipitous drop in 4-VP yields and PDC activity. The chemical route requires bio-pHCA of high purity; the biocatalyzed process is not constrained by impurities in a crude bio-pHCA substrate such as water and proteinaceous components and as such may offer some process flexibility.

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