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Chemicals from agricultural biomass: chemoenzymatic approach for production of vinylphenols and polyvinylphenols from phenolic acids[1)]

Abstract: A two-step chemoenzymatic process for the preparation of polyvinylphenols from phenolic acids (PAs), being abundant aromatic constituents found in agricultural biomass, was developed. In the first step, conversion of 4-hydroxycinnamic acid derivatives to the corresponding vinylphenols, mediated by a recombinant phenolic acid decarboxylase, was evaluated using a variety of bioprocessing technologies that include biphasic whole cell and cell free extract biotransformations, a combination of biocatalyst with adsorbent resins for *in situ* product recovery, and fixed bed reactors using immobilized whole cells. The best yield (90%) with a high space time yield of 4.83 g/l/h was the result of a combination of crude enzyme extracts of the recombinant *Escherichia coli* (*E. coli*) with water immiscible organic solvents such as toluene. In the second step, cationic and radical polymerizations were tested to produce polyvinylguaiacol (PVG) from vinyl phenols. Characterization of PVG by thermogravimetric analysis (TGA), differential scanning calorimetry (DSC) and nanoindentation test are reported here for the first time. The feasibility of the chemoenzymatic process for the production of aromatic polymers from biomass was demonstrated by the production of polymers from a mixture of ferulic acid (FA) and *p*-coumaric acid (*p*CA), obtained from alkaline hydrolysis of corn bran. Interestingly, nanoindentation tests showed that both PVG and "mixed" PVG polymers showed significantly higher performances than a commercial polystyrene polymer.

Keywords: biorefinery; biotransformation; chemoenzymatic synthesis; green polymers; phenolic acids.

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1 Introduction

In the last few years, commercial and ecological interests by government agencies and the chemical industry have initiated multifaceted research on the use of biomass for the production of biofuels and chemicals [1, 2]. Through advances in numerous disciplines including biotechnology, molecular biology, and metabolic engineering, great progress has been made in the development and implementations of technologies for the preparation of a wide array of small aliphatic carbon building blocks and production of biobased products from carbohydrates [3–7].

In a biorefinery setting, lignin is considered to be the primary source of aromatic chemicals [4–8]. However, current technologies have not yet satisfied the needs for the economical production of aromatic platform chemicals in acceptable purities, mainly due to the complex nature of the lignin material [3, 4, 6]. Phenolic acids (PAs) which are covalently bound to polysaccharides in plant cell walls, mostly through ester linkages, represent an alternative and valuable source of renewable oxygenated aromatic compounds from biomass feedstock (Figure 1) [7–15]. Suffice it to say, this is a path "less travelled by" vis-à-vis the high road of the lignocellulosic feedstock.

Most abundant among PAs in graminaceous cell walls, are ferulic acid (FA) and *p*-coumaric acid (*p*CA), the former identified as one of the two top aromatic building blocks from lignocellulosic materials and biomass [6]. The content of FA varies from 5 g kg^{-1} in wheat bran to 9 g kg^{-1} in sugar-beet pulp and 50 g kg^{-1} in corn kernel [16].

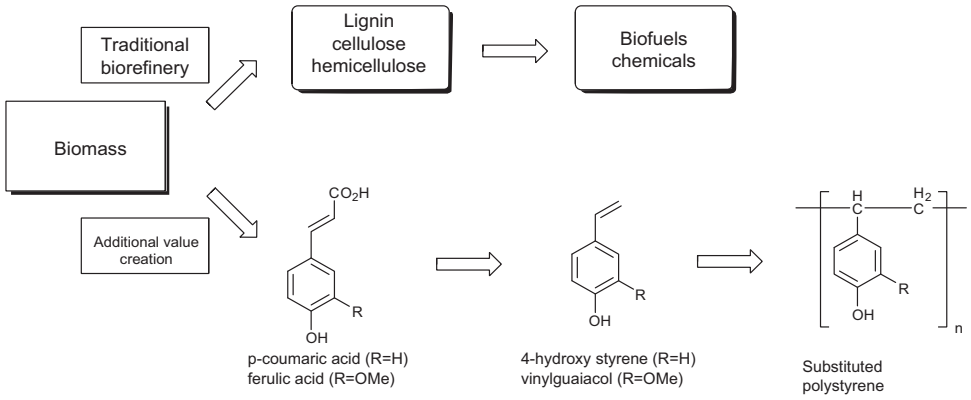


Figure 1 Integration of phenolic acid product stream in a traditional biorefinery model.

Functionally, FA is a multi-faceted molecule, mostly used as a food preservative due to its antioxidant properties and as an active ingredient in sunscreens [16–18]. In addition, processes have been developed for the production of vanillin from FA [19–21], and several research groups including our own, have reported on either chemical [22–25] or enzymatic [26–32] conversion of FA into vinylguaiacol (VG) [33], a compound of industrial importance in the food industry as a flavoring substance. Interestingly, two reports on the preparation of polyvinylguaiacol (PVG) from VG as simplified models for softwood lignins were published in the 1980s and revealed that PVG obtained by radical polymerization is biodegradable [34, 35].

The potential high availability of PAs from biomass prompted this study to explore various bioprocessing conditions, in order to optimize the enzymatic decarboxylation of FA to VG, using a previously cloned phenolic acid decarboxylase (PAD) derived from *Bacillus pumilus* strain UI-670 [32, 36]. A chemoenzymatic route for an efficient extraction of FA from corn bran was established. In addition, we reinvestigated the application of VG in polymerization to produce PVG as a potential biodegradable alternative to polyvinylstyrene. Characteristics of PVG by TGA, DSC and nanoindentation tests are described for the first time.

2 Materials and methods

2.1 Chemicals and methods

All chemicals and reagents were purchased from Sigma Aldrich (Mississauga, ON, Canada), Alfar Aesar (Ward Hill, MA, USA), TCI America (Portland, OR, USA) or Thermo Fisher Scientific (Waltham, MA, USA) and were used without further purification. Corn bran was generously provided by Bunge Milling Inc. (Danville, IL, USA). For the determination of the content of PAs and vinyl derivatives, appropriate calibration curves and response factors for product/substrate mixtures were prepared [37]. H and ^{31}P nuclear magnetic resonance (NMR) analysis was performed on a Bruker (500 MHz) spectrometer (Milton, ON, Canada).

2.2 Preparation of whole cells and crude cell free extracts of PAD

Escherichia coli (*E. coli*) JM109 harboring the PAD-containing plasmid was maintained on LB medium containing glycerol (50%, vol/vol) at -80°C. For biotransformation experiments, a fresh LB agar plate (1.5% agar) containing ampicillin (100 μg/ml) was prepared from the stock culture, and one colony was transferred to a preculture (10 ml) containing LB medium supplemented with ampicillin (100 μg/ml) and grown at 30°C at 200 rpm on an orbital shaker overnight. An aliquot of the suspension (10 ml) was used to inoculate 1 l of Terrific Broth (TB) medium in a 2 l fermenter (DASGIP, Julich, Germany). The temperature was set to 30°C and the pH was controlled at 7.2, by the addition of concentrated ammonium hydroxide. The cell broth was aerated at 1.0 vvm and stirred at 400–900 rpm, to maintain 30% dissolved oxygen. If necessary, the airflow was supplemented with oxygen. After 7 h of growth (OD_{600} of ~8) glucose (33% w/w aqueous solution) was fed at an initial rate of 3 ml/h and gradually increased to 6 ml/h. After 24 h of growth (OD_{600} of ~8) the cells were harvested by centrifugation (6300×*g*, 4°C, 20 min) and washed with 100 mM sodium phosphate buffer pH 7 followed by centrifugation. Whole cells were resuspended in the appropriate amount of buffer, and used for either immobilization studies or the preparation of cell free extracts as follows; the centrifuged and washed cells were resuspended in 20 mM

sodium phosphate buffer (OD_{600}~70) and broken using a French press cell operating at 20,000 psi. The crude extract was obtained by centrifugation (20,000×*g*, 45 min) followed by sterile filtration using a 0.22 µm membrane. Protein concentration was determined by Bradford assay [38] and the activity was determined as described below.

2.3 Immobilization of recombinant whole cells overexpressing PAD with alginate

Immobilization of recombinant *E. coli* cells overexpressing PAD with alginate was performed by a modified procedure from the literature [39]. Resuspended cells (55 ml, OD_{600} 60, equivalent to 100 ml cell suspension obtained from DASGIP fermentation) in an aqueous solution of sodium chloride (0.9%) were mixed with an aqueous solution of alginate (2.4%, 275 ml) and the suspension was added dropwise to a solution of $CaCl_2$ (2%, 3 l) *via* a syringe pump. Beads were formed with a diameter of 2.2–2.6 mm and cured in $CaCl_2$ solution at 4°C for 16 h. The beads were washed and resuspended in the reaction buffer used for further experiments.

2.4 Continuous reaction for the decarboxylation of FA using alginate beads

Alginate beads (240 ml, equivalent to 75 ml of whole cells obtained by DASGIP fermentation) were packed into a Pharmacia XK50/30 column and a solution of FA (2 g/l) in 25 mM MOPS buffer supplemented with 5 mM $Ca(OAc)_2$ was pumped through the immobilized cells at a continuous flow of 0.35 ml/min, using a Waters 600 controller and Waters 610 fluid unit (Mississauga, ON, Canada). Aliquots of the product stream were used to analyze the conversion of FA to VG by HPLC at defined time points. Quantitative analysis of the reaction was carried out by addition of the aqueous stream to a vessel containing EtOAc (300 ml). Every 24 h, this vessel was replaced by another containing EtOAc (300 ml) and the layers of the obtained biphasic mixtures were separated. The pH of the aqueous layer was adjusted to 3, using 1N HCl. The acidified aqueous layer was extracted three times with EtOAc and the organic layers were combined, washed with 100 mM phosphate buffer (pH 8) and brine. The organic layer was dried over anhydrous sodium sulfate, filtered and the solvent was evaporated under reduced pressure to give a VG. This extraction procedure was repeated for the other three biphasic mixtures collected over the following reaction time, to give an overall isolated yield of 49% of VG (1.53 g). Analytical data of the product were in agreement with data obtained from a commercial sample.

2.5 Optimized procedure for the transformation of cell free extract transformation of FA in biphasic reaction medium

FA (3.00 g, 15.5 mmol) was suspended in 100 mM phosphate buffer (pH 7, 45 ml) and the free acid was dissolved by the addition of 6 M NaOH, until all FA was dissolved and the pH of the solution was 7 (total volume 47 ml). Toluene (50 ml) was added and the reaction mixture was warmed to 30°C. The reaction was started by the addition of a cell free extract of PAD (25 U/ml, 3 ml) and was stirred at 30°C for 16 h. The layers were separated and the pH of the aqueous layer was adjusted to 3, using 1N HCl. The acidified aqueous layer was extracted three times with EtOAc and the organic layers were combined, washed with 100 mM phosphate buffer (pH 8) and brine. The organic layer was dried over anhydrous sodium sulfate, filtered and the solvent was evaporated under reduced pressure to give VG (2.03 g) as a slightly yellow oil in 88% yield. Analytical data of the product were in agreement with data obtained from a commercial sample.

2.6 Radical polymerization of VG using azobisisobutyronitrile (AIBN)

A solution of AIBN in toluene (0.2 M, 0.15 ml, 5 mg, 0.03 mmol) was added to a degassed (N_2) solution of VG (500 mg, 3.33 mmol) in toluene (0.49 ml) and the reaction mixture was placed in a preheated oil bath at 100°C and heated for 18 h, before it was cooled down to room temperature and an aliquot of reaction was used for HPLC analysis. The solvent was evaporated under reduced pressure and the residue was dissolved in the minimum amount of MeOH (0.7 ml). This solution was added dropwise to water (100 ml) and the resulting suspension was centrifuged. The supernatant was decanted and the white residue was freeze dried to give PVG (452 mg) as a slightly yellow solid in 90% yield. Mn=8415, Mw=18,658; T_g=86–92°C.

2.7 Cationic polymerization of VG using $BF_3{\cdot}(Et_2O)_2$

VG (8.00 g, 53.3 mmol) was dissolved in dichloromethane (DCM) (54 ml, 72 g) in a flame dried flask and the solution

was cooled to -40°C. $BF_3{\cdot}(Et_2O)_2$ (0.08 g, 0.6 mmol) dissolved in DCM (100 µl) was added dropwise over 3 min. The reaction mixture was allowed to stir at the same temperature for 1 h, before it was quenched by the addition of methanol (10 ml). The solvent was evaporated under reduced pressure and the residue dissolved in the minimum amount of MeOH (60 ml). This solution was added dropwise to water (1.5 l) and the resulting suspension was centrifuged. The supernatant was decanted and the white residue was freeze dried to give PVG (7.75 g) as an off-white solid, in 97% yield. Mn=12,077, Mw=27,149; Tg=106–111°C; FTIR (KBr) ν_{max}: 3418, 3012, 2999, 2918, 2841, 1609, 1512, 1454, 1450, 1431, 1362, 1267, 1229, 1120, 1032, 853, 816, 787 cm^{-1}; ^{1}H NMR (MeOH-d4, 500 MHz) δ: 7.53 (bs, 1H), 6.05–7.10 (m, 3H), 3.75–4.12 (m, 3H); 2.11–2.65 (m, 1H), 1.48–2.00 (m, 2H); ^{31}P NMR: 139.8 (bs).

2.8 Preparation of polyvinyl phenol from corn bran

Milled corn bran (100 g) was suspended in 2N NaOH (2 l) and the reaction mixture was shaken at 30°C at 200 rpm on an orbital shaker for 16 h. The suspension was centrifuged (9000×*g*, 20 min) and the pH of the supernatant was adjusted to 8, using concentrated HCl and centrifuged again. Then, the pH of the supernatant was acidified to 3 and extracted three times with EtOAc. The organic layers were combined, dried over anhydrous sodium sulfate, filtered, and the solvent was evaporated under reduced pressure to give a brown semi-solid (2.9 g). The residue was suspended in 100 mM phosphate buffer (pH 8, 300 ml) and 5 M NaOH was added to readjust the pH to 8. The aqueous layer was extracted three times with a mixture of hexanes and EtOAc (1:1) and the aqueous layer was acidified to pH 3 and again extracted three times with EtOAc. The organic layers were combined, dried over anhydrous sodium sulfate, filtered and the solvent was evaporated to yield a brownish solid (2.2 g). Trituration of the residue with EtOAc and hexanes yielded a slightly yellow solid [1.9 g, 88% FA (w/w), 8% pCA (w/w), trace amounts of cinnamic acid] corresponding to a recovery of 72% of total alkali extractable phenolic acids.

The obtained mixture of phenolic acids [1.70 g; FA: *p*CA, (10:1)] was suspended in 100 mM phosphate buffer (pH 7, 34 ml) and dissolved by the addition of 2N NaOH. Toluene (34 ml) was added and the reaction was carried out as described before, using a cell free extract of PAD (25 U/ml, 2.1 ml) to give 0.99 g (75%) of a mixture of VG to 4-hydroxystyrene in a ratio of 10:1 in >95% purity, as analyzed by HPLC and ^{1}H NMR. This sample of vinyl phenols was used for cationic polymerization without further purification. This mixture of vinyl phenols (900 mg) was applied to cationic polymerization using DCM (9.1 ml, 12.15 g) and $BF_3{\cdot}(Et_2O)_2$ (9 mg, 0.06 mmol) at -20°C, as described before, to yield 872 mg of an off-white solid (97%). The ratio of VG units to 4-hydroxystyrene units was determined by derivatization of an aliquot of the polymer (14 mg) with 2-chloro-4,4,5,5-tetramethyldioxaphospholane, as described in the literature (guaiacol:4-hydroxy phenol; 9:1). [25]Mn=10,543, Mw=22,670; FTIR (KBr) ν_{max}: 3413, 3040, 3019, 2917, 2841, 1611, 1510, 1454, 1449, 1430, 1360, 1264, 1227, 1121, 1033, 851, 816, 787 cm^{-1}; ^{1}H NMR (MeOH-d4, 500 MHz) δ: 9.7 (bs, 0.3H), 9.08–9.51 (m, 2H), 6.17–7.64 (m, 7 H), 4.22 (bs, 6H), 1.60–2.97 (m, 7H); ^{31}P NMR: 139.8 (bs, 9P), 137.0 (bs, 1P).

2.9 Size exclusion chromatography

Size exclusion chromatography (SEC) was performed using a multi-detection system from Viscotek (Houston, TX, USA) consisting of a Model 302 Triple Detector Platform, including a refractive index detector, a four capillary viscometer and a two angles laser light scattering detector, and a GPCmax integrated pump, autosampler, and degasser. Molar masses of the samples were determined with universal calibration with the refractive index detector and the viscosity detector. The universal calibration curve was based on polystyrene standards [Polymer Laboratories (Amherst, MA, USA) and Aldrich (Oakville, ON, Canada)] using the molar masses determined by the manufacturer and the intrinsic viscosities measured by the apparatus. The software OmniSEC software from Viscotek was used for data collection and calibration. Separation in tetrahydrofuran (THF) was performed by injecting 100 µl of 1.5–2 mg/ml solutions of standards or samples into thermostatically controlled SupeRes columns [35°C; PAS-102, PAS-102.5, PAS-103-L, each 300 mm×8 mm; PolyAnalytik (London, ON, Canada)]. The flow rate was 1 ml/min.

2.10 Solid-state properties characterization

The thermal properties of PVG were characterized by TGA and DSC. TGA measurements were performed with a Metler Toledo (Mississauga, ON, Canada) TGA SF model analyzer in a nitrogen atmosphere at a heating rate of 20°C/min in the temperature range of 30°C–800°C. The glass transition temperature, T_g, of the samples was measured using a TA Q200 differential scanning calorimeter (Brossard, Quebec, Canada). The DSC runs were conducted over

the temperature range of -70°C–230°C, with a three part sequence that included heating, cooling and reheating. The underlying heating rate and cooling rate was 20°C/min. The second heat curve was used for the T_g determination. Precise T_g values were obtained by measuring peaks on the derivative graph of the enthalpy vs. temperature curve.

2.11 Nanoindentation test

Polymer powders were compressed at 150°C. The resulting samples were embedded in epoxy and polished with a colloidal silica suspension of 0.04 μm. The equipment used was an indenter G200 (Agilent/MTS, Mississauga, ON, Canada) with the XP head and a Berkovich tip. The continuous stiffness measurement (CSM) method was employed, using standard conditions (harmonic frequency target of 45 Hz; harmonic displacement target of 2 nm; and strain rate target of 0.05/s). Ten indents were done to a maximum depth of 2000 nm.

The reported values of the modulus (E) and hardness (H) are an average of the values taken between 800 and 2000 nm.

3 Results and discussion

3.1 Optimization of the enzymatic decarboxylation of FA

Based on previous reports on the enzymatic decarboxylation of FA to VG, and *p*CA to 4-hydroxystyrene [27, 28], as well as on our work on the recombinant whole cell mediated decarboxylation of FA (25 g/l) in a biphasic bioreactor [32], which allowed for the recovery of VG in 70% isolated yield and a productivity of 6.9 g/l reaction volume, we investigated a variety of bioprocesses in order to increase the productivity, as well as the overall isolated yield. Bacterial cells were grown in TB medium with additional glucose feed at pH 7 in a DASGIP Parallel Bioreactor system for 24 h [40] and the recombinant cells were harvested at an OD_{600} of 35 after 24 h and further used in different applications.

At first, various immobilization, matrices [41] using alginate [39], chitosan [42], and polyacrylamide [43] were investigated to assess the potential benefits of the immobilized recombinant whole cells (for experimental details, please refer to the Supplementary Information). Alginate immobilized whole cells (OD_{600} 10) in a 3-(N-morpholino) propanesulfonic acid (MOPS) buffer supplemented with calcium acetate were shown to be the superior biocatalyst system (Supplementary Table 1). After six reaction cycles, we observed full conversion of FA (2.5 g/l initial concentration) after 18 h of biotransformation.

The alginate immobilized cells (OD_{600} 10) were further applied to the design of a continuous reactor on a preparative scale. FA (2 g/l), dissolved in buffer system consisting of MOPS and calcium acetate, was continuously pumped through a Pharmacia XK column (26/15) loaded with alginate beads (240 ml) (Figure 2). The conversion of FA was acceptable (~80%) for the first 48 h, but then dropped significantly. The overall productivity of 1 l recombinant cells was estimated to yield 40.3 g of VG/l of recombinant cells, a significant improvement to the use of whole cells in the biphasic reactor (13.8 g/l) [32].

Crude enzyme extracts of PAD were then considered as an option for the decarboxylation of PA to VG. This, however, led to a low % yield or space time yield. Inhibition of the enzyme at even low concentrations of VG (0.25 g/l) appeared to present a major problem, as seen previously with whole cells system [32]. It was also established that PAD works most efficiently at high concentrations of FA (>10 g/l) (Supplementary Figure 1).

We revisited the use of a biphasic bioreactor system for *in-situ* extraction of the water-insoluble VG, except this time crude enzyme extracts were used instead of whole cells. Long chain aliphatic hydrocarbons and toluene enhanced the activity of PAD considerably and acted as sinks for the produced vinyl derivative, whereas chlorinated solvents such as dichloromethane and chloroform, as well as ethyl acetate, inhibited the efficiency of PAD when compared to experiments run in conventional buffer (Supplementary Table 2). Preparative scale experiments (100 ml scale) with the best performing solvents from the biphasic enzyme assay (toluene, decane and ethyl acetate as "green" solvent) were then carried out with defined amounts of enzyme and FA concentrations of 25 g/l, shown in Table 1. To showcase the beneficial effects of the co-solvents used, one reaction was carried out in aqueous buffer and listed for comparison.

The best results for these preparative scale experiments (100–200 ml total reaction volume) were obtained with toluene as a second layer (Table 2). In contrast, decane and heptane, which performed best in the activity assay (Supplementary Table 2), gave considerably lower conversions of VG to FA. The use of ethyl acetate did not have any beneficial effect on the outcome of the reaction and the conversion of FA to VG was similar to the use of cell free extract in buffer. In the presence of toluene, complete conversion of starting material was observed for up to 33 g/l FA, enzyme loadings of 0.75 U/ml, and a reaction time of 4 h, and the product was easily isolated after extraction in

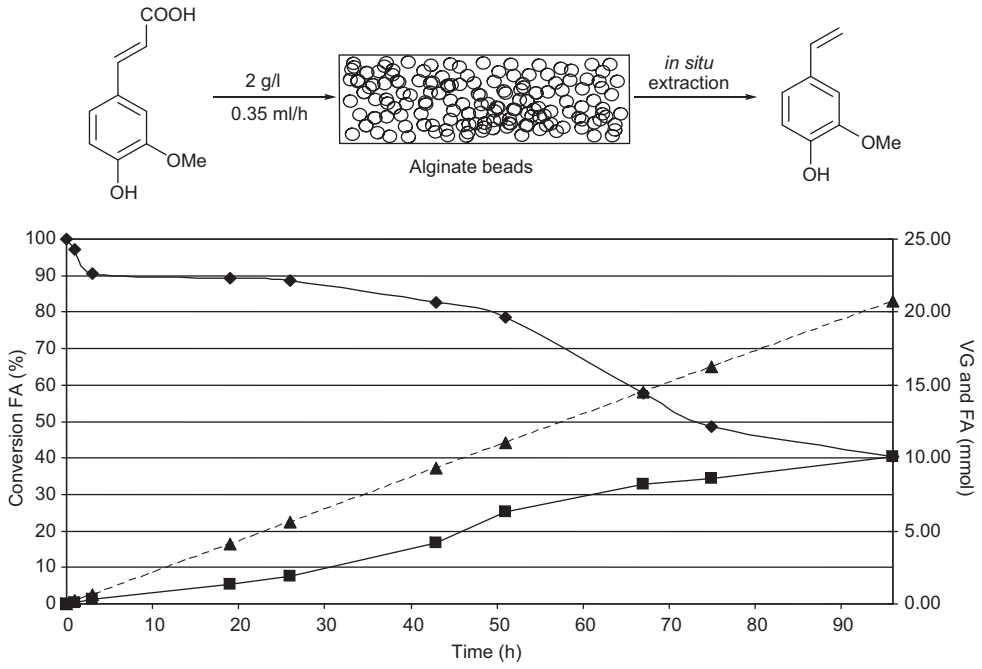


Figure 2 Design of a continuous reactor for the decarboxylation of ferulic acid (FA) to vinylguaiacol (VG) using alginate beads. Graph illustrates the amount of FA added (triangles), conversion of FA as determined by HPLC (diamonds) and isolated yield of VG (rectangle) over 4 days.

high yield and purity (Table 1). Even at a concentration of 40 g/l FA, we observed 98% conversion within 16 h. The use of toluene in a ratio of 1:2 with aqueous buffer still gave complete conversion of FA (33 g/l) after 4 h, however, minor amounts of impurities were detected in the crude reaction mixture. In addition to the conversion of FA to VG, we applied the biphasic process to the conversion of the structurally closely related *p*CA and 4-hydroxystyrene was isolated in good yield (73%) using 0.75 U/ml of PAD (based on FA consumption) and 20 g/l of *p*CA. Although biochemical data suggested that *p*CA has a higher affinity to PAD than FA [Km (*p*CA)=3.6 mM; Km (FA)=6.5 mM] only 90% conversion of *p*CA (20 g/l) was observed after 16 h. Nevertheless, the vinyl derivative was easily separated from its corresponding acid by extraction of the organic layer with an aqueous solution of pH 8.

In terms of space time yield and productivity/l of cells, these were established to be respectively, 4.83 g (VG)/l h

Table 1 Optimization of the crude enzyme mediated decarboxylation of ferulic acid (FA) to vinylguaiacol (VG) in biphasic media.

Co-solvent[a]	PAD (U/ml)[b]	PA (g/l)[c]	Time (h)	Conversion (%)[d]	Isolated yield (%)[e]
Toluene	0.25	10 (FA)	1	85	72
Toluene	0.38	10 (FA)	1	100	90
Toluene	0.75	25 (FA)	4	100	93
Decane	0.75	25 (FA)	4	85	55
Heptane	0.75	25 (FA)	4	60	n.d.
Ethyl acetate	0.75	25 (FA)	4	10	n.d.
None	0.75	25 (FA)	4	10	n.d.
None	2	25 (FA)	16	55	35
Toluene (33% v/v)	1	33 (FA)	4	100	76
Toluene	0.75	30 (FA)	16	100	88
Toluene	0.75	40 (FA)	16	98	67
Toluene	0.75	25 (*p*CA)	4	85	69
Toluene	0.75	20 (*p*CA)	16	90	73

[a]Ratio of co-solvent to buffer was 1:1 (v:v) unless otherwise stated; [b]PAD was used as crude enzyme extract; [c]concentration of phenolic acid in reaction; [d]determined by HPLC; [e]product purity >95% as determined by HPLC and ^{1}H NMR.

Table 2 Comparison of different bioprocesses for their efficiencies.

Bioprocess	Concentration ferulic acid (g/l)	Time (h)	Yield (%)	Space time yield (g/l h)	Productivity/l cells OD_{600} 35 (g/l)
Whole cells – biphasic (toluene)	12.5	2	70	3.39	13.8
Alginate immobilized cells – continuous reactor	8	96	49	0.03	40.3
Cell free extract (2.0 U/ml)	20	4	36	1.39	24.2
Cell free extract (0.75 U/ml) – biphasic toluene (4 h)	25	4	93	4.83	224.1
Cell free extract (2.0 U/ml) – resin	20	4	72	2.78	48.3

and 224 g (VG)/l of cell suspension produced in the DASGIP fermentation system (Table 2). The use of cell free extract in a toluene biphasic system turned out to be most productive among the various processes examined, including the possible use of resins, in an attempt to circumvent the use of organic solvents (Table 2; Supplementary Table 3). In brief, non-ionic resins such as Sepabeads SP850 gave the highest yield of VG (72%), followed by Lewatit VPOC 1163 of 68%. Amberlite XAD 4 and 8 rendered between 51% and 54% yield (Supplementary Table 3). Nonetheless, among the processes examined, the use of resins as a possible green alternative to the use of toluene in a biphasic system came out second in terms of productivity/l cells (Table 2).

3.2 Polymerization of VG and characterization of PVG

The easy access to large amounts of VG from FA led us to explore potential industrial applications of VG, besides the use as a flavor substance or as a starting material for the production of vanillin. Encouraged by previous reports on the biodegradability of PVG and the industrial applications of the structurally closely related polyvinyl phenol in lithographic applications [44, 45], we tested the polymerization of VG under radical or cationic conditions using conventional proven chemistry shown in Table 3.

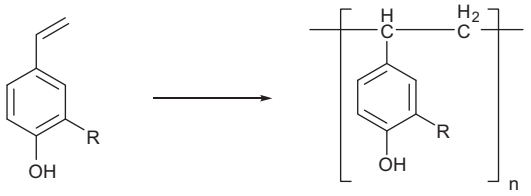
The tendency of VG to polymerize without the addition of an initiator was shown by a blank experiment, where a solution of VG in toluene (50% w/w) was heated to 100°C. After a reaction time of 2 days, only 38% of the initial concentration of the hydroxystyrene derivative was detected in the reaction mixture by HPLC and SEC showed the formation of a polymer with a number average molecular weight (Mn) of 4469 and a polydispersity of 1.5. The use of the sulfide radical generating initiator tetraethylthiuram disulfide (TETD) led to shorter polymer chains as determined by SEC, with 90% conversion of the starting material. Benzoylperoxide induced polymerization gave similar chain lengths to experiments without any initiator and the highest Mn values were obtained with the two carbon radical generating initiators AIBN and 1,1′-azobis (cyclohexanecarbonitrile). Thermal analysis of the AIBN catalyzed polymer (PVG_{rad}) revealed a T_g of 86°C–92°C, as determined by DSC and a thermal stability of up to 350°C. In addition, we wanted to investigate Lewis acids as catalysts in the cationic polymerization of VG [46]. Titanium tetrachloride and aluminum trichloride appeared to be unsuitable catalysts, whereas as boron trifluoride gave a smooth conversion of VG to PVG. We found that lower reaction temperatures allowed for the preparation of polymers with higher molecular weights (Mn=12,077). Since boron trifluoride etherate-catalyzed reactions yielded polymers with the highest Mn values, we subjected the obtained samples (PVG_{cat}) to thermal analysis and preliminary mechanical testing. DSC showed a T_g in the range of 106°C–111°C, with a similar thermal stability to samples obtained by radical polymerization.

Preliminary investigations of the structure of the obtained polymers was performed by ^{31}P-NMR after derivatization with an excess of 2-chloro-4,4,5,5-tetramethyldioxaphospholane, following a literature procedure [47]. The spectrum of PVG showed one broad signal at 139.8 ppm, indicating that polymerization of VG proceeded through the expected vinyl mechanism and no additional cross linking between the phenol units had occurred. For comparison, a commercially available sample of polyvinylphenol gave a peak at 137.0 ppm after derivatization.

3.3 Preparation of polymers from biomass

In order to showcase the usability of both, the optimized enzymatic decarboxylation of phenolic acids using PAD, and our studies on the polymerization of VG, we wanted to apply our technologies to the preparation of polyvinylphenol derivatives from biomass directly. Corn bran was chosen as a starting material, due to its large content of FA and *p*CA [16, 48]. First, we investigated the action of PAD in a biphasic reaction mixture on hydrolysates of corn bran prepared by alkaline hydrolysis (for economic

Table 3 Comparison of cationic and radical polymerization conditions of vinylguaiacol (VG) and mixtures of VG and 4-hydroxystyrene.



Solvent	Monomer concentration (%)	Initiator[a]	Temperature (°C)	Time (h)	Conversion[b] (%)	Number average molecular weight (Mw)[c]	Weight average molecular weight (Mn)[c]	Polydispersity (Mw/Mn)
Toluene	50	None	100	48	62	4469	6810	1.5
Toluene	50	AIBN[d]	100	48	95	8415	18,658	2.2
Toluene	50	ABCN[e]	100	48	89	5243	10,615	2.0
Toluene	50	TETD[f]	100	48	92	1401	2055	1.5
Toluene	50	Benzoylperoxide	100	48	90	5534	9510	1.7
None	100	AIBN	100	48	92	3452	6472	1.9
DCM	10	$BF_3{\cdot}Et_2O$	22	0.1	100	4873	10,556	2.2
DCM	10	$BF_3{\cdot}THF$	22	0.1	100	4430	7667	1.7
DCM	10	$TiCl_4$	22	0.1	86		Dimers, trimer and tetramers	
DCM	10	$AlCl_3$	22	0.1	61		Dimers, trimer and tetramers	
DCM	10	$BF_3{\cdot}(Et_2O)_2$	0	0.1	100	5177	11,112	2.1
DCM	10	$BF_3{\cdot}(Et_2O)_2$	-40	1	100	12,077	27,149	2.2
DCM	10	$BF_3{\cdot}THF$	-40	1	100	5200	10,387	2.0
DCM	20	$BF_3{\cdot}(Et_2O)_2$	-40	1	100	5460	19,884	3.6

[a]1% initiator based on monomer was used for all reaction; [b]determined by HPLC and SEC; [c]determined by SEC; [d]azobisisobutyronitrile; [e]1,1′-azobis(cyclohexanecarbonitrile); [f]tetraethylthiuram disulfide.

reasons) followed by adjustment of the pH, since this process would avoid the isolation of phenolic acids and therefore simplifying the reaction setup considerably. We observed quantitative conversion of phenolic acids (1 g/l initial concentration), however the vinyl derivatives were isolated in low purity, due to contamination with fatty acids from the biomass and the purification of the desired vinyl phenols was arduous. Therefore, we considered a more classical approach and the biomass was treated with diluted sodium hydroxide, centrifuged, extracted with ethyl acetate, acid-base washed and recrystallized from ethyl acetate and hexanes to give a mixture of FA and *p*CA in a ratio of 10:1 in high purity. Starting from 100 g of milled corn bran, we were able to isolate 1.90 g of a mixture of phenolic acids, which accounts for 72% of total alkali extractable phenolic acids (2.65 g/100 g corn bran). Decarboxylation of the mixture of phenolic acids was achieved by applying the optimized conditions for the enzymatic decarboxylation using a cell free extract of PAD (0.75 U/ml) in combination with toluene for *in-situ* product recovery. After a reaction time of 4 h, a mixture of VG and 4-hydroxy vinyl phenol in a ratio of 10:1 was recovered in good yield (0.99 g, 75%) and in high purity (<95%). This mixture was further subjected to cationic polymerization at a low temperature, to yield a mixed polymer consisting of VG and 4-hydroxystyrene in 97% isolated yield. The ratio of monomer units in the polymer was determined by ^{31}P-NMR and was in close agreement with the ratio of the applied mixture, indicating that the copolymerization of VG and 4-hydroxystyrene proceeded smoothly. SEC confirmed the existence of a polymer with an Mn of 10,543 and a polydispersity of 2.15.

From TGA analysis, it can be observed that degradation of the two PVG polymers and the mixed PVG polymer began at around 350°C, which is a good thermal characteristic, but slightly lower than polystyrene [49]. The three PVG polymers have additional OMe and OH groups, which show higher reactivity, hence less thermal stability is expected. DSC analysis showed that these polymers are amorphous with T_g of 86°C for PVG_{rad}, 112°C for PVG_{cat}, and 123°C for PVG_{mixed}. This T_g temperature range is similar to that of the high molecular weight polystyrene and poly (methyl methacrylate), PMMA. The PVG_{cat} and PVG_{mixed} polymers are similar in Mn and polydispersity, however the 9:1 ratio of guaiacol:4-hydroxy phenol monomer units in the PVG_{mixed} polymer has contributed to a higher T_g. As presented in Table 4, nanoindentation characterization of the two PVG polymers performed on polymer films compressed at 150°C

Table 4 Nanoindentation tests of polyvinylguaiacol (PVG) samples.

Sample	Modulus, E (GPa)	Hardness, H (GPa)
PVG_{rad}	6.66±0.05	0.421±0.003
PVG_{cat}	6.75±0.05	0.463±0.006
PVG_{mixed}	6.5±0.1	0.384±0.012
PS 1301	4.15±0.02	0.249±0.001

following the CSM, showed that the two PVGs present similar H and E values, which are significantly higher than the performance measured on commercial polystyrene, PS 1301 (NOVA Chemicals) (H=0.249±0.001 GPa and E=4.15±0.02 GPa) that was also analyzed for comparison purposes. The presence of the OMe and OH groups are certainly responsible for the improved performance. The mixed PVG polymer, containing a 9:1 ratio of guaiacol: 4-hydroxy phenol monomer units, had slightly lower E and H values than the two PVG polymers, but a significantly higher performance than the PS 1301 polymer.

4 Conclusions

For the first time, we showcased the production of polyvinylphenols primarily derived from a biobased feedstock. Optimization of the enzymatic decarboxylation of PAs extracted from an agricultural biomass, paved the way for an efficient production of vinyl phenols. Comparison of five different bioprocesses revealed the use of cell free extracts of recombinant PAD, in combination with toluene, as the most efficient process, due to the fact that any generated product was removed by the second organic layer most effectively. The use of nonionic adsorbent resins as an alternative to the use of organic solvents for *in-situ* product recovery, representing a "greener" process, appeared to be a less efficient procedure when compared to the use of whole cells in biphasic media or immobilized cells. Polymerization of the produced 4-hydroxy vinyl derivative could be easily achieved by either cationic or radical reaction conditions. The produced polymers showed T_gs of 112°C for boron trifluoride mediated polymers of VG and 86°C for polymers derived with radical initiators and both polymers seemed to be stable up to 350°C. Based on the ease of both procedures, we demonstrated the feasibility for the chemoenzymatic production of hydroxylated polystyrene derivatives from biomass. Aqueous alkaline extraction of corn bran led to the isolation of 75% of total alkali extractable PAs and the obtained mixture consisting of FA and *p*CA was further subjected, first to enzymatic decarboxylation, followed by cationic polymerization, to give a "mixed" polymer consisting of a 9:1 ratio VG and 4-hydroxystyrene units. The T_g of the mixed polymer was significantly higher (123°C) than that obtained for the cationic VG polymer. In addition, the mixed PVG polymer had slightly lower E and H values than the radical and cationic PVG polymers, but significantly higher performance than a commercial polystyrene polymer. It is anticipated that the production of PAs, VG and 4-hydroxy styrene and polymers, would add significant value to a biorefinery setting. In addition, 4-hydroxy styrene derivatives may eventually be used for substituting significant amounts of styrene in the current polymer industries, similar to the addition of bioethanol to oil derived fuels. The future of a biobased platform polymer showcased in this study appears to hold great promise [50].

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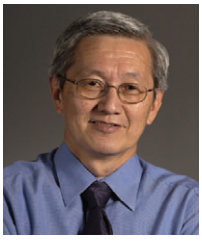

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