

# Biocatalytic Synthesis of Polycatechols from Toxic Aromatic Compounds

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A process is described in which toxic aromatic compounds are converted by toluene dioxygenase and in turn toluene *cis*-dihydrodiol dehydrogenase to catechols which are further polymerized by peroxidase-catalyzed oxidation producing polycatechols. Three approaches for obtaining catechols were employed: (1) addition of halogenated aromatics to *P. putida* F1, resulting in the accumulation of halogenated catechols; (2) inhibition of catechol 2,3-dioxygenase of *P. putida* F1 by known aromatic and aliphatic inhibitors; and (3) overexpression of toluene dioxygenase and toluene *cis*-dihydrodiol dehydrogenase genes in *E. coli* JM109. The process is suitable for producing novel catechols that upon oxidation may yield polymers with unique properties, presenting a tool for producing tailor-made biopolymers. Formation of 3-chlorocatechol from chlorobenzene, 3,4-dichlorocatechol from 1,2-dichlorobenzene, and catechol from benzene and their subsequent oxidation and polymerization was demonstrated. Oxidation of catechol yielded polymers with molecular weights of up to 4000 Daltons. Their apparently high water solubility eliminates the need for water-miscible solvents. In aqueous solution oxidation of catechols was rapid, yet the presence of 20%, 30%, and 40% ethanol, resulted in a rate decrease of 31%, 95%, and 93%, respectively. The advantage is that significantly less peroxidase is required for performing the reactions if miscible solvents are not employed. Furthermore, water-soluble polymers may be desirable for many applications.

## Introduction

The toluene dioxygenase (TDO) of *P. putida* F1, which initiates the degradation of aromatic compounds, is known to oxidize on the order of 100 substrates (1). Dioxygenation by TDO is followed by dehydrogenation by toluene *cis*-dihydrodiol dehydrogenase (TDD) resulting in the formation of a catechol (Figure 1). TDD can oxidize a wide range of dihydrodiol substrates (2). The catechol 2,3-dioxygenase (C2,3O) that catalyzes cleavage of the aromatic ring in the next step of the pathway also has a broad substrate specificity (3). However, when the aromatic ring contains a halogen

substituent, C2,3O is either inactivated by the halogenated catechol itself (4) or its ring cleavage product, an acyl chloride (5). As a result, accumulation of halogenated catechols results when *P. putida* F1 acts on halogen-substituted aromatics (6). Recombinant *E. coli* JM109(pDTG602), containing the *todC1C2BAD* genes from *P. putida* F1 encoding TDO and TDD has also been shown to yield catecholic transformation products upon addition of aromatic substrates that are substrates for TDO (7–9). Inhibition of C2,3O in *P. putida* F1 would similarly be expected to result in the accumulation of catecholic transformation products from aromatic substrates. A range of aromatic and aliphatic compounds are known to be inhibitors of C2,3O (4, 10, 11).

Peroxidases are heme enzymes, which catalyze the oxidation of a variety of aromatic substrates utilizing H<sub>2</sub>O<sub>2</sub> as an electron acceptor (12). Phenols are oxidized to generate phenoxy radicals, which have a tendency to couple forming oligomeric and polymeric products. This phenomenon has been considered for the biocatalytic production of useful oligomers and polymers (12, 13). The major advantages of employing enzymes for the synthesis of oligomers and polymers are as follows: (i) enzyme-catalyzed polymerization of phenols proceeds under mild reaction conditions without the use of toxic reagents (environmentally benign process); (ii) phenol monomers having various substituents can be polymerized to give new classes of functional polyaromatics; (iii) the structure and solubility of the polymer can be controlled by changing the reaction conditions; (iv) the polymerization procedure as well as the polymer isolation are very facile. Phenolic polymers synthesized by peroxidase-catalyzed reactions possess electrical and optical properties due to the conjugated backbone (14–16). These unique properties could enable novel applications in the area of adhesives, biosensors, electronics, molding, photonics, photolithography, rechargeable batteries, and thermoresistant filters (12, 13, 17). Moreover, polymers formed by peroxidases could be used as an alternative to phenolic-formaldehyde resins (14). These resins show excellent toughness and temperature-resistant properties. However, the toxic nature of formaldehyde causes problems in their manufacture and practical use.

The current research tested the conception of a process in which catechols, obtained upon transformation of monoaromatic compounds by TDO and in turn TDD, were further polymerized by peroxidase-catalyzed oxidation to polycatechols as schematically represented in Figure 1. Three approaches for producing catechols were studied. The microbial-enzymatic process highlights the possibility of effectively extending the range of substrates available for peroxidase-catalyzed oxidation and offers a tool for designing tailor-made biopolymer production.

## Materials and Methods

**Strains.** *Pseudomonas putida* F1 (18) and recombinant *Escherichia coli* JM109(pDTG602) (19) were kindly provided by D. T. Gibson.

**Growth and Maintenance of Microorganisms.** *P. putida* F1 was maintained on nutrient agar and grown in minimal medium (MSB) (20) containing 40 mM pyruvate as carbon source at 30 °C in a rotary shaker at 200 rpm. TDO was induced by toluene in the vapor phase. Filter paper, soaked in toluene, was placed in a sidearm molded to the Erlenmeyer flask.

Recombinant *E. coli* JM109(pDTG602) carrying the *todC1C2BAD* genes was maintained on agar solidified Luria-Bertani (LB) medium containing ampicillin (200 mg/L) (9) and cultivated on LB medium, supplemented with 200 mg/L

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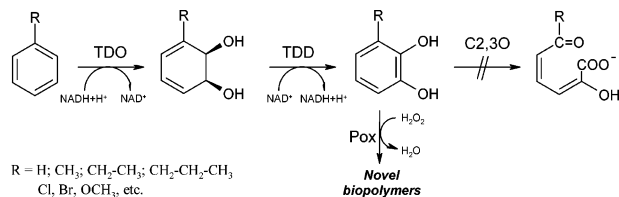


FIGURE 1. Schematic representation of the biocatalytic synthesis of polycatechols. The reaction is initiated by toluene dioxygenase (TDO). Subsequent dehydrogenation by toluene *cis*-dihydrodiol dehydrogenase (TDD) results in the formation of a catechol which is oxidized by peroxidase (Pox) resulting in the formation of polymeric products.

ampicillin. Cells were grown to an OD  $\approx$  0.7 at 35 °C and 200 rpm and then induced for 2 h with 100  $\mu$ M IPTG.

**Resting Cell Studies with *P. putida* F1.** *P. putida* F1 was harvested by centrifugation (6000 rpm, 10 min) and washed with 50 mM Na–K phosphate buffer, pH 7.0, before being resuspended in the same buffer to give an OD of 6.4. Resting cell experiments were then performed by adding varying amounts of chlorobenzene (0, 0.2, 0.4, 0.6, 0.8, 1 mM) from 100 mM stock solutions in DMSO along with 0.2% pyruvate as energy source. Aliquots of 25 mL were added to 125 mL serum bottles, which were then sealed with butyl rubber stoppers and incubated in the dark at 30 °C on a rotary shaker at 200 rpm for 2 h. Cells were then precipitated by centrifugation, and the supernatant was subjected to reverse phase-HPLC analysis as described below.

The influence of competitive inhibitors of C2,3O (1-pentanol, 2-pentanone, benzyl alcohol, and 1,10-phenanthroline) as well as the noncompetitive inhibitor, 4-chlorocatechol on the accumulation of catechol from 1 to 2 mM benzene was similarly studied.

**Preparative Biocatalytic Synthesis of Dead End Products by *P. putida* F1.** 1.5 L of toluene induced culture was equally divided into three 1 L Erlenmeyer flasks. The cultures were then incubated with 1 mM of chlorobenzene at 30 °C and 200 rpm for 2 h. Subsequently, cells were removed by centrifugation at 6000 rpm for 10 min, and the supernatant was acidified with a few drops of concentrated HCl and extracted with ethyl acetate in a ratio of 1:5 (solvent: supernatant). After evaporation to dryness, the products were redissolved in 2 mL of methanol and fractionated by semipreparative reverse phase-C18 HPLC. Peaks corresponding to dead end products were collected, acidified with a few drops of concentrated HCl, and re-extracted with ethyl acetate (ratio 1:5). After evaporation to dryness, the products were stored in the dark at –20 °C before being examined by <sup>1</sup>H NMR or oxidized by HRP.

**Whole-Cell Biotransformations with Recombinant *E. coli* JM109 (pDTG602).** After induction with 100  $\mu$ M IPTG for 2 h, the pH was adjusted to 7.0 with KOH. Then, 1 mM transformation substrate was added, and the cultures were incubated overnight at 35 °C and 200 rpm. Cells were then precipitated by centrifugation, and the supernatant was subjected to reverse phase-HPLC analysis as described below.

**Enzymatic Reactions.** HRP, type I with an RZ ( $A_{409}/A_{280}$ ) of 1.0 and an activity of 78 U/mg solid, was purchased from Sigma (Rehovot, Israel), and its concentration was measured at 403 nm using an extinction coefficient of 100 mM<sup>–1</sup> cm<sup>–1</sup> (21).

HRP reactions were carried out in 50 mM Na–K phosphate buffer, pH 7.0 at 25 °C. Conditions, including enzyme, reducing substrate, and H<sub>2</sub>O<sub>2</sub> concentrations are given in the figure legends.

Reaction rates for the oxidation of catechol were measured as previously described (22). Reactions contained 0.02  $\mu$ M

HRP, varying concentrations of catechol, and 2.5 mM H<sub>2</sub>O<sub>2</sub> in 50 mM Na–K phosphate buffer, pH 7.0 at 25 °C.

**Spectrophotometry.** Spectral changes during oxidation by HRP were monitored using a Hewlett-Packard HP8453 diode array spectrophotometer.

**HPLC Analysis.** HPLC analysis was conducted using a Hewlett-Packard HPLC (HP1100 series) equipped with a diode array detector. All solvents were of far-UV-quality HPLC-grade purity where available.

**Analytical Reverse Phase HPLC.** Analytical reverse phase-HPLC was conducted as previously described (23), with a few modifications. The mobile phase consisted of component A (10%, v/v, aqueous acetonitrile, 1 mM trifluoroacetic acid) and component B (40%, v/v, aqueous methanol, 40%, v/v, aqueous acetonitrile, 1 mM trifluoroacetic acid) in the following program: initially 100% A; linear gradient over 15 min to 100% B; held isocratically at 100% B for 5 min; linear gradient to 100% A over 5 min. The flow rate in all cases was maintained at 1 mL/min. Peak detection was at 210, 254, and 280 nm. Catechol was quantified by integration of peak-areas at 280 nm, with reference to calibrations, which were made using known amounts of authentic catechol.

**Semipreparative Reverse Phase HPLC.** Transformation products were fractionated using a semipreparative reverse phase Lichrospher 100 reverse phase-C18 column (25 cm  $\times$  10 mm i.d., 10  $\mu$ m; Lichrocart) employing the previously described gradient system. A flow rate of 6 mL min<sup>–1</sup> ensured an elution profile similar to that of the analytical column, and transformation products were collected using a fraction collector (Gilson, Model 203) (24).

**Gel Permeation HPLC.** Gel permeation analysis was performed as previously described (24).

**NMR Spectroscopy.** <sup>1</sup>H NMR experiments were performed using a 500 MHz – Bruker Avance 500 with the broadband inverse probe.

**Chemicals.** H<sub>2</sub>O<sub>2</sub> (30% v/v solution) was obtained from Sigma (Rehovot, Israel). The concentration of stock solutions of H<sub>2</sub>O<sub>2</sub> was determined at 240 nm using an extinction coefficient of 39.4 M<sup>–1</sup>cm<sup>–1</sup> (25).

Benzene, benzyl alcohol, chlorobenzene, 4-chlorocatechol, 1,2-dichlorobenzene, 2-pentanone, 1,10-phenanthroline, and phenol were all obtained from Aldrich (Rehovot, Israel). Catechol and 1-pentanol were obtained from Fluka (Rehovot, Israel).

## Results

Analysis by reverse phase HPLC of the cell-free supernatant obtained after incubation of chlorobenzene with resting cells of *P. putida* F1 previously induced with toluene revealed a peak with a retention time of 11.0 min, the intensity of which increased with increasing chlorobenzene concentration. The <sup>1</sup>H NMR spectrum of the fractionated peak presenting chemical shifts at H6: 6.75 ppm, 1H, dd, 8.1 and 1.6 Hz; H5: 6.62 ppm, 1H, t, 8.0 Hz; H4: 6.69 ppm, 1H, dd, 8.1 and 1.6 Hz is identical to that of 3-chlorocatechol (26).

When cell free supernatants containing 3-chlorocatechol were incubated with HRP and varying amounts of H<sub>2</sub>O<sub>2</sub>, reverse phase HPLC analysis revealed a gradual decrease in the area of the peak corresponding to the 3-chlorocatechol with increasing H<sub>2</sub>O<sub>2</sub> concentration. In the absence of either peroxidase or H<sub>2</sub>O<sub>2</sub>, no decrease in peak area was observed, demonstrating that the disappearance of 3-chlorocatechol was a result of enzymatic oxidation. The oxidation of 3-chlorocatechol by HRP was accompanied by instantaneous formation of pink-colored products.

Gel permeation analysis of the same reactions revealed an increase in the molecular weight upon oxidation (Figure 2). As can be seen, a gradual decrease in intensity of the peak corresponding to a molecular weight of 144 (3-chlorocatechol) was noticed upon addition of increasing amounts of

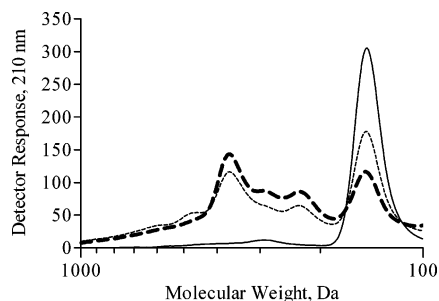


FIGURE 2. Gel permeation analysis of cell free supernatant containing 3-chlorocatechol after incubation with 0.5  $\mu$ M HRP and varying amounts of  $\text{H}_2\text{O}_2$  in 50 mM phosphate buffer, pH 7.0. Initial  $\text{H}_2\text{O}_2$  concentrations were 0 mM (—), 0.25 mM (---), and 0.5 mM (— · —). One hour after the reactions were initiated, they were acidified to pH 2.5, frozen at  $-78^\circ\text{C}$ , lyophilized, redissolved in tetrahydrofuran, and subjected to gel permeation analysis.

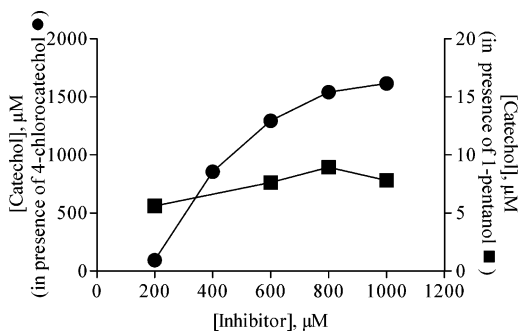


FIGURE 3. Influence of catechol 2,3-dioxygenase inhibitors on the accumulation of catechol by *P. putida* F1 following oxidation of benzene (1–2 mM).

$\text{H}_2\text{O}_2$ , which was accompanied by the appearance of peaks corresponding to higher molecular weight products. Those corresponding to molecular weights of  $\sim 250$  Da and  $\sim 400$  Da could possibly be dimers and trimers of 3-chlorocatechol, respectively. No difference was noticed if HRP was incubated with purified 3-chlorocatechol or with cell free supernatants containing 3-chlorocatechol, indicating that the cell free supernatant did not contain components that interfered with the peroxidase-catalyzed oxidation of 3-chlorocatechol.

Similar findings were witnessed when 1,2-dichlorobenzene was added to resting cells, and the supernatant was subsequently treated with HRP and  $\text{H}_2\text{O}_2$ .  $^1\text{H}$  NMR analysis revealed the formation of 3,4-dichlorocatechol upon transformation of 1,2-dichlorobenzene, which was oxidized by HRP to form colored products accompanied by an increase in molecular weight (data not shown). Furthermore, the enzymes lignin peroxidase and soybean peroxidase were also efficient at oxidizing the formed catechols.

Clearly these findings demonstrate that halogenated aromatics can be specifically transformed into oligomeric and possibly polymeric products in a two stage process, the first involving dihydroxylation and dehydrogenation by TDO and TDD of *P. putida* F1, respectively, resulting in the formation of dead-end catechol products, which are subsequently oxidized and polymerized in the second stage.

Another approach for transforming toxic aromatics to catechols involves inhibiting C2,3O of *P. putida* F1. A range of aromatic and aliphatic compounds are known to be competitive inhibitors of C2,3O (4, 10, 11). When 1-pentanol, previously shown to have a  $K_i$  of 0.58 mM for C2,3O (10), was added at concentrations between 0.2 and 1 mM to resting cells in the presence of 1–2 mM benzene, less than 10  $\mu\text{M}$  catechol accumulated at all concentrations of 1-pentanol (Figure 3). In similar experiments in which the alleged competitive inhibitors benzyl alcohol ( $K_i = 1.4$  mM (10)),

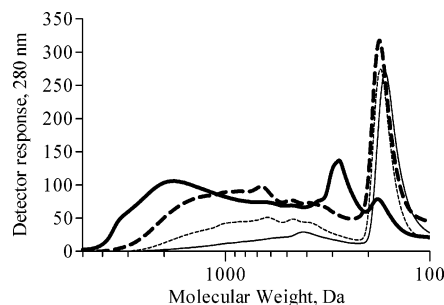


FIGURE 4. Gel permeation analysis of 2 mM catechol following oxidation by 0.5  $\mu$ M HRP and varying concentrations of  $\text{H}_2\text{O}_2$  in 50 mM phosphate buffer, pH 7.0. Initial  $\text{H}_2\text{O}_2$  concentrations were 0 (—, thin line), 0.25 (---), 0.5 (— · —), and 2 (—, thick line) mM. One hour after the reactions were initiated, they were acidified to pH 2.5, frozen at  $-78^\circ\text{C}$ , lyophilized, redissolved in tetrahydrofuran, and subjected to gel permeation analysis.

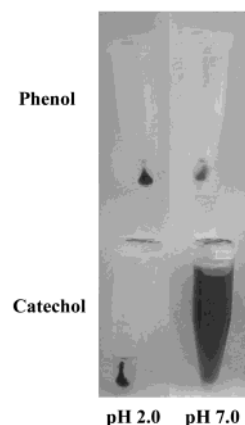


FIGURE 5. Photos demonstrating the fate of the polymeric products obtained upon oxidation of 2 mM phenol and 2 mM catechol by 1  $\mu$ M HRP and 5 mM  $\text{H}_2\text{O}_2$  in 50 mM phosphate buffer, pH 7.0. One hour after the reactions were initiated, the pH was either reduced to pH 2.0 or not adjusted (pH 7.0), and samples were subjected to centrifugation at 14000 rpm for 5 min.

2-pentanone ( $K_i = 0.15$  mM (10)) and 1,10-phenanthroline were added to resting cells at a concentration of 1 mM, little if any catechol was detected. On the other hand, in the presence of concentrations of between 0.2 and 1 mM of the noncompetitive inhibitor, 4-chlorocatechol, considerably catechol accumulated in the extracellular fluid. C2,3O is either inactivated by the halogenated catechol itself (4) or its ring cleavage product, an acyl chloride (5).

The most promising approach for producing catechols involves the use of recombinant *E. coli* JM109(pDTG602), which overexpresses TDO and TDD. This organism has been shown to give high effective mass yields of catechols from a number of aromatic compounds (7–9). We demonstrated the effective production of 3-chlorocatechol, 3,4-dichlorocatechol, and catechol, upon addition of either chlorobenzene, 1,2-dichlorobenzene, or benzene, respectively, to growing cells of *E. coli* JM109(pDTG602) (data not shown). Oxidation of the resulting catechol by HRP leads to formation of polymeric products (Figure 4). The molecular weight of the products obtained increased with increasing  $\text{H}_2\text{O}_2$  concentration, and products possessing molecular weights of up to 4000 Daltons were obtained in aqueous reactions, without the need for water-miscible organic solvents. In Figure 5, it is evident that the polymeric products obtained from catechol at pH 7.0 remained in solution, whereas those obtained from oxidation of phenol under identical conditions clearly precipitated. The polycatechol products precipitated only after the reaction mixture was acidified to pH 2.0.



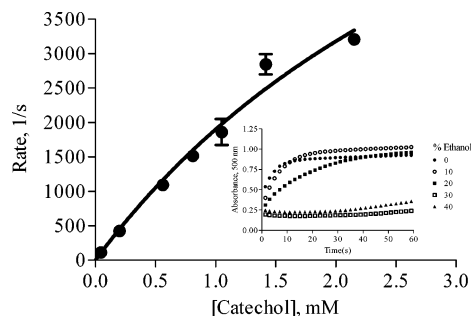


FIGURE 6. Influence of initial substrate concentration on the rate of oxidation of catechol by  $0.02 \mu\text{M}$  HRP and  $2.5 \text{ mM}$   $\text{H}_2\text{O}_2$  in  $50 \text{ mM}$  phosphate buffer, pH 7.0. Inset: Influence of ethanol on the time dependent oxidation of  $2 \text{ mM}$  catechol by  $0.5 \mu\text{M}$  HRP and  $6 \text{ mM}$   $\text{H}_2\text{O}_2$ , monitored spectrophotometrically at  $500 \text{ nm}$ .

The apparent high water solubility of the polymers obtained upon oxidation of catechol presumably eliminates the need for water miscible solvents to obtain high molecular weight polymeric products (as is the case for oxidation of phenol). Because water miscible solvents are detrimental to peroxidase activity, a significant increase in the amount of enzyme is required. In aqueous solution oxidation of catechols by peroxidase proceeds rapidly. To highlight this, the influence of substrate concentration on the rate of oxidation of catechol by  $0.02 \mu\text{M}$  HRP and  $2.5 \text{ mM}$   $\text{H}_2\text{O}_2$  in  $50 \text{ mM}$  phosphate buffer, pH 7.0 is shown in Figure 6. The overall rate of oxidation is significantly decreased by the addition of small amounts of water-miscible solvents, and the inset of Figure 6 shows the influence of ethanol on the time dependent oxidation of  $2 \text{ mM}$  catechol by  $0.5 \mu\text{M}$  HRP and  $6 \text{ mM}$   $\text{H}_2\text{O}_2$  in  $50 \text{ mM}$  phosphate buffer, pH 7.0, monitored spectrophotometrically at  $500 \text{ nm}$ . From the initial slope, the presence of 20%, 30%, and 40% ethanol resulted in a decrease of 31%, 95%, and 93% in the rate of oxidation, respectively. Addition of ethanol at 5% or 10% had little or no effect on product formation.

## Discussion

The current research demonstrates the potential of employing TDO and in turn TDD for obtaining novel catechols, which are subsequently polymerized by peroxidase-catalyzed oxidation to polycatechols in aqueous solution.

Three approaches may be adopted for obtaining catechols: (1) addition of halogenated aromatics to *P. putida* F1, which results in the accumulation of halogenated catechols due to inactivation of C2,3O (4, 5); (2) inhibition of C2,3O of *P. putida* F1 by known inhibitors of C2,3O (4, 10, 11); and (3) overexpression of TDO and TDD in *E. coli* JM109 (7–9).

Oxidation of the accumulating catechols results in the formation of polymers. The formation of colored products in this stage is the basis for a recently reported bioassay for the detection of bioavailable benzene, toluene, ethyl benzene, and xylenes (BTEX) (27). The intensity of the colored products was correlated with the concentration of the BTEX compounds. However, the formation of polymers during the HRP-catalyzed oxidation of the catechols formed from BTEX was overlooked. In fact, there is very little information on the pattern of polymerization of catechols by peroxidases, although a recent study does relate to the production of polycatechol by peroxidase in two types of solvent systems (28).

Polyphenols synthesized by peroxidase-catalyzed reactions possess electrical and optical properties due to the conjugated backbone (14–16). These unique properties could

enable novel applications in the area of biosensors, electronics, photonics, photolithography, and rechargeable batteries.

Previous reports indicate that enzymatic production of polymers from phenols in the aqueous phase does not appear to be practical for two main reasons (29): (i) Most phenols are poorly soluble in water, thus necessitating working with dilute solutions, which leads to low productivities. (ii) The initially formed phenolic dimers and trimers are insoluble in water and readily precipitate, preventing further polymerization. The findings reported here indicate that polycatechols exhibit high water solubility (Figures 4 and 5), a property that could eliminate the need for the addition of water miscible solvents to obtain the desired polymeric products. Such solvents are detrimental not only to the environment but also to peroxidase activity as demonstrated here (Figure 6). The advantage is that significantly less peroxidase is required for performing the reactions if miscible solvents are not required. Furthermore, water soluble polymers may be desirable for many applications.

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