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Applied Biocatalysis

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https://doi.org/10.1002/9781119487043.ch8

Vanillyl alcohol oxidase-catalysed production of (*R*)-1-(4′-hydroxyphenyl) ethanol

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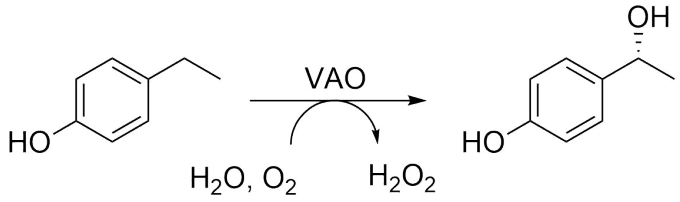
The introduction of a chiral centre into a non-chiral starting material is hard to achieve using traditional methods of chemical synthesis. Nevertheless, it is very important to obtain efficient methods by which to do so, as pure enantiomers of chiral compounds are of great importance to e.g. the pharmaceutical industry. One way in which chirality may be created is through the application of enzymes that are capable of enantioselectively functionalising a target molecule. For example, the flavin-dependent oxidoreductase vanillyl alcohol oxidase from *Penicillium simplicissimum* (VAO) can catalyse the enantioselective hydroxylation of 4-alkylphenols at the Cα position, leading to the formation of the (*R*)-enantiomer of the corresponding alcohol in high enantiomeric excess.[1] To demonstrate that this enzyme can be used to produce chiral secondary alcohols on a synthetically relevant scale, we employed it in the enantioselective hydroxylation of the non-chiral aromatic compound 4-ethylphenol to yield (*R*)-1-(4′-hydroxyphenyl)ethanol (Scheme 1).[2] The protocol described here provides a detailed practical description of how this synthesis can be performed at a scale of 10 g of starting material. Readers interested in the production of the (*S*)-enantiomer are referred to recent work describing its enzymatic preparation[3].



Scheme 1: VAO-catalysed conversion of 4-ethylphenol to (*R*)-1-(4′-hydroxyphenyl)ethanol

Procedure 1: Production of *Escherichia coli* cells expressing the His-VAO biocatalyst

Materials and Equipment

- pJ404-His-VAO plasmid[4] (available from authors upon request)
- Chemically competent BL21 *E. coli* cells (commercially available)
- LB medium mix (commercially available)
- LB agar mix (commercially available)
- Ampicillin
- Isopropyl β-D-1-thiogalactopyranoside (IPTG)
- Deionised water
- 30 mL Sterile syringe
- 0.45 μm Pore size sterile syringe filter
- 50 mL Sterile conical tube
- 2 x 100 mL Schott bottles

- 12 x 2 L Erlenmeyer flasks (no baffles)
- Centrifuge buckets
- Plastic cuvettes
- Petri dishes
- Cell spreader
- Parafilm
- Aluminium foil
- Plate incubator
- Autoclave
- Orbital shaker
- Spectrophotometer
- Tabletop Eppendorf centrifuge
- Centrifuge

Procedure

1. Dissolve Ampicillin (2 g) in deionised water (20 mL, final concentration 100 $mg.mL^{-1}$). Transfer the solution to a 30 mL syringe and sterilise by passing it through a 0.45 µm sterile syringe filter. Aliquots of this stock solution can be stored at -20 °C for up to one year.
2. Dissolve IPTG (3.8 g) in deionised water (20 mL, final concentration 190 $mg.mL^{-1}$, 0.8 M). Sterilise as described above. Store at -20 °C.
3. Prepare 100 mL LB agar according to manufacturer's instructions, transfer to 100 mL Schott bottle. Sterilise by autoclaving for 20 min at 120 °C. Remove the bottle from the autoclave and allow it to cool. Once it has cooled sufficiently to be comfortable to the touch, but before the LB agar has set, add 100 µL of the 100 $mg.mL^{-1}$ Ampicillin stock solution. Mix by gently swirling the solution, being careful not to introduce air bubbles into the liquid. Once mixed, pour into petri dishes (approx. 15 mL per dish) and allow to set. Plates are best prepared as fresh as possible, but can be stored at 4 °C for up to two weeks if necessary.
4. Transform the pJ404-His-VAO plasmid into competent BL21 *E. coli* cells according to the supplier's instructions. Using the cell spreader, plate out the transformed cells on the prepared LB agar plates for selection and incubate them in the plate incubator at 37 °C for 16 hours. Isolated colonies should be visible on the plates after this time, wrap the edge with parafilm and store at 4 °C until it is time to pick a colony (preferably as soon as possible, at most one week).
5. Prepare 6 L LB medium according to the supplier's instruction. Divide equally over the twelve 2 L Erlenmeyer flasks. Cover the neck of the flasks with a piece of aluminium foil, folded over once. Separately, prepare 100 mL LB medium and transfer it to a 100 mL Schott bottle. Sterilise the medium by autoclaving for 20 min at 120 °C.
6. Transfer 10 mL of LB from the Schott bottle into a 50 mL conical tube. Add 10 µL of the 100 $mg.mL^{-1}$ Ampicillin stock solution. Inoculate the medium with a colony of BL21 *E. coli* containing the pJ404-His-VAO plasmid by picking a colony using a sterile pipette tip

and transferring the cells to the medium. Transfer the tube to an orbital shaker and incubate at 37 °C for 16 h, shaking at 200 rpm.

7. After 16 h, the tube should contain a dense cell suspension. Centrifuge for 15 min at 4000 x *g*, 21 °C. This should yield a clear supernatant and a cell pellet. Remove the supernatant and resuspend the pellet in 10 mL fresh LB medium. Add 0.5 mL 100 $mg.mL^{-1}$ Ampicillin solution to each of the 2 L Erlenmeyer flasks. Subsequently, add 0.5 mL of the cell suspension. Transfer the Erlenmeyer flasks to an orbital shaker and incubate at 37 °C, shaking at 200 rpm. Periodically, take a 1 mL sample of the culture from one of the flasks, transfer it to a cuvette and measure the optical density at 600 nm using the spectrophotometer.
8. Once the optical density of the cultures is 0.6, remove all Erlenmeyer flasks from the shaker and set the shaker temperature to 25 °C. Add 0.5 mL of the 0.8 M IPTG stock solution to each Erlenmeyer flask. Return the flasks to the shaker and incubate at 25 °C for 16 h, shaking at 200 rpm.
9. After 16 h, transfer the cultures to centrifuge buckets and centrifuge for 15 min at 4000 x *g*, 4 °C. This should yield a clear supernatant and a cell pellet (35-40 g). Discard the supernatant. Now, one can proceed immediately to procedure 2 with the cell pellet. Alternatively, the cell pellet can be stored at -20 °C and procedure 2 can be performed at a later point.

Procedure 2: Extraction of the His-VAO biocatalyst

NOTE: To prevent inactivation of the His-VAO biocatalyst, keep biocatalyst-containing solutions on ice whenever possible.

Materials and Equipment

- Potassium phosphate monobasic (KH_2PO_4)
- Potassium phosphate dibasic (K_2HPO_4)
- Dithiothreitol (DTT)
- cOmplete™ protease inhibitor cocktail pills (Roche)
- DnaseI (Roche)
- Magnesium sulphate heptahydrate ($MgSO_4 \cdot 7\ H_2O$)
- Ultrapure water
- 1.5 mL Eppendorf tubes
- 50 mL Conical tubes
- 250 mL Graduated cylinder
- 2 x 250 mL Schott bottle
- Magnetic stirring bar
- Ice bucket
- French pressure cell press
- Centrifuge
- Centrifuge tubes suitable for use at 39,000 *g*
- pH Meter

Procedure

1. Dissolve DTT (770 mg) in Ultrapure water (10 mL, final concentration 77 $mg.mL^{-1}$, 0.5 M). Aliquots of this solution can be stored at -20 °C.
2. Dissolve $MgSO_4 \cdot 7\ H_2O$ (2.46 g) in Ultapure water (10 mL, final concentration 246 $mg.mL^{-1}$, 1 M). Store this solution at 4 °C.
3. Prepare a stock solution of 100 mM potassium phosphate buffer, pH 7.0. Dissolve KH_2PO_4 (2.72 g) in Ultrapure water (200 mL, final concentration 13.6 $g.L^{-1}$, 100 mM). Dissolve K_2HPO_4 (3.48 g) in Ultrapure water (200 mL, final concentration 17.4 $g.L^{-1}$, 100 mM). Mix the KH_2PO_4 solution (80 mL) with the K_2HPO_4 solution (120 mL) in the 250 mL Schott bottle. Measure the pH of the solution, it should be around 7. Adjust the pH to 7.0 by adding KH_2PO_4 solution to adjust it downwards or K_2HPO_4 solution to adjust it upwards as appropriate. Store the 100 mM potassium phosphate buffer, pH 7.0, stock solution at room temperature.
4. To prepare 200 mL lysis buffer, mix 100 mL 100 mM potassium phosphate buffer, pH 7.0, stock solution, 80 mL Ultrapure water, 200 µL DTT solution and 100 µL $MgSO_4$ solution in a 250 mL graduated cylinder. Add four cOmplete™ protease inhibitor cocktail pills and DNaseI (4 mg) and stir using a magnetic stirring bar until they have dissolved. Remove the stirring bar and adjust to 200 mL by adding Ultrapure water. Transfer the buffer to a 250 mL Schott bottle and put it in a bucket of ice to cool. Prepare this lysis buffer just before use.
5. Add cold lysis buffer to the cell pellets from step 1. The volume of lysis buffer added should be minimised in order to obtain a biocatalyst with a high activity per volume. A volume of 70-100 mL (2-2.5 mL/g cells) should be sufficient to obtain a suitable suspension.. Re-suspend the cells in the lysis buffer by pipetting up and down. Keep the cells on ice.
6. Pass the cell suspension through a pre-cooled French pressure cell press three times. Cool well between cycles by putting the samples on ice in order to prevent inactivation of the biocatalyst. Collect the suspension obtained after lysis in 50 mL conical tubes.
7. Transfer the suspension to centrifuge tubes and centrifuge for 45 min at 39,000 x *g* and 4 °C. After centrifugation, a clear yellow/brownish supernatant and a beige pellet should be obtained. Remove the supernatant and transfer it to 50 mL conical tubes. Typically, the volume of the obtained enzyme solution is 50-70 mL. Take a sample (0.5 mL) and transfer it to a 1.5 mL Eppendorf tube. This sample will be used to analyse the activity of the extract. Freeze the remainder of the supernatant using liquid nitrogen and store it at -20 °C. This is the His-VAO biocatalyst.

Procedure 3: Determination of the activity of the His-VAO biocatalyst

Materials and Equipment

- Vanillyl alcohol (3-methoxy-4-hydroxybenzyl alcohol, 308 mg, 2 mmol)
- Potassium phosphate monobasic (KH_2PO_4)
- Potassium phosphate dibasic (K_2HPO_4)
- Ethanol

- Ultapure water
- His-VAO biocatalyst (prepared as described above)
- pH Meter
- Parafilm
- Spectrophotometer with a thermostatted cell holder
- Hamilton syringe
- Quartz cuvettes, pathlength 1 cm, volume 600 µL

Procedure

1. Prepare 50 mM potassium phosphate buffer, pH 7.5. Dissolve KH_2PO_4 (0.68 g) in Ultrapure water (100 mL, 6.8 $g.L^{-1}$, 50 mM). Dissolve K_2HPO_4 (0.87 g) in Ultrapure water (100 mL, 8.7 $g.L^{-1}$, 50 mM). Mix 40 mL of the K_2HPO_4 solution with 10 mL KH_2PO_4 solution. Measure the pH of the solution, it should be around 7.5. Adjust the pH to 7.5 by adding KH_2PO_4 solution to adjust it downwards or K_2HPO_4 solution to adjust it upwards as appropriate.
2. Dissolve vanillyl alcohol (308 mg, 2 mmol) in ethanol (10 mL, final concentration 30.8 $mg.mL^{-1}$, 200 mM). Add 100 µL of the vanillyl alcohol stock solution to 9.9 mL of the 50 mM potassium phosphate buffer, pH 7.5, to give a 2 mM vanillyl alcohol stock solution.
3. Transfer 590 µL of the 2 mM vanillyl alcohol stock solution to a quartz cuvette. Cover the cuvette with parafilm and incubate it in the thermostatted cell holder of the spectrophotometer set at 25 °C for 10 min.
4. Using a Hamilton syringe, add 10 µL of the His-VAO biocatalyst sample to the cuvette, puncturing the parafilm with the syringe. Mix by inverting the cuvette three times and transfer to the cell holder of the spectrophotometer, thermostatted at 25 °C. Monitor the conversion of vanillyl alcohol to vanillin by measuring the absorption of the solution at 340 nm (ε = 14.000 M^{-1} cm^{-1}). The rate of the reaction should be such that an initial reaction rate can be determined by fitting a linear curve to the trace of the absorption. If the reaction rate is too high, dilute the His-VAO biocatalyst sample in 50 mM potassium phosphate buffer, pH 7.5, and repeat the measurement using this sample. Once an adequate dilution has been performed, repeat the measurement twice.
5. To calculate the activity of the His-VAO biocatalyst solution, first determine the average initial rate of change in absorption at 340 nm from the reaction traces by fitting linear curves to the data. Subsequently, calculate the activity of the biocatalyst in $U.mL^{-1}$, where 1 U is the amount of enzyme required to convert 1 µmol of vanillyl alcohol to vanillin per minute. The enzyme solution prepared as described above typically has an activity of 5-8 $U.mL^{-1}$.

Procedure 4: Enzymatic synthesis of (*R*)-1-(4′-hydroxyphenyl)ethanol

NOTE: This procedure involves working under a pure oxygen atmosphere. This represents a fire and explosion hazard. Please ensure appropriate safety measures are taken.

Materials and equipment

- 4-Ethylphenol (10 g, 82 mmol)

- His-VAO biocatalyst (prepared as described above)
- Potassium phosphate monobasic (KH_2PO_4)
- Potassium phosphate dibasic (K_2HPO_4)
- Ultapure water
- Acetone
- Ethyl acetate
- Hexane
- Anhydrous sodium sulphate (Na_2SO_4)
- Brine (saturated aqueous NaCl solution)
- Oxygen cylinder
- 4 L Three-neck round-bottom flask
- Thermometer
- Three-way balloon adapter
- Vacuum pump
- Balloon
- Overhead stirrer
- Büchner funnel
- Erlenmeyer flask
- Filter papers
- Separation funnel
- Water bath
- Rotary evaporator
- Oil bath
- Vacuum oven
- pH meter

Procedure

1. Prepare 100 mM potassium phosphate buffer, pH 7.5. Dissolve KH_2PO_4 (13.6 g) in Ultrapure water (1 L, 13.6 $g.L^{-1}$, 100 mM). Dissolve K_2HPO_4 (69.6 g) in Ultrapure water (4 L, 17.4 $g.L^{-1}$, 100 mM). Mix 3.2 L of the K_2HPO_4 solution with 800 mL of the KH_2PO_4 solution. Measure the pH of the solution, it should be around 7.5. Adjust the pH to 7.5 by adding KH_2PO_4 solution to adjust it downwards or K_2HPO_4 solution to adjust it upwards as appropriate.
2. Dissolve 4-ethylphenol (10 g, 82 mmol) in acetone (345 mL) and transfer into a 4 L three-neck round-bottom flask, thermostatted at 30 °C using a water bath. Check the temperature using a thermometer inserted through one of the side necks of the flask. Add 100 mM potassium phosphate buffer, pH 7.5 (3075 mL) and incubate until the temperature is stable at 30 °C. Then add 202 U of His-VAO biocatalyst (volume depends on the activity of the extract, typically 25-40 mL). Insert the overhead stirrer through the top neck of the flask.

3. Attach the vacuum pump and a balloon filled with oxygen from the oxygen cylinder to the three-way balloon adapter. Attach the adapter to the remaining side neck of the flask. Evacuate the reaction vessel and fill it with oxygen from the balloon. Repeat the process of evacuation and filling with oxygen twice.
4. Incubate the reaction mixture at 30 °C for 9 h under stirring at 230 rpm. After 9 h, the oxygen atmosphere can be removed. Incubate the reaction under atmospheric pressure for a further 15 h.
5. Using a rotary evaporator, remove the solvent under reduced pressure while heating to 50 °C using a water bath to reduce the volume of solvent. Some white solid may be formed during the concentration process.
6. Once the volume has been reduced sufficiently (to approximately 1 L), filter the solution through a Büchner funnel. Transfer to a separation funnel and add 1 L of ethyl acetate. Mix, allow separating and collect the aqueous and organic phases separately. Return the aqueous phase to the separation funnel and repeat the extraction process three times with a further 500 mL ethyl acetate each.
7. Pool the organic phases and transfer them to the separation funnel. Add 1 L of brine, mix, allow to separate and collect the organic phase. To the organic phase, add anhydrous Na_2SO_4 until dry. Remove the Na_2SO_4 by filtration.
8. Remove the solvent by distillation under reduced pressure to give a solid crude product (green beige solid). Dissolve the crude product in ethyl acetate (40 mL) then heat to 90 °C using an oil bath. Subsequently, allow the solution to cool to room temperature. A white solid should crystallise.
9. Filter the suspension through a Büchner funnel. Subsequently, wash the remaining solid with a 1:1 ethyl acetate:hexane mixture. Collect the solid. Dry the solid in a vacuum oven until no further decrease in weight is observed (4.10 g, 36%, 97% ee).

Analytical methods (GC and ^{1}H-NMR analysis)

Materials and equipment

Gas chromatograph with FID detector
Hydrodex β-6TBDM chiral GC column (25 m x 2.45 mm, Machery-Nagel)
400 MHz NMR spectrometer

Procedure

Analyse the product by GC and ^{1}H-NMR to confirm its identity and purity.

GC method

Gas chromatography experiments were performed on a GC-2010 plus (Shimadzu) with an FID detector (260 °C) and a Hydrodex β-6TBDM column (25 m x 2.45 mm, Machery-Nagel). Hydrogen was used as a carrier gas at a flow rate of 1.5 mL/min and the injector temperature was 80 °C. Runs were performed using a 20 min gradient from 80 to 220 °C followed by 10 min at 220 °C. (*R*)-1-(4′-hydroxyphenyl)ethanol had a retention time of 22.3 min.

^{1}H-NMR

(400 MHz, d6-DMSO) δ = 9.10 (s, 1H, Ar-OH), 7.12 (dt, J = 8.02 Hz, 2H, Ar-H), 6.68 (dt, J = 8.69 Hz, 2H, Ar-H), 4.85 (d, J = 4.29 Hz, 1H, aliph.-OH), 4.63-4.57 (m, 1H, CH), 1.27 (d, J = 6.44 Hz, 3H, CH_3).

Conclusion

This protocol enables the enzymatic synthesis of the chiral secondary alcohol (*R*)-1-(4′-hydroxyphenyl)ethanol from the achiral starting material 4-ethylphenol. A 94 % conversion of 10 g 4-ethylphenol was achieved after 24 h and the product was obtained in 36 % isolated yield and 97 % ee. The described protocol could potentially also be used for the production of chiral secondary alcohols from other short-chain 4-alkylphenols.[1,4]

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