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# Biocatalysis with In-Situ Product Removal Improves *p*-Coumaric Acid Production

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Natural and pure *p*-coumaric acid has valuable applications, and it can be produced via bioprocessing. However, fermentation processes have so far been unable to provide sufficient production metrics, while a biocatalytic process decoupling growth and production historically showed much promise. This biocatalytic process is revisited in order to tackle product inhibition of the key enzyme tyrosine ammonia lyase. In situ product removal is proposed as a possible solution, and a polymer/salt aqueous two-phase system is identified as a suitable system for extraction of *p*-coumaric acid from an alkaline solution, with a partition coefficient of up to 13. However, a 10% salt solution was found to reduce tyrosine ammonia lyase activity by 19%, leading to the need for a more dilute system. The cloud points of two aqueous two-phase systems at 40 °C and pH 10 were found to be 3.8% salt and 9.5% polymer, and a 5% potassium phosphate and 12.5% poly(ethylene glycol-*ran*-propylene glycol) mW~2500 system was selected for in situ product removal. An immobilized tyrosine ammonia lyase biocatalyst in this aqueous two-phase system produced up to 33 g/L *p*-coumaric acid within 24 hours, a 1.9-fold improvement compared to biocatalysis without in situ product removal.

## 1. Introduction

*p*-Coumaric acid (*p*CA) is a plant natural product with antioxidant and antimicrobial properties, with potential industrial applications as a precursor to poly(4-vinylphenol), a promising material for manufacturing organic semiconductors with uses in e.g. organic LED (OLED) displays for smartphones. As a plant natural product, *p*CA can be extracted from agricultural biomass, but yields are low and the extracts are contaminated by ferulic acid, making it difficult to achieve high purities. Bioprocessing offers an alternative production- route to natural products with a combination of high titers, yields, and purities, with reasonable volumetric rates from renewable feedstocks. The production of 12.5 g/L *p*CA via fermentation has been demonstrated in laboratory scale,[1] but this suffers from product toxicity, which limits microbial growth rates and production titers. *p*CA toxicity has been studied in bacteria, where it has been shown to both disrupt the cell membrane and bind DNA.[2] Bacteria seem especially susceptible to *p*CA toxicity, while yeast strains are inefficient at producing the tyrosine precursor needed to fuel efficient *p*CA production. An extensive comparison of different bioprocesses for phenylpropanoid production was recently published by us,[3] and to date the most successful bioprocess for *p*CA production decouples tyrosine fermentation and *p*CA biocatalysis in two separate steps (Figure 1).[4] However, even this process was insufficient at making an industrially viable process, although we can only speculate on which of the production metrics did not meet the economic targets. Their production titers were approximately 40 g/L *p*CA from 50 g/L tyrosine, or a 88% mol/mol yield, which is not ideal considering the value of the product and substrate are around 20 and 10 $US kg$^{-1}$ respectively. In our attempt to tackle this process, we decided to use another variant of the TAL enzyme from *Flavobacterium Johnsoniae* (*Fj*TAL), which has the advantage of having a high substrate specificity towards tyrosine compared to other TAL variants,[5] which may also be related to its relatively low affinity towards the product *p*CA.[6] Competitive product inhibition can dramatically reduce the catalytic rate of *p*CA production by occupying the TAL active site and potentially catalyzing the reverse reaction, even when a highly specific variant like *Fj*TAL is used. This is in part due to the low solubility of the substrate tyrosine, even at moderately alkaline pH. We believe product inhibition may explain the incomplete conversion of the DuPont process, and we therefore considered different approaches for mitigating it.

Enzyme engineering has previously been used to improve the Michaelis constant $K_M$ and/or catalytic rate constant $K_{cat}$ of PAL/TAL enzymes,[7–9] and could potentially be used to improve the substrate to product affinity ratio $K_M/K_i$ of TAL. However, determining this variable with sufficient precision for a single variant requires scores of replicates at multiple substrate and product concentrations,[6] making it unamenable to high-throughput screening.

One potential strategy is to decrease the *p*-coumaric acid concentration via in situ product removal (ISPR).[10,11] One of the difficulties with implementing ISPR in this case is the structural

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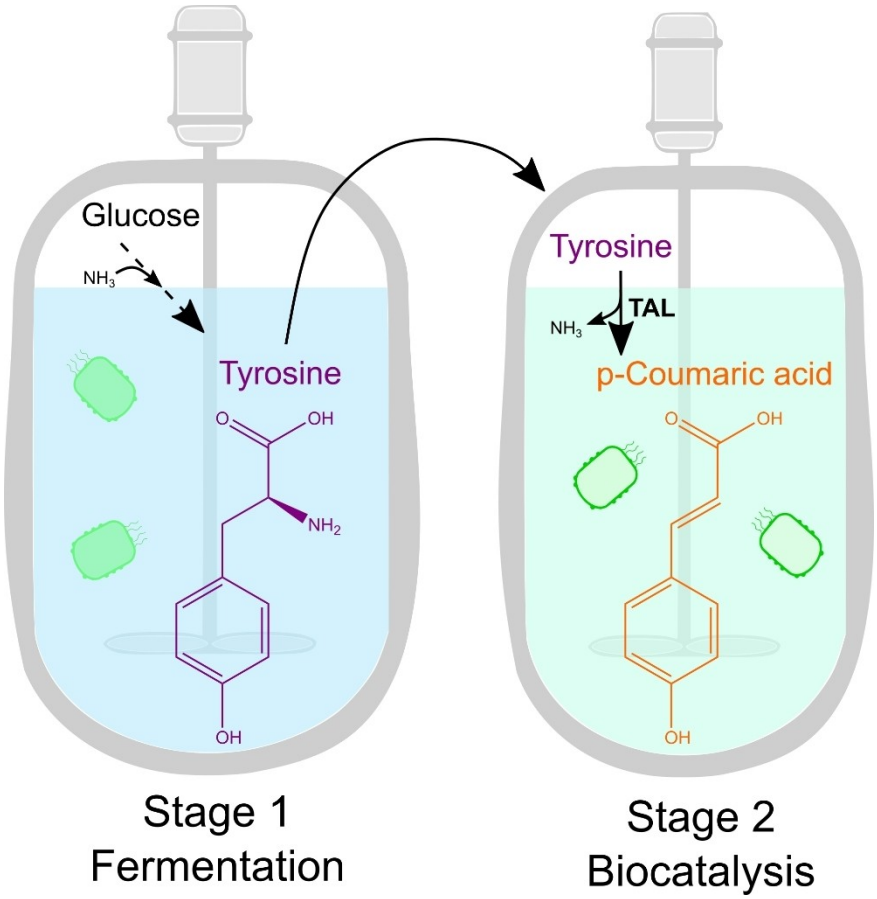


**Figure 1.** Overview of the two-stage bioprocess for *p*CA.Fed-batch fermentation using a tyrosine overproducing *E. coli* strain. Batch biocatalysis in an alkaline solution using permeabilized and immobilized *E. coli* cells overexpressing TAL.

and chemical similarities between the substrate and the product, which is likely to lead to co-removal of both. A possible solution to this would be to operate the ISPR truly in situ and not in stream. Rather than removing the product (and substrate) from the reactor, it is removed to a separate phase in the reactor. Then, the conversion of tyrosine and the removal of *p*CA might drive the concurrent release of tyrosine back into the bulk liquid phase. The charges (z) for tyrosine and *p*CA at pH 10 were calculated with the Henderson-Hasselbach equation, and the charge of *p*CA is −0.47 lower than that of tyrosine at pH 10 (Supporting information 1). Thus, we considered whether *p*CA might not have a higher affinity than tyrosine for anion-exchange resins, and could displace bound tyrosine. However, the capacity of ion-exchange resins operated in-situ was found to be insufficient (Supporting information 1).

*p*CA has an improved solubility in some organic solvents,[12] and thus the use of an organic overlay has also been developed for the ISPR of *p*CA.[11] However, this is only effective at low pH where *p*-coumaric acid is neutral,[13] and where TAL has very little activity.[5] We considered whether an aqueous two-phase system (ATPS) might be used instead at alkaline pH, in order to have high TAL activity and remove *p*CA into a relatively more hydrophobic aqueous polymer phase. ATPSs have mainly been studied for the removal of proteins,[14,15] but have in some cases also been tested for the extraction of small molecules.[16]

## 2. Results

### 2.1. Aqueous Two-Phase Systems

Initially we tested a 15% w/w PEG 8000: 15% w/w Dextran ATPS with 100 μM *p*CA at room temperature, and in this system the partition coefficient for *p*CA in the PEG phase was ~20 (Supporting information 2). However, high Mw dextran is prohibitively expensive, and a dextran solution was also found to be incompatible with the use of hydrogels for biocatalyst immobilization. We therefore aimed at using a salt:polymer ATPS instead, and after much consideration decided that alkaline salts with phosphate or carbonate anions would be preferable due to their ability to make a basic solution, in addition to their good phase forming properties according to the Hofmeister series.[17] A 15% w/w PEG 8000: 15% w/w $K_2HPO_4$ solution with 100 μM *p*CA at room temperature had a partition coefficient for *p*CA of ~13 (Supporting information 2).

In order for the polymer/salt ATPS to be effective for ISPR, the TAL enzyme needs to be present and active in the salt

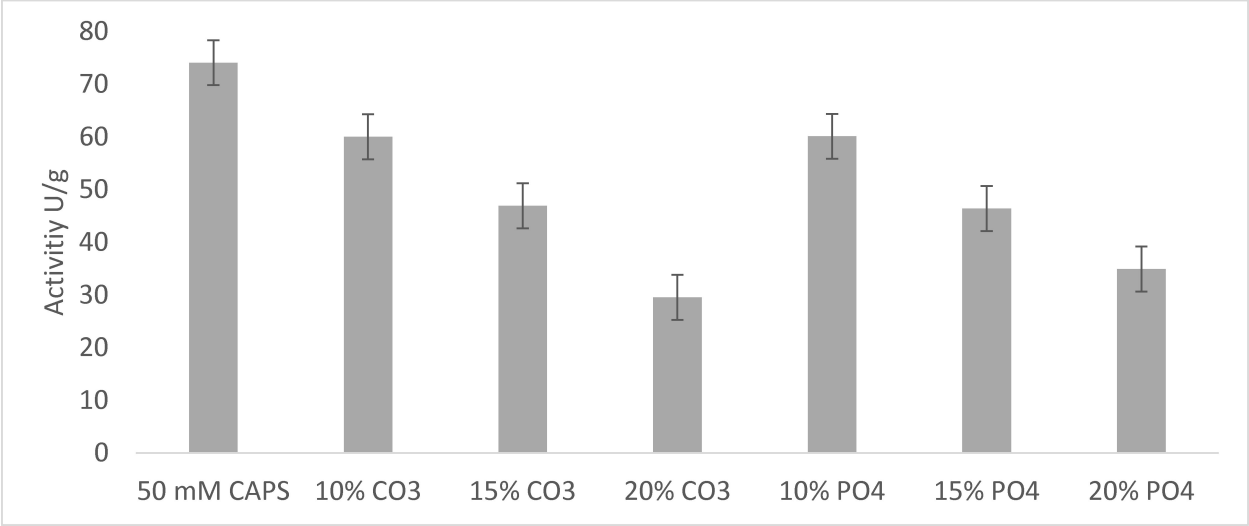


**Figure 2.** Specific activity of FjTAL in buffer and various salt concentrations. CO3, potassium carbonate. PO4, potassium phosphate. Means of duplicates. Error bars indicate the 95% confidence intervals based on the pooled standard deviation.

phase. We tested the activity of purified *Fj*TAL in a range of 10–20% w/w potassium phosphate or carbonate (Figure 2). The activity of *Fj*TAL decreased by 19% in 10% salt, by 37% in 15% salt, and by more than 50% in 20% salt. This highlights that the salt concentration should be as low as possible to limit the activity loss of *Fj*TAL.

With regards to the polymer phase, we chose to test four different polyethers with varying hydrophobicities; poly(propylene glycol) (PPG) Mw ~1000, poly(ethylene glycol-ran-propylene glycol) (PEGRPG) Mw ~2500, PEG Mw ~8000, and PEGRPG Mw ~12000. After testing the ATPS at the proposed operating conditions, PEG 1000 was excluded since it formed a thin organic overlay, rather than an ATPS. PEG-ran-PPG 12000 was excluded due to its very high viscosity which made it difficult to handle. Rather than determine the binodal curves of the four remaining ATPSs, we identified their cloud points starting from the same initial compositions of 20% polymer 8% salt 4% *p*CA. Here the cloud points of the phosphate systems were at lower concentrations of components than that of the carbonate systems. The cloud points were the same for the two polymers with phosphate (Table 1).

**Table 1.** Potassium salt and polymer concentrations of different ATPS at 40 °C pH 10 at their cloud points upon dilution. Results of a single experiment.

| ATPS | Carbonate PEGRPG 2500 | Carbonate PEG 8000 | Phosphate PEGRPG 2500 | Phosphate PEG 8000 |
|---|---|---|---|---|
| Salt, w/w | 5.7% | 5% | 3.8% | 3.8% |
| Polymer, w/w | 14.3% | 12.5% | 9.5% | 9.5% |

We made new ATPSs with 8% potassium phosphate and 20% polymer including 4% *p*CA, and analyzed the partition coefficients of *p*CA. They were found to be lower than the initial measurement with 100 μM *p*CA, presumably due to the much higher concentration of *p*CA and the lower salt concentration. Diluting the systems with water to 5% potassium phosphate, 12.5% polymer, and 2.5% *p*CA reduced the partition coefficients still further (Figure 3). However, the volumes and concentrations of *p*CA of the salt phases remained relatively

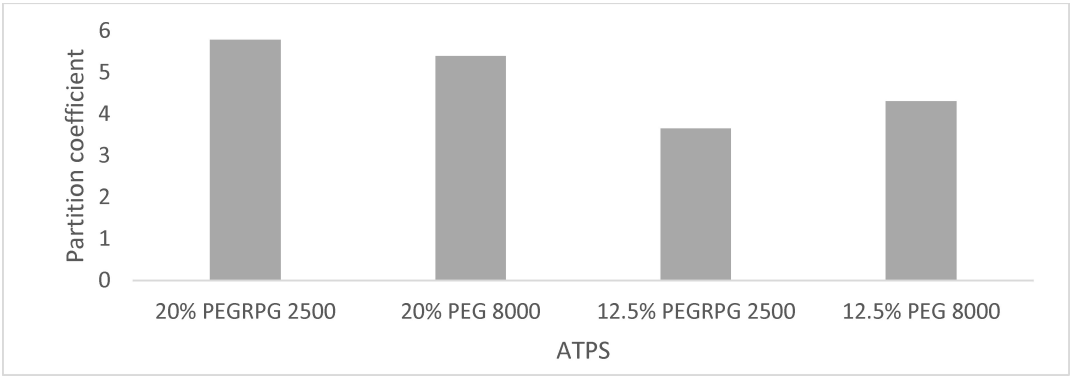


**Figure 3.** Partition coefficients of *p*CA in different ATPSs.20% polymer/8% potassium phosphate 4% *p*CA, or 12.5% polymer/5% potassium phosphate 2.5% *p*CA. Results of a single experiment.

unchanged (data not shown), while the polymer phases were diluted. This highlights that the concentration of the phase components is more important than the concentration of *p*CA.

Because the two polymers performed similarly with potassium phosphate in terms of phase separation and partition coefficients, we settled on using PEGRPG 2500 based on the convenience of it being a liquid polymer at room temperature, making it easier to dissolve than the solid PEG 8000 powder. PEGRPG 2500 also has a lower cloud point, which may aid downstream processing. pH measurements in a PEGRPG 2500 solution were found to be offset, which was corrected for as described in Supporting Information 3.

### 2.2. Whole-Cell Immobilization

While we never tested the partition of *Fj*TAL in the ATPS experimentally, proteins often partition into polymer phases at alkaline pH.[15] Rather than trying to optimize the partition of both the enzyme and product, we decided to immobilize the enzyme in a solid form to place it solely in the salt phase. An easy, cheap, and effective strategy is to immobilize whole-cells containing the enzyme intracellularly.[18] Whole-cell immobilization in natural hydrogels is a mild procedure, with calcium-alginate being the most commonly used hydrogel. However, calcium-alginate beads are unstable in the salt phase due to the ion-exchange of calcium with potassium, and the formation of insoluble calcium phosphate or calcium carbonate (Supporting Information 4). Other natural hydrogels for immobilization include agarose and *k*-carrageenan, and the latter has the advantage of a lower gelling temperature to avoid thermal enzyme denaturation, and better mechanical strength when cross-linked with potassium ions and locust bean gum (LBG).[19,20] The *k*-carrageenan beads were found to be stable for 23 hours in a 25% w/w potassium carbonate solution at pH 9.9 40 °C vortexed at 300 rpm (Supporting Information 4), making them superior to Ca-alginate beads under the proposed conditions. We produced *E. coli* cells expressing *Fj*TAL and immobilized up to 17 g wet cells in 100 mL of a 2% w/v 2:1 *k*-carrageenan:locust bean gum hydrogel, and cross-linked it with potassium chloride, producing ~100 g beads per batch.

### 2.3. Biocatalysis in an Aqueous Two-Phase System

The beads were loaded into a 1 L rotating bed reactor (RBR, Spinchem) with a slurry of tyrosine adjusted to 40 °C and pH 10 with $K_3PO_4$. After ~22 hours, the titer of *p*CA was 11.4 g/L. When the experiment was repeated with the same batch of cells, but with added PEGRPG and salt for ATPS formation, the initial rate was slightly lower but the final titer was similar (Figure 4A). We suspected the titers were insufficient to cause significant product inhibition, but we repeated the experiment with another batch of cells with higher activity. The initial rate of production was in this case similar with and without the ATPS, but the final titer after ~23 hours without the ATPS was 17.7 g/L, while it was almost double for the ATPS at 33.3 g/L (Figure 4B). The results of the two experiments are summed up in Supporting Information 5.

Following each experiment the ATPS was allowed to separate, and the volume and *p*CA concentration of both phases was determined. In the last batch the concentration of *p*CA in the salt phase was 24.57 g/L, and that of the polymer phase 47.58 g/L, giving a partition coefficient of 1.94 (table 2). The overall concentration was calculated to be 33.1 g/L based on the individual concentrations in the two phases and their volumes, in agreement with the direct sampling from the dispersion. This gives a yield of 0.724 $g_{pCA}/g_{tyrosine}$. The final volume in the second experiment was slightly larger than that of the first, due to more base being added to maintain the pH.

## 3. Discussion

### 3.1. Biocatalysis with ATPS

The initial *p*CA production rate was similar regardless of the presence or absence of an ATPS, but it seems that the ATPS

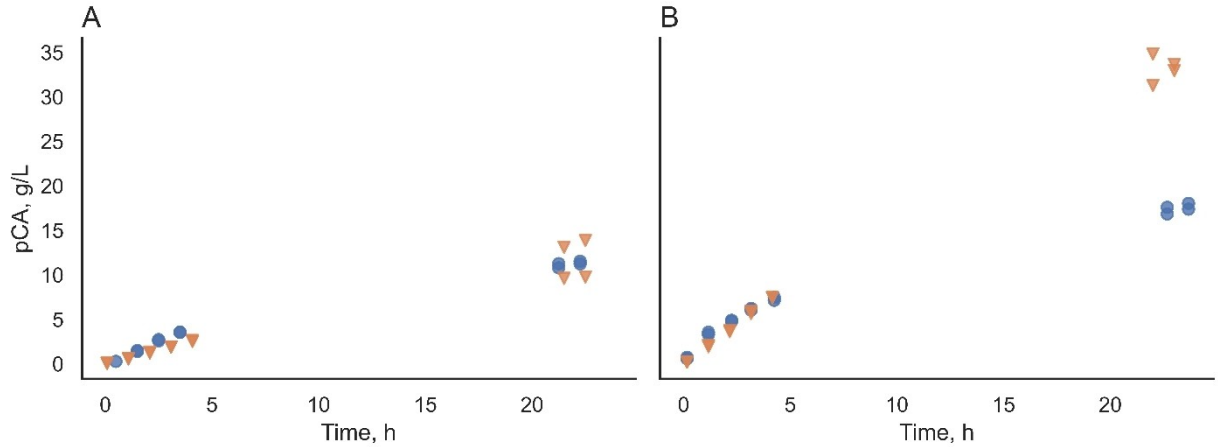


**Figure 4.** TAL biocatalysis without (●) and with (▼) ATPS. A. First experiment. B. Second experiment. Duplicate samples.

**Table 2.** Summary of the aqueous two-phase systems in the two experiments. Means of duplicates. 95% confidence intervals based on the pooled standard deviation of all four groups.

| Experiment | Phase | Phase volume, mL | Phase concentration, g/L | Partition coefficient | Overall concentration, g/L | Yield, $g_{pCA}/g_{tyrosine}$ |
|---|---|---|---|---|---|---|
| 1 | Salt | 500 | 8.2 +/− 1.9 | 2.19 | 11.9 | 0.238 +/− 0.053 |
| | Polymer | 300 | 18.0 +/− 1.9 | | | |
| 2 | Salt | 550 | 24.6 +/− 1.9 | 1.94 | 33.1 | 0.724 +/− 0.116 |
| | Polymer | 325 | 47.6 +/− 1.9 | | | |

helped maintain high *p*CA productivity. One possible interpretation of this is that the ATPS was efficient at ISPR, such that product inhibition did not reduce the catalytic rate of the TAL biocatalyst. However, this interpretation is not fully supported by the data. In the second experiment the final *p*CA concentration in the salt phase of the ATPS was 24.5 g/L, higher than the 17.7 g/L of the monophase without the ATPS. Thus, if product inhibition was the only factor behind the drop in activity, we should not expect the concentration of the salt phase to exceed that of the monophase without the ATPS. However, the pore size of a *k*-carrageenan hydrogel is too small to allow the PEGRPG 2500 polymer with an average molecular mass of 127.5 kDa and a random coil structure from entering, when the pore size is too small to allow globular enzymes from leaking out.[20,21] Thus it is possible that there is a third partition coefficient of *p*CA between the hydrogel and the bulk salt phase.

### 3.2. Immobilization

The *k*-carrageenan-LBG hydrogels were stable for up to 23 hours in an alkaline ~4-6% potassium phosphate solution at 40 °C with 200 rpm stirring, without any visible deterioration or swelling. This is a significant improvement compared to the use of ca-alginate immobilized beads, which are unstable in alkali, and which specifically require calcium ions in solution to prevent leeching from the beads.[22,23] The calcium ions can easily precipitate with most anions including phosphates and carbonates, while soluble potassium salts are a lot more numerous. While unnecessary for the single batch conversion tested here, further crosslinking with glutaraldehyde and poly(ethyleneimine) is likely to add additional stability to the gel.[18] The stability of the immobilized enzyme was also higher than that of the purified enzyme at the same pH and temperature.[6]

### 3.3. ATPS

A striking result is that the partition coefficient for *p*CA dropped throughout the development of the ATPS. While the initial partition coefficient in a PEG:Dextran ATPS was ~20, this fell slightly when moving to a salt:polymer system. It also fell dramatically when the *p*CA concentration was increased, when the temperature was increased, and when the concentrations of phase forming components were decreased. Eventually it was as low as 3.4, and this dropped even further to ~2 in the actual bioreactor, almost a 10-fold reduction compared to the first test. This obviously speaks to the sensitivity of ATPS to every operational parameter, and highlights the importance of screening ATPSs under realistic operating conditions. This can also make continuous process development and optimization difficult, because introducing changes to one process variable which has a favorable impact on one hand may negatively influence the ATPS on the other hand.

### 3.4. Polymer Recycle

While the use of an alkali salt for pH control is mandatory regardless, the use of a polymer to form an ATPS adds to the cost of the process. At a cost of ~2 $US/kg polymer, the cost of 120 g polymer/L adds up to 0.24 $US/L, while the value of even the target 40 g/L *p*CA at a proposed cost of ~20 $US/kg is only 0.8 $US/L. Thereby 30% of the value of the product is offset by the cost of the polymer, indicating that polymer recycle is required for its use to be economically feasible. Simply adding acid to the polymer phase to crystallize *p*CA would contaminate the polymer phase with anions. Another idea is to raise the temperature of the polymer phase above its cloud point to make a new two-phase system, but preliminary testing indicated that *p*CA remained in the polymer phase (not shown). It may be possible to combine the two, first crystallizing *p*CA with acid, and then heating the polymer phase beyond its cloud point to separate out the contaminating anions via the new aqueous phase. Alternatively, all salts including *p*CA could be separated from the polymer by ultrafiltration using a membrane with a Mw-cutoff below that of the polymer. The economic feasibility of such recycling strategies would also have to be evaluated.

## 4. Conclusions

We considered several different strategies for improving the biocatalytic process for *p*-coumaric acid, and made progress in this pursuit. We developed an aqueous two-phase system for *in-situ* product removal, which almost doubled the titer and yield of *p*-coumaric acid compared to a control, suggesting product inhibition was alleviated. We also immobilized a whole-cell tyrosine ammonia lyase biocatalyst in a *k*-carrageenan

hydrogel, which was stable in an alkaline solution, unlike calcium alginate beads or the pure enzyme. This may contribute to the productivity of a TAL biocatalyst. These strategies need to be further optimized and tested with a more efficient TAL biocatalyst in repeated batches, in order to properly assess their long-term effectiveness. This work may contribute to the development of an industrially viable *p*CA production process.

## Materials and Methods

### Aqueous Two-Phase Systems

Solutions of 5% *p*CA and 10% $K_3PO_4$-$K_2HPO_4$ or 10% $K_2CO_3$-$KHCO_3$ pH 10 (40 °C) were made in microcentrifuge tubes and mixed with polymers to 20% w/w polymer. ATPSs were formed by vortexing the mixtures and centrifuging them briefly. Samples were pipetted from either phase, but after aspirating from the bottom phase and before dispensing, the pipette tip was wiped with a paper towel to remove any polymer solution adhering to the tip.

### TAL Activity Assay

The TAL purification and activity assay was carried out essentially as described previously.[6] Briefly, 1 μg purified *Fj*TAL was added to buffer in a UV-vis microplate containing 2 mM tyrosine and adjusted to pH 10 at 30 °C. The standard buffer was 50 mM CAPS adjusted with NaOH, while the 10–20% salt buffers were adjusted by mixing 10–20% solutions of the base and conjugate acid to the desired pH. The absorbance of *p*CA was followed over time at 325 nm, and the activity is reported as μmol *p*CA $min^{-1}$ (U) $g^{-1}$ TAL.

### Strain Engineering, Fermentation, and Whole Cell Immobilization

*E. coli* cells were cloned to express *Fj*TAL with an arabinose inducible promoter from a plasmid (Supporting Information 6). The strain was cultivated in a fed-batch fermentation, and harvested as described in Supporting Information 7.

Wet cells (~14 g) were thawed and resuspended in 20 mL of saline, before mixing them with 80 mL of a ~50 °C warm solution of 1.33% w/V *k*-carrageenan 0.66% w/V locust bean gum (Sigma-Aldrich) 0.9% NaCl. The cell mixture was extruded through a 9-needle die (Mesoram) with 1 mm inner diameter needles using a peristaltic pump with a pump speed of 33 mL/min, placed ~20 cm above a 2% w/V KCl solution warmed to ~37 °C and stirred slowly with a magnetic bar. The resulting hydrogel beads were crosslinked in the KCl solution for 30 minutes without additional heating, drained, and stored overnight at 4 °C. In the second experiment ~17 g wet cells from a different fermentation batch were used.

### Biocatalysis in a Rotating Bed Reactor

For the reaction without the ATPS, ~100 g of immobilized biocatalyst was loaded into a 1 L rotating bed reactor (RBR, Spinchem) containing a slurry of 850 mL deionized water (DI) and 50 g tyrosine adjusted to 40 °C and pH 9.9 +/− 0.05 with $K_3PO_4$ (~10 g). The RBR was rotated at 200 rpm, the pH was controlled automatically at the setpoint with a 50% w/V $K_3PO_4$ solution, and the reaction was allowed to proceed. For the reaction with the ATPS, the RBR was instead loaded with a slurry containing 730 mL DI water and 120 mL poly(ethylene glycol-ran-propylene glycol) with an average Mw of ~2500 (Sigma-Aldrich), 50 g tyrosine, and 30 g $K_2HPO_4$ adjusted to 40 °C and pH 10.11 +/− 0.05 with $K_3PO_4$ (~10 g). The reactor was initially sampled every hour, and again after 21 h. After 22 h, the RBR was removed from the vessel, and the ATPS was allowed to settle before sampling both the salt and polymer phase of the ATPS. Finally, the entire contents were drained into a preheated measuring cylinder and allowed to settle again, and the volumes of the phases were determined. Samples were quenched with an equal volume of 1 M NaOH and stored in the dark at 4 °C, the absorbance spectra of the samples were measured, and the absorbance at 385 nm was compared to a standard curve of *p*CA, after diluting samples and *p*CA standards 20-fold in a 50 mM CAPS buffer pH 10 at 30 °C. Some values in table 2 are based on the following calculations:

$$\text{Partition coefficient} = [pCA]_{polymer}/[pCA]_{salt}.\ g_{pCA} = [pCA]_{polymer} {}^{*}V_{polymer} + [pCA]_{salt} {}^{*}V_{salt}.\ \text{Yield} = g_{pCA}/g_{tyrosine}.$$

$$\text{Overall concentration} = \frac{g_{pCA}}{V_{polymer} + V_{salt}}.$$

## Acknowledgements

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## Conflict of Interests

Alex Toftgaard Nielsen has ownership in Cysbio ApS, a company which holds a patent related to the production of p-coumaric acid using FjTAL (Patent No. US20200399665A1).

## Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Keywords:** Biphasic catalysis · Biocatalysis · immobilization · In-situ product removal · ammonia lyase

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