

Whole cell biosynthetic activity of *Komagataella phaffii* (*Pichia pastoris*) GS115 strains engineered with transgenes encoding *Chromobacterium violaceum* ω -transaminase alone or combined with native transketolase

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ABSTRACT

Whole cell biocatalysis is an ideal tool for biotransformations that demand enzyme regeneration or robustness to fluctuating pH, osmolarity and bio-contaminant load in feedstocks. The methylotrophic yeast *Komagataella phaffii* (*K. phaffii*) is an attractive alternative to *Escherichia coli* (*E. coli*) for whole cell biocatalysis due to its genetic tractability and capacity to grow to up to 60% wet cell weight by volume. We sought to exploit high cell density *K. phaffii* to intensify whole-cell chiral amino-alcohol (CAA) biosynthesis. We engineered two novel *K. phaffii* GS115 strains: one by inserting a *Chromobacterium violaceum* ω -transaminase CV2025 transgene, for strain PpTAmCV708, and a second strain, PpTAm-TK16, by also inserting the same CV2025 transgene plus a second transgene for a native transketolase. At high cell density, both strains tolerated high substrate concentrations. When fed three low cost substrates, 200 mM glycolaldehyde, 200 mM hydroxypyruvate and 150 mM methylbenzylamine, PpTAm-TK16 whole cells achieved 0.29 g L⁻¹ h⁻¹ space-time yield of the acetophenone by-product, a 49-fold increase of the highest levels reported for *E. coli* whole cells harbouring the equivalent pathway. When fed only the low-cost substrate, 150 mM methylbenzylamine, strain PpTAmCV708 achieved a 105-fold increase of reported *E. coli* whole cell biocatalysis performance, with a space-time yield of 0.62 g L⁻¹ h⁻¹ of the CAA, 2-amino-1,3,4-butanetriol (ABT). The rapid growth and high biomass characteristics of *K. phaffii* were successfully exploited for production of ABT by whole-cell biocatalysis at higher levels than the previously achieved with *E. coli* in the presence of the same substrates.

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Introduction

Whole cell biocatalysis enables the design^{1,2} of self-renewing, multi-enzyme micro-machines capable of dynamically maintaining controlled internal environments sequestered from

exterior conditions. These three key features define the utility of whole cell biocatalysis for use with low value or crude feedstocks and for production of complex products. Whole cell biocatalysis performance depends largely on cellular viability, cellular metabolism and the synthesis, trafficking and localisation of recombinant proteins within cells.³ Within eukaryotic cells, protein abundance can result from complex control of DNA transcription and RNA translation⁴ and enzyme activity may not directly correlate to enzyme concentration.^{5,6} By contrast, biocatalysis with purified enzymes is defined solely by the density and biochemistry of the proteins, their cofactors and coenzymes, and their deployment in a reaction chamber, such as being in solution or immobilised. The differing nature and applications of biocatalysis using whole cells or purified enzymes led Schrewe *et al.*³ to propose a set of distinct performance metrics for whole cell biocatalysts that make no assumptions with respect to expression of transgenes within whole cells (see also Table 1, this study).

Chiral amino-alcohols (CAAs) have aroused great industrial interest as they can form key groups within the structure of industrially significant small molecules such as of antibiotics and protease inhibitors. One such CAA is 2-amino-1,3,4-butanetriol (ABT), an important synthon in the production of several drugs, including the protease inhibitor, pepstatin,⁷ and the antiretroviral drug, Nelfinavir, which is effective in the treatment of HIV/AIDS.⁸ Conventional chemical routes to synthesis of optically pure batches of these molecules frequently require extremes of pH and temperature, which are expensive to maintain, and multiple reactions, each of which involve the formation of unwanted secondary products.⁹ Biological routes for CAA production^{10,11} have been sought with the hope that they will enable the use of more mild, therefore more sustainable conditions for synthesis. For the biosynthesis of ABT, a *de novo* metabolic pathway has been designed, which does not occur naturally in any existing organism,¹¹ coupling the biocatalytic action of two enzymes, a transketolase and a transaminase, in which the product of the first reaction is a suitable substrate for the second reaction.

Transketolases are enzymes capable of catalysing the transfer of a two-carbon group from a ketose sugar to an aldose sugar forming an asymmetric carbon-carbon bond. They have been used for whole cell biocatalysis in *E. coli*,¹² and *K. phaffii*¹³ cells, for asymmetrisation of hydroxypyruvate and glycolaldehyde to generate the ketoalcohol, L-erythrulose. Transketolases require two cofactors;¹⁴ thiamine pyrophosphate (TPP) and a divalent cation such as magnesium (II). The formation of the new carbon-carbon bond is highly stereospecific and the reaction can be made effectively irreversible if a ketol donor substrate is employed to produce carbon dioxide.^{15,16} Due to the high demand for stereoselective properties, transketolases have been successfully used as a biocatalyst for the synthesis of chiral compounds such as L-Gluco-heptulose¹⁷ and (3R)-1,3-dihydroxypentan-2-one.¹⁸ Both *E. coli* and yeast transketolases have been investigated for their ability to form asymmetric carbon-carbon bonds.^{11,14,19}

Transaminase enzymes are ubiquitous in nature as they play an important role in nitrogen utilisation for all cellular life and have been successfully co-expressed with other enzymes in *E. coli* as part of *de novo* assembly of metabolic biosynthetic pathways.^{11,20,21} Class III transaminases encompass β -transaminases, γ -transaminases, and ω -transaminases and are especially sought after as they have the widest substrate range.²² The ω -type transaminases are the only type known to perform stereo-selective amination of ketones. Moreover, Class III ω -type transaminases are typically highly active and also stable to a broad range of pH, temperature and concentration of substrate and product. As such, they have been investigated extensively for industrial production of amino acids, chiral amines and amine alcohols, all of which are valuable key intermediates for chemical synthesis of chiral, small-molecule therapeutics.²³ Class III ω -type transaminases have been studied in *Vibrio fluvialis*;^{24,25} *Pseudomonas putida*,²⁶ *Chromobacterium violaceum*²¹ and *Saccharomyces cerevisiae*.²⁷

With the necessary substrates and cofactors, transketolase and transaminase can perform two coupled reactions within host cells to catalyse the conversion of achiral substrates into complex chiral molecules. Ingram *et al.*¹¹ assembled such a *de novo* multi-step pathway (Figure 1) consisting of a native *E. coli* transketolase and the β -alanine:pyruvate transaminase from *Pseudomonas aeruginosa* (*P. aeruginosa*). Whole *E. coli* cells harbouring the pathway achieved of 3 mM ABT. Working on the same system, Rios-Solis *et al.*²⁸ substituted the *P. aeruginosa* transaminase for the *Chromobacterium violaceum* (*C. violaceum*) CV2025 transaminase to achieve a four-fold ABT yield increase.

The methylotrophic yeast, *P. pastoris*, recently reclassified as *K. phaffii*,²⁹ readily grows to high cell densities, up to 60% v/v wet cell weight, when cultivated using chemically defined culture media and bioreactors. *K. phaffii* can use methanol as its only carbon and energy source, making its cultivation economically favourable. We previously engineered a recombinant *K. phaffii* strain for overexpression of a native transketolase¹³ and characterised its whole-cell biocatalysis performance for the production of the chiral sugar L-erythrulose from the pro-chiral compounds hydroxypyruvate (HPA) and glycolaldehyde (GA).

K. phaffii is an established work horse in recombinant protein production due to its rapid growth to high biomass. The application of *K. phaffii* in whole cell biocatalysis continues to be explored, including the use of dead³⁰ and permeabilised^{31,32} whole cells, and cells with enzyme displayed on their surface.^{33,34} Tools are emerging for the optimisation of whole cell biocatalysis steps, such as mathematical modelling of reactions and pathways,³⁵ the application of machine learning to interrogate proteomics and metabolic flux data,² and precise control over genomic location and dosage of transgenes.³⁶ To consider only the typical properties of *K. phaffii* as a methylotrophic host cell capable of growth to high cell density, we have elected to investigate strains prior to the performance of any optimisation steps, by using strains selected only on the basis of growth performance. We intend that the

pre-optimisation data captured in this study can inform future studies in which the influence of factors such as transgene dosage, genomic location and expression, reaction configuration and pathway analytics are also mapped.

The motivation for this study is to demonstrate that *K. phaffii*, previously exploited in the area of recombinant protein production, can also be applied to whole cell biocatalysis. This is important as *E. coli*, which is more commonly used for whole cell biocatalysis, is susceptible to lysis by bacteriophage, which are naturally ubiquitous in the biosphere and therefore also in agricultural feed streams. *E. coli* cells also experience lethal stress below pH 5.58 and above pH 8.9. Yeasts such as *K. phaffii* are in theory a viable alternative, with no pathogens equivalent to bacteriophage and a broader range of pH tolerance, from pH 3 to pH 7.11. To validate *K. phaffii* as an alternative biocatalytic host cell, we further modify the transketolase *K. phaffii* strain of Wei *et al.*¹³ by inserting transgenes encoding the native transketolase and the *C. violaceum* transaminase CV2025, shown by others^{21,28,37} to be the best-performing transaminase for the production of ABT within the equivalent *de novo* pathway in *E. coli* whole cell biocatalysis (Figure 1). We also engineer a further *K. phaffii* strain to harbour a transgene encoding the *C. violaceum* transaminase CV2025 only. When fed appropriate substrates, we demonstrate that these *K. phaffii* whole cell biocatalyst strains are capable of higher levels of biocatalytic activity than those previously reported for *E. coli*.

Materials and Methods

Molecular biology and general reagents

Restriction enzymes, polymerase chain reaction (PCR) and ligation reagents were obtained from New England Biolab (Hitchin, UK). One Shot TOP10 *E. coli* chemically competent cells were obtained from Life Technologies (Paisley, UK). *P. pastoris* (*K. phaffii*) strains: GS115 wild-type, GS115 Human Serum Albumin and GS115 β -Galactosidase, and the *K. phaffii* expression plasmid, pAO815, were obtained from Invitrogen Corporation (Paisley, UK). The CV2025 transaminase gene from *C. violaceum* (Uniprot Accession number Q7NWX4) was

codon-optimised and synthesised by GenScript (Piscataway, NJ, USA). The proprietary GenScript OptimumGene™ algorithm was used for the codon-optimisation. Details of the outputs of the codon optimisation, including the optimised coding sequence, are available as a public data set (Supplementary Material). Oligonucleotides (Supplementary Material) were purchased from Eurofins Genomics (Ebersberg, Germany). Nutrient broth, nutrient agar, (S)- α -methylbenzylamine (MBA), L-erythrulose (ERY), pyridoxal-5'-phosphate (PLP), acetophenone (ACP), dimethyl sulfoxide (DMSO), glycolaldehyde (GA), hydroxypyruvate (HPA), thiamine pyrophosphate (TPP) and magnesium chloride ($MgCl_2$) were obtained from Sigma-Aldrich (Gillingham, UK). All plasmids for which construction included a PCR step were sequenced using the services of Eurofins (Ebersberg, Germany) and primers assigned the following codes: 5AOX1JH, JH3AOX1 and CV2025.opt.Mid_Rev for plasmid p8TAmCV708; and 5AOX1JH, JH3AOX1, TK151_Mid_FWD and TK151_Mid_REV for plasmid pAOX0150-TK-bm (primer sequences provided in Supplementary Material).

Construction of plasmid p8TAmCV708 for expression of CV2025 transaminase

The codon-optimised transaminase CV2025 was PCR-amplified using the plasmid provided by GenScript and the primers CV2025FWD and CV2025REV (primer sequences in Supplementary Material), which incorporate *Eco RI* restriction sites (indicated by lower case sequence). The resultant 1.4 kb amplicon featured the transaminase CV2025 open reading frame (ORF) flanked by *Eco RI* restriction sites. This was subcloned into the *K. phaffii* expression plasmid, pAO815,³⁸ using standard molecular biology techniques, generating the 9.1 kb transaminase CV2025 expression plasmid, p8TAmCV708.

Construction of plasmid p8TAm-TK16 for co-expression of CV2025 transaminase and native transketolase

Previously, Wei *et al.*¹³ used pAO815 as the backbone for construction of the plasmid, pAOX0150-TK, which encodes a gene for expression of a native *K. phaffii* transketolase (Genbank: CAY67980.1) under control of the methanol-inducible AOXI promoter. For DNA

assembly purposes, we used standard molecular biology techniques to perform *Dpn* I-mediated, site directed mutagenesis of pAOX0150-TK to remove a *Bgl* II site from the transketolase ORF while preserving amino acid sequence encoding. Primers FTK150sdm and TK150sdmR (primer sequences in Supplementary Material) were used for the mutagenesis. The resultant plasmid, with the removed *Bgl* II site, was designated pAOX0150-TK-bm. We next performed a *Bgl* II and *Bam*H I double digest of pAOX0150-TK-bm to obtain a 3.3 kb DNA fragment, encoding the *K. phaffii* AOXI promoter followed by the transketolase ORF and a transcription termination (TT) element, flanked upstream by a *Bgl* II overhang and downstream by a *Bam*H I overhang. This fragment was inserted non-directionally into a one *Bam*H I site present in p8TAmCV708, downstream of the transaminase CV2025 expression cassette. This ligation, and subsequent orientation screening, generated the new 12.5 kb plasmid, p8TAm-TK16, which co-encodes expression cassettes for both native transketolase and transaminase CV2025, both under control of AOXI promoters.

Genomic integration of transgenes

Generation of all recombinant *K. phaffii* strains was performed using genetic elements present in the pAO815 backbone (sequence provided in Supplementary Material). The expression cassette of this vector is composed of an AOX1 promoter upstream of a TT element, both separated by a unique *Eco* RI cloning site and also flanked upstream by a *Bgl* II restriction site and downstream a *Bam*HI restriction site. The expression cassette was designed in this way to favour integration into the genome of the host by homologous recombination. The histidine dehydrogenase gene, *HIS4*, present in the pAO815 backbone downstream of the TT, was used for plasmid linearization by digestion at a unique restriction sites located within *HIS4*. *K. phaffii* GS115 strains sourced from Invitrogen with a deficient copy of the histidine dehydrogenase gene, *his4*, were then used for transformation. A single crossover of linearised plasmid at the *his4* locus is designed to result in a genomic

integration and the conversion from the *his4* to *HIS4* genotype. This will cause a phenotypic shift from being unable to grow on histidine-free media (*his4*) to being able to grow on histidine-free media (*HIS4*). In this way histidine-free solid media plates can be used to screen for transformant colonies.

Generation of *K. phaffii* PpTAmCV708 and PpTAm-TK16 strains

K. phaffii GS115 strains cells were subjected to a standard procedure prior to transformation to ensure their electro-competence. An initial culture was prepared in a 50 mL conical tube with 5 mL Yeast extract peptone dextrose medium (YPD), inoculated with 50 μ L GS115 glycerol stock, and incubated at 30 °C under agitation at 250 rpm. After at least 16 hours of culture, 500 μ L were used to inoculate a second culture prepared with 500 mL YPD in a 2 L flask, and incubated until OD₆₀₀ 1.3-1.5. Cells were harvested through centrifugation at 4,000 rpm for 5 minutes at 4 °C and pellet was resuspended with 500 mL of ice-cold sterile purified water. A further three centrifugation steps were performed under the same conditions; firstly with pellet resuspension in 250 mL of ice-cold sterile purified water, secondly resuspension in 20 mL of ice-cold 1 M sorbitol and finally resuspension in 1 mL of ice-cold sorbitol (1 M). The final volume (1.5 mL) of electro-competent cells was kept on ice ready for transformation. Tips, tubes and any material for these resuspension steps were pre-chilled. To favour homologous recombination with the *K. phaffii* competent cells, plasmids p8TAmCV708 and p8TAm-TK16 were first linearised using unique restriction sites located within the *HIS4* gene. To generate a *K. phaffii* GS115 strain harbouring only a *C. violaceum* CV2025 transaminase transgene, p8TAmCV708 was linearized with *Sal* I restriction site, before transformation to generate the new strain, designated PpTAmCV708. To generate a *K. phaffii* GS115 strain harbouring a transgene encoding both *C. violaceum* CV2025 transaminase and native TK151 transketolase, p8TAm-TK16 was linearized with *Stu* I before transformation to generate the new strain, designated PpTAm-TK16. After linearization digests, plasmids were purified and 5-20 μ g were mixed with 80 μ L competent

K. phaffii GS115 cells into an ice-cold electroporation cuvette. The cuvette with the mixture was incubated on ice for 5 min and then inserted and placed in a Bio-Rad Micropulser Electroporation Apparatus (Biorad), set up at 2.0 kV and 1 pulse. Immediately after, 1 mL of ice-cold 1 M sorbitol was added to the cuvette and the transformation solution spread onto histidine-deficient minimal dextrose (MD) agar plates which were incubated at 30 °C for two days to score the *HIS4* phenotype of resultant colonies. The *K. phaffii* GS115 β -Galactosidase strain, with the methanol utilization phenotype (Mut+), and *K. phaffii* GS115 Human Serum Albumin strain, with the methanol utilization slow phenotype (MutS), were assessed alongside experimental strains for verification of the Mut+ phenotype in transformants. Diagnostic PCR was used to confirm the presence of genome-integrated forms of p8TAmCV708 in strain pTAmCV708 (using primer combinations 5AOX1JH with CV2025REV, and CV2025FWR with JH3AOX1) and p8TAm-TK16 in strain PpTAm-TK16, using primer combinations 5AOX1JH plus CV2025REV, TK151_Mid_FWR plus JH3AOX1 and CV2025FWR plus TK.Sapl.R (primer sequences in Supplementary Material).

Shake flask cultivation

Glycerol was used for cryopreservation of liquid cultures. For cultivation, typically 50 mL of nutrient broth BMGY (1% w/v yeast extract, 2% w/v peptone, 100 mM potassium phosphate buffer pH 6.0, 1.34% Yeast nitrogen base, 0.4 $\mu\text{g mL}^{-1}$ biotin and 1% v/v glycerol), in a 250 mL conical baffled flask, was inoculated with 100 μL of a glycerol stock vial to a typical initial OD_{600} of 0.5. After 16-18 hours of agitation at 250 rpm and 30 °C, cells were harvested by centrifuging at 5,000 rpm for 5 min at room temperature. The supernatant was discarded, pellet washed twice with 100mM potassium phosphate buffer (pH 6.0), and then re-suspended in a 1 L conical baffled flask with 200 mL of BMMY medium (1% w/v yeast extract, 2% w/v peptone, 100 mM potassium phosphate buffer pH 6.0, 1.34% Yeast nitrogen base, 0.4 $\mu\text{g mL}^{-1}$ biotin and 2% v/v methanol), at a typical initial OD_{600} 1.5-2.0. Induction

phase was carried out with BMMY medium for at least 24 hours and up to 5 days, during which methanol evaporation was compensated by addition of pure methanol every 24 hours. For bioconversion reactions using cells cultivated in shake flask, cells were harvested after 48 hours of induction, typically at OD₆₀₀ 15-20. Harvesting was performed by centrifugation at 13,000 rpm for 10 min at 4 °C, the supernatant was removed and the pellet stored at -20 °C. Cell pellets were typically thawed by re-suspension in 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (HEPES) buffer (pH 7.5, 50 mM), in volumes necessary to reconstitute the original OD₆₀₀ or a higher OD₆₀₀.

Bioreactor cultivation

Bioreactor cultivation was performed using a 1 L Infors HT Multifors 1 (Infors AG, Switzerland) device and an Invitrogen regime.³⁹ Cells were cultured at 30 °C, pH 5, and dissolved oxygen (DO) was maintained at 30% via a cascade control system, whereby in order to increase the transference of oxygen, impeller speed rose from an initial 300 rpm to a maximum of 1,100 rpm. When increasing agitation was no longer sufficient to maintain DO level, gas blending was employed to increase the proportion of oxygen in the inlet air to a maximum of 100% oxygen, at a rate of 1 volume of 100% oxygen per volume of medium per min (VVM).

The Invitrogen regime involves three phases. In the first phase 'glycerol batch' growth is employed to maximise the rate of biomass accumulation, as glycerol also acts to repress the AOXI promoter. The bioreactor was filled with 600 mL of sterile fermentation basal salt medium (BSM), which consists of 26.7 mL 85% w/v H₂PO₄, 0.93 g CaSO₄, 18.2 g K₂SO₄, 14.9 g MgSO₄·7H₂O, 4.13 g KOH, and 40.0 g glycerol per litre) and 4.35 mL of trace salts solution, known as Pichia trace salts for media 1 (PTM1), which consists of 6.0 g/L CuSO₄·5H₂O, 0.08 g/L NaI, 3.0 g/L MnSO₄·H₂O, 0.2 g/L Na₂MoO₄·2H₂O, 0.02 g/L H₃BO₃, 0.5 g/L CoCl₂, 20.0 g/L ZnCl₂, 65.0 g/L FeSO₄·7H₂O, 0.2 g/L biotin, and 5.0 mL/L 96% v/v H₂SO₄ per litre of BSM, and inoculated from a seeding culture for an initial OD₆₀₀=1. This

phase was run until a DO spike was observed, indicating the carbon source (glycerol) depletion, approximately 18-20 hours post-inoculation.

The second phase, 'glycerol fed-batch', was initiated when 50% w/v glycerol (supplemented with 12 mL PTM1/L) was added at a rate of 18.15 mL/h per litre of initial fermentation volume. This feed was maintained for 6-8 hours and followed by a brief starvation period of one hour to ensure the total glycerol consumption and thus, to avoid inhibition of the transgene expression.

The final phase, 'induction' was initiated by switching to a pure methanol feed, starting at a feed rate of 3.6 mL/h per litre of initial volume for two hours, to enable cells to adjust to the changed carbon source. Following this, the feed rate was increased to 7.3 mL/h per litre of initial volume during one hour, and finally increased again to 10.9 mL/h per litre and maintained until completing 48 hours of induction phase. Throughout bioreactor cultivation, OD₆₀₀ readings, wet cell weight (WCW) and dried cell weight (DCW) were monitored, and samples were periodically taken for further enzyme activity analysis.

Whole-cell bioconversion by PpTAmCV708 strain after shake-flask cultivation

Bioconversions were performed in 2 mL glass vials with screw cap to avoid evaporation during incubation, in a total reaction volume of 900 µL. During bioconversion reaction and cofactor incubation, temperature was maintained at 30 °C, agitation at 500 rpm in a ThermoMixer C (Eppendorf, UK), and pH was maintained at 7.5, for which all dilutions were prepared using HEPES buffer (50 mM, pH 7.5).

The required mass of frozen pellet material was re-suspended to OD₆₀₀ 1300 in a PLP-buffer (PLP 0.2 mM) in a final volume of 300 µL and incubated for 20 min at 30 °C and 500 rpm in a thermomixer (Eppendorf ThermoMixer® C) for co-factor loading. The reaction was initiated by addition of a 600 µL pH 7.5 solution containing substrates (MBA and ERY), HEPES buffer (pH 7.5, 50 mM) and DMSO to give final substrate concentration as indicated in the results section, and a final DMSO concentration of 4% v/v. Aliquots of 100 µL were taken at several

time intervals during the reaction, quenched with 100 μL 0.1% v/v trifluoroacetic acid (TFA), and then centrifuged for 5 min at 13,000 rpm and 4 $^{\circ}\text{C}$. The supernatant was stored at -20 $^{\circ}\text{C}$ for HPLC analysis.

Whole-cell bioconversion by PpTAmCV708 and PpTAm-TK16 strains after bioreactor cultivation

Transamination reactions by strains PpTAmCV708 and PpTAm-TK16 were performed as with shake flask cultures except for the details that follow. Reactions using PpTAmCV708 cells concentrated to up to 518.06 g DCW L^{-1} (OD_{600} predicted to be 5600), and PpTAm-TK16 cells at up to 553.32 g DCW L^{-1} (OD_{600} predicted to be 5600), were performed with initial cell re-suspension and cofactor loading in a 600 μL reaction volume for 20 min at 30 $^{\circ}\text{C}$ and 500 rpm in a thermomixer. This reaction mixture was then centrifuged, supernatant removed and cell pellet re-suspended to a final volume of 900 μL in substrate (MBA and ERY), HEPES buffer (pH 7.5, 50 mM) and DMSO, with 0.2 mM PLP to match the PLP concentration present in shake flask reactions.

For measuring the activity of the *de novo* pathway in strain PpTAm-TK16, cell pellet material was initially re-suspended in 160 μL of HEPES buffer (pH 7.5, 50 mM), with indicated concentration of HPA and GA and 2.4 mM TPP, and incubated for two hours at 30 $^{\circ}\text{C}$ and 500 rpm agitation in a thermomixer. The transaminase reaction was then initiated by addition of 555 μL of MBA-buffer to a final concentration of 150 mM, and 0.2 mM PLP before a further two hour incubation at 30 $^{\circ}\text{C}$ and 500 rpm agitation in a thermomixer. Prior to mixing with cells, the pH of the substrate-containing buffers was adjusted to 7.5 by addition of NaOH or HCl as required. Aliquots of 100 μL were taken at several time intervals during the reaction and processed for HPLC analysis in the same way as shake flask samples.

HPLC detection methods

MBA, ACP and ABT levels were analysed using a Dionex Ultimate 3000 HPLC system (Camberley, UK) with an ACE 5 C18 reverse phase column (150 mm x 4.6 mm, 5 μm particle

size), controlled by Chromeleon client 7.0 software (Advance Chromatography Technologies, UK). For MBA and ACP detection, stored samples from bioconversion were thawed, centrifuged for 15 min at 4 °C and 13,000 rpm, and mixed with TFA 0.1% v/v to a final 20-fold dilution, and transferred to an HPLC vial for MBA and ACP detection. The HPLC program was run at 30 °C, employing 0.1% TFA as mobile phase A and 100% acetonitrile as mobile phase B. The program consisted of a gradient of 85% to 28% solvent A for 9 min (flow rate 1 mL/min), after which, the system returns to the initial mobile phase for re-equilibration of the column for 2 min before the next sample. ACP detection occurred after 7.8 min at 260 nm, and MBA detection after 3.6 min at the same wavelength.

For ABT detection, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) was used as derivatization agent. In AQC derivatisation⁴⁰ the nitrogen of amine group in the target compound forms a single covalent bond with the carbon atom present in the amino group within 6-amino-quinoline. AQC was synthesised by Dr Fabiana Subrizi at UCL Department of Chemistry using the standard Cohen and Michaud⁴¹ method, and prepared for derivatization by solubilisation in dry acetonitrile to a concentration of 10 mg/mL. Stored samples from bioconversion reactions were thawed, centrifuged for 15 min at 4 °C and 13,000 rpm, and diluted 40-fold with purified water from an Elix 5 water purification system (Millipore, France). 40 µL aliquots were taken and derivatized with 80 µL AQC, followed by addition of 120 µL of 0.2 M borate buffer pH 8.8. The mixture was centrifuged for 5 min at 4 °C and 13,000 rpm, and then transferred to an HPLC vial. For ABT detection the solvent program employed 140 mM sodium acetate (pH 5.05) as mobile phase A, and 100% acetonitrile as mobile phase B. The program used a gradient of 85% to 100% solvent A for 10 min (flow rate 0.5 mL/min), followed by a 2.5 min wash phase at 40% solvent A (flow rate 1 mL/min) and 2.5 min of 100% solvent A (flow rate 1 mL/min). A re-equilibration phase was run with 85% solvent A for 5.0 min before the next sample was applied. The ABT detection peak was at 254 nm after 6.1 min.

Biomass concentration measurement

For growth monitoring, samples were taken during incubation, and biomass concentration was measured as optical density at 600 nm (OD_{600}) in an Aquarius spectrophotometer (CECIL Elegant Technology, UK). For determining DCW, 1 mL samples were taken into pre-weighted micro-centrifuge tubes and centrifuged at 13,000 rpm (Eppendorf 5415R centrifuge, Eppendorf UK, Ltd, Cambridge, UK). Supernatant was removed and the weight of the harvested pellet recorded as WCW. The cell pellet was then dried for three days in a pre-weighted tube using a Memmert Modell 400 (Memmert GMBH, Schwabach, Germany) oven at 100 °C. Final pellet weight was recorded as DCW once it stabilised, using a Mettler AT261 analytical balance.

Results and Discussion

Establishing transaminase activity in engineered K. phaffii. We previously demonstrated that insertion of a transgene encoding native transketolase under the control of the strong AOX1 promoter in *K. phaffii* strain GS115 can provide a whole cell biocatalyst tolerant of high substrate concentrations and capable of high levels of production of ERY, up to 46.58 g L⁻¹ h⁻¹ space-time yield, when fed 1.5 M HPA and 1.5 M GA as prochiral substrates.¹³ In this study we tested if addition of a transgene encoding the *C. violaceum* transaminase CV2025 under the control of the strong AOX1 promoter in *K. phaffii* strain GS115 could also result in an active whole cell biocatalyst. We first designed a version of the *C. violaceum* transaminase CV2025 open reading frame (ORF) open reading frame (ORF) codon-optimized for *K. phaffii* and sub-cloned it into the pAO815 expression plasmid. This plasmid was transformed into strain GS115. Four colonies were tested by colony PCR for correct insertion of the integrative plasmids. Cultivation in shake flasks showed similar growth performance. No further analysis of transformants was performed with respect to

transgene dosage, location or expression level. One clone was then chosen at random for further study and designate as strain PpTAmCV708.

To test if methanol-inducible, whole-cell transaminase activity (Figure 1, Step 2) was present in strain PpTAmCV708, we first cultivated cells in shake flasks in the presence or absence of methanol (Figure 2). Cell suspensions were incubated with 10 mM MBA only with no additional ERY added. Disappearance of MBA and appearance of ACP was then monitored over a 7 hour reaction duration (Figure 2A-B). Methanol-inducible transaminase activity in these reactions would indicate that the AOXI expression cassettes were functioning as intended and that either a natural pool of ERY, or an alternative abundant cellular ketone, such as pyruvate,⁴² was being utilised as substrate.

Significant MBA disappearance was observed only when methanol was present in the growth media for PpTAmCV708 cells (Figure 2A). MBA measurements fluctuated for the unmodified parental strain, GS115, in the presence or absence of methanol with a general trend of little or no loss. Fluctuation in the MBA levels was also observed for strain PpTAmCV708 in the absence of methanol. This variation of up to 8.3% increase and 16% decrease from the starting MBA concentration may have been due to some interference with the assay due to ingress and egress of MBA from the whole cells present or components of the complex media used for shake flask cultivation. As this MBA variability was not observed in subsequent experiments using cells cultivated with chemically defined media in a bioreactor, this phenomenon was not investigated further.

ACP accumulation was observed in the presence of methanol-induced PpTAmCV708 cells (Figure 2B). The parental strain, GS115, caused no ACP production, in the presence or absence of methanol. For un-induced PpTAmCV708, despite little MBA disappearance being observed (Figure 2A), ACP production was measured (Figure 2B). This may be due to leakiness in the AOXI promoter and utilisation of alternative sources of MBA available from cells or the complex growth medium.

We next fed cells with 30 mM ERY in addition to 10 mM MBA. The PpTAmCV708 strain showed conversion of approximately 50% of the MBA to ACP over 2 hours, even when un-induced (Figure 2C). In light of the activity observed in Figure 2B, we attributed this observation to leakiness of the AOXI promoter. When methanol-induction was provided, conversion of MBA to ACP completed within the first 30 minutes of the 2-hour reaction period (Figure 2D).

Bioreactor cultivation of strains to high cell density. We concluded from the observations in Figure 2 that the genetic modification steps we had taken in engineering strain PpTAmCV708 had resulted in the intended transaminase whole cell biocatalytic activity. Wei *et al.*¹³ had previously shown that transketolase whole cell biocatalytic activity could be achieved by insertion of a native transketolase transgene into *K. phaffii* strain GS115. Informed by these observations, we constructed plasmid p8TAm-TK16, which encodes AOXI promoter-based cassettes for the native transketolase ORF plus the *C. violaceum* CV2025 transaminase ORF, and used it to generate strain PpTAm-TK16, which we intended to exhibit both transketolase and transaminase whole cell biocatalytic activity. Clones of strains PpTAm-TK16 and PpTAmCV708 were selected only based on rapid cell growth and no other metric. Studies involving analysis, or even matching, of transgene locus, copy number and expression across the strain pair, were outside the scope of this investigation.

Both PpTAmCV708 (Figure 3A) and PpTAm-TK16 (Figure 3B) strains were grown to high cell density in a 1 L bioreactor following a commercial protocol. Briefly, a batch of glycerol was provided as carbon source and completely consumed to achieve an initial increase in biomass followed by fed-batch cultivation, with methanol as the growth-limiting carbon source. This protocol has been empirically developed to maximise activity of the AOXI promoter.⁴³ Both strains achieved high cell density (Figure 3), each of a level within 20% of the other, which is within the typical variation observed between bioreactor runs. Both strains

achieved $g\ DCW\ L^{-1}$ biomass level, at 75 hours post-inoculation, of approximately $140\ g/L$, which was comparable to that observed by Wei *et al.*¹³ for their strain that overexpressed native transketolase only.

Biocatalytic activity of high density PpTAm-TK16 whole cells fed GA, HPA and MBA.

Feeding strain PpTAm-TK16 cells with GA, HPA and MBA should be sufficient for production of ACP and ABT if the transketolase and transaminase were functioning as a two-step *de novo* pathway. However, when GA, HPA and MBA were fed as substrates, multiple HPLC peaks were observed upon AQC-derivatization of ABT, preventing its quantification. 1-fluoro-2,4-dinitrobenzene (DNFB) was used as an alternative derivatization agent following the protocol described by Cardenas-Fernandez *et al.*,⁴⁴ but peak multiplication was still observed. We suggest the observed peak multiplication was related only to the presence of GA and HPA resulting in derivatization species that obscured the ABT peak, but further work will be required to resolve this. When GA and HPA were absent from reactions AQC-derivatization of ABT produced a clear singlet.

Due to the putative substrate interference with the ABT assay, we measured only ACP production from PpTAm-TK16 cells fed with GA, HPA and MBA. Whole cells were used in reactions either at their original density (Figure 3B) or concentrated to $285.36\ g\ DCW\ L^{-1}$ or $553.32\ g\ DCW\ L^{-1}$ (Figure 4A). ACP production levels were then followed as a function of the concentration of GA and HPA added to the reaction mixture. As no ERY was added to the reaction mixtures, the only source of ERY would be that produced due to the presence of the transketolase transgene, plus any inherent cellular ERY pool. Using a PpTAm-TK16 whole cell density of $132.72\ g\ DCW\ L^{-1}$ yielded up to $6\ mM$ ACP (fed with $50\ mM$ HPA and GA). At a cell density to $285.36\ g\ DCW\ L^{-1}$, and using $200\ mM$ HPA and GA, the maximum ACP yield increased to $9.53\ mM$ (see also time course in Figure 4B) but a further cell density increase to $553.32\ g\ DCW\ L^{-1}$ increased ACP yield only to $10.17\ mM$. This suggested that

there was an upper limit to the cell density that could be usefully applied to increase the intensity of the whole cell biocatalysis.

For bioreactor-derived cells, MBA was provided at 150 mM to demonstrate activity in the presence of high concentration of feed chemicals and to drive the reversible reaction to reach a higher yield of the desired aminated product (Figure 4). This, however, resulted in lower percentage conversion of MBA to products than had been observed for shake flask-derived cells (Figure 2). As such it seems likely the 150 mM is in excess of the maximum concentration the cells can convert. Further optimisation in future work will be required to identify conditions that maximise activity of high density, whole cell biocatalysis reactions with the strains engineered in this study.

Biocatalytic activity of high density PpTAm-TK16 and PpTAmCV708 whole cells fed MBA, ERY or both. The assay interference caused by GA and HPA meant that, although we could not directly measure ABT production from the two-step *de novo* pathway in PpTAm-TK16 cells, we could cautiously interpret ACP production (Figure 4A-B) as a proxy for ABT production. Furthermore, we were encouraged by our Figure 2B observation that strain PpTAmCV70 whole cells produced ACP when fed only MBA with no additionally fed ketone. We hypothesised that an alternative intracellular pool of ketone or keto acid, likely to be pyruvate, may be a viable input into the pathway for efficient production of ABT.

To test if PpTAm-TK16 and PpTAmCV708 whole cells at high density could also produce ABT while fed only MBA and no ketone, we used 268.02 g DCW L⁻¹ and 285.36 g DCW L⁻¹ respectively for each strain, in reactions with both, or one, of 150 mM MBA and 100 mM ERY. When 150 mM MBA was absent and only 100 mM ERY provided, no ACP (Figure 4C, 4D), or ABT (Figure 4E, 4F) was produced by either strain, indicating no alternative pool of MBA was available within cells or buffer.

PpTAm-TK16 whole cells produced 11.4 mM ACP (Figure 4C) and 10.4 mM ABT (Figure 4D) when both 150 mM MBA and 100 mM ERY were present in the reaction. Approximately

30% of those levels of ACP and ABT production occurred even when only 150 mM MBA and no ERY was provided (Figure 4C, 4D). The observation that ACP production by strain PpTAm-TK16 whole cells was higher, 9.5 mM ACP (Figure 4A), when cells were fed GA, HPA and MBA than when they were fed MBA only, 3.2 mM ACP (Figure 4C), was consistent with the two-step, *de novo* pathway functioning as intended in these cells.

Strain PpTAmCV708 whole cells produced 20.3 mM ACP (Figure 4E) and 22.9 mM ABT (Figure 4F) when fed 150 mM MBA and 100 mM ERY, with approximately 50% of the ACP ABT levels maintained even when MBA and no ERY was provided as substrate (Figure 4E, 4F). With respect to yield performance, strain PpTAmCV708 produced 22.9 mM ABT, approximately double the 10.4 mM ABT yield of strain PpTAm-TK16, when both strains were fed MBA and ERY (compare Figure 4D and 4F).

Strain performance against whole cell biocatalysis metrics

Schrewe *et al.*³ proposed a set of performance metrics for useful comparison and characterisation of whole cell biocatalysts. Table 1 provides a selection of Schrewe metrics for the engineered *K. phaffii* strains cultivated in a 1 L bioreactor, after 48 hour of induction, followed by concentration to 268.02 g DCW L⁻¹ for strain PpTAmCV708 and 285.36 g DCW L⁻¹ for strain PpTAm-TK16.

To test the activity of the two step *de novo* pathway in strain PpTAm-TK16 whole cells, we fed cells with the transketolase substrates GA and HPA only MBA for the anticipated transaminase step (Table 1, reaction A). Due to the constraint of being unable to directly measure ABT production in this reaction mixture, we used the ACP yield for comparing the biocatalytic performance of PpTAm-TK16 to equivalently engineered *E. coli* cells reported by others such as Ingram *et al.*¹¹ who reported no interference when measuring ABT via AQC-derivatisation in *E. coli* growth media. We suggest it is not unreasonable to use ACP as a proxy in this way as ABT production was approximately equimolar to ACP production for both strains across reactions B to I (Table 1).

The resultant $0.29 \text{ g L}^{-1} \text{ h}^{-1}$ space-time yield (STY) of ACP achieved by PpTAm-TK16 whole cells represented a 49-fold increase of the highest ACP levels reported by Ingram *et al.*¹¹ for *E. coli* whole cells harbouring the equivalent pathway and fed the same substrates. Rios-Solis *et al.*³⁵ reported the activity of *E. coli* whole cells harbouring the equivalent transketolase-transaminase pathway but fed alternative substrates that yielded isopropylamine (IPA) as a by-product. The ACP STY of $0.29 \text{ g L}^{-1} \text{ h}^{-1}$ we observed with *K. phaffii* strain PpTAm-TK16 represented 688% of the IPA STY reported by Rios-Solis *et al.*³⁵ when *E. coli* whole cells were fed glycolaldehyde (GA) and hydroxypyruvate (HPA), and 318% of the IPA STY achieved by *E. coli* fed propionaldehyde as an alternative to GA. Notably, strain PpTAmCV708 still yielded 10.2 mM ABT when fed only MBA and no other substrates (Table 1, reaction I). This yield was comparable to the 10.4 mM ABT yield achieved by strain PpTAm-TK16 when both MBA and ERY were fed (Table 1, reaction C). The ABT STY of $0.62 \text{ g L}^{-1} \text{ h}^{-1}$ achieved when feeding PpTAmCV708 only one substrate represents a 105-fold increase of that reported by Ingram *et al.*¹¹ for *E. coli* whole cells harbouring the equivalent pathway and fed three substrates. These data are particularly encouraging in light of the fact that the reaction conditions were intended for proof of principle only, so were non-optimised, and the host cells have been selected only on the basis of growth performance and not transgene expression or dosage. This suggests *K. phaffii* is a promising host organism for whole cell biocatalysis.

Conclusions

Transketolase and transaminase overexpression in whole cell biocatalysts represent an effective biocatalytic tool for production of CAAs (Figure 1). In this report we observed that an engineered *K. phaffii* GS115 strain, PpTAmCV708, harbouring a *C. violaceum* transaminase CV2025 transgene, could convert MBA to ACP, whereas the parental *K. phaffii* GS115 strain was unable to do so (Figure 2). We concluded from these observations

that strain PpTAmCV708 possessed whole cell transaminase activity due to the presence of the transgene. A second strain, PpTAm-TK16, encoding transgenes for *C. violaceum* transaminase CV2025 plus a native transketolase, was also generated and showed similar growth performance to PpTAmCV708 when cultivated at high cell density in a bioreactor (Figure 3). We conclude from this that the presence of two transgenes in strain PpTAm-TK16 has not compromised the industrial growth performance of the strain relative to the single-transgene strain, PpTAmCV708.

Strain PpTAm-TK16 produced 3.2 mM ACP when fed only MBA, but up to 10.17 mM ACP when GA and HPA were co-fed alongside MBA (Figure 4). This observation was consistent with enhanced transketolase activity within the PpTAm-TK16 whole cells. We have demonstrated that the ability of *K. phaffii* to grow to high cell densities can be exploited to intensify whole cell biocatalysis. This is evidenced by the fact that even the lowest level of performance recorded in this study (Table 1) exceeded performance levels reported for comparable *E. coli* whole cell biocatalysts.

The strain construction and activity data reported here provide a baseline study for future investigations into exploiting *K. phaffii* as a host cell biocatalyst. Factors that could further intensify *K. phaffii* whole cell performance include screening for novel low cost substrates, feedstocks and reaction configurations and also cell design factors such as transgene dosage, location and expression level.

The strains PpTAm-TK16 and PpTAmCV708 represent a toolset of whole cell transaminase activity compatible with three different substrate feeding regimes: MBA alone, MBA with ERY or MBA with GA plus HPA (Table 1). This flexibility with respect to substrate feeding could provide valuable process options for responding to changes in the cost profile of different substrates.

Figure 1. Proposed native transketolase:*Chromobacterium violaceum* ω -transaminase *de novo* pathway in *K. phaffii*. In this route Step 1 is catalysed by the native TK (Genbank: CAY67980.1) transferring a ketol group from hydroxypyruvate (HPA) to glycoaldehyde (GA) to produce L-erythrulose (ERY) and carbon dioxide. Step 2 is catalysed by TAm CV2025 and involves the transference of an amino group from (S)- α -methylbenzylamine (MBA) to ERY, previously generated in the first reaction, to form the chiral product 2-amino-1,3,4-butanetriol (ABT). Adapted from Ingram *et al.*¹¹

Figure 2. Transaminase activity of engineered strain PpTAmCV708 cultivated in shakes flasks. Cells of PpTAmCV708 and the unmodified GS115 parental strain were cultivated in shake flasks in the presence (induced) or absence (uninduced) of methanol for 48 hours. Cells were then pelleted by centrifugation, re-suspended at an $OD_{600}=1300$ for bioconversion reactions of seven hours with 10 mM (S)- α -methylbenzylamine (MBA) and 0 mM L-erythrulose (ERY). Levels of (A), MBA, and (B), acetophenone (ACP), were then monitored by HPLC. Key on the right of plot B applies to plots A and B). Black open square symbols indicate data points for induced parental strain GS115 and grey triangles uninduced parental strain GS115. Grey open circles symbols indicate data points for induced strain PpTAmCV708 and grey open diamonds uninduced strain PpTAmCV708. For bioconversion reactions 10 mM MBA and 30 mM ERY were provided. Levels of MBA and ACP during a 2-hour reaction period were then plotted for un-induced (C) and induced (D) strain PpTAmCV708. Error bars, indicating standard deviation between two biological repeats, did not exceed the area covered by the symbol for each data set. Key on the right of plot D applies to plots C-D. Black open square symbols indicate data points for ACP levels. Black open circles indicate data points for MBA levels.

Figure 3. Cultivation of engineered strains to high cell density in a 1 L bioreactor.

Engineered strains, PpTAmCV708 (**A**) and PpTAm-TK16 (**B**) were cultivated for a period of 75 hours in a 1 L bioreactor, with glycerol as carbon source for the first 27 hours followed by methanol feeding for the remaining 48 hours. Dry cell weight (DCW) was determined for each monitoring sample. Bioreactor cultivation was performed in three phases: Phase 1, a glycerol batch carried out during approximately 18-20 h until a dissolved oxygen spike was detected; Phase 2, a glycerol fed-batch carried out feeding glycerol (50% w/v) for approximately 6-8 h at 18.15 mL/h per L; and Phase 3, a methanol fed-batch carried out feeding methanol at 3.6 mL/h per L for 2 h, then at 7.3 mL/h per L for 1 h, and finally at 10.9 mL/h per L maintained to the end of the fermentation. PpTAmCV708 (**A**) was harvested at 0.145 g DCW mL⁻¹ (145.50 g DCW L⁻¹) and PpTAm-TK16 (**B**) at 0.132 g DCW mL⁻¹ (132.72 g DCW L⁻¹). Error bars indicate standard deviation between two experimental repeats.

Figure 4. Transaminase and transketolase activity of engineered strains

PpTAmCV708 and PpTAm-TK16 cultivated in 1 L bioreactors. For each strain, cells were cultivated in a 1 L bioreactor and harvested at the final time point, as indicated in Figure 3. Strain PpTAm-TK16 was fed (**A**) with 150mM (S)- α -methylbenzylamine (MBA) plus glycoaldehyde (GA) and hydroxypyruvate (HPA) each at the concentration indicated in the X axis, with cells either at their original concentration at harvest, 132.72 g DCW L⁻¹ (triangles), or after concentration to 285.36 g DCW L⁻¹ (circles) or 553.32 g DCW L⁻¹ (squares). The two-hour reaction profile of a single reaction at 285.36 g DCW L⁻¹ is plotted in graph **B**, with substrate concentrations as indicated in the key next to graph **B**. Error bars indicate standard deviation between two biological repeats of the experiment. For strain PpTAm-TK16 used at 285.36 g DCW L⁻¹, 150 mM MBA and 100 mM L-erythrulose (ERY) were each individually present or omitted from reaction mixtures and resultant (**C**) acetophenone (ACP), and (**D**) 2-amino-1,3,4-butanetriol (ABT) concentration plotted during a 2-hour reaction period.

Resultant ACP (**E**) and ABT (**F**) were also plotted for strain PpTAmCV708 used at 268.02 g DCW L⁻¹ with the same substrate combinations. Key on the right of plot **E** applies to plots **C-F**.

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Table 1. Schrewe metrics of whole-cell biocatalyst performance. Performance of five best-performing reactions identified in this study (**A-E**) for *K. phaffii* strains PpTAmCV708 and PpTAm-TK16 cells cultivated in a 1 L bioreactor, after 48 hour of induction followed by concentration to 268.02 g dry cell weight (DCW) L⁻¹ for PpTAmCV708 and 285.36 g DCW L⁻¹ for PpTAm-TK16. MW acetophenone (ACP) =120.15, MW 2-amino-1,3,4-butanetriol (ABT) =121.135. The following feed chemicals were provided in reaction mixtures: hydroxypyruvate (HPA), glycoaldehyde (GA), L-erythrulose (ERY) and (S)- α -methylbenzylamine (MBA)

REACTI ON VALUES	STRAIN								
	PpTAm -TK16	PpTAm-TK16		PpTAm-TK16		PpTAMCV708		PpTAMCV708	
[GA]	200 mM	0 mM		0 mM		0 mM		0 mM	
[HPA]	200 mM	0 mM		0 mM		0 mM		0 mM	
[MBA]	150 mM	150 mM		150 mM		150 mM		150 mM	
[ERY]	0 mM	100 mM		0 mM		100 mM		0 mM	
DCW in 900 μ L reaction	33.1 g/L	99.15 g/L		99.15 g/L		90.8 g/L		90.8 g/L	
Time of reaction (h)	4 h	2 h		2 h		2 h		2 h	
PRODUC TS	ACP	ACP	ABT	ACP	ABT	ACP	ABT	ACP	ABT
[Product] after hours of complete reaction	9.5 mM 1.14 g L^{-1}	11.4 mM 1.37 g L^{-1}	10.4 mM 1.26 g L^{-1}	3.2 mM 0.38 g L^{-1}	2.8 mM 0.34 g L^{-1}	20.3 mM 2.44 g L^{-1}	22.9 mM 2.77 g L^{-1}	9.3 mM 1.12 g L^{-1}	10.2 mM 1.24 g L^{-1}
μ M product per min	39.58 μ M min ⁻¹	95 μ M min ⁻¹	86.67 μ M min ⁻¹	26.67 μ M min ⁻¹	23.33 μ M min ⁻¹	169.17 μ M min ⁻¹	190.83 μ M min ⁻¹	77.5 μ M min ⁻¹	85.0 μ M min ⁻¹
STY ($g_{product} L^{-1} h^{-1}$)	0.29 ($g_{product} L^{-1} h^{-1}$)	0.69 ($g_{product} L^{-1} h^{-1}$)	0.63 ($g_{product} L^{-1} h^{-1}$)	0.19 ($g_{product} L^{-1} h^{-1}$)	0.17 ($g_{product} L^{-1} h^{-1}$)	1.22 ($g_{product} L^{-1} h^{-1}$)	1.39 ($g_{product} L^{-1} h^{-1}$)	0.56 ($g_{product} L^{-1} h^{-1}$)	0.62 ($g_{product} L^{-1} h^{-1}$)
Specific activity (U g_{bcw}^{-1}) (U = μ mol min ⁻¹)	1.20 U g_{bcw}^{-1}	0.96 U g_{bcw}^{-1}	0.87 U g_{bcw}^{-1}	0.27 U g_{bcw}^{-1}	0.24 U g_{bcw}^{-1}	1.86 U g_{bcw}^{-1}	2.10 U g_{bcw}^{-1}	0.85 U g_{bcw}^{-1}	0.94 U g_{bcw}^{-1}

$Y_{p/s}$ ($\frac{\text{mol}_{\text{product}}}{\text{mol}_{\text{substrate}}}$ $^{-1}$)	0.06 mol mol ⁻¹	0.11 mol mol ⁻¹	0.10 mol mol ⁻¹	0.021 mol mol ⁻¹	0.018 mol mol ⁻¹	0.20 mol mol ⁻¹	0.23 mol mol ⁻¹	0.062 mol mol ⁻¹	0.068 mol mol ⁻¹
$Y_{p/x}$ ($\frac{\text{g}_{\text{product}}}{\text{g}_{\text{DCW}}}$ $^{-1}$)	34.44 mg g _{DCW} ⁻¹	13.82 mg g _{DCW} ⁻¹	12.71 mg g _{DCW} ⁻¹	3.83 mg g _{DCW} ⁻¹	3.43 mg g _{DCW} ⁻¹	26.87 mg g _{DCW} ⁻¹	30.51 mg g _{DCW} ⁻¹	12.33 mg g _{DCW} ⁻¹	13.66 mg g _{DCW} ⁻¹
	A	B	C	D	E	F	G	H	I

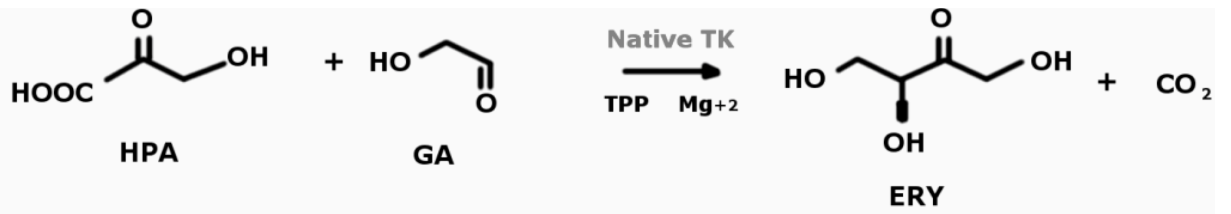
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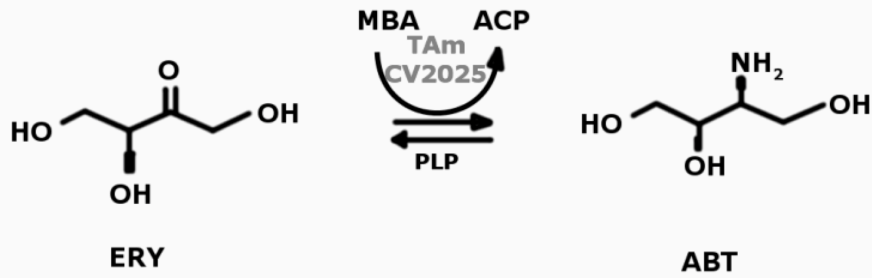
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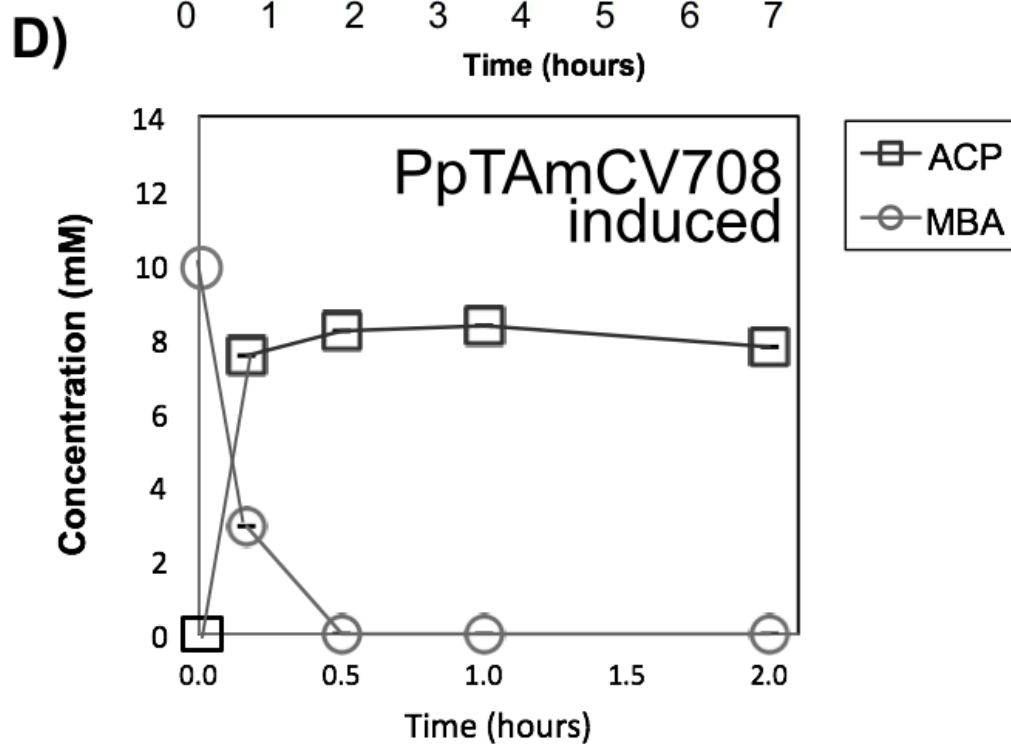
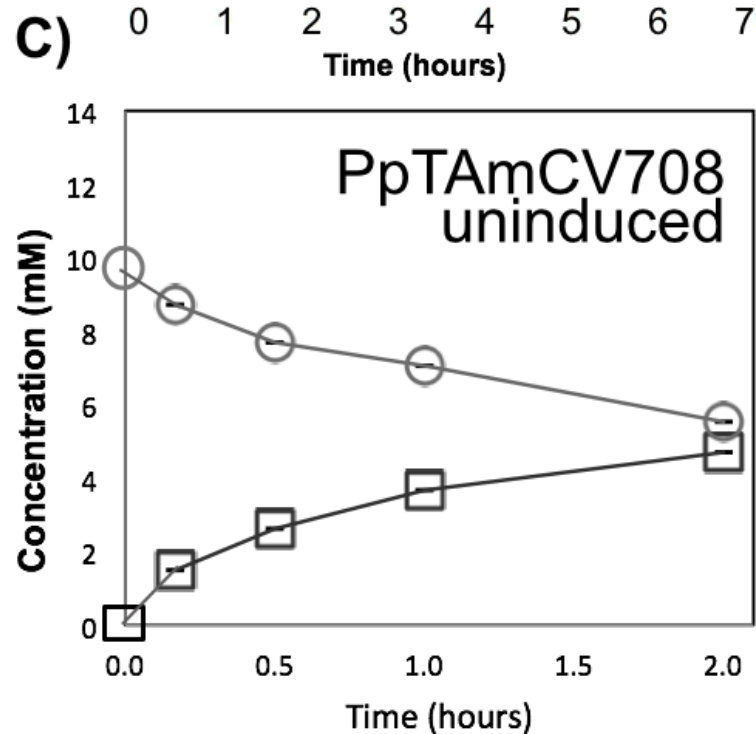
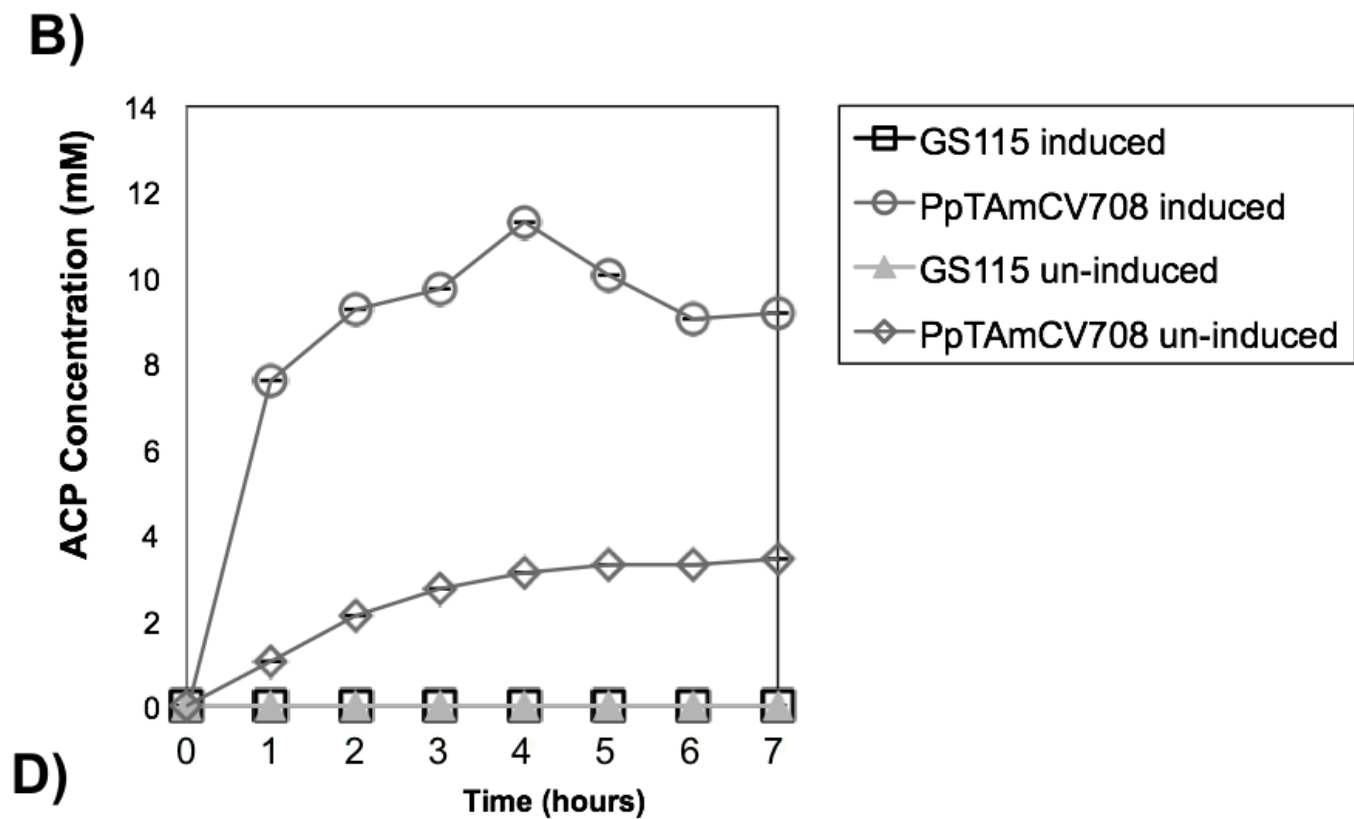
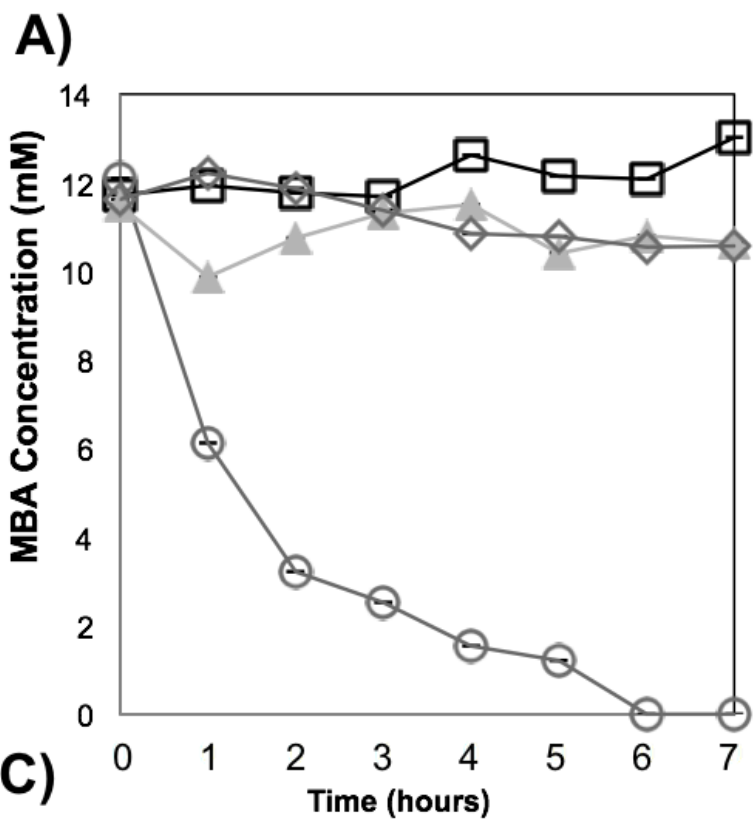
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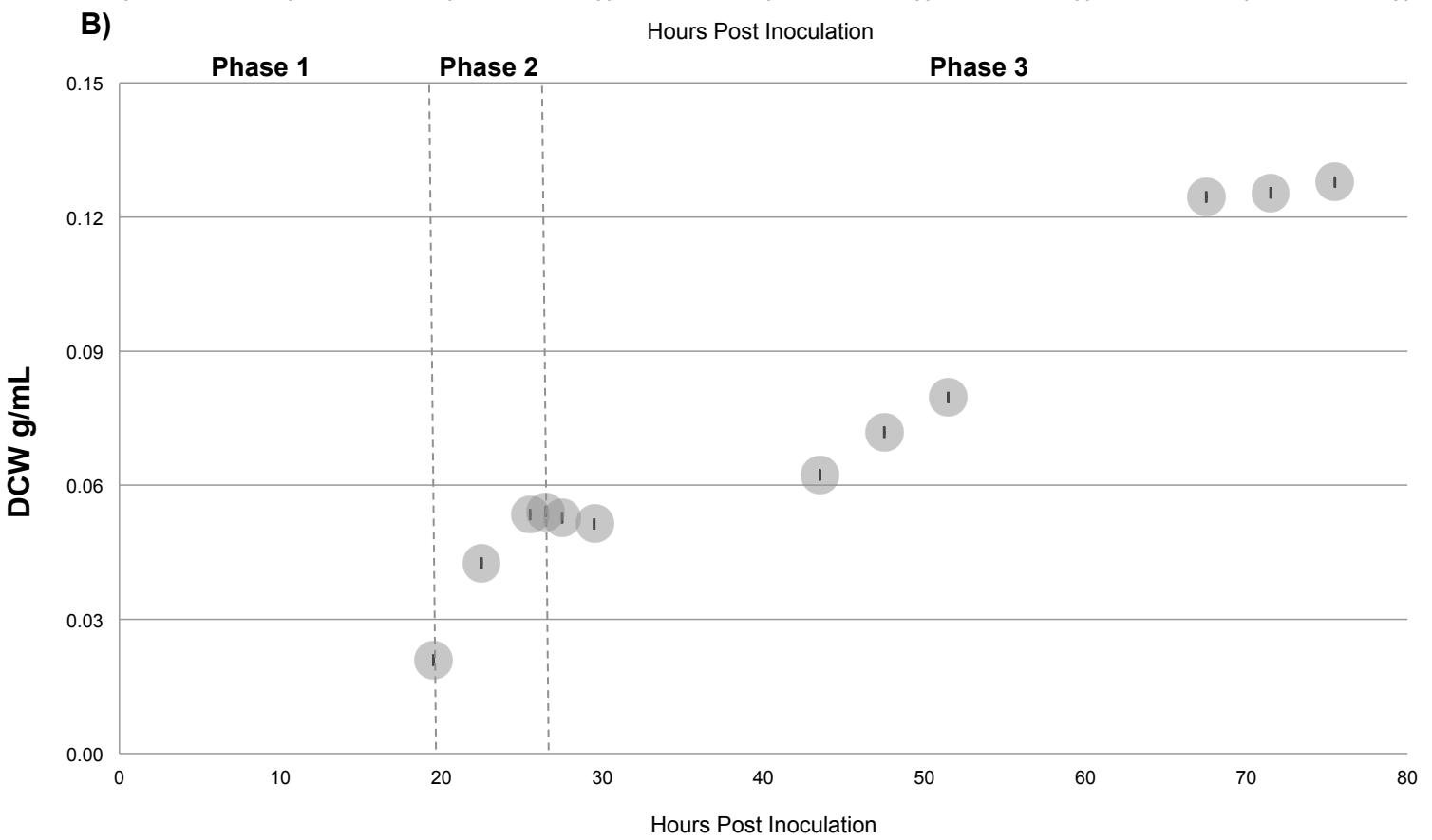
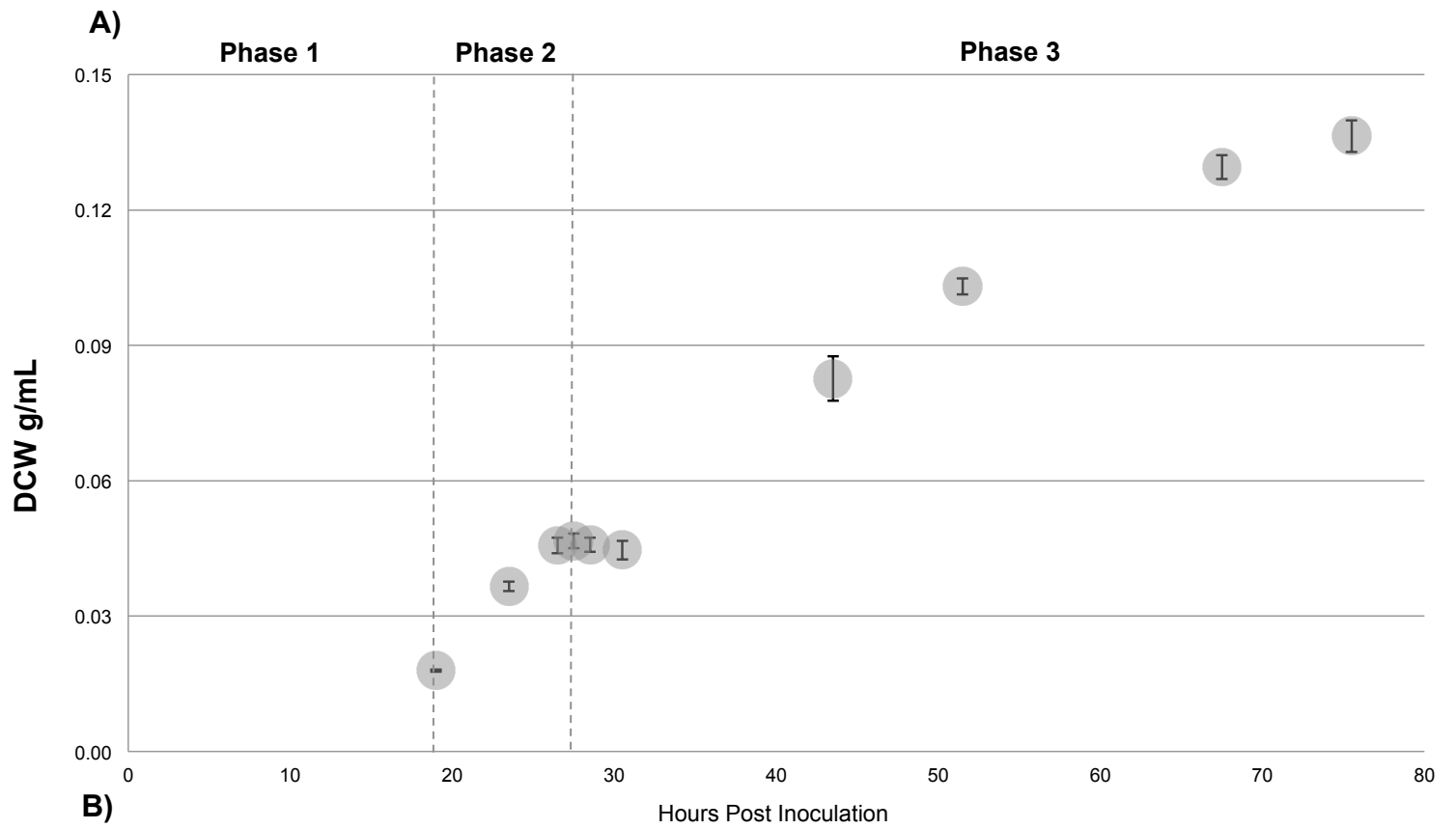
**Step 1:
TK reaction**

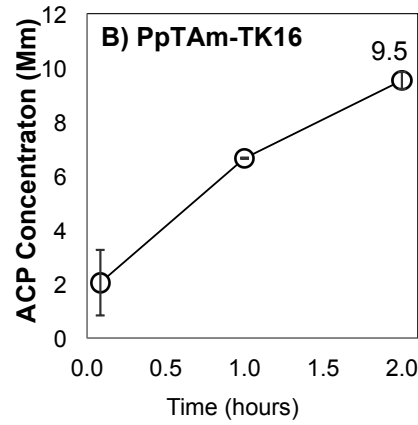
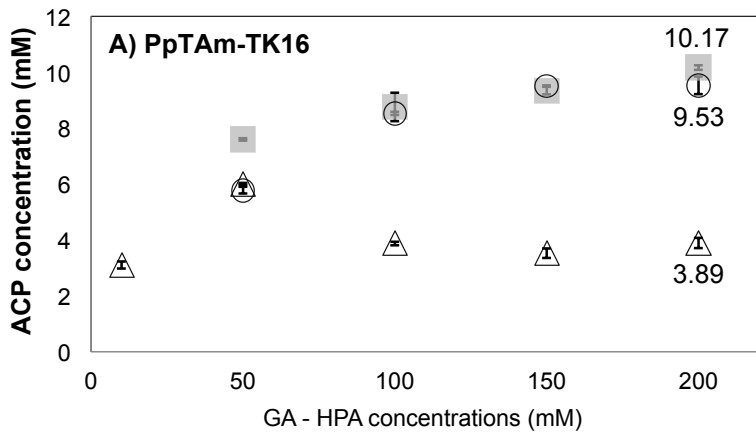


**Step 2:
TAm reaction**

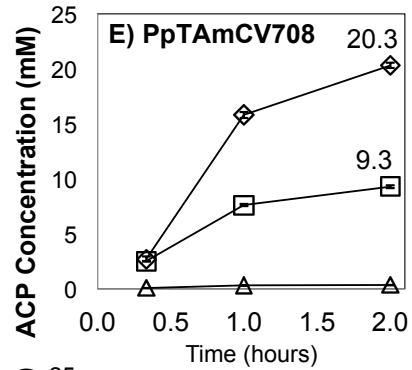
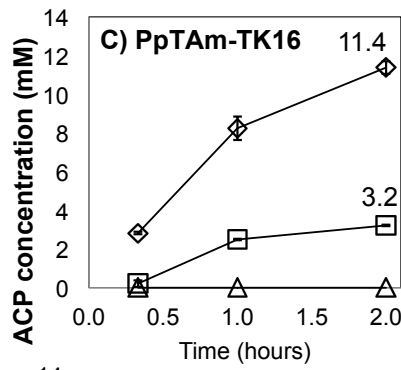








GA = 200 mM
HPA = 200 mM
MBA = 150 mM



△ MBA = 0 mM
ERY = 100 mM
□ MBA = 150 mM
ERY = 0 mM
◇ MBA = 150 mM
ERY = 100 mM

