A toolbox approach for the rapid evaluation of multi-step enzymatic syntheses comprising a 'mix and match' *E. coli* expression system with microscale experimentation‡.

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Keywords : transketolase, transaminase, biocatalyst, multi-step enzymatic synthesis, chiral amino alcohols, *de novo* pathway

‡ Manuscript prepared for Biocatalysis and Biotransformation (submitted November 2010).

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ABSTRACT

This work describes an experimental 'toolbox' for the rapid evaluation and optimisation of multi-step enzymatic syntheses comprising a 'mix and match' *E. coli*-based expression system and automated microwell scale experimentation. The approach is illustrated with a *de novo* designed pathway for the synthesis of optically pure amino alcohols using the enzymes transketolase (TK) and transaminase (TAm) to catalyze asymmetric carbon-carbon bond formation and selective chiral amine group addition respectively. The *E. coli* expression system, based on two compatible plasmids, enables pairs of enzymes from previously engineered and cloned TK and TAm libraries to be evaluated for the sequential conversion of different initial substrates. This is complemented by the microwell experimentation which enables efficient investigation of different biocatalyst forms, use of different amine donors and substrate feeding strategies.

Using this experimental 'toolbox', one-pot syntheses of the diastereoisomers (2*S*,3*S*)-2-aminopentane-1,3-diol (APD) and (2*S*,3*R*)-2-amino-1,3,4-butanetriol (ABT) were designed and performed which gave final product yields of 90% mol/mol for APD and 87% mol/mol for ABT (relative to the initial TK substrates) within 25 hours. For the synthesis of APD, the TK mutant D469E was paired with the TAm from *Chromobacterium violaceum* 2025 while for ABT synthesis the wild-type *E. coli* TK exhibited the highest specific activity and *ee* of >95%. For both reactions, whole-cell forms of the TK-TAm biocatalyst performed better than cell lysates while isopropylamine (IPA) was a preferable amine donor than methylbenzylamine (MBA) since side reactions with the initial TK substrates were avoided. The available

libraries of TK and TAm enzymes and scalable nature of the microwell data suggest this ‘toolbox’ approach provides an efficient approach to early stage bioconversion process design in the chemical and pharmaceutical sectors.

1. INTRODUCTION

Biological catalysts have repeatedly demonstrated their usefulness for chemical organic synthesis due to their moderate reaction conditions, reduced environmental impact and high regio- and stereo selectivity (Whitesides and Wong, 2003). This has led to the successful investigation of multi-step, metabolically engineered pathways toward application as pharmaceutical production platforms (Wilkinson and Bachmann, 2006). It is now possible to design *de novo* non-native pathways in heterologous hosts, to carry out specific non-natural bioconversions producing chiral compounds difficult to obtain by existing biosynthetic pathways or chemical synthesis (Lye *et al.*, 2002; Burkart, 2003; Roessner and Scott, 2003; Prather and Collin, 2008; Dalby *et al.*, 2009). These studies have led to the rapid expansion of molecular pathway construction for the synthesis of a range of commodity fine chemicals and pharmaceutical intermediates (Wilkinson and Bachmann, 2006).

The *de novo* design of synthetic pathways has recently been applied for the sequential two-step reaction of carbon-carbon bond formation using a transketolase (TK) enzyme, followed by a transaminase (TAm) to create chiral amino alcohols from achiral substrates (Ingram *et al.*, 2007). The aminodiol moiety is a useful industrial synthon, necessary in the synthesis of several protease inhibitors (Kaldor *et al.*, 1997; Kwon and Ko, 2002), or broad spectrum antibiotics like chloramphenicol and thiamphenicol (Bhaskar *et al.*, 2004; Boruwa *et al.*, 2005). It also serves as chiral starting material for the synthesis of optically active molecules such as (*S*)-amphetamine (Rozwadowska, 1993).

The standard chemical synthesis route to produce optically pure aminodiols is usually complex, requiring many steps in order to obtain the final product, resulting in low overall productivities (Hailes *et al*., 2009; Smithies *et al*., 2009).

Using erythrulose (ERY) and glycolaldehyde (GA) as achiral substrates, the one-pot synthesis of the single syn-diastereoisomer (2*S*,3*R*)-2-amino-1,3,4-butanetriol (ABT), which is a moiety found in the core of the protease inhibitor drug Nelfinavir™ (Kaldor et al., 1997) was previously demonstrated using *E. coli* whole cells expressing both wild type TK and a β-alanine-pyruvate TAm as shown in Scheme 1 (Ingram *et al*., 2007). The main limitation of this two step synthesis was the low activity of the β-alanine-pyruvate TAm causing a low overall yield of the reaction. Consequently we have constructed libraries of engineered TKs and novel TAms which could be used to produce a range of the desired amino alcohols with higher yields and stereoselectivities (Hibbert *et al*., 2007; Kaulmann *et al*., 2007; Hibbert *et al*., 2008; Smith *et al*., 2008). Subsequent work has shown that the synthesis of (2*S*,3*S*)-2-aminopentane-1,3-diol (APD) (Scheme 2) could be achieved in two separate TK and TAm bioconversions, where the enzymes selected were the *E. coli* TK mutant D469T and the *Chromobacterium violaceum* CV2025 TAm, reaching more than 90% mol/mol yield in each of the reaction steps (Smith *et al.,* 2010). In this case, the required isolation of the TK product (3*S*)-1,3-dihydroxypentan-2-one (PKD) to perform the TAm bioconversion, led to a loss of more than 30% mol/mol of the intermediate. This could be potentially avoided by using a one-pot synthesis strategy, where the activities of the enzymes could be matched leading to higher yields of final product (Chen *et al*., 2006).

The aim of this work is to demonstrate a toolbox approach for the rapid evaluation of different variants of TK and TAm for the one-pot synthesis of a range of amino alcohols. The toolbox is based on an *E. coli* host where it is possible to systematically evaluate different pairs of the enzymes, screening them against different ketol donors, acceptors and amino donors. All the experiments are performed in parallel at the microwell scale and could be readily automated (Micheletti and Lye, 2006). The one-pot syntheses of ABT (Scheme 1) and (APD) (Scheme 2) were successfully demonstrated at 87 and 90 % mol/mol yields respectively, using the most appropriate forms of TK-TAm expressing biocatalysts.

2. MATERIALS AND METHODS

2.1. MATERIALS

Molecular biology enzymes were obtained from New England Bio-laboratories (NEB, Hitchin, UK). Nutrient broth and nutrient agar were obtained from Fisher Scientific (Leicestershire, UK). Competent *E. coli* BL21-Gold (DE3) cells were obtained from Stratagene (Amsterdam, NL). All other reagents were obtained from Sigma-Aldrich (Gillingham, UK) unless noted otherwise, and were of the highest purity available.

2.2. SYNTHESIS OF SUBSTRATES AND PRODUCTS

HPA was synthesized by reacting bromopyruvic acid with LiOH following a previously described method (Morris *et al*., 1996). PKD was synthesized in a 100 ml scale bioconversion with 300 mM HPA, 300 mM PA, 9 mM $MgCl_2$, 2.4 mM TPP, pH

7.0 and 30% v/v of D469E TK lysate (final TK concentration of 0.3 mg ml^{-1}). The reaction was stirred for 10 hours at room temperature in a sealed flask and the pH was maintained at 7.0 using a 718 STAT Titrino pH controller (Metrohm Ion Analysis, Switzerland). The solution was dried on silica and purified by column chromatography (ethyl acetate: hexane, 1:1) to yield PKD as a colorless oil that crystallized on standing. ABT and APD products were prepared in a multi-step chemical synthesis described elsewhere (Ingram *et al*., 2007, Smith *et al.*, 2010).

2.3. PLASMIDS

2.3.1 TRANSKETOLASE PLASMID AND MUTAGENESIS

Plasmid pQR412 contained the complete *E. coli* TK gene, *tktA*, with its native promoter and an N-terminal His6-tag. It was constructed using the expression vector pMMB67HE (8.8kb), which has a RSF1010 origin of replication, the *tac* promoter, the Lac repressor and codes for resistance to ampicillin (Fürste *et al*., 1986). Even if the plasmid contained the inducible *tac* promoter, the expression of the *E. coli tktA* was constitutive because of the presence of its native promoter (Ingram *et al*., 2007). Site directed mutagenesis of the TK gene was performed as described previously (Hibbert *et al*., 2007).

2.3.2 TRANSAMINASE PLASMID

Plasmid pQR801 contained the complete *Chromobacterium violaceum* 2025 TAm gene with a His6-tag (GenBank accession no. NP_901695). Plasmid pQR801 was

constructed using the expression vector pET29(a)+ (5.3kb), which contains an inducible T7 promoter, the Lac repressor and codes for resistance to kanamycin (Kaulmann *et al.* ,2007).

2.4. BIOCATALYSTS

2.4.1. TK WHOLE CELL AND LYSATE PREPARATION

Competent *E. coli* BL21-Gold (DE3) cells were transformed with the plasmid pQR412 using the heat shock technique described by the supplier (Stratagene, Amsterdam, NL). An overnight culture of the transformed cells was obtained in a 100 ml shake flask (10 ml working volume) of LB-glycerol broth (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl and 10 g l^{-1} glycerol) containing 150 mg ml^{-1} ampicillin. Growth was performed at 37 ^{o}C with orbital shaking at 250 rpm using an SI 50 orbital shaker (Stuart Scientific, Redhill, UK). The total volume of this culture was used to inoculate a 1 litre shake flask (100 ml working volume) which was left to grow for 8 hours. The cells were harvested and stored at -20 ^{o}C following the removal of broth by centrifugation. When a TK lysate was needed, the cells were resuspended in 50 mM TRIS buffer, pH 7.5 and sonicated with a Soniprep 150 sonicator (MSE, Sanyo, Japan). The lysate suspension was centrifuged at 5000 rpm in Falcon tubes for 5 min and then stored at -20 ^{o}C and used within 1 month.

2.4.2. TAm WHOLE CELL AND TK-TAm BIOCATALYST PREPARATION

Transformation of *E. coli* BL21-Gold (DE3) cells with the plasmid pQR801 and inoculum preparation were performed in the same way as described in Section 2.4.1, except that 150 mg ml^{-1} of kanamycin was used for the single transformed cells and 50 mg ml^{-1} of both kanamycin and ampicillin were used for the double transformed strain. After inoculation of a 1 litre shake flask (100 ml working volume), when the OD_{600} reached a value of 1.5-2, isopropylthiogalactopyranoside (IPTG) was added to final concentration of 0.2 mM. After 4 hr induction, the cells were harvested and following the removal of broth by centrifugation, they were resuspended in 50 mM HEPES buffer, pH 7.5 and used for whole cell bioconversions. When lysates were needed, after the resuspension, the cells were sonicated and stored using the same procedure described in Section 2.4.1.

2.5. BIOCONVERSION KINETICS

2.5.1. MICROSCALE EXPERIMENTAL PLATFORM

All bioconversions were performed in a glass 96-well, flat-bottomed microtiter plate with individual wells having a diameter of 7.6 mm and height of 12 mm (Radleys Discovery Technologies, Essex, UK). The microplate was covered with a thermo plastic elastomer cap designed to work with automated equipment (Micronic, Lelystad, Netherlands). All the bioconversions were performed using 300 µl total volume at 30 ^{o}C, pH 7.5 unless noted otherwise, and shaking was provided at 400 rpm with a Thermomixer Comfort shaker (shaking diameter of 3 mm, Eppendorf, Cambridge, UK). TK single step reactions were performed in 50 mM TRIS buffer and the concentration of cofactors $MgCl_2$ and thiamine pyrophosphate (TTP) were 9 mM

and 2.4 mM respectively for all reactions. TAm single step reactions and all the two step syntheses were carried out in 50 mM HEPES buffer, and the concentration of TAm cofactor pyridoxal-5-phosphate (PLP) was 0.2 mM in all cases. (*S)*-(α)-Methyl benzylamine (MBA) or isopropylamine (IPA) were added as amino donors as indicated in the results section at final concentrations of 10 mM and 100 mM respectively unless noted otherwise. A final concentration of 0.3 mg ml^{-1} of TK or TAm was used in all the single enzyme reactions. The whole cell suspension or lysate with the cofactor solutions were always added first in the well and left to incubate for 20 min at 30 ^{o}C, prior to initiation of the reaction with the addition of the substrate solutions. Aliquots of 20 µl were taken at various time intervals and quenched with 380 µl of a 0.1% v/v trifluoroacetic acid (TFA) solution. They were then centrifuged for 5 min at 5000 rpm and transferred into an HPLC vial for further analysis. All experiments were performed in triplicate. The specific activities were determined as the amount of PKD, ERY, acetophenone (AP), APD and ABT formed per unit of time normalized by the amount of enzyme used in the reaction.

2.6 ANALYTICAL METHODS

Biomass concentration was measured as optical density at 600 nm (OD_{600}) using a spectrophotometer (Thermo Spectronic, Cambridge, UK) and converted to dry cell weight (DCW) using a calibration curve where 1 OD_{600} = 0.5 g_{DCW} l^{-1}. Protein concentrations were obtained using Bradford assay and SDS-PAGE as described previously (Kaulmann *et al*, 2007). A Dionex HPLC system (Camberley, UK) with a Bio-Rad Aminex HPX-87H reverse phase column (300 x 7.8 mm, Bio-Rad Labs., Richmond, CA, USA), controlled by Chromeleon client 6.60 software was used for

the separation and analysis of PKD, ERY and HPA. The system comprised a GP50 gradient pump, a FAMOS autosampler, an LC30 chromatography column oven and an AD20 UV/Vis absorbance detector. The HPLC method used has been described previously (Chen *et al*., 2008). To quantify MBA, AP, APD and ABT, an integrated Dionex ultimate 3000 HPLC system (Camberley, UK) with an ACE 5 C18 reverse phase column (150mm×4.6 mm, 5 µm particle size; Advance Chromatography Technologies, Aberdeen, UK) controlled by Chromeleon client 6.60 software was employed. The HPLC method has been reported elsewhere (Kaulmann *et al*, 2007). To analyse ABT and APD, the samples were derivatized by addition of an excess of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate. The derivatizing reagent was made in house following the protocol of Cohen and Michaud (1992), and the HPLC method used has been described previously (Ingram *et al*, 2007). The enantiomeric excess (*ee*) of the ketodiols was determined by derivatization via dibenzoylation for satisfactory peak resolution by chiral HPLC, the method has been described in more detail elsewhere (Cázares *et al*., 2010).

3. RESULTS AND DISCUSSION

3.1. BIOCATALYST PRODUCTION

In order to separately regulate expression of the TK and TAm enzymes in *E. coli*, a dual plasmid system was selected to facilitate the "mix and match" approach first proposed by Hussain and Ward (2003). This is based on two plasmids with different origins of replication and antibiotic resistance genes that would enable the rapid evaluation of different variants of TK and TAm. To establish the metabolic burden of

the two plasmids on the host cells, five different whole cell biocatalysts were initially generated and evaluated in shake flask fermentations. Growth kinetics were obtained with cultures of *E. coli* BL21-Gold (DE3) containing either no plasmid, the single TK or TAm gene expressing plasmids, or both the TK and TAm plasmids, in each case either induced or non induced (Figure 1).

Single transformed strains with plasmid pQR412 (TK) or pQR801 (TAm) reached biomass concentrations of 2.1 and 1.8 g_{DCW} l^{-1} respectively. These were 12.5% and 25% less than for the non transformed strain respectively. The double transformed and induced strain obtained a final biomass concentration of 1.2 g_{DCW} l^{-1}, representing nearly a 50% decrease compared with the non transformed strain. This decrease in growth, however, is compensated for by the increase in enzyme synthesis which was expected due to the metabolic burden caused by the maintenance and expression of two plasmids.

In terms of enzyme expression levels, TK and TAm (induced with IPTG) in the single transformed cells were found to represent 20% and 40% w/w of the total protein respectively. For the double transformed strain, the TK concentration diminished compared to the single TK plasmid strain, and after inducing at a biomass concentration of 1 g_{DCW} l^{-1} for 4 hours, the final enzyme expression of TK and TAm represented 8% and 36% w/w of the total protein. Because of the significantly lower activity of the TAm compared to the TK (Chen *et al*., 2006; Ingram *et al*., 2007), this method of induced expression of TK and TAm was considered acceptable and was used to prepare all the biocatalysts for the later two-step bioconversions. Related work

in our group is focusing on fine control of the expression levels of the two enzymes, so that the optimum proportions can be obtained for specific bioconversions.

3.2. CONSIDERATIONS FOR TWO-STEP TK-TAm SYNTHESES

The use of multi-step enzymatic syntheses necessarily requires some compromises to be made regarding the optimum reaction conditions for each enzyme. This is particularly acute with isolated enzymes but can be alleviated, to some extent, by the use of whole cell biocatalysts. Optimum conditions reported for TK-catalyzed ketodiol syntheses were pH 7.0 using TRIS buffer at 25 ^{o}C (Chen *et al*., 2006; Hibbert *et al*., 2007; Cázares *et al*., 2010). The best conditions for the CV2025 TAm to catalyze syntheses of various amino alcohols were at pH 7.5 using HEPES buffer at 37 ^{o}C (Kaulmann *et al*., 2007; Smithies *et al*., 2009). In order to evaluate the process compromises to be made, the synthesis of 10 mM ABT using MBA as amino donor was used as a reference reaction (Scheme 1). Control TK reactions in the presence of the working concentration of the TAm cofactor PLP demonstrated that this did not have any effect on the TK reaction. The same finding was obtained for the effect of TPP and Mg^{2+} on the TAm bioconversion. Reactions of TK carried at a pH of 7.5 showed a decrease of TK activity of 30% (data not shown), nevertheless this pH was selected for subsequent studies considering the generally higher activity of TK compared to TAm as described above.

In terms of reaction temperature, a control reaction using a lysate of untransformed *E. coli* BL21-Gold (DE3) showed that at 37 ^{o}C up to 50% of the HPA substrate was consumed by side reactions after 2 hours, while at 30 ^{o}C, less than 5% of HPA was

consumed over the same time (data not shown). Consequently the TK-TAm reactions were performed at 30 ºC to avoid HPA metabolism. With regards to buffer selection, control experiments using TRIS and phosphate buffer inhibited the CV2025 TAm. We have previously shown that HEPES buffer catalyzes several side reactions between HPA and aldehydes at 37 ºC (Smith *et al*., 2006b), nevertheless at 30 ºC, control experiments showed that less than 5% of HPA was lost by the biomimetic activity of 50 mM HEPES after two hours. Based on all these considerations, the reaction conditions selected for the *in vitro* evaluation of the different TK-TAm variants were 30 ºC and pH 7.5 in 50 mM HEPES buffer.

3.3. BIOCONVERSIONS WITH TK

Both the TK and TAm bioconversions were initially studied individually under conditions suitable for later two-step reactions (Section 3.2). Information on specific activities, *ee* and final yields gathered from those experiments will then enable us to successfully "mix and match" the best pair of enzymes to synthesize specific amino alcohols. Initial TK kinetics experiments showed that the activities and final yields obtained with *E. coli* TK lysates and whole cells were similar (data not shown), therefore data reported here for TK bioconversions were performed only with lysates containing the different TK variants (Table 1).

From TK mutant libraries developed previously (Hibbert *el al*, 2008; Smith *et al*., 2008), the TK variants D469E, D469T and the wild type TK were selected as candidates due to their catalytic potential for the synthesis of ERY and/or PKD. Using HPA, the TK reactions were irreversible due to the release of CO_2 as a side product,

and yields of more than 90% mol/mol were obtained using concentrations up to 300 mM of HPA and the aldehyde GA or PA (data not shown). In contrast, TAm reaction high yields were limited to around 10 mM using MBA as amino donor, hence initial TK bioconversions studies were performed at this lower concentration.

Figure 2 shows the kinetics of ketodiol production for different combinations of TKs and aldehyde acceptors. The quantified activities, yields and measured *ee* values are summarized in Table 1. The wild type TK was selected for ERY synthesis because it presented the highest activity and *ee* of 6.6 µmol min^{-1} mg^{-1} and >95% respectively. For the synthesis of PKD, the highest activity was 1.98 µmol min^{-1} mg^{-1} using the TK mutant D469T, while no activity after 70 min of reaction was detected using the wild type enzyme. The *ee* of PKD obtained with TK D469E was >90%, compared to a 64% *ee* of D469T. Due to the importance of the *ee* in the synthesis of optically pure amino alcohols, the TK mutant D469E was selected even though it had a 25% lower activity than D469T. The D469E mutation is in the pyrimidine binding domain of TK, and is assumed to enable activity towards PA because it replaces the original interaction of Asp with the C-2 hydroxyl group of GA, by creating a specific interaction between the Glu and the methyl group of the new substrate (Hibbert *et al*., 2008; Cázares *et al*., 2010).

3.4. BIOCONVERSIONS WITH TAm

Single step TAm bioconversions focused on the CV2025 TAm as it had already been shown to be the best candidate for amino alcohol synthesis among several other transaminases cloned by us (Kaulmann *et al*., 2007). The comparison of *in vitro* and

in vivo catalytic activity of CV2025 TAm has not previously been reported, so it is investigated here where a whole cell TAm biocatalyst is compared with a lysate form for syntheses of a number of ketodiols.

Figure 3 shows product formation kinetics using either MBA or IPA as amino donors and PKD as amino acceptor. Due to the volatility of IPA, an excess concentration of 100 mM IPA was used instead of 10 mM MBA to compensate for any loss due to evaporation. The results of activities and final yields of both amino alcohols are summarized in Table 2. Similar yields were obtained using ERY or PKD as substrates, but the specific activities using ERY were between 17% and 42% higher than those of PKD. For synthesis of both amino alcohol products, the whole cell biocatalysts performed better than the lysates demonstrating the beneficial effect of using TAm *in vivo* for these bioconversions. This phenomenon has also been reported in the literature, where higher yields using whole cell TAm biocatalysts have been attributed to better stability of the enzyme inside the cell (Shin and Kim, 1997), to metabolism consumption of inhibitory compounds (Shin and Kim, 1999), or to the effect of the cell membrane in partitioning inhibitory components between the inside and outside of the cell (Yun *et al*, 2004). In this work, the inhibitory product acetophenone (AP) was not further metabolized by the whole cells. MBA and AP where found to be more inhibitory than IPA and acetone (data not shown), nevertheless lower yields using TAm lysates were obtained using IPA (75 % and 71 % mol/mol for APD and ABT respectively) compared to MBA (83 % and 86 % mol/mol respectively). Addition of more enzyme and PLP after the initial reaction had stopped partially restored the biocatalytic activity, suggesting that the improved yield using whole cells could be attributed to an increase in stability of the enzyme. Higher

concentration of substrates (up to 100 mM) could also be used with IPA as amino donor without compromising dramatically the final yield. This was not the case using MBA, where the yield diminished significantly at concentrations above 10 mM, showing evidence of the strong inhibition by MBA (data not shown). The inhibitory effect of MBA has also been reported in the literature with transaminases from *Klebsiella pneumonia* and *Vibrio fluvialis* (Shin and Kim, 1997; Yun *et al*., 2004).

Based on the results in Table 2, the whole cell TAm biocatalyst was selected for further study because of its superior performance compared to the lysate. IPA appears preferable as the amino donor because of its advantages in terms of economy, downstream processing and reduced inhibition compared to MBA. However, both amino donors were evaluated for one-pot synthesis reactions in the next section.

3.5. ONE-POT SYNTHESIS OF AMINO ALCHOHOLS

In order to achieve the one-pot synthesis of amino alcohols, two whole cell biocatalysts were constructed both with plasmid pQR801 expressing the CV2025 TAm, and with either pQR412 expressing the TK mutant D469E or the wild type variant for APD and ABT syntheses respectively. The first one-pot synthesis of APD using MBA as amino donor gave a poor yield (15 % mol/mol) of amino alcohol after 20 hours but another product was detected by HPLC analysis. This compound was identified as serine, which was possibly produced by the transamination of HPA. To overcome this problem, MBA was subsequently added in fed batch mode after the TK reaction was complete (Figure 4). The volume added of the solution of MBA was equal to the volume of the reaction mixture, hence the original concentration of the

reactants was diluted 2-fold after MBA addition. The first step comprising the TK reaction reached completion after 4 hours with a specific activity of 2.68 μmol min^{-1} mg^{-1}. The second step TAm reaction, had a specific activity of 0.067 μmol min^{-1} mg^{-1} and reached a maximum APD yield of 90 % mol/mol after 21 hours of reaction with MBA. This measured activity is in agreement with that found for the single TAm reaction (Table 2).

When using IPA as amino donor at the compromise temperature of 30 ^{o}C (Section 3.2), the TAm did not show any noticeable activity towards HPA, GA or PA over 5 hours (data not shown). This was not the case at 37 ^{o}C, where activity towards TK substrates was detected in that period of time. This suggested it was possible to perform the bioconversion of APD using IPA in a true one-pot synthesis at 30 ^{o}C as shown in Figure 5, were the TK reaction was completed after 5 hours, leading to a 90 % mol/mol yield of APD after 25 hours. The specific activities of TK and TAm in the one-pot synthesis were 1.46 and 0.022 μmol min^{-1} mg^{-1} respectively, which were also in agreement with the corresponding TK and TAm specific activities for the single bioconversions (Table 1 and 2). Similar results were obtained for the one-pot synthesis of ABT using IPA as amino donor, reaching a final yield of 87 % mol/mol after 25 hours (Figure 6). The TK reaction for the ABT synthesis was completed in 1.5 hours instead of the 5 hours required the APD synthesis. This was in agreement with the higher activity of the wild type TK enzyme towards ERY synthesis in comparison to the activity of the D469E variant towards PKD synthesis (Table 1).

Taking all these results together demonstrates the utility of the ‘mix and match’ microscale experimental system for rapidly evaluating pairs of enzymes and the

interaction of reaction conditions. IPA is also seen to be a preferable amino donor compared to MBA, and the whole cell biocatalyst performed better than the lysate. The 87% mol/mol yield for ABT synthesis represents an improvement of 4-fold in yield in a quarter of the reaction time compared to our previous work (Ingram *et al*, 2007). The 90 % mol/mol yield for APD represents a 2-fold improvement yield in a third of the reaction time compared to our previous two-step synthesis (Smith *et al.*, 2010).

4. CONCLUSIONS

The microscale 'toolbox' described in this work has been shown to enable the efficient "mix and match" evaluation of pairs of TK and TAm enzymes for the one-pot synthesis of optically pure amino alcohols. In particular the CV2025 TAm was found to display better catalytic activity when used *in vivo* leading to the selection of whole cell biocatalysts for the synthesis of the single diastereoisomers of both ABT and APD. IPA was identified as the most suitable amino donor for the one-pot reactions because side reactions with the initial TK substrates (catalysed by TAm) were negligible. Our current work is seeking to increase the size of the available TK and TAm libraries and to expand the available microscale methods for evaluation of subsequent product recovery and purification operations.

AKNOWLEDGEMENTS

The Mexican National Council for Science and Technology (CONACYT), The Mexican Ministry of Public Education (SEP), The Royal Thai Government and the are acknowledge for studentships to the authors of this work. The authors also would like to thank the UK Engineering and Physical Sciences Research Council (EPSRC) for support of the multidisciplinary Biocatalysis Integrated with Chemistry and Engineering (BiCE) programme (GR/S62505/01) at University College London (London, UK). Financial support from the 13 industrial partners supporting the BiCE programme is also acknowledged.

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LIST OF TABLE LEGENDS

Table 1. Measured TK specific activities, product yields and *ee* for the lysate catalyzed bioconversions shown in Figure 2.

Table 2. Measured specific activities and product yields for the TAm catalyzed conversion of PKD and ERY.

LIST OF REACTION SCHEMES

Scheme 1. Reaction scheme of the *de-novo* TK-TAm pathway for the synthesis of chiral aminodiol (2*S*,3*R*)-2-amino-1,3,4-butanetriol (ABT), from achiral substrates glycolaldehyde (GA) and hydroxypyruvate (HPA).

Scheme 2. Reaction scheme of the *de-novo* TK-TAm pathway for the synthesis of chiral aminodiol (2*S*,3*S*)-2-aminopentane-1,3-diol (APD) from achiral substrates propionaldehyde (PA) and hydroxypyruvate (HPA).

LIST OF FIGURES LEGENDS

Figure 1. Shake flask fermentation kinetics for *E. coli* BL21-Gold (DE3) grown in LB-glycerol medium at 37 ^{o}C with (▼) untransformed cells, and cells transformed with (Δ) pQR412 (TK), (●) pQR801 (CV2025 TAm), (o) double transformed

uninduced strain with pQR801 and pQR412, (□) double transformed strain induced with 0.2 mM IPTG after 6 hours of cell growth. Error bars represent one standard deviation about the mean (n=3).

Figure 2. Bioconversion kinetics showing ketodiol synthesis using different TK lysates and aldehyde acceptors: (●) ERY with wild type TK, (▼) ERY with D469T TK, (■) ERY with D469E TK, (Δ) PKD with D469T TK, (□) PKD with D469E TK, (o) PKD with wild type TK. Reaction conditions: 10 mM HPA and GA, 2.4 mM TTP, 9 mM $MgCl_2$, 0.3 mg ml^{-1} TK, pH 7.5 in 50 mM TRIS, 30 ^oC. Error bars represent one standard deviation about the mean (n=3).

Figure 3. Bioconversion kinetics showing ADP synthesis using CV2015 TAm in either lysate or whole cell form with different amino donors: (●) whole cell with MBA, (▲) whole cell with IPA, (○) lysate with MBA, (Δ) lysate with IPA. Reaction conditions : 10 mM MBA or 100 mM IPA, 10 mM PKD, 0.2 mM PLP, 0.3 mg ml^{-1} TAm, pH 7.5 in 50 mM HEPES, 30 ^oC. Error bars represent one standard deviation about the mean (n=3).

Figure 4. Typical bioconversion kinetics for the one pot, whole cell TK-TAM catalytic synthesis of APD with addition of amino donor (MBA) after 4 hours : (▼) PKD, (●) HPA, (Δ) APD, (◊) MBA and (o) AP. Reaction conditions (TK step): 20 mM HPA and PA, 0.4 mM PLP, 2.4 mM TTP, 9 mM Mg^{2+}, 0.095 mg ml^{-1} TK, 0.43 mg ml^{-1} TAm, pH 7.5 in 50 mM HEPES, 30 ^oC. TAm step was initiated by adding MBA solution which resulted in a 2 fold dilution of reactants.

Figure 5. Typical bioconversion kinetics for the one pot, whole cell TK-TAm catalytic synthesis of APD using IPA as amino donor: (▼) PKD, (●) HPA, and (Δ) APD. Reaction conditions: 10 mM HPA and PA, 100 mM IPA, 0.2 mM PLP, 2.4 mM TTP, 9 mM Mg^{2+}, 0.095 mg ml^{-1} TK , 0.42 mg ml^{-1} TAm, pH 7.5 in 50 mM HEPES, 30 ^{o}C.

Figure 6. Typical bioconversion kinetics for the one pot, whole cell TK-TAm catalytic synthesis of ABT using IPA as amino donor: (▼) ABT, (●) HPA, and (o) ERY. Reaction conditions: 10 mM HPA and GA, 100 mM IPA, 0.2 mM PLP, 2.4 mM TTP, 9 mM Mg^{2+}, 0.095 mg ml^{-1} TK , 0.42 mg ml^{-1} TAm, pH 7.5 in 50 mM HEPES, 30 ^{o}C.

LIST OF TABLES

Table 1

TK variant	Aldehyde	Specific activity[a] (µmol min^{-1} mg^{-1})	*ee (%)*	Final yield[b] (% mol/mol)
Wild type	GA	6.62 ± 0.13[c]	>95	100
Wild type	PA	-	-	-
D469T	GA	5.13 ± 0.43	85	95
D469T	PA	1.98 ± 0.27	64	98
D469E	GA	4.96 ± 0.39	nd	100
D469E	PA	1.49 ± 0.26	>90	90

[a]The specific activity is based on the measured mass of TK in each bioconversion.

[b]Yield was determined at t=70 min.

[c] The error of the specific activity represents one standard deviation about the mean (n=3).

Table 2

Biocatalyst form	Amino donor	Amino acceptor	Specific activity[a] (µmol min^{-1} mg^{-1})	Final yield[b] (% mol/mol)
Whole cell	MBA	PKD	0.074 ± 0.006[c]	95
Lysate	MBA	PKD	0.034 ± 0.003	83

Whole cell	IPA	PKD	0.029 ± 0.0035	96
Lysate	IPA	PKD	0.015 ± 0.003	75
Whole cell	MBA	ERY	0.096±0.005	92
Lysate	MBA	ERY	0.045±0.003	86
Whole cell	IPA	ERY	0.035±0.05	91
Lysate	IPA	ERY	0.026±0.04	71

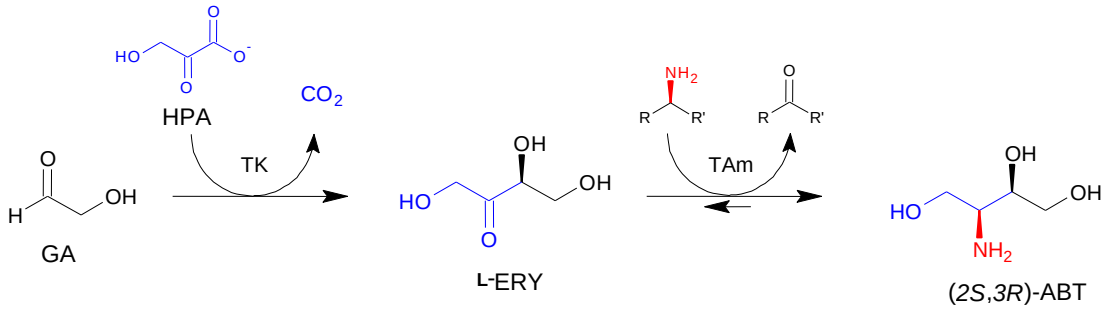
[a]The specific activity was based on the measured mass of TAm present in each bioconversion. For whole cell experiments, it was calculated based on 50% of the dry cell weight of the cells being protein (Watson, 1972), and SDS-PAGE analysis that showed that 40% of the protein was TAm.

[b]Yield was determined at t=30 hours.

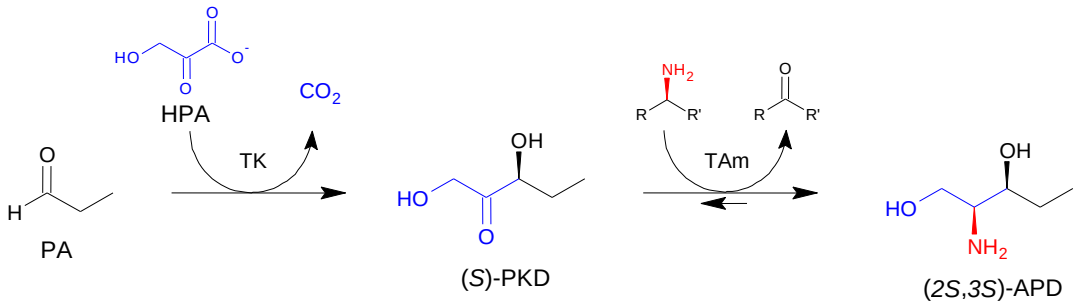
[c] The error of the specific activity represents one standard deviation about the mean (n=3).

LIST OF SCHEMES

Scheme 1



Scheme 2



LIST OF FIGURES

Figure 1

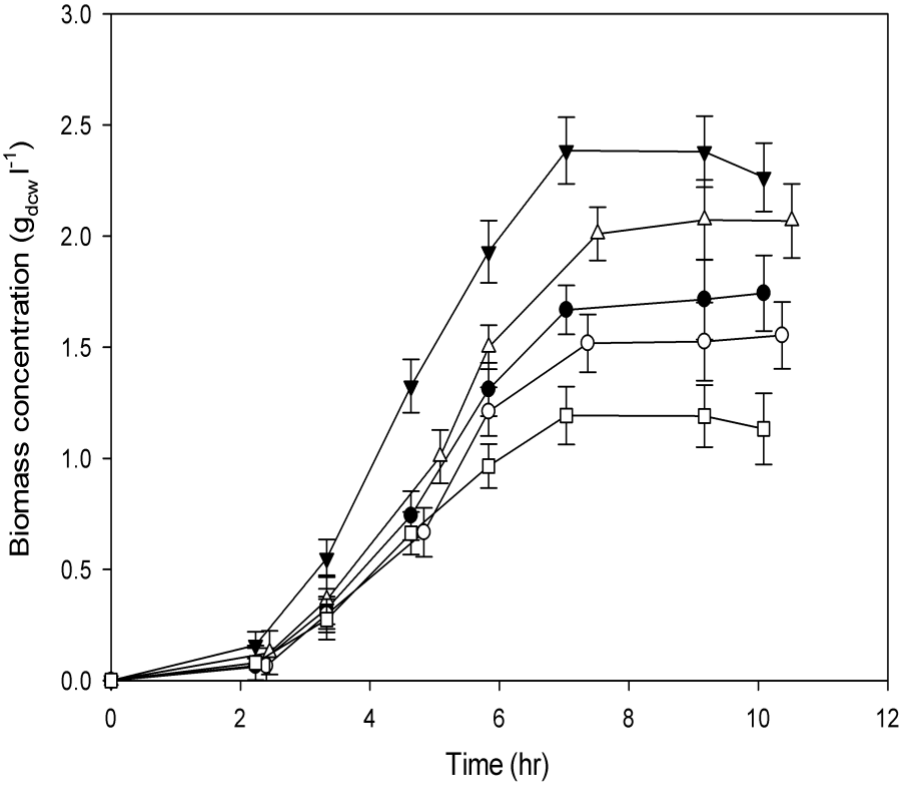
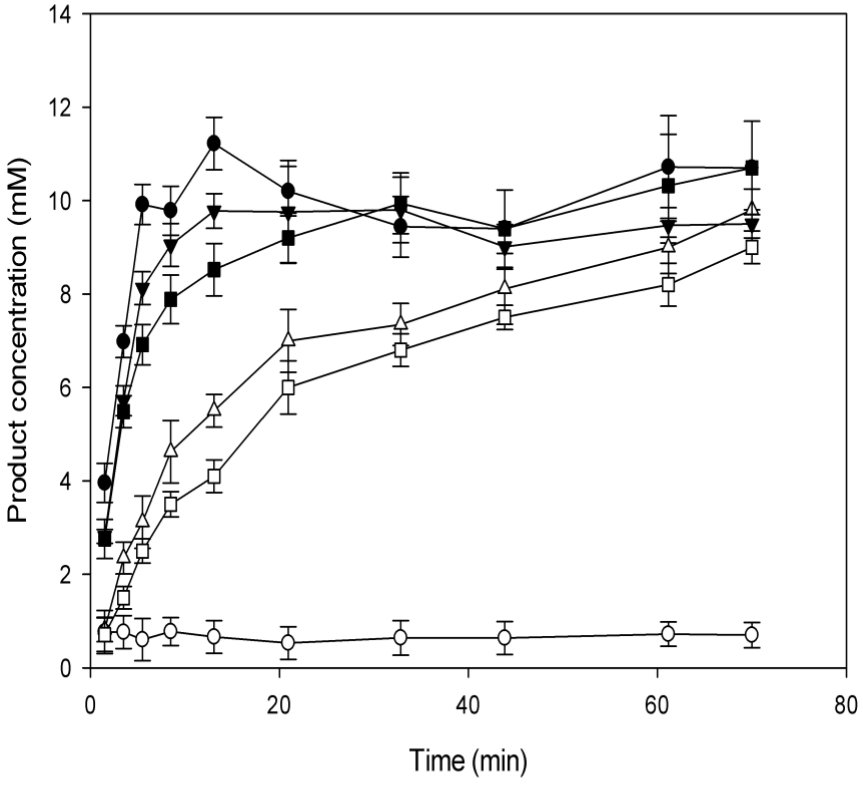


Figure 2



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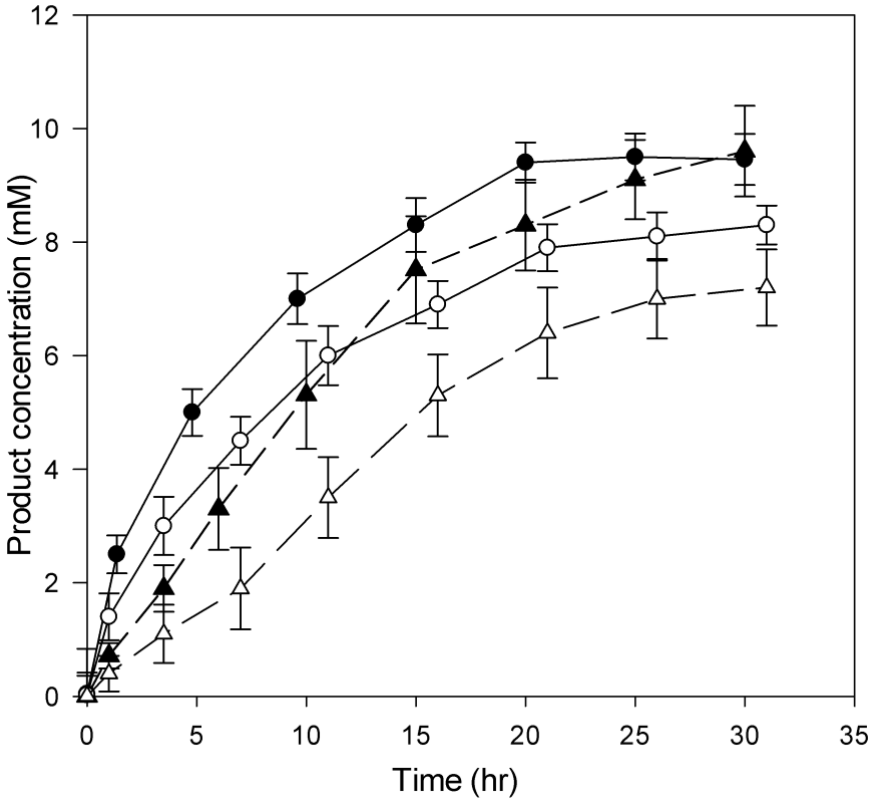
Product concentration (mM)
Time (hr)
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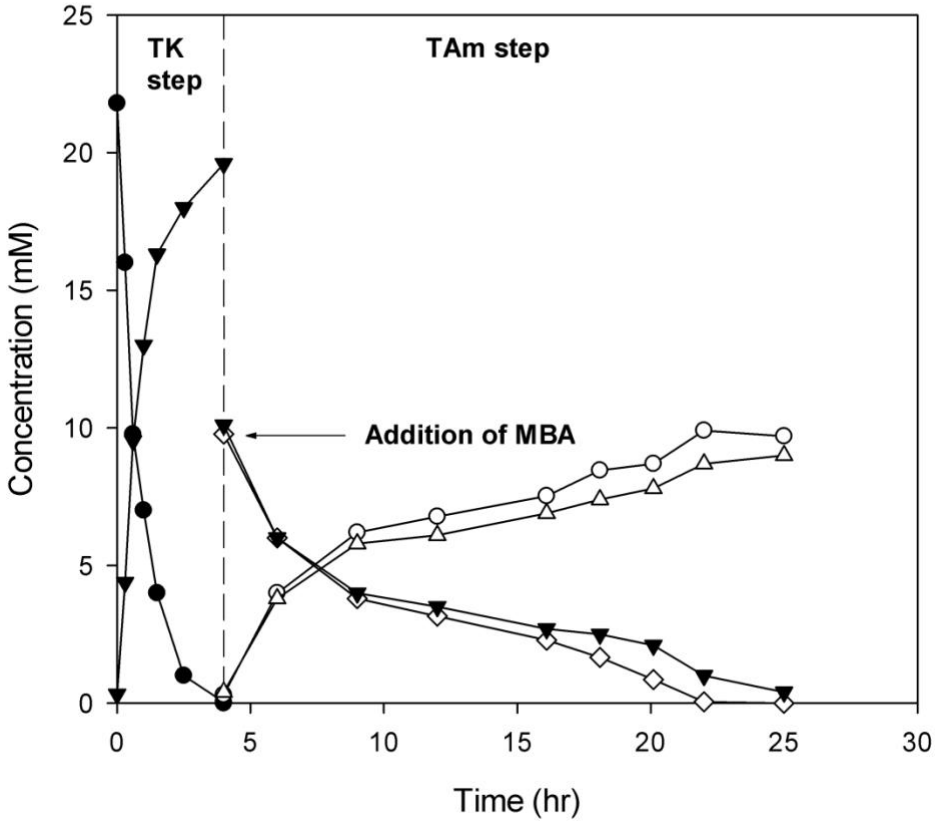
2
4
6
8
10
12
0
5
10
15
20
25
30
35

Figure 4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TAm step
Addition of MBA
Concentration (mM)
Time (hr)
0
5
10
15
20
25
30

Figure 5

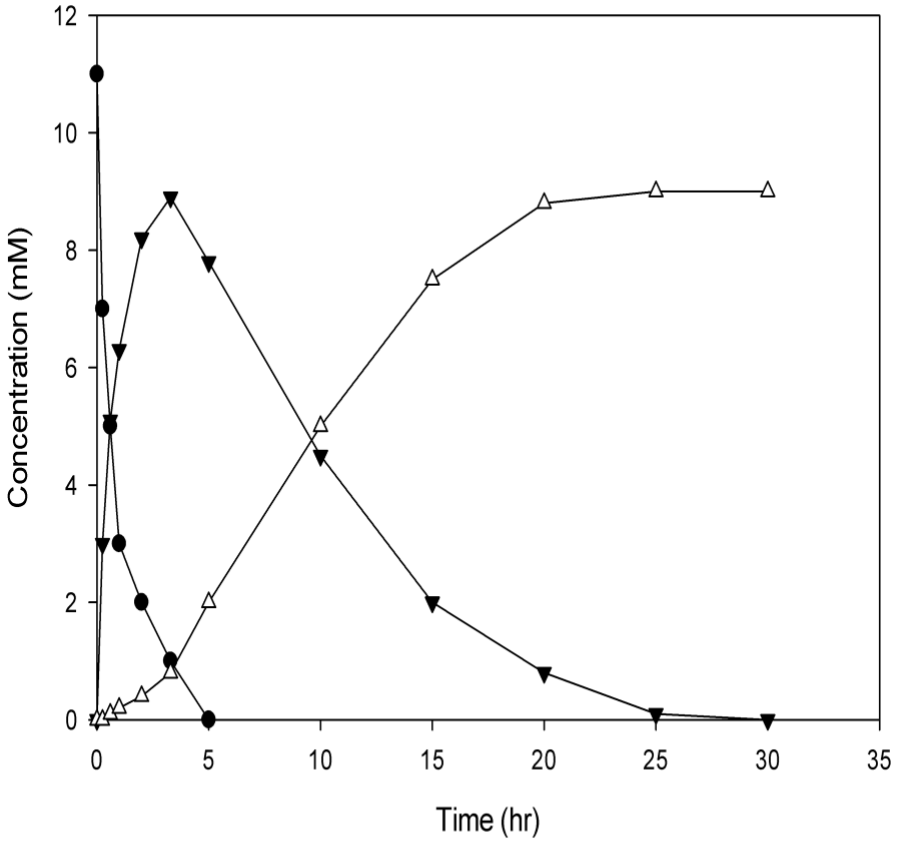

Figure 6