NON-LINEAR KINETIC MODELLING OF REVERSIBLE BIOCONVERSIONS: APPLICATION TO THE TRANSAMINASE CATALYSED SYNTHESIS OF CHIRAL AMINO-ALCOHOLS‡

L. Rios-Solis[1], N. Bayir[1], M. Halim[1], C. Du[1], J.M. Ward[2], F. Baganz[1], G.J. Lye[1+]

[1] Department of Biochemical Engineering, University College London, Torrington Place, London, WC1E 7JE, UK

[2]Institute of Structural and Molecular Biology, Darwin Building, University College London, Gower Street, London, WC1E 6BT, UK

Keywords: nonlinear kinetic modelling, transaminase, reversible bioconversions, amino-alcohols, microscale high-throughput, reaction mechanism selection

‡ Manuscript prepared for Biochemical Engineering Journal.

+Corresponding author (Email: g.lye@ucl.ac.uk).

ABSTRACT

This work describes the establishment of a full kinetic model, including values of apparent kinetic parameters, for the whole cell *E. coli* mediated synthesis of the chiral amino-alcohol (2*S*,3*R*)-2-amino-1,3,4-butanetriol (ABT), using (S)-(-)-α-methylbenzylamine (MBA) as amino donor. The whole cell biocatalyst expressed the CV2025 ω-transaminase from *Chromobacterium violaceum*. Establishment of the most suitable reaction mechanism and determination of the complete forward and reverse kinetic parameter values for the reversible bioconversion where obtained using a hybrid methodology. This combined traditional initial rate experiments to identify a solution in the vicinity of the global minimum, with nonlinear regression methods to determine the exact location of the solution. The systematic procedure included selection and statistical evaluation of different kinetic models that best described the measured reaction rates and which ultimately provided new insights into the reaction mechanism; in particular the possible formation of a dead end complex between the amino donor and the cofactor enzyme complex. The hybrid methodology was combined with a microscale experimental platform, to significantly reduce both the number of experiments required as well as the time and material required for full kinetic parameter estimation. The equilibrium constant was determined to be 849, and the forward and reverse rate constants were found to be 97 and 13 min^{-1} respectively, which greatly favoured the asymmetric synthesis of chiral ABT. Using the established kinetic model, the asymmetric synthesis of ABT was simulated, and excellent agreement was found between the experimental and predicted data over a range of reaction conditions. A sensitivity analysis combined with various simulations suggested the crucial bottleneck of the reaction was the second half reaction of the ping pong bi-bi mechanism, in part due to the low Michaelis constant of substrate L-erythrulose (ERY). The toxicity of MBA towards the transaminase was identified as another major bottleneck. The kinetic model was useful to give early insights into the most appropriate bioconversion conditions, which can improve the rate and yield of ABT formation, as well as minimizing the toxicity and inhibition effects of the substrates and products. The systematic methodology developed here is considered to be generic and useful in regard to speeding up bioconversion process design and optimisation.

1. INTRODUCTION

Chiral amino-alcohols are of great interest to the pharmaceutical and fine-chemical industries, as they are useful building blocks in the synthesis of optically pure pharmaceuticals like HIV protease inhibitors (Kaldor *et al*., 1997; Kwon & Ko 2002), active molecules such as (S)-amphetamine (Rozwadoska, 1993) or broad spectrum antibiotics like chloramphenicol and thiamphenicol (Bhaskar, 2004; Boruwa et *al.*, 2005). The standard chemical synthesis of optically pure amino-alcohols is usually complex, requiring many steps and resulting in low overall productivities (Hailes *et al*., 2009; Smithies *et al*., 2009). Transaminases (TAm), are a group of enzymes that can directly convert a carbonyl to an amino group (Christen & Metzler, 1985), and have been shown to be a promising alternative for the asymmetric synthesis of chiral amino-alcohols (Kaulmann *et al*., 2007; Kirsty Smithies *et al.,* 2009; Smith et *al*., 2010; Rios-Solis et *al*., 2011). Compared to other enzymes performing the same conversion, like the acid or amine dehydrogenases (Brunhuber & Blanchard 1994), TAm has some clear advantages including broad substrate specificity, no need for redox cofactor recycling and high stereoselectivity (Taylor *et al*., 1998; Stewart, 2001).

Transaminases have been classified into 4 different groups based on primary structure, and into different subgroups depending of the classification of the substrates utilised (Mehta *et al*., 1993). The ω-TAms, belonging to Group II, can catalyse substrates not generally accepted by the other TAms, including primary amine compounds not bearing a carboxylic group (Stirling, 1992). Among them, the ω-TAm from *Chromobacterium violaceum* 2025 (CV2025 TAm) showed the best performance for amino-alcohol synthesis from a pool of several other TAms cloned by us in previous work (Kaulmann *et al.,* 2007). Applications of TAms have been hindered due to the low equilibrium constants and product or substrate inhibition (Stewart, 2001; Yun *et al.,* 2005). Strategies to overcome these problems have included coupling a TAm reaction with other enzymes, using a biphasic system or a membrane reactor, performing the reaction under vacuum or using whole cell biocatalysts (Bartsch *et al*., 1996; Shin & Kim 1997; Chao *et al*., 1999; Truppo *et al*., 2010). Selection of the best strategy to overcome inhibition and/or low equilibrium constants and yield requires an accurate understanding of the TAm kinetics. Determination of a TAm kinetic model would also allow accurate bioconversion

simulations, enabling determination of the optimum reaction conditions and suitable bioreactor designs (Chen *et al*., 2006; Shin & Kim, 1998).

This work reports kinetic modelling and simulation of the TAm mediated synthesis of the chiral amino-alcohol (2*S*,3*R*)-2-amino-1,3,4-butanetriol (ABT) using (S)-(-)-α-methylbenzylamine (MBA) as amino donor (Scheme 1). The CV2025 TAm was used in whole cell form, as this has been previously shown to be more stable than pure TAm, or TAm lysates (Rios-Solis *et al.*, 2011). To aid in data generation, kinetic parameters were obtained using a model driven microscale experimental methodology based on nonlinear regression (Lye *et al*., 2002; Katare *et al*., 2004; Chen *et al.*, 2008). These approaches are extended here to: (i) aid identification of the most appropriate reaction mechanism and (ii) to deal with the increased number of kinetic parameters to be determined. As shown in Scheme 1, this is a reversible reaction for which a number of possible reaction mechanisms exist, and kinetic parameters must be determined in both forward and reverse reaction. Therefore it was necessary to include both forward and reverse reaction data in the kinetic parameter determination routine, in order to establish the appropriate kinetic model. Using this new approach allowed rapid determination of the kinetic parameters and gave insight into the mode and extent of various reactant inhibitions. Subsequent simulations were needed to identify major reaction bottlenecks which where validated experimentally.

2. EXTENDED METHODOLOGY FOR KINETIC MODEL AND PARAMETER IDENTIFICATION

Determination of the required data for establishment of full kinetic models can be time and resource consuming, especially when using traditional linear data fitting methods (Ranaldi 1999; Chen *et al*., 2009). This problem is further accentuated for non-natural bioconversions used in the pharmaceutical industry that often exhibit strong substrate and product inhibition (Yazbeck *et al.*, 2004). Nonlinear regression methods can use programmable optimization algorithms to determine the kinetic parameters and are consequently relatively less time and resource consuming (Blackmond, 2005). However, model parameters determination can suffer from global convergence problems, in particular the estimation procedure can be strongly dependent on the initial values of the parameters (Moros *et al*., 1996). Hybrid methods combine traditional initial rate experiments to identify a solution in the vicinity of the global minimum,

with nonlinear methods to determine the exact location of the solution (Katare *et al*., 2004). Those hybrid methods have been successfully applied to bioconversions suffering from substrate and product inhibition as the TAm (Al-Haque *et al*., 2012). This represents a potentially more efficient way forward especially when combined with high-throughput experimental methods to establish complex kinetic models and determine the parameter values (Lye *et al*., 2002; Doig *et al*., 2002; Chen *et al.*, 2008).

Many reported TAm reactions are reversible and usually present different forms of strong substrate and/or product inhibition, which can lead to the existence of many potential kinetic models (Koszelewski *et al*., 2010). Consequently we have extended our previously developed method for nonlinear parameter estimation (Chen *et al*., 2009) to deal with equilibrium bioconversions and to enable testing and identification of the most appropriate reaction mechanism. The new methodology incorporates 5 main steps as described in Figure 1 which are as follows (enhancements over our previous work are shown in italic):

1. Determination of the linear region between the enzyme concentration and initial rate bioconversion.
2. *Preliminary testing and statistical evaluation of different possible kinetic models.* This is based on high-throughput, initial rate experiments, in order to elucidate the most promising reaction mechanism and calculate the preliminary Michaelis and rate constants.
3. Determination of the *enzyme stability* under actual bioconversion conditions.
4. Determination of *preliminary values of equilibrium constant* and inhibition constants through nonlinear regression of experimental progress curves for forward *and reverse reactions*.
5. Reconciliation of calculated parameters via an additional round of nonlinear regression of the progress curves using preliminary values of all the kinetic parameters for the final regression, and *verification of the selected reaction mechanism through statistical analysis* (statistical comparison with other proposed models).

3. MATERIALS AND METHODS

3.1. MATERIALS

Competent *Escherichia 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.

3.2. TRANSAMINASE PLASMID

Plasmid pQR801 contained the complete *Chromobacterium violaceum* 2025 TAm gene with a N-Terminal 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).

3.3. TAm WHOLE CELL BIOCATALYST PREPARATION

Competent *E. coli* BL21-Gold (DE3) cells were transformed with the plasmid pQR801 using the heat shock technique described by the supplier (Stratagene, Amsterdam, NL). For whole cell bioconversions, fresh cells were always used and they were produced as follows: 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 µg ml^{-1} kanamycin. 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) and when the OD_{600} reached a value of 0.5, isopropylthiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM. After 6 hr induction, the cells were harvested and following the removal of broth by centrifugation, they were resuspended in 200 mM HEPES buffer, pH 7.5 and used for whole cell bioconversions. When a TAm lysate was needed, the cells were sonicated with a Soniprep 150 sonicator (MSE, Sanyo, Japan). The sonicated lysate was then centrifuged at 5000 rpm in Falcon tubes for 5 min to remove cell debris, and the clarified

supernatant was finally aliquoted into 1.5 ml Eppendorf vials to be stored at -20 ºC and used within 1 month.

3.4. 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 bioconversions were performed using 300 µl total volume at 30 ºC, pH 7.5 unless noted otherwise. Shaking occurred at 400 rpm using a Thermomixer Comfort shaker (Eppendorf, Cambridge, UK) (shaking diameter of 3 mm) situated on the deck of a Tecan Genesis laboratory robotic platform (Micheletti & Lye 2006). TAm bioconversions were carried out in 200 mM HEPES buffer, and the concentration of TAm cofactor pyridoxal 5´-phosphate (PLP) used was 0.2 mM. In all cases, the whole cell suspension and the cofactor solution were always added first in the well and left to incubate for 20 min at 30 ºC, prior to initiation of the reaction by addition of the substrate solutions.Previous studies have shown that initial incubation with cofactor was necessary to allow the enzyme to bind to PLP (Davies *et al*., 1960, Van Ophem *et al*., 1998). This practice also enabled more consistent measurement of specific activity data, by avoiding initial nonlinear variation of product concentration believed to be caused in some cases by the binding of the enzyme and cofactors. 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 acetophenone and ABT formed per unit of time normalized by the amount of enzyme used in the reaction. The specific activity was calculated 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), combined with quantitative SDS-PAGE analysis of the percentage of the total protein represented by the overexpressed TAm.

3.5. 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} = 0.4 g_{DCW} l^{-1}. Protein concentrations of the lysates were obtained using a 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 ERY. 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 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, 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). ABT standards were prepared in a multi-step chemical synthesis described elsewhere (Ingram *et al*., 2007, Smith *et al.,* 2010). The *ee* of ABT was determined by first derivatizing the amino-alcohol to the respective benzoate form. The assay was performed against the four diastereomer samples of the benzoate synthesized as described elsewhere (Smith *et al*., 2010) using chiral HPLC: Chiracel-OD column (Daicel); mobile phase, isopropanol/hexane (5:95); flow rate, 0.8 ml/min, detection, UV 210 nm. Examples of the different HPLC profiles have been published elsewhere (Rios-Solis *et al.*, 2011).

3.6. NONLINEAR REGRESION METHODS

In order to implement the procedure illustrated in Figure 1, a programme was developed using Matlab® software (MathWorks, Natick, MA, USA) in order to automatically perform all the nonlinear regressions and statistical analyses. All the nonlinear regressions were performed

using the mesh adaptive pattern search algorithm in Matlab® known as: ‘‘The Genetic Algorithm and Direct Search Toolbox’’. This method was previously shown to be more likely to achieve global optimization than gradient-based methods (Chen *et al*., 2008).

4. RESULTS

4. 1. Proportionality between reaction rate and enzyme concentrations

As described in Figure 1, the first step to obtain the kinetic parameters is to determine the region of linear proportionality between TAm concentration and the measured initial reaction rate. This was necessary to ensure that any increase in enzyme concentration would contribute fully to the measured kinetics (Chen *et al.*, 2008). It is common that increasing the enzyme concentration in a reaction does not correspond to a proportional increase in the initial bioconversion rate due to mass transfer limitations or certain forms of inhibition (Law *et al*., 2006). For the synthesis of ABT (Scheme 1), the substrate concentrations used were 10 mM MBA and 50 mM ERY for each enzyme concentration. Under these conditions the linear relationship could be maintained up to a TAm concentration of 0.8 mg ml^{-1} as can been in Figure 2. This value was thus set as the upper limit for all subsequent experiments.

This limit of 0.8 mg ml^{-1} is one order of magnitude smaller when compared to other types of enzymes like transketolase (Chen *et al*., 2009). The observation of a lower limit of proportionality among TAms has been reported before (Banks *et al.* 1959). A possible explanation is that the enzyme can show self-inhibition where an end amino group of one TAm molecule might bind the pyridoxal phosphate associated with the active site of another enzyme molecule (Ellis & Davies, 1961). In the case of whole cell forms of TAm, mass transfer limitations across the cell wall might also be the cause (Ni & Chen, 2004; Woodley, 2006). However, provided that this TAm concentration of 0.8 mg ml^{-1} is not exceeded, such effects did not need to be accounted for.

4.2. Preliminary reaction mechanism identification and quantification of initial rates for forward and reverse reactions

Following the second step of the procedure (Figure 1), a set of initial rate data varying the concentration of one substrate, while maintaining the other one fixed was obtained for both forward and reverse reactions for the TAm mediated synthesis of ABT and MBA respectively (Scheme 1). Figure 3 shows the measured initial rate of ABT formation for the forward reaction as a function of varying concentration of substrate ERY while maintaining the concentration of MBA fixed at 10 mM. Also shown in Figure 3 are results of the variation of initial rate as a function of the concentration of MBA while maintaining ERY at 200 mM. Strong inhibition of MBA can be observed at concentrations higher than 10 mM of the amino donor. In a similar way for the reverse reaction, Figure 4 shows the measured initial reaction rate as a function of varying concentration of substrate ABT, while maintaining the concentration of AP fixed at 35 mM. Also shown in Figure 4 is the initial rate of the reverse reaction as a function of AP concentration while maintaining ABT concentration at 100 mM. Most noticeable when comparing the forward and reverse reaction rates is that the reverse reaction is an order of magnitude slower than the forward rate.

In terms of elucidating the appropriate reaction mechanism for the TAm mediated synthesis of ABT, it is known that TAm requires the cofactor pyridoxal 5′-phosphate and that it catalyses enzymatic amino group transfer by a ping-pong bi–bi mechanism (Bulos & Handler 1965; Kuramitsu *et al.*, 1990). It has been reported for other TAm bioconversions that a substrate or product can bind an incorrect enzyme form, creating dead end complexes that cannot react further, causing a potentially strong form of inhibition (Bulos & Handler, 1965; Shin & Kim, 1998; Shin & Kim, 2002). For the CV2025 TAm mediated synthesis of ABT, the presence of abortive complexes in the reaction mechanism was not previously been shown, hence it provides a good test system on which to elaborate the methodology shown in Figure 1. Consequently different kinetic models were initially fitted by nonlinear regression to the experimental initial rate data shown in Figures 3 and 4. These explored the possibility of different dead end complex formation with the aim of identifying the most suitable preliminary kinetic model (which would be further verified in Step 5 of the proposed methodology). Among all the models tested, the reaction mechanism which included substrate inhibition by the formation of the dead end complex E-PMP-MBA gave the best statistical fitting, with an R^2 of 0.98 and a sum of squares of 0.24. This was significantly better than the R^2 of 0.87 and sum of squares of 14.2 for the second best fitting model which did not include any substrate inhibition by a dead end complex. The resulting King-Altman figure for the selected reaction pathway is

presented in Figure 5, and the corresponding rate model is derived in Equation 1 (Shin & Kim 1998; Bisswanger 2002).

$$v = \left\{ k_f k_r E_{iTAm} \left([MBA][ERY] - \frac{[AP][ABT]}{K_{eq}} \right) \right\} / den \qquad \text{Equation 1}$$

$$den = k_r K_{MBA}[ERY] + k_r K_{ERY}[MBA] + k_r[ERY][MBA] + \frac{k_f K_{AP}[ABT]}{K_{eq}} + \frac{k_f K_{ABT}[AP]}{K_{eq}} + \frac{k_f[AP][ABT]}{K_{eq}} + \frac{k_r K_{MBA}[ERY][ABT]}{K_{iABT}} + \frac{k_f K_{ABT}[MBA][AP]}{K_{eq} K_{iMBA}} + \frac{k_f K_{AP}[ABT][MBA]}{K_{iMBAc}} + \frac{k_r K_{ERY}[MBA]^2}{K_{eq} K_{iMBAc}}$$

Where v represents the reaction rate, k_f and k_r represents the catalytic rate constants for the forward and reverse reaction respectively, K_{ERY}, K_{AP}, K_{ABT} and K_{MBA} are the Michaelis constants of ERY, AP, ABT and MBA respectively, K_{iABT} and K_{iMBA} are the inhibition constants of ABT and MBA respectively, K_{iMBAc} is the inhibition constant for the MBA-PMP abortive complex, E_{iTAm} represents the TAm concentration and K_{eq} is the overall equilibrium constant.

In the absence of products for the forward reaction, Equation 1 can be simplified to Equation 2:

$$\frac{1}{v} = \frac{1}{k_f E_{iTAm}} \left(1 + \frac{K_{MBA}}{[MBA]} + \frac{K_{ERY}}{[ERY]} + \frac{K_{ERY}[MBA]}{K_{iMBAc}[ERY]} \right) \qquad \text{Equation 2}$$

The initial rate data from Figure 3 was fitted using nonlinear regression to Equation 2 obtaining an R^2 of 0.98 and sum of squares of 0.24. The calculated preliminary values of the constants k_f, K_{MBA}, K_{ERY} and K_{iMBAc} were 97.2 mM min^{-1}, 0.5, 101.2 and 23.9 mM respectively.

At this point, a statistical F-test (Markowski & Markowski, 1990) was performed in order to determine if the addition of the parameter K_{iMBAc} gave a significantly better fit to the data than in the absence of any dead end complex formation. The result of the F-test was equal to 282.4, which was considerably higher than the critical value of 6.6 from the F distribution tables for

a false-rejection probability of 0.05. This shows that the addition of the parameter K_{iMBAc} was statistically significant and gives preliminary confidence that the most appropriate kinetic model has been selected.

The plots of initial rate for the reverse reaction as a function of substrate concentration shown in Figure 4 did not suggest the presence of strong inhibition that could be caused by a dead end complex. This was corroborated by nonlinear fitting regression, where all the models including the formation of dead end complexes in the reverse reaction failed to give a statistically better fit than the model represented by Equation 3, which is a simplification of Equation 1 without including products of the reverse reaction.

$$\frac{1}{v} = \frac{1}{k_r E_{iTAm}}\left(1 + \frac{K_{AP}}{[AP]} + \frac{K_{ABT}}{[ABT]}\right) \qquad \text{Equation 3}$$

The initial rate data was fitted to Equation 3 by nonlinear regression, and the preliminary parameters of k_r, K_{AP} and K_{ABT} were found to be 13.1 min^{-1}, 19.6 and 39.4 mM respectively with an R^2 of 0.98. Both nonlinear regressions for the forward and reverse reaction had upper and lower bounds of 0.1 and 800 and both converged with any arbitrary initial value.

A set of experiments to obtain initial reaction rates using pure TAm purified to homogeneity by His tag affinity column chromatograph were performed and the results are also shown in Figure 4. It had been previously reported that the CV2025 TAm biocatalyst *in vivo* form was more stable than *ex vivo* (Rios-Solis *et al*., 2011). This stability effect was negligible for initial rate data, where both plots using TAm in pure and whole cell were statistically identical (Figure 4). This suggested three things: (i) that there are not additional mass transfer resistances imposed on substrate/product transfer when using whole cells, (ii) that there does not appear to be any significant interaction of the reactants and products with the host cell metabolism for the timescale of the experiments and consequently (iii) the reaction mechanism of the TAm both *in vitro* and *in vivo* are likely to be similar. This justifies the use of whole cells in this work. Similar results were found for the initial rates of Figure 3 comparing lysates and whole cells (data not shown).

4.3. Enzyme stability under bioconversion conditions

An underlying assumption in our methodology is that the TAm activity should be stable for the duration of the progress curve experiments. These are needed in order to determine the remainder of the unknown inhibition and equilibrium constants (Figure 1). Good stability of the TAm biocatalyst would demonstrate that the total concentration of the active enzyme was constant during the complete reaction, and therefore that any decrease in the reaction rate could be attributed to inhibition or equilibrium effects and not to irreversible enzyme deactivation. To test the enzyme stability, the whole cell TAm biocatalyst was incubated with each substrate or product for incubation times spanning the likely duration of a bioconversion reaching completion. The enzyme activity was determined at intervals and is plotted as a function of the incubation time in Figure 6. Forward reaction rate data was used for incubation with ERY or MBA, and reverse reaction rate data for incubation with ABT or AP.

No significant decrease in enzyme activity was detected for up to 35 hr of incubation with ERY, ABT and AP. In contrast there was a 15% decrease in initial reaction rate for the whole cell TAm while incubated with MBA. This was considered acceptable over the extended incubation period for kinetic parameters determination, and care was taken to collect several data points in the early stage of the reaction when the enzyme deactivation would be minimized. When performing the stability studies it was noticed that when incubating the enzyme with MBA, further PLP had to be added after the incubation so that the enzyme activity would be restored. This was not necessary when incubating the enzyme with ERY, AP or ABT. This could be attributed to a full conversion of PLP to PMP through completion of the first half reaction, due to the absence of amino acceptor, as has been shown previously using the CV2025 TAm (Schell *et al.*, 2009).

4.4. Kinetic parameter identification using progress curves

Step 4 in the proposed methodology (Figure 1) involves obtaining complete progress curves at higher substrate concentrations, where equilibrium as well as substrate and product inhibition effects would have a stronger weight. A set of 9 progress curves for the forward reaction, each with 12 sampling points at different intervals were obtained to ensure the accuracy of the model fit to the experimental data. This set was complemented with the addition of 3 reverse reaction progress curves, to ensure that the model would fit both forward and reverse reaction profiles.

The substrate concentrations chosen are shown in Table 1, and included those under which the final bioconversion process might be performed. Also included are experiments covering the lower and higher range of the concentration spectrum, to ensure that the inhibition and equilibrium constants would be accurately determined over the widest possible range of bioconversion conditions.

Initial nonlinear regression analysis highlighted the importance of the data points before 60 min of reaction. These points involving low product concentration were found to be crucial in giving adequate weight to the preliminary parameters k_f, k_r, and the Michaelis constants previously obtained, thus allowing a better convergence of the optimization algorithm. Therefore care was taken to include several points within that time range, in order for the optimization to converge around the vicinity of the preliminary values determined in Section 4.2.

The preliminary results for k_f and k_r, of 97.2 and 13.1 min^{-1}, and for K_{MBA}, K_{ERY}, K_{iMBAc}, K_{AP} and K_{ABT} of 0.5, 101.2, 23.9, 19.6 and 39.4 mM respectively obtained in Section 4.2 were used as fixed values in the full kinetic model (Equation 1). This was done in order to be able to determine the rest of the kinetic constants by nonlinear regression of the progress curves. The equilibrium constant was calculated simultaneously in the iteration process using the Haldane equation :

$$Keq = \left(\frac{k_f}{k_r}\right)^2 \frac{K_{ABT}K_{AP}}{K_{MBA}K_{ERY}} \qquad \text{Equation 4}$$

The two remaining inhibition constants K_{iERY} and K_{iAP} were calculated using the following Haldane equations:

$$K_{iAP} = \frac{K_{eq}k_r K_{ERY}K_{iMBA}}{k_f K_{ABT}} \qquad \text{Equation 5}$$

$$K_{iERY} = \frac{k_f K_{AP}K_{iABT}}{K_{eq}k_r K_{MBA}} \qquad \text{Equation 6}$$

The partial equilibrium constant K_1 for the first half reaction, where MBA reacts with E-PLP to produce AP and E-PMP (Figure 5) was calculated using Equation 7:

$$K_1 = \frac{k_1 k_3}{k_2 k_4} = \frac{K_{iAP}}{K_{iMBA}} \qquad \text{Equation 7}$$

In the same way, the partial equilibrium constant K_2 for the second half reaction, where E-PMP is converted to E-PLP through the formation of ABT from ERY (Figure 5) was obtained using Equation 8:

$$K_2 = \frac{k_5 k_7}{k_6 k_8} = \frac{K_{iABT}}{K_{iERY}} \qquad \text{Equation 8}$$

It should be noted that the overall equilibrium constant K_{eq} can also be expressed as the product of the two partial constants as shown in Equation 9, and the value obtained should be the same as the one obtained with Equation 4.

$$K_{eq} = K_1 K_2 = \frac{[AP]_{eq}[ABT]_{eq}}{[ERY]_{eq}[MBA]_{eq}} \qquad \text{Equation 9}$$

Global optimization was next performed using the pattern search algorithm described in Section 3.6. Following this approach, the optimization solution was relatively independent of the initial values of the inhibition constants, because only 3 parameters needed to be estimated. The preliminary values of K_{iMBA}, K_{iABT} and K_{eq} were determined to be 0.004 and 2.2 mM and 830 respectively. The lower and upper bounds for both nonlinear regressions were set at 0.0001 and 800, and the optimization was always found to converge to the same result for any given initial value within the bounds.

4.5. Reconciliation and final kinetic parameter values

The preliminary kinetic parameters obtained up to this point are believed to be close to the "true" values, yet they still present some inaccuracies because they are based on the catalytic and Michaelis-Menten constants determined using the simplified kinetic models in Equations

2 and 3. Any error obtained using such simplified models in Step 2 would be propagated to the values of all the next parameters determined in Step 4. Therefore in Step 5 of the methodology described in Figure 1, the kinetic constants were reconciled by using the corresponding full kinetic model (Equation 1) combined with nonlinear regression of the 12 progress curves, where all the preliminary parameters were used as initial values for the optimization.

The lower and upper bounds were set as 0.1 and 800 (except for K_{MBA} and K_{iMBA} where the lower bound was set to 0.01 and 0.0001 respectively), and because the initial estimates were close to the final parameter values, the global optimization was rapidly achieved around the vicinity of the initial estimates. The preliminary and "reconciled" kinetic values obtained this way are summarized in Table 2, where a description in terms of rate constants of each parameter is also included. The preliminary and final values were relatively similar with the major difference (33%) was found for the inhibition constant K_{APi}. Such changes to the final values of the kinetic parameters at this point was considered acceptable for a nonlinear regression based methodology (Moros *et al*., 1996; Blackmond, 2005; Chen *et al*., 2008).

In order to perform a complete statistical analysis, the full methodology described in Figure 1 was also performed using the kinetic model without including substrate inhibition by the formation of dead end complexes (Equation 1 without including the last two terms). The final nonlinear regression performed with this model in Step 5 to reconcile the parameters failed to converge successfully. Instead, it reached the upper limit for K_{ERY} and showed relatively high discrepancies between the initial and "reconciled" values (data not shown), presenting a residual of 16.3 compared to 0.55 using the full model of Equation 1. A statistical F-test was performed in order to determine if the addition of the parameter K_{iMBAc} gave a significantly better fit to the progress curve data. The result of the F-test was equal to 15.7, which was higher than the critical value of 3.9 from the F distribution tables with false-rejection probability of 0.05. This confirms that the addition of the parameter K_{iMBAc} was also statistically significant to fit the progress curves. These statistical analyses, in addition to those in Section 4.2, showed that the kinetic model described by Equation 1 represents the best reaction mechanism for describing both initial rate data and the complete progress curves. This finding strongly supports the formation of the abortive complex E-PMP-MBA for the TAm reaction studied here. Further experiments using mass spectrometry would be required to confirm this.

4.6. Validation of kinetic parameters

In order to validate the kinetic parameters obtained in Table 4.2, experimental and predicted data of reactant concentrations as a function of time were compared. Very good agreement was found for all the forward and reverse reaction conditions described in Table 1. Figure 7 shows a set of experimental and model data comparisons for the forward reaction, using progress curves from Table 1 that were used to determine the kinetic parameters. Figure 7(a) specifically compares experimental ABT data points from different progress curves with the predicted model where excellent agreement is seen. Figure 7(b) focuses on the initial period until 60 min where, as described in Section 4.4, several key data points were collected. Good agreement was again found between the experimental values and model predictions for reaction occurring over both short and long periods. Figure 8 shows experimental data points for MBA formation during the reverse reaction conditions described in Table 1. Again excellent agreement is observed between the data and the model.

To further test the kinetic model and the calculated parameters, Figure 9 shows the comparison of model predictions with additional sets of experimental progress curve data (under conditions not included in Table 1 to establish the kinetic parameters). Good agreement is also found between these additional independent progress curves and the model predictions, verifying the appropriate determination of the kinetic model (Equation 1) and its corresponding parameters (Table 2).

5. DISCUSSION

5.1. Kinetic constants

The value of the Michaelis constant of K_{AP} was determined to be two orders of magnitude higher than K_{MBA}. A similar low K_{MBA} and higher K_{AP} value was determined for the ω-TAm from *Vibrio fluvialis* (Shin & Kim, 2002) which shares 38% sequence identity towards the CV2025 TAm (Kaulmann *et al*., 2007). Interestingly, the opposite result was found for the ω-TAm from *Bacillus thuringiensis,* where the Michaelis constant for MBA and AP were two orders of magnitude higher and lower respectively than the ones determined in this work (Shin & Kim, 1998). The Michaelis constant of ERY was 200 times bigger than the one for MBA,

while the Michaelis constant of ABT was half that for AP. In contrast to the reverse reaction, the kinetic parameters of the forward reaction did not follow the general trend where the Michaelis constant of each amino donor were higher than the corresponding amino acceptor (Henson & Cleland, 1964; Christen & Metzler, 1985; Shin & Kim, 1998; Lain-guelbenzu *et al.,* 1991; Lo *et al.,* 2005; Martin *et al.,* 2007).

5.2. Equilibrium constants

The uses of transaminases to synthesize chiral amines have been generally hampered by equilibrium constants near unity or constants which favours the ketone starting material (Taylor *et al*., 1998; Stewart, 2001; Truppo *et al*., 2010). For the conversion studied here however, the value of the partial equilibrium constants K_1 and K_2 for the first and second half reactions were 275 and 3.1 as determined by Equations 7 and 8 respectively. The high value of K_1 has also been suggested in literature for the synthesis of pyridoxamine 5´-phopsate using CV2025 TAm (Schell *et al*., 2009). The values of K_1 and K_2 lead to a global equilibrium constant of 843 (Equation 9), which strongly favoured the asymmetric synthesis of ABT.

In addition, the catalytic constant for the forward reaction was almost one order of magnitude higher than the constant for the reverse reaction (Table 2). Similar differences in catalytic constants have been found for the ω-TAm from *B. thuringiensis* to produce L-alanine from pyruvate and MBA (Shin & Kim, 1998), yet in general these results are not common among other TAms where k_f and k_r do not differ greatly (Henson & Cleland, 1964; Bulos & Handler, 1965; Christen & Metzler, 1985; Kuramitsu *et al*., 1990; Hayashi *et al.,* 1993; Taylor *et al.,* 1998). In general, due to the low equilibrium constants of the TAm bioconversions, the commonly used method in industry for amine synthesis has been by kinetic resolution, which is hampered by a 50% theoretical yield. The previously mentioned results are thus relevant, as they indicate that the kinetics and equilibrium constants of the TAm mediated synthesis of specific amino-alcohols will be suitable for asymmetric syntheses where >95% yields could be achieved directly.

5.3. Bottlenecks of the bioconversion and optimum reaction conditions

After evaluating the kinetic constants in Table 2, and performing a sensitivity analysis for each parameter (data not shown), it was found that one of the key obstacles to improve the amino-alcohol productivity was the high value of K_{ERY} of 96 mM. For similar reaction conditions, Michaelis constants for pyruvate were reported to be one order of magnitude smaller than K_{ERY} using the ω-TAm from *V. fluvialis* and *B. thuringiensis* (Shin & Kim, 1998; Shin, 2002).

The higher stability of the complex E-PLP-MBA compared to E-PMP-ERY was evidenced by the value of the dissociation constant K_{iMBA} of 4.0×10^{-3} mM, which was 3 orders of magnitude smaller compared to the value of K_{iERY} of 1.0 mM. By analyzing the expressions of the parameters as rate constants in Table 2, it can be concluded that the higher values of K_{ERY} and K_{iERY} compared to K_{MBA} and K_{iMBA} make the second half reaction the limiting step of the overall bioconversion. Mutagenesis improving this step would be very beneficial in improving the catalytic rate of the TAm synthesis of ABT, provided that the screening method used specifically covers the second step of the bioconversion.

The value of the kinetic parameter k_f, which is a function of the forward catalytic constant rates k_3 and k_7, was found to be 97.4 min^{-1}. This value of k_f was of the same order of magnitude as that reported for a similar ω-TAm using MBA and pyruvate as substrates (Shin & Kim, 1998; Shin & Kim, 2002), but it was found to be several orders of magnitude lower compared with other classes of TAms when used with natural substrates *ex vivo* (Kuramitsu *et al.*, 1990; Markova *et al.,* 2005). Product inhibition in the forward reaction was found to be not so severe compared to other similar ω-TAm bioconversions (Shin & Kim, 1998; Shin & Kim, 2002). In those works, some of the product inhibition constants were found to be one order of magnitude smaller than the value of K_{iAP} and K_{iABT} of 1.1 and 3.1 mM obtained in this work respectively (Table 2). A low product inhibition of AP and ABT was also suggested in literature using a TAm from *Pseudomonas aeruginosa* for the asymmetric synthesis of ABT *(*Ingram *et al., 2007)*.

Several simulations predicted that high reaction yields of more than 95% should be obtained using high equimolar concentrations of substrates, with the condition that longer reactions times were used than 35 hr. However, the experimental data started to diverge gradually from the predicted model data after longer reaction times (data not shown). This may have been due to general toxicity effects of the MBA on the whole cells or to the irreversible enzyme inactivation by MBA As it was shown in Figure 6, 15 % of the TAm was deactivated after

being incubated for 35 hr with 50 mM MBA. This deactivation percentage was considered negligible for the purposes of determining the kinetic parameters, however the deactivation may become more significant if longer residence times and also higher concentrations of MBA were used. TAm inactivation by the amino donor has also been reported in literature (Yun *et al.*, 2004), and in some cases whole cell TAm biocatalysts have been shown to maintain the TAm more stable versus the inactivation of the amino donor (Yun *et al.*, 2004; Rios-Solis *et al.*, 2011). Concentrations of MBA of up to 50 mM have been used without noticeable toxic effect with growing cells (Shin et al., 1997). Nevertheless in this work concentrations of up to 250 mM where used. Further addition of PLP or TAm (individually) when the reaction stopped did not restore the catalytic activity. In contrast, addition of PLP and TAm (together) did restore partially the activity, suggesting both enzyme and PLP were inactivated or consumed after longer reaction times.

Finally, taking into account all the above findings, it was identified that maintaining a high concentration of ERY while minimizing the concentration of MBA would enhance the final yield of ABT. The simulation in Figure 9 that considered an excess of ERY (220 mM of ERY and 40 mM MBA with 0.4 mg ml^{-1} of TAm) predicted a 99% conversion with respect to the limiting substrate MBA in 500 min corresponding to the maximum theoretical yield achievable for the reaction. In contrast, only a 35% conversion was predicted using an excess of MBA at the same enzyme concentration ((250 mM of MBA and 40 mM ERY with 0.4 mg ml^{-1} of TAm). The experimental results in Figure 9 confirmed the predictions, showing very good agreement between the experimental and predicted data sets.

6. CONCLUSIONS

A systematic, microscale high-throughput procedure has been developed in this work to rapidly determine the kinetic parameters of challenging reversible enzymatic reactions with strong inhibition and where the reaction mechanism has not been previously elucidated. The methodology was applied to the *C. violaceum* TAm mediated synthesis of chiral amino-alcohol ABT, combining traditional initial rates experiments to identify a solution in the vicinity of the global minimum, with nonlinear regression methods to determine the exact location of the solution for kinetic parameter determination. Statistical comparisons of different kinetic models were also included, which allowed new insights into the reaction mechanism to be

determined and in particular the formation of a dead end complex between the amino donor and the cofactor enzyme complex.

Excellent agreement was found between the experimental data and the determined kinetic model, which revealed that the forward reaction was strongly favoured. The optimum conditions to maximize the reaction rate were found to be using an excess of the amino acceptor. Current work is evaluating a reactor configuration which includes a fed batch addition of substrate MBA, to minimize the toxic and inhibitory effect of the amino donor and hence increase bioconversion rate and yield.

7. ACKNOWLEDGEMENTS

The Mexican National Council for Science and Technology (CONACYT), The Mexican Ministry of Public Education (SEP) and The Ministry of Higher Education of Malaysia (MOHE) are acknowledged for support of LR-S and MH. NB and CD acknowledge support from the UCL Department of Biochemical Engineering. The UK Engineering and Physical Sciences Research Council (EPSRC) is thanked for the 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.

REFERENCE

N. Al-Haque, P. A. Santacoloma, W. Neto, P. Tufvesson, R. Gani and J. M. Woodley. A robust methodology for kinetic model parameter estimation for biocatalytic reactions. Biotechnology Progress. 28 (2012) 1186-96.

B. E. C. Banks, K. G. Oldham E. M. Thain, and C. A. Vernon. Glutamic–aspartic transaminase of pig heart muscle. *Nature,* 183 (1959)1187-1187.

K. Bartsch, R. Schneider, and A. Schulz. Stereospecific production of the herbicide phosphinothricin (glufosinate): purification of aspartate transaminase from *Bacillus stearothermophilus*, cloning of the corresponding gene, aspC, and application in a coupled transaminase process. *Applied and Environmental Microbiology,* 62 (1996) 3794-3799.

H. Bisswanger. Enzyme kinetics. Principles and methods. Wiley VCH, 2nd Edition (2008) 138-145.

D. G. Blackmond. Reaction progress kinetic analysis: a powerful methodology for mechanistic studies of complex catalytic reactions. *Angewandte Chemie*, 44 (2005) 4302-4302.

J. Boruwa, J. C. Borah, S. Gogoi, and, N. C. Barua. A short asymmetric total synthesis of chloramphenicol using a selectively protected 1,2-diol. *Tetrahedron Letters,* 46 (2005) 1743-1746.

N. M. Brunhuber and, J. S. Blanchard. The biochemistry and enzymology of amino acid dehydrogenases. *Critical Reviews in Biochemistry and Molecular Biology,* 29 (1994) 415-467.

B. Bulos, and P. Handler. Kinetics of beef heart glutamic-alanine transaminase. *Journal of Biological Chemistry*, 240 (1965) 3283.

Y. P. Chao, Z. J. Lai, P. Chen, and J. T. Chern. Enhanced conversion rate of L-phenylalanine by coupling reactions of aminotransferases and phosphoenolpyruvate carboxykinase in *Escherichia coli* K-12. *Biotechnology Progress,* 15 (1999) 453-458.

B. H. Chen, A. Sayar, U. Kaulmann, P. A. Dalby, J. M. Ward, and J. M. Woodley. Reaction modelling and simulation to assess the integrated use of transketolase and ω-transaminase for the synthesis of an aminotriol. *Biocatalysis and Biotransformation,* 24 (2006) 449-457.

B. H. Chen, E. G. Hibbert, P. A. Dalby, and J. M. Woodley. A new approach to bioconversion reaction kinetic parameter identification. *American Institute of Chemical Engineering Journal*, 54 (2008) 2155–2163.

B. H. Chen, M. Micheletti, F. Baganz, J. Woodley, and G. J. Lye. An efficient approach to bioconversion kinetic model generation based on automated microscale

experimentation integrated with model driven experimental design. *Chemical Engineering Science,* 64 (2009) 403-409.

B. K. Cho, H. Y. Park, J. H. Seo, J. Kim, T. J. Kang, B. S. Lee, and B. G. Kim. Redesigning the substrate specificity of omega-aminotransferase for the kinetic resolution of aliphatic chiral amines. *Biotechnology and Bioengineering*, 99 (2008) 275–284.

P. Christen, and D. E. Metzler. Transaminases. Wiley. New York USA (1998).

D. D. Davies and R. J. Ellis. Glutamic-oxaloacetic transaminase of cauliflower. *Biochemical Journal*, 78 (1961) 623

R. J. Ellis, and D. D. Davies. Glutamic-oxaloacetic transaminase of cauliflower. 1. Purification and specificity. *The Biochemical Journal,* 78 (1961) 615-623.

H. Hayashi, K. Inoue, T. Nagata, S. Kuramitsu, and H. Kagamiyama. *Escherichia coli* aromatic amino acid aminotransferase: characterization and comparison with aspartate aminotransferase. *Biochemistry,* 32 (1993) 12229-12239.

C. P. Henson, and W. Cleland. 1964. Kinetic studies of glutamic oxaloacetic transaminase isozymes. *Biochemistry, ACS Publications,* 3 (1964) 338–345.

C. U. Ingram, M. Bommer, M. E. B. Smith, P. A. Dalby, J. M. Ward, H. C. Hailes, and G. J. Lye. One-pot synthesis of amino-alcohols using a *de-novo* transketolase and b-alanine: pyruvate transaminase pathway in *Escherichia coli*. *Biotechnology and Bioengineering,* 96 (2007) 559-569.

S. W. Kaldor, V. J. Kalish, J. F. Davies, B. V. Shetty, J. E. Fritz, K. Appelt, J. A. Burgess, K. M. Campanale, N. Y. Chirgadze, D. K. Clawson, B. A. Dressman, S. D. Hatch, D. A. Khalil, M. B. Kosa, P. P. Lubbehusen, M. A. Muesing, A. K. Patick, S. H. Reich, K. S. Su, and J. H. Tatlock. Viracept (nelfinavir mesylate, AG1343): a potent, orally bioavailable inhibitor of HIV-1 protease. *Journal of Medicinal Chemistry,* 40 (1997) 3979-3985.

S. Katare, A. Bhan, J. M. Caruthers, W. N. Delgass, and V. Venkatasubramanian. 2004. A hybrid genetic algorithm for efficient parameter estimation of large kinetic models. *Computers and Chemical Engineering,* 28 (2004) 2569-2581.

U. Kaulmann, K. Smithies, M. Smith, H. Hailes, and J. Ward. Substrate spectrum of ω-transaminase from *Chromobacterium violaceum* DSM30191 and its potential for biocatalysis. *Enzyme and Microbial Technology,* 41 (2007):628-637.

D. Koszelewski, K. Tauber, K. Faber and W. Kroutil. Omega-Transaminases for the synthesis of non-racemic alpha-chiral primary amines. *Trends in Biotechnology,* 28 (2010) 324-332.

S. Kuramitsu, K. Hiromi, H. Hayashi, Y. Morino, and H. Kagamiyama. 1990. Pre-steady-state kinetics of *Escherichia coli* aspartate aminotransferase catalyzed reactions and thermodynamic aspects of its substrate specificity. *Biochemistry,* 29 (1990) 5469-5476.

S. Kwon, and S. Ko. Synthesis of statine employing a general syn-amino alcohol building block. *Tetrahedron Letters,* 43 (2002) 639-641.

B. Lain-guelbenzu, J. Cardenas, and J. Muroz-blanco. Purification and properties of L-alanine aminotransferase from *Chlamydomonas reinhardtii*. *Enzyme,* 887 (1991) 881-887.

H. Law, C. Baldwin, B. Chen, and J. Woodley. Process limitations in a whole-cell catalysed oxidation: Sensitivity analysis. *Chemical Engineering Science,* 61 (2006) 6646-6652.

W. Liu, P. E. Peterson, R. J. Carter, X. Zhou, J. A. Langston, A. J. Fisher, and M. D. Toney. 2004. Crystal structures of unbound and aminooxyacetate-*bound Escherichia coli* gamma-aminobutyrate aminotransferase. *Biochemistry,* 43 (2004) 10896-10905.

H. H. Lo, S. K. Hsu, W. D. Lin, N. L. Chan, and W. H. Hsu. Asymmetrical synthesis of L-homophenylalanine using engineered *Escherichia coli* aspartate aminotransferase. *Biotechnology Progress,* 21 (2005) 411-415.

M. E. Lombardo, L. S. Araujo, A. A. Juknat, and A. M. Batlle. Glutamate:4,5-dioxovaleric acid transaminase from *Euglena gracilis*. *Kinetic studies*. *European Journal of Biochemistry,* 182 (1989) 657-660.

G. J. Lye, P. A. Dalby, and J. M. Woodley. Better Biocatalytic Processes Faster: New Tools for the Implementation of Biocatalysis in Organic Synthesis. *Organic Process Research & Development,* 6 (2002) 434-440.

M. Markova, C. Peneff, M. J. E. Hewlins, T. Schirmer, and R. A John. Determinants of substrate specificity in omega-aminotransferases. *Journal of Biological Chemistry,* 280 (2005) 36409-36416.

C.A. Markowski, and Markowski, E.P. Conditions for the effectiveness of a preliminary test of variance. *American Statistician*, 44 (1990) 322–326.

A. R. Martin, D. Shonnard, S. Pannuri, and S. Kamat. Characterization of free and immobilized (S)-aminotransferase for acetophenone production. *Applied Microbiology and Biotechnology,* 76 (2007) 843-851.

P. K. Mehta, T. I. Hale, and P. Christen. Aminotransferases: demonstration of homology and division into evolutionary subgroups. *European Journal of Biochemistry,* 214 (1993) 549-561.

R. Moros, H. Kalies, H. G. Rex, and S. Schafvarczy. 1996. A genetic algorithm for generating initial parameter estimations for kinetic models of catalytic processes. *Computers and Chemical Engineering,* 20 (1996) 1257–1270.

Y. Ni, and R. R. Chen. Accelerating whole-cell biocatalysis by reducing outer membrane permeability barrier. *Biotechnology and Bioengineering,* 87 (2004) 804-811.

P. W. van Ophem, S. D. Erickson, A. Martinez del Pozo, I. Haller, B. T. Chait, T. Yoshimura, K. Soda, D. Ringe, G. Petsko and J. M. Manning. Substrate inhibition of d-amino acid transaminase and protection by salts and by reduced nicotinamide adenine dinucleotide: isolation and initial characterization of a pyridoxo intermediate related to inactivation. *Biochemistry***,** *37 (*1998) 2879-2888.

F. Ranaldi. What students must know about the determination of enzyme kinetic parameters. *Biochemical Education*, 27 (1999) 87-91.

L. Rios-Solis, M. Halim, A. Cázares, P. Morris, J. M. Ward, H. C. Hailes, P. A. Dalby, F. Baganz, and G. J. Lye. A toolbox approach for the rapid evaluation of multi-step enzymatic syntheses comprising a “mix and match” *E. coli* expression system with microscale experimentation. *Biocatalysis and Biotransformation*, 29 (2011) 192-203.

M. D. Rozwadoska. An efficient synthesis of S-(+)-amphetamine. *Tetrahedron: Asymmetry,* 4 (1993)1619-1624.

U. Schell, R. Wohlgemuth, and J. M. Ward. Synthesis of pyridoxamine 5′-phosphate using an MBA:pyruvate transaminase as biocatalyst. *Journal of Molecular Catalysis B: Enzymatic,* 59 (2009) 279-285.

I. H. Segel. Enzyme kinetics: behavior and analysis of rapid equilibrium and steady state . Wiley New York USA (1975).

J. S. Shin, and B. G. Kim. Kinetic resolution of alpha-methylbenzylamine with omicron-transaminase screened from soil microorganisms: application of a biphasic system to overcome product inhibition. *Biotechnology and Bioengineering,* 55 (1997) 348-358.

J. S. Shin, and B. G. Kim. Exploring the active site of amine:pyruvate aminotransferase on the basis of the substrate structure-reactivity relationship: how the enzyme controls substrate specificity and stereoselectivity. *Journal of Organic Chemistry*, 67 (2002) 2848-2853.

J. S. Shin, and B. G. Kim. Kinetic Modeling of ω-Transamination for Enzymatic Kinetic Resolution of a-Methylbenzylamine. *Biotechnology and Bioengineering,* 60 (1998) 534-540.

J. S. Shin, and B. G. Kim. Substrate Inhibition Mode of ω -Transaminase from *Vibrio fluvialis* JS17 is dependent on the chirality of substrate. *Biotechnology,* 77 (2002) 882-887.

M. E. B. Smith, B. H. Chen, E. G. Hibbert, U. Kaulmann, K. Smithies, J. L. Galman, F. Baganz, P. A. Dalby, H. C. Hailes, G. J. Lye, J. M. Ward, J. M. Woodley, and M. Micheletti. A multidisciplinary approach toward the rapid and preparative-scale biocatalytic synthesis of chiral amino alcohols: a concise transketolase-/ω-transaminase-mediated synthesis of (2 S ,3 S)-2-aminopentane-1,3-diol. *Organic Process Research and Development,* 14 (2010) 99-107.

K. Smithies, M. E. B. Smith, U. Kaulmann, J. L. Galman, J. M. Ward, and H. C. Hailes. 2009. Stereoselectivity of an ω-transaminase-mediated amination of 1,3-dihydroxy-1-phenylpropane-2-one. *Tetrahedron: Asymmetry,* 20 (2009) 570-574.

J. D. Stewart. Dehydrogenases and transaminases in asymmetric synthesis. *Current Opinion in Chemical Biology,* 5 (2001) 120-129.

P. P. Taylor, D. P. Pantaleone, R. F. Senkpeil, and I. G. Fotheringham. Novel biosynthetic approaches to the production of unnatural amino acids using transaminases. *Trends in Biotechnology,* 16 (1998) 412-418.

M. D. Truppo, J. D. Rozzell, and N. J. Turner. 2010. Efficient Production of Enantiomerically Pure Chiral Amines at Concentrations of 50 g/L Using Transaminases. *Organic Process Research & Development,* 14(2010) 234-237.

J. M. Woodley. Choice of biocatalyst form for scalable processes. *Biochemical Society Transactions,* 34 (2006) 301-303.

D. R. Yazbeck, C. A. Martinez, S. Hu and J. Tao. Challenges in the development of an efficient enzymatic process in the pharmaceutical industry. *Tetrahedron: Asymmetry*, 15 (2004) 2757-2763.

H. Yun, B. K. Cho, and B. G. Kim. Kinetic resolution of (R,S)-sec-butylamine using omega-transaminase from *Vibrio fluvialis* JS17 under reduced pressure. *Biotechnology and* Bioengineering, 87 (2004) 772-778.

H. Yun, B. Y. Hwang, J. H. Lee, and B. G. Kim. Use of Enrichment Culture for Directed evolution of the Vibrio fluvialis JS17 transaminase which is resistant to product inhibition by aliphatic ketones. Applied and Environmental Microbiology, 71 (2005) 4220-4224.

LIST OF TABLE LEGENDS

Table 1. Initial substrate and enzyme concentrations used for experimental progress curve determination (data obtained was used to determine the kinetic parameters in Steps 4 and 5 of the methodology described in Figure 1). The first 9 progress curves represent forward reaction bioconversions, while the last 3 represent reverse reaction bioconversions.

Table 1. Initial and final (reconciled) values of apparent kinetic parameters. Values were obtained in Step 5 of Figure 1 using the full kinetic model represented by Equation 1.

LIST OF SCHEME LEGENDS

Scheme 1. Reaction scheme of the TAm mediated asymmetric synthesis of chiral amino-alcohol (2*S*,3*R*)-2-amino-1,3,4-butanetriol (ABT), from substrates L-erythrulose (L-ERY) and (S)-(-)-α-methylbenzylamine (MBA). PLP is cofactor pyridoxal 5′-phosphate and AP refers to acetophenone.

LIST OF FIGURE LEGENDS

Figure 1. Overview of the systematic procedure for rapid kinetic parameter determination for a reversible bioconversion. The approach is illustrated for the whole cell TAm mediated synthesis of ABT from ERY and MBA (Scheme 1).

Figure 2. Initial rate of TAm mediated synthesis of ABT (Scheme 1) as a function of TAm concentration in whole cell form. Reaction conditions: 0.2 mg ml^{-1} [TAm] in whole cell form, 10 mM [MBA] and 50 mM [ERY], 0.2 mM PLP, 30^{o}C and pH 7.5 in 200 mM HEPES buffer. Solid line fitted by linear regression (R^2 0.998). Error bars represent one standard deviation about the mean (n=3).

Figure 3. Initial rate of the forward reaction for the TAm mediated synthesis of ABT (Scheme 1) as a function of substrate concentration: (●) [MBA] varied while maintaining initial [ERY] at 200 mM, (Δ) [ERY] varied while maintaining initial [MBA] at 10 mM. In all experiments 0.2 mg ml^{-1} [TAm] in whole cell form was used with 0.2 mM [PLP], 30^{o}C and pH 7.5 in 200 mM HEPES buffer.

Figure 4. Apparent initial rate of the TAm reverse reaction for the synthesis of ABT and MBA as function of the concentration of substrates: (Δ) varying [ABT] while maintaining [AP] fixed at 35 mM using pure enzyme and (▲) TAm in whole cell form, (○) varying [AP] while maintaining [ABT] fixed at 100 mM using pure enzyme and (●) TAm in whole cell form. In all experiments 0.2 mg ml^{-1} of TAm in whole cell or pure form was used with 0.2 mM PLP, 30^{o}C and pH 7.5 in 200 mM HEPES. Error bars represent one standard deviation about the mean (n=3).

Figure 5. Proposed King-Altman figure for the TAm mediated synthesis of ABT. Solid lines represent the basic kinetic model without the formation of abortive complexes. The dashed arrows represent substrate inhibition via formation of dead end complex E-PMP-MBA.

Figure 6. Evaluation of whole cell TAm biocatalyst stability: forward initial reaction rates after incubation with (●) 50 mM [ERY] or (○) 50 mM [MBA]; reverse initial reaction rates after incubation with (▼) 100 mM [ABT] or (Δ) 35 mM [AP]. In all experiments 0.2 mg ml^{-1} of TAm in whole cell form was used with 0.2 mM PLP, 30ºC and pH 7.5 in 200 mM HEPES buffer. Reaction conditions for forward reaction were 50 mM equimolar concentration of substrates, and for the reverse reaction 100 mM [ABT] or 35 mM [AP] were added to start the reaction. Error bars represent one standard deviation about the mean (n=3).

Figure 7. Example experimental and fitted progress curves for the forward reaction indicating ABT formation (a) for the entire bioconversions and (b) for the first 50 min of reaction. Initial substrate concentrations were (●) 70 mM [ERY], 250 mM [MBA] and 0.4 mg ml^{-1} [TAm], (▲) 50 mM [ERY], 100 mM [MBA] and 0.3 mg ml^{-1} [TAm] and (■) 10 mM [ERY], 120 mM [MBA] and 0.3 mg ml^{-1} [TAm]. Dotted lines show fitted model based on Equation 1 and final kinetic parameters in Table 2. For all the experiments 0.2 mM PLP was used at 30ºC and pH 7.5 in 200 mM HEPES buffer. Error bars represent one standard deviation about the mean (n=3).

Figure 8. Example experimental and fitted progress curves for the reverse reaction following MBA formation with initial substrate concentrations of: (○) 10 mM [AP], 120 mM [ABT] and 0.15 mg ml^{-1} [TAm], (Δ) 20 mM [AP], 200 mM [ABT] and 0.44 mg ml^{-1} [TAm], (□) 40 mM [AP], 100 mM [ABT] and 0.3 mg ml^{-1} [TAm]. For all the experiments 0.2 mM PLP was used at 30ºC and pH 7.5 in 200 mM HEPES buffer. Dotted lines show model predictions based on Equation 1 and final kinetic parameters in Table 2.

Figure 9. Examples of model verification comparing model predictions with additional experimental data sets. Progress curves for TAm mediated synthesis of ABT using initial concentrations of: (▲) 220 mM [ERY] and 40 mM [MBA], (○) 250 mM [MBA] and 40 mM [ERY]. Dashed lines represent kinetic model predictions based on Equation 1 and the parameters listed in Table 2. [TAm] concentration was 0.4 mg ml^{-1} in whole cell form, 0.2 mM PLP, 30ºC and pH 7.5 in 200 mM HEPES buffer. Error bars represent one standard deviation about the mean (n=3).