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Modelling and optimisation of the one-pot, multi-enzymatic synthesis of chiral amino-alcohols based on microscale kinetic parameter determination ☆

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HIGHLIGHTS

- Integration of numerical methods and microscale tools for kinetic characterisation.
- Synthetic biology principles for 'mix and match' expression of pairs of enzymes.
- Model predictions for the multi-enzymatic syntheses were verified experimentally.
- Reaction simulations were used to identify key process bottlenecks.
- Optimum conditions for fed-batch bioreactor operation were identified.

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ABSTRACT

Advances in synthetic biology are facilitating the *de novo* design of complex, multi-step enzymatic conversions for industrial organic synthesis. This work describes the integration of multi-step enzymatic pathway construction with enzyme kinetics and bioreactor modelling, in order to optimise the synthesis of chiral amino-alcohols using engineered *Escherichia coli* transketolases (TK) and the *Chromobacterium violaceum* transaminase (TAm). The specific target products were (2*S*,3*S*)-2-aminopentane-1,3-diol (APD) and (2*S*,3*R*)-2-amino-1,3,4-butanetriol (ABT). Kinetic models and parameters for each of the enzymatic steps were first obtained using automated microwell experiments. These identified the TK-catalysed conversions as being up to 25 times faster than the subsequent TAm conversions and inhibition of TAm by the amino-donor used, (*S*)-(−)-α-methylbenzylamine (MBA), as limiting the overall conversion yields. In order to better 'match' the relative rates of the two enzymes an *E. coli* expression system, based on two compatible plasmids, was constructed to produce both enzymes in a single host. By control of induction time and temperature it was possible to produce six times more recombinant TAm than TK to help balance the reaction rates. To overcome MBA inhibition and an unfavourable reaction equilibrium, fed-batch addition of the amino-donor was introduced as well as the use of isopropylamine as an alternate amino-donor. Adopting these strategies, and using the kinetic models to optimise feeding strategies, the one pot syntheses of APD and ABT were successfully scaled-up to preparative scales. Excellent agreement was found between the kinetic profiles and yields predicted and those achieved experimentally at the larger scale. In this case the integration of these multi-disciplinary approaches enabled us to achieve up to a 6 fold greater yield using concentrations an order of magnitude higher than in previous preparative scale batch bioconversions carried out sequentially.

1. Introduction

With the recent advances in synthetic biology (McArthur and Fong, 2010), there is increasing interest in the design of multi-step enzymatic conversions for the synthesis of speciality chemicals and pharmaceutical intermediates. Irrespective of whether such *de*

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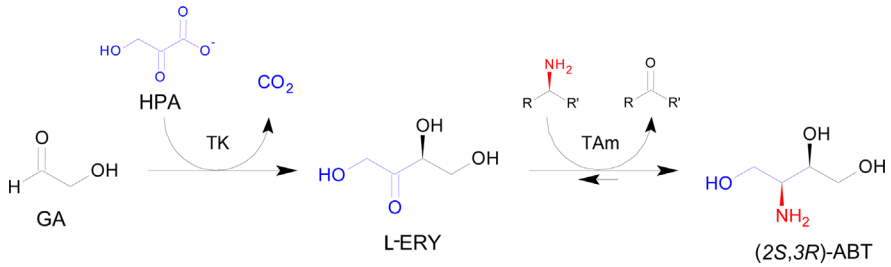
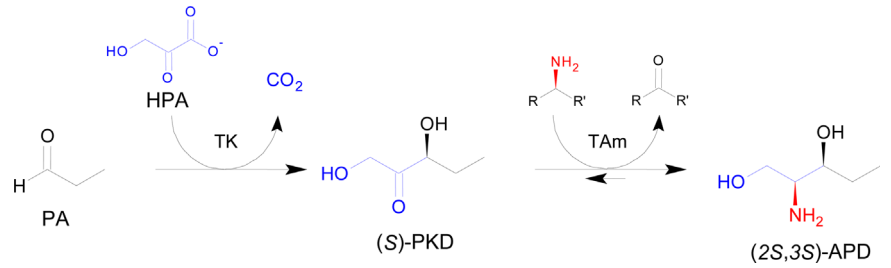


Scheme 1. Reaction scheme of the *de-novo* transketolase (TK)–transaminase (TAm) pathway for the synthesis of chiral amino-alcohol (2*S*,3*R*)-2-amino-1,3,4-butanetriol (ABT), from achiral substrates glycolaldehyde (GA) and hydroxypyruvate (HPA). TK intermediate product L-(+)-erythrulose (ERY). Amino-donor (*S*)-(−)-α-methylbenzylamine (MBA) R=Ph and R′=Me, isopropylamine (IPA) R=R′=Me.



Scheme 2. Reaction scheme of the *de-novo* TK (D469E)-TAm pathway for the synthesis of chiral amino-alcohol (2*S*,3*S*)-2-aminopentane-1,3-diol (APD) from achiral substrates propionaldehyde (PA) and hydroxypyruvate (HPA). TK intermediate product is (3*S*)-1,3-dihydroxypentan-2-one (PKD). Amino-donor (*S*)-(−)-α-methylbenzylamine (MBA) R=Ph and R′=Me, isopropylamine (IPA) R=R′=Me.

novo pathways are ultimately applied *in vivo* (as a whole cell biocatalyst) or *in vitro* (as isolated or immobilised enzymes), there is a need to understand the mechanisms of the enzymes involved and to model reaction kinetics in order to optimise the overall conversion yield. This can be particularly challenging when designing synthetic routes involving engineered enzymes and when using non-natural reactants as frequently encountered in industrial syntheses (Meyer et al., 2007). In these cases appropriate reaction mechanisms must first be established and then kinetic parameters determined for multiple enzymes and a variety of potential starting reactants and pathway intermediates.

A particular target for *de novo* designed multi-step enzymatic conversions is the synthesis of complex chiral compounds from simple starting reactants (Roessner and Scott, 1996; Prather and Martin, 2008; Dalby et al., 2009). Ideally such reactants would be cheap, achiral and available from renewable sources (Keasling, 2010). Products such as chiral amino-alcohols are of considerable industrial interest and represent an important target for multi-step enzymatic syntheses. They are useful building blocks in the synthesis of a range of optically pure pharmaceuticals such as HIV protease inhibitors (Kaldor et al., 1997; Kwon and Ko, 2002), active molecules such as (*S*)-amphetamine (Rozwadoska, 1993) or broad spectrum antibiotics like chloramphenicol and thiamphenicol (Bhaskar, 2004).

Previously we have established a series of automated, microwell-based methods to inform early stage bioconversion process design and aid in quantitative prediction of larger scales process kinetics (Lye et al., 2003; Micheletti and Lye, 2006). We have also established 'mix and match' expression systems to rapidly trial pairs of enzymes for use in *de novo* engineered pathways. The utility of this approach was illustrated for a sequential, two-step synthesis comprising carbon-carbon bond formation using a transketolase (TK), followed by a transaminase (TAm) to create chiral amino-alcohols from achiral substrates (Rios-Solis et al., 2011). In that work the wild type *Escherichia coli* TK and the *Chomobacterium violaceum* 2025 (CV2025) TAm were selected for the one-pot synthesis of diastereoisomer (2*S*,3*R*)-2-amino-1,3,4-butanetriol (ABT) (Scheme 1), while the mutant *E. coli* TK D469E and the CV2025 TAm were selected for the synthesis of (2*S*,3*S*)-2-aminopentane-1,3-diol (APD) (Scheme 2). Nevertheless, the kinetic modelling and optimisation of multi-step biocatalytic processes has been poorly explored, due to the complexities of integrating the kinetic models for multiple enzymes and the number of kinetic parameters involved (Xue and Woodley, 2012). Improved modelling and simulation would enable hypothetical changes to be explored in *silico*, speeding up process development and allowing the evaluation of process control strategies, to ensure stability and the desired efficiency (Sin et al., 2009).

The aim of this work was to establish detailed kinetic models for each of the individual enzymatic reactions, exploiting our previously developed experimental methodologies, in order to optimise the overall conversion yields of ABT and APD (Schemes 1 and 2). Kinetic models for the various TK and TAm bioconversions were first obtained from microscale experimental data using previously established numerical techniques for rapid kinetic parameter determination (Chen et al., 2009; Rios-Solis et al., 2013). These combine 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 thus reducing the number of experiments required. Once established, the kinetic models were validated against preparative scale (50 mL) bioconversion data before being used to identify key reaction constraints (Pollard and Woodley, 2007) and simulate scenarios for optimal bioreactor operation. These were again verified experimentally illustrating the importance of kinetic modelling to underpin the design and optimisation of multi-enzymatic systems in synthetic biology.

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).