



# Characterization and multi-step transketolase- $\omega$ -transaminase bioconversions in an immobilized enzyme microreactor (IEMR) with packed tube<sup>☆</sup>



Amanatuzzakiah Abdul Halim<sup>a,b</sup>, Nicolas Szita<sup>a</sup>, Frank Baganz<sup>a,\*</sup>

<sup>a</sup> The Advanced Centre for Biochemical Engineering, University College London, Torrington Place, London WC1E 7JE, United Kingdom

<sup>b</sup> Biotechnology Engineering Department, International Islamic University Malaysia (IIUM), P.O. Box 10, 50728 Kuala Lumpur, Malaysia

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## ABSTRACT

The concept of *de novo* metabolic engineering through novel synthetic pathways offers new directions for multi-step enzymatic synthesis of complex molecules. This has been complemented by recent progress in performing enzymatic reactions using immobilized enzyme microreactors (IEMR). This work is concerned with the construction of *de novo* designed enzyme pathways in a microreactor synthesizing chiral molecules. An interesting compound, commonly used as the building block in several pharmaceutical syntheses, is a single diastereoisomer of 2-amino-1,3,4-butanetriol (ABT). This chiral amino alcohol can be synthesized from simple achiral substrates using two enzymes, transketolase (TK) and transaminase (TAm). Here we describe the development of an IEMR using His<sub>6</sub>-tagged TK and TAm immobilized onto Ni-NTA agarose beads and packed into tubes to enable multi-step enzyme reactions. The kinetic parameters of both enzymes were first determined using single IEMRs evaluated by a kinetic model developed for packed bed reactors. The  $K_{m(app)}$  for both enzymes appeared to be flow rate dependent, while the turnover number  $k_{cat}$  was reduced 3 fold compared to solution-phase TK and TAm reactions. For the multi-step enzyme reaction, single IEMRs were cascaded in series, whereby the first enzyme, TK, catalyzed a model reaction of lithium-hydroxypyruvate (HPA) and glycolaldehyde (GA) to L-erythrulose (ERY), and the second unit of the IEMR with immobilized TAm converted ERY into ABT using (S)- $\alpha$ -methylbenzylamine (MBA) as amine donor. With initial 60 mM (HPA and GA each) and 6 mM (MBA) substrate concentration mixture, the coupled reaction reached approximately 83% conversion in 20 min at the lowest flow rate. The ability to synthesize a chiral pharmaceutical intermediate, ABT in relatively short time proves this IEMR system as a powerful tool for construction and evaluation of *de novo* pathways as well as for determination of enzyme kinetics.

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## 1. Introduction

Microreactor technology has immense potential to notably improve chemical and biological analyses as well as abundant applications in medical diagnostics and therapeutic devices. Owing to their miniature size, they require only a small volume of reagent, shorter processing time and can be parallelized using lab-on-a-chip technology (Hong et al., 2009; Mark et al., 2010).

The integration of immobilized enzymes in microreactor systems has great impact in many emerging technologies employing

biocatalytic and biorecognition events. Several reports show that a microreactor can be an ideal platform for enzyme reactions (Hickey et al., 2007, 2009; Ngamsom et al., 2010; Alam et al., 2011; Matsuura et al., 2011). Rapid screening can be done with the capacity of multiple usage of the immobilized enzyme (Forsberg et al., 2004; Matosevic et al., 2011a). In addition to that, enzymes are found to gain higher stability facilitating their application under harsh environmental conditions of temperature, pH and organic solvents (Sakai-Kato et al., 2002; Nomura et al., 2004; Berne et al., 2006). Enzyme immobilization may also result in changes in enzymatic activity, optimum pH or affinity for the substrate (Krenková and Foret, 2004; Ladero et al., 2006) and must be considered when designing enzyme microreactors.

Several enzyme immobilization methods are available. Nidetzky (2013) have summarized typical techniques for preparation of enzymes immobilized in microreactors. A generically applicable and well established approach for the immobilization of oriented

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\* Corresponding author.

E-mail address: [f.baganz@ucl.ac.uk](mailto:f.baganz@ucl.ac.uk) (F. Baganz).

protein molecules is the use of recombinant tags, particularly poly-histidine tags (His-tag). This technique stemmed from immobilized metal ion affinity chromatography (IMAC) and has been applied extensively to protein immobilization (Block et al., 2009; Cheung et al., 2012). It offers fast and specific binding and allows protein immobilization without pre-purification steps. Moreover, the His-tag does not interfere with the structure or function of proteins and does not affect the secretion, compartmentalization, or folding of fusion proteins within cells (Cha et al., 2004). Application of the immobilization of protein molecules via His-tag has been successfully demonstrated in numerous studies. Site-specific immobilization which orientates all proteins uniformly was performed by Abad et al. (2005), whereby His-tagged horseradish peroxidase and ferredoxin- NADP<sup>+</sup> reductase confirmed full functional attachment of the protein to the NTA-Co(II)-matrix. Immobilization of His-tagged *Rhodotorula gracilis* D-amino acid oxidase (RgDAO) on Ni-NTA magnetic beads was demonstrated by Kuan et al. (2008). The immobilization of RgDAO enhanced its stability against thermal inactivation where the relative activity of the immobilized enzyme was 56% while the free enzyme was completely inactivated after incubation at 50 °C for 1 h. The residual activity and the storage stability were also improved in the immobilized form of the enzyme. The work by Cha et al. (2005) showed that sulfotransferases enzymes immobilized through poly-His tag exhibited similar activities as in the free solution as a result of highly specific orientation offered by the His-tag linkage.

We recently developed an IEMR based on the reversible immobilization of His-tagged enzyme via Ni-NTA linkage to surface derivatized silica (Matosevic et al., 2009). This IEMR was first characterized and used to quantify the immobilized transketolase kinetics in a stop-flow mode. In the current work, we focus on a single diastereoisomer of 2-amino-1,3,4-butanetriol (ABT). This chiral amino alcohol is one of the building blocks of several antibiotics, antivirals and sphingolipids (Contestabile and John, 1996). ABT can be produced using two enzymes, transketolase (TK) and transaminase (TAm), that catalyze asymmetric carbon-carbon bond formation and amine group addition, respectively (Fig. 1a). Studies have been carried out by our research group to screen for different enzyme combinations to synthesize the optically pure amino alcohol. Ingram et al. (2007), demonstrated the synthesis of ABT, where *E. coli* TK converts achiral lithium-hydroxypyruvate (HPA) and glycolaldehyde (GA) into L-erythrose (ERY), and subsequently β-alanine:pyruvate TAm from *Pseudomonas aeruginosa* catalyzes the reaction between (S)-α-methylbenzylamine (MBA) and Ery, producing ABT in a one-pot synthesis. In addition, Rios-Solis et al. (2011) used wild-type *E. coli* TK paired with an ω-TAm from *Chromobacterium violaceum* 2025 (Kaulmann et al., 2007) to synthesize ABT in a whole-cell system. And more recently Matosevic et al. (2011b), further investigated this reaction in an IEMR. To demonstrate the feasibility of performing the continuous flow TK-TAm enzymatic reaction using immobilized enzymes Matosevic et al. (2011b) derivatized the inner surface of a fused silica capillary (“open-tubular” reactor).

In this work, we characterize a packed tube IEMR prototype to further facilitate the *in vitro* study of this dual enzyme pathway in a continuous flow system. This microreactor system, based on the reversible immobilization of His<sub>6</sub>-tagged enzymes was implemented for the quantitative evaluation of TK-TAm bioconversion kinetics. Higher loading of enzyme was achieved using microbeads compared to the previous system (Matosevic et al., 2011b) which were packed into a tube that works as a packed bed reactor. The strong coupling method produces a highly stable and reusable IEMR. First, the stability of both enzymes on the immobilized form was evaluated and reusability of the microreactor was demonstrated over several cycles of immobilization and elution. The IEMR prototype was then used for determining the kinetic parameters

for both TK and TAm enzymes before demonstrating the synthesis of the chiral amino alcohol ABT by coupling the TK and TAm IEMRs.

## 2. Materials and methods

### 2.1. Reagents and chemicals

All reagents were of analytical grade and were obtained from Sigma-Aldrich (Gillingham, UK) unless stated otherwise. Reverse osmosis (RO) water was used in all of the experiments.

### 2.2. Biocatalyst production

#### 2.2.1. Inoculum preparation

Stocks of *E. coli* BL21gold (DE3) containing plasmid pQR791 (His<sub>6</sub>-TK) (Martinez-Torres et al., 2007) or pQR801 (His<sub>6</sub>-TAm) (Kaulmann et al., 2007) (previously cloned from expression constructs provided by Prof. John Ward, Department of Biochemical Engineering, UCL) were used. Cells were grown for 16 h at 37 °C on agar plate containing LB-glycerol nutrient broth (yeast extract 5 g/L, tryptone 10 g/L, NaCl 10 g/L, glycerol 10 g/L), 15 g/L agar and 0.15 g/L selection antibiotic ampicillin (TK cultures) or kanamycin (TAm cultures). A single colony was inoculated into a sterile 250 mL shake flask containing 20 mL of the LB-glycerol medium and grown on an orbital shaker (Stuart Scientific, Redhill, UK) at 250 rpm for 12–16 h at 37 °C.

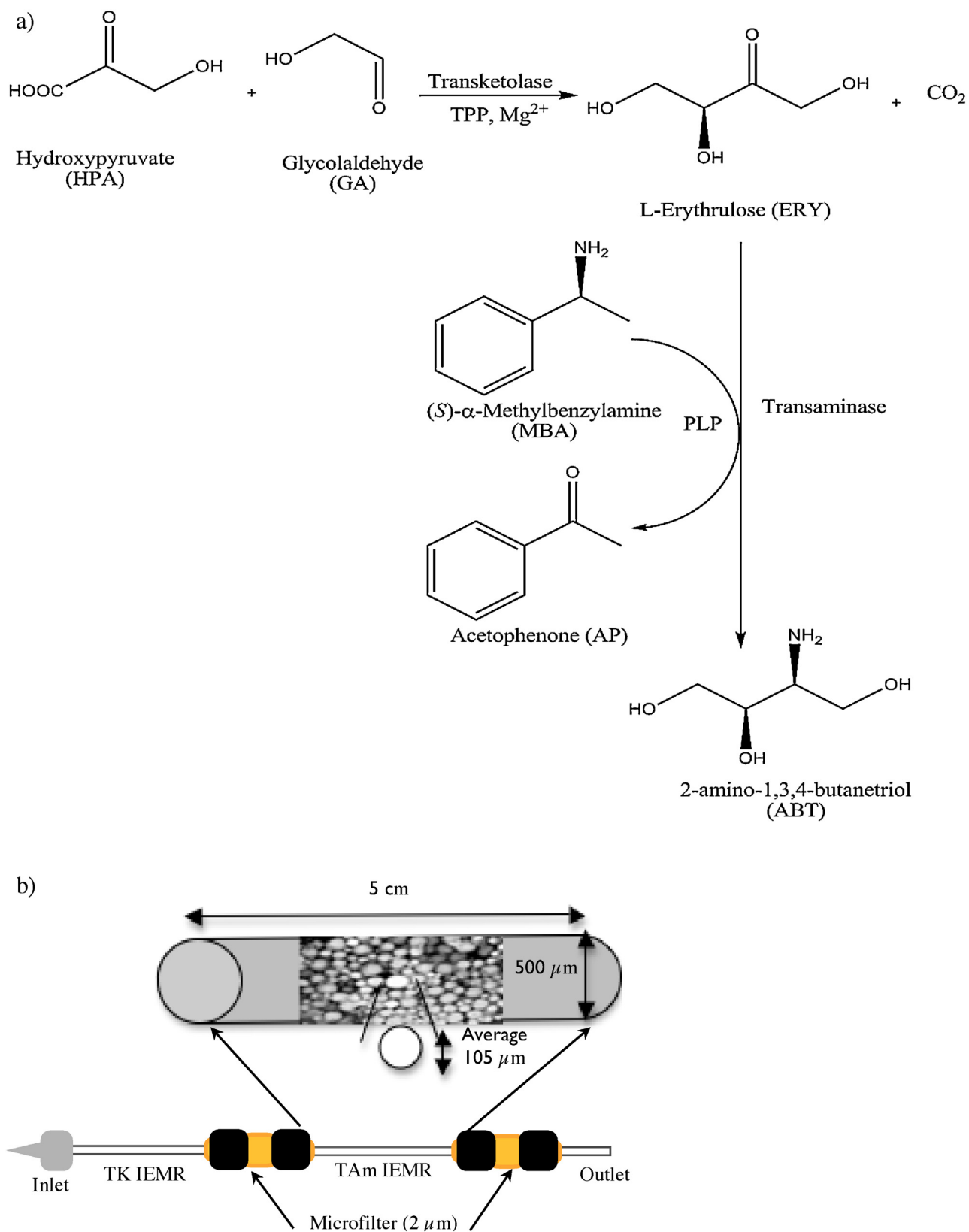
#### 2.2.2. Enzyme production

20 mL of inoculum prepared earlier was transferred into 1 L shake flask containing 180 mL of LB-glycerol with 0.15 g/L antibiotic. The culture was incubated for 7–8 h at 37 °C and 250 rpm on an orbital shaker. For TAm production isopropyl β-D-1-thiogalactopyranoside (IPTG) of 0.2 mM was added after 3 h of fermentation at optical density (OD): 0.6. The OD<sub>600nm</sub> of the solution was measured every hour to check bacterial growth until it reached stationary phase. Following the fermentation, culture broth was centrifuged at 4000 rpm for 10 min at 4 °C. The supernatant was discarded and the pellets were stored at –20 °C.

#### 2.2.3. Quantification of protein concentration

2.2.3.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and densitometry analysis. Pre-cast gels (1.0 mm x 12 wells) of SDS 12% Tris-Glycine (Novex, Paisley, UK) were used for SDS-PAGE protein analysis as described by King and Laemmli (King and Laemmli, 1971). Gels were run in Tris-Glycine buffer (Novex, Paisley, UK) using a Mini-155 Protean II system (Bio-Rad Laboratories Inc., Hemel Hempstead, UK) at a power of 80–150 V for about 90 min. The gel was then rinsed twice for 5 min with 100 mL of RO water. Next, the gel was stained with sufficient SimplyBlue SafeStain (~20 mL) (Novex, Paisley, UK) to cover the gel for 1 h at room temperature with gentle shaking. Then the gel was de-stained with de-staining buffer (40%, v/v methanol, 10%, v/v acetic acid and 50%, v/v RO water) for 2–3 h. Finally, the mini gel was washed with 100 mL of RO water for 1–3 h.

2.2.3.2. Protein concentration determination. Quantification of protein was carried out by UV absorbance of the purified enzyme at 280 nm using ATI Unicam UV/VIS spectrophotometer (Spectronic, Leeds, UK). The extinction coefficient used were 93,905 M cm<sup>-1</sup> for transketolase and 78,000 M cm<sup>-1</sup> for transaminase. The Bradford protein assay was carried out based on the method of Bradford (Bradford, 1976). Bradford Reagent was used with bovine serum albumin (BSA) as the standard protein. Absorbance measurements



**Fig. 1.** (a) Reaction scheme for the synthesis of 2-amino-1,3,4-butanetriol (ABT) by the TK–TAm enzyme pathway. Dual enzyme pathway showing the TK-catalyzed production of L-erythrulose (ERY) followed by the TAm-catalyzed production of ABT. (b) Schematic illustration of the IEMR used for analysis of TK and TAm reactions. An IEMR with inner diameter (ID) of 500  $\mu\text{m}$  and length of 5 cm gives a total reaction volume of  $\sim 10 \mu\text{L}$ . The prototype consisted of single IEMRs that were cascaded in series to allow multi-step enzymatic reaction. TK catalyzed the reaction of hydroxypyruvate (HPA) and glycolaldehyde (GA) to L-erythrulose (ERY), and the second unit of the IEMR with immobilized TAm converted ERY into ABT using (S)- $\alpha$ -methylbenzylamine (MBA) as amine donor.

at 595 nm were carried out on an ATI Unicam UV/VIS spectrophotometer (Spectronic, Leeds, UK).

### 2.3. Purification of His<sub>6</sub>-tagged enzymes

Frozen cell pellets from the fermentation were thawed at room temperature. The pellet was resuspended in a small quantity of 50 mM Tris-HCl (1:25, v/v) and vortexed. The suspension was sonicated on ice using a Soniprep 150 sonicator (MSE, Sanyo, Japan, 10 cycles of 10 s ON, 10 s OFF). The solution was centrifuged using a Hettich Universal 320R mid-bench centrifuge (DJB Labcare, Newport Pagnell, UK) for 10 min at 4000 rpm and 4 °C. The supernatant was decanted and the pellets were discarded. The supernatant was filtered using a 0.2 µm pore filter (GE Healthcare, Buckinghamshire, UK). Purification of His<sub>6</sub>-tagged enzymes was performed using Ni-NTA superflow cartridges (Qiagen, Sussex, UK). The cartridge was equilibrated with 10 mL of binding buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl and 10 mM imidazole). The clarified lysate was loaded into the cartridge and subsequently washed with 20 mL of wash buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl and 20 mM imidazole). The enzyme was eluted with 2.5 mL of elution buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl and 250 mM imidazole).

### 2.4. Microreactor set-up and bioconversions

#### 2.4.1. Packing of a FEP tube with microbeads

HIS-Select Nickel Affinity agarose beads (Sigma–Aldrich, UK) with diameter of 45–165 µm were used as the packing material. They were first washed with 50 mM of Tris-HCl and left in suspension in the same buffer. A Nanobaume (Presearch Ltd., UK) pressurized container was used to pack the beads in the tube. The bead slurry containing 5% (v/v) beads was placed in a glass vial with total volume of 1.5 mL. A stir bar was put in the vial and the vial was placed into the pressurized container. The container was then set on top of a magnetic stirrer. The fluorinated ethylene propylene (FEP) tube (500 µm ID, Presearch Ltd., UK) was attached to the container while a microfilter assembly (2 µm, Presearch Ltd., UK) was connected at the end of the tube. A two-way valve was connected to the pressurized container while the other end was connected to a nitrogen gas cylinder. A pressure of 5 bar was applied to the system and the pressure drop (0.6–1 bar) was monitored by the pressure gauge installed. The packing took 5–10 min.

#### 2.4.2. Enzyme immobilization and elution

The packed tube IEMR was first equilibrated with binding buffer (500 mM NaCl, 20 mM Tris-HCl, 5 mM imidazole; pH 7.0 or 7.5) at 5 µL/min for 15 min. Enzyme concentrations used were 0.05–2.0 mg/mL. The purified enzyme (100 µL) was loaded into the tube at 5 µL/min and was left to incubate for an additional 1 h following immobilization. Non-specific binding of the enzyme on the beads was removed by treating the tube with washing buffer (50 mM Tris-HCl; pH 7.0 or 7.5) at 25 µL/min for 20 min. Bound enzyme was removed by treating the tube with elution buffer (1 M imidazole, 500 mM NaCl and 20 mM Tris-HCl; pH 7.0 or 7.5) at 5 µL/min for 10 min.

#### 2.4.3. Transketolase reaction in IEMR

The packed tube IEMR (5 cm length) was connected to a KDS100 syringe pump (KD Scientific, Holliston, MA, USA) on one end and connected to a microfilter assembly (2 µm, Presearch Ltd., UK) at the outlet. The TK reaction was carried out at room temperature in 50 mM Tris-HCl buffer (pH 7.0). One unit of TK enzyme was defined as the amount that catalyses the formation of 1 µmol of erythrose (ERY) per minute using 50 mM of glycolaldehyde (GA) and lithium-hydroxypyruvate (HPA) at pH 7.0. TK reactions were conducted using model substrates, 50 mM of HPA and 50 mM

of GA unless stated otherwise. The enzyme was incubated with cofactors, thiamine pyrophosphate (TPP) of 2.4 mM and MgCl<sub>2</sub> of 9 mM at 5 µL/min for 30 min to reconstitute the holoenzyme. Substrate solutions of HPA and GA were loaded continuously at various flow rates and 20 µL samples were taken from the outlet of the tube at regular time intervals. The samples were quenched with 0.1% trifluoroacetic acid (TFA) and analyzed by HPLC. Reactions were monitored for EYR production by HPLC assay.

#### 2.4.4. Transaminase reaction in IEMR

The reaction was carried out at room temperature in 50 mM Tris-HCl buffer (pH 7.5). One unit of TAm enzyme was defined as the amount that catalyses the production of 1 µmol of acetophenone (AP) per minute using 100 mM L-erythrose (ERY) and 10 mM (S)-α-methylbenzylamine (MBA) at pH 7.5. TAm reactions were conducted using model substrates, 10 mM of MBA and 100 mM of ERY unless stated otherwise. Substrate solutions of MBA and ERY and 0.2 mM cofactor pyridoxal-5'-phosphate (PLP) were loaded continuously at various flow rates and 20 µL samples were taken from the outlet of the tube at regular time intervals. The samples were quenched with 0.1% TFA and analyzed by HPLC. Reactions were monitored for acetophenone (AP) production by HPLC assay.

#### 2.4.5. Coupled TK–TAm reaction in IEMR

The set-up of the IEMR cascade is shown in Fig. 1b. The enzyme immobilization of individual IEMRs was conducted separately before assembly. Two separate (2 cm × 5 cm) packed tubes of IEMR were connected in series where the beads were retained using a microfilter assembly (2 µm, Presearch Ltd., UK). The second packed tube IEMR was connected to the first using a microferrule (Presearch Ltd.). The two-tube system was connected to a KDS100 syringe pump (KD Scientific, Holliston, MA, USA) so that the inlet of the first tube was directly connected to the pump while its outlet was used to collect the samples for further off-line HPLC analysis. Coupled reactions were carried out using the TK–TAm model reaction. Solution substrates of GA, HPA and MBA at desired concentrations and cofactors for both enzymes at pH 7.5 were loaded together into the first tube containing packed beads immobilized with TK. The erythrose required by the immobilized TAm was provided by the TK reaction step. The reaction was carried out continuously at flow rates between 1 and 30 µL/min. 20 µL samples were taken at different time intervals and quenched with 0.1% TFA. Reactions were monitored for ABT and AP production by HPLC assay.

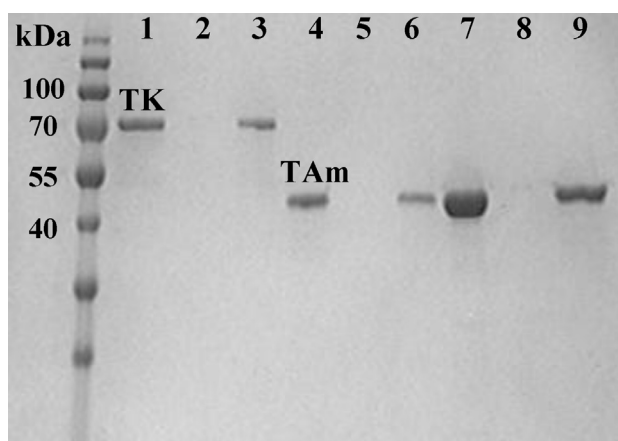
### 2.5. HPLC analysis

#### 2.5.1. HPLC assay for TK

HPLC analysis was conducted using a microbore HPLC system (Dionex, Surrey, UK) controlled by Chromeleon 6.8 software with a multiwell plate autosampler (Spark, Emmen, Holland). The HPLC system contained an Aminex 87H column (Bio-Rad, Hertfordshire, UK) and analysis of ERY was conducted at 60 °C using a mobile phase of 0.1% (v/v) TFA in water at 0.6 mL/min. Detection was at 210 nm and the retention time was around 11.5 min for ERY.

#### 2.5.2. HPLC assay for TAm

AP and ABT were analyzed using an ACE5 C18 reverse phase column (150 mm × 4.6 mm, Advanced Chromatography Technologies, Aberdeen, UK) on the Dionex HPLC system. The mobile phase for AP detection comprised of 0.1% TFA at 1.0 mL/min with a gradient of acetonitrile from 15% to 72% over 9 min. The gradient was followed by a 2 min equilibration. UV detection was carried out at 250 nm with retention time for AP was around 7.3 min. For ABT analysis, the samples were derivatized by addition of an excess of



**Fig. 2.** SDS-PAGE gel of enzyme immobilization-elution process. Purification performed as described in Section 2.3. (1) Purified TK (0.2 mg/mL); (2) flow through from the IEMR; (3) eluted TK (0.1 mg/mL); (4) purified TAm (0.2 mg/mL); (5) flow through from the IEMR; (6) eluted TAm (0.1 mg/mL); (7) purified TAm (1.9 mg/mL); (8) flow through from the IEMR; (9) eluted TAm (1.1 mg/mL).

6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. The derivatizing reagent was made in house based on Cohen and Michaud (1993) protocol and the HPLC method reported by Ingram et al. (2007) was used.

### 3. Results and discussion

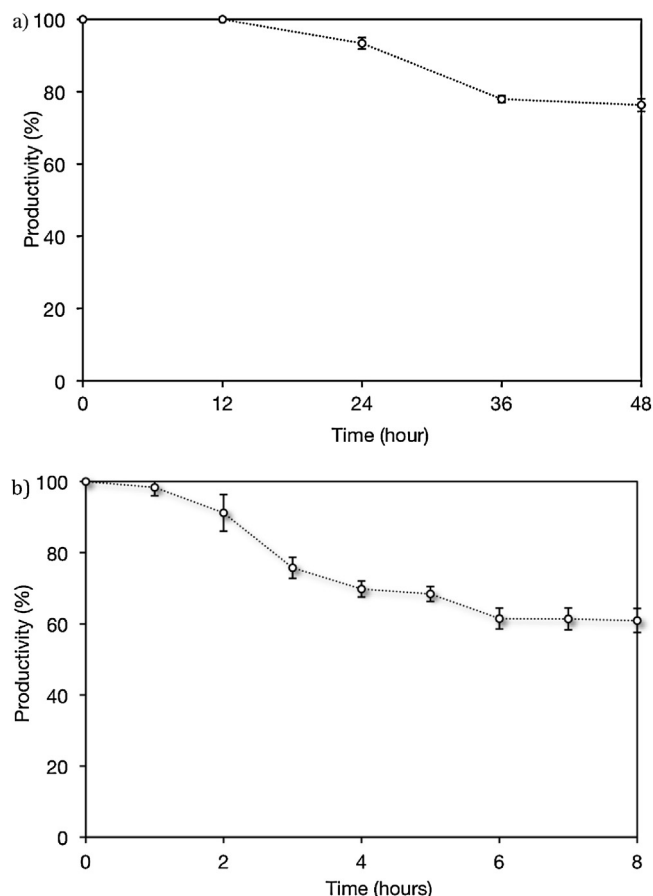
#### 3.1. Microreactor set-up and enzyme immobilization

A schematic illustration of the IEMR used in this study is shown in Fig. 1b. Packing of the tube was carried out using a Nanobaume pressurized container. This method produced consistent and reproducibly packed tubes according to microscope images. The dense packing in the tube was formed as a result of the compressibility of the agarose beads used.

Purified enzymes were used in all immobilization processes. Samples collected from the feed and elution steps were run on SDS-PAGE and quantified by the Bradford assay. The eluted enzyme purity was >95%, observed as a single band on the gel for TK and TAm (Fig. 2). Specific activity of purified TK and TAm calculated were  $20.3 \pm 4.5$  U/mg and  $0.13 \pm 0.025$  U/mg respectively. Following immobilization of 0.2 mg/mL of the enzyme feed,  $0.1 \pm 0.01$  mg/mL (10  $\mu$ g) of the enzyme was recovered during the elution process. This shows approximately 50% of the enzyme feed was retained inside the microreactor while the remaining proportion was removed during the washing process. In order to test if the maximum enzyme loading capacity was reached, the concentration of TAm in the feed was increased to 1.9 mg/mL, which resulted in a 10-fold increase of immobilized enzyme in the microreactor ( $1.1 \pm 0.15$  mg/mL). This suggests that enzyme loading increases gradually with higher enzyme concentration in the feed until it reaches the maximum capacity of the beads. The IEMR with higher TAm loading was later used in the dual enzyme reaction. In addition, no adsorption of protein on the surface of the FEP tube was detected when a similar immobilization-washing-elution process was conducted on an empty tube. This eliminates any errors that might mask the actual enzyme activity in the IEMR.

#### 3.2. Operational and storage stability of IEMR

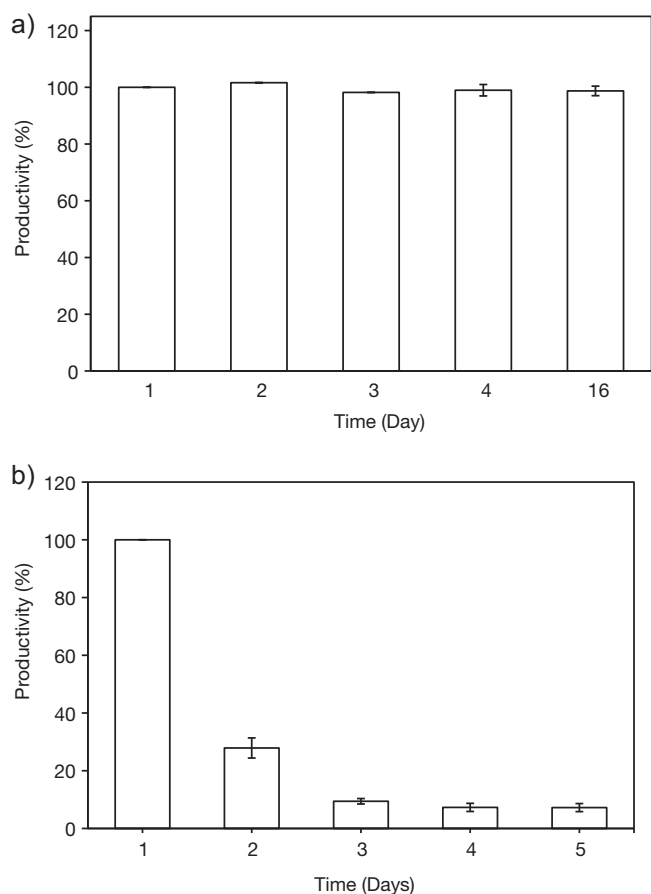
The operational stability of the immobilized TK was evaluated continuously for 48 h at a flow rate of 1  $\mu$ L/min. Samples were taken every 12 h to measure the conversion of HPA and GA to ERY product. Immobilized TK was found to remain stable during the first 12 h of



**Fig. 3.** Operational stability of TK and TAm in IEMR. The productivity is defined as the amount of product generated over 12 h (TK) or 1 h (TAm) (per amount of enzyme) divided by the product formed of the starting enzyme activity determined in the same way. A productivity of 100% indicates no deactivation and degradation of the immobilized enzyme in the reactor. (a) Continuous reaction of TK in packed tube IEMR at 1  $\mu$ L/min for 48 h. (b) Continuous reaction of TAm in packed tube IEMR at 1  $\mu$ L/min for 8 h. Concentration of immobilized TK and TAm was  $\sim 0.2$  mg/mL and 0.3 mg/mL respectively. Error bars represent one standard deviation about the mean ( $n=3$ ).

reaction with 100% productivity (Fig. 3a). However the productivity dropped to 76% after 48 h of reaction. A similar test was performed with immobilized TAm where the productivity was determined continuously every hour at 1  $\mu$ L/min. In contrast to TK, after only 8 h of continuous reaction, the productivity of the immobilized TAm was reduced to 60% (Fig. 3b).

The storage stability of immobilized TK in the packed tube stored at 4  $^{\circ}$ C in 50 mM of Tris-HCl was measured over a period of 16 days. Immobilized TK retained almost 100% productivity after 16 days, when compared to the first day of reaction (Fig. 4a). This consistency in activity of the immobilized TK enzyme, proved the high stability of TK in the immobilized form whilst  $\sim 8\%$  of activity drop was observed with free enzyme (data not shown). By contrast, immobilized TAm only retained  $\sim 7\%$  of its initial productivity after 5 days (Fig. 4b) compared to  $\sim 85\%$  residual activity in free enzyme (data not shown). The sharp decrease in productivity showed that immobilization significantly affect enzyme stability (Krenková and Foret, 2004; Ladero et al., 2006). This low catalytic stability of TAm was most likely due to the alteration in the three-dimensional conformation of the protein upon immobilization, which led to rapid inactivation of TAm after repeated use. Besides that, the stability of an enzyme was found to be highly dependent on the type of immobilization support used.  $\omega$ -TAm from *Vibrio fluvialis* immobilized on chitosan beads exhibited higher stability compared to Eupergit<sup>®</sup> C,



**Fig. 4.** Storage stability at 4 °C in 50 mM Tris-HCl of (a) TK IEMR and (b) TAm IEMR. The productivity of the IEMR was determined according to the standard TK and TAm assays (Sections 2.4.3 and 2.4.4). The productivity is defined as the amount of product generated (per amount of enzyme) divided by the product formed of the starting enzyme activity determined in the same way. Slight fluctuations may be due to small differences in the concentration of the immobilized enzyme. Concentration of immobilized TK and TAm was ~0.2 mg/mL and 0.3 mg/mL respectively. Error bars represent one standard deviation about the mean ( $n=3$ ).

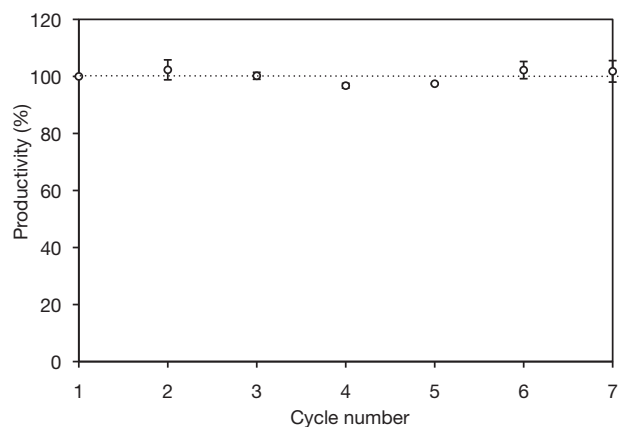
whereby 70% of residual activity was retained after 3.5 weeks and only 10% for the latter one (Yi et al., 2007).

### 3.3. Reusability of IEMR

The reusability of the microreactor was investigated by using the same packed tube reactor in seven TK immobilization-elution processes. The activity of the immobilized enzyme was then tested to verify that the repeated immobilization-elution process in the microreactor does not affect the activity of the enzyme. Based on the results shown in Fig. 5, the productivity of the IEMR was retained over seven cycles of the immobilization-elution process within a 4-week period. This is similar to the findings from work of Matosevic et al. (2009), however the reusability of the packed tube was made simpler compared to the surface derivatized capillary as it does not require a regeneration step before the subsequent immobilization process. The same concentration of enzyme was used throughout the experiments to allow a direct comparison between each cycle of the immobilization.

### 3.4. TK and TAm kinetics in IEMR

The kinetic parameters of the immobilized enzyme may result in different values compared to the solution-phase reaction due to mass transfer effects and enzyme deactivation (Kerby et al., 2006;



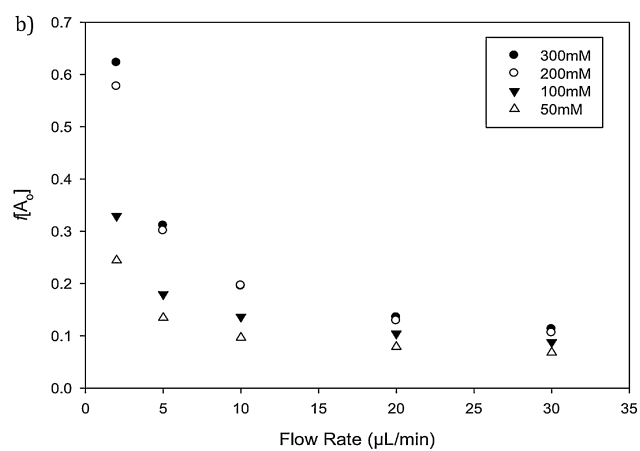
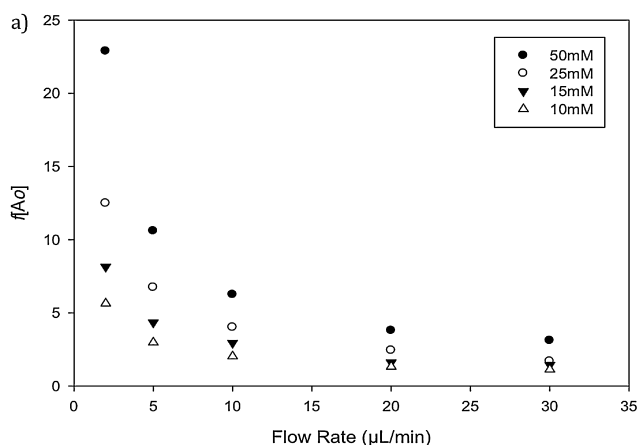
**Fig. 5.** Reusability test of the IEMR. Reusability of packed tube was measured by productivity of the immobilized TK over seven cycles of immobilization-elution processes using a single IEMR. Productivity of the IEMR was measured according to the standard TK assay (Section 2.4.3) over a 4-week period. Slight fluctuations may be due to small differences in the concentration of the immobilized enzyme. Concentration of immobilized TK and TAm was ~0.2 mg/mL and 0.3 mg/mL respectively. Error bars represent one standard deviation about the mean ( $n=3$ ).

Ozdural et al., 2001). Under these conditions, apparent kinetic constants are used to evaluate the kinetics of the immobilized enzyme. The Lilly et al. (1966) model is commonly employed to determine kinetic parameters for continuous packed-bed reactors. Furthermore, the model is able to account for any mass transfer effects that may be present and can alter the overall enzyme kinetics. Under steady state conditions the apparent kinetic parameters in a packed-bed enzyme reactor can be determined by the following equation:

$$f[A_0] = K_{m(app)} \ln(1-f) + \frac{C}{Q}$$

where  $f$  is the fraction of substrate reacted,  $[A_0]$  is the initial substrate concentration,  $K_{m(app)}$  is the apparent Michaelis constant,  $C$  is the reactor capacity and  $Q$  is the flow rate.

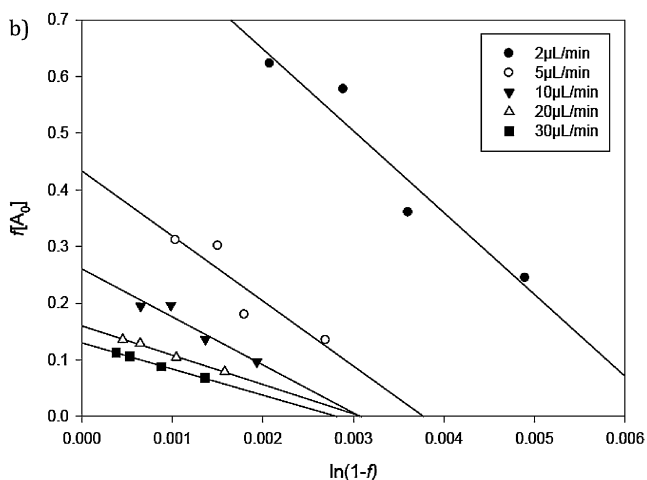
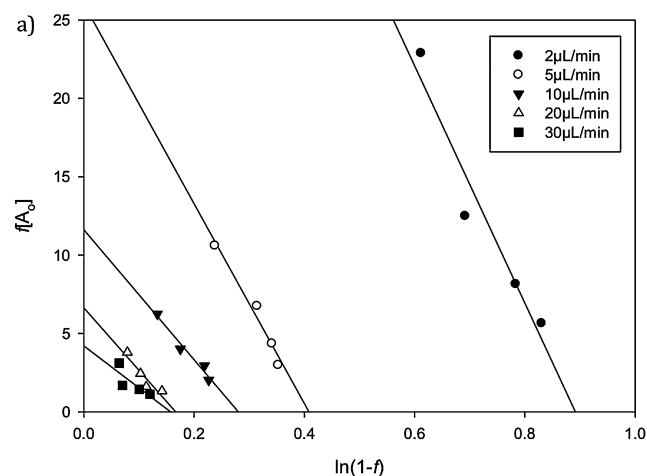
Determination of kinetic parameters of the continuous flow mode packed tube IEMR was conducted for both enzymes at flow rates ranging from 2 to 30  $\mu\text{L}/\text{min}$ . For TK, the concentration of GA was varied from 10 to 50 mM while HPA was kept at 100 mM and for TAm the concentration of ERY was varied from 50 to 300 mM with MBA kept at 10 mM. The progression curve for each substrate concentration was shown to follow the standard TK and TAm reaction profile in solution-phase, with conversion yield increasing proportionately to substrate concentration (Fig. 6). Data from these graphs were then used to plot the Lilly et al. (1966) kinetic model and determine the kinetic parameters for the continuous flow reaction (Fig. 7). The slopes of the fitted lines were evaluated to give the  $K_{m(app)}$  for each flow rate. From Fig. 8, a pronounced effect of flow rate was observed on  $K_{m(app)}$ , where the values increased with lower flow rates. This can be explained by the presence of a diffusion layer surrounding the immobilized enzyme (Lilly et al., 1966). At high flow rates, the mass transfer effect is less pronounced as the diffusion layer around the bead gets thinner, while the effect is more significant at lower flow rates, which could be observed as an elevation of the  $K_{m(app)}$  values. This diffusion layer theory fits the trend displayed in Fig. 8, where an approximately linear decrease was seen for  $K_{m(app)}$  between 2 and 10  $\mu\text{L}/\text{min}$ , and then the values started to level-off between 10 and 30  $\mu\text{L}/\text{min}$ . Toda (1975) addressed the problem of inter-particle mass transfer in connection with the overall kinetics of gel-entrapped enzymes packed in a fixed bed reactor. He suggested that the increase in the  $K_{m(app)}$  value at a low flow rate is due to film diffusion or liquid channelling factor. In addition, several experimental studies have shown



**Fig. 6.** Bioconversions using different substrate concentration at flow rates ranging from 2  $\mu\text{L}/\text{min}$  to 30  $\mu\text{L}/\text{min}$ . (a) Continuous-flow TK-catalyzed reaction at different GA concentration (10–50 mM) with HPA concentration was kept at 100 mM. (b) Continuous-flow TAM-catalyzed reaction at different ERY concentration (50–300 mM) with MBA concentration was kept at 10 mM. Concentration of immobilized TK and TAM was  $\sim 0.05$  mg/mL and 0.4 mg/mL respectively.

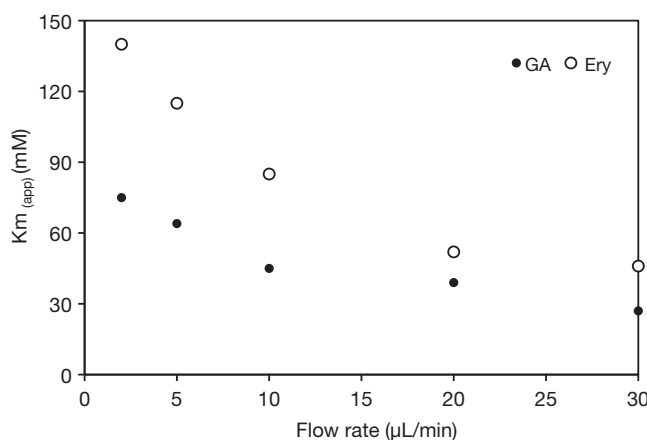
similar flow dependency of  $K_{m(\text{app})}$  (Lilly et al., 1966; Ozdural et al., 2001; Shiraishi et al., 1996). They explained the importance of mass transfer outside a solid catalyst with the fact that the apparent Michaelis constant determined in a packed column reactor varied with the flow rate of the liquid. On the contrary, Matosevic et al. (2011b) found an increase in  $K_{m(\text{app})}$  with increasing flow rate of the substrate through a surface derivatised capillary containing immobilized TK enzyme. With lower operating flow rates of 0.33–3  $\mu\text{L}/\text{min}$  and reduced enzyme concentration compared to the packed tube reactor, the mass transfer effect observed was possibly not as pronounced as in our system.

The reactor capacity ( $C$ ) values obtained were nearly constant for all experiments, with an average of  $12.88 \pm 0.27$  mM/min and  $0.28 \pm 0.027$  mM/min for GA and ERY, respectively. These values were then used to determine the turnover number,  $k_{\text{cat}}$  which was  $32 \text{ s}^{-1}$  for TK and  $0.058 \text{ s}^{-1}$  for TAM. These values were approximately 3 times less than  $k_{\text{cat}}$  values in solution-phase (data not shown). This apparent loss in activity may be due to the inaccessibility of some enzyme molecules that were immobilized in the packed tube, to the substrate molecules. The intrinsic compressibility of porous agarose beads resulted in highly compact packing, which may have created a barrier to the accessibility of the substrate to those enzyme molecules located within the beads packing. In addition, non-uniform enzyme distributions within the support would also affect the apparent kinetic parameters where the catalyst immobilized on the surface can render far greater enzyme

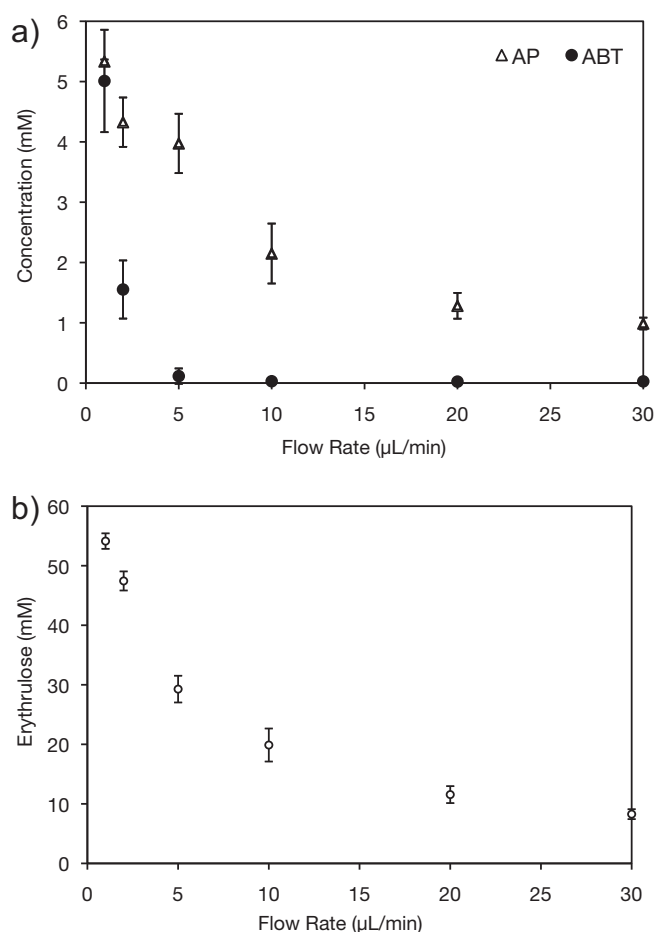


**Fig. 7.** Kinetics determination using Lilly–Hornby model for continuous-flow mode reaction. Data to obtain these plots was taken from Fig. 6. (a) TK-catalyzed reaction and (b) TAM-catalyzed reaction. Solid lines fitted by linear regression. Values of  $K_{m(\text{app})}$  and  $C$  for the continuous flow reactions could be obtained from these plots.

utilization efficiency compared to the catalyst in the interior core configuration (Dalvie and Baltus, 1992; Juang and Weng, 1984). Furthermore, altered conformation of enzyme and steric hindrance could also contribute to the loss of activity of the immobilized enzyme (Delouise and Miller, 2005; Kerby et al., 2006; Krenková and Foret, 2004). Overall, further investigations are required to



**Fig. 8.**  $K_{m(\text{app})}$  values for GA (TK-catalyzed reaction) and ERY (TAM-catalyzed reaction) ranging from 2  $\mu\text{L}/\text{min}$ –30  $\mu\text{L}/\text{min}$ . Data to obtain this plot was taken from Fig. 7.



**Fig. 9.** Multi-step TK-TAm reaction in IEMR. (a) Multi-step TK-TAm reaction with initial substrate concentration of 60 mM (HPA and GA) and 6 mM (MBA), and (b) single TK IEMR running with similar condition as in coupled reaction. The concentration of immobilized enzyme was  $1.1 \pm 0.15$  mg/mL for TAm and  $0.15 \pm 0.006$  mg/mL for TK. Error bars represent one standard deviation about the mean ( $n = 3$ ).

evaluate the effect of the external and internal mass transfer on the enzyme behaviour in this packed tube IEMR.

### 3.5. Demonstration of dual enzyme reaction: TK-TAm bioconversion

The multi-step enzyme reaction was executed by connecting single TK and TAm IEMR in series (see Fig. 1b). The substrates used for each enzyme were a mixture of 60 mM (GA and HPA, each) and 6 mM (MBA), were fed together in the presence of cofactors. The coupled reaction was performed at pH 7.5, which was previously shown to be suitable for both enzymes (Kaulmann et al., 2007; Mitra et al., 1998). In the first reaction, TK catalyzed the conversion of the achiral substrates HPA and GA to form ERY as a product, which was then fed to the second IEMR. The dual reaction was completed with the bioconversion of ERY and MBA by TAm into AP and ABT.

With initial substrate concentration of 6 mM MBA, the coupled reaction reached approximately 83% conversion in 20 min at the lowest flow rate (Fig. 9a). This corresponds to a specific activity of  $0.03 \mu\text{mol}/\text{min}/\text{mg}_{\text{TAm}}$ . However, this analysis only gives information on the final product yield of the coupled reaction. In order to estimate the conversion yield of GA and HPA to ERY in the first reaction a separate experiment was conducted. A single IEMR was prepared with immobilized TK. Similar enzyme and substrate concentrations were used to match the condition in the coupled reaction. Based on Fig. 9b, the reaction reached a conversion of

90% in 10 min at the lowest flow rate with a specific activity of  $8.20 \mu\text{mol}/\text{min}/\text{mg}_{\text{TK}}$ . This suggested that a total of 54 mM ERY was fed to the second IEMR in the coupled reaction. Therefore, about 9% of the relative amount of ERY supplied by the TK step was aminated by TAm in the second reaction. Based on these results, it is apparent that transaminase has a much slower conversion rate compared to the transketolase enzyme. A similar trend was also observed in the rates of catalysis of the two enzymes in batch solution as well as studies with whole cells (Ingram et al., 2007; Matosevic et al., 2011; Rios-Solis et al., 2011). Nevertheless, low specific activity of TAm was not only due to the slow amination rate of the enzyme but also due to the presence of side reactions with residual HPA and GA. Kaulmann et al. (2007) reported that TAm has the affinity to catalyze the amination of both HPA and GA leading to the reduced formation of ABT at higher flow rates when higher residual concentrations of HPA and GA from the TK-catalyzed reaction were present.

## 4. Conclusion

The packed tube microreactor system described here represents a prototype IEMR that allows multi-step enzymatic synthesis of complex molecules such as ABT. Characterization of the IEMR was first demonstrated using TK and TAm enzymes. Storage stability tests of both enzymes were performed and results showed that immobilized TK retained its activity up to 16 days, while immobilized TAm lost ~93% of its activity after 5 days. Furthermore, the reusability of the IEMR was tested, whereby a single IEMR could be used for at least seven TK immobilization-bioconversion-elution processes without having any reduction in binding property and enzyme activity.

In addition, kinetic properties of immobilized TK and TAm were determined in continuous flow mode at flow rates ranging from  $2 \mu\text{L}/\text{min}$  to  $30 \mu\text{L}/\text{min}$ . The kinetic analysis relied on the Lilly et al. (1966) model, which has been previously used to evaluate apparent kinetic parameters in packed-bed enzyme reactors under steady-state conditions.  $K_{m(\text{app})}$  for all substrates appeared to be flow rate dependent indicating that for these heterogeneous enzyme reactions, the  $K_{m(\text{app})}$  value may be affected by mass transfer resistance that masked the actual enzyme kinetics. The increase in  $K_{m(\text{app})}$  at low flow rates was possibly due to the diffusional resistance imposed by the liquid layer surrounding each particle. Besides that, the turnover rate  $k_{\text{cat}}$  was found to be significantly lower than that in solution-phase. Imperfect immobilization, altered enzyme conformation and steric hindrance were suggested to be the causes for the observed difference.

In the final part of the work, we demonstrated the TK-TAm coupled reaction in continuous flow mode to synthesize the chiral amino alcohol, ABT. Based on the solution-phase data, specific activity of TAm was found to be significantly lower than that of TK. Based on previous results (Matosevic et al., 2011b), it was expected that the amination rate of TAm would be slower compared to the TK catalyzed reaction in the two-step enzymatic conversion. Therefore a 7-fold higher TAm concentration ( $1.1 \pm 0.15$  mg/mL) was loaded compared to  $0.15 \pm 0.006$  mg/mL of TK to compensate for the much lower reaction rate of TAm, which improved the yield of the coupled reaction synthesizing ABT. Moreover, while the overall reaction was mainly affected by the low catalytic activity of TAm, the amount of ERY produced in the first step of the reaction was also affecting the rate of amination in the TAm step as well as minimizing the side reaction with residual substrates from the TK step. Therefore, identifying the best matching enzyme concentration for loading would be a suitable strategy to optimize this dual-step enzyme reaction.

The packed tube IEMR described here has shown the ability to synthesize the chiral pharmaceutical intermediate, ABT. The facile



His tag-immobilization method provides a stable heterogeneous catalyst, which can be easily incorporated into a FEP tube and is cost-effective due to the low material cost and reusability property of the IEMR. The versatility of the immobilization method makes the system amenable to a wide range of biocatalysts to be integrated in order to create a variety of synthetic pathways for rapid synthesis and screening purposes. Finally, this packed tube IEMR system could potentially be used as model for a microfluidic reactor on a chip (Boehm et al., 2013).

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