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Microfluidic multi-input reactor for biocatalytic synthesis using transketolase☆

James Lawrence[a], Brian O'Sullivan[a], Gary J. Lye[a], Roland Wohlgemuth[b], Nicolas Szita[a,*]

[a] Department of Biochemical Engineering, University College London, Torrington Place, London WC1E 7JE, UK
[b] Sigma–Aldrich, Industriestrasse 25, 9470 Buchs, Switzerland

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ABSTRACT

Biocatalytic synthesis in continuous-flow microreactors is of increasing interest for the production of specialty chemicals. However, the yield of production achievable in these reactors can be limited by the adverse effects of high substrate concentration on the biocatalyst, including inhibition and denaturation. Fed-batch reactors have been developed in order to overcome this problem, but no continuous-flow solution exists. We present the design of a novel multi-input microfluidic reactor, capable of substrate feeding at multiple points, as a first step towards overcoming these problems in a continuous-flow setting. Using the transketolase-(TK) catalysed reaction of lithium hydroxypyruvate (HPA) and glycolaldehyde (GA) to L-erythrulose (ERY), we demonstrate the transposition of a fed-batch substrate feeding strategy to our microfluidic reactor. We obtained a 4.5-fold increase in output concentration and a 5-fold increase in throughput compared with a single input reactor.

1. Introduction

Microfluidic reactors are of increased interest for preparative synthetic work due to a variety of advantages such as improved heat and mass transfer, mixing, safety, process intensification, scalability and reproducibility. As such they are being applied to a growing number of reactions in research and industry [1–4]. The use of microreactors for catalytic asymmetric synthesis has a high potential among the many different methodologies for the efficient and sustainable synthesis of valuable chiral compounds with improved control of reaction conditions and fast optimisation [5]. As biocatalytic asymmetric synthesis has proven to be a sustainable and viable methodology in organic synthesis and industrial manufacturing processes [6–9], efforts to combine the advantages of biocatalysis and micro-reactor technology in a mutually beneficial way have started to create numerous new application areas of interest, including resolution of racemic mixtures, reactions using hazardous substrates or two-phase systems [10–21].

Transketolases have been shown to be useful biocatalysts for creating new carbon–carbon bonds between aldehydes and the irreversible two-carbon donor lithium hydroxypyruvate (HPA) with high selectivity, broad substrate specificity and a high degree of conversion. They therefore represent a highly attractive and versatile biocatalytic platform technology with an ever-increasing range of applications [22–27]. Since the scale-up to viable manufacturing processes requires also work on the process design, reaction engineering and the optimisation of reaction conditions [28,29], the application of microreactor technology to transketolase-catalyzed carbon-carbon bond formation reactions is of fundamental interest [30].

In a previous publication, we have described the cascading of a microfluidic reactor and a filtration system for biocatalytic asymmetric synthesis using transketolase [31]. The system was able to fully convert HPA to the chiral product L-erythrulose (ERY) with high enantiomeric purity, as well as separating out the transketolase (TK) from the product, synthesising a pure pharmaceutical intermediate in a continuous-flow setting. However, the yield of ERY was low relative to the typical output that would be expected from chemical synthesis processes. Therefore it would be of great value to investigate improvements of yield and throughput in such devices.

The maximum product yield achievable with a T-channel reactor design, such as that used in our previous publication, is heavily dependent upon the effects of increased substrate concentration on the biocatalyst. These effects include inhibition and denaturation of the biocatalyst, with the latter causing clogging in continuous-flow reactors. In batch reactors, such effects are overcome by controlled feeding of the substrate solution at defined time points (fed-batch mode). Miniaturised fed-batch reactors with operation volumes of 12 mL [32] and 0.5 mL [33] exist, and could be employed to maintain the concentration of the substrate below

* Corresponding author. Tel.: +44 0207 679 4418; fax: +44 0207 916 3943.
E-mail address: n.szita@ucl.ac.uk (N. Szita).

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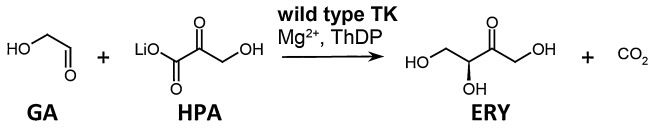

Scheme 1. Reaction scheme. The transketolase-catalysed reaction of lithium hydroxypyruvate (HPA) and glycolaldehyde (GA) to L-erythrulose (ERY).

inhibitory levels, allowing a higher product output. However these reactors cannot be operated in continuous-flow fashion, which limits their applicability for multi-step process integration. Additionally, the significant space-time-yield increases, characteristic of continuous-flow microreactors, cannot be exploited [34].

Reactor systems allowing injection of substrates at multiple points have been demonstrated for the purpose of controlling exothermic chemical reactions. These systems were designed for the continuous synthesis of allylcarbinol and of organometallic compounds, using multi-point feeding to control the formation of impurities and the generation of heat respectively [35–37]. However, such systems have not been applied to the problem of substrate inhibition in biocatalytic reactions.

Continuous-flow 'loop-type' microreactors designed to allow the recycling of unconverted substrates have previously been demonstrated [38,39]. It is possible to use these reactors to gradually feed substrate, however the continuous injection of substrate into the recycling loop at a single point means that a pure product stream is fundamentally difficult to achieve. The continuous removal of product also requires that the biocatalyst is either immobilised or removed by an in-line separation system, necessitating a more complex reactor design.

In this contribution, we combine a continuous-flow reactor and the principles of a fed-batch substrate feeding strategy for the first time at the microfluidic scale. We present the design of a novel microfluidic reactor capable of substrate feeding at multiple points. We demonstrate the application of the reactor to the TK-catalysed reaction of lithium hydroxypyruvate (HPA) and glycolaldehyde (GA) to L-erythrulose (ERY; Scheme 1).

2. Experimental

2.1. Reagents and analysis

Unless otherwise stated, all chemicals and reagents (Sigma–Aldrich, Gillingham, UK) were used without further purification.

Transketolase concentrations were measured by SDS-PAGE electrophoresis with 12% Tris–glycine resolving gel, using bovine serum albumin standards. 20 μg of total protein was applied to each lane and the samples were stained with Coomassie Blue R-250.

HPLC quantification of lithium hydroxypyruvate (HPA) and L-erythrulose (ERY) was performed on Aminex (Biorad, Hemel Hempstead, UK) ion-exchange column (HPX-87H, 300 mm × 7.8 mm), mobile phase: 0.1% (v/v) aqueous trifluoroacetic acid (TFA) at 0.6 mL min^{-1}. HPA and ERY were detected by UV absorption at 210 nm.

2.2. Fabrication of the microfluidic multi-input reactor

The channels and cut-outs of the multi-input reactor (MIR) were fabricated in three layers of 1.5 mm poly(methylmethacrylate) (PMMA) using a CO_2 laser marking head (Laserlines, Banbury, UK) with a maximum power of 25 W. The features were ablated with a power of 50% and a mark speed (laser tracking speed) of 200 mm s^{-1} and 10 mm s^{-1} for channels and cut-outs, respectively. The three layers were cleaned and thermally bonded (1.5 h, 105 °C, 90 min).

The interconnect blocks of the MIR were fabricated in 5 mm polycarbonate (PC) with a micromilling machine (Folken IND, Glendale, USA), using a 2 mm end mill (Kyocera, Kyoto, Japan) with a spindle speed of 10,000 rpm and feed rate of 80 mm min^{-1}. M3 and M6 taps were used to prepare the interconnect blocks for use. Standard connection fittings were used to attach tubing (P-221, Upchurch Scientific, Oak Harbour, WA, USA).

Plugs used to seal unused auxiliary inputs were fabricated in poly(dimethylsiloxane) (PDMS; Sylgard 184, Dow Corning, Midland, USA). A mould was milled from 5 mm PMMA with a 2 mm tool, a spindle speed of 7000 rpm and feed rate of 40 mm min^{-1}. The liquid polymer was prepared in a ratio of 10:1 (monomer to curing agent), cast, degassed and then cured at 90 °C for 2 h.

2.3. Preparation of transketolase lysate

Transketolase lysates were prepared according to the method of Matosevic et al. [40]. Overnight cultures of *E. coli* BL21gold (DE3) (with transketolase-producing plasmid pQR791) were grown in 2 L shake-flasks from inoculation of 400 mL Lysogeny Broth (LB) with 1 mL of concentrated cell suspension in LB-glycerol stock solution (25%, v/v, glycerol, stored at −80 °C until inoculation). This was incubated for 20–24 h at 37 °C, until the bacterial growth had reached stationary phase as confirmed by optical density measurements. The contents of the flask were transferred to 50 mL falcon tubes and centrifuged at 5000 rpm for 10 min. The supernatant was discarded and the cell paste was frozen at −80 °C until needed for lysis and purification.

For lysis, the cell paste was resuspended in 2 mL 50 mM Tris–HCl buffer, cooled on ice and sonicated (10 cycles of 10 s on, 10 s off) with a sonication probe (Soniprep 150, Sanyo, Japan). The suspension was then centrifuged at 5000 rpm for 10 min and the supernatant containing the enzyme was stored at −20 °C until required.

2.4. Continuous-flow microfluidic reaction of HPA to ERY (with multiple GA inputs)

Two separate solutions were used to perform the reaction. The main reaction mixture (solution A) consisted of 0.069 mg mL^{-1} clarified transketolase lysate, different HPA concentrations (211/316/421/526 mM HPA, depending on experiment), 2.53 mM thiamine diphosphate (ThDP) and 10.3 mM $MgCl_2$ in 50 mM Tris–HCl buffer, pH 7. The concentrations of the solutes were chosen such that they would be diluted to the following concentrations once combined with the first GA input: 0.066 mg mL^{-1} clarified transketolase lysate, 200/300/400/500 mM HPA, 2.4 mM thiamine diphosphate (TDP) and 9.8 mM $MgCl_2$. The supplementary GA solution (solution B) consisted of 1 M GA in 50 mM Tris–HCl buffer, pH 7 (Scheme 2).

The MIR was primed with Tris–HCl buffer (50 mM, pH 7). Solution A was pumped into the first primary input of the reactor with a single-drive syringe pump. Solution B was pumped into the second primary input, along with a number of auxiliary inputs, using a dual-drive syringe pump adapted to fit ten 1 mL syringes. The flow rates and the number of auxiliary inputs used were dependent upon the desired residence time and input HPA concentration (Table 1).

The reactor was allowed to equilibrate for 2.5 residence times before sampling began. Samples were collected in pre-weighed vials containing 270 μL 0.1% (v/v) aqueous trifluoroacetic acid (TFA). The quenched samples were weighed, centrifuged and the supernatant was diluted 1:1 with 0.1% TFA before being analysed by HPLC as described in Section 2.1.