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n-Butylamine as an alternative amine donor for the stereoselective biocatalytic transamination of ketones

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ABSTRACT

Formal reductive amination has been a main focus of biocatalysis research in recent times. Among the enzymes able to perform this transformation, pyridoxal-5′-phosphate-dependent transaminases have shown the greatest promise in terms of extensive substrate scope and industrial application. Despite concerted research efforts in this area, there exist relatively few options regarding efficient amino donor co-substrates capable of allowing high conversion and atom efficiency with stable enzyme systems. Herein we describe the implementation of the recently described *spuC* gene, coding for a putrescine transaminase, exploiting its unusual amine donor tolerance to allow use of inexpensive and readily-available *n*-butylamine as an alternative to traditional methods. *Via* the integration of SpuC homologues with tandem co-product removal and cofactor regeneration enzymes, high conversion could be achieved with just 1.5 equivalents of the amine with products displaying excellent enantiopurity.

1. Introduction

Chiral amine motifs are rapidly becoming common in both pharmaceutical and agrochemical industries and almost half of the active pharmaceutical ingredients (APIs) contain at least one chiral centre with a chiral amine unit. Approximately 95% of all drugs are predicted to be chiral by 2020 and share a market of almost $5bn together with agrochemical, flavour and fragrance industries [1]. Special emphasis is now placed on the development of safe and efficient chiral amine synthetic methods. Complementing the traditional chemical synthetic methods, biocatalysis offers a distinct approach to the synthesis of high-value enantiopure amines, often using small, low-cost achiral starting materials. Biocatalytic routes to chiral amines have evolved significantly during the past decade, from kinetic resolution of amines using lipases, to the successful use of transaminases, imine reductases and ammonia lyases, amongst others [2].

Transaminases (**EC 2.6.1.18**) perform the transfer of an amino group from an amino donor to a prochiral ketone or aldehyde acceptor, employing pyridoxal-5′-phosphate PLP as a cofactor in this process. Asymmetric chiral amine synthesis catalysed by transaminases is a robust and simple method, but it has specific challenges in becoming a green and cost effective approach.

Particular attention has been drawn to overcome problems in the synthesis reaction; in which, transamination of prochiral ketones using alanine as the amino donor is hindered by a poor equilibrium constant and the occurrence of an inhibitory co-product [3]. In the last decade, several *in situ* pyruvate product removal strategies have been devised to displace the equilibrium, such as coupling the enzymatic reaction with a secondary concomitant irreversible reaction with oxidoreductases that recycles cofactor NAD(P)H. The most well-known method involves the use of lactate dehydrogenase (LDH) or formate dehydrogenase (FDH) in the presence of excess alanine and a sacrificial reducing agent (formate or glucose) to generate faster reaction rates (Scheme 1, I) [3,4]. Recently the use of diamine donors has been the focal point to displace the equilibria *via* ring aromatisation/cyclisation (Scheme 1, II) [5]. Although still in its infancy these diamine donors have proven to be successful in the transamination reaction, even though their

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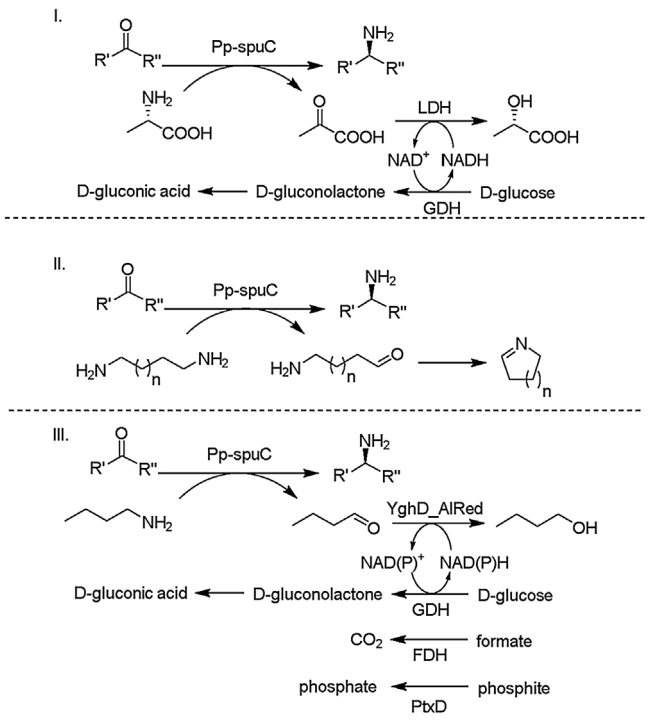

Scheme 1. Summary of transamination equilibrium-driving strategies involving the novel SpuC bifunctional transaminases. Each class of amino donor presents a specific method of co-product recycling/removal. **I. Amino acids**- alanine is used together with the LDH/GDH system for the removal of pyruvate; **II. Short-chain aliphatic diamines**- the spontaneous cyclization of the reactive amino-aldehyde intermediate provides the driving force of the reaction; **III. *n*-Butylamine**- the inhibitory butanal is removed *via* enzymatic reduction coupled with various NAD(P)$^+$ recycling systems.

full potential has yet to be achieved [5,6]. Other amino donors such as isopropylamine are the preferred industrial choice since the amine is readily available and economically efficient; however, a 50–100 fold amine donor excess is required with further downstream processing required utilising specialised temperature platforms to remove highly soluble volatile coproducts *via* evaporation [7]. The selection of the amine donor is crucial and ideally must satisfy requirements of both atom (near-stoichiometric amounts to be used) and economic efficiency (low cost). In addition, the co-product has to be easy to remove or recycle and should not complicate the main product purification step.

Previous cost effective and environmentally benign strategies were attempted to replace isopropylamine or alanine with *n*-butylamine **1** as amino donor with commercially available transaminases but only negligible product formation was detected. Presumably, the reactive aldehyde product (butanal **2**) can bind irreversibly to the enzyme affecting the overall catalytic activity [8]. Herein we circumvented the limitations and implemented a novel enzyme cascade that harnesses the high activity of recently published Pp-spuC transaminase in tandem with a well-studied aldehyde reductase YqhD from *E. coli* [9,10].

The *yqhD* gene product is described as a scavenger of reactive aldehydes in non-metabolic pathways and has NADPH-dependent reductase activity towards simple aldehydes with a high turnover number for *n*-butanal **2** and was also shown to be effective in the industrial production of isobutanol [11]. Aldehyde reductase (YqhD) requires NADPH as cofactor to reduce the aldehyde efficiently; however, an alternative cofactor recycling system was sought after previous experience with glucose dehydrogenase led to some reduction of the ketone substrates. Our attention was focused on the phosphite dehydrogenase (PtxD) enzyme [12] as part of an NAD(P)$^+$ recycling cascade that has rarely been exploited and presents itself with several advantages over the ubiquitous formate dehydrogenase and/or glucose dehydrogenase systems. Such difficulties include low specific activities and high production costs of FDHs [13,14], and the generation of acidic byproducts (gluconic or carbonic acid) that require a controlled addition of base not suitable for pH sensitive reactions. PtxD presents an attractive alternative that catalyzes a near irreversible reaction with a thermodynamically favorable equilibrium constant in order to reduce nicotinamide cofactors NAD(P)$^+$, with the generation of innocuous phosphate as the byproduct and with negligible effects on pH.

The present work describes the implementation of a novel enzyme cascade transaminase/aldehyde reductase/phosphite dehydrogenase, which explores the use of *n*-butylamine as an alternative and inexpensive amino donor. The toxic co-product butanal is converted to the environmentally benign *n*-butanol by the YqhD aldehyde reductase and phosphite dehydrogenase PtxD is used as part of NAD(P)H recycling system. The novel enzyme system is tested on a diverse panel of ketones substrates and the substitution of NADP$^+$ with more the more economically affordable NAD$^+$ is also investigated.

2. Materials and methods

2.1. Chemicals

The ketones (**4a-l**), amines (**5a-l**) and buffers were purchased from Sigma Aldrich (St Louis, MO, USA) or Alfa Aesar (Haverhill, MA, USA) and were used throughout without further purification. The HPLC solvents were purchased from Sigma Aldrich. Restriction enzymes, T4 ligase, *Taq* polymerase, dNTPs and broad protein marker (2–212 kDa) were purchased from New England Biolabs (Ipswich, MA, USA). *Escherichia coli* DH5α and BL21 (DE3) cells were purchased from New England Biolabs (Ipswich, MA, USA). Phusion polymerase wese purchased from Thermo Fisher (Waltham, MA, USA). Expression vector pET-28b was purchased from Novagen (Darmstadt, Germany) and was used for gene expression. *Pseudomonas fluorescens* (KX954133), *Pseudomonas chlororaphis* subsp. *aureofaciens* 30–84, (KX954134), *Pseudomonas putida* NBRC 14161 (T2HES1) were obtained from NCIMB culture collection. The phosphite dehydrogenase (PtxD) gene from *P. stutzeri* was previously subcloned into a pBAD expression vector and was gratefully received from Professor Nigel Scrutton of University of Manchester. Commercially available transaminases ATA-113 and ATA-117 were purchased from Codexis in the form of lyophilised cell extract.

2.2. Cloning of the YqhD gene from E. coli K-12

The coding region of the *yqhD* gene (UNIPROT acc. no. Q46856) was amplified using Phusion polymerase *via* colony PCR from *E. coli* K-12 using the oligonucleotide primers as follows: (i) Forward 5′-ATATGGCTAGCATGAACAACT-TTAATCTGCACACCC-3′ and (ii) Reverse complementary 5′- TGGTGCTCGAGTTAGCG-GGCGGCTTCGTATATACGG −3′ with *Nhe*I and *Xho*I restriction sites underlined respectively. The following PCR protocol was used: 5 min denaturation at 95 °C and then 30 cycles of 30 s denaturation at 95 °C, 30 s annealing at 58 °C and 90 s elongation at 68 °C with a 5 min final extension time at 68 °C. The PCR product was cloned into ZERO Blunt TOPO PCR cloning vector (K2830-20) following the manufacture's protocol. The *yqhD* gene was subcloned into pET-28b expression vector containing a *N*-terminal His_6-tag with a thrombin linker. The inserted gene was in frame and downstream from the ribosome binding site as confirmed *via* DNA sequencing (Eurofins) and gave plasmid pET-28b-yqhD. The pET-

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