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One-pot, two-step transaminase and transketolase synthesis of L-*gluco*-heptulose from L-arabinose

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ABSTRACT

The use of biocatalysis for the synthesis of high value added chemical building blocks derived from biomass is becoming an increasingly important application for future sustainable technologies. The synthesis of a higher value chemical from L-arabinose, the predominant monosaccharide obtained from sugar beet pulp, is demonstrated here via a transketolase and transaminase coupled reaction. Thermostable transketolases derived from *Deinococcus geothermalis* and *Deinococcus radiodurans* catalysed the synthesis of L-*gluco*-heptulose from L-arabinose and β-hydroxypyruvate at elevated temperatures with high conversions. β-Hydroxypyruvate, a commercially expensive compound used in the transketolase reaction, was generated in situ from L-serine and α-ketoglutaric acid via a thermostable transaminase, also from *Deinococcus geothermalis*. The two steps were investigated and implemented in a one-pot system for the sustainable and efficient production of L-*gluco*-heptulose.

1. Introduction

The increasing production of bio-based chemicals and products is paving the way for a greener, sustainable future away from a fossil-based economy. Conversion of biomass into high value bio-based chemicals is frequently explored via biocatalysis, which is a green, environmentally friendly technology. The high enantio- and regioselectivities that enzymes naturally exhibit towards their substrates are ultimately superior to conventional chemical synthetic routes [1]. In addition, there is increasing interest in the construction of multi-enzyme routes and cascades to enhance the efficiency and commercial viability of the biocatalytic approach towards the synthesis of pharmaceutical intermediates and speciality chemicals [2].

L-arabinose is the main monosaccharide found in sugar beet pulp (SBP), a by-product stream of sucrose extraction, that can be recovered enzymatically or via acid hydrolysis from pectin [3,4]. The upgrading of L-arabinose using a transketolase (TK) enzyme was previously performed to produce a higher value added compound, L-*gluco*-heptulose (Scheme 1) [4]. Ketoheptoses such as L-*gluco*-heptulose are of pharmaceutical interest with therapeutic applications in hypoglycaemia and cancer due to their ability to inhibit sugar metabolism [5–7].

TKs (EC 2.2.1.1.) are thiamine diphosphate (ThDP) dependent enzymes which in vivo, are in the pentose phosphate pathway which provides the cell with reduced NADPH cofactors for anabolic reactions and the starting compounds for aromatic amino acid biosynthesis [8]. TKs catalyse the stereospecific formation of carbon-carbon bonds by the reversible transfer of the C1-C2 ketol unit from a ketose phosphate to an aldose acceptor to form a new ketose phosphate. In many synthetic applications however, TKs are used with lithium β-hydroxypyruvate (Li-HPA) as the donor substrate, which renders the reaction irreversible due to the release of carbon dioxide (CO_2). For this reason, TK catalysed carbon-carbon bond formation is considered to be a useful and desirable tool in ketose synthesis.

Transaminases (TAm, EC 2.6.1-) are a highly diverse family of enzymes that have gained significant attention in applied organic synthesis due to their ability to produce a wide variety of single isomer chiral amines. These pyridoxal 5′-phosphate (PLP) dependent enzymes catalyse the reversible transfer of an amino group from an amine donor to a keto acceptor, often from low cost substrates, to produce enantiopure amines [9]. β-Hydroxypyruvate (HPA) is a natural intermediate in serine biosynthesis and glycerate metabolism and is present in the cell in both the phosphorylated and non-phosphorylated forms. It was previously demonstrated that serine within *E. coli* was produced via the transamination of phosphorylated HPA to produce phosphorylated serine by a phosphoserine TAm [10]. The amino group on serine can also be transferred to glyoxylate to form HPA and glycine by a TAm in

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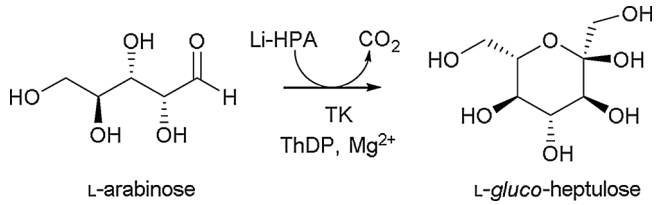


Scheme 1. TK reaction for the L-*gluco*-heptulose synthesis from Li-HPA (lithium β-hydroxypyruvate) and L-arabinose.

many bacteria [10]. These TAm steps are useful biocatalytic tools to facilitate further HPA-dependent TK reactions instead of using commercially expensive Li-HPA, thus reducing the overall synthetic costs of target compounds.

Enzymatic TK and TAm reaction coupling continues to be explored with an early example being the two-step synthesis of the building block, 2-amino-1,3,4-butanetriol (ABT) [11,12]. ABT was synthesised via the transamination of l-erythrulose by an alanine:pyruvate TAm from *Pseudomonas aeruginosa*, whilst the l-erythrulose was synthesised by a TK from *E. coli* using the achiral substrates glycolaldehyde (GA) and synthetic HPA. Subsequently, this was ultimately achieved via a one-pot process [12]. A more recent investigation was carried out in which strategies to supply the enzyme cascade with non-phosphorylated HPA for the synthesis of ABT were achieved [13]. ABT was synthesised by a recycling pathway with a TAm catalysed amination of l-erythrulose using serine as the amino donor, giving ABT and HPA. The HPA was then used as a substrate with GA and TK for the synthesis of L-erythrulose.

Presented here is an enzymatic one-pot, two-step cascade for the synthesis of L-*gluco*-heptulose from L-arabinose, a major monosaccharide found in SBP. The first step of the one-pot reaction utilised a thermostable TAm to produce HPA in situ from L-serine as the amino donor and α-ketoglutaric acid as the amino acceptor. The HPA produced was then used as the ketol donor in the second step of the reaction via a thermostable TK for the production of L-*gluco*-heptulose from L-arabinose. In addition, two new TKs expressed from *Deinococcus radiodurans* DSM 20539 and *D. geothermalis* DSM 11300 and two new TAms from *D. geothermalis* and *Geobacillus stearothermophilus* DSM 22 are described. In line with the extremophilic properties of *D. radiodurans* [14] and the thermophilic properties of *D. geothermalis* [15] and *G. stearothermophilus* [16], optimum temperature and pH of the TK and TAm enzymes were also investigated.

2. Materials and methods

2.1. Bacterial strains and vectors

Strains were obtained from DSMZ, *E. coli* strains DH5-α and BL21 (DE3) were obtained from Invitrogen. Reagents for molecular biology were obtained from New England BioLabs and Qiagen. Oligonucleotides for PCR were obtained from Eurofins Scientific. Chemicals were supplied by Sigma Aldrich.

2.2. Construction of plasmids

E. coli DH5-α and *E. coli* BL21 (DE3) were routinely grown in Luria-Bertani (LB) medium and used as the cloning and expression hosts, respectively. The TK_{Dgeo} (Q1IW07) and TAm_{Dgeo} (Q1IZC2) genes were amplified by PCR from genomic DNA of *Deinococcus geothermalis* DSM 11300 with the following primers: TK_{Dgeo}- forward primer CATAGGA ATTCATGAGTCCCGAACAGCAGG and reverse primer- CATAGCTCGA GCCTCTGCAAAACGCCCTT, TAm_{Dgeo}- forward primer- CATAGGGATC CATGTTCGAGGACACGCCC and reverse primer- CATAGCTCGAGGGC CGCGACGCCCAGCGC. The TK_{Drad} (Q9RS71) gene was amplified by PCR from genomic DNA of *Deinococcus radiodurans* DSM 20539 with the following primers: TK_{Drad}- forward primer- CATAGCATATGACAGACC AGAGCGTTTCC and reverse primer-CATAGCTCGAGCAGCACGGAGTG GACCACCT. The TAm_{Gste} gene was amplified by PCR from genomic DNA of *Geobacillus stearothermophilus* DSM 22 with the following primers: TAm_{Gste}- forward primer- CATAGCATATGAAATTGGCAAAAC GGG and reverse primer- CATAGCTCGAGAGCGCGCGCTTCCATAAA.

Plasmid pET29 (+) (Novagen, USA) was used for the direct cloning and expression of PCR products with kanamycin (50 μg/mL) required for the selection of recombinant strains in *E. coli*. TAm and TK genes were amplified with various restriction sites. Restriction enzymes were supplied by New England BioLabs. Amplified DNA was directly cloned into corresponding cloning sites within pET29a (+) (all genes amplified with *Nde*I-*Xho*I sites apart from TK_{Dgeo} and TAm_{Dgeo} which used *Eco*RI-*Xho*I and *Bam*HI-*Xho*I respectively) and introduced into DH5-α before being transformed into *E. coli* BL21 (DE3) cells. A hexa-histidine tag was included at the C-terminus of the protein to facilitate protein purification.

2.3. Expression of recombinant TAm and TK

Recombinant protein was expressed in *E. coli* BL21 (DE3) cells transformed with pET29a (+). Cells were grown at 37 °C, 220 rpm in 400 mL LB medium containing 50 μg/mL kanamycin. When the OD_{600} of the culture reached 0.6–1, 1 mM IPTG was added to the cells to induce protein expression. The temperature was then reduced to 25 °C and the culture was incubated for a further period of 16 h. Cells were harvested by centrifugation after which the cell pellet was resuspended in 5 mL 50 mM sodium phosphate buffer, pH 7. Cells were sonicated (Soniprep 150 sonicator, MSE, Sanyo Japan) on ice using 4 cycles of 30 s on, 60 s off at an amplitude of 10 μm (μm). The remaining cell lysate was centrifuged at 11,000 × *g* at 4 °C for 50 min. Enzyme expression was confirmed by SDS-PAGE using 12% Tris-Glycine Precast Gels (Bio-Rad, USA).

2.4. Protein purification

Recombinant TAm and TK were purified using affinity chromatography by immobilised metal affinity chromatography (IMAC). The expressed His_6-tagged proteins were applied to a Ni^{2+} chelating affinity column for purification. TAm and TK proteins were harvested from 400 mL of culture and suspended in 5 mL suspension buffer containing imidazole (10 mM), Na_2HPO_4 (50 mM) and NaCl (0.5 M) at pH 7.4. The cells were lysed via sonication for 135 s on ice and the insoluble pellet was discarded after centrifugation at 12,000 × *g*, 30 min, 4 °C. The cell lysate was loaded onto an open tubular mini column containing Chelating Sepharose™ Fast Flow resin (GE Healthcare) charged with nickel ions, $NiSO_4$, which was equilibrated with the above buffer. After the column was washed with the same buffer, the protein was eluted using elution buffer containing 500 mM imidazole, 50 mM Na_2HPO_4 and 0.5 mM NaCl. Protein concentration was determined using protein absorbance at 280 nm and Beer-Lambert's law. Purified proteins were then precipitated using ammonium sulfate (3.2 M) and stored in this solution at 4 °C.

2.5. High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis method

Quantitative HPA and L-*gluco*-heptulose detection was carried out by injecting 10 μL samples into a Reagent-Free Ion Chromatography System (ICS 5000+, Dionex) equipped with a Dionex Aminopac™ PA1 anion exchange column 4 × 250 mm fitted with a Dionex Aminopac™ PA1 guard column 4 × 50 mm, an electrochemical detector system, and an eluent generator with a KOH 500 cartridge. Analysis was performed with an isocratic flow using 30 mM KOH mobile phase with a flow rate of 0.25 mL/min for 18 min at 30 °C. The retention time of the detected compounds were Li-HPA: 8.9 min and L-*gluco*-heptulose: 6.5 min.

Standard calibration curves with Li-HPA (Sigma-Aldrich) and L-*gluco*-heptulose were used for quantification purposes. The identity of L-*gluco*-heptulose was confirmed by HPAEC-PAD using a L-*gluco*-heptulose standard that had been synthesised, isolated and characterised in a previous study by NMR spectroscopy and GC–MS [4].

2.6. TK activity

TK activity was initially studied between 25 and 70 °C in 50 mM HEPES, pH 7.0, 5.1 mM $MgCl_2$, 1.6 mM ThDP, 30 mM Li-HPA, and 30 mM L-arabinose with 0.10 mg/mL TK crude extract when investigating the single TK reaction. After 24 h, 50 µL aliquots were quenched with 50 µL of 0.5% sulphuric acid. All samples were analysed by HPAEC-PAD to determine L-*gluco*-heptulose production.

2.7. TK optimum temperature and pH

The effect of temperature on TK_{Dgeo} and TK_{Drad} was investigated in the range of 25–70 °C in HEPES buffer (50 mM, pH 8) with L-arabinose and Li-HPA as substrates. The temperature of the reaction was controlled using an Eppendorf Thermomixer®. Enzymes were mixed with $MgCl_2$ (4.5 mM) and ThDP (1.25 mM) cofactors and L-arabinose and placed at various temperatures for 15 min. When the desired temperature was reached, Li-HPA was added to the mixture to initiate the reaction. The samples were taken at different time intervals and activity was measured via HPAEC-PAD analysis. All reactions were performed in triplicate.

The optimum pH of TK_{Dgeo} and TK_{Drad} was determined in the range of pH 7–10 with L-arabinose and Li-HPA with buffers HEPES (pH 7–8), sodium borate (pH 8–9), and sodium carbonate (pH 9–10) at a final concentration of 50 mM. Reactions were incubated at 25 °C and residual activities were measured at various time points using HPAEC-PAD. All reactions were done in triplicate.

2.8. TK kinetic parameters

Kinetic parameters were determined for L-arabinose by measurement of initial velocities of TK_{Dgeo} and TK_{Drad} by varying L-arabinose concentration from 10 to 150 mM while Li-HPA concentration was constant at 50 mM. The reaction rate for each concentration was calculated. This data was applied in a nonlinear regression to the Michaelis-Menten model using GraphPad Prism version 7.03 for Windows (GraphPad Software, La Jolla California USA) to determine kinetic parameter values. All measurements were performed in triplicate.

2.9. TAm activity

TAm reactions were carried out at 50 °C in 50 mM HEPES, pH 7.0 containing 0.2 mM PLP, 10 mM L-serine, 10 mM pyruvate or α-ketoglutaric acid with crude extract. After 16 h, 50 µL aliquots were quenched with 50 µL of 0.5% sulfuric acid. All samples were analysed by HPAEC-PAD to determine HPA production.

2.10. TAm kinetic parameters

Kinetic parameters were determined for L-serine by measurement of initial velocity of TAm_{Dgeo} (0.2 mg/mL) by varying L-serine concentration from 10 to 150 mM while α-ketoglutaric concentration was constant at 50 mM. Initial velocities were determined using data collected at multiple time points for each substrate concentration. This data was applied in a nonlinear regression to the Michaelis-Menten model using GraphPad Prism version 7.03 for Windows (GraphPad Software, La Jolla California USA) to determine kinetic parameter values. All measurements were performed in triplicate.

2.11. TAm optimum pH

The pH spectrum was investigated by measuring TAm_{Gste} and TAm_{Dgeo} activity in the range of pH 7–10 with L-serine and α-ketoglutaric acid in different buffers: Bis-Tris (pH 6–6.5), HEPES (pH 7–8), sodium borate (pH 8–9) and sodium carbonate (pH 9–10) at a final concentration of 50 mM. The activity of TAm_{Dgeo} was determined at different time points using HPAEC-PAD. All reactions were carried out in triplicate.

2.12. One-pot TAm and TK reaction, general procedure

To investigate the transaminase-transketolase coupled reaction, all reactions were performed at 50 °C in 50 mM buffer ranging from pH 7–8.5 containing 0.125 mM PLP, 30 mM L-serine, 30 mM α-ketoglutaric acid, 10 mM L-arabinose, 4.5 mM $MgCl_2$, 1.25 mM ThDP, 0.42 mg/mL TAm and 0.2 mg/mL TK. TK enzyme was pre-incubated with the 4.5 mM $MgCl_2$ and 1.25 mM ThDP at 25 °C for 10 min prior to addition into reaction mixture. Substrates and buffer were incubated at 50 °C before the addition of enzymes. After the reaction, 50 µL aliquots were quenched with 50 µL 0.5% sulfuric acid and samples were analysed using HPAEC-PAD.

3. Results and discussion

3.1. Cloning, expression and purification of recombinant TK and TAm

For this study, TK genes from in-house thermophiles were selected, cloned and expressed (see Table 1 for DNA/protein sequences accession codes in Ref. [17]). Also, a panel of thermostable TAms were selected, cloned and expressed on the basis of their homologies to known TAms that displayed specificity to serine from an in-house library of strains (see Table 2 in Ref. [17]). Notably, the characterised TAms serine:pyruvate aminotransferase (SPAT) and CV2025 from *Sulfolobus solfataricus* and *Chromobacterium violaceum* respectively have been shown to accept L-serine but at low rates while TAm DGEO_0713 from *D. geothermalis* has previously shown high specificity towards L-serine [13,18,19]. These TAms were therefore appropriate candidates for use in TAm selection from thermophiles available (see Table 2 for similarity analysis of homologous sequences applied to thermostable TAms in Ref. [17]). Thermostable enzymes were selected due to their many advantages in industrial processes including increased resistance to chemical denaturants and utilisation of high substrate concentration from improved substrate solubility at elevated temperatures [20].

Table 1
Activities of TK_{Dgeo} and TK_{Drad} at various temperatures towards L-arabinose.

TK	Temperature (°C)	Conversion yield[a] (%)	Initial rate[b] ($mM\,h^{-1}$)
TK_{Dgeo}	25	6	0.11 ± 0.02
	40	21	0.35 ± 0.06
	50	35	0.48 ± 0.09
	60	22	0.34 ± 0.01
	70	6	0.21 ± 0.03
TK_{Drad}	25	8	0.14 ± 0.03
	40	34	0.38 ± 0.05
	50	39	1.0 ± 0.2
	60	3	0.26 ± 0.01
	70	3	0.11 ± 0.02

[a] Conversion yields were determined based on L-*gluco*-heptulose production at 24 h by HPAEC-PAD with purified TK.

[b] Initial rates were determined by monitoring the production of L-*gluco*-heptulose using multiple time points over 60 min using HPAEC-PAD. Reactions carried out in duplicate. The remaining three TKs expressed showed either very low or no activity with Seliwanoff reagent so no further work was carried out with these.

Table 2
Kinetic parameters for L-arabinose by TK_{Dgeo} and TK_{Drad} at 50 °C.

TK	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M ($mM^{-1} \times min^{-1}$)
TK_{Dgeo}	100.5 ± 9.2	189.1 ± 9.5	1.88 ± 0.08
TK_{Drad}	517.1 ± 22.3	542.1 ± 25.3	1.048 ± 0.004

3.2. Identification and characterisation of thermostable TK activity with arabinose

TKs generally have a low affinity towards non-phosphorylated sugars compared to their natural phosphorylated substrates erythrose-4-phosphate and D-ribose-5-phosphate, which can be explained by the architecture of the substrate channel of the TK active site [21]. Highly conserved TK residues for substrate binding are observed in close proximity to the phosphate group of modelled substrates. Indeed, replacement of any of the three conserved residues, R533, S395 and H474 within the active site of the TK from *Saccharomyces cerevisiae*, has been shown to substantially increase the K_M values for phosphorylated substrates such as ribose-5-phosphate [22].

Initial screening of TK activity towards L-arabinose was carried out with five new thermostable TKs. A colorimetric assay using Seliwanoff's reagent [23] was utilised, which detects ketoses by giving a red xanthenone product (results not shown). TKs found to give a positive result for the ketose product were then monitored in reactions via high anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The yields of L-*gluco*-heptulose formed from L-arabinose at temperatures ranging from 25 to 70 °C by TKs from *D. geothermalis* (TK_{Dgeo}) and *D. radiodurans* (TK_{Drad}) were analysed (Table 1). These two TKs exhibited the highest activity towards L-arabinose at 50 °C, which was reflected in their increased initial rates of reaction and conversion yields at this temperature: a 5-fold increase in conversion yield of L-*gluco*-heptulose was observed compared to the activity measured at 25 °C. Furthermore, TK_{Dgeo} appeared to still be relatively active at 60 °C. Conversions and initial velocities at 50 °C were comparable to those observed by TK variants from *E. coli* towards L-arabinose at room temperature [4]. These active-site variants had previously been selected for L-*gluco*-heptulose production on the basis that they could accept polyhydroxylated substrates.

3.3. TK_{Dgeo} and TK_{Drad} characterisation, pH optimisation and kinetic parameters

The effect of pH on TK activity with L-arabinose was determined in the pH range 7–10 at 25 °C, as an increase in solution temperature will change the pH of many buffers [3] (Fig. 1). TK_{Dgeo} and TK_{Drad} both exhibited the highest activity at pH 8, which is similar to the optimum pH for microbial TK activity characterised to date. Specific activities were relatively low due to the reaction being carried out at 25 °C rather than 50 °C. Similar to this, the thermostable TK from *G. stearothermophilus* displayed maximum activity at pH 7–8 [24] whilst TK from *E. coli* had an optimum pH in the range of 8–8.5 [25].

Michaelis-Menten kinetic parameters of the TKs at 50 °C were determined to understand the differences between TK_{Dgeo} and TK_{Drad} towards L-arabinose (Table 2). TK_{Dgeo} was the most efficient enzyme using L-arabinose, showing the highest affinity towards the compound in comparison to TK_{Drad} (4 times lower K_M) which has the highest catalytic constant. The data supports the theory that enzyme-substrate affinity and positioning of the substrate are major factors to overcome when attempting to improve the catalytic activity of poorly accepted non-phosphorylated sugars in TKs. However, the K_M values showed that the TKs can tolerate high substrate concentrations such as 0.5 M L-arabinose which are within the range of sugar concentrations seen when sugar beet hydrolysates are prepared [26]. The substrate scope of other TKs is considered to be a limiting factor for their wider use, and non-phosphorylated compounds have been reported to have 10–100 times lower affinity for most TK enzymes [27].

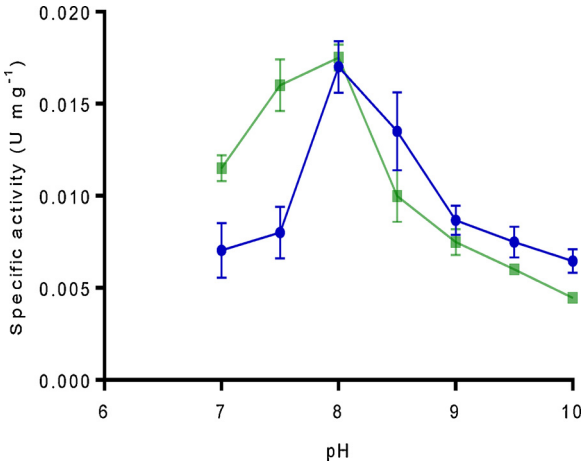


Fig. 1. Effect of pH on TK_{Dgeo} (blue) and TK_{Drad} (green) activity. Activity was measured over 60 min in the presence of ThDP (1.25 mM), $MgCl_2$ (4.5 mM), L-arabinose (10 mM), Li-HPA (10 mM), TK (0.1 mg/mL). Buffers (50 mM) used were HEPES (pH 7–8), sodium borate (pH 8–9) and sodium carbonate (pH 9–10). A unit of activity is defined as 1 μmol of product per minute. All measurements were performed in triplicate and error bars are standard deviation of the mean. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

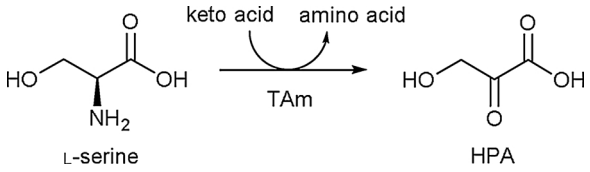
3.4. Identification and characterisation of TAm activity with L-serine

The two-step cascade envisaged was dependent on a TAm to aminate pyruvate or another amino acceptor using L-serine as an amino donor to produce HPA for the TK reaction. However, the TAm for the proposed cascade was required to be active at 50 °C to match the preferred temperature of TK_{Dgeo} and $TK_{Drad.}$ Eight new TAms were cloned and screened from the in-house collection of microbial thermophiles using L-serine as the donor substrate and two common keto acids as the acceptor substrate to produce HPA (Scheme 2). The assay was carried out at 50 °C for 16 h and monitored using HPAEC-PAD. The results showed that two of the TAms produced significant yields of HPA with both α-keto acid acceptors pyruvate and α-ketoglutaric acid. These enzymes were cloned from *G. stearothermophilus* and *D. geothermalis* and named respectively TAm_{Gste} and TAm_{Dgeo} (Fig. 2).

The remaining six TAms gave HPA yields of 7–22% at 16 h (Fig. 2). From the amino acid sequences, TAm_{Gste} and TAm_{Drad} are classified as α-transaminases indicating that they would have a strong preference for α-keto acids, which was observed [28]. TAm_{Gste} and TAm_{Drad} exhibited a similar specific activity towards both pyruvate and α-ketoglutaric acid at 50 °C, although slightly higher activities were observed with α-ketoglutaric acid (Table 3).

3.5. Enzyme characterisation, kinetic parameters and pH profiles: TAm_{Gste} and TAm_{Drad}

The kinetic constants for TAm_{Gste} and TAm_{Dgeo} were determined for the preferred reaction conditions using a Michaelis-Menten plot



Scheme 2. TAm reaction for the synthesis of HPA from L-serine and α-keto acid.

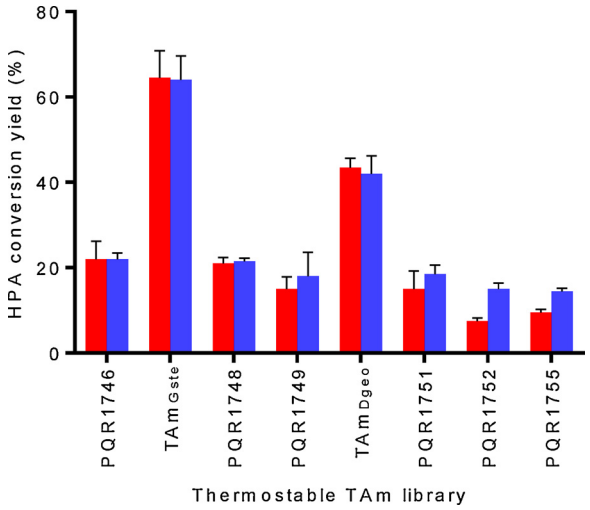


Fig. 2. Screening of thermostable TAm activities towards L-serine as amino donor and α-keto acid (pyruvate and α-ketoglutaric acid) as amino acceptor over 16 h. HPA production quantified via HPAEC-PAD. Reaction conditions: L-serine (10 mM), α-keto acids (pyruvate-red, α-ketoglutaric acid-blue, 10 mM), PLP (0.2 mM), HEPES buffer (50 mM, pH 7), TAm clarified lysates (50 μL), 50 °C. All measurements were performed in duplicate and error bars are standard deviation of the mean. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Amino acceptors profile of TAm_{Gste} and TAm_{Drad}.

TAm	Amino acceptor	Specific activity (U mg^{-1})
TAm_{Gste}	Pyruvate	3.61 ± 0.04
	Ketoglutaric acid	3.95 ± 0.01
TAm_{Dgeo}	Pyruvate	3.51 ± 0.01
	Ketoglutaric acid	4.09 ± 0.01

Table 4
Kinetic parameters relative to L-serine of TAm_{Gste} and TAm_{Drad} at 50 °C.

TAm	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M ($mM^{-1} \times min^{-1}$)
TAm_{Gste}	22.8 ± 3.2	2.9 ± 0.4	0.127 ± 0.002
TAm_{Dgeo}	12.7 ± 2.8	3.7 ± 0.5	0.33 ± 0.03

(Table 4). The K_M values obtained in the present study for both TAm_{Gste} and TAm_{Dgeo} were in similar ranges to that of TAm_{tca} from *Thermosinus carboxydivorans* (22.4 mM) and SGAT from *Arabidopsis thaliana* (5 mM) [29,30]. TAm_{Dgeo} appeared to possess a higher affinity for L-serine in comparison to TAm_{Gste} with a larger turnover number of 3.7 min^{-1}. Furthermore, there appears to be no inhibition of the TAms by either α-keto acid which was also reported for a different TAm from *D. geothermalis* [13].

The optimal activity of TAm_{Gste} was determined to be at pH 7-7.5 and this is not unlike that of the archaeal TAm, SPAT from *S. solfataricus* which was found to be pH 7.1 [18]. However, the optimum pH of TAm_{Dgeo} was measured as pH 8.5 (Fig. 3) and this is similar to other reported thermostable TAms such an ω-transaminase from *Sphaerobacter thermophilus* (ω-TAST) [31] and a taurine-pyruvate transaminase (TPTA) from *G. thermodenitrificans* [32]. Furthermore, like ω-TAST and TPTA, for TAm_{Dgeo} the optimum pH range is very narrow as activity is drastically reduced at pHs below 8, with almost no activity at pH 6. The differences in pH between these TAms maybe due to the pH required for optimal growth of the organisms or cytoplasmic pH, in relation to *S. solfataricus* [33]. Specific activities were relatively low due to the reaction being carried out at 25 °C rather than 50 °C.

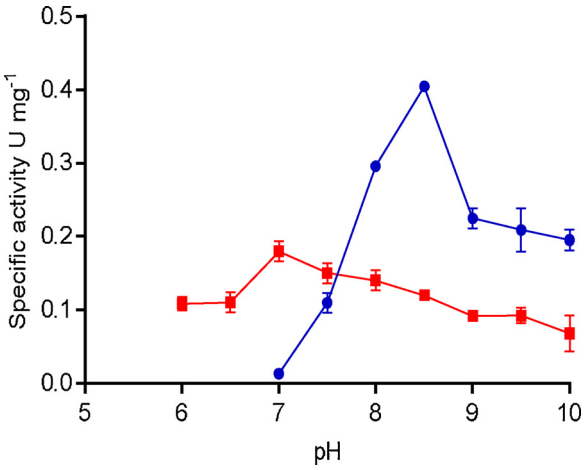


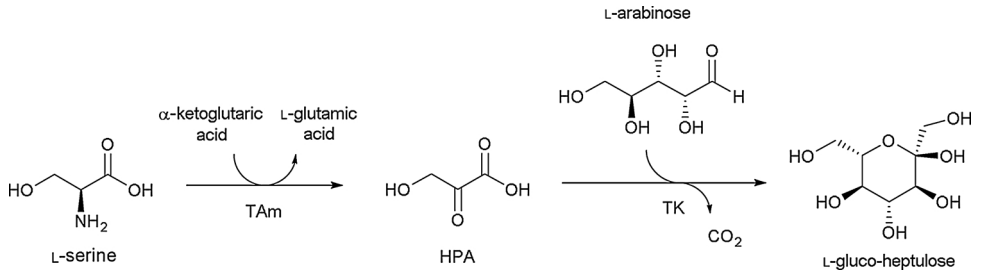
Fig. 3. Effect of pH on TAm_{Gste} (red) and TAm_{Dgeo} (blue) activity. Activity was measured over 60 min and HPA production quantified via HPAEC-PAD. Reaction conditions: L-serine (20 mM), α-ketoglutaric acid (4 mM), PLP (0.125 mM), Buffers (50 mM) used were Bis-Tris (pH 6–6.5), HEPES (pH 7–8), sodium borate (pH 8–9) and sodium carbonate (pH 9–10), TAm clarified lysates (50 μL), 50 °C. A unit of activity is defined as 1 μmol of product per minute. All measurements were performed in triplicate and error bars are standard deviation of the mean. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.6. *One-pot synthesis of L-gluco-heptulose*

The coupling of TAm and TK in a single one-pot reaction at 50 °C was then investigated for the integrated synthesis of L-*gluco*-heptulose from L-arabinose, L-serine and α-ketoglutaric acid (Scheme 3). The process used to enable the one-pot synthesis employed either TK_{Dgeo} or TK_{Drad} and TAm_{Dgeo} simultaneously as either cell lysates or purified enzymes. One-pot reactions were set up with TAm_{Dgeo}, amino donor and acceptor substrates, L-serine and α-ketoglutaric acid, in the presence of the TK substrate, L-arabinose and cofactors for both enzymes. It should be noted that the one-pot reaction with TAm_{Gste} and either of the TKs failed to produce L-*gluco*-heptulose from L-arabinose, under the preferred reaction conditions and therefore the focus of the one-pot reaction was with TAm_{Dgeo}. Investigations into the one-pot synthesis showed that the conversion yield of L-*gluco*-heptulose was consistently higher when using purified enzyme (62–88%) than the yield achieved with clarified cell lysates under comparable conditions (Table 5). Activity of both TK and TAm is not only dependent on temperature but also the pH and considering all the data it is possible to combine the two enzymes in a one-pot reaction as pH optima for both types of enzyme are quite similar. The one-pot reaction was examined at pH 8–8.5 and Table 5 shows preferred pH conditions for both of the one-pot reactions.

For purified TK, the yields of L-*gluco*-heptulose obtained by the two-step cascade were significantly higher than those obtained when synthetic Li-HPA was used for the single step TK reaction under comparable conditions. Li-HPA stability has been extensively studied under various conditions showing that while Li-HPA appears to be stable in pure water, it decomposes over time in buffered solutions with pHs above 7 at high temperatures [24,29,34]. Such instability would inhibit the maximum activity of the TK towards L-arabinose, a poor acceptor substrate which requires a longer reaction time. However, HPA produced in situ would be continuously consumed by the TK and hence decomposition of the HPA is not a significant problem. This may explain why the higher yields of L-*gluco*-heptulose were observed in the two-step reaction. This interestingly makes the two-step reaction more efficient than the single TK step.

It was demonstrated using synthetic Li-HPA that both TK_{Dgeo} and TK_{Drad} were thermostable enzymes working efficiently at 50 °C for the



Scheme 3. Enzymatic cascade synthesis of L-*gluco*-heptulose from L-arabinose and L-serine.

production of L-*gluco*-heptulose from L-arabinose. An application such as this would be beneficial in industrial processes where thermophilic enzymes possess certain advantages over their mesophilic counterparts [20]. However the high expense of Li-HPA makes it an undesirable keto-donor thus hindering its use. Therefore, the identification of a TAm producing HPA from L-serine at 50 °C was a crucial part of the application. The coupling of the TAm and TK in a single system at 50 °C for the synthesis of L-*gluco*-heptulose from L-arabinose and L-serine could significantly reduce the cost of overall application and furthermore reduces the problem of HPA instability at high temperatures as previously mentioned.

The rate of reaction with purified TAm_{Drad} and either TK was seen to slow markedly after 24 h with little product formation up to 72 h (Fig. 4). The addition of further TK and TAm enzymes was conducted after 24 h. Upon the addition of more TK, no increase in L-*gluco*-heptulose production was observed whilst the addition of more TAm at 24 h led to a 15% increase in L-*gluco*-heptulose production in both one-pot reactions with either TK_{Dgeo} and TK_{Drad}. This suggested that the reaction yield was limited by the loss of TAm enzyme activity which has previously been reported for this class of enzyme [11,35]. The initial rates for TAm_{Dgeo} (1.24 mM h^{-1}), TK_{Dgeo} (0.48 mM h^{-1}) and TK_{Drad} (1.02 mM h^{-1}) were within the same orders of magnitude. To this, one equivalent of L-arabinose and three equivalents of L-serine were added to shift the equilibrium of the first step towards excess HPA generation for the subsequent TK reaction, which resulted in high conversion yields of 62% and 88% for L-*gluco*-heptulose in the TK_{Dgeo} and TK_{Drad} one-pot reaction respectively with purified enzyme. Furthermore, it appeared that TAm_{Dgeo} was specific for L-serine and not the L-arabinose or the product, L-*gluco*-heptulose. This means that the two-step reaction was working towards maximum conversion without any limitations, unlike the two-step recycling cascade for the production of ABT [13]. The preferred substrate of the TAm in that study was the GA, resulting as a significant side reaction rather than amination of L-erythrulose to give ABT.

As well as in the upgrading of sugar beet pulp monosaccharides, it would be useful to explore the in situ production of ketol donor for other TK reactions and compounds. Indeed, the in situ synthesis of HPA has very recently been shown to be efficient in the two-step cascade reaction towards highly valuable L-*erythro* (3*S*,4*S*) ketoses at elevated temperatures [29]. Moreover, the recycling of HPA in a two-step cascade has proved to be feasible for the synthesis of chiral amines such as ABT [13]. This further emphasises the importance and sustainability of such procedures as an alternative to the conventional chemical synthesis of HPA.

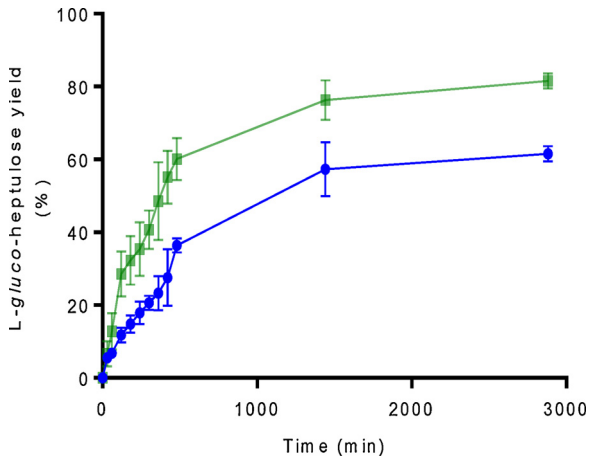


Fig. 4. Time scale of L-*gluco*-heptulose production in TAm_{Dgeo}/TK_{Dgeo} (blue) and TAm_{Dgeo}/TK_{Drad} (green) one-pot reaction. Yield of L-*gluco*-heptulose was measured over 72 h in presence of L-serine (30 mM), α-ketoglutaric acid (30 mM), PLP (0.125 mM), L-arabinose (10 mM), $MgCl_2$ (4.5 mM), ThDp (1.25 mM), HEPES buffer (50 mM, pH 8). All measurements were performed in triplicate and error bars are standard deviation of the mean. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Conclusions

The synthesis of the value added compound L-*gluco*-heptulose from L-arabinose, was successfully demonstrated at 50 °C, catalysed by thermostable TAms and TKs. The construction of this process was achieved in a one-pot reaction using either cell lysates or purified enzymes. The two-step reaction process showed improved efficiency as conversion yields of the target product are higher compared to when a single TK step was investigated for L-*gluco*-heptulose production. The efficiency of the two-step cascade approach was based on the characterisation of novel thermostable TAm and TK for the in situ synthesis of HPA and L-*gluco*-heptulose respectively. It would also be useful to demonstrate this synthesis as a whole cell biocatalyst instead of using isolated enzymes. The two-step cascade could utilise natural substrates that reside in the cell, such as L-serine and α-ketoglutaric acid with externally added L-arabinose and this would lead to whole cell catalysts and thus provide a route to batch bioconversions of L-arabinose to product.

Table 5
Reaction conditions of one-pot synthesis of L-*gluco*-heptulose.

One-pot reaction	TAm/TK (mg/mL)	pH	Reaction Temp (°C)	Reaction time (h)	Conversion yield of L-*gluco*-heptulose (%)
TAm_{Dgeo} + TK_{Dgeo} (clarified lysate)	0.2/0.2	8	50	24	45
TAm_{Dgeo} + TK_{Drad} (clarified lysate)	0.2/0.2	8	50	24	43
TAm_{Dgeo} + TK_{Dgeo} (purified)	0.42/0.29	8	50	16	62
TAm_{Dgeo} + TK_{Drad} (purified)	0.42/0.29	8	50	16	88

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