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Stabilization of *Escherichia coli* uridine phosphorylase by evolution and immobilization

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ABSTRACT

Mutation and immobilization techniques were applied to uridine phosphorylase (UP) from *Escherichia coli* in order to enhance its thermal stability and hence productivity in a biocatalytic reaction. UP was evolved by iterative saturation mutagenesis. Compared to the wild type enzyme, which had a temperature optimum of 40 °C and a half-life of 9.89 h at 60 °C, the selected mutant had a temperature optimum of 60 °C and a half-life of 17.3 h at 60 °C. Self-immobilization of the native UP as a Spherezyme showed a 3.3 fold increase in thermostability while immobilized mutant enzyme showed a 4.4 fold increase in thermostability when compared to native UP. Combining UP with the purine nucleoside phosphorylase from *Bacillus halodurans* allows for synthesis of 5-methyluridine (a pharmaceutical intermediate) from guanosine and thymine in a one-pot transglycosylation reaction. Replacing the wild type UP with the mutant allowed for an increase in reaction temperature to 65 °C and increased the reaction productivity from 10 to 31 g l^{-1} h^{-1}.

1. Introduction

Nucleoside analogues are widely used as antiviral and anticancer drugs, where they act as inhibitors of viral replication or cellular DNA replication. The antiviral compounds stavudine and AZT (azidothymidine) can be synthesized from β-thymidine, which can in turn be synthesized from 5-methyluridine (5-MU). The traditional synthetic routes for these compounds are often complex and inefficient multi-stage processes [1]. We have previously demonstrated that a combination of the purine nucleoside phosphorylase (PNP, EC 2.4.2.1) from the thermotolerant alkalophile *Bacillus halodurans* (BHPNP1) with the *Escherichia coli* uridine phosphorylase (EcUP, EC 2.4.2.3) in a one-pot cascade reaction can produce 5-MU in high yield [2,3] (Fig. 1.). The optimal operating conditions, with loadings based on mass of substrate per reaction mass (m m^{-1}), were found to be 9% guanosine (378 mM) and 4.7% thymine (439 mM) at 60 °C with an enzyme loading of 2000 U l^{-1} operating in a low shear environment. Under these conditions, a final product concentration of 84 g l^{-1}, a guanosine conversion of >95% and a 5-MU yield of 85% were achieved. An overall productivity of 10 g l^{-1} h^{-1} 5-MU was possible, approaching the figure of 15.5 g l^{-1} h^{-1} that Straathof et al. [4] indicate is the average for economic viability.

This reaction productivity could be significantly improved by increasing reaction temperature. Due to the low solubility of the reaction components the biocatalytic reaction medium is a slurry with limited solid–liquid mass transfer [3]. However, the current optimal reaction temperature of 60 °C is constrained by the low thermostability of the UP at 60 °C and higher enzyme loading is required to offset the rate of thermal deactivation. Hence it is desirable to improve the volumetric productivity of the transglycosylation reaction by enhancing the thermostability of EcUP by mutation or immobilization.

Of particular interest for rapid evolution of enzyme stability is the method developed by Reetz and co-workers [5–7] known as iterative saturation mutagenesis (ISM). The method combines the randomization of saturation mutagenesis with rational design in that the saturation is targeted at or areas of the protein that are likely to create an enhanced phenotype based on structural or catalytic information. In addition, this method represents a "rapid" form of evolution in that the libraries created are small and focused and therefore do not require extensive screening programs. Analysis of mesophilic and thermophilic enzymes shows

Abbreviations: PNP, purine nucleoside phosphorylase; PyNP, pyrimidine nucleoside phosphorylase; UP, uridine phosphorylase; BHPNP1, *Bacillus halodurans* PNP; 5-MU, 5-methyluridine; SZ, Spherezyme; EcUP, *Escherichia coli* UP; ISM, iterative saturation mutagenesis; NP-4, Nonoxyl 4.

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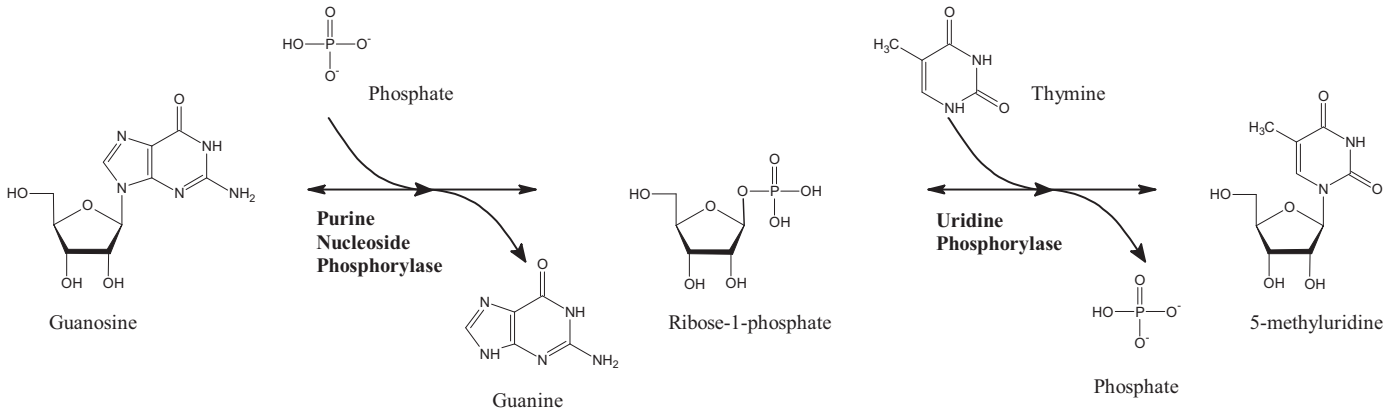

Fig. 1. Transglycosylation reaction for the production of 5-methyluridine from guanosine and thymine.

that extremophilic enzymes have a higher degree of surface rigidity. Reetz et al. [6] therefore targeted amino acids with the highest degree of flexibility indicated by atomic displacement parameters available from X-ray data, namely B-factors. The B-Factor Iterative Test (B-FIT) highlights the amino acids with the highest flexibility and thereby creates targets for mutagenesis. EcUP is a good candidate for directed evolution through ISM as the crystal structure has been determined [8,9], which simplifies the process of determining saturation targets and, as a native *E. coli* enzyme, expression of EcUP mutants is well suited for an *E. coli* expression system. Previous research of mutagenesis on pyrimidine nucleoside phosphorylases (PyNP), of which EcUP is a sub-class, was directed at discovering residues critical to folding [10] and for determining active site residues [11]. To date no mutagenesis studies have been reported for the specific enhancement of physical or catalytic characteristics of PyNP.

An alternative route to stabilization is through immobilization [12,13]. The *E. coli* UP and PNP have been co-immobilized previously by covalent linkage to epoxy-activated Sepabeads for the biocatalytic preparation of a variety of natural and modified purine nucleosides [14]. Similarly, the nucleoside phosphorylase from *Geobacillus stearothermophilus* was covalently immobilized on aminopropylated macroporous glass [15]. These preparations showed increased thermal stability and high levels of activity retention (>80%) when immobilized. Of particular interest is the work of Hori and co-workers, who immobilized PNP and PyNP from *G. stearothermophilus* by ionic binding to DEAE-Toyopearl 650 M anion exchange resin [16]. Using the immobilized biocatalysts, they were able to design a continuous reaction for the production of 5-methyluridine from inosine and thymine which was run for 17 days at 60 °C. Self-immobilization techniques, such as the Spherezyme method, are particularly suited to multimeric enzymes as they eliminate the potential of only one of the monomers binding to a carrier [12]. This study aims to show that stabilization of EcUP, through either enzyme evolution, immobilization or a combination thereof, can lead to increased reaction productivity for the synthesis of 5-MU.

2. Experimental

2.1. Materials

Thymine, guanosine, 5-methyluridine and guanine standards were purchased from Sigma (Missouri, USA). The enzymes purine nucleoside phosphorylase from *B. halodurans* (BHPNP1), uridine phosphorylase from *E. coli* (EcUP) and mutant *E. coli* UP (UPL8) were expressed in *E. coli* as *E. coli* JM109[pMSPNP], *E. coli* BL21(DE3)[pETUP] and *E. coli* BL21(DE3)[pETUPL8], respectively. The enzymes were produced by fermentation as according to methods previously described [2,3].

2.2. Choice of saturation mutagenesis targets

The crystal structure of *E. coli* UP (1LX7) [17] was used to determine surface residues with the highest degree of flexibility, indicating potential areas of structural instability [6]. Target amino acids were identified using "B-fitter" [6]. Six regions of interest (mutant libraries 1–6) were identified for saturation mutagenesis (Fig. 2).

2.3. Mutagenesis

A QuikChange II Mutagenesis Kit (Stratagene, USA) was used to perform plasmid based mutagenesis. Primers were obtained from Inqaba Biotech (Pretoria, South Africa). To initiate the reaction, 1 μl of *PfuTurbo* DNA polymerase (2.5 U μl^{-1}) was added to the reaction mixes. The PCR reaction was as follows. A single hold at 95 °C for 1 min was followed by 18 cycles at 95 °C for 50 s, 55 °C for 50 s, and 68 °C for 5 min, followed by a hold at 68 °C for 7 min. *Dpn*I restriction enzyme (5 μl) was then added to each reaction and incubated for 5 h at 37 °C to digest the parental (i.e., the nonmutated) supercoiled dsDNA. The mutated plasmid was then cleaned and concentrated (Zymogen DNA clean up kit, Fermentas). Between 100 and 250 ng of this material was used to transform competent *E. coli* XL1 blue cells by heat shock (42 °C, 45 s).

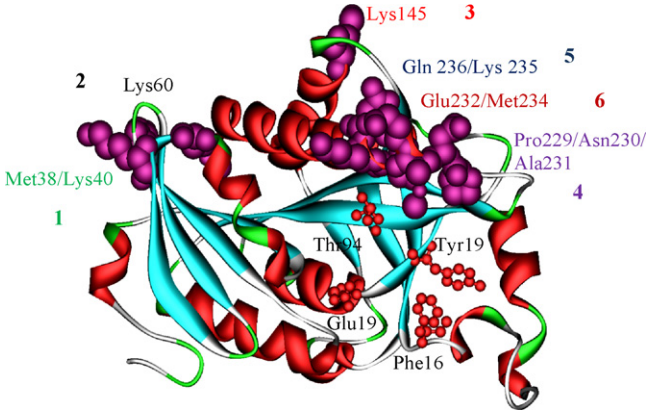

Fig. 2. Ribbon representation of *E. coli* uridine phosphorylase based on the 1LX7 structure [9]. Catalytic residues are shown in ball and stick format and sites targeted for saturation mutagenesis (1–6) based on high B-factors are in CPK format.

2.4. Preparation of mutant screening libraries

Mutant libraries were plated onto Luria agar (100 μg ml^{-1} ampicillin) in Q-trays (Genetix, UK) and incubated overnight at 37 °C. Colonies were picked and inoculated into Luria Bertani (LB) medium (60 μl, 384 well microtitre plates) using the QPix2 colony picker (Genetix, UK). The number of colonies picked ranged from 600 to 3500 per library depending on the number of colonies required to obtain coverage of all the possible mutations. A total of 12,300 clones were picked across the 6 initial libraries. After an overnight incubation, duplicate plates were prepared using the replication function of the QPix2. The replicate microtitre plates were incubated overnight and served as the back-up cultures. To the master plates, IPTG was added to a final concentration of 1 mM. These plates were incubated for a further 20 h to facilitate mutant protein expression. Cells were then harvested by centrifugation (3000 × *g*, 20 min). The cells were broken by the addition of 15 μl B-Per (Pierce, USA) directly to the cell pellet followed by 60 min incubation at room temperature. Cell debris was removed by centrifugation (3000 × *g*, 20 min).

2.5. Library screening

p-Nitrophenol-β-D-ribofuranoside, prepared according to the methods of Schramm et al. [18], was used as the substrate for UP screening. For 96 and 384 well microtitre plates a volume of 240 μl or 40 μl, respectively, was added to an aliquot of crude cell extract. The change in absorbance due to the release of *p*-nitrophenol was measured at 410 nm using a Powerwave HT microtitre plate reader (Biotek, USA).

Primary screening (set point residual activity): Activity of the samples was measured before and after incubation at 70 °C for 15 min. The wild type *E. coli* UP showed 10% residual activity under these conditions. Hits from each of the libraries were selected based on the highest percentage residual activity.

Secondary screening (thermostability profile): Primary hits were re-inoculated into 5 ml LB broth and incubated overnight. The plasmid harboring the mutated gene was then extracted (QIAprep Spin Miniprep Kit, Qiagen, USA). This plasmid was used to transform *E. coli* XL1 blue. This new culture was then grown (50 ml LB 100 μg ml^{-1} ampicillin) and protein expression induced (0.1 mM IPTG, 3.5 h). Cells were harvested by centrifugation (3000 × *g*, 20 min) and disrupted by addition of B-Per (4 ml per gram wet weight). After removal of cellular debris, the expressed protein was further purified by ultrafiltration through a 100 kDa membrane (Amicon, USA). The resultant protein solutions were then incubated at temperatures between 40 and 80 °C for 60 min to determine the temperature at which 50% of the initial activity was retained ($T_{50\ (\%)}^{60\ (\mathrm{min})}$ value).

2.6. Iterative mutagenesis

The plasmid expressing the mutated enzyme showing the highest stability after the first round of mutagenesis was used as the template for the second round of mutagenesis. In this case a strain from library 5 showed the highest residual activity after a 15 min incubation at 70 °C (95% activity retained). The plasmid harboring this mutated gene was used in a PCR with the mutation primers for library 4 and library 1, which had given the next two best hits, respectively. The second round of saturation mutagenesis and subsequent screening was performed as described above. Plasmid DNA from the best results from each of the mutation experiments was isolated and sequenced as before (Inqaba Biotech).

The plasmid for the best mutant (UPL8 from library 8) was isolated from the *E. coli* XL1 blue strain (QIAprep Spin Miniprep Kit, Qiagen, USA) and retransformed by heat shock (45 °C, 45 s) into competent *E. coli* BL21 (DE3) for over expression and production of the mutant enzyme. This strain was designated *E. coli* BL21 (DE3)[pETUPL8].

2.7. Production and characterization of UPL8

The mutant enzyme was produced in two 10 l fermentations and purified as described previously [3]. Characterization of UPL8 was performed according to a modified method of Hammer-Jespersen et al. [20] wherein a suitably diluted broth sample (10 μl) was added to 190 μl of 50 mM sodium phosphate buffer containing 2.5 mM uridine, in 96 well polypropylene microtitre plates. After 10 min incubation at 40 °C, the reaction was stopped by addition of 100 μl of 0.5 M perchloric acid. The samples were then incubated on ice for 20 min and centrifuged for 20 min (7000 × *g*) to remove residual protein. Sample (100 μl) was then transferred to a UV compatible microtitre plate and combined with 100 μl of 1 N NaOH. The change in absorbance at 290 nm due to the liberation of uracil was measured on a Powerwave HT microplate spectrophotometer. One unit (U) of UPase was defined as the enzyme required for liberation of 1 μmol of uracil from uridine. The extinction coefficient under these conditions was determined to be 3240 M^{-1} cm^{-1}. For pH profiling the phosphate buffer in the standard assay was replaced with Universal buffer [21] (50 mM Tris, 50 mM boric acid, 33 mM citric acid, 50 mM Na_2PO_4, adjusted with either HCl or NaOH to pH values between 3 and 11). Temperature profiling was performed using the standard assay between temperatures of 30 °C and 90 °C. Thermostability was determined by incubating enzyme solutions (wild type UP and UPL8) at 60 °C or 70 °C. Samples were analyzed for activity over a 6 h period. UPL8 kinetic parameters were determined using the standard assay, with uridine initial concentrations varying between 0.1 mM and 5.0 mM. The reaction was stopped at 1, 2, 3, 4, 6 and 10 min for selection of data within the linear range. Michaelis–Menten plots and the linear transformations (Lineweaver–Burk, Hanes–Woolf and Eadie–Hofstee) were used to determine kinetic parameters.

2.8. Enzyme immobilization

The enzymes were immobilized as Spherezymes [22]. This technique uses a water in oil emulsion and addition of a protein cross-linking agent to generate spherical self-immobilized macromolecular biocatalysts. Solutions (2 ml) of EcUP (100 mg ml^{-1}), UPL8 (100 mg ml^{-1}) and BHPNP1 (70 mg ml^{-1}) were prepared. In addition, mixtures (2 ml) of EcUP and BHPNP1 (60 and 70 mg, respectively) as well as UPL8 and BHPNP1 (85 and 70 mg, respectively) were prepared for co-immobilization studies. Active site protectants (50 mM inosine and/or 50 mM uridine) were combined to the solution directly prior to cross linking. To these solutions, 320 μl of the cross linker, which consisted of equal volumes of glutaraldehyde (25% solution) and polyethyleneimine (5% solution), was added, mixed and then directly added to 20 ml of the oil phase (mineral oil with 0.05% NP-4). The solutions were stirred at 700 rpm with a magnetic stirrer for 1 min to ensure a proper emulsion. Stirring was then decreased and the emulsion was allowed to incubate overnight at 4 °C. The emulsion was then broken and the particles recovered by centrifugation (Beckman J-21, 1000 × *g*, 10 min). Immobilized enzyme particles were washed 4 times with 50 mM Tris–HCl, pH 8.0, containing 1 mM ethanolamine. Excess ethanolamine was washed off with the same Tris buffer. Finally, the immobilized enzyme particles were recovered by filtration under vacuum (Whatman No. 1). The immobilized enzyme particles were then dried at room temperature under high vacuum (Virtis Genesis 25L freeze dryer, USA).

Table 1
Best hits from libraries UP 7 and UP 8 based on residual activities observed after incubation of the enzyme preparations for 1 h at 75 °C.

Library	Mutant	Observed mutation	% Residual activity
Control	n/a	n/a	3.70%
7	UPL7	Met38Val; Lys40Asp Lys235Arg; Gln236Ala	88.5%
8	UPL8	Lys235Arg; Gln236Ala	80.2%

2.9. Transglycosylation by stabilized enzyme preparations

A series of transglycosylation experiments were performed to compare various combinations of biocatalysts. Reactions (100 ml) contained 1.5% m m^{-1} loading of guanosine and thymine in 50 mM sodium phosphate buffer (pH 8.0) with 200 U l^{-1} of each of the biocatalysts. Reactions were performed at 60 °C and 70 °C in round bottomed flasks immersed in an oil bath controlled at the set temperatures. Flasks were fitted with condensers to negate the effects of evaporation. Mixing was achieved with magnetic stirrers at 500 rpm.

2.10. Synthesis of 5-MU

The reaction (65 °C, 100 ml) contained 9.0% m m^{-1} guanosine and 4.7% m m^{-1} thymine suspended in 50 mM sodium phosphate buffer, pH 8.0, in a round bottomed flask fitted with a condenser. A 1000 U l^{-1} biocatalyst loading was used. Samples (100 μl) were removed (in triplicate) hourly. The sample was diluted in 900 μl of 10 M NaOH to stop the reaction and fully dissolve the nucleosides. This solution was then further diluted in 1 M NaOH for analysis so as to ensure that the sample concentration was within the linear region of the calibration curve. Guanosine, guanine, thymine and 5-MU were quantitatively analyzed by HPLC, using a Waters Alliance Model 2609 instrument with a Synergi 4 μm Max-RP 150 mm × 4.6 mm column and compared to pure standards (Sigma–Aldrich). Components were detected using a UV detector at 260 nm. The eluent was 25 mM ammonium acetate (pH 4.0), at a flow rate of 1 ml min^{-1} and a run time of 20–30 min at 25 °C. Elution times for guanine, thymine, 5-MU and guanosine were 6.53, 9.38, 17.20 and 19.66 min, respectively.

3. Results and discussion

3.1. Mutagenesis

The *E. coli* UP was mutated using iterative saturation mutagenesis guided by the B-Fit method [5–7], with the aim of improving thermal stability, and hence permitting application at higher temperatures with the intention of enhancing biocatalytic reaction productivity. The best hits after the primary screening were from libraries 1, 4 and 5 based on their retained activity after incubation at 70 °C for 1 h (32%, 51% and 96%, respectively). Mutation of the best hit from library 5 (Lys235Arg; Gln236Ala) with the primers for library 4 (giving library 7) and library 6 (giving library 8) again resulted in positive results in initial screening (Table 1), now performed at the elevated temperature of 75 °C for 15 min.

Determination of residual activities after incubating the mutant enzymes at set temperature for 1 h (Fig. 3) showed good stability at 70 °C for both mutants but no activity at 80 °C, skewing the final stability values. The mutant from library 8 (UPL8) showed better activity retention at 70 °C and it was therefore decided to determine the stability of that enzyme at 60 and 70 °C to get a better indication of improved thermostability (Fig. 4). These results showed marked improvements in stability at both 60 and 70 °C compared to the wild type UP.

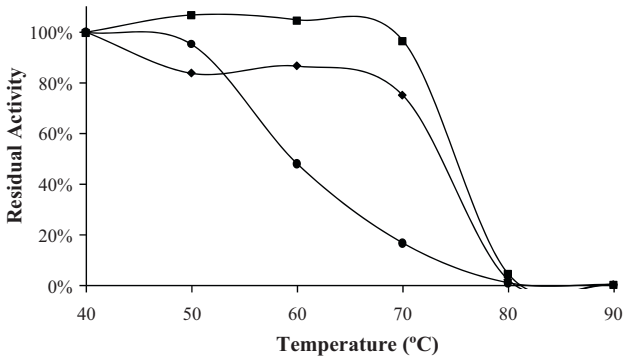

Fig. 3. Plot of residual activity for mutants UPL7 (♦) and UPL8 (■) compared to wild type UP (-●-). Residual activity was determined after incubation for 60 min at the set temperatures.

The characterization we performed previously [19] indicated that BHPNP1 would operate most effectively between 60 °C and 70 °C for the duration of the biocatalytic reaction. The target for directed evolution was therefore to enhance EcUP thermostability to match that of the BHPNP1. The results in Fig. 4 clearly show that this was achieved with UPL8. Although further stabilization could possibly be obtained by further rounds of mutation, it was unnecessary since further enhancements in stability would then outperform BHPNP1. It was decided therefore to continue with characterization of this mutant.

3.2. Characterization of the mutant UPL8

UPL8 showed a pH optimum of 7.0, retaining 60% activity between pH 5.6 and 8.4 which is similar to the wild type UP (optimum of 7.0, retaining 60% activity between pH 6.0 and 8.2). UPL8 has a significantly improved temperature optimum (60 °C) and a broader activity range, retaining 60% activity between 37 and 67 °C. In contrast, native UP had an optimum of 40 °C with a narrow activity range (retaining 60% activity) between 30 and 52 °C. The thermal characteristics of the modified enzyme were now similar to those of BHPNP (optimum of 70 °C, range of 30–74 °C). Wild type UP showed a half life of 9.9 h at 60 °C and inactivated almost instantaneously at 70 °C. The mutant enzyme had a half life at 60 °C of 17.3 h and 3.3 h at 70 °C (Table 2). The thermal characteristics of the modified enzyme were now similar to those of BHPNP1 (optimum of 70 °C, range of 30–74 °C) [17].

Data obtained for varying uridine concentrations also showed good linear regression fit ($R^2 \geq 0.95$). From the plots (Lineweaver–Burk, Eadie–Hofstee and Hanes–Woolf), K_M and V_{max} were determined with less than 5% deviation in the values cal-

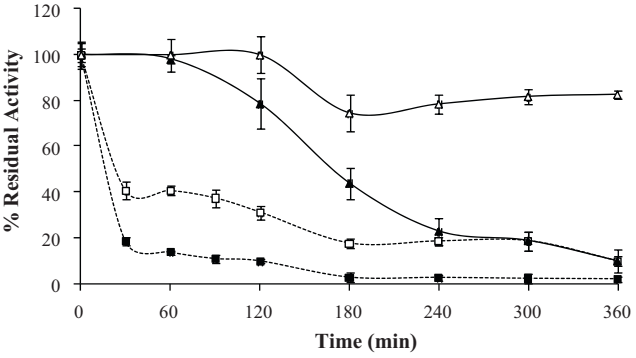

Fig. 4. Thermostability comparison for EcUP (■) and mutant UPL8 (▲). Enzyme preparations were incubated at 60 °C (open symbols) and 70 °C (closed symbols) for 6 h. Data averaged from triplicate results.

Table 2
Physical and kinetic characteristics of UPL8 and EcUP characterized using uridine as the substrate at 40 °C.

Parameter	Unit	EcUP	UPL8
Specific activity	U mg^{-1}	30.69	19.18
K_M	μM	233.9	464.3
V_{max}	mol s^{-1}	4.57×10^{-5}	6.46×10^{-5}
k_{cat}	s^{-1}	2.73×10^{7}	2.81×10^{7}
Specificity constant	M^{-1} s^{-1}	1.17×10^{11}	6.28×10^{10}
pH optimum	–	7.0	7.0
pH range	–	6.0–8.2	5.6–8.4
Temp optimum	°C	40	60
Temp range	°C	30–52	38–67
Temp stability ($t_{1/2}$ at 60 °C)	h	9.9	17.3
Temp stability ($t_{1/2}$ at 70 °C)	h	–	3.3

culated from the three plots. Subsequently the turnover number (k_{cat}) and the specificity constant were calculated. The data is summarized in Table 2.

3.3. Sequence and homology model analysis of the mutant UPL8

The best mutant identified from the first round of mutation was from library UP5, which targeted Lys235 and Gln236. The subsequent mutations (those from libraries UP4 and UP6) targeted Pro229, Asn230, Ala231; and Glu232, Met234 in two separate experiments, respectively. The expectation therefore would be to achieve between 2 and 7 mutations in the final mutants. The best mutant from library UP7 showed a total of 4 mutations (Table 1). These additional mutations were not necessarily beneficial as the UPL8 mutant showed only the original mutations at position 235 (Lys → Arg) and 236 (Gln → Ala), yet UPL8 was shown to be the superior mutant. This was unexpected as the Lys235Arg mutation is an exchange of similar, basic amino acids. The larger arginine should also have increased flexibility (and therefore decrease stability) at the site due to it being a longer side chain. This longer side chain may however be interacting with the neighboring α-helix, thereby conferring rigidity to the overall structure. The Gln236Ala mutation does fit with the theory of decreased flexibility due to alanine having a smaller side chain and being non-polar as opposed to the polar glutamine. Why just these two amino acid changes should have such a marked effect on the stability of the protein is unknown. Both are positioned on the α-helix leading to the N-terminal of the protein. This entire domain may have created instability in the native protein and it is plausible that these mutations stabilized that region. This is further confirmed by the mutation in library 4, where removal of the entire α-helix yielded good thermostability characteristics. The mutations are also situated in close proximity to the entrance of the binding pocket and not associated with subunit binding, indicating that this enzyme is thermally denatured due to distortion of the active site rather than dissociation of the subunits. To prove this, an experiment was performed to determine the primary mode of thermal inactivation of the native enzyme by incubating different concentrations of the enzyme at 60 °C. Results of this experiment (data not shown) showed that the rate of inactivation is independent of enzyme concentration, indicating that distortion (and not subunit dissociation) is the primary mode of thermal inactivation. Mutations that decrease distortion would therefore show the improvement in stability noted in this research.

This mutant UP is compared in Table 3 to the few characterized wild type enzymes reported in the literature. Additionally PyNP from *B. subtilis* [23] and *T. thermophilus* [24] have been purified for crystallography studies, but no characterization was reported. The PyNP from *G. stearothermophilus* has the highest temperature optimum and thermal stability reported to date. *E. coli* UPL8 is then the next most stable PyNP. The substrate affinity of the mutant enzyme (K_M = 0.46 mM) is lower than both the native *E. coli* and the *G. stearothermophilus* enzymes, but is still within the micromolar range, making it significantly active towards uridine.

3.4. Enzyme immobilization

Immobilization of enzymes can lead to enhanced thermal stability [15], and hence could result in improved reactor productivity at higher temperatures where the enzyme would otherwise denature. As the enzymes EcUP, UPL8 and BHPNP1 are all multimeric, it was decided to use an immobilization method that could provide both inter-subunit bonds (to enhance multimer stability) and inter-enzyme bonds. The method used was the Spherezyme self-immobilization technique that does not require any carrier. EcUP, UPL8 and BHPNP1 were all successfully immobilized with varying degrees of activity retention using this method (Table 4). Both the immobilized EcUP (EcUP-SZ) and the EcUP co-immobilized with BHPNP1 (EcUP/BHPNP1-SZ) showed improved temperature

Table 3
Physical and kinetic characteristics of reported prokaryotic UP.

Organism	K_M (mM) (uridine)	pH optimum	Temperature optimum	Ref.
E. coli	0.15	7.5	37	[25]
L. casei	3.8	7.0	–	[26]
E. carotovora	–	–	60	[27]
UPL8	0.46	7.0	60	This study
E. aerogenes	0.7	8.52	65	[28]
G. stearothermophilus	0.19	7.2	70	[29,30]

Table 4
Characteristics of free and immobilized (Spherezyme) forms of EcUP, UPL8 and BHPNP1. Data for co-immobilized enzymes was determined using the uridine phosphorylase assay.

Biocatalyst	Specific activity (U mg^{-1})	Activity retention (%)	pH optimum	Temp optimum (°C)	Temp range (°C)
EcUP	18.3	–	7.0	40.0	30–52
EcUP-SZ	2.7	4.5	7.0	60.0	40–67
EcUP–BHPNP1-SZ	2.4	13.9	7.0	60.0	40–80
UPL8	12.3	–	7.0	60.0	40–67
UPL8-SZ	1.8	2.2	7.0	60.0	40–80
UPL8/BHPNP1-SZ	3.2	40.9	7.0	60.0	40–80
BHPNP1[a]	8.7	–	7.0	70.0	32–74
BHPNP1-SZ[a]	1.0	25.4	7.0	50.0	40–80

[a] Data determined using guanosine as the substrate.

optima and had activity at 70 and 80 °C, which had not been noted with the free enzyme. UPL8-SZ did not show an increase in the temperature optimum but did exhibit a broader thermal range, maintaining significant activity at 70 and 80 °C. Both preparations maintained the pH optimum profiles seen for the free enzymes. No significant changes were noted in either the temperature or pH optimum for BHPNP1, although the preparation did show greater activity at 80 °C than that noted for the free enzyme. In addition to the single enzyme preparations, co-immobilized combinations were also evaluated. Co-immobilizing UP with BHPNP1 seemed to increase the cross-linking efficiency and activity retention of the UP, with UPL8 and EcUP showing increase to 13.9% and 40.9% in activity retention, respectively, when immobilized with BHPNP1. The physical characteristics of the co-immobilized enzymes were similar to that of the single-immobilized preparations.

Hori and co-workers [16] immobilized 0.42 units of crude cell extract (containing PNP and PyNP) from *G. stearothermophilus* on anion exchange resin for production of 5-MU and showed no loss on activity through immobilization. The PNP and PyNP from *G. stearothermophilus* were immobilized on a glass solid support [15] with only 30% loss in initial activity. Similar activity loss was noted for the immobilization of *E. coli* PNP and PyNP on Sepabeads [14]. In contrast, between 51 and 86% of the activity was lost on Spherezyme formation although this figure may be improved upon further optimization of the immobilization process. The advantage of immobilization by Spherezymes, however, is the high specific activity compared to other preparations. In the study by Zuffi and co-workers, specific activities (per mg of immobilized biocatalyst) were 0.18 and 0.04 U mg^{-1} for PNP and UP, respectively. In comparison, co-immobilized BHPNP1 and UPL8 showed specific activities (per mg Spherezyme) of 0.6 and 2.6 U mg^{-1}, respectively.

3.5. Production of 5-MU by transglycosylation using free enzyme preparations

The control reaction (using BHPNP1 and EcUP at 60 °C) showed similar results to those obtained previously [2], indicating that the reaction conditions were similar (Fig. 5). Use of the mutant uridine phosphorylase (UPL8), however, showed a marked improvement in reaction productivity (5.0 g l^{-1} h^{-1} compared to 1.29 g l^{-1} h^{-1} for the control) while maintaining the same yield (73% yield compared to 75% for the control, Table 5).

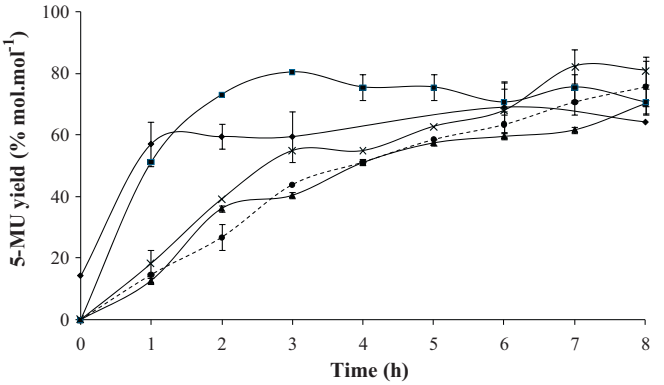

Fig. 5. Selected transglycosylation experiment showing the 5-MU yield obtained over time when using 200 U l^{-1} free EcUP (●) or free UPL8 (■) in combination with free BHPNP1; 200 U l^{-1} separately immobilized EcUP and BHPNP1 (×) and co-immobilized UPL8 and BHPNP1 (▲); and 1000 U l^{-1} separately immobilized EcUP and BHPNP1 (♦). All reactions were performed using 1.5% m m^{-1} substrate loading at 60 °C. Data averaged from triplicate samples.

3.6. Production of 5-MU by transglycosylation using immobilized preparations

The use of immobilized enzymes for this reaction could potentially have two advantages, namely an increase in stability of mesophilic enzymes allowing a higher reaction temperature, and the ability to recycle the biocatalyst to decrease the catalyst cost. The results obtained for the use of single immobilized enzymes demonstrated increased stability compared to the native EcUP, indicated by the production of 5-MU at 70 °C (Table 5). This increased stability however did not lead to a significant increase in reaction productivity at 60 °C (1.50 g l^{-1} h^{-1} compared to 1.29 g l^{-1} h^{-1} for the free enzyme control. Higher 5-MU yield was noted when using UPL8-SZ (70%, Reaction 8) compared to using EcUP-SZ (29%, Reaction 6) at 70 °C.

Co-immobilizing enzymes could be advantageous in that the proximity of the two enzymes could enhance the mass transfer characteristics of the system, thereby increasing the reaction rate while maintaining the other potential advantages discussed above. Using Spherezyme technology it was indeed possible to co-immobilize two multimeric enzymes. However similar yields and reaction productivities were seen for the co-immobilized enzymes (Reactions 10–13) when compared to the single immobilized preparations. Immobilized preparations did show higher yields at

Table 5
Comparative figures for guanosine conversion, 5-MU yield and reaction productivity for transglycosylation reactions using free enzyme, immobilized enzyme and co-immobilized enzyme combinations.

Rxn[a]	Biocatalysts[b]		Temp (°C)	Reaction time (h)	Guanosine conversion (% mol/mol)	5-MU yield (% mol/mol)	5-MU productivity (g l^{-1} h^{-1})
	PNP	PyNP					
1	BHPNP1	EcUP	60	8	88.9	75.6	1.29
2	BHPNP1	EcUP	70	8	44.4	0.0	0.00
3	BHPNP1	UPL8	60	2	91.1	73.1	5.00
4	BHPNP1	UPL8	70	8	44.4	0.0	0.00
5	BHPNP1-SZ	EcUP-SZ	60	7	86.7	76.8	1.50
6	BHPNP1-SZ	EcUP-SZ	70	8	57.8	29.2	0.50
7	BHPNP1-SZ	UPL8-SZ	60	8	93.3	69.5	1.19
8	BHPNP1-SZ	UPL8-SZ	70	8	75.6	70.6	1.21
9	BHPNP1-SZ	UPL8-SZ	60	2	85.7	62.7	4.16
10	BHPNP1–EcUP-SZ		60	7	82.2	65.8	1.29
11	BHPNP1–EcUP-SZ		70	8	53.3	41.4	0.71
12	BHPNP1–UPL8-SZ		60	8	86.7	80.4	1.38
13	BHPNP1–UPL8-SZ		70	8	57.8	51.2	0.88
14	BHPNP1	UPL8	65	2	79.8	76.8	31.50

[a] Reactions 1–12 contained 1.5% m m^{-1} (53 mM) guanosine and 1.5% m m^{-1} (119 mM) thymine. Reactions 13 and 14 contained 9.0% m m^{-1} (378 mM) Guanosine and 4.6% m m^{-1} (439 mM) thymine.
[b] Biocatalyst loading for Reactions 1–12 was 200 U l^{-1} of each. For reactions 9, 13 and 14, 1000 U l^{-1} was used.

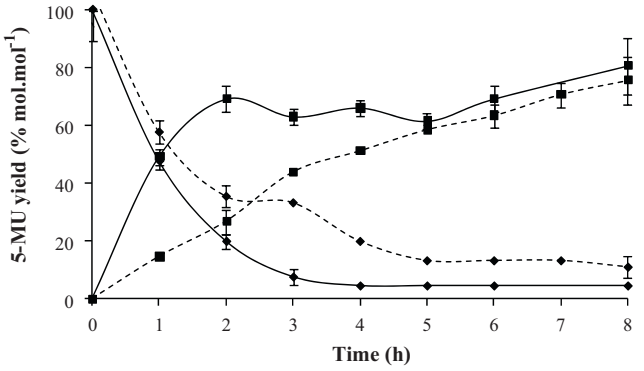

Fig. 6. Yield of 5-MU (■) and guanosine conversion (♦) over time by transglycosylation using either 2000 U l^{-1} EcUP (broken lines) or 1000 U l^{-1} UPL8 with equivalent amounts of BHPNP1. Reactions were performed at 60 °C (EcUP) or 65 °C (UPL8) in 50 mM sodium phosphate buffer, pH 7.5, with 9% m m^{-1} guanosine and 4.6% m m^{-1} thymine as the starting substrate concentrations. Data averaged from triplicate samples.

70 °C compared to free enzyme systems indicating that immobilization improved the thermal stability of the enzymes. The lower productivity observed is likely due to mass transfer limitations. An experiment was therefore performed at 1.5% m m^{-1} substrate loading using 5 fold higher loading of UPL8-SZ and BHPNP1-SZ (1000 U l^{-1} compared to 200 U l^{-1}) to prove that the low productivities could be improved by higher enzyme loading. This resulted in an increase in productivity to 4.16 g l^{-1} h^{-1} compared to 1.19 for the same reaction using 200 U l^{-1} (Reactions 7 and 9 in Table 5, respectively).

Free UPL8 with BHPNP1 were then tested under the optimum reaction conditions determined for this process [3], namely using 9% m m^{-1} guanosine and 4.6% m m^{-1} thymine as starting substrate concentrations. In this experiment, however, the temperature was increased slightly to 65 °C as previous results had shown that all the biocatalysts would be stable at this temperature. In addition, the enzyme load was decreased to 1000 U l^{-1} (compared to optimized reaction described in [3] where 2000 U l^{-1} was used) as it was felt that the high enzyme load used in the optimized reaction was not necessary due to the increased stability of the mutant enzyme. The results in Fig. 6 and Table 5 (Reaction 14) show that use of UPL8 as free enzyme biocatalysts leads to similar 5-MU yields (76.8%) at much higher reactor productivities. The reaction was essentially complete within 2 h leading to a productivity of 31.5 g l^{-1} h^{-1}, which is a 3-fold improvement on the optimized reaction using the native EcUP (10 g l^{-1} h^{-1}).

4. Conclusions

Increasing the temperature of the reaction could increase productivity of 5-MU production. This required a catalyst that was more thermostable. This stability enhancement was attempted through mutagenesis and immobilization. We have shown here that it is possible to increase the thermal stability of *E. coli* UP by directed evolution, without the need for extensive screening. The mutation shown here increased the thermostability of the enzyme two-fold at 60 °C and gave a ten-fold improvement at 70 °C. This was achieved after screening fewer than 20000 clones. Small scale experiments showed that the mutant enzyme UPL8 is a superior catalyst for the production of 5-MU. The increase in stability of the mutant enzyme lead to a significant (three-fold) increase in reactor productivities while maintaining the high yields (75–80%) in the free enzyme system.

Immobilization of the enzyme led to an increase in stability for EcUP and a further increase in stability for UPL8. The yields obtained with immobilized enzymes were similar to the free enzyme preparations at 60 °C and higher than the free enzymes at 70 °C. Co-immobilized enzymes (PNP and UP), provided higher yields at 70 °C. Reactor productivity was not equivalent to the free enzyme systems at equal enzyme loading, indicating a potential mass transfer limitation. Increasing the immobilized enzyme loading however resulted in the high productivity observed in the free enzyme reaction. Considering the possibility of recycling the immobilized catalysts, such a system would then be more cost-effective than the use of free enzymes. Optimization of the immobilization method with the aim of improving activity retention will be performed in future work.

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References

[1] E.S. Lewkowicz, A.M. Iribarren, Curr. Org. Chem. 10 (2006) 1197.
[2] D.F. Visser, K.J. Rashamuse, F. Hennessy, G.E.R. Gordon, P.J. Van Zyl, K. Mathiba, M.L. Bode, D. Brady, Biocatal. Biotransformation 28 (2010) 245.
[3] G.E.R. Gordon, D.F. Visser, D. Brady, N. Raseroka, M.L. Bode, J. Biotechnol., doi:10.1016/j.jbiotec.2010.11.013.
[4] A.J.J. Straathof, S. Panke, A. Schmid, Curr. Opin. Biotechnol. 13 (2002) 548.
[5] M.T. Reetz, J.D. Carballeira, J. Peyralans, H. Hobenreich, A. Maichele, A. Vogel, Chem.: Eur. J. 12 (2006) 6031.
[6] M.T. Reetz, J.D. Carballeira, A. Vogel, Angew. Chem. Int. Ed. Engl. 118 (2006) 7909.
[7] M.T. Reetz, J.D. Carballeira, Nat. Protoc. 2 (2007) 891.
[8] E.Y. Morgunova, A.M. Mikhailov, A.N. Popov, E.V. Blagova, E.A. Smirnova, B.K. Vainshtein, C. Mao, S. Armstrong, S.E. Ealick, A.A. Komissarov, FEBS Lett. 367 (1995) 183.
[9] T.T. Caradoc-Davies, S.M. Cutfield, I.L. Lamont, J.F. Cutfield, J. Mol. Biol. 337 (2004) 337.
[10] I. Oliva, G. Zuffi, G. Orsini, G. Tonon, L. De Gioia, D. Ghisotti, Enzyme Microb. Technol. 35 (2004) 309.
[11] D.V. Chebotaev, L.B. Gul'ko, V.P. Veiko, Russ. J. Bioorg. Chem. 27 (2001) 160.
[12] D. Brady, J. Jordaan, Biotechnol. Lett. 31 (2009) 1639.
[13] R. Fernandez-Lafuente, Enzyme Microb. Technol. 45 (2009) 405.
[14] G. Zuffi, D. Ghisotti, I. Oliva, E. Capra, G. Frascotti, G. Tonon, G. Orsini, Biocatal. Biotransformation 22 (2004) 25.
[15] S.A. Taran, K.N. Verevkina, S.A. Feofanov, A.I. Miroshnikov, Bioorg. Khim. 35 (2009) 822.
[16] N. Hori, M. Watanabe, K. Sunagawa, K. Uehara, Y. Mikami, J. Biotechnol. 17 (1991) 121.
[17] F.T. Burling, R. Kniewel, J.A. Buglino, T. Chadha, A. Beckwith, C.D. Lima, Acta Crystallogr. D: Biol. Crystallogr. 59 (2003) 73.
[18] V. Schramm, R.H. Furneaux, P.C. Tyler, K. Clinch, US Patent US2002/0132263 A1 (2002).
[19] D.F. Visser, F. Hennessy, K. Rashamuse, M.E. Louw, D. Brady, Extremophiles 14 (2010) 185.
[20] K. Hammer-Jespersen, A. Munch-Petersen, M. Schwartz, P. Nygaard, Eur. J. Biochem. 19 (1971) 533.
[21] D.D. Perrin, B. Dempsey, Buffers for pH and Metal Ion Control, Halsted Press, New York, 1974, p. 38.
[22] D. Brady, J. Jordaan, C. Simpson, A. Chetty, C. Arumugam, F.S. Moolman, BMC Biotechnol. 8 (2008) 8.
[23] X.F. Gao, X.R. Huang, C.C. Sun, J. Struct. Biol. 154 (2006) 20.
[24] K. Shimizu, N. Kunishima, Acta Crystallogr. F: Struct. Biol. Cryst. Commun. 63 (2007) 308.
[25] J.C. Leer, K. Hammer-Jespersen, M. Schwartz, Eur. J. Biochem. 75 (1977) 217.
[26] Y. Avraham, J. Yashphe, N. Grossowicz, FEMS Microbiol. Lett. 56 (1988) 29.
[27] A. Zaks, D.R. Dodds, Drug Discov. Today 2 (1997) 513.
[28] T. Utagawa, H. Morisawa, S. Yamanaka, A. Yamazaki, F. Yoshinaga, Y. Hirose, Agric. Biol. Chem. 49 (1985) 3239.
[29] N. Hori, M. Watanabe, Y. Yamazaki, Y. Mikami, Agric. Biol. Chem. 53 (1989) 2205.
[30] T. Hamamoto, T. Noguchi, Y. Midorikawa, Biosci. Biotechnol. Biochem. 60 (1996) 1179.