STABILISATION E. COLI UP by EVOLUTION and IMMOBILISATION

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Abstract

Uridine phosphorylase from *Escherichia coli* was evolved by iterative saturation mutagenesis. The best mutant showed a temperature optimum of 60°C and a half-life of 17.3 h at 60°C. The mutant enzyme, as well as a purine nucleoside phosphorylase from *Bacillus halodurans*, were immobilised as Spherezymes™. Immobilisation of the mutant enzyme provided a further increase in thermostability. When combined with the purine nucleoside phosphorylase from *B. halodurans*, productivity of 5-methyluridine, a pharmaceutical intermediate, was increased from 10 to 31 $g.l^{-1}.h^{-1}$.

Keywords: biocatalysis, transglycosylation, directed evolution, immobilisation, spherezyme, 5-methyluridine.

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 can be synthesised from β–thymidine, which can in turn be synthesised 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 (BHPNP1) from the thermotolerant alkalophile *Bacillus halodurans* 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]. The optimal operating conditions were found to be a loading of 378 mM (9% $m.m^{-1}$) guanosine and 439 mM (4.7% $m.m^{-1}$) thymine at 60°C at 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, but Straathof *et al*. [4] indicate that an economically viable process is typically 15.5 $g.l^{-1}.h^{-1}$. Moreover the optimal reaction temperature of 60°C is within the thermostability range of BHPNP, but the stability of the UP is only 40°C. This requires higher enzyme loading to offset the rate of thermal deactivation. Moreover, due to the low solubility of the reaction components the biocatalytic reaction medium is a slurry reaction with limited solid-liquid mass transfer. Hence, in order to increase the productivity, one would need to increase the reaction temperature to increase substrate solubility. The aim of this research was to improve the volumetric productivity of the transglycosylation reaction by enhancing the thermostability of UP by mutation and immobilisation. Of particular interest for rapid evolution of enzyme stability is the method developed by Reetz and co-workers [5, 6, 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 an area 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. Selecting the amino acids or areas of the protein to target represents a challenge. Analysis of mesophillic and thermophillic enzymes shows that extremophillic enzymes have a higher degree of surface rigidity. Reetz *et a.l* [6] therefore targeted amino acids with the highest degree of flexibility indicated by atomic displacement parameters available from X-ray data, namely B-factors (also called B-values). They developed a method called B-Factor Iterative Test (B-FIT), which highlights the amino acids with the highest flexibility and thereby creates targets for mutagenesis. Using the ISM method, Reetz and co-workers increased the thermostability of *Bacillus subtilis* lipase from 48°C to 93°C after screening only 8 000 clones [6]. Similarly, the same group applied ISM to sites of interest in the active site of *Aspergillus niger* epoxide hydrolase. After a total screening of 20 000 clones, they managed to increase the enantioselectivity from an E value of 4.6 to a value of 115.

EcUP is a good candidate for directed evolution through ISM. It is a multimeric protein, which complicates rational design; the crystal structure of uridine phosphorylase has been determined [8, 9], which simplifies the process of determining saturation targets; and, as a native *E. coli* enzyme, expression of mutants is well suited for an *E. coli* expression system. The only reports of mutagenesis on pyrimidine nucleoside phosphorylases PyNP is 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 nucleoside phosphorylases.

The *E. coli* UP and PNP have been co-immobilised by covalent linkage to epoxy-activiated Sepabeads for the biocatalytic preparation of a variety of natural and modified purine nucleosides [12]. Similarly, nucleoside phosphorylase from *Geobacillus stearothermophilus* were covalently immobilised on aminopropylated macroporous glass [13]. These preparations showed increased thermal stability high levels of activity retention (>80%) when immobilised. Of particular interest is the work done by Hori and co-workers, who immobilised PNP and PyNP from *Bacillus stearothermophilus* by ionic binding to DEAE-Toyopearl 650M anion exchange resin [14]. Using the

immobilised 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. This study aims to show that stabilization of EcUP, through either enzyme evolution, immobilization or a combination of both, can lead to increased reaction productivity for the production of 5-MU.

Methods and Materials

Materials

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

Saturation Mutagenesis

The crystal structure of *E. coli* UP (1LX7) [15] 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" (http://www.mpi-muelheim.mpg.de/kofo/institut/arbeitsbereiche/reetz/englisch/reetz_research1.html). Six regions of interest were identified for saturation mutagenesis (See Fig. 1). The primers designed for mutagenesis of these regions are given in Table 1.

Table 1

Primers for saturation mutagenesis targets for *E. coli* UP (only forward primers shown). Degenerate IUB codes in bold.

Library	Target amino acids	Primer
1	Met38Lys40	AAGATCGCCGCGCTG**NNK**GAT**NNK**CCGGTTAAGCTG
2	Lys60	AGCTGGATGGT**NNK**CCTGTTATCGT
3	Lys145	TTGAAGCTGCG**NNK**TCCATTG
4	Pro229Asn230 Ala231	CAAGAGATC**DNKVDKNRT**GAGACGATGAAACAA

5	Lys235 Gln236	AATGCTGAGACGATG**NNKNNK**ACCGAAAGCCATGCG
6	Glu232Met234	CAAGAGATCCCGAATGCT**NNK**ACG**NNK**AAACAAACC

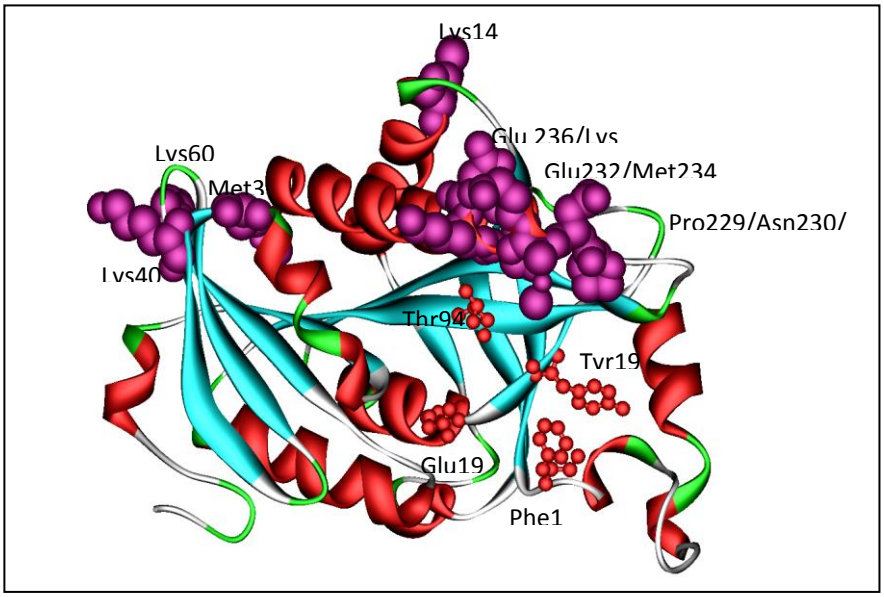

Fig. 1. Ribbon representation of *E. coli* uridine phosphorylase. Catalytic residues are shown in red (ball and stick format) and residues targeted by ISM in purple (CPK format) based on the 1LX7 structure [15].

Mutagenesis

A Stratagene 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) was added to reaction mixes containing dNTP's, plasmid harbouring target gene and mutagenic primers. The PCR reaction was as follows: a single cycle at 95°C for 1 minute, 18 cycles at 95°C for 50 seconds, 55°C for 50 seconds, and 68°C for 1 minute per kb of plasmid length, followed by a cycle at 68°C for 7 minutes. *Dpn*I restriction enzyme (5 µl) was then added to each reaction and incubated for 5 hours 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).

Preparation of mutant screening libraries

Mutant libraries were plated on to 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 plates) using the QPix2 colony picker (Genetix). The number of colonies picked ranged from 600 to 3500 per library depending on the number of clones required to obtain coverage of all the possible mutations. A total of 12300 colonies were picked across the 6 initial libraries. After an overnight incubation, duplicate plates were prepared using the replication function of the QPix2. The replicate 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 x 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 x g, 20 min).

Library Screening

p-Nitrophenol-β-D-ribofuranoside was used as the substrate for UP screening. The substrate was prepared according to the methods of Schraam *et al.* [16]. A suspension of 10 g 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose, 5.6 g *p*-nitrophenol, 1.2 ml boron trifluoride diethylether in 100 ml dry $CHCl_2$ was allowed to stir overnight at room temperature. The solution was washed with aq. bicarbonate. The aqueous phase was separated and the resultant organic portion was reduced to half the initial volume under reduced pressure before separation of the *p*-nitrophenol-2,3,5-tri-*O*-benzoyl-β-D-ribofuranoside from the reactants by flash chromatography (1:2:8 EtOAc:$CHCl_3$:Hexane). Fractions showing the desired product were pooled yields and dried under vacuum. This product was then suspended in 100 ml methanol, adjusted to pH 10 with NaOH and left to stir overnight. The solution was then concentrated under reduced pressure. The residue was dissolved in dissolved in 1:1 MeOH/CH_2Cl_2 and filtered. The eluant was concentrated and purified by EtOAc trituration. A yield of 3.88 g of product (*p*-nitophenol-β-D-ribofuranoside) was achieved. The product and its purity were confirmed by ^{3}H-NMR. An amount equivalent to 0.01% m.v^{-1} of the substrate was dissolved in a minimal volume of warm methanol, and made up to volume with 50 mM sodium phosphate buffer (pH 7.5). For 96-well and 384-well microtitre plates a volume of 240 µl or 40 µl, respectively, was added to an aliquot of crude extract. The change in absorbance due to the release of *p*-nitrophenol was measured at 410 nm using a Powerwave HT microtitreplate reader (Biotek, USA).

Primary Screening: Cellular extracts (5 µl) from each of four wells were pooled to single destination wells on each of two plates using an EpMotion 5025 liquid handling station (Eppendorf, Hamburg, Germany). Activity of the combined 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. The original four samples corresponding to the hits were then assayed in the same way to determine the original culture giving the highest residual activity.

Secondary Screening: Single culture hits were re-inoculated into 5 ml cultures and incubated overnight. The plasmid harbouring 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 medium, 100 µg.ml^{-1} ampicillin) and protein expression induced (0.1 mM IPTG, 3.5 h). Cells were harvested by centrifugation (3000 x 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). The resultant protein solutions were then incubated at temperatures between 40 and 80°C degrees for 60 min to determine the temperature at which 50% of the initial activity was retained ($T_{50\,(\%)}^{60\,(min)}$ value).

Iterative Mutagenesis: The strain containing the mutated enzyme showing the highest stability after the first round of screening was used as the template for the second round of screening. In this case a strain from library 5 (Table 1) showed the highest residual activity after a 15 min incubation at 70°C (95% activity retained). The plasmid harbouring this mutated gene was used in PCR reaction with the mutation primers for library 4 and library 1, which had given the next two best hits, respectively. Saturation mutagenesis and subsequent screening was performed as described above. Plasmid from the best strains 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 transformed by heat shock (42°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].

Production and characterisation of UPL8

The mutant enzyme was produced in two 10 l fermentations and as described previously [17]. Characterisation of UPL8 was performed according to a modified method of Hammer-Jespersen *et al* [18] wherein a suitably diluted 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 time at 40°C, the reaction was stopped by addition of 100 µl 0.5 M perchloric acid. The samples were then incubated on ice for 20 min and centrifuged for a further 20 min (7000 x g) to remove residual protein. Sample (100 µl) was then transferred to a UV compatible microtitre plate and combined with 100 µl 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 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 [19] (50 mM Tris, 50 mM Boric Acid, 33 mM Citric acid; 50 mM Na_2PO_4, adjusted with either HCl or NaOH), adjusted 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 and 70°C. Samples were analysed 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.

Spherezyme preparation

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-immobilisation studies. Active site protectants (50 mM inosine and/or 50 mM uridine) were added to the solution directly prior to cross linking. To these solutions, 320 µl of the cross linker (1:1 Gluteraldehyde:5% Polyethyleneimine) 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 over night at 4°C. The emulsion was then broken and the particles recovered by centrifugation (Beckman J-21, 1000 x g, 10 min). Immobilised 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 immobilised enzyme particles were recovered by filtration under vacuum (Whatman No. 1). The immobilised enzyme particles were then dried at room temperature under high vacuum (Virtis Genesis 25L freeze dryer, UK).

Transglycosylation by stabilised 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.

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 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 analysed by HPLC, using a Waters Alliance Model 2609 instrument with a Synergi 4 µm Max-RP 150 x 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 minutes at 25°C.

Results and discussion

Round 1 Mutation

All mutation targets seemed to show some degree of improvement on thermostability. The best hits after the primary screening are given in

Table 2. These values were used as the basis for the best hit selection, as the T_{50}^{60} (Fig. 2) proved inconclusive for determining differences between the mutants. No activity was observed after incubation at 80°C regardless of the stability of the enzyme at 70°C, a result which skewed the potential stability values.

Table 2

Best hits from the first six libraries based on residual activities observed after incubation of the enzyme preparations for 1 h at 70°C

Library	Mutant	Observed Mutation	% Residual Activity
1	UPL1-2/A11	Met38Leu; Lys40Ser	31.7%
2	UPL2-2/G15	Lys60Lys	12.2%
3	UPL3-1/G9	Lys145Lys	12.9%
4	UPL4-3/F2	Pro229*Ter*; Asn230Asn; Ala231Asp	51.1%
5	UPL5-10/F9	Lys235Arg; Gln236Ala	95.5%
6	UPL6-2/H10	Glu232Glu; Met234*	30.9%

*Sequence inconclusive

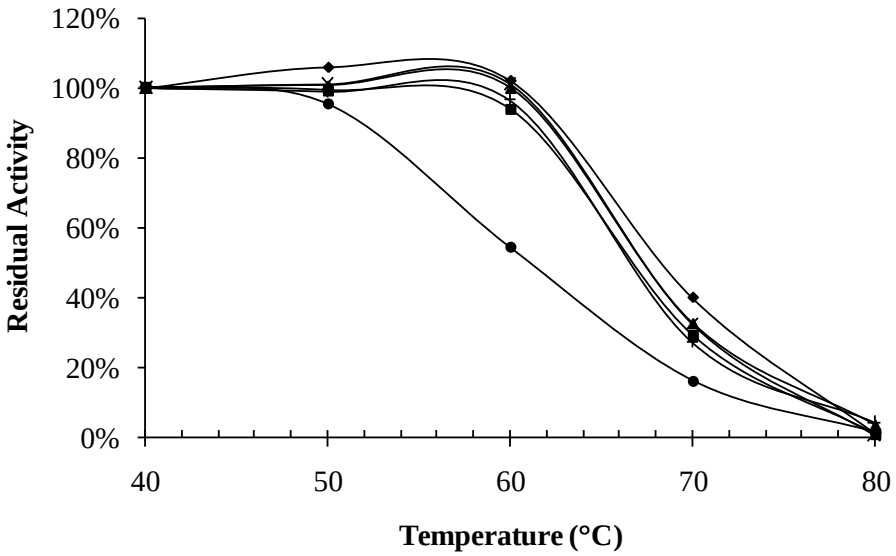

Fig. 2. Plot of residual activity for the best mutants from each library in the first round of mutation. Residual activity was determined after incubation for 60 min at the set temperatures (37, 50, 60, 70 and 80°C). (UPL1-2/A11 (-♦-); UPL3-1/G9 (-■-); UPL4-3/F2 (-×-); UPL5-10/F9 (-♦-); UPL6-2/H10 (-+-); Wild type UP (-●-))

Round 2 mutation

Mutation of the best hit from library 5 (round 1) with the primers for library 4 (giving library 7) and library 6 (giving library 8) again gave positive results in initial screening (Table 3). Screening, however, was now performed at 75°C for 15 min.

Table 3

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
7	UPL7-2/C15	Met38Val; Lys40Asp Lys235Arg; Gln236Ala	88.5%
8	UPL8-4/I5	Lys235Arg; Gln236Ala	80.2%

Determination of T_{50}^{60} values (Fig. 2) showed good stability at 70°C but again no activity at 80°C, skewing the final values. It was decided to determine the stability of the enzyme from library 8 at 60 and 70°C to get a better indication of improved stability (Fig. 3). These results showed marked improvements in stability at both 60 and 70°C.

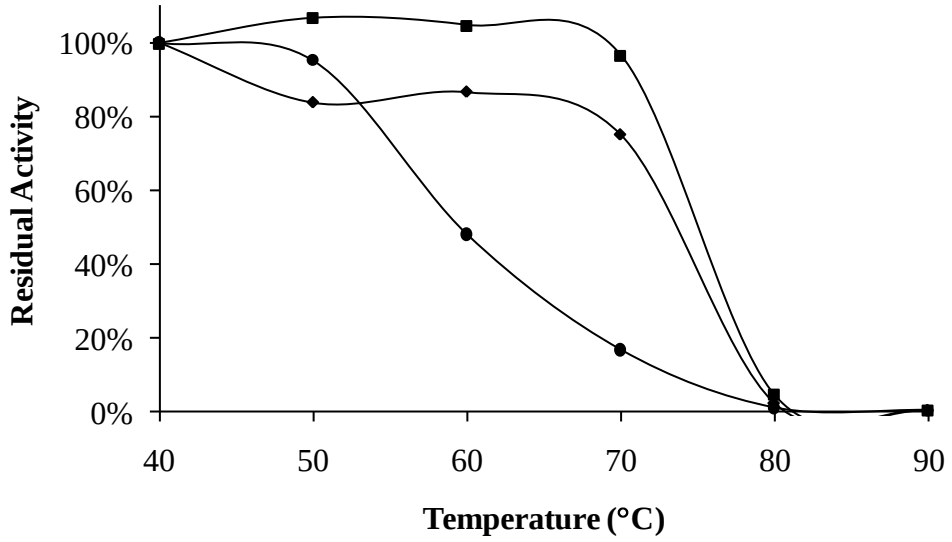

Fig. 3. Plot of residual activity for mutants UPL7-2/C15 (-♦-) and UPL8-4/I5 (-■-) compared to wild type UP (-●-). Residual activity was determined after incubation for 60 min at the set temperatures.

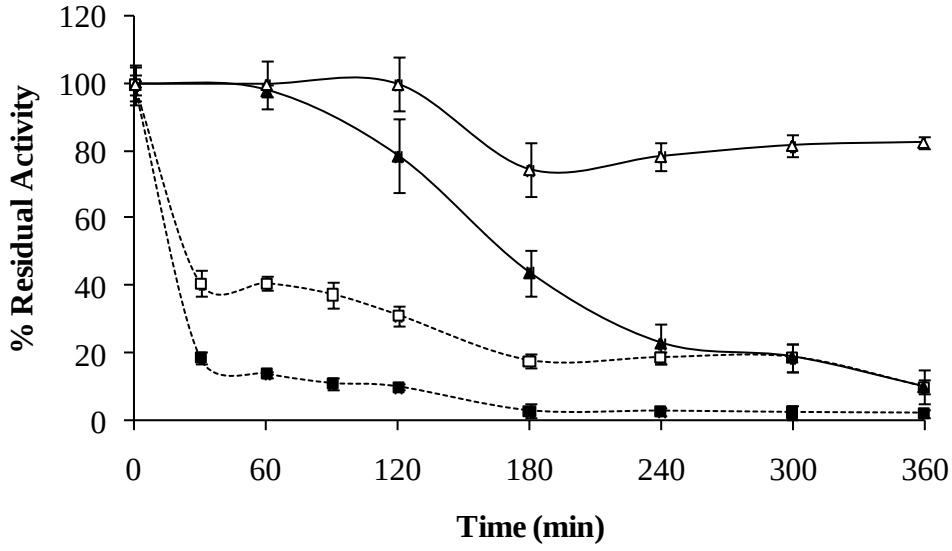

Fig. 4. Thermostability comparison for EcUP (-▲-) and mutant UPL8 (¨■¨). Enzyme preparations were incubated at 60°C (open symbols) and 70°C (closed symbols) for 6 h.

The aim of the directed evolution was to increase the thermostability of *E. coli* UP. The characterisation in [17] indicated that BHPNP would operate best at 60°C for the duration of the biocatalytic reaction. The target for this evolution was therefore to enhance EcUP thermostability at 60°C. The results in Fig. 3 clearly show that this has been achieved. While further stabilisation could be achieved by further rounds of mutation, it is not necessary at this point since further enhancements in stability would then out perform BHPNP and thus be redundant. It was decided therefore to continue with production and characterisation of this mutant.

Characterisation of UPL8

pH Optimum: UPL8 showed a pH optimum of 7.0, retaining 60% activity between pH 5.6 and 8.4 (Fig. 5), which is similar to the wild type UP (optimum of 7.0, retaining 60% activity between pH 6.0 and 8.2).

Temperature Optimum: UPL8 has a significantly improved temperature optimum (60°C) and a broader activity range, retaining 60% activity between 37 and 67°C (Fig. 6). 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 BHPNP1 (optimum of 70°C, range of 30 to 74°C).

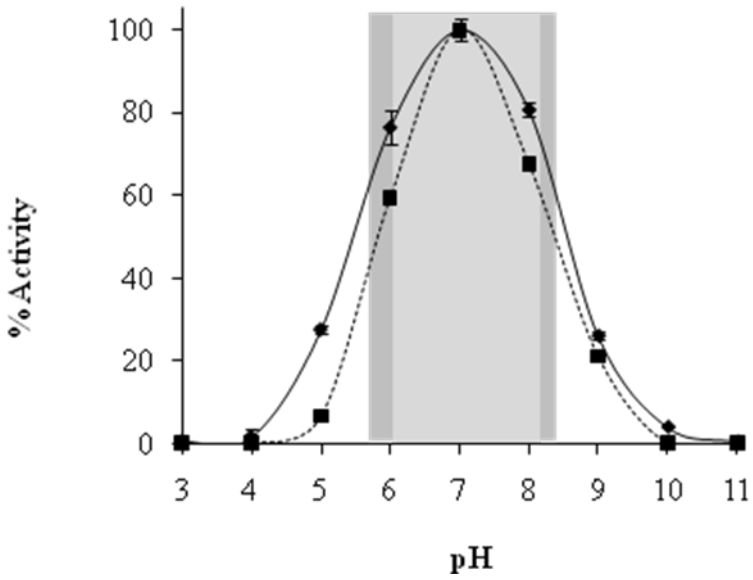

Fig. 5. Activity profiles of EcUP (■) and the mutant UPL8 (♦) as a function of pH. Shaded areas indicate the pH range of the enzymes (> 60% activity). A moderate increase in the pH range is noted for UPL8 while pH optima remain the same at pH 7.0.

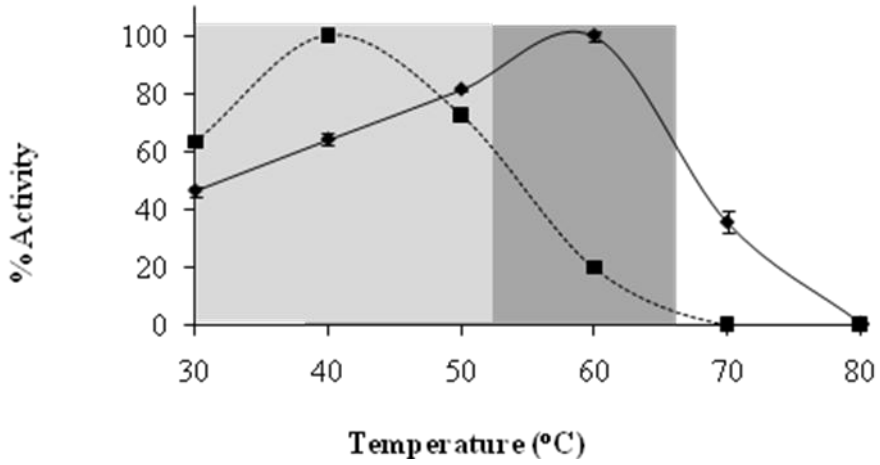

Fig. 6. Activity profiles of EcUP (■) and the mutant UPL8 (♦) and as a function of temperature. The shaded areas indicate the temperature range of the enzymes (> 60% activity).

Temperature Stability: Wild type UP showed a half life of 9.9 h at 60°C, albeit at 20% of its optimum activity. At 40°C, the half-life was 37 h. The mutant enzyme has a half life at 60°C of 17.3 h and 3.3 h at 70°C (Fig. 4). This again is comparable to the stability data for BHPNP.

Kinetic Characterisation: 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 calculated from the three plots. Subsequently the turnover number (k_{cat}) and the specificity constant were calculated. The values are summarised in Table 4.

Table 4

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

Parameter	Unit	EcUP	UPL8
Specific Activity	$U.mg^{-1}$	30.69	19.18
K_m	µM	233.91	464.3
V_{max}	$mol.s^{-1}$	4.57×10^{-5}	2.85×10^{-5}
k_{cat}	s^{-1}	4.55×10^{4}	4.55×10^{4}
Specificity Constant	$M^{-1}.s^{-1}$	1.94×10^{8}	1.94×10^{8}
pH Optimum	-	7.0	7.0
pH range (60%)	-	6.0-8.2	5.6 - 8.4
Temp Optimum	^{o}C	40	60
Temp Range (60%)	^{o}C	30-52	38-67
Temp Stability ($t_{1/2}$ @ $60^{o}C$)	h	9.89	17.3
Temp Stability ($t_{1/2}$ @ $70^{o}C$)	h	3.3	37

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 3). 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) (Fig. 7). Yet UPL8 was shown to be the superior mutant. The

Lys235Arg mutation should not have had a dramatic affect as they are both basic amino acids. The larger argenine should also have decreased flexibility at the site due to it being a longer side chain. This longer side chain however may be interacting with the neighbouring α-helix, thereby conferring rigidity to the overall structure. The Gln236Ala mutation however does fit with the theory of decreased flexibility due to Ala having a smaller side chain and being non-polar as opposed to the polar Gln. 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 stabilised 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. It is plausible that an increase in rigidity at this point would limit flexibility and therefore conformational changes during binding of the substrate.

The data obtained shows that a relatively small, unpredictable mutation can have a marked effect on the physical characteristics of an enzyme. While it would be of interest to study the effects of the observed mutation and further mutations further, the desired increase in thermostability has been achieved. It was decided, therefore, to continue with immobilisation studies to determine whether mutation or immobilisation, alone or in combination, would lead to a vastly improved biocatalyst.

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UP      151  THVGVTASSDTFYPGQERYDTYSGRVVRHFKGSMEEWQAMGVMNYEMESA  200
             ||||||||||||||||||||||||||||||||||||||||||||||||||
UPL8    151  THVGVTASSDTFYPGQERYDTYSGRVVRHFKGSMEEWQAMGVMNYEMESA  200

Up      201  TLLTMCASQGLRAGMVAGVIVNRTQQEIPNAETMKQTESHAVKIVVEAAR  250
             ||||||||||||||||||||||||||||||||:.|||||||||||||||
UPL8    201  TLLTMCASQGLRAGMVAGVIVNRTQQEIPNAETMRATESHAVKIVVEAAR  250

Up      251 RLLX  254
            |||
UPL8    251 RLLX  254
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Fig. 7. Amino acid alignment (residues 150 to end) of the native *E. coli* uridine phosphorylase (UP) and the mutant *E. coli* uridine phosphorylase (UPL8). Mutated amino acids are highlighted.

Saturation mutagenesis proved to be an effective method for rapid evolution of a multimeric enzyme. Fewer than 15000 clones were screened across 8 libraries of mutants to obtain an enzyme with a 20°C shift in optimum temperature and vastly improved stability at 60°C and 70°C compared to the wild type enzyme. The mutant enzyme retained its pH activity characteristics but showed a moderate drop in substrate specificity. UPL8 was successfully produced by batch fermentation to high expression levels (52 $kU.l^{-1}$) and was subsequently purified using the methodology established for the wild type enzyme. There have been no reports of engineering for enhanced physical characteristics on any prokaryotic UPs. It is only therefore possible to compare this mutant UP to other wild type enzymes, as listed in Table 5. Very few PyNPs and particularly UPs from prokaryote sources have been fully characterised. Other than those listed, PyNPs from *B. subtilis* [20] and *T. thermophilus* [21] have been purified for crystallography studies, but no characterization was reported. The PyNP from *B. 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 *B. stearothermophilus* enzymes, but is still within the micromolar range, making it significantly active towards uridine. The pH optimum for most reported PyNPs is around 7.0.

Table 5

Physical and kinetic characteristics of reported prokaryotic UPs

Organism	K_m (mM) (uridine)	pH optimum	Temperature optimum	Ref.
E. coli	0.15	7.5	37	[22]
L. casei	3.8	7.0	-	[23]
E. carotovora	-	-	60	[24]
E. coli UPL8	0.46	7.0	60	This study
E. aerogenes	0.7	8.52	65	[25]
B. stearothermophilus	0.19	7.2	70	[26]; [27]

Enzyme Immobilisation

EcUP, UPL8 and BHPNP were all successfully immobilised with varying degrees of activity retention (

Table 6). UP showed less cross-linking efficiency (indicated by mass recovery) as well as lower activity retention, which would indicate that the immobilisation affects the binding capacity of the enzyme. Immobilisation however did have a stabilising effect on both EcUP and UPL8. EcUP-SZ and showed a new temperature optimum at 60°C and activity at 70 and 80°C which was not noted with the free enzyme. UPL8-SZ did not show an increase in the optimum temperature but did exhibit a broader activity range, maintaining significant activity at 70 and 80°C. Both preparations maintained the pH optimum profiles seen for the free enzymes. The BHPNP1 showed higher cross-linking efficiency as well as activity retention. No significant changes were noted in either the temperature or pH optimum, 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-immobilised combinations were also developed. Co-immobilising UP with BHPNP1 seemed to increase the cross-linking efficiency and activity retention of the UP, with UPL8 and EcUP showing increases of 9% and 38% in activity retention, respectively, when immobilised with BHPNP1. The physical characteristics of the co-immobilised enzymes were similar to that of the single-immobilised preparations.

Table 6

Physical characteristics of free and immobilized (Spherezyme) forms of EcUP, UPL8 and BHPNP1.

Biocatalyst	Specific activity	Activity retention	pH optimum	pH range	Temp optimum	Temp range
	U.mg^{-1}	%			°C	°C
EcUP	18.3	-	7.0	6.0 - 8.2	40.0	30 - 52
EcUP-SZ	2.7	4.5	7.0	6.0 - 8.2	60.0	40 - 67
EcUP/BHPNP-SZ	2.4	14.7	7.0	4.6 - 8.8*	60.0	40 - 80
UPL8	12.3	-	7.0	5.6 - 8.4	60.0	40 - 67
UPL8-SZ	1.8	2.2	7.0	6.0 - 8.2	60.0	40 - 80
UPL8/BHPNP-SZ	3.2	33.7	7.0	4.6 - 8.2*	60.0	40 - 80
BHPNP	8.7	-	7.0	5.7 - 8.4	70.0	32 - 74
BHPNP-SZ	1.0	25.4	7.0	5.7 - 8.4	50.0	40 - 80

* Based on activity towards uridine

Hori and co-workers [14] immobilised 0.42 units of crude enzyme from *B. stearothermophilus* on anion exchange resin for production of 5-MU and showed no loss on activity through immobilisation. The PNP and PyNP from *G. stearothermophilus* were immobilised on a glass solid support [13] with only 30% loss in initial activity. Similar activity loss was noted for the immobilisation of *E. coli* PNP and PyNP on Sepabeads [12]. In contrast, between 80 and 90 % of the activity was lost on Spherezyme formation. This figure may be improved upon further optimisation of the immobilisation process. The advantage of immobilisation 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 immobilised biocatalyst) were 0.18 and 0.04 U.mg^{-1} for PNP and UP, respectively. In comparison, co-immobilised BHPNP1 and UPL8 showed specific activities (per mg spherezyme) of 2.6 and 1.8 U.mg^{-1}, respectively, which is approximately 15 fold higher.

5-MU production 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. 8). 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, see Table 7).

5-MU production by transglycosylation using Spherezyme preparations

The use of immobilised enzymes for this reaction could potentially have two advantages; namely an increase in stability of mesophillic enzymes allowing a higher reaction temperature, and recycle of the biocatalyst to decrease the catalyst cost. The results obtained for the use of single immobilised enzymes show that the immobilised enzymes conferred increased stability to the native *E. coli* UP, indicated by the production of 5-MU at 70°C (Table 7). 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). Similar results were obtained when using the immobilised mutant enzyme (UPL8-SZ) where production of 5-MU was noted at both 60°C and 70°C, but at reaction productivities lower than those seen for the free enzyme control reaction. Higher 5-MU yield was noted when using UPL8-SZ (69%) compared to using EcUP-SZ (29 %) at 70°C.

Co-immobilising 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. However similar yields and reactor productivities were seen for EcUP co-immobilised with BHPNP1 compared to the free enzymes. The results at 70°C did show higher yields and productivities than the single immobilised enzyme which would indicate a higher degree of stability in the co-immobilised system. The UPL8 enzyme co-immobilised with BHPNP1 gave the best yields at both 60°C (80.4%) and 70°C (51.2%) of all the systems tested. This indicates that this system had the highest degree of stability all the biocatalysts tested. The reaction productivities however (1.38 and 0.88 $g.l^{-1}.h^{-1}$ at 60°C and 70°C, respectively) were still not significantly different or higher than the free enzyme control reaction.

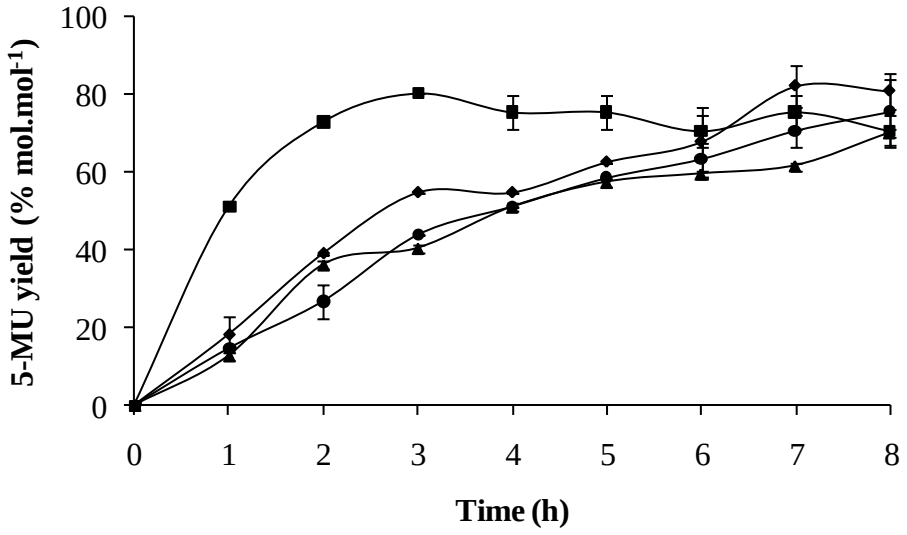

Fig. 8. Selected transglycosylation experiment showing the 5-MU yield obtained when using free EcUP (●) or free UPL8 (■) in combination with BHPNP1. Also shown are the the combinations of separately immobilised EcUP and BHPNP1 (♦) and co-immobilised UPL8 and BHPNP1 (▲). All reactions were performed using 1.5 % $m.m^{-1}$ substrate loading at 60°C. Data averaged from triplicate samples.

5-MU production by transglycosylation using 9% m.m^{-1} guanosine and 4.6% m.m^{-1} thymine as starting substrate concentrations

The previous section showed that free UPL8 with BHPNP1 and co-immobilised UPL8 with BHPNP1 gave the highest productivities for free and immobilised biocatalyst systems respectively (Fig. 9).

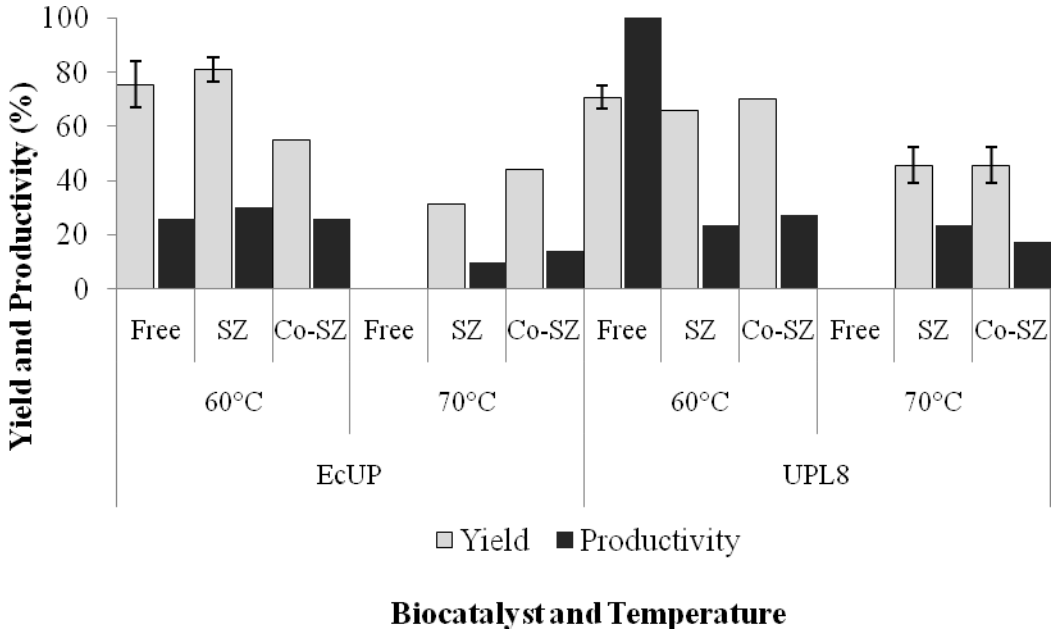

Fig. 9. Analysis of 5-MU yield (% mol.mol^{-1}) and productivity (% relative to maximum productivity observed) for 1.5% m.m^{-1} substrate loading reactions. Different biocatalyst configurations: Free – free enzyme; SZ – Single immobilised enzyme; Co-SZ – co-immobilised enzymes. See text for further reaction details.

These systems were therefore tested under the optimum reaction conditions determined for this process, namely using 9% m.m^{-1} guanosine, 4.6% m.m^{-1} thymine as starting substrate concentration with increased enzyme loading. 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} as it was felt that the high enzyme load used in the optimised reaction was not necessary due to the increased stability of the mutant enzyme.

The results in Fig. 10 and Table 7 (Reactions 13 and 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 optimised reaction using the native EcUP (10 $g.l^{-1}.h^{-1}$). The co-immobilised enzyme system also showed a good yield of 5-MU (75.1%) but at a lower productivity (7.7 $g.l^{-1}.h^{-1}$) as was seen at lower substrate loading.

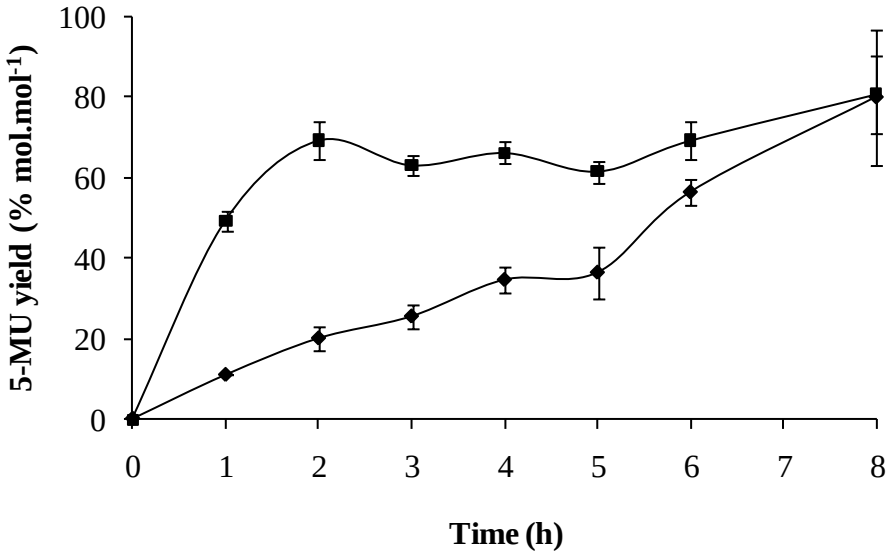

Fig. 10. 5-MU yield by transglycosylation from guanosine (9% $m.m^{-1}$) and thymine (4.6% $m.m^{-1}$) using free BHPNP1 and UPL8 (■) or a combination of BHPNP-SZ and UPL8-SZ (♦) as biocatalysts at 65°C.

Table 7

Comparative figures for guanosine conversion, 5-MU yield and reaction productivity for transglycosylation reactions using a variety of biocatalyst combinations

	Biocatalysts*		Temp	Reaction time	Guanosine Conversion	5-MU Yield	5-MU Productivity
Rxn†	PNP	PyNP	°C	h	% mol/mol	% mol/mol	$g.l^{-1}.h^{-1}$
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	UPL9	70	8	44.4	0.0	0.00
5	BHPNP-SZ	EcUP-SZ	60	7	86.7	76.8	1.50
6	BHPNP-SZ	EcUP-SZ	70	8	57.8	29.2	0.50
7	BHPNP-SZ	UPL8-SZ	60	8	93.3	69.5	1.19
8	BHPNP-SZ	UPL8-SZ	70	8	75.6	69.5	1.19
9	BHPNP1/EcUP-SZ		60	7	82.2	65.8	1.29
10	BHPNP1/EcUP-SZ		70	8	53.3	41.4	0.71
11	BHPNP1/UPL8-SZ		60	8	86.7	80.4	1.38
12	BHPNP1/UPL8-SZ		70	8	57.8	51.2	0.88
13	BHPNP1	UPL8	65	2	79.8	76.8	31.50
14	BHPNP-SZ	UPL8-SZ	65	8	47.1	75.1	7.70

†Reactions 1 – 12 contained 1.5% $m.m^{-1}$ (53 mM) GuO and 1.5% $m.m^{-1}$ (119 mM) Thy. Reactions 13 and 14 contained 9.0% $m.m^{-1}$ (318 mM) GuO and 4.6% $m.m^{-1}$ (365 mM) Thy.

*Biocatalyst loading for Reactions 1 – 12 was 200 $U.l^{-1}$ of each. For reactions 13 and 14, 1000 $U.l^{-1}$ was used.

The best transglycosylation reaction for the preparation of 5-MU using thymine and guanosine in the presence of PNP and UP was described by Ishii *et al*. [28]. Reasonable yields of 5-MU (74%) were achieved at relatively high guanosine and thymine concentrations of 300 mM each. The reactions were carried out at 60°C using whole cells of *Erwinia carotovora*. The chief disadvantage of this process was the long reaction time which resulted in a low reactor productivity of 1.19 $g.l^{-1}.h^{-1}$; an order of magnitude below an economically desirable level [4]. Enzyme preparations generally have

higher specific activities than whole cells and do not have the limitations associated with membrane transport [29], potentially leading to higher mass transfer rates and therefore higher reaction productivities.

Conclusions

We have shown here that it is possible to increase the thermal stability of *E. coli* UP by directed evolution in a relatively short period time, without the need for extensive screening. The mutation shown here doubled the thermostability of the enzyme at 60°C and gave a ten-fold improvement at 70°C. This was achieved after screening fewer than 15000 clones.

Small scale experiments showed that the mutant enzyme UPL8 is a superior catalyst for the production of 5-MU. As expected, the increase in stability of the mutant enzyme lead to a significant (3-fold) increase in reactor productivities while maintaining the high yields (75- 80%) in the free enzyme system. The increase in productivity was achieved within the constraints of the reaction operating window [3], implying that no further reaction optimisation would be necessary to implement the mutant enzyme. The productivity achieved was nearly 30 fold higher than the process described by Ishii *et al.* [28]. The biotransformation demonstrated here also compares well to other commercial biotransformations (as summarised in [4]). Within the 64 biotransformations for the production of fine chemicals analysed in that study, the average productivity was 15.5 $g.l^{-1}.h^{-1}$, the average product yield was 78 % and the average final product concentration was 108 $g.l^{-1}$. The transglycosylation reaction in this study has an above average productivity (31.5 $g.l^{-1}.h^{-1}$) and an acceptable yield (77%). The final product concentration (74 $g.l^{-1}$) is lower than the commercial average, but is equivalent to the average for commercial nucleotide production (65 $g.l^{-1}$).

Immobilisation of the enzyme did lead to the expected increase in stability for both EcUP and UPL8, but this did not lead to increased reactor productivity. This could only be explained by the immobilisation having a detrimental effect on mass transfer in the slurry system. This was further confirmed with the use of co-immobilised enzymes, where higher yields were noted at 70°C, but no improvements on reaction productivity were observed for the reactions run at 60°C or 70°C. The yields obtained with immobilised enzymes were similar to the free enzyme preparations at 60°C and higher than the free enzymes at 70°C. Increasing the immobilised enzyme loading is likely to increase the reaction productivity while maintaining high yields (> 75%). Considering the possibility of recycling the immobilized catalysts, such a system would then be more cost-effective than the use of free enzymes.

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