High yielding cascade enzymatic synthesis of 5-methyluridine using a novel Purine Nucleoside Phosphorylase, from *Bacillus halodurans* Alk36

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INTRODUCTION

5-Methyluridine (5-MU) is a non-natural nucleoside that can be used as an intermediate in the synthesis of thymidine, and in the synthesis of nucleoside analogues AZT and stavudine, both of which are used in Highly Active Anti-Retroviral Treatment (HAART) of HIV/AIDS patients. 5-MU can be synthesised through the transglycosylation of D-ribose-1-phosphate, using guanosine as a donor, and thymine as receptor (Figure 1). However, the reagents guanosine and thymine are relatively insoluble, resulting in particulate substrates with poor reaction kinetics, and the most effective method of solubilising these materials is in hot aqueous solutions. It would therefore be preferable to utilize thermostable enzymes. The present work investigated the purine nucleoside phosphorylase (BHPNP1) present in the moderately thermophilic and alkaliphilic organism, *Bacillus halodurans* Alk36[1,2]. We report on the combination of that enzyme with the uridine phosphorylase from *E. coli* in a one-pot cascade reaction to produce 5-methyluridine in high yield.

METHODOLOGY

- ***Cloning and Expression of Nucleoside Phosphorylases*** - BHPNP1 and *E.coli* UP were amplified as previously described[3,4]. *E.coli* BL21(DE3) was used as the expression host.
- ***Production of Enzymes in Batch Fermentation*** - *E. coli UP and B. halodurans* BHPNP1 were produced in batch fermentations using GMO media[5]. Crude extracts of the enzymes were prepared by a combination of high-pressure cell disruption, ultrafiltration (30 kDa cut-off membrane) and lyophilisation.
- ***Characterization of BHPNP1*** - Kinetic and physical characteristics of BHPNP1 were determined using guanosine as a substrate[3].
- ***Transglycosylation for the preparation of 5-MU*** - Reaction (650 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 (*E. coli* UP and BHPNP1). Reactions were performed at 40°C in round bottomed flasks with stirring at 500 rpm. Reaction components were measured by HPLC.

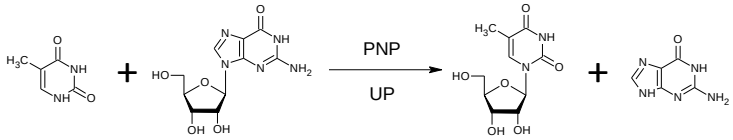

Figure 1: Biocatalytic production of 5-methyluridine by transglycosylation. UP – uridine phosphorylase. PNP – purine nucleoside phosphorylase.

CHARACTERISATION OF BHPNP1

BHPNP1 was successfully expressed and purified. Physical and kinetic characteristics of the enzyme are given in Table 1. BHPNP1 showed highest amino acid similarity to bovine PNP, and was therefore homology modeled on that structure (Figure 2)[3] .

Table 1 Physical and kinetic characteristics of BHPNP1

Parameter	Unit	Inosine	Guanosine
Specific Activity	U.mg^{-1}	26.28	
K_m	µM	236	206
V_{max}	mol.s^{-1}	4.76 x 10^{-6}	2.03 x 10^{-9}
k_{cat}	s^{-1}	2.844 x 10^{2}	1.214 x 10^{1}
Specificity Constant	M^{-1}.s^{-1}	1.203 x 10^{8}	5.869 x 10^{4}
pH Optimum	-	7.0	
pH range (60%)	-	5.7 - 8.4	
Temp Optimum	°C	70	
Temp Range (60%)	°C	32 - 74	
Temp Stability ($t_{1/2}$ @ 60°C)	h	20.8	

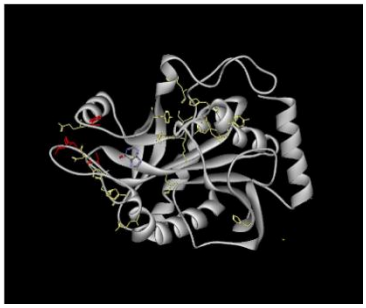

Figure 2: Ribbon representation of the homology modelled three dimensional structure of BHPNP1 . Modelling of the BHPNP1 structure was performed using the bovine structure 1VFN[6] as a template. The monomeric subunit was modelled along with the substrate, hypoxanthine using Accelrys Discovery Studio 2.0.

TRANSGLYCOSYLATION

Application of a crude enzyme preparation of BHPNP1 in combination with *E. coli* UP to the transglycosylation reaction gave a 5-MU yield of 79% mol.mol^{-1} in under 8 h in a slurry-based reaction medium (Figure 3)

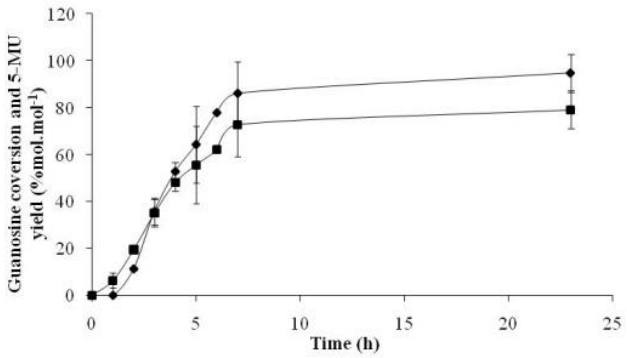

Figure 3 Bench scale (650 ml) biocatalytic production of 5-MU containing thymine (127 mM), guanosine (53 mM), BHPNP1 and UP in 50 mM sodium phosphate buffer (pH 7.8) at 40°C. Guanosine conversion (◆) and 5-MU yield (■) are shown. Error bars are calculated from the mole balance of the complete reaction (Reproduced from (7))

CONCLUSIONS

- The biocatalytic reaction described here indicates that a novel combination of nucleoside phosphorylases (*B. halodurans* PNP1 and *E. coli* UP) can facilitate the production of pyrimidine nucleosides from purine nucleosides in high yields.
- Partially purified enzyme preparations were applied in a two step transglycosylation reaction for the production of 5-methyluridine in a one-pot synthesis step with a yield of 79.1% mol/mol on guanosine at a productivity of 1.37g.l^{-1}.h^{-1}.
- This represents the first example of a free-enzyme transglycosylation giving high yields in a slurry-based reaction for the production of 5-MU.

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