Biocatalysis and Biotransformation, July–August 2010; 28(4): 245–253

informa healthcare

ORIGINAL ARTICLE

High-yielding cascade enzymatic synthesis of 5-methyluridine using a novel combination of nucleoside phosphorylases

DANIEL F. VISSER, KONANANI J. RASHAMUSE, FRITHA HENNESSY, GREGORY E. R. GORDON, PETRUS J. VAN ZYL, KGAMA MATHIBA, MOIRA L. BODE & DEAN BRADY

CSIR Biosciences, Private Bag X2, Modderfontein, 1645, South Africa

Abstract

A novel combination of *Bacillus halodurans* purine nucleoside phosphorylase (BhPNP1) and *Escherichia coli* uridine phosphorylase (EcUP) has been applied to a dual-enzyme, sequential, biocatalytic one-pot synthesis of 5-methyluridine from guanosine and thymine. A 5-methyluridine yield of >79% on guanosine was achieved in a reaction slurry at a 53 mM (1.5% w/w) guanosine concentration. 5-Methyluridine is an intermediate in synthetic routes to thymidine and the antiretroviral drugs zidovudine and stavudine.

Keywords: *Purine nucleoside phosphorylase, uridine phosphorylase, biocatalysis, 5-methyluridine, fermentation, transglycosylation*

Introduction

Nucleoside analogs are widely used as antiviral and anticancer drugs, where they act as inhibitors of viral replication or cellular DNA replication. The traditional synthetic routes for these compounds are often complex and inefficient multi-stage processes (Lewkowicz & Iribarren 2006). Zidovudine (AZT) and stavudine (d4T) are thymidine analogs that are approved by regulatory bodies such as the South African Medicines Control Council as part of an HIV/AIDS treatment regimen. Due to the current high cost of antiretrovirals and high incidence of HIV/AIDS in southern Africa, low-cost syntheses are needed. Chemical synthesis of both zidovudine and stavudine can be achieved using 5-methyluridine (5-MU) as a precursor (Chen et al. 1995; Shiragami et al. 1996).

Early 5-MU syntheses used toxic thyminylmercury and tri-*O*-acetyl-D-ribofuranosyl chloride in toluene, and subsequent deacetylation using methanolic hydrogen chloride gave low product yields (5–25%). An alternative coupling protocol using tri-*O*-benzoyl-D-ribosyl halide (chloride or bromide) and dithyminylmercury, followed by quantitative debenzoylation using alcoholic ammonia, resulted in enhanced yields of 50% and 36%, respectively (Fox et al. 1956; Stepanenko et al. 1973). Subsequently, in the 1980s, alternative biocatalytic syntheses for nucleosides became the focus of research (Hanrahan & Hutchinson 1992; Prasad et al. 1999; Utagawa 1999; Lewkowicz & Iribarren 2006; Mikhailopulo 2007). 5-MU can be synthesized by means of selective biocatalytic transglycosylation of guanosine and thymine (Utagawa 1999; Figure 1). Biocatalytic transglycosylation reactions between purines and pyrimidines require the combination of pentosyltransferases such as a purine nucleoside phosphorylase (PNPase; EC 2.4.2.1) and a pyrimidine nucleoside phosphorylase (PyNPase; EC 2.4.2.2), both of which catalyze the reversible phosphorolysis of nucleosides. Other enzymes that have a similar catalytic function to PyNPases (and can be referred to as PyNPases) are uridine phosphorylase (UPase; EC 2.4.2.3) and thymidine phosphorylase (TPase; EC 2.4.2.4). A particular benefit of nucleoside synthesis by enzymatic transglycosylation is the *in situ* activation of the 1′ position by phosphorylation with an anomeric selectivity that results in formation of only the β-anomer of the nucleoside.

Correspondence: D. F. Visser, CSIR Biosciences, Private Bag X2, Modderfontein, 1645, Johannesburg, South Africa, 1645, Tel: +27-11-605-2748. Fax: +27-11-608-3020. E-mail: dvisser@csir.co.za

(Received 25 January 2010; revised 18 March 2010; accepted 11 May 2010)

ISSN 1024-2422 print/ISSN 1029-2446 online © 2010 Informa UK Ltd. (Informa Healthcare, Taylor & Francis AS)
DOI: 10.3109/10242422.2010.493210

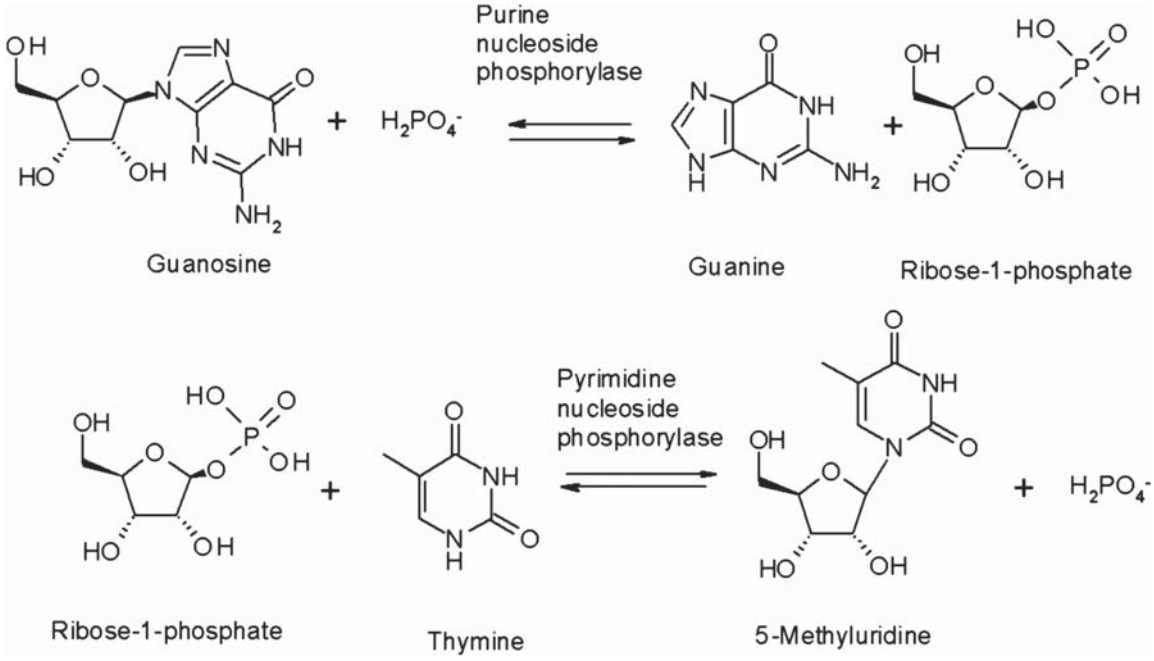

Figure 1. The component reactions in the synthesis of 5-MU from guanosine and thymine.

Other synthetic methods typically yield a mixture of the α- and β-anomers (Freskos et al. 1990; Lewkowicz & Iribarren 2006), which then need to be chromatographically separated. The equilibrium for PNPase is towards nucleoside formation for natural substrates, while PyNPase favors the phosphorolysis reaction (Erion et al. 1997; Bzowska et al. 2000; Lewkowicz & Iribarren 2006; Figure 1), and hence the majority of the work to date has focused on synthesis of purine nucleosides from pyrimidine nucleosides.

The anticipated adverse reaction equilibrium and the very low solubility of the starting substrates would suggest that synthesis of 5-MU (a pyrimidine nucleoside) would suffer from low yield and productivity. Studies using inosine as the glycosyl donor, thymine and crude enzyme were performed by Hori et al. (1989a,b), but the reaction yielded only 22% 5-MU at low substrate concentrations. Further work by the same group (Hori et al. 1991a) using immobilized enzymes showed improvements, with a continuous conversion of inosine and thymine at an initial concentration of 75 mM in the feed to give a 5-MU yield of 33%. The poor equilibrium constant of 0.24 of the overall transglycosylation reaction limited the conversion to 5-MU (Hori et al. 1991b), indicating that the reaction lacks an overall driving force towards pyrimidine synthesis. However the potential of transglycosylation was demonstrated by Ishii et al. (1989), who showed that by using guanosine in combination with thymine and whole cells of *Erwinia carotovora* it was possible to produce 5-MU at a yield of 74% from high starting substrate concentrations (300 mM), albeit over a 48 h period. Another potentially limiting factor for enzymatic conversion is that the substrates guanosine and thymine are only sparingly soluble in aqueous solutions. As heating the aqueous solution improves the solubility, it would be preferable to utilize moderately thermostable nucleoside phosphorylases in heated reactions. In general prokaryotic PyNPase and PNPase tend to be more thermostable and have broader specificity than their mammalian counterparts (Tonon et al. 2004). A few thermostable PNPase enzymes from extremophiles have been reported and applied to the production of nucleosides (Hori et al. 1991a; Cacciapuoti et al. 2005, 2007).

Previously, we have expressed and isolated the purine nucleoside phosphorylase (BhPNP1) from the thermotolerant alkalophile *Bacillus halodurans* (Visser et al. 2010), formerly *Bacillus brevis* (Louw et al. 1993). Here we report on the combination of that enzyme with the *Escherichia coli* UPase in a one-pot cascade reaction to produce 5-MU in high yield.

Materials and methods

Assessment of nucleoside phosphorylases for production of 5-methyluridine

Demonstration of 5-methyluridine synthesis by enzymatic transglycosylation. Reactions (3 mL) with nucleoside and/or base at concentrations of 2.5 mM (Table I) were performed in phosphate buffer, pH

7.4, at 25°C over 3 h with agitation. TPase (Sigma catalog no. T2807), bacterial PNPase (Sigma catalog no. N8264) and xanthine oxidase (XO) (Sigma catalog no. X2252) were evaluated, as well as a freshly prepared crude enzyme extract of *E. coli* containing both PNPase and UPase activity.

Native enzyme production. A strain of *E. coli* was used to provide a crude native enzyme solution for initial experiments. An inoculum culture of *E. coli* JM109 was grown in 100 mL Luria broth (LB) (NaCl 10 g L^{-1}, tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}) overnight at 37°C with shaking at 200 rpm. Fifteen milliliters of this culture was used to inoculate 5 × 400 ml LB in Fernbach flasks. These cultures were grown for 4 h at 37°C with shaking at 220 rpm. Two liters of culture broth were centrifuged for 10 min at 17 000*g*. The resultant pellet was re-suspended in 100 mL sonication buffer (20 mM Tris–HCl, pH 7.2, 5 mM ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid, 1 mM dithiothreitol) and chilled on ice for 20 min. This suspension was sonicated for 10 min at 4°C and then centrifuged for 10 min at 17 000*g*. Ammonium sulfate was added to the supernatant to a saturation of 40% (w/v) and stirred at 4°C for 20 min. This was centrifuged as before and additional ammonium sulfate was added to the supernatant to obtain 70% (w/v) saturation, which was again stirred on ice for 20 min. After centrifugation the pellet containing the enzymes of interest was re-suspended in 100 mL Tris–HCl buffer at pH 7.2. This preparation was desalted by ultrafiltration through a 10 kDa filtration membrane. The concentrated sample was washed with water and filtered to aid desalting. The resulting solution was lyophilized (50 mL) and a total of 710 mg of lyophilized material was obtained, which constituted the crude extract sample.

Similarly *B. halodurans*, *Klebsiella pneumoniae* and *Bacillus licheniformis* were cultivated in TYG medium (tryptone 5 g L^{-1}, yeast extract 2 g L^{-1}, glucose 1 g L^{-1}) at 40°C with shaking at 200 rpm overnight for isolation of native PyNPase (BhPyNP, KpPyNP and BlPyNP, respectively). *K. pneumoniae* and *B. licheniformis* had been identified as good PyNPase producers in a previous screening experiment (unpublished).

Biocatalytic screening. Enzyme stock solutions (0.02 U mL^{-1}) of the enzymes described above were prepared in water. Each of the enzymes was tested for the ability to produce 5-MU. The total enzyme concentration was maintained at 0.004 U mL^{-1} for each of the experiments. Enzyme solutions and assay reagent (100 µL containing 5 mM guanosine and 5 mM thymine in 50 mM phosphate buffer, pH 8.0) were aliquoted into a 96-well microtiter plate using an EpMotion 5075 liquid handler (Eppendorf, Hamburg, Germany). The microtiter plate was incubated for 1 h at 40°C with shaking at 900 rpm (Labsystems shaker; Thermomix, Helsinki, Finland). Results were analyzed by TLC (5 µL spot, mobile phase chloroform–methanol, 85:15 (v/v), UV_{254} Silica plates (Merck, Darmstadt, Germany)).

Over-expression and preparation of selected nucleoside phosphorylases

Isolation of E. coli DNA

E. coli XL1 blue was grown overnight at 37°C in a 10 mL culture volume. A 1.5 mL aliquot of this was pelleted by centrifugation, and genomic DNA was isolated using a genomic DNA isolation kit (Fermentas Canada, Inc., Burlington, ON, Canada).

Oligonucleotides, plasmids and microbial strains

E. coli JM109 (DE3) was used as the expression host for *E. coli* PNPase1 (EcPNP1), PNPase2 (EcPNP2) and *B. halodurans* PNPase (BhPNP1). *E. coli* BL21 (DE3) was used as the production host for *E. coli* UPase (EcUP).

The PNPase gene designated *BhPNP1* was amplified and cloned as described previously (Visser et al. 2010). Isolation and cloning of the *E. coli* genes encoding PNPase and UPase was carried out as described by Lee et al. (2001) and Spoldi et al. (2001), respectively. The *E. coli* PNP2 (EcPNP2, product of the *xapA* gene) was cloned according to the methods of Dandanell et al. (2005). The amplified PCR products were subcloned initially into pGEM-T Easy and subsequently into pMS470 (EcPNP1, EcPNP2, BhPNP1) and pET20b (EcUP). The expression plasmids were then transformed into their respective expression hosts by heat shock (Sambrook & Russell 2001).

Preparation of extracts of over-expressed enzymes

Recombinant strains producing selected nucleoside phosphorylases were prepared at 700 mL scale using defined growth medium (K_2HPO_4 14.6 g L^{-1}, $(NH_4)_2SO_4$ 2 g L^{-1}, Na_2HPO_4 3.6 g L^{-1}, citric acid 2.5 g L^{-1}, $MgSO_4$ 0.25 g L^{-1}, NH_4NO_3 5 g L^{-1}, yeast extract 10 g L^{-1}, glucose 30 g L^{-1}, ampicillin 100 µg mL^{-1}). Overnight cultures (100 mL) of each strain were used as the inocula for 600 mL media in 2L Fernbach flasks. Cultures were grown for 4 h at 37°C with shaking at 200 rpm before enzyme expression was induced with a final concentration of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG).

Cultures were then harvested after a further 2 h growth under the same conditions.

Culture broth was centrifuged for 10 min at 17 000*g*. The resultant pellet was re-suspended in Bugbuster HT (Novagen, Merck KGaA, Darmstadt, Germany) containing lysozyme (USB, Cleveland, OH, USA) at 3 mg mL^{-1} and incubated for 2 h at 30°C. Cell debris was removed by centrifugation (16 000*g*, 10 min). The supernatant was diluted with 20 mM Tris–HCl buffer, pH 7.2, containing 50 mM NaCl. Samples were dialyzed against the same buffer overnight. Anion-exchange chromatography of each sample was performed on an AKTA Prime (Amersham Biosciences, Bucks, UK) using Tosoh BioSep SuperQ650m resin (Tosoh, Tokyo, Japan). Proteins were eluted using a gradient of 50 to 350 mM NaCl in 20 mM Tris–HCl, pH 7.2, over 400 mL (4 mL min^{-1}). PNPase and UPase activity was assayed on all fractions (5 mL fractions collected). Fractions identified in this step for UPase and PNPase activity were separately pooled and concentrated to 2 mL by ultrafiltration (30 kDa membrane).

Enzyme production by fermentation

Organism maintenance

E. coli JM109 (pMSPNP) and *E. coli* BL21 (pETUP) were maintained as cryopreserved cultures at –70°C.

Inoculum train

Fernbach flasks containing 650 mL LB medium with 100 μg ampicillin mL^{-1} were inoculated with 2 mL of cell bank cultures. The cultures were grown overnight and used as inocula for the fermentations. The production strain had a maximum growth rate of between 0.80 and 0.88 in the exponential phase.

Batch fermentations

Batch fermentors (B. Braun Biotech International GmbH, Melsungen, Germany) containing 10 L of GMO 20 medium were inoculated with 650 mL inoculum. The composition of the GMO 20 medium was according to Visser et al. (2010). The temperature was controlled at 37°C and the aeration set to 1 vvm (volume of air per volume of reactor per minute). The starting agitation was set at 300 rpm and ramped up manually to control the pO_2 above 30% saturation. Growth, enzyme activity and glucose utilization were measured using 10 mL samples taken at hourly intervals.

Initially *E. coli* JM109 (pMSPNP) fermentations were induced at a residual glucose concentration of between 1 and 3 g L^{-1} at an IPTG concentration of 1.0 mM. Upon further investigation at 1L scale (data not shown), it was determined that targeting induction at mid-log growth phase based on measurements of optical density at 660 nm ($OD_{660} \approx 7$) and at an IPTG concentration of 0.5 mM was more effective. Induction using 0.5 mM IPTG of *E. coli* BL21 (pETUP) in fermentations was at an OD_{660} of approximately 13, which was reached at 4 h.

Enzyme recovery

After fermentation, the broth was harvested and allowed to settle overnight at 4°C. The biomass was separated from the supernatant by decanting, and subjected to a freeze–thaw cycle alternating between +20°C and –20°C. Liberated soluble protein was stored at 4°C, after separation by centrifugation (14 000*g*, 10 min, Beckman Avante; Beckman Coulter, Inc., Fullerton, CA, USA). The pelleted biomass was re-suspended in 1 L deionized water and further disrupted using a pressure-based cell disruptor (2 Plus; Constant Systems, Daventry, UK) with one pass at 276 MPa (40 ksi) to release additional enzyme. Cellular debris was again removed by centrifugation. The combined resultant protein solutions (supernatants from freeze–thaw and cell disruption processes) were concentrated and simultaneously washed with water by ultrafiltration using a Prostak cross-flow filtration unit (30 kDa cut-off membrane; Waters Corp., Milford, MA, USA). The final preparation was lyophilized in the presence of 1% w/w maltose and 1% w/w PEG 8000 (Vertis Genesis 25 L freeze drier, Gardiner, NY, USA).

Biocatalytic reactions

Except where stated otherwise, reactions were carried out at 40°C with agitation in sodium phosphate buffer (50 mM, pH 7.4) using equivalent molar concentrations of thymine and guanosine (53 mM) and incorporating appropriate amounts of PNPase and UPase.

Analytical

Biocatalysis reaction samples were prepared by dissolving the required amount of sample in sodium hydroxide (10 M, 0.5–1 mL) and samples were then made up to the required volume so as to ensure the sample concentration was within the linear region of the calibration curve. Reaction components were quantitatively analyzed by HPLC, using a Waters Alliance model 2609 instrument (Waters Corp.) with a Synergi 4 μm Max-RP 150 mm × 4.6 mm column. Components were detected using a UV detector at 260 nm. The eluent was ammonium acetate (NH_4OAc, 25 mM), pH 4.00, flow rate 1 mL min^{-1} and run time of 20–30 min at 25°C. Elution times were 6.5, 9.4, 17.2 and 16.7 min for guanine, thymine, 5-MU and guanosine, respectively, using authentic materials as reference standards. Guanine (98% pure, catalog no. G11950) and guanosine

(99% pure, catalog no. G-6752) were supplied by Sigma-Aldrich (St Louis, MO, USA). 5-MU (98% pure) and thymine (99% pure) were supplied by NSTU Chemicals (Hangzhou, China).

An indirect method of ribose-1-phosphate (R-1-P) analysis was used based on the acidic decomposition of R-1-P to ribose and phosphate ion. Released ribose was measured by ion chromatography at ambient temperature and run time of 10 min on a CarboPac PA10 column (4 mm×250 mm; Dionex, Bannockburn, IL, USA) using a Dionex GP40 pump fitted with a TSP AS 3500 autosampler (ThermoFinnigan, San Jose, CA, USA) and a Dionex ED40 electrochemical detector. To validate the method, the released ribose was compared with the molar concentration of guanine released in the same reaction since guanine and R-1-P are produced in equimolar concentrations during the phosphorolysis reaction.

Fermentation sampling, growth and analysis

Growth was measured by determining the optical density at 660 nm and dry cell weight (dcw) in triplicate. A volume of 2 mL of the sample was centrifuged, washed with 0.1 M HCl to remove precipitated salts, and the pellet was then used for dry cell weight determination by drying to constant weight at 110°C. Glucose concentration was measured using an Accutrend sensor (Boehringer Mannheim, Mannheim, Germany).

For determination of the enzyme activity of the biomass, triplicate samples of 1 mL were centrifuged, re-suspended in a minimum volume of the cell disruption solution B-Per (Pierce, Rockford, IL, USA) and vortexed briefly to re-suspend the pellet. After incubation at room temperature for 5 min the samples were centrifuged and the supernatant analyzed for nucleoside phosphorylase activity using the standard enzyme assays.

Enzyme assays. The method of Hwang & Cha (1973) was modified for PNPase determination wherein a suitably diluted sample (10 µL) was added to 190 µL of 50 mM sodium phosphate buffer containing 0.5 mM inosine and 0.2 U XO mL^{-1} in UV-compatible, 96-well microtiter plates (Thermomix). The change in absorbance at 293 nm due to the liberation of uric acid was measured on a PowerWave HT microplate spectrophotometer (Biotek Instruments, Inc., Winooski, VT, USA). One unit of PNPase was defined as the enzyme liberating 1 µmol of uric acid from inosine per minute, in the presence of excess XO. The extinction coefficient under these conditions was determined to be 7454 cm^2 mol^{-1}.

The method of Hammer-Jespersen et al. (1971) was modified for UPase determination, 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 microtiter plates. After 10 min incubation time at 40°C, the reaction was stopped by addition of 100 µL of 0.5 N perchloric acid. The samples were then incubated on ice for 20 min and centrifuged for a further 20 min (7000*g*) to remove residual protein. Samples (100 µl) were then transferred to a UV-compatible microtiter 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 (Biotek Instruments, Inc.). 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 cm^2 mol^{-1}. Nucleosides were purchased from Sigma-Aldrich.

Results and discussion

Assessment of nucleoside phosphorylases for production of 5-methyluridine

The aim of the research was to develop an enzyme-based high-yielding synthesis for 5-MU. To this end initial reactions were performed to confirm the relevant enzyme activities and demonstrate the transglycosylation reaction. Results were analyzed by HPLC (Table I).

Reactions 1 and 2 were performed to confirm the reversibility and direction of equilibrium of the pyrimidine phosphorolysis, while reaction 3 confirmed purine nucleoside phosphorolysis when using PNPase in the presence of XO. XO was used to convert the co-product hypoxanthine to uric acid to prevent the reverse reaction. Reaction 4 successfully demonstrated transglycosylation using commercial enzyme preparations with transfer of deoxyribose from thymidine to hypoxanthine. However, use of the same commercial enzyme preparations for transglycosylation involving ribose transfer failed (reactions 5 and 6). This failure was presumably due to the strict requirement of TPase for deoxyribose-1-phosphate rather than R-1-P, a result that was anticipated, but required confirmation. The enzyme UPase can utilize R-1-P, but was not commercially available, and on this basis we decided to isolate UPase from *E. coli*. Through the use of native *E. coli* cell extract (which contained both PNPase and UPase activities of 0.017 and 0.012 U mg^{-1}), it was possible to generate 5-MU (reactions 7 and 8).

Combinations of partially purified nucleoside phosphorylases were screened for 5-MU production. While all combinations tested demonstrated 5-MU production (Figure 2), reactions containing either EcPNP1 or BhPNP1 combined with EcUP showed the highest production levels (lanes 1, 5, 9).

Table I. Demonstration of 5-MU synthesis by enzymatic transglycosylation (data extracted from Visser et al. 2009).

Reaction	Expected product	Starting reagents	Enzymes	Product peak (% of total peak area)
1	Thymine	Thymidine	TPase	78.5
2	Thymidine	Thymine, deoxyribose-1-phosphate	TPase	19.5
3	Hypoxanthine, xanthine	Inosine	XO, PNPase	61.7
4	2-deoxyinosine	Hypoxanthine, thymidine	TPase, PNPase	33.4
5	5-methyluridine	Thymine, Guanosine	TPase, PNPase	0
6	5-methyluridine	Inosine, thymine	XO, TPase, PNPase	0
7	5-methyluridine	Inosine, thymine	Crude extract, XO	21.8
8	5-methyluridine	Guanosine, thymine (16 h)	Crude extract	8.7

A series of experiments was then conducted to identify which enzyme system (EcPNP1/EcUP or BhPNP1/EcUP) provided the best 5-MU yield. The enzymes were over-expressed as stated in the Materials and methods section by shake-flask cultivation. EcPNP (0.85 U mg^{-1}), EcUP (0.52 U mg^{-1}) and BhPNP1 (1.41 U mg^{-1}) at final concentrations of 0.15 U mL^{-1} each were tested in 75 mL reactions at 40°C for 25 h. 5-MU yields of 51% on guanosine were observed for the combination of the *E. coli* UPase and PNPase. However, a combination of the *B. halodurans* PNPase (BhPNP1) and the *E. coli* UPase (EcUP) gave a dramatically improved yield of 80% (Figure 3). This enzyme combination was used in all subsequent reactions.

Influence of the second reaction on the reaction equilibrium: Effect of decoupling the first reaction step

Transglycosylation can in theory occur as either a one-pot or a two-pot process, and it was of interest to determine the influence of the second reaction on overall reaction equilibrium. A phosphorolysis experiment was conducted using 53 mM guanosine, sodium phosphate buffer and PNPase enzyme (200 U, 5.14 U mg^{-1}) to investigate the decoupling of the biocatalytic reaction.

The precipitation of guanine due to low solubility, approximately 0.01% w/v at 40°C, was expected to drive the phosphorolysis reaction to completion. However the results (Figure 4) show that a guanosine conversion of only 37% occurred. The utilization of R-1-P in the coupled reaction system thus plays a far greater role in driving the phosphorolysis reaction to completion than was anticipated. The conclusion based on these results is that the only practical means of conducting the reaction is as a coupled process. The results also indicated that ribose-1-phosphate was relatively stable under the biocatalytic reaction conditions for the duration of the reaction.

Effect of co-solvents and surfactants

At 53 mM (1.5% w/w) the substrates are well above their solubilities and therefore form slurries. The low solubility of guanosine and thymine, both around 0.1% w/v at 40°C, was considered to be a possible limiting factor in the success of the reaction. The rate of conversion of substrates in aqueous suspensions may be increased by the addition of co-solvents to increase substrate solubility. Co-solvents

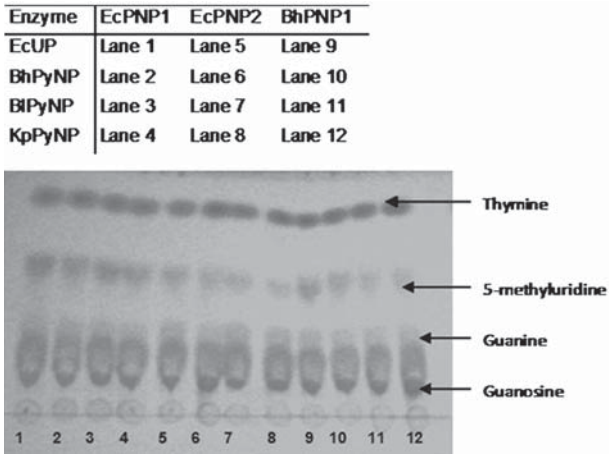

Figure 2. Comparative efficiencies of combinations of purine and pyrimidine nucleoside phosphorylases in the production of 5-MU (combinatorial enzyme reactions listed in table insert; adapted from Visser et al. 2009).

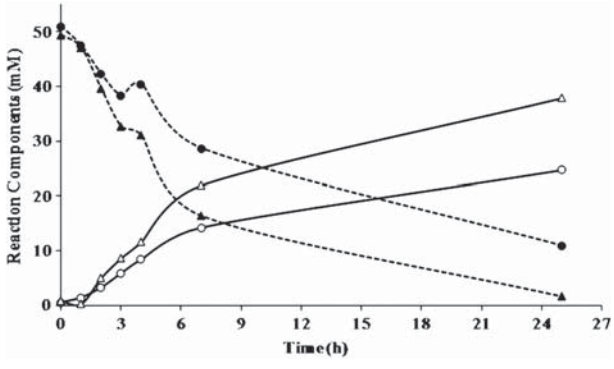

Figure 3. Guanosine conversion (●, ▲) and 5-MU production (○, △) for a combination of *E. coli* PNPase and UPase (●, ○) and *B. halodurans* PNPase and *E. coli UPase* (▲, △).

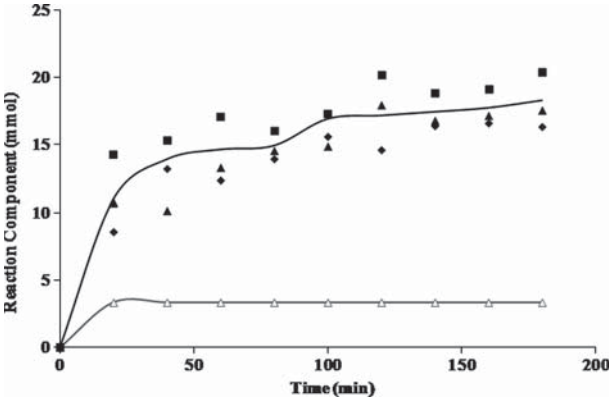

Figure 4. Guanosine phosphorolysis by PNPase in a decoupled reaction. (—) Average of guanosine converted (■), guanine (◆) and total ribose (▲). No increase in free ribose (△) in the suspension indicates that R-1-P remains stable throughout the reaction.

Table II. Effect of co-solvents and surfactants on the transglycosylation reaction.

Co-solvent	Guanosine conversion (%)	5-MU yield[a] (%)	Mole balance[b] (%)
Aqueous	95.1±1.3	56.4±1.7	92.3±7.4
20% v/v MeOH	90.7±2.5	61.4±4.4	92.2±7.5
20% v/v EtOH	87.7±2.3	59±9.8	87.2±14.9
20% v/v DMSO	94.3±1.1	63.3±14.0	84.4±18.4
2.5% Triton X-100	97.2±0.3	46.4±1.1	78.5±6.9
2.5% Tween 80	96.7±0.8	50.8±10.0	80.8±16.5

Experimental conditions: 53 mM guanosine, 122 mM thymine, sodium phosphate buffer (50 mM, pH 7.5–8.0), 40°C. The triplicate reactions were catalyzed by BhPNP1 0.27 U (5.14 U mg-protein^{-1}) and EcUP 0.27 U (0.2 U mg-protein^{-1}).
[a]5-MU yield on guanosine at 24 h reaction.
[b]Mole balance for reaction, including guanosine, guanine, thymine and 5-MU.

and surfactants have previously been demonstrated to improve reaction yields in nucleoside (Hori et al. 1991b) as well as in slurry-based reactions (Brady et al. 2004; Steenkamp & Brady 2008). Hydrophilic solvents such as methanol and ethanol, which showed moderate guanosine wetting properties, were tested. These solvents are of interest owing to their ease of removal with boiling points of 64.7°C (methanol) and 78°C (ethanol). At 53 mM, guanosine was completely soluble in 20% v/v DMSO in water, indicating that this could be a suitable reaction medium. However DMSO is generally difficult to remove from the product as high distillation temperatures are required (DMSO boiling point 189°C).

The use of non-miscible hydrophobic solvents such as toluene was not considered since this would further increase the complexity of the biocatalytic reaction, resulting in both biphasic liquids (water and toluene) and solids (guanosine, guanine and thymine).

These reactions (3 mL) were tested by adding guanosine and thymine to mixtures of co-solvent (20% v/v in water) or surfactant (2.5% v/v in water). The reaction mixtures were stirred at room temperature for 1 h before addition of enzyme, and then incubated for 24 h. However, addition of co-solvent or surfactant did not significantly improve the conversion of guanosine (Table II), while the influence on the 5-MU analytical results obscured any marginal increase in 5-MU yield. What is obvious is that although the guanine was completely soluble in the DMSO solution the conversion was similar to the control reaction, again indicating that precipitation of this co-product is not the main driving force of the coupled reaction.

Enzyme production

In order to prepare sufficient enzyme for larger-scale reactions, optimized fermentations (10 L) were performed for the production of BhPNP1 and EcUP (Figure 5). High levels of enzyme were produced within 8 to 10 h of fermentation. The fermentation results are summarized in Table III. Data

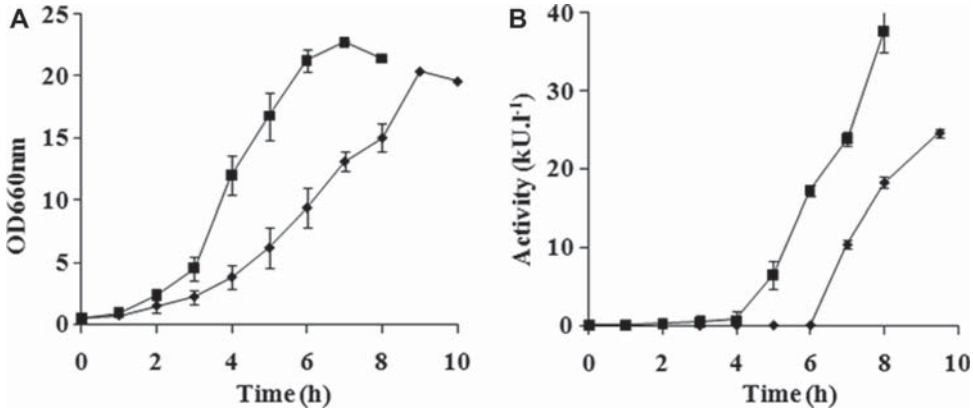

Figure 5. Growth (A) and enzyme activity (B) profiles of *E. coli* (pMSPNP) (◆) and *E. coli* (pETUP) (■) in duplicate batch fermentations.

presented are averages of duplicate fermentations. The lyophilized BhPNP1 activity (5.41 U mg^{-1}) was similar to that of the preparation used earlier (5.14 U mg^{-1}), while the EcUP preparation (4.3 U mg^{-1}) showed a more than 20-fold improvement in purity when compared to the previous preparation (0.2 U mg^{-1} used in the screening experiments).

Bench-scale biocatalytic reaction

To demonstrate the biocatalysis at bench scale, a 650 mL reaction was performed. The results obtained showed a guanosine conversion of 94.7% and a 5-MU yield of 79.1% (Figure 6) within 7 h, at a 5-MU productivity of 1.37 g L^{-1} h^{-1}. The yield of this non-optimized reaction was comparable to the 74% reported by Ishii et al. (1989) using whole cells of an *Erwinia* wild-type organism, but in a much shorter time. This also demonstrated that cell free extracts are tolerant of high substrate concentrations as slurries, using starting substrate concentrations in excess of 0.1 M, which has previously only been applied in whole-cell biocatalytic reactions (Ishii et al. 1989).

Finally, to confirm the structure of the reaction product, a sample was washed with hot isopropyl alcohol, filtered and vacuum dried. NMR data for this compound: ^{1}H NMR (400 MHz, D_2O), δ (ppm) 7.69 (1H, s), 5.89 (1H, d, $\mathcal{J}$=4.7 Hz), 4.33 (1H, t, $\mathcal{J}$=5.1 Hz), 4.23 (1H, *t*, $\mathcal{J}$=5.4 Hz), 4.11 (1H, dd, $\mathcal{J}$=4.2 and 8.2 Hz), 3.91 (1H, dd, $\mathcal{J}$=2.9 and 12.8 Hz), 3.81 (1H, dd, $\mathcal{J}$=4.2 and 12.8 Hz), 1.87 (3H, s); ^{13}C NMR (100 MHz, D_2O), δ (ppm) 166.3, 151.6, 137.2, 111.2, 88.8, 84.0, 73.4, 69.2, 60.5, 11.4. Spectroscopic data were identical to that of a commercial standard (see Supplementary Figure S1 for ^{1}H NMR spectrum).

Conclusions

The biocatalytic reaction described here shows that a novel combination of nucleoside phosphorylases (*B. halodurans* PNPase and *E. coli* UPase) can facilitate the production of pyrimidine nucleosides from purine nucleosides in high yields. Partially purified enzyme preparations were applied in a two-step one-pot transglycosylation reaction for the production of 5-MU in a synthesis step with a molar yield of 79.1% on guanosine. Reaction engineering is anticipated to improve yield and productivity further.

Table III. Summary of fermentation data for the production of BhPNP1 and EcUP.

Value	BhPNP1	EcUP
Maximum OD (660 nm)	14.4	20.59
μ_{max}	0.43	0.60
Yield (g-dcw g-glucose^{-1})	0.53	0.55
Biomass (g L^{-1})	9.45	12.37
Productivity (g-dcw L^{-1} h^{-1})	1.16	1.62
Enzyme yield (kU L^{-1})	26.9	37.7
Enzyme productivity (kU L^{-1} h^{-1})	3.3	5.8
Enzyme yield (kU)[a]	215	211
Specific activity (kU g^{-1})[b]	5.41	4.30

[a]Total recovered units after downstream processing.
[b]Units per gram dry product after lyophilization.

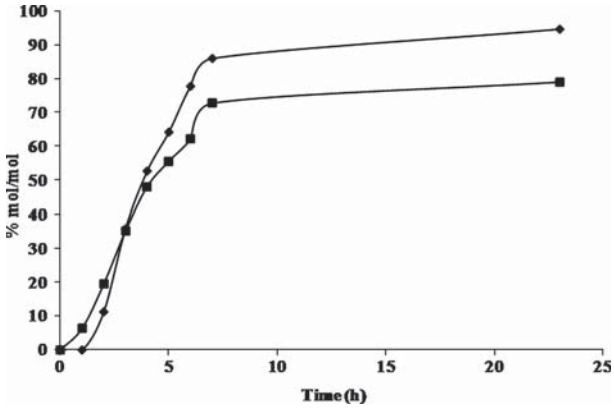

Figure 6. Bench-scale (650 mL) biocatalytic production of 5-MU containing 127 mM thymine (1.6% w/w,), 53 mM guanosine (1.5% w/w), BhPNP1 (105 U) and EcUP (75 U) in 50 mM sodium phosphate buffer (pH 7.8) at 40°C. Guanosine conversion (◆) and 5-MU yield (■) are shown.

Acknowledgements

The authors would like to thank N. Gumede, S. Ramchuran (LIFElab), R. Lalloo, C. van der Westhuyzen, V. Moodley, K. Pillay, D. Mabena, S. Machika and H. Manchidi (CSIR Biosciences) for technical assistance. They gratefully acknowledge Dr D. R. Walwyn (CEO of Arvir Technologies) and Dr H. Roman (CSIR) for management of the project. Financial support for this work was provided by CSIR Biosciences, LIFElab, DST and Arvir Technologies. Thanks also go to Professor D. Litthauer (University of the Free State) for the *K. pneumoniae* and *B. licheniformis* cultures. This paper is dedicated to the analytical chemist and colleague Mr Simon Machika, who passed away during this research.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Brady D, Steenkamp L, Skein E, Chaplin JA, Reddy S. 2004. Optimisation of the enantioselective biocatalytic hydrolysis of

naproxen ethyl ester using ChiroCLEC-CR. Enzyme Microb Technol 34:283–291.

Bzowska A, Kulikowska E, Shugar D. 2000. Purine nucleoside phosphorylases: properties, functions, and clinical aspects. Pharmacol Ther 88:349–425.

Cacciapuoti G, Forte S, Moretti MA Brio A, Zappia V, Porcelli M. 2005. A novel hyperthermostable 5′-deoxy-5′-methylthioadenosine phosphorylase from the archaeon *Sulfolobus solfataricus*. FEBS J 272:1886–1899.

Cacciapuoti G, Gorassini S, Mazzeo MF, Siciliano RA, Carbone V, Zappia V, Porcelli M. 2007. Biochemical and structural characterization of mammalian-like purine nucleoside phosphorylase from the archaeon *Pyrococcus furiosus*. FEBS J 274:2482–2495.

Chen B-C, Quinlan SL, Gregory Reid J. 1995. A new synthesis of the anti-AIDS drug AZT from 5-methyluridine. Tetrahedron Lett 36:7961–7964.

Dandanell G, Szczepanowski RH, Kierdaszuk B. Shugar D. Bochtler M. 2005. *Escherichia coli* purine nucleoside phosphorylase II, the product of the *xapA* gene. J Mol Biol 348: 113–125.

Erion MD, Takabayashi K, Smith HB, Kessi J, Wagner S, Hönger S, Shames SL, Ealick SE. 1997. Purine nucleoside phosphorylase. 1. Structure–function studies. Biochemistry 36:11725–11734.

Fox JJ, Yung N, Davoll J, Brown GB. 1956. Pyrimidine nucleosides. I. A new route for the synthesis of thymine nucleosides. J Am Chem Soc 78:2117–2122.

Freskos JN, Senaratne K, Pushpananda A. 1990. Synthesis of beta-thymidine. US patent 4,914,233.

Hammer-Jespersen K, Munch-Petersen A, Schwartz M, Nygaard P. 1971. Induction of enzymes involved in the catabolism of deoxyribonucleosides and ribonucleosides in *Escherichia coli* K 12. Eur J Biochem 19:533–538.

Hanrahan JR, Hutchinson DW. 1992. The enzymatic-synthesis of antiviral agents. J Biotechnol 23:193–210.

Hori N, Watanabe M, Yamazaki Y, Mikami Y. 1989a. Synthesis of 5-methyluridine by a thermophile, *Bacillus stearothermophilus* JTS 859. Agric Biol Chem 53:197–202.

Hori N, Watanabe M, Yamazaki Y, Mikami Y. 1989b. Purification and characterization of thermostable purine nucleoside phosphorylase from *Bacillus stearothermophilus* JTS 859. Agric Biol Chem 53:2205–2210.

Hori N, Watanabe M, Sunagawa K, Uehara K, Mikami Y. 1991a. Production of 5′-methyluridine by immobilized thermostable purine nucleoside phosphorylase and pyrimidine nucleoside phosphorylase from *Bacillus stearothermophilus* JTS 859. J Biotechnol 17:121–131.

Hori N, Watanabe M, Mikami Y. 1991b. The effects of organic solvent on the ribosyl transfer reaction by thermostable purine nucleoside phosphorylase and pyrimidine nucleoside phosphorylase from *Bacillus stearothermophilus* JTS 859. Biocatal Biotransform 4:297–304.

Hwang WI, Cha S. 1973. A new enzymatic method for the determination of inorganic phosphate and its application to the nucleoside diphosphatase assay. Anal Biochem 55:379–387.

Ishii M, Shirae H, Yokozeki K. 1989. Enzymatic production of 5-methyluridine from purine nucleosides and thymine by *Erwinia cartovora* AJ-2992. Agric Biol Chem 53:3209–3218.

Lee J, Filosa S, Bonvin J, Guyon S, Aponte R, Turnbull J. 2001. Expression, purification, and characterization of recombinant purine nucleoside phosphorylase from *Escherichia coli*. Protein Expr Purif 22:180–188.

Lewkowicz ES, Iribarren AM. 2006. Nucleoside phosphorylases. Curr Org Chem 10:1197–1215.

Louw ME, Reid SJ, Watson TG. 1993. Characterization, cloning and sequencing of a thermostable endo-(1,3-1,4) β-glucanase-encoding gene from an alkalophilic *Bacillus brevis*. Appl Microbiol Biotechnol 38:507–513.

Mikhailopulo IA. 2007. Biotechnology of nucleic acid constituents – state of the art and perspectives. Curr Org Chem 11:317–335.

Prasad AK, Trikha S, Parmar VS. 1999. Nucleoside synthesis mediated by glycosyl transferring enzymes. Bioorg Chem 27:135–154.

Sambrook J, Russell DW. 2001. Molecular cloning: A laboratory manual. Cold Spring Harbor (NY): Cold Spring Harbor Press.

Shiragami H, Ineyama T, Uchida, Y, Izawa K. 1996. Synthesis of 1-(2,3-dideoxy-β-D-glycero-pent-2-enofuranosyl)thymine (d4t; stavudine) from 5-methyluridine. Nucleosides Nucleotides Nucleic Acids 15:47–58.

Spoldi E, Ghisotti D, Cali S, Grisa M, Orsini G, Tonon G, Zuffi G. 2001. Recombinant bacterial cells as efficient biocatalysts for the production of nucleosides. Nucleosides Nucleotides Nucleic Acids 20:977–979.

Steenkamp L, Brady D. 2008. Optimisation of stabilised carboxylesterase NP for enantioselective hydrolysis of naproxen methyl ester. Process Biochem 43:1419–1426.

Stepanenko BN, Kaz'mina EM, Dubinkina ZS. 1973. Methods of synthesis of pyrimidine nucleosides. Russ Chem Rev 42:494–508.

Tonon G, Capra E, Orsini G, Zuffi G. 2004. Novel immobilized biocatalysts usable for the production of natural nucleosides and modified analogs by enzymatic transglycosylation reactions. US Patent Application 20040142438.

Utagawa T. 1999. Enzymatic preparation of nucleoside antibiotics. J Mol Catal B: Enzym 6:215–222.

Visser DF, Gordon G, Bode ML, Hennessy F, Rashamuse K, Louw ME, Brady D. 2009. A biocatalytic method for synthesis of 5-methyluridine. Patent number: WO2010055369.

Visser DF, Hennessy F, Rashamuse K, Louw M, Brady D. 2010. Cloning, purification and characterisation of a recombinant purine nucleoside phosphorylase from *Bacillus halodurans* Alk36. Extremophiles 14:185–192.

Supplementary material available online

Supplementary Figure S1. Proteon NMR spectrum of 5-MU produced by the biocatalytic method described in this paper.