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 and Dean Brady.

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

Abstract

Bacillus halodurans purine nucleoside phosphorylase (PNPase) and *Escherichia coli* uridine phosphorylase (UPase) were expressed in *E. coli* as *E. coli* [pMSPNP] and *E. coli* [pETUP] to yields of 26.9 and 37.7 $kU.l^{-1}$ respectively in batch fermentations, partially purified, and subsequently applied as a novel combination to the dual-enzyme sequential biocatalytic one pot synthesis of 5-methyluridine from guanosine and thymine. A 5-methyluridine yield of 79% was achieved at a 52 mM (1.5% m/m) guanosine concentration. 5-Methyluridine is an intermediate in synthetic routes to β-thymidine and the antiretrovirals Zidovudine (AZT) and stavudine (d4T).

Key words: Purine nucleoside phosphorylase, uridine phosphorylase, biocatalysis, 5-methyluridine (5-MU), fermentation, transglycosylation

Introduction

Nucleoside analogues 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, inefficient, multi-stage processes (Lewkowicz and Iribarren, 2006).

Early 5-MU syntheses used toxic thyminylmercury and tri-*O*-acetyl-D-ribofuranosyl chloride in toluene, and subsequent deactylation using methanolic hydrogen chloride gave low product yields (5 - 25%), while 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 1980's alternative biocatalytic syntheses for nucleosides became the focus of research (Lewkowicz and Iribarren, 2006; Mikhailopulo, 2007; Hanrahan, 1992; Utagawa, 1999; Prasad et al, 1999). 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 (PyNP; EC 2.4.2.2), both of which catalyse the reversible phosphorolysis of nucleosides by *in situ* activation by phosphorylation of the 1′ position. Of particular value is the the anomeric selectivity of the enzyme which yields only the β-anomer. Other synthetic methods typically yield a mixture of the α- and β-anomers (Freskos et al 1990; Lewkowicz and Iribarren, 2006), which then need to be chromatographically separated. The equilibrium for PNPase is towards nucleoside formation for natural substrates, while PyNPase favours the phosphorolysis reaction (Bzowska et al 2000; Erion et al, 1997; Lewkowicz and Iribarren, 2006; Scheme 1), and hence the majority of the work to date has focused on synthesis of purine nucleosides from pyrimidine nucleosides. This represents a challenge in the synthesis of pyrimidine nucleosides.

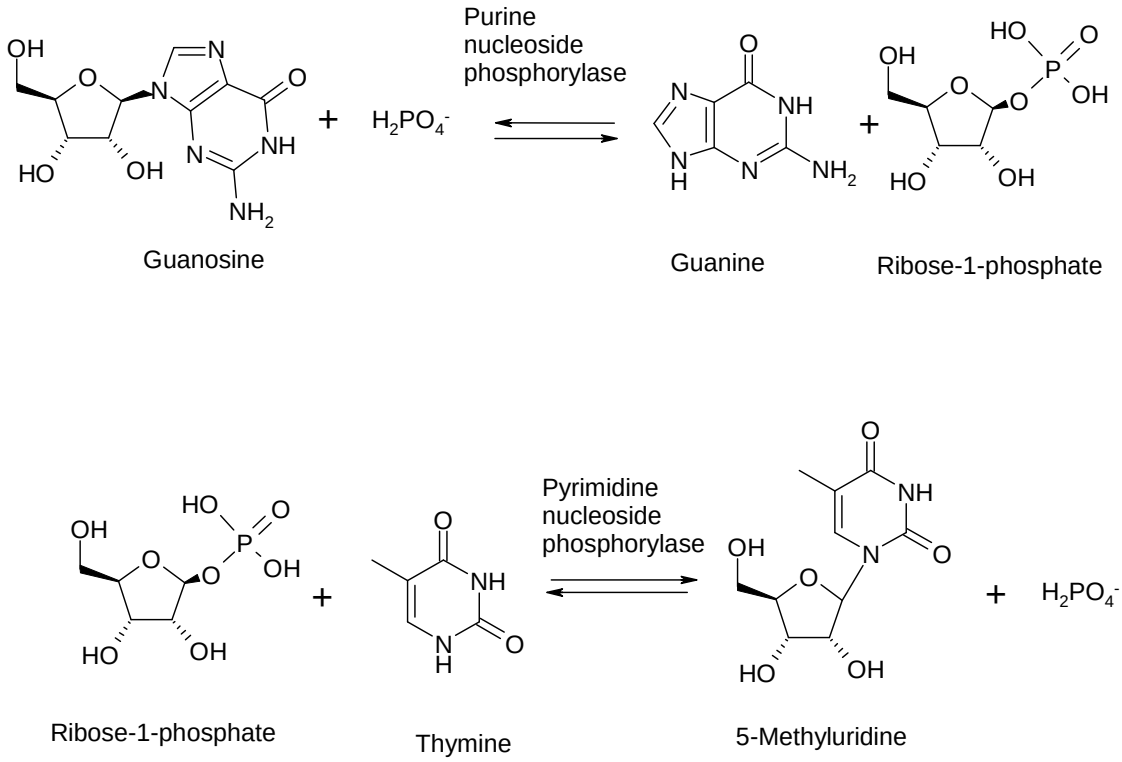
Zidovudine and (AZT) Stavudine (d4T) are thymidine analogues that are approved by regulatory bodies such as the South African Medicines Control Council as part an HIV/AIDS treatment regimen. Due to the current high cost of antiretrovirals and high incidence of HIV/AIDS in southern Africa, low cost syntheses are needed. Chemical synthesis of both AZT and Stavudine can be achieved using 5-methyluridine (5-MU) as a precursor (Chen et al 1995; Shiragami et al 1996). 5-Methyluridine itself can be synthesised by means of selective biocatalytic transglycosylation of guanosine and thymine (Utagawa T,

1999; Scheme 1). However, the anticipated adverse reaction equilibrium and the very low solubility of the starting substrates would suggest that synthesis of this 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 immobilised enzymes showed improvements with a continuous conversion of 33% inosine (at an initial concentration of 75 mM in the feed). The poor equilibrium constant of 0.24 limited the conversion (Hori et al. 1991b). However Ishii et al. (1989) 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. This reaction negates the requirement for an additional enzyme for the conversion of the base by-product to drive the reaction in the desired direction, such as xanthine oxidase for inosine derived hypoxanthine). Moreover the liberated guanine is extremely insoluble in aqueous solutions, and hence the precipitation moves the reaction equilibrium towards the pyrimidine. This yield may have been possible because the liberated guanine is extremely insoluble in aqueous solutions, and hence the precipitation moves the overall reaction equilibrium towards the pyrimidine.

The substrates guanosine and thymine are however only sparingly soluble in aqueous solutions and this would appear to be a potential limiting factor. Heating the aqueous solutions improves the solubility, and therefore it would be preferable to utilise moderately thermostable nucleotide phosphorylases in heated reactions. In general prokaryotic UPase and PNPase tend to be more thermostable and have broader specificity than their mammalian counterparts (Tonon et al. 2004). Moreover, a few thermostable PNPase from extremophiles have been reported and applied to the production of nucleotides (Hori *et al*, 1991a; Cacciapuoti *et al*, 2005 and 2007).

Previously, we have expressed and isolated the purine nucleoside phosphorylase (BhPNP1) from the thermotolerant alkalophile *Bacillus halodurans*

(Visser et al. 2010) (previously *B. brevis*; Louw et al. 1993). Here we report on the combination of that enzyme with the uridine phosphorylase (EC 2.4.2.3) from *E. coli* in a one-pot cascade reaction to produce 5-methyluridine in high yield.



Scheme 1. The component reactions in the synthesis of 5-methyluridine from guanosine and thymine.

Materials and methods

Isolation of E. coli DNA

Genomic DNA was isolated from *E. coli* according to the method of Lovett and Keggins (1979). *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).

Oligonucleotides, plasmids and microbial strains

E. coli JM109 (DE3) [*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd* R17, (rK-, mK+), *rel*A1, *sup*E44, l-, D(*lac-pro*AB), [F', *tra*D36, *pro*A+B+, *lac*IqZDM15] (DE3)] was used as the expression host for *E. coli* PNPase1 (EcPNP1), PNPase2 (EcPNP2) and *B. halodurans* PNPase (BhPNP1). *E. coli* BL21 (DE3) [*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd* R17, (rK-, mK+), *rel*A1, *sup*E44, l-, D(*lac-pro*AB), [F', *tra*D36, *pro*A+B+, *lac*IqZDM15] (DE3)] was used as the production host for *E. coli* Upase (EcUP). Both the pMS470Δ8 and the pET20b plasmids conferred ampicillin resistance in the host. *Bacillus halodurans* Alk36 was used as a source of UPase from that organism (BhUP). *Klebsiella Pneumoniae* and *Bacillus Lichniformis* were identified as good UPase producers in a previous screening experiment (unpublished).

The PNPase gene designated BhPNP1 was amplified and cloned as described previously (Visser et al. 2010). Isolation and cloning of the *E. coli* PNPase and UPase genes was carried out as described by Lee et al. (2001) and Spoldi et al. (2001) respectively. The *E.coli* PNP2 (EcPNP2, product of xapA gene) was cloned according to the methods of Dandanell et al. (2005).

The amplification *of deoD, upr, and* xapA genes (encoding EcPNP1, EcUP and EcPNP2 respectively) from *E. coli* JM109 genomic DNA was at 55°C using the High Fidelity Polymerase from Roche (Germany). The amplified PCR products were subcloned

initially into pGEM-T Easy and subsequently into pMS470 (EcPNP1, EcPNP2) and pET20b (EcUP). The expression plasmids were then transformed into their respective expression hosts by heat shock (Sambrook and Russel 2001).

E. coli crude enzyme production

An inoculum culture of *E. coli* was grown in 100 ml Luria broth (LB) (10 $g.l^{-1}$ NaCl; 10 $g.l^{-1}$ Tryptone, 5 $g.l^{-1}$ yeast extract) overnight at 37°C with shaking at 200 rpm.

Fifteen millilitres of this culture was used to inoculate 5 x 400 ml LB in Fernbach flasks. These cultures were grown for 4 h at 37°C with shaking at 220 rpm. The two litres of culture broth was centrifuged for 10 min at 17000 x g. The resultant pellet was resuspended in 100 ml sonication buffer (20 mM Tris-HCl, pH 7.2, 5 mM EDTA, 1 mM DTT) and chilled on ice for 20 min. This solution was sonicated for 10 min at 4°C and then centrifuged for 10 min at 17000 x g. Ammonium sulphate was added to the supernatant to a saturation of 40% and stirred at 4ºC for 20 min. This was centrifuged as before and ammonium sulphate was added to the supernatant to obtain 70% saturation, which was again stirred on ice for 20 min. After centrifugation the pellet containing the enzymes of interest was resuspended 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). A total of 710 mg of lyophilized material was obtained which constituted the crude extract sample.

Expression and preparation of nucleoside phosphorylases

Recombinant strains producing selected nucleoside phosphorylases were prepared at 700 ml scale using defined growth media (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 $g.l^{-1}$ and ampicillin, 100 $\mu g.ml^{-1}$). An overnight culture (100 ml) of each strain was used as the inoculums for 600 ml media in 2 L 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 IPTG. Cultures were then harvested after a further 2 h growth under the same conditions.

B. halodurans, Klebsiella pneumoniae and *Bacillus licheniformis* were cultivated in TYG media (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 UPase (BhUP, KpUP and BlUP respectively).

Preparation of Crude Extracts. Culture broth was centrifuged for 10 min at 17000 x g. The resultant pellet was resuspended in Bugbuster HT (Novagen) containing 3 mg/ml lysozyme (USB) and incubated for 2 h at 30^{o}C. Cell debris was removed by centrifugation (13000 rpm, 10 min, Heraeus Biofuge Pico). The supernatant was diluted with 20 mM Tris-HCl buffer, pH 7.2, containing 50 mM NaCl. Samples were dialysed against the same buffer overnight. Anion exchange chromatography of each sample was performed on an AKTA Prime (Amersham Biosciences) using TosohBioSep SuperQ650m resin. Proteins were eluted using a gradient of 50 mM – 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).

Assessment of different nucleoside phosphorylases for production of 5-methyl uridine

Enzyme stock solutions (0.02 U.ml^{-1}) of the above mentioned enzymes were prepared in water. Each of the enzymes were tested for their 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 pH 8.0 phosphate buffer) were aliquoted into a 96-well microtitre plate using an EpMotion 5075 liquid handler. The microtitre plate was incubated for 1 h at 40^{o}C with shaking at 900 rpm (Labsystems Thermomix). Results were analysed by TLC (5 µl spot, 85:15 chloroform : methanol mobile phase, UV_{254} Silica plates).

Enzyme Production by Fermentation

Organism maintenance. E. coli JM109 [pMSBhPNP1] and *E. coli* BL21 [pETUP] were maintained as cryopreserved cultures at -70^{o}C.

Inoculum train. Fernbach flasks containing 650 ml LB media with 100 µg.ml^{-1} ampicillin were inoculated with 2 ml of cell bank cultures. The cultures were grown overnight and used as the inoculum for the fermentations. The production strain had maximum growth rates of between 0.8 and 0.88 in the exponential phase.

Batch fermentations. Batch fermentors (Braun C, Braun Germany) containing 10 litres of GMO 20 medium (Ramchuran et al. 2002) 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 v.v.$^{-1}$min^{-1}. 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 was 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 1 l scale (data not shown), it was determined that targeting induction at mid-log growth phase based on measurements at 660 nm (OD ~ 7) and at an IPTG concentration of 0.5 mM was more effective. Induction of the *E. coli* BL21 [pETUP] fermentations was at an OD 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^{o}C and –20^{o}C. Liberated soluble protein was stored at 4°C, after separation by centrifugation (14000 x g, 10 min, Beckman Avante, Beckman Coulter, Inc. CA, USA). The pelleted biomass was resuspended in 1 l deionised water and further disrupted using a pressure based cell disruptor (2 Plus, Constant Systems, UK)

with 1 pass at 40 kpsi 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 H_2O by ultrafiltration using a Prostak cross-flow filtration unit (30 kDa cut-off membrane, Waters USA). The final preparation was lyophilized in the presence of 1% maltose and 1% PEG 8000 (Vertis Genesis 25 L freeze drier).

Analytical

Samples were prepared by dissolving the required amount of sample in sodium hydroxide (10 M, 0.5 – 1 ml) and then made up to the required volume so as to ensure the sample concentration was within the linear region of the calibration curve. Guanosine, guanine, thymine and 5-methyluridine were quantitatively analysed by HPLC, using a Waters Alliance Model 2609 instrument (Waters, USA) with a Synergi 4 µMax-RP 150 x 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 a run time of 20-30 minutes at 25°C.

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 using a Dionex GP40 pump fitted with a TSP AS 3500 autosampler, a Carbo Pac PA10, 4 x 250 mm, at ambient temperature using an Electrochemical Dionex ED40 detector and a run time of 10 min. To validate the method the released ribose was compared to the molar concentration of guanine released in the same reaction since guanine and R-1-P are produced in equi-molar concentrations during the phosphorolysis reaction. An 86% correlation was observed based on the amount of guanine (HPLC analysis) released and ribose (ion chromatography) detected after acidic hydrolysis of the ribose-1-phosphate.

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 Accutrend@ (Boehringer Mannheim).

For enzyme activity determination of the biomass triplicate samples of 1 ml were centrifuged and resuspended in a minimum volume of B-Per (Pierce, USA) and vortexed briefly to resuspend the pellet. After incubation at room temperature for 5 min the samples were centrifuged and the supernatant analysed for nucleoside phosphorylases activity using the standard enzyme assays.

Enzyme Assays - The method of Hwang and 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 $U.ml^{-1}$ xanthine oxidase, in UV compatible 96 well microtitre 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, USA). One Unit of PNPase was defined as the enzyme liberating 1 µmol of uric acid from inosine per minute, in the presence of an excess of xanthine oxidase. 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 microtitre plates. After 10 min incubation time at 40°C, the reaction was stopped by addition of 100 µl 0.5 N 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 $cm^2.mol^{-1}$. Nucleosides were purchased from Sigma.

Biocatalysis Reactions

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

Results and Discussion

Assessment of different nucleoside phosphorylases for production of 5-methyluridine

The aim of the research was to develop an enzyme based high yielding synthesis for 5-methyluridine. To this end initial reactions were performed to confirm the relevant enzyme activities. Reactions (3 ml) at a 2.5 mM nucleoside concentration were performed in phosphate buffer, pH 7.4 at 25°C for one to three hours, with agitation (Table I). Thymidine phosphorylase (TP) [Sigma cat no T2807], PNPase [Sigma cat no N8264] and xanthine oxidase (XO) [Sigma cat no X2252] were evaluated, as well as a freshly prepared crude enzyme extract of E. coli containing both PNPase and UPase activity.

Table I: Summary of results obtained for reactions[a]

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

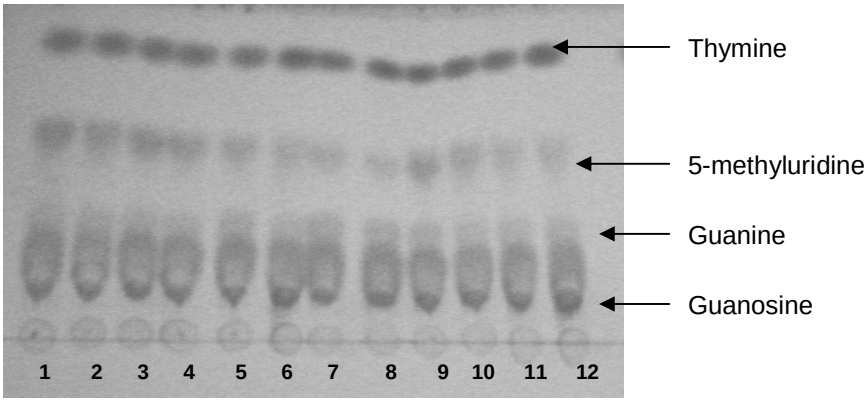
[a]Reactions were carried out at 3 ml scale in sodium phosphate buffer (50 mM, pH 7.4) with equivalent molar concentrations of thymine and guanosine (2.5 mM). The reactions were performed over 3 hours at 25°C using commercially available enzymes: 0.1 U of both PNPase, *ex.* Sigma Aldrich, 10 $U.mg^{-1}$ protein and approximately 60% protein and recombinant thymidine phosphorylase from *E. coli ex.* Sigma Aldrich.

Reactions 1 and 2 were performed as control reactions to confirm the reversibility and direction of equilibrium of the pyrimidine phospholysis, while Reactions 3 and 4 were performed to confirm that forward and reverse purine reactions can occur when using XO and PNPase. Xanthine oxidase was used to convert the co-product hypoxanthine to uric acid to prevent the reverse reaction.

Our aim was to transfer the ribose group from guanosine to thymine (i.e. from a purine to a pyrimidine), yielding 5-methyluridine, but this was unsuccessful using the commercial preparation (Reaction 5). An alternative purine nucleoside, inosine, was evaluated as a ribose donor, but this was also unsuccessful (Reaction 6). Reactions 5 and 6 could not progress due to the requirement of TP for deoxyribose-1-phosphate rather than R-1-P. The enzyme UPase can utilise R-1-P, but was not commercially available. On this basis we decided to isolate UPase from *E. coli* as this is known to have a broader substrate range. Through

the use of *E. coli* cell extract (which contained both PNPase and UPase activities 0.017 and 0.012 $U.mg^{-1}$), it was possible to generate 5-methyluridine (Reactions 7 and 8).

Combinations of partially purified nucleosides were screened 5-methyluridine production. While all combinations tested demonstrated 5-methyl uridine production (Figure 1), reactions containing EcPNP1 (1-4), BHPNP (9-12) and EcUP (1, 5, 9) showed the highest production levels. It was decided therefore to perform larger scale tests using these enzymes.



Enzyme	EcPNP1	EcPNP2	BhPNP1
EcUP	Lane 1	Lane 5	Lane 9
BhUP	Lane 2	Lane 6	Lane 10
BlUP	Lane 3	Lane 7	Lane 11
KpUP	Lane 4	Lane 8	Lane 12

Figure 1**.** Comparative efficiencies of combinations of purine and pyrimidine nucleoside phosphorylases in the production of 5-methyluridine (combinatorial enzyme reactions listed in Table insert)**.**

A series of experiments were 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 methods by shake-flask cultivation. EcPNP (0.85 U/mg), EcUP (0.52 U/mg) and BhPNP1 (1.41 U/mg) at final concentrations of 0.15 U/ml each were tested in 75 ml reactions at 40°C for

25 h. Improved yields to 51% on 52 mM (1.5% m/m) guanosine in the presence of 127 mM (1.5% m/m) thymine were observed for the combination of the *E. coli* enzymes. However, a combination of BhPNP1 and EcUP gave a improved yield of 80% (Figure 2). At these concentrations the substrates for the transglycosylation are well above their solubilities and therefore form slurries. The guanine co-product is also highly insoluble and contributes to the slurry as it is formed.

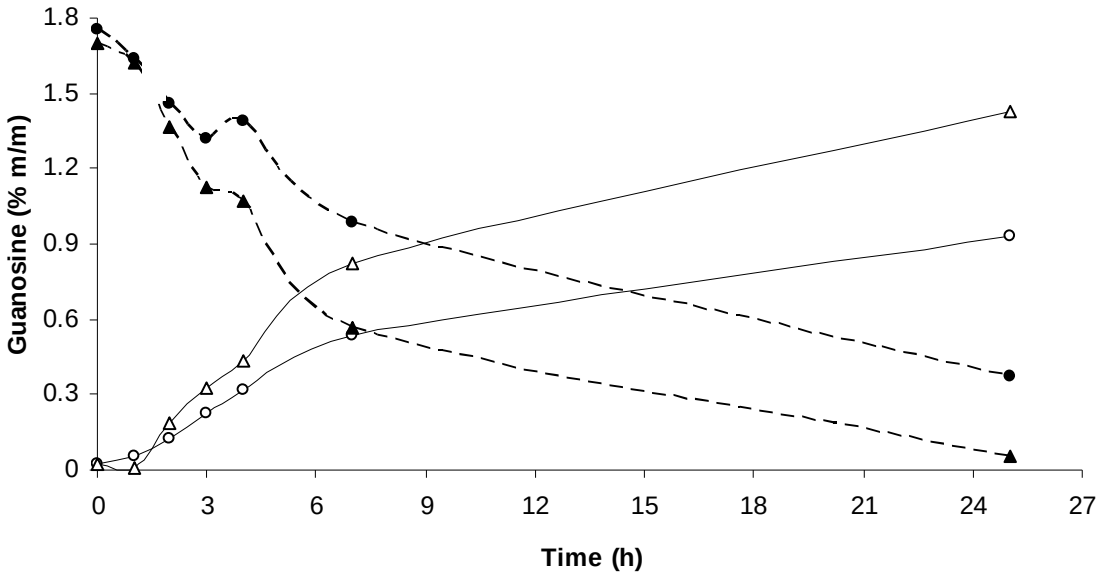


Figure 2. Guanosine conversion (closed) and 5-MU production (open) for *E. coli* PNPase and UPase (●),*B. halodurans* PNPase and *E. coli* UPase (▲).

Influence of the second reaction on the reaction equilibrium: effect of decoupling the first reaction step.

Transglycosylation can in theory occur either as a one pot or 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 a guanosine concentration of 1.5% m/m, sodium phosphate buffer (50 mM) and PNP enzyme (200 U, 5.14 U/mg protein) to investigate the decoupling of the biocatalytic reaction.

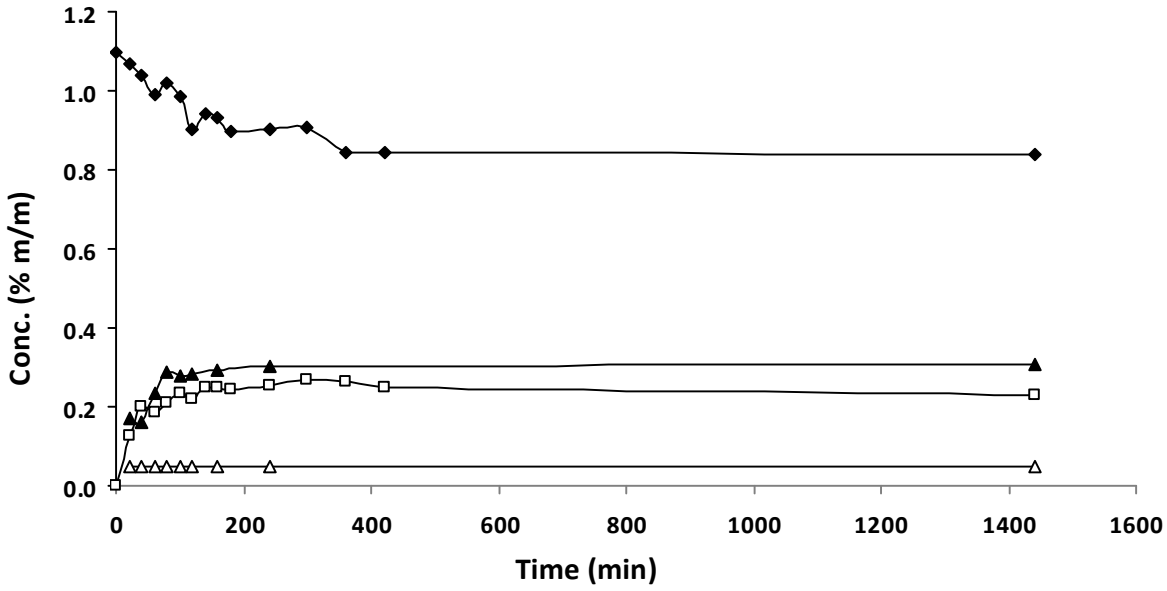


Figure 3. Guanosine hydrolysis by PNPase in a decoupled reaction. (♦) Guanosine, (□)Guanine, and (Δ) free Ribose in the reaction solution. (▲) Total ribose (free ribose and ribose released from R-1-P as released by acid hydrolysis).

The precipitation of guanine due to low solubility, approximately 0.01% at 40°C, was expected to drive the phosphorolysis reaction to completion; however the results (Fig. 3) show that this did not occur, with guanosine conversion of

only 44% and guanine yield of 33% obtained. Thus, the utilization of R-1-P in the coupled reaction system plays a far greater role in driving the phosphorolysis reaction to completion than was anticipated. This view is supported by the fact that small scale results have shown that in the presence of EcUP an equilibrium was reached at 20% conversion of 1.5% m/m 5-MU to thymine. The results also indicated that ribose-1-phosphate was relatively stable under the biocatalytic reaction conditions for the duration of the reaction. Thus the only practical means of conducting the reaction is as a coupled process.

Effect of co-solvents and surfactants

The low solubility of guanosine and thymine, both around 0.1% at 40°C, was considered to be a possible limiting factor in the success of the reaction. Increasing the rate of an enzymatic conversion of substrates in aqueous suspensions may be achieved by increasing the solubility of the substrate, and therefore increasing the available concentration, by the addition of co-solvents. Co-solvents and surfactants have previously been demonstrated to improve reaction yields in other slurry based reactions (Brady et al. 2004; Steenkamp and Brady 2008). A 1.5% m/m of guanosine was completely soluble in 20% v/v DMSO indicating that this could be a suitable reaction medium, while the use of hydrophilic solvents such as methanol and ethanol which showed moderate guanosine wetting properties were of interest due to their ease of removal with boiling points of 64.7^{o}C (methanol) and 78^{o}C (ethanol). 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 bi-phasic liquids (water and toluene) and solids (guanosine, guanine and thymine).

At the 3 ml scale mixtures of co-solvent (20% v/v aqueous) or surfactant (2.5% v/v aqueous) were prepared to which guanosine and thymine were added at the required levels, and the reaction mixture was stirred at room temperature for 1 hour before addition of enzyme, and incubated for 24 h. However, addition

of co-solvent or surfactant did not significantly improve the conversion of guanosine (Table II). The guanine was also completely soluble in the DMSO solution, again indicating that precipitation of this co-product is not the main driving force of the coupled reaction. Although 5-MU yield appears to have increased in some examples (relative to the aqueous control), the absolute value is uncertain due to the higher standard deviation in 5-MU concentration results in the presence of co-solvent. Moreover the impact of using a co-solvent with respect to cost and additional processing required should be evaluated against the possible benefits derived. For example when using DMSO as a co-solvent, high distillation temperatures are required (DMSO boiling point 189^{o}C) and the solvent is generally difficult to remove.

Table II: Effect of co-solvents and surfactants[a]

Co-Solvent	Guanosine conversion (%)	5-MU yield on guanosine[b] (%)	Reaction Mole balance[c] (%)
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

[a] Experimental conditions: Guanosine conc. ~1.5% m/m, thymine conc. ~1.5% m/m, thymine: guanosine mole ratio ~2.3: 1, sodium phosphate buffer (50 mM, pH ~7.5-8.0). Temperature 40^{o}C, BhPNP1 0.27U, EcUP 0.27U (PNPase: UPase: 1:1) from preparations of BhPNP1 5.14 U.mg^{-1} protein and EcUP 0.2 U.mg^{-1} protein.

[b] 5-MU yield on guanosine at 24 h reaction (single point evaluation).

[c] Mole balance for reaction, including guanosine, guanine, thymine and 5-MU.

Enzyme Production

In order to prepare sufficient enzyme for larger scale reactions, optimised fermentations (10 l) were performed for the production of BhPNP1 and EcUP (Figure 3). High levels of enzyme were produced within 8 to 10 h of fermentation. The fermentation results are summarised in Table III. Data presented are averages of duplicate fermentations in each case (those with less than 10% standard deviation). The expression of EcUP was 37.7 kU.l^{-1}, which was 10 fold that of the wild type. The lyophilised BhPNP1 activity (5.41 U.mg^{-1}) was similar to 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 $U.mg^{-1}$).

Table III. Summary of fermentation data

Value	BhPNP1	EcUP
Maximum OD (660nm)	14.4	20.59
μ_{max}	0.43	0.60
Yield ($g_{dcw}.g^{-1}_{glucose}$)	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 lyophilisation

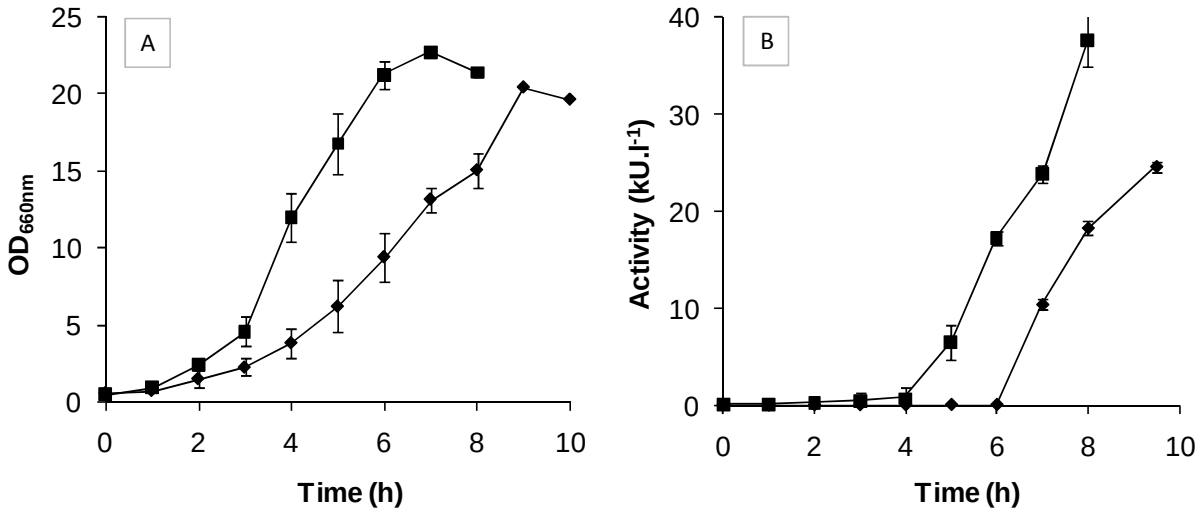


Figure 3. Growth (A) and Activity (B) profiles of *E. coli* [pMSPNP] (◆) and *E. coli* [pETUP] (■) in duplicate batch fermentations.

Benchscale biocatalytic reaction

To demonstrate the overall reaction at bench scale an experiment was conducted at 650 ml using an enzyme load of 105 U BhPNP1 and 75 EcUP and a thymine to guanosine mole ratio of 2.3 : 1 (1.5% m/m guanosine and 1.6% m/m thymine; 53 mM to 127 mM) performed at 40°C over 23 h, with Trizma Base (50 mM) and sodium phosphate buffer (50 mM) at pH 7.8, to ensure both adequate buffering while providing phosphate for the reaction. The results obtained showed a guanosine conversion of 94.7% and a 5-methyluridine yield of 79.1% (Figure 4). An increase in the rate of reaction was observed as the reaction was complete within 7 hours, at a 5-MU productivity of 1.37 $g.l^{-1}.h^{-1}$. The yield of this non-optimised reaction was comparable to those reported by Ishii et al. (1989) (74% 5-MU) using whole cells of an *Erwinia* wild type organism. 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).

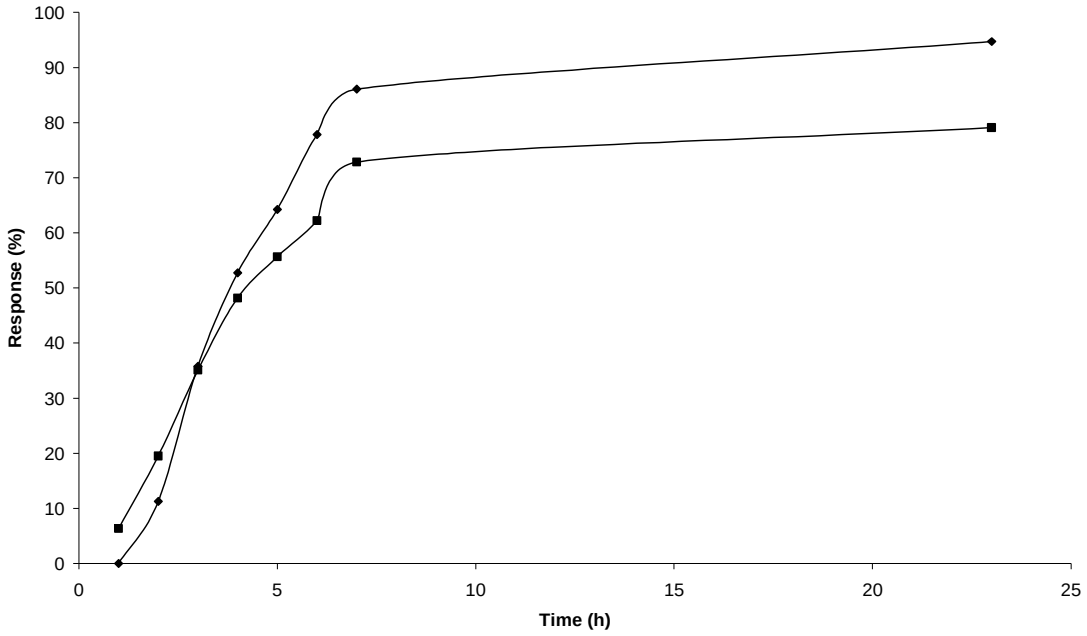


Figure 4. Thymine to guanosine mole ratio (2.3:1) on guanosine conversion (◆) and 5-methyl uridine yield (■)

Conclusions

The biocatalytic reaction described here indicates that a novel combination of nucleoside phosphorylases can facilitate the production of pyrimidine nucleosides from purine nucleosides in high yields. Partially purified enzyme preparations could be applied directly to a two step transglycosylation reaction for the production of 5-methyluridine in a one-pot synthesis step with a yield of 79.1%. Reaction engineering is anticipated to improve yield and productivity further.

Production of *B. halodurans* purine nucleoside phosphorylase and *E. coli* uridine phosphorylase, which were used in the synthesis of 5-methyluridine, was demonstrated in batch fermentations using the production strains *E. coli* JM109

[pMSPNP] and *E. coli* BL21 [pETPyNP] resulting in enzyme yields of 26.9 and 37.7 $kU.l^{-1}$ for PNPase and UPase respectively.

Acknowledgements

We would like to thank R. Lalloo, N. Gumede, S. Ramchuran (LIFElab) V. Moodley, K. Pillay, D. Mabena, S. Machika and H. Manchidi (CSIR Biosciences) for technical assistance. We gratefully acknowledge Dr. D. R. Walwyn (CEO of ARVIR Technologies) and Dr Henry Roman (CSIR) for management of the project. Financial support for this work was provided by CSIR Biosciences, LIFElab, DST and Arvir Technologies. We thank Prof. Derek Litthauer (University of the Free State) for loan of the *Klebsiella pneumoniae* and *Bacillus licheniformis* cultures. This paper is dedicated to the analytical chemist and colleague Mr Simon Machika, who passed away during this research.

References

1. 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 Microbial Technol 34: 283-291.
2. Bzowska A, Kulikowska E, Shugar D, (2000). Pharmacology and Therapeutics, 88, 369.
3. 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 Journal 272: 1886-1899.
4. 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 Journal 274: 2482-2495.
5. Chen B-C, Quinlan SL, Gregory Reid J (1995). A new synthesis of the anti-AIDS drug AZT from 5-methyluridine. Tetrahedron Letters 36: 7961-7964.

6. Dandanell,G, Szczepanowski,R.H., Kierdaszuk,B. Shugar,D. Bochtler,M (2005), Escherichia coli purine nucleoside phosphorylase II, the product of the xapA gene, J.Mol.Biol. 348(1):, 113-125
7. Erion MD, Takabayashi K, Smith HB, Kessi J, Wagner S, Hönger S, Shames SL, and Ealick SE (1997) Purine Nucleoside Phosphorylase. 1. Structure−Function Studies. Biochem 36:, 11725-11734
8. Freskos JN, Senaratne K, Pushpananda A (1990). Synthesis of beta-thymidine US Pat 4914233.
9. 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.
10. 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.
11. Hanrahan JR, Hutchinson DW (1992). The Enzymatic-Synthesis of Antiviral Agents, *J. Biotech*. 23: 193-210.
12. Hori N, Watanabe M, Yamazaki Y, Mikami Y (1989a) Synthesis of 5-methyluridine by a thermophile, *Bacillus stearothermophilus* JTS 859. Agr Biol Chem 53:197-202.
13. Hori N, Watanabe M, Yamazaki Y, Mikami Y (1989b), Purification and characterization of thermostable purine nucleoside phosphorylase from *Bacillus stearothermophilus* JTS 859, Agri. Bio. Chem, 53(8): 2205-2210.
14. 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. 121-131.
15. Hori N, Watanabe M and 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. Biocat. Biotrans. 4: 297 – 304.

16. 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(2):379-87.
17. 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.
18. 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 Expression and Purification 22: 180-188.
19. Lewkowicz, ES and Iribarren AM (2006). Nucleoside phosphorylases. Current Organic Chemistry 10: 1197-1215.
20. Louw, M.E., Reid, S.J., Watson, T.G. (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.
21. Lovett PS, Keggins KM (1979) Bacillus subtilis as a host for molecular cloning. Method Enzymol 68:342–357
22. Mikailopulo I.A, (2007), Biotechnology of Nucleic Acid Constituents – State of the Art and Perspectives, Curr. Org. Chem. 11, 317-335
23. Prasad A. K., Trikha S., Parmar V.S., (1999), Nuceloside Synthesis Mediated by Glycosyl Transferring Enzymes, *Biorg. Chem.* 27, 135-154.
24. Ramchuran SO, Nordberg Karlsson E, Velut S, de Maré L, Hagander P and Holst O (2002) Production of heterologous thermostable glycoside hydrolases and the presence of host-cell proteases in substrate limited fed-batch cultures of *Escherichia coli* BL21(DE3). Appl. Microbiol. Biotechnol. 60:408-416.
25. Sambrook J and Russell DW (2001), Molecular Cloning: A laboratory manual, Cold Spring Harbor Press, Cold Spring Harbor, New York
26. 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 and Nucleotides 15: 47-58.

27. 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(4-7):, 977-979
28. Steenkamp L and Brady D (2008). Optimisation of stabilised Carboxylesterase NP for enantioselective hydrolysis of naproxen methyl ester. Process Biochem 43: 1419-1426
29. Stepanenko BN, Kaz'mina EM, Dubinkina ZS (1973) Methods of Synthesis of Pyrimidine Nucleosides Russian Chemical Reviews, 42: 494-508.
30. Tonon G, Capra E, Orsini G, Zuffi G (2004). Novel Immobilized biocatalysts usable for the production of natural nucleosides and modified analogues by enzymatic transglycosylation reactions. US 2004/0142438.
31. Utagawa T, Morisasa H, Yamanaka S, Yamazaki A, and Yoshinaga F, (1986) Agric Biol Chem 50: 121-126.
32. Utagawa T (1999). Enzymatic preparation of nucleoside antibiotics. J Molecular Catal B Enzymatic 6: 215-222.
33. Visser DF, Hennessy F, Rashamuse K, Louw M and Brady D (2010). Characterisation of the purine nucleoside phosphorylase, BH1531, from *Bacillus halodurans* expressed in *Escherichia coli*. Extremophiles (2010) in press.