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# Nitroreductase from *Bacillus licheniformis*: A stable enzyme for prodrug activation

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ABSTRACT

5-Aziridinyl-2,4-dinitrobenzamide (CB1954) has potential applications in enzyme/prodrug targeted anti-cancer therapies since it can be activated by nitroreductases to form a cytotoxic, bifunctional hydroxylamine derivative. A nitroreductase that can activate CB1954 has been previously isolated from *Escherichia coli*, but its usefulness is limited by its poor stability and low catalytic efficiency for CB1954. We now report the identification and characterization of a nitroreductase enzyme from the thermophilic bacterium *Bacillus licheniformis*. Although there is only 28% amino acid sequence identity between this enzyme and the previously isolated *E. coli* nitroreductase, the two enzymes have a number of characteristics in common. Both enzymes have been shown to reduce both CB1954 and menadione in the presence of NADH and NADPH. However, whereas *E. coli* nitroreductase produces equimolar amounts of the 2- and 4- hydroxylamine derivative of CB1954, the *B. licheniformis* enzyme produces only the desired 4-hydroxylamine derivative. It has a preference for NADPH as cosubstrate, and is also active with a range of CB1954 derivatives as substrate and reduced pyridinium cofactor analogues. Moreover, the enzyme is much more thermostable than the *E. coli* nitroreductase and shows maximum activity at 30 °C. These characteristics suggest that the *B. licheniformis* nitroreductase may be a possible candidate enzyme for enzyme/prodrug therapies due to its bacterial origin, the high activity observed with CB1954 and its enhanced stability.



## 1. Introduction

FMN-dependent nitroreductases (NTRs) are a group of structurally homologous enzymes that includes *Escherichia coli* NTR, *Enterobacteria cloacae* NTR, *Salmonella typhimurium* NTR and *Vibrio fischeri* flavin oxidoreductase 1 [1–4]. The enzymes catalyse the reduction of nitro groups in a wide range of substrates to form the corresponding hydroxylamine product [5,6]. They also catalyse the reduction of quinones, such as menadione, and are inhibited by dicoumarol. The enzyme-mediated reduction of nitro groups to the corresponding hydroxylamines, which can be further metabolised to form cytotoxic DNA cross-linking agents, is the basis for the pharmaceutical application of nitroreductases as prodrug activators. An example of this is the activation of the aziridinyl prodrug CB1954 (5-aziridinyl-2,4-dinitrobenzamide) by *E. coli* NTR, forming a hydroxylamine derivative that undergoes further reaction with a thioester, such as acetyl CoA, to form a final DNA-reactive species [1,7,8]. As a result of this, CB1954 has potential applications in cancer prodrug therapy including antibody-directed enzyme prodrug therapy (ADEPT) [7,8], gene-directed enzyme prodrug therapy (GDEPT) [9] and Clostridia-directed enzyme prodrug therapy (CDEPT) [10].

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The use of any prodrug activating enzyme is, however, limited by factors such as its stability and the level of activity achievable when, for example, conjugated to an antibody. In this respect, enzymes from thermophilic organisms have considerable potential as they are stable and remain fully active under conditions that would denature most enzymes of mesophilic origin. Not only can these enzymes withstand higher temperatures, they also show increased stability towards other factors [11,12]. However, such enzymes would need to have sufficient catalytic activity at 37 °C if they are to be used in prodrug therapy.

We now report the identification and characterization of a nitroreductase from the Gram-positive thermophilic bacterium *Bacillus licheniformis*, which has an optimum growth temperature around 50 °C. Properties of this enzyme, including its ability to activate CB1954 and its analogues, are presented and compared with the nitroreductase previously isolated from *E. coli*.

## 2. Materials and methods

### 2.1. Materials

All chemicals and reagents were supplied by Sigma Aldrich (Poole, UK) unless otherwise stated. CB1954 was supplied by Morvus Technology Ltd. (Llanarthne, UK). The *B. licheniformis* was obtained from the CAMR culture collection, currently held in the Centre for Extremophile Research at the University of Bath, UK.

### 2.2. Cloning and expression of a nitroreductase enzyme from B. licheniformis

A potential nitroreductase gene was identified from the genome sequence of *B. licheniformis* [13] by a BLAST search [14] with the sequence of *E. coli* nitroreductase (nfs1). PCR primers incorporating *Nde*1 and *Bam*H1 restriction sites (underlined) were designed for amplification of the identified gene, and were supplied by MWG Biotech (London, UK).

Forward primer: 5′-CGCCATATGATGACAGAGCAATCCAA-GAAGC
Reverse primer: 5′-GCGGGATCCTTATTCGACCCATTTCAC-GATT

*B. licheniformis* was grown in TS broth (3 ml) overnight at 60 °C and genomic DNA was obtained using a GenElute™ Bacterial Genomic DNA kit (Sigma Aldrich, Poole, UK). PCR was carried out using *B. licheniformis* genomic DNA as a template. The PCR reactions consisted of 5× Green GoTaq Flexi™ Buffer (1×) (Promega, Southampton, UK), $MgCl_2$ (2 mM), PCR nucleotide mix (0.2 mM each dNTP), forward primer (0.1 μM), reverse primer (0.1 μM), GoTaq™ DNA Polymerase (1.25 U, Promega), genomic DNA (0.2 μg) and nuclease-free water to a final volume of 50 μl. The PCR reaction, carried out in an Eppendorf Mastercycler™, involved an initial denaturation step of 2 min (95 °C), 30 cycles of denaturation (95 °C) for 1 min, annealing (53 °C) for 1 min, and extension (72 °C) for 1 min, followed by a final denaturation (72 °C) for 5 min. The amplified DNA was cloned into pGEM-T Easy™ vector (Promega) and transformed into JM109 competent cells (Promega). Plasmid DNA was purified from transformants using a QIAprep Spin Miniprep™ kit (Qiagen, Crawley, UK). A sample of the purified plasmid DNA was sequenced (Geneservice, Cambridge, UK) to confirm the identity of the amplification product.

The nitroreductase coding sequence was excised from the pGEM-T construct using the *Bam*H1 and *Nde*1 restriction sites and cloned into the expression vector pET28a (Novagen, Nottingham, UK). The pET28a construct was then transformed into BL21 (DE3) competent cells (Novagen) and expression of the His-tagged recombinant protein was carried out using Overnight Express™ Autoinduction Instant TB Medium (Novagen). A single colony was used to inoculate 100 ml of medium, which was incubated at 30 °C and 180 rpm until stationary phase was reached. The resulting cell pellet, obtained via centrifugation at 8000 × *g* for 20 min, was resuspended in 5 ml phosphate buffered saline (PBS) and sonicated on ice using a Sanyo Soniprep 150 ultrasonic disintegrator. Sonication was performed at 15 μm for five 30 s pulses with 1 min intervals between each pulse. Cellular debris was removed via centrifugation at 20000 × *g* for 30 min at 4 °C.

Expressed protein was purified from the supernatant using a HiTrap™ Chelating HP column (GE Healthcare, Amersham, UK). The column was loaded with 0.1 M $NiSO_4$ and equilibrated with 20 mM sodium phosphate buffer (pH 7.4), 0.5 M NaCl. The supernatant was applied and the bound protein eluted with a gradient of 0–500 mM imidazole over five column volumes. Fractions (1 ml) were collected and analysed for nitroreductase activity. The active fractions were pooled and buffer exchanged into PBS using a PD-10 desalting column (GE Healthcare).

### 2.3. Enzyme assays

Nitroreductase activity was determined by incubation with CB1954 (100 μM) and NADH or NADPH (500 μM) in 10 mM sodium phosphate buffer (pH 7) at 37 °C. Samples were removed every 6 min, and aliquots (10 μl) were injected onto a Partisil ™10 SCX HPLC column (4.2 × 150 mm, Whatman, Maidstone, UK) and eluted isocratically with 0.13 M sodium phosphate (pH 5) at 1.5 ml $min^{-1}$. The column eluate was monitored by absorption at 325 nm, and nitroreductase activity was calculated from the decrease in CB1954 concentration, determined from the area of the appropriate elution peak.

Quinone reductase activity was determined by a spectrophotometric method using menadione as substrate and cytochrome *c* as terminal electron acceptor. Stock solutions of menadione and dicoumarol were made up in DMSO. All other solutions were made up in PBS. The final reaction mixture contained menadione (10 μM), cytochrome C (70 μM) and NAD(P)H (500 μM) in PBS. The reaction was started by the addition of nitroreductase, and the activity of the enzyme was determined at 37 °C from the linear increase in absorbance at 550 nm.

Protein concentrations were determined using the conventional protein assay (Bio-Rad) calibrated against bovine serum albumin.

### 2.4. Molecular size analysis and gel filtration

The $M_r$ of the purified nitroreductase was determined by mass spectrometry, carried out at the BMS Mass Spectrometry and Proteomics Facility, University of St. Andrews, UK.

Gel filtration was carried out using a Superdex™ 100 10/300 GL column (GE Healthcare). The column was eluted at 0.5 ml $min^{-1}$ using 0.2 M phosphate buffer (pH 7), 0.15 M NaCl, and was calibrated using a low molecular weight gel filtration kit (GE Healthcare).

### 2.5. Flavin cofactor analysis

The purified enzyme was heated to 100 °C for 5 min and 8 M urea was added. The released flavin was separated from the enzyme by ultrafiltration using a Vectraspin™ Micro centrifugal filter (Whatman) and identified via HPLC using a method based on Knox et al., [16]. The flavin: protein ratio was determined from the absorption of the purified enzyme at 445 nm and 280 nm, using absorption coefficients of 12,500 $M^{-1}$ $cm^{-1}$ for the flavin (FMN) and 29450 $M^{-1}$ $cm^{-1}$ for the protein.

## 3. Results

### 3.1. Properties of the B. licheniformis nitroreductase

A BLAST search of the genome sequence of *B. licheniformis* (ATCC14580) using the amino acid sequence of *E. coli* nitroreductase nfs1 identified the *B. licheniformis* protein Yfk0 (*nfsB* gene product) as the closest match, with 28% amino acid sequence identity. The *B. licheniformis* gene was successfully amplified and, after confirmation of the correct nucleotide sequence, cloned into the vector pET28a. A recombinant protein was expressed using *E. coli* BL21(DE3) cells and, although approximately 95% of the expressed product was insoluble, purification of nitroreductase from the soluble fraction was possible, producing 10 mg of pure enzyme per 100 ml culture.

The subunit $M_r$ of the enzyme was determined as 28,350 via mass spectrometry, in agreement with the value of 28,341 predicted from the gene sequence with the addition of the vector-derived His-tag. Gel filtration of the purified enzyme yielded a single active peak of $M_r$ = 55,000, indicating that the enzyme is active as a dimer.

The active enzyme has a yellow colouration, which is indicative of the presence of a flavin coenzyme. The flavin, which was shown to be FMN by HPLC and spectral analysis, was present in the purified enzyme in a ratio of approximately 0.4 mol of FMN per mole of nitroreductase. This ratio was increased to 1 by incubation of the purified nitroreductase with excess FMN (1 mM) followed by removal of unbound FMN by gel filtration.

### 3.2. Kinetic parameters

Using the HPLC assay, the purified enzyme was shown to be active with CB1954 using either NADH or NADPH as cofactor. The sole product of the reduction of CB1954 was shown to be 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide by HPLC analysis of the reaction products. In the spectrophotometric assay, menadione was also shown to be a substrate for the enzyme, again using either NADH or NADPH as cofactor.

With both substrates the *B. licheniformis* nitroreductase followed Michaelis–Menten kinetics, and the determined kinetic parameters in comparison with those for the *E. coli* nitroreductase are presented in Table 1. The specific activity of *B. licheniformis* nitroreductase under standard assay conditions with CB1954 was 2.5 μmol $min^{-1}$ $mg^{-1}$, and 72 μmol $min^{-1}$ $mg^{-1}$ with menadione. These values were increased by 3-fold when the FMN:protein ratio was increased to 1 by the addition of excess FMN to the enzyme.

For two substrate (A and B) enzyme-catalysed reactions, patterns of primary plots of [A]/velocity versus [A] at fixed [B], and *vice versa*, allow the enzyme mechanism to be determined. In the case of a ternary complex mechanism, primary plots of [A]/velocity versus [A] at fixed [B] result in a series of lines that intersect to the left of the *y*-axis, whereas for a ping-pong mechanism the lines intersect on the *y*-axis [15]. As shown in Fig. 1, in the case of *B. licheniformis* NTR the pattern of intersections corresponds to that of a ternary complex mechanism. The compound dicoumarol was found to be an inhibitor of the enzyme. With respect to menadione, dicoumarol was shown to be an uncompetitive inhibitor with a $K_i'$ of 8 μM, but with respect to NADH, it showed mixed inhibition with a $K_i$ of 10 μM and $K_i'$ of 20 μM.

**Table 1 – Kinetic parameters for nitroreductase from *B. licheniformis* and *E. coli*.**

| Substrate/cofactor | CB1954 | Menadione | NADH | NADPH |
|---|---|---|---|---|
| *B. licheniformis* | | | | |
| $K_M$ (μM) | 30 (±3.4) | 5.0 (±0.7) | 820 (±40) | 160 (±10) |
| $k_{cat}$ ($min^{-1}$) | 6.4 (±0.52) × $10^4$ | 7.4 (±0.40) × $10^3$ | 4.3 (±0.20) × $10^3$ | 7.1 (±0.20) × $10^3$ |
| $k_{cat}/K_M$ ($\mu M^{-1}$ $min^{-1}$) | 2.1 (±0.29) × $10^3$ | 1.5 (±0.23) × $10^3$ | 5.2 (±0.35) | 44 (±3.0) |
| *E. coli* | | | | |
| $K_M$ (μM) | 860 | 80 | 6 | 6 |
| $k_{cat}$ ($min^{-1}$) | 3.6 × $10^2$ | 4.2 × $10^4$ | - | - |
| $k_{cat}/K_M$ ($\mu M^{-1}$ $min^{-1}$) | 0.42 | 530 | - | - |

Data for the *B. licheniformis* enzyme were analysed via the direct linear plot [26], using Enzpack software (Biosoft, Cambridge). Values for the *E. coli* enzyme were taken from [1]. Values for menadione, NADH and NADPH were obtained using the standard menadione assay, where the concentration of one substrate was varied and the other held at a fixed concentration of 10 × $K_M$. Values for CB1954 were obtained using the standard nitroreductase assay, where CB1954 concentrations were varied and NADPH was held at a fixed concentration of 10 × $K_M$.

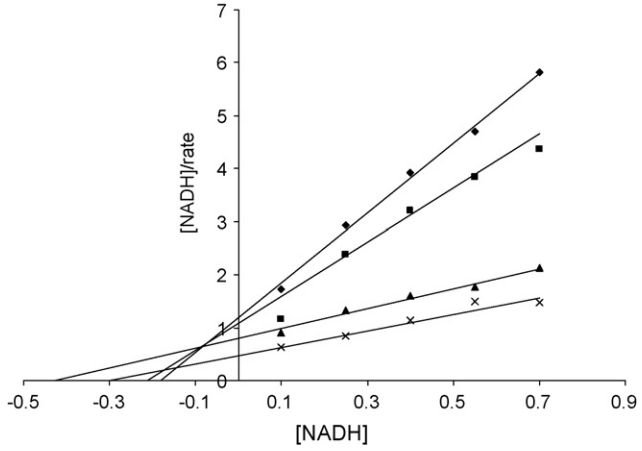



**Fig. 1 – Primary plot of [NADH]/rate against [NADH] at different menadione concentrations. Enzyme activity was determined at 37 °C using varying concentrations of NADH at fixed concentrations of menadione: 0.5 mM (◆), 0.75 mM (■), 1.0 mM (▲), 3.0 mM (×).**

### 3.3. *Activity with alternative substrates and cosubstrates*

As shown in Tables 2A and 2B, the recombinant *B. licheniformis* NTR is active with a range of alternative dinitrobenzamide-related substrates and nicotinamide cosubstrate analogues.

### 3.4. *Temperature optimum and thermal stability*

When the temperature dependence of nitroreductase activity was determined using an extract of *B. licheniformis* cells, maximum activity was observed at 55 °C (Fig. 2A). However, the optimum temperature for activity of the purified His-tagged recombinant nitroreductase was shown to be 30 °C. Non-tagged nitroreductase, produced using the expression vector pET3a, showed a similar temperature-activity profile, indicating that the presence of a His-tag had no effect on the temperature optimum of the recombinant *B. licheniformis* enzyme. The temperature optimum of *E. coli* nitroreductase was shown to be 40 °C.

The stability of the purified recombinant *B. licheniformis* nitroreductase to irreversible thermal inactivation was also investigated, as shown in Fig. 2B. The half-life of the enzyme at 50 °C was 400 min, whereas the half-life of the *E. coli* enzyme was 6 min at this temperature.

## 4. Discussion

We report the gene cloning, expression, purification and characterisation of a nitroreductase enzyme from the thermophilic bacterium *B. licheniformis*. Although several nitroreductases have been identified in the annotation of the *B. licheniformis* genome, the YfkO protein shows the closest identity with *E. coli* NTR (nfs1). An amino acid sequence alignment (Fig. 3) shows that the majority of flavin cofactor-binding residues identified in the crystal structures of *E. coli* NTR and its complexes [17] are conserved in both the *B. licheniformis* nitroreductase and in other related nitroreductases. Residues that interact with the substrate (CB1954 or menadione) are less well conserved in the *B. licheniformis* enzyme.

The purified *B. licheniformis* NTR is an FMN-containing flavoprotein of subunit $M_r$ = 28,350 and is active as a dimer. The FMN can be removed by heating and treatment with urea, showing that the flavin is tightly associated with, but not covalently bound to, the protein. The ratio of FMN:protein in the purified recombinant enzyme was determined to be 0.4, but this value could be increased to a molar ratio of 1 by the addition of excess FMN to the enzyme.

Although the enzyme shows only 28% amino acid sequence identity to the previously reported *E. coli* nitroreductase, both enzymes are capable of reducing CB1954, using either NADH or NADPH as cofactor. Both enzymes are also capable of reducing menadione. Reduction of CB1954 by *E. coli* nitroreductase results in the generation of equimolar amounts of 5-(aziridin-1-yl)-2-hydroxylamino-4-nitrobenzamide and 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide [5,12], whereas reduction of CB1954 with *B. licheniformis* nitroreductase yields only the biologically-active 4-hydroxylamine product.

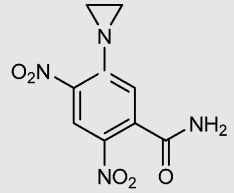
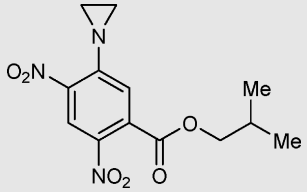
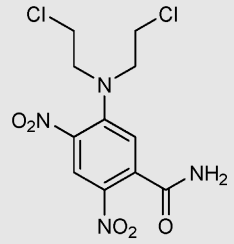
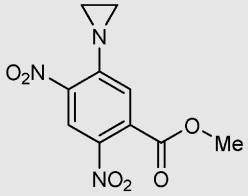
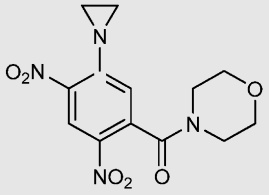
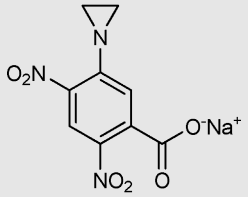
Kinetic parameters were obtained for *B. licheniformis* nitroreductase and were compared to those of the *E. coli* enzyme. The *B. licheniformis* enzyme showed a higher $k_{cat}$ with CB1954 as substrate but a lower $k_{cat}$ with menadione as substrate when compared with the *E. coli* enzyme, whereas the $K_M$ values for both substrates were lower with the *B. licheniformis* enzyme. In contrast, the $K_M$ values obtained with respect to NADH and NADPH were significantly higher for the *B. licheniformis* enzyme than the values for the *E. coli* enzyme. The $K_M$ value for NADH (0.82 mM) was approximately five times greater than the value for NADPH (0.16 mM), with both cofactors giving the same specific activity value (2.5 $\mu mol^{-1}\ min^{-1}\ mg^{-1}$) with menadione as substrate. Thus, comparison of the $k_{cat}/K_M$ values indicates that NADPH is the preferred cofactor for the *B. licheniformis* enzyme. This is a desirable feature as NADPH is the dominant form of the cosubstrate in human cells [18].

With respect to menadione and NADH, the kinetics conform to those of a ternary complex mechanism. In the case of the *E. coli* enzyme, however, the reaction proceeds via a ping-pong Bi–Bi reaction mechanism, with electron transfer from NAD(P)H as the first step [17]. Amino acid sequence alignment (Fig. 3) shows that residues that interact with the substrate are not highly conserved and this may explain the difference in mechanisms observed.

Dicoumarol, an anticoagulant that is structurally similar to a dimer of the substrate menadione, has been shown to be an uncompetitive inhibitor of reductases including NAD(P)H quinine reductase NQO1 and *E. coli* nitroreductase with respect to menadione [5]. Similarly, dicoumarol was shown to be an uncompetitive inhibitor of *B. licheniformis* nitroreductase with menadione as substrate.

*B. licheniformis* has an optimum growth temperature of 50 °C and maximum nitroreductase activity of a *B. licheniformis* extract was achieved at 55 °C. The *E. coli* enzyme showed maximum activity at 40 °C. Unexpectedly, the optimum temperature for catalytic activity of the recombinant *B.*

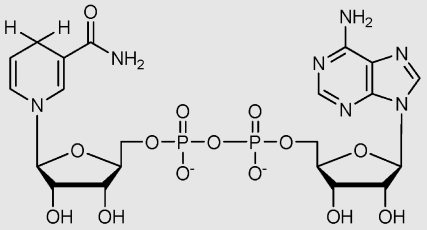
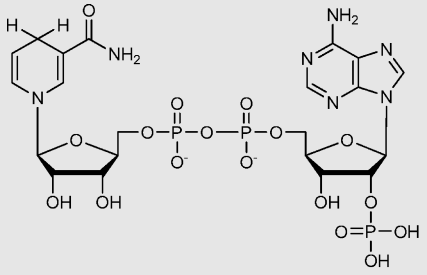
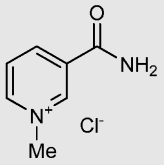
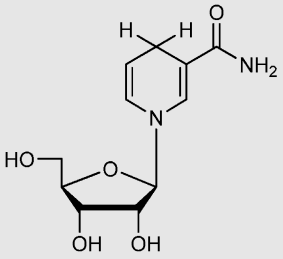
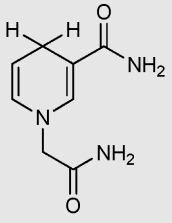
**Table 2A – Specific activity of *B. licheniformis* nitroreductase with alternative substrates.**

| Substrate | Structure | Specific activity ($\mu$mol min$^{-1}$ mg$^{-1}$) |
|---|---|---|
| CB1954 5-(aziridin-1-yl)-2,4-dinitrobenzamide |  | 2.4 |
| CB10-200 isobutyl 5-(aziridin-1-yl)-2,4-dinitrobenzoate |  | 26.7 |
| EP202 (SN23872) 5-(bis(2-chloroethyl)amino)-2,4-dinitrobenzamide |  | 12.0 |
| SN26723 methyl 5-(aziridin-1-yl)-2,4-dinitrobenzoate |  | 7.1 |
| SN23897 (5-(aziridin-1-yl)-2,4-dinitrophenyl)(morpholino)methanone |  | 1.7 |
| AP143 (SN26399) sodium 5-(aziridin-1-yl)-2,4-dinitrobenzoate |  | 0 |

Activity was investigated using standard nitroreductase assays with NADH as cofactor and substituting 100 $\mu$M CB10-200, AP143, SN26723, SN23897 and EP202 for CB1954.

*licheniformis* nitroreductase was found to be 30 °C, well below that of the native enzyme. The presence or absence of a His-tag on the recombinant enzyme had no effect on the temperature optimum. It is possible that the recombinant enzyme does not fold into an optimal conformation when expressed in *E. coli* or that there were other enzymes present in the unpurified cell lysate of *B. licheniformis* that have the ability to reduce menadione, and these could account for the higher

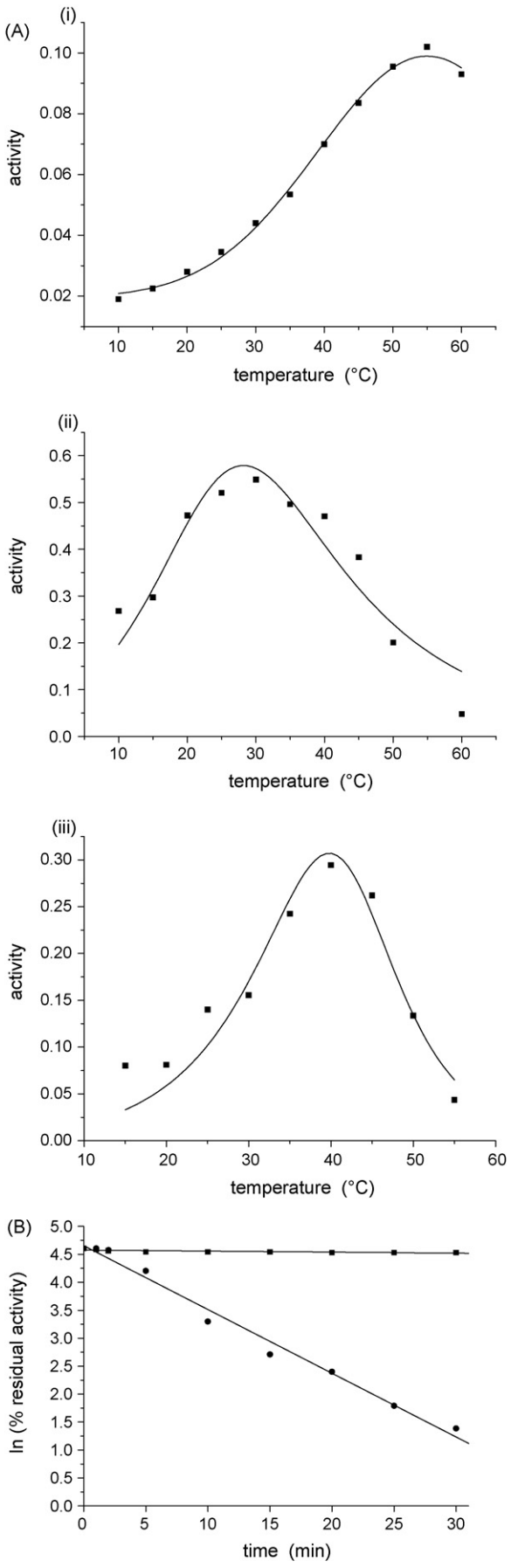
**Table 2B – Specific activity of *B. licheniformis* nitroreductase with alternative cosubstrates.**

| Cofactor | Structure | Specific activity ($\mu$mol min$^{-1}$ mg$^{-1}$) |
|---|---|---|
| NADH |  | 2.4 |
| NADPH |  | 2.4 |
| 1-methylnicotinamide chloride |  | 0.73 |
| NRH |  | 0.21 |
| EP0152R 1-(2-amino-2-oxoethyl)-1,4-dihydropyridine-3-carboximide |  | 0.16 |

Activity was determined using the standard nitroreductase assay with CB1954 as substrate and substituting 500 $\mu$M reduced nicotinamide riboside, 1-methylnicotinamide and EP0152R for NADH.

activity observed at temperatures above 30 °C. The presence of other proteins in the unfractionated *B. licheniformis* cell lysate, such as chaperones, may also have an effect on the temperature/activity profile of the native nitroreductase.

Irreversible thermal inactivation of the recombinant *B. licheniformis* nitroreductase was investigated and, although optimum activity was achieved at 30 °C, the enzyme was shown to be stable for more than 6 h at temperatures up to 50 °C. Thus, the observed temperature optimum is not a result of irreversible thermal inactivation of the enzyme, a phenomenon that has been observed previously with other enzymes [19]. This shows that the *B. licheniformis* NTR is active and would be stable over an extended period at 37 °C, which is a requirement for the use of the enzyme in targeted anti-cancer




therapies. The thermal stability of the *Bacillus* enzyme may also make it a desirable enzyme for use in biocatalytic processes, although operating temperatures may be higher than the optimum for this enzyme.

The *B. licheniformis* NTR was shown to be active with a number of alternative cofactors (Table 2B). For use in targeted anticancer therapies, a cofactor would be required to be present in order for the nitroreductase enzyme to catalyse the reduction of the prodrug. The cofactors NADH and NADPH are not suitable in this case, as they become recognised and rapidly oxidised by serum proteins on the cell surface [20]. The *B. licheniformis* NTR showed activity with alternative, simple synthetic reduced pyridinium compounds, and the activity observed with 1-methylnicotinamide shows that there is little requirement for the adenine nucleotide and phosphate portions of NAD(P)H by the enzyme. However, activity is significantly reduced in their absence. Reduced nicotinic acid riboside (NRH) is stable to metabolism by serum proteins and can be produced *in vivo* by the administration of nicotinic acid 5′-*O*-benzoyl riboside (reduced); therefore, the requirement for a cofactor would not be a limiting factor in the use of this enzyme in targeted anticancer therapies. The *B. licheniformis* NTR was also active with a number of CB1954 derivatives (Table 2A), which is desirable as alternative prodrugs are being investigated that can be activated more efficiently by nitroreductases [21,22,23].

In conclusion, we have purified and characterised a nitroreductase enzyme from *B. licheniformis*. Although there is only 28% amino acid sequence identity between this enzyme and the previously isolated *E. coli* nitroreductase, the two enzymes have a number of things in common. Both enzymes have been shown to reduce both CB1954 and menadione in the presence of NADH and NADPH. However, *E. coli* nitroreductase produces equimolar amounts of the 2- and 4- hydroxylamine derivative, whereas *B. licheniformis* nitroreductase produces only the desired 4-hydroxylamine derivative. The *B. licheniformis* nitroreductase also has a preference for NADPH as cosubstrate. These characteristics suggest that *B. licheniformis* nitroreductase may have a role in cancer therapy, as CB1954 in combination with a nitroreductase can be used in enzyme directed prodrug therapy, for example in virus directed enzyme prodrug therapy (VDEPT) and in ADEPT [8,24]. It is a requirement of ADEPT that the enzyme involved has no equivalent activity in humans, is active under physiological conditions and remains active when conjugated to an antibody [25]. The *B. licheniformis* nitroreductase may, therefore, be a possible candidate enzyme due to its bacterial origin, the high activity observed with CB1954 and its enhanced thermal stability.

**Fig. 2 – (A) The activity of *B. licheniformis* NTR was determined at a series of temperatures using the menadione assay. (i) *B. licheniformis* cell extract; (ii) cell extract of recombinant *E. coli* expressing *B. licheniformis* NTR and (iii) pure *E. coli* NTR. (B) The thermal stability of pure *E. coli* (●) and recombinant *B. licheniformis* NTR (■) was determined at 50 °C. The enzyme was incubated at 50 °C and the remaining catalytic activity was measured after various time intervals using the standard menadione assay at 37 °C.**

```
                                          ###                                ##+
E.cloacae_NTR_[nfnB/nfs1]        -------MDIISVALKRHSTKAFDASKKLTAEEAEKIKTLLQYSPSSTNS
S.typhimurium_NTR                -------MDIVSVALQRYSTKAFDPSKKLTAEEADKIKTLLQYSPSSTNS
E.coli_NTR                       -------MDIISVALKRHSTKAFDASKKLTPEQAEQIKTLLQYSPSSTNS
B.licheniformis YfkO             MTEQSKKQEILDAFQFRHATKEFDPDRKISDEDFQFILEAGRLSPSSVGL
                                        :*:..   *::** **..:*:: *: : *    : ****..

                                                +#
E.cloacae_NTR_[nfnB/nfs1]        QPWHFIVASTEEGKARVAKSAAGTYVFNERKMLDASHVVVFCAKTAMDDA
S.typhimurium_NTR                QPWHFIVASTEEGKARVAKSAAGNYTFNERKMLDASHVVVFCAKTAMDDA
E.coli_NTR                       QPWHFIVASTEEGKARVAKSAAGNYVFNERKMLDASHVVVFCAKTAMDDV
B.licheniformis YfkO             EPWQFVVVVQNKELREKLRQVSWG----AQGQLPTASHFVLLLGRTAKEMR
                                 :**:*:*...:* : :: : : *     : ::  ***.*:: .:** :

                                                      +
E.cloacae_NTR_[nfnB/nfs1]        WLERVVDQEEADGRFNTPEAKAANHKGRTYFADMHRVDLKDDD------Q
S.typhimurium_NTR                WLERVVDQEDADGRFATPEAKAANDKGRRFFADMHRVSLKDDH------Q
E.coli_NTR                       WLKLVVDQEDADGRFATPEAKAANDKGRKFFADMHRKDLHDDA------E
B.licheniformis YfkO             RDSGYVADQLKHVKKMPEDIIENMLKEDGVLESFQDGDFHLYESDRAMFD
                                   .  * ::  . :  . :      *    : .::  .::         :

                                       ##                ## ##
E.cloacae_NTR_[nfnB/nfs1]        WMAKQVYLNVGNFLLGVGAMGLDAVPIEGFD----AAILDEEFGLKEKGF
S.typhimurium_NTR                WMAKQVYLNVGNFLLGVAAMGLDAVPIEGFD----AEVLDAEFGLKEKGY
E.coli_NTR                       WMAKQVYLNVGNFLLGVAALGLDAVPIEGFD----AAILDAEFGLKEKGY
B.licheniformis YfkO             WVSKQTYIALANMMTAAALIGIDSCPIEGFNYDKVHDILEKEGVLEDGRF
                                 *::**.*: :.*:: ... :*:*: *****:      :*: *  *::  :

                                                  +     # #
E.cloacae_NTR_[nfnB/nfs1]        TSLVVVPVGHHSVEDFNATLPKSRLPLSTIVTEC-
S.typhimurium_NTR                TSLVVVPVGHHSVEDFNAGLPKSRLPLETTLTEV-
E.coli_NTR                       TSLVVVPVGHHSVEDFNATLPKSRLPQNITLTEV-
B.licheniformis YfkO             DISVMAAFGYRVKEPR----PKTRRALDQIVKWVE
                                    *:...*::  *      **:* . .  :.
```

**Fig. 3 – Alignment of *B. licheniformis* YfkO with other nitroreductase sequences. The amino acid sequences of nitroreductase (NTR) from *E. coli*, *E. cloacae* and *S. typhimurium* were aligned with the sequence of nitroreductase YfkO from *B. licheniformis* using Clustal W. Residues identified as interacting with the substrates CB1954 and menadione (+), and the flavin cofactor (#), in the *E. coli* enzyme [15] are identified.**

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