Special Issue

Process Optimisation Studies and Aminonitrile Substrate Evaluation of *Rhodococcus erythropolis* SET1, A Nitrile Hydrolyzing Bacterium

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A comprehensive series of optimization studies including pH, solvent and temperature were completed on the nitrile hydrolyzing *Rhodococcus erythropolis* bacterium SET1 with the substrate 3-hydroxybutyronitrile. These identified temperature of 25 °C and pH of 7 as the best conditions to retain enantioselectivity and activity. The effect of the addition of organic solvents to the biotransformation mixture was also determined. The results of the study suggested that SET1 is suitable for use in selected organo-aqueous media at specific ratios only. The functional group tolerance of the isolate with unprotected and protected β-aminonitriles, structural analogues of β-hydroxynitriles was also investigated with disappointingly poor isolated yields and selectivity obtained. The isolate was further evaluated with the α- aminonitrile phenylglycinonitrile generating acid in excellent yield and ee (>99% (*S*) – isomer and 50% yield). A series of pH studies with this substrate indicated pH 7 to be the optimum pH to avoid product and substrate degradation.

1. Introduction

Because of the inherent enantioselectivity of enzymes and the relatively benign reaction conditions used, biotransformations are under active research by many academic researchers and major pharmaceutical multinationals as routes to novel chiral drug molecules.[1] An additional advantage of biocatalytic synthesis is that it offers sustainable green technology with reduced requirement for solvents and other reagents, helping to decrease the environmental impact of the processes when compared to traditional chemical syntheses. Nitriles as substrates in biocatalytic processes can offer routes to synthetically important acids and amides *via* hydrolysis reactions. Traditional chemical methods of nitrile hydrolysis often utilize strong acids and bases and employ high temperatures, which can lead to degradation of other labile groups that may be present. Nitrile hydrolyzing enzymes such as nitrilase, nitrile hydratase and amidase offer the potential to generate such enantiopure acids and amides *via* single step (in the case of nitrilase) or multi step enzymatic cascade (in the case of nitrile hydratase/amidase) using milder, more efficient processes.[2] Various strains of *Rhodococci* contain nitrilase, nitrile hydratase and amidase enzymes and have been applied as efficient biocatalysts in several key industrial processes.[3]

Enantiopure β-amino acids are useful precursor building blocks for synthetic peptides, peptidomimetics, agrochemicals and pharmaceuticals.[1e,4] In contrast to α-amino acids, β-amino acids are not typically available in nature, the exceptions being β-alanine and β-aminoisobutyric acid. In addition, β-peptides can exhibit higher *in vivo* stability compared to α-peptides and when β-residues are present in mixed α/β-peptides they can protect closely situated amides from proteolytic cleavage. This offers promise for the development of novel peptide-based drugs which are not rejected or degraded in the human body.[5]

The synthesis of enantiopure β-amino acids can be difficult and currently several types of biocatalysts are employed, for example, transaminase and reductase enzymes.[6] Nitrile metabolizing enzymes also offer the potential for the synthesis of single enantiomer β-amino acids from β-amino nitriles and hence this prompted the work undertaken by our group.[2l,7] Such enzymes have also been used in tandem with other enantioselective enzyme systems in a non-asymmetric hydrolysis step.[8]

The ability to generate single enantiomer *a*-amino acids, highly important building blocks in organic synthesis, medicinal chemistry and pharmacology remains a challenge with methods such as diastereomeric salt resolution, the use of chiral auxiliaries and enzymatic synthesis as options.[9] Nitrile hydrolyzing enzymes can also be highly efficient in generating enantiopure α-amino acids and their derivatives.[10] There are limited examples of nitrilases being used in such routes however,[11] although they offer great potential to generate highly selective reaction products and the ability for possible

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Special Issue *Biosynthesis*

additional *in-situ* racemization of the unwanted isomer. Both Chaplin and Wei have reported high yield synthesis of D-phenylglycine and its derivatives by nitrilase mediated dynamic kinetic resolution.[11b] This prompted us to also evaluate this substrate as a model for the synthesis of α-amino acids.

A strategy for the rapid identification of isolates demonstrating nitrile-hydrolysing activity was previously published by our research group[7h] and a whole cell catalyst *Rhodococcus erythropolis* SET1 was found to catalyse the hydrolysis of 3-hydroxybutyronitrile with remarkably high enantioselectivity. This was subsequently screened against 34 related nitriles.[12] The aim of the work presented in this publication was to optimise reaction conditions with 3-hydroxynitrile and investigate the solvent tolerance of the bacterial isolate with this substrate. In addition, we wanted to assess the functional group tolerance of the isolate with β-aminonitriles, which are structural analogues of β-hydroxynitriles and which can upon reaction with the enzyme potentially generate enantiopure β-amino acids. Finally, as D-phenyl glycine is an important intermediate in the synthesis of medicinal products and in particular antibiotics,[13] and its precursor nitrile is a further amino analogue of previously studied substrates mandelonitrile and 2-phenylpropionitrile, we wanted to examine the reaction of the isolate with phenylglycinonitrile as a model α-amino nitrile. This would further expand the substrate scope of this isolate.

2. Results and Discussion

2.1. Effect of Temperature and pH

The effect of temperature on SET1 activity and enantioselectivity towards 3-hydroxybutyronitrile **1** was first investigated,[14] as shown in Scheme 1. Relative activity was calculated based on the activity observed and concentration of ammonia produced during the hydrolysis of nitrile (10 mM) in potassium phosphate buffer at pH 7 at 25 °C. Observations from this work were that a minor decrease in activity was observed when the temperature was reduced from 25 °C to 10 °C with 82% relative activity maintained, while 104% relative activity was retained when the temperature was increased from 25 °C to 30 °C. A further increase in temperature from 30 °C to 40 °C significantly increased the relative activity to 132%.

Although lower temperature (10 °C) was successful in maintaining the enantioselectivity, lower solubility of the nitrile was observed, and the reaction activity decreased. In contrast, above 30 °C the nitrilase enantioselectivity was rapidly lost. The biotransformation at 30 °C caused an approximate 63% loss of the initial enantioselectivity with a further decrease to 84% at 40 °C as shown in Figure 1.

When compared to other nitrile metabolising strains, SET1 appears to follow the trend that biotransformations at higher temperatures (30–40 °C) can result in higher activity.[15] Although higher temperatures resulted in higher activity, enantioselectivity was lost, however. When comparing the results of this particular study with initial screening work and substrate evaluation as described in our earlier papers, which were performed at 25 °C, enantioselectivity at this temperature was also >99% towards 3-hydroxybutyronitrile following incubation of cells at 25 °C for 24 hours. Since optimum activity and enantioselectivity seemed to be almost identical at 20 and 25 °C, with an apparent significant drop in ee when temperature rose above this we deemed 25 °C to be optimum for further substrate studies and to allow direct comparisons.

The alkaline and acid tolerance of *R. erythropolis* SET1, were further determined with the results presented in Figure 2. Activity was maximal at pH 7.0, falling sharply at both higher and lower values. Isolate SET1 was found to have a pH optimum

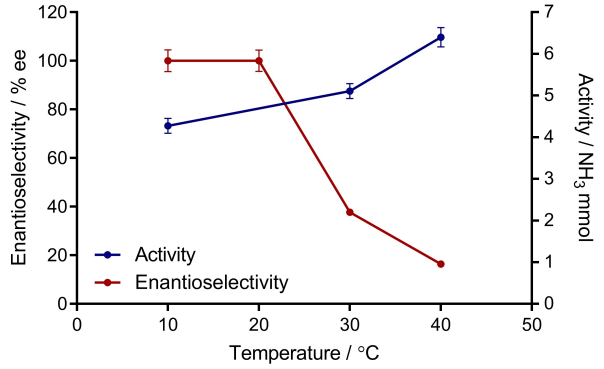


Figure 1. Effects of temperature on the nitrilase activity of R. erythropolis SET1. Reactions were run for 24 hours in potassium phosphate (100 mM, pH 7.0) at 10, 20, 30 and 40 °C. Activity was determined in triplicate using the Nesslers microscale colorimetric assay. Enantioselectivity was determined in triplicate by HPLC analysis using a chiral column.

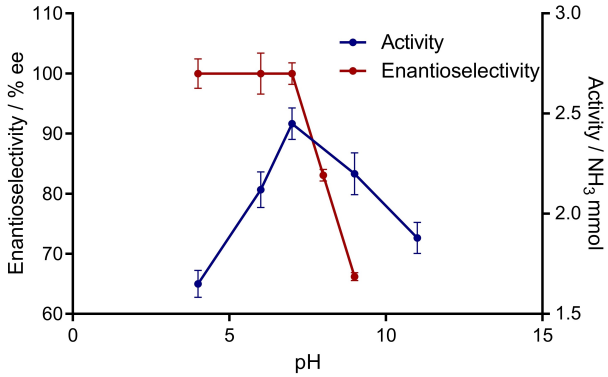
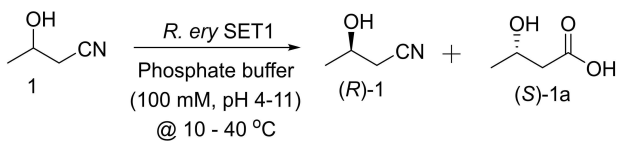


Figure 2. Effect of pH on the nitrilase activity of *R. erythropolis* SET1. Reactions were run for 24 hours at 25 °C in potassium phosphate (100 mM) buffered at pH 4, 6, 7, 9 and 11. Activity was determined in triplicate using the Nessler's microscale colorimetric assay. Enantioselectivity was determined in triplicate by HPLC analysis using a chiral column.



Scheme 1. An investigation into the effect of temperature and pH on the SET1 nitrilase catalysed hydrolysis of 3-hydroxybutyronitrile.

of 7, with maximum activity and enantioselectivity both retained. When the pH was reduced to 6, 86% of the maximum activity relative to that observed at pH 7 (25 °C) was maintained. A further decrease of pH resulted in a further loss of activity with 67% relative activity observed at pH 4.

A similar trend is observed with biotransformations carried out under alkaline conditions where activity is lost at higher pH values. However, the drop in activity is not as sharp under basic conditions, with 89% relative activity at pH 9 and 76% at pH 11. The enantioselectivity of the biotransformation however was less dramatically influenced by acid pH; >99.9% ee was retained under acidic conditions even at pH 4. A significant decrease in ee was found however under alkaline conditions, with approximately 83% and 66% of the initial enantioselectivity remaining at pH 8 and pH 9 respectively. To compare isolate SET1 with other nitrilases, the pH optimum of nitrilases can lie within the range of 7.5 to 9. In contrast for example, *R. rhodochrous K22* demonstrated a maximum activity in the more acidic region of pH 5.5 towards crotononitrile.[15a,c,16] Therefore, the relatively high activity observed under acidic pH conditions with SET1, which enabled the biotransformation to be performed without any substantial loss in enantioselectivity, is promising for biotransformations of nitrile analogues which may decompose at a higher pH.

2.2. Effect of Organic Solvents on Nitrilase Activity of SET1

Aqueous media can limit the use of nitrile hydrolysing enzymes in the hydration of inherently hydrophobic nitriles, though added quantities of organic solvents can be used to aid substrate availability to the enzyme in some cases.[17] Several solvent based approaches have been reported in the literature including monophasic systems with solvents such as DMSO[18] and biphasic systems such as toluene/H_2O and hexane/H_2O[1d] and some systems can operate in both buffer-organic solvent monophasic or biphasic mixtures.[18] In several cases, enhancement of the enzyme activity and enantioselectivity of hydrolysis has also been observed in organic solvent systems.[1f,6e,19] However it has been noted that the mechanism of solvent interaction with nitrile hydrolysing reactions is much less studied than in the case of other hydrolytic enzymatic systems.[7b]

In order to assess the solvent tolerance of SET1 and determine an optimum solvent system, a series of solvent studies were carried out. An important criterion for the selection of a potential organic solvent is its biocompatibility towards the enzyme. Eight organic solvents were examined in this work. This included toluene, hexane, ethyl acetate, butanol, IPA, ethanol, DMSO and THF. Initial work examined water miscible organic solvents, such as IPA, DMSO, ethanol and THF for the effect on the activity retention of the whole cells and results are presented in Figure 3. In this study we chose to focus on the effect of activity of the enzyme in the various solvent mixtures monitoring this using the technique of Nesslerisation for ease of reaction handling.

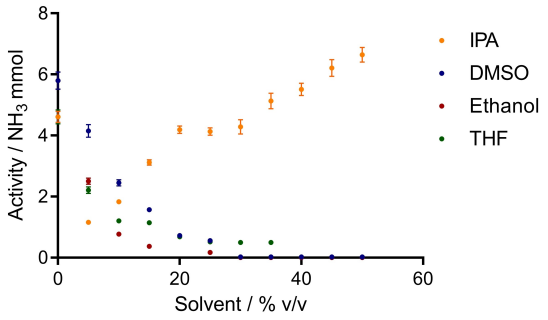


Figure 3. Effects of monophasic solvents on the nitrilase activity of *R. erythropolis* SET1. Reactions were run in duplicate for 24 hours in potassium phosphate (100 mM, pH 7.0) solvent mixture at 25 °C. Activity was determined in triplicate using the Nesslers microscale colorimetric assay

Whole cells of *R. erythropolis* SET1 were incubated with 3-hydroxybutyronitrile dissolved in aqueous buffer with various additional co-solvents at quantities between 5 and 50% (v/v). The reaction mixture was incubated at 25 °C for 24 hours. The appropriate cell blanks in the various solvent ratios allowed for careful determination of the nitrilase activity. Each biotransformation and subsequent activity assay were performed in triplicate. Relative activity was calculated based on the activity observed from the standard reaction conditions without added solvent, using the concentration of ammonia produced during the hydrolysis of 3-hydroxybutyronitrile (10 mM) in potassium phosphate buffer at pH 7.

The addition of 35% (v/v) IPA resulted in retention of 100% relative activity. As the ratio of IPA increased (50% v/v) so too did the activity (144%). This is in contrast to DMSO, ethanol and THF, where the activity decreases when the percentage of organic solvent is increased; this is also shown in Figure 3. The addition of 5% ethanol and 5% THF resulted in a decrease in relative activity of 54% and 47% respectively. The relative activity decreased as the percentage of organic solvent increased and the enzyme appeared to be inhibited (0% relative activity) at concentrations of 50% v/v ethanol and THF. This significant drop in enzyme activity may indicate that the proteins became denatured in the presence of higher concentrations of these organic solvents.

Low concentrations of DMSO (5% v/v) resulted in retention of 89% relative activity, while higher concentrations of DMSO (50% v/v) further reduced the activity (4%). In summary, although the presence of IPA (35% v/v) maintained the initial activity, higher concentration of the solvent (50% v/v) increased the activity to 144%. Other co solvents examined decreased the relative activity of the reaction in all cases.

In order to relieve the biocatalyst from substrate inhibition in the aqueous monophasic reaction system, a water organic biphasic system may provide an attractive alternative. In this case the hydrophobic substrate will be mainly retained in the organic phase which can act as a reservoir for the toxic or insoluble substrate, thus regulating the substrate concentration around the biocatalyst and minimizing the substrate inhibition.[7f,20] Taking into account the poor water solubility of

some nitriles, this allows higher substrate concentrations and also facilitates the product recovery.

Four water immiscible organic solvents; ethyl acetate, toluene, butan-1-ol and hexane between 5–50% v/v were examined for the effect on the activity retention of the whole cells and the results are presented in Figure 4.

The highest relative activities were obtained in hexane and toluene which had Log P values of 3.5 and 2.5 respectively. The maximum activity retention was obtained in 50% (v/v) hexane/aqueous which was 89% of that in neat aqueous buffer. This may be due to the more rigid and stable conformational structure of the enzyme in this solvent.[20] Although toluene has a similar Log P value to hexane of 2.5, the activity in 50% (v/v) solvent/aqueous decreased to 48%. It was observed that the incorporation of low concentrations of ethyl acetate (5% v/v) and butan-1-ol (5% v/v) decreased the relative activity of the reaction to 4.8% and 9.7% respectively.

This result indicates that a biphasic system Log P $\geq$ 2.5 is preferable and that water immiscible solvents with low Log P values such as ethyl acetate and butan-1-ol (0.68 and 0.88 respectively) may inhibit the activity of SET1. The optimum percentage of relative activity retained during the biotransformation of nitrile in the presence of various organic solvents (water miscible and immiscible) is shown in Figure 5. The results of the study suggested that SET1 may be suitable for use in selected organo-aqueous media at selected ratios only.

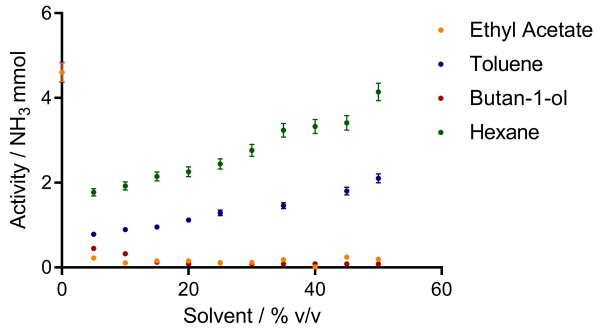


Figure 4. Effects of biphasic solvents on the nitrilase activity of *R. erythropolis* SET1. Reactions were run in duplicate for 24 hours in potassium phosphate (100 mM, pH 7.0) and up to 50% (v/v) solvent mixture at 25 °C. Activity was determined in triplicate using the Nesslers microscale colorimetric assay.

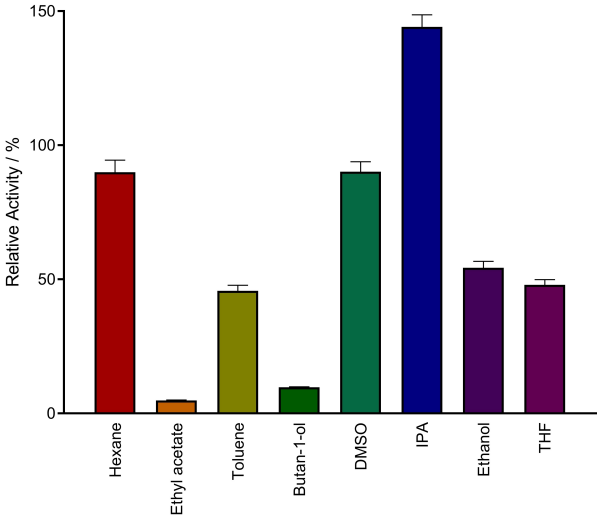


Figure 5. Effects of organic solvents on the nitrilase activity of *R. erythropolis* SET1. Reactions were run in duplicate for 24 hours in potassium phosphate (100 mM, pH 7.0) solvent mixture at 25 °C. Activity was determined in triplicate using the Nesslers microscale colorimetric assay.

Preliminary studies were carried out to probe the enantioselectivity observed in the presence of organic solvents however, it was difficult to extract the remaining nitrile and product from the reaction solvents for derivatisation and analysis by normal phase HPLC. This will be evaluated further in future studies.

2.3. Further Substrate Evaluation of SET1 with β-Amino Nitriles

Given that bacterial isolate SET1 could selectively hydrolyse β-hydroxy nitriles to carboxylic acids and amides, with the aliphatic substrate 3-hydroxybutyronitrile (3-HBN) giving high yield and ee of acid product without a docking group, it was thought this may be a promising isolate suitable for the hydrolysis of analogous β-amino nitriles. In particular the transformation of aliphatic β- amino nitriles is a much less explored area and requires further knowledge. A series of protected and unprotected aliphatic and aromatic β-amino nitriles **2**–**9** Figure 6 were prepared for evaluation of SET1.

Both aliphatic and aromatic structures were included to compare to previous work and thus offer comparisons to the known preferences of isolate SET1. Commonly used amine protecting groups were also evaluated to provide insight into the conformation of the active site of the enzyme and also for ease of recovery of the amino acid products.

The unprotected aliphatic, 3-aminobutyronitrile **2** was obtained commercially and protected amino acid derivatives were prepared from this by employing standard protection protocols from literature as outlined in the supplementary information.[2i,7a,21] The aromatic analogue 3-amino-3-phenylpropionitrile **6** was prepared by adapting a procedure developed by Brady *et al*,[2i] to employ sodium triacetoxyborohydride in place of sodium cyanoborohydride for enamine reduction as.[2j] While this method did furnish **6** in appropriate quantities for evaluation, reproducibility of the STAB reduction proved difficult.

In order to develop respective chiral HPLC analytical methods quantities of amide, and acid standards were

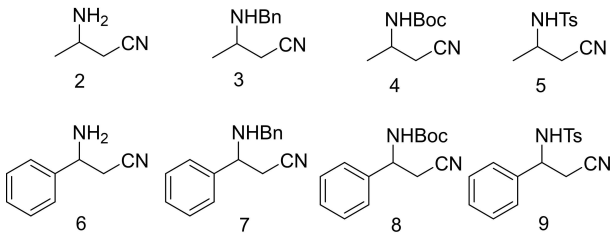


Figure 6. Unprotected and protected aliphatic and aromatic β-aminonitrile analogues evaluated with SET1.

synthesised by hydrolysis. In developing HPLC methods for ee determination, for the majority of the compounds, including the starting nitriles, derivatisation was required.

Initial work focused on the unprotected 3-aminobutyronitrile **2** and 3-amino-3-phenylpropionitrile **6** which we hoped to transform without the need for a protecting group to ensure maximum efficiency. In discussing the biotransformation the high water solubility of both the unprotected acid and nitrile must be highlighted. While this is desirable from a reaction point of view, it severely hampered the recovery process by solvent extraction and has been noted with unprotected 3-aminobutyronitrile,[7a] and other aliphatic unprotected β-amino nitriles and acids.[22] It must also be mentioned that the lack of a UV- chromophore in both the starting material and product in the case of 3-aminobutyronitrile also proved complex for HPLC analytical method development and reaction determination using TLC.

Attempts to recover and analyse the unprotected 3-aminobutyric acid **2b** and 3-aminobutyronitrile **2** products post biotransformation *via in-situ* Cbz-protection with Cbz-OSu generated complex mixtures of products not easily separated by flash chromatography. For accurate yield determination it was necessary to use HPLC with concentration of product determined by comparison to standard curves prepared from authentic standards completed in triplicate. For ee determination of the acid product due to availability of suitable separating columns a GITC derivatisation method was developed which is outlined in the experimental section.

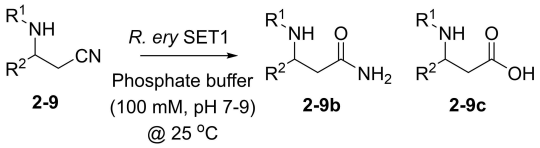
Protocols and biotransformation methods from the process optimisation work outlined above were employed as a starting point for investigation of the aminonitriles. Parameters that were deemed optimum such as temperature of 25 °C for enantioselectivity and activity were retained. In terms of the pH however, in the case of 2 biotransformations were carried out at pH 7, 8 and 9 to investigate the effect of pH on the amino group. In these studies cells were induced on 3-hydroxybutyronitrile prior to biotransformation.

Unfortunately, disappointingly low yields and ee were observed for the hydrolysis of unprotected **2** and **6** in all reactions and no nitrile and only minor quantities of acid were recovered after the biotransformation of **2**, with no acid or nitrile recovered in the case of the aromatic analogue **6** (Table 1, entries 1–3, entry 8). The result for **2** follows observations noted in prior work with SET1 and 3-hydroxybutyronitrile where nitrile was not recovered post biotransformation. This may be due to the nitrile becoming trapped within the cell or in its outer membrane. Another possibility is the presence of another enzymatic pathway that is competing for the nitrile and causing degradation of the acid and nitrile products. In addition, the recovery of both product and starting material were hampered by their water solubility.

It can be tentatively observed from these studies that as pH increases that the enantioselectivity of isolate SET1 falls rapidly (Table 1, entries 1, 2 and 3). This result further highlighted the observations outlined in Section 1.1 above, that the optimum temperature and pH for the microbial isolate are 25 °C and pH 7. Investigation of induction conditions of the isolate were carried out in order to improve the activity and selectivity.[23] Here, **2** was used to induce isolate SET1 rather than **1**. The biotransformation was subsequently carried out at pH 7 with **2** as substrate. In this instance again disappointingly low yield of acid and nitrile was recovered. (Table 1, entry 4).

In the case of **2**, it has been previously observed that *N*-benzyl protection of the amino group significantly enhanced ee and yield in a nitrile hydratase/amidase system after the addition of the *N*-benzyl protecting group[7a] . As noted by Klempier *et al* the choice of protecting group for β-aminonitriles should be governed by the ease of protection and removal of the group, the ability to improve analytical reaction monitoring

Table 1. Results of the biotransformation of unprotected and protected racemic β-aminoalkane and β-aminoaromatic nitriles 2–9.



Entry	Substrate	pH	R^1	R^2	Recovered Nitrile Yield (%)[b]	ee (%)[c]	Product Amide **b** Yield (%)[b]	ee (%)[c]	Product Acid **c** Yield (%)[b]	ee (%)[c]
1	**2**	7	H	CH_3	–	–	–	–	<1	29 *(S)*
2	**2**	8	H	CH_3	–	–	–	–	<1	18 *(S)*
3	**2**	9	H	CH_3	–	–	–	–	<1	5 *(S)*
4*	**2**	7	H	CH_3	8	2	–	–	5	7 *(S)*
5	**3**	7	Bn	CH_3	-	-	3	90	6	75 *(S)*
6	**4**	7	Boc	CH_3	55	79[d]	–	–	1	10 *(S)*
7	**5**	7	Ts	CH_3	74	0	9	1	<1	>99 *(S)*[d]
8	**6a**	7	H	C_6H_5	17	11	–	–	–	–
9	**7**	7	Bn	C_6H_5	78	0	–	–	–	–
10	**8**	7	Boc	C_6H_5	66	1	–	–	–	–
11	**9**	7	Ts	C_6H_5	96	1	–	–	–	–

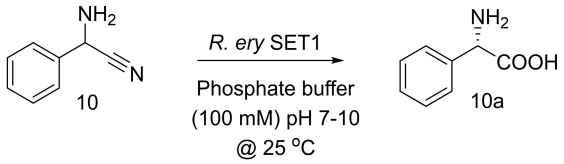
The biotransformations were carried out by incubating the nitrile (10 mmol/L) in a suspension of *R. erythropolis* SET1 ($OD_{600nm=1}$) in phosphate buffer at 25°C. [b] Isolated yield. [c] Determined by HPLC analysis using a chiral column. The configuration was determined by comparison of HPLC traces with prepared single enantiomer standards and literature. N.d: not detected. [d] Chiral HPLC analysis performed on the corresponding derivative. * Isolate induced on 3-aminobutryonitrile.

and product detection and also substrate solubility and these were considered for this study.[22]

We chose to evaluate *N*-benzyl, *N*-Boc carbamate and *N*-Tosyl derivatives for this work. We anticipated that some of the *N*-protected nitriles would exhibit poor solubility in phosphate buffer, in particular the *N*-Tosyl and *N*-Boc protected substrates and measures were taken to ensure 100% availability of the substrate to the bacterial isolate. The earlier organic solvent studies shown above in Figure 5 suggest that the addition of 5% DMSO could allow for the retention of activity of SET1 with 3-hydroxybutyronitrile and this was chosen as a starting point for this work. In all cases the pH was maintained at 7 for the biotransformations. Each biotransformation was carried out in a suspension of phosphate buffer (10 mM) with 5% DMSO, containing induced cells ($OD_{600nm} = 1$) at pH 7 and enantioselectivity was determined using HPLC employing a chiral column with comparison to synthesised standards of acid and amide.

The optimum enantioselectivity was obtained with the *N*-benzyl protected alphatic substrate **5** where ee of 75% of (*S*)-acid was observed in the very low yield of 6% (Table 1 entry 5). Again isolation of the product proved difficult and nitrile was not recovered from the reaction mixture despite using published methods. In addition a minor quantity of amide was also recovered with ee of 90%. The *N*-Boc protected aliphatic nitrile **4** did not yield any acid product after biotransformation but did furnish enantioenriched starting nitrile (Table 1, entry 6). In the case of the *N*-tosyl protected derivative a trace quantity of acid was recovered with unreacted nitrile predominantly recovered post biotransformation. The size of the protecting group may have played a role in the lack of observed acid product in this case. None of the protected aromatic variants **7**–**9** yielded product from the reaction with racemic unreacted starting material obtained. (Table 1, entries 9–11). The presence of amide as product in two of the cases along with acid is not unusual in reactions with this bacterial isolate. In our previous work, we observed the formation of amide in cases where the substrate contained an electron withdrawing group at the α position to the nitrile, and this has also been noted in several other similar studies.[12]

In a final separate study of the substrate scope of SET1, as the bacterial isolate had shown activity and selectivity with mandelonitrile in previous studies, we also preliminarily investigated the ability of the enzyme to transform the α-aminonitrile phenylglycinonitrile **10**. Nitrilase enzymes have previously been shown to successfully transform α-aminonitrile substrates with relatively high selectivity[24,25,26]. Particular consideration must be taken with phenylglycinonitrile as a substrate as it undergoes spontaneous decomposition into benzaldehyde, HCN and ammonia *via* retro-Strecker reaction. Meanwhile mandelonitrile can arise from the spontaneous reaction of HCN and benzaldehyde,[27] therefore, choice of reaction conditions require careful consideration. To this end, the influence pH had on the reaction outcome of SET1 with 2-phenylglycinonitrile was examined. In these reactions, enzyme activity and enantioselectivity were determined after 96 h incubation with racemic 2-phenylglycinonitrile (10 mM) at pH ranging from 7 to 10 (Scheme 2) by chiral HPLC analysis following acidification



Scheme 2. Effect of pH on the nitrilase activity of *R. erythropolis* SET1 towards phenylglycinonitrile. Reactions were run for 96 hours at 25 °C in potassium phosphate (100 mM) buffered at pH 7, 8, 9 and 10. Activity and enantioselectivity were determined by HPLC analysis using a CR+ chiral column.

and removal of the biomass by centrifugation. Standard curves were completed with standard and runs in triplicate for acid, amide and nitrile to allow for product quantitation.

The results in Figure 7 demonstrate the effect of pH on nitrilase activity and enantioselectivity. In contrast to the results for β-aminonitriles isolate SET1 was found to efficiently transform the α-aminonitrile 2-phenylglycinonitrile **10** at pH 7, with maximum yield and enantioselectivity obtained for the (*S*)-acid product. In this case the bacterial isolate was extremely selective generating acid in >99% ee and in addition nitrile was detected in 10% yield and 44% ee. When the pH increased the yield of acid product decreased from 51% at pH 7 to 36% at pH 10, however, the high enantioselectivity was retained. The decreasing acid yield may be due to enzyme inhibition caused by benzaldehyde or deactivation of the nitrilase under alkaline conditions. Detection of the remaining nitrile increased from 10% at pH 7 to 15% at pH 10, while the enantioselectivity data for the nitrile along with low conversions indicate possible partial racemisation of the aminonitrile as previously described by Chaplin *et. al.*.[28] They established that pH values >10 allowed for the rapid racemisation of amino nitriles. The loss in mass balance for the nitrile and acid product may be attributed to the decomposition of the starting material by such a mechanism..[28] The yields and ee values are influenced by the competition of the hydrolysis, decomposition and racemisation and vary greatly depending on the reaction conditions. It is plausible that spontaneous decomposition of the starting material to produce benzaldehyde, HCN and ammonia may cause enzyme inhibition. However, it is also likely that the decreased quantity of remaining phenylglycinonitrile may also

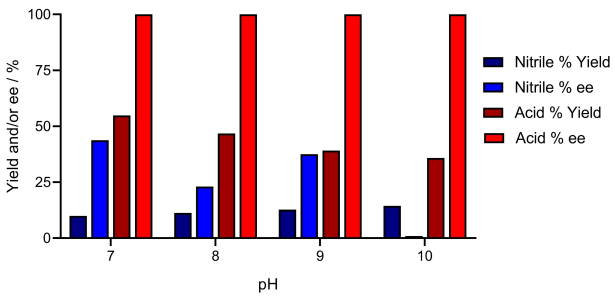


Figure 7. Effect of pH on the nitrilase activity of *R. erythropolis* SET1 towards phenylglycinonitrile Reactions were run for 96 hours at 25 °C in potassium phosphate (100 mM) buffered at pH 7, 8, 9 and 10. Yield and enantioselectivity were determined by HPLC analysis using a CR+ chiral column.

account for reduced yields, this may be supported by the loss in mass balance for the nitrile and acid product.

In all reactions amide was not detected.[12] This is in contrast to previous results obtained when examining the reaction of SET1 with mandelonitrile, an α-hydroxy nitrile in which both acid (8.2%) and amide (44.1%) were formed and thought to be a result of the electron-withdrawing nature of the hydroxy substituent (and proposed stabilisation of the tetrahedral intermediate).[12] The sole formation of acid in the case of phenylglycinonitrile may be due to the electronic and steric interactions because of the NH_2 and phenyl group (π-π interactions). It is also interesting to compare the ability of the isolate to selectively transform an unprotected α-aminonitrile with the lack of detected selectivity with the analogous and related unprotected β-aminonitrile substrates and with selectivity observed in β-hydroxyl derivatives. This further indicates that both substrate functionality type and its distance from the nitrile group, along with the presence of either aromatic or aliphatic sterically demanding neighbouring groups may have an effect on the selectivity of such biocatalytic reactions. This final preliminary study also offers significant potential for the transformation of other aminonitrile substrates, and the generation of further amino acid products and will be evaluated further in future studies.

3. Conclusion

We have presented detailed substrate optimisation studies on the bacterial isolate *R. erythropolis* SET1 with 3-hydroxybutyronitrile **1** outlining the key parameters of pH, temperature and also solvent compatibility for use with this substrate. The reaction system was screened with a series of β- amino nitriles but does yield sufficient isolated quantities of enantioenriched product. It is clear that a protecting group is necessary for any appreciable product recovery and detection in these reactions. Both the yield and ee values for amide and acid product appear to be a result of the structure and the size of the protecting group on the amino moiety. This work also presents the inherent difficulties associated with handling such β-aminonitrile substrates in contrast to β-hydroxy analogues and indicates possible alternative metabolic pathways as evident in the low/unrecoverable yield of the starting substrate. In additional studies, the position of the free amino group α- or β- to the nitrile was observed to have a dramatic effect on the reaction outcome. In contrast to the β-amino nitrile substrates the biotransformation of α- substituted 2-phenylglycinonitrile with SET1 resulted in excellent yields and ee of (*S*)-acid (51% yield, $>$99% ee). This was observed to be pH dependent with lower yield observed at higher pH. This offers significant potential for the transformation of other amino nitrile substrates and the generation of valuable amino acid products.

Experimental Section

Materials

Racemic 3-hydroxybutyronitrile (**1**) and 2-phenylglycinonitrile were purchased from Sigma-Aldrich and 3-aminobutyronitrile (**2**) was purchased as a hydrochloride salt from Enamine. All other chemicals were of analytical grade and obtained from various commercial sources. NMR spectra were recorded with a JEOL ECX 400 MHz spectrometer. The chemical shifts (expressed in ppm) of the ^{1}H and ^{13}C NMR spectra are referenced to the solvent peaks. TLC was carried out on aluminium-backed sheets with silica gel 60 F54 (Merck). Column chromatography was performed with silica gel 60 (0.04–0.063 mm, Merck). HPLC analysis was undertaken with a Hewlett-Packard 1050 series instrument, LCMS was carried out with an Agilent technologies 1200 series instrument with an LC/MSD Trap XCT detector and GC-MS with a Varian 450 GC coupled with a 220 MS.

Bacterial Isolates

The bacterial isolate used in this study was previously described in Coady *et al.*[7h] The isolate SET1 has been deposited in the National Collection of Marine, Food and Marine Bacteria (NCIMB), Patent Deposit number NCIMB 41986.

General Method for Nesslers Microscale Ammonia Assay

Isolate SET1 was examined in the hydrolysis of 3-hydroxybutyronitrile using the Nesslers colorimetric assay [5], in 96-well microtitre plates (Sarstedt Ltd). Fresh cultures were grown in M9-minimal media containing 10 mM nitrile (before washing 3 times with 500 μL of phosphate buffer). Each 150 μl reaction contained 10 mM nitrile and cells ($OD_{600nm} = 0.1$) in potassium phosphate buffer (100 mM, pH 7.0). Microtitre plates were sealed using adhesive film (Sarstedt) and incubated at various temperatures at 250 rpm for 24 hours. The reaction was then quenched by adding 37.5 μL of 250 mM HCl. Plates were centrifuged at 500 g for 10 minutes to pellet the cell debris. 20 μl of the supernatant was transferred to a microtitre plate, 181 μL of assay mastermix was added (155 μL deionised water, 1 μL 10 N NaOH, 25 μL Nesslers reagent (Merck)). The reaction was allowed to stand for 10 minutes and the absorbance was read at 425 nm. Cell blanks contained cells @ $OD_{600nm} = 0.1$ in phosphate buffer. Nitrile blanks contained 150 μL of 10 mM nitrile in phosphate buffer.

General Procedure for Enantioselectivity Screening Towards 3-Hydroxybutyronitrile

Racemic nitrile (5.1 mg, 5.9μL, 10 mM) was added in one portion to a solution of potassium phosphate buffer (0.1 M, 6 mL) containing induced cells ($OD_{600nm} = 1$), and activated at the chosen temperature for 30 minutes with orbital shaking (250 rpm). The reaction was quenched after 24 hours by removal of the biomass by centrifugation at 3,000 g. The resulting aqueous solution was acidified by the addition of 1 M HCl (200 μL). The aqueous portion was then extracted with ethyl acetate, the extracts were dried over $MgSO_4$ and the solvent removed under vacuum. The biotransformation products were first derivatised to their corresponding β-benzyloxyethers before analysis. Silver oxide (1 equiv, 0.06 mmol, 13.6 mg), benzylbromide (4 equiv, 0.24 mmol, 28 μL) and dichloromethane (2 mL) were added and the mixture stirred in the dark for 24 hours. The reaction mixture was diluted with acetone and filtered through a 0.45 μm filter and solvent was removed under vacuum. 1 mL of mobile phase (90% hexane: 10% IPA and 0.1% TFA) was added

before the solution was injected on the Chiral HPLC system. Chiralcel AD–H stationary phase was used for the resolution of β-hydroxyacids. Analytical conditions applied: 90% hexane, 10% IPA and 0.1% TFA, with a flow rate of 0.8 mL/min and a detection wavelength of 215 nm. All experiments were performed in triplicate. % enantiomeric excess is calculated from the ratio of the enantiomer by the difference in peak area divided by the sum of the peak areas for the major and minor enantiomers.

Temperature Studies

Nitrilase activities towards 3-hydroxybutyronitrile (10 mM) was assayed as described above using whole cells (SET1, $OD_{600nm} = 1$) in KH_2PO_4 (100 mM, pH 7.0). The hydrolysis was performed at 0 °C, 10 °C, 20 °C, 30 °C, and 40 °C. Chiral HPLC analysis as outlined in the previous procedure was used to measure enantiomeric excess.

pH Studies

Nitrilase activities towards 3-hydroxybutyronitrile (10 mM) was assayed as described above using whole cells (SET1, $OD_{600nm} = 1$) in KH_2PO_4 (100 mM). The hydrolysis was performed at pH 4, 5, 7, 9 and 10 at 25 °C. Chiral HPLC analysis as outlined in the previous procedure was used to measure enantiomeric excess.

Solvent Studies

Whole cells of *R. erythropolis* SET1 were incubated with 3-hydroxybutyronitrile dissolved in aqueous buffer with various additional co-solvents at quantities between 5 and 50% (v/v). The reaction mixture was incubated at 25 °C for 24 hours and enzyme activity was monitored using the technique of Nesslerisation, along with cell blanks and solvent blanks.

Synthesis of β- Aminonitrile Substrates

Compounds (**3**), (**4**), (**5**), (**7**), (**8**) and (**9**) were prepared using literature procedures and are detailed in the supplementary information.[7a,2i]

3-Amino-3-Phenylpropionitrile (6 a)

Solid $NaBH_4$ (10 mmol, 0.39 g) was added portion-wise with stirring to glacial acetic acid (0.18 mmol, 10 mL) at 10 °C. The mixture was stirred for 30 min. A solution of 3-amino-3-phenylacrylonitrile (3.4 mmol, 0.49 g) in glacial acetic acid (3.4 mL) was added portion-wise. The solution was left to stir at room temperature for 2 h. The mixture was concentrated down *in vacuo* then NaOH (1 M, 40 mL) was added. The mixture was extracted with EtOAc (3×40 mL) and the combined organic extracts were washed with brine, dried over Na_2SO_4, and the solvent removed *in vacuo*. The product was purified by silica flash chromatography (Hex:EtOAc 70:30) to give the title compound as a pale yellow oil (1.9 mmol, 0.27 g, 54%).^{1}H NMR (400 MHz, $CDCl_3$), δ 7.36 (d, $J = 4.3$ Hz, 4H, Ar–H), 7.34–7.27 (m, 1H, Ar–H), 4.31 (dd, $J = 7.2$, 5.7 Hz, 1H, CH), 2.75–2.58 (m, 2H, CH_2), 1.79 (s, 2H, NH_2). ^{13}C NMR (100 MHz, $CDCl_3$), δ 142.5 (CN), 129.0 (C–Ar), 128.5 (C–Ar), 126.2 (C–Ar), 118.1 (C-5), 52.8 (C-4), 28.6 (C-3).ESI-MS, low res, *m/z* 168.9 ($M + Na^+$), 147.0 ($M + H^+$), 130, $C_9H_{10}N_2$

Acid and amide standards of all substrates were also prepared by hydrolysis using methods provided in the literature,[7i] and are detailed in the supplementary information along with chiral HPLC methods for analysis of nitriles, amides and acids.

Biotransformation Procedures for β-Aminonitrile Substrates

General Procedure for Biotransformations of β-Aminonitrile Substrates

Each biotransformation was carried out in a suspension of phosphate buffer (100 mM) set to the required pH, containing SET1 cells, previously induced on 3-hydroxybutryonitrile ($OD_{600nm} = 1$). Racemic β-aminobutyronitrile (10 mM) was added to the flask and the mixture was incubated at 25 °C for the required time with mechanical shaking (200 RPM), and monitored by TLC. The appropriate derivatisation, extractive work up or work-up with cation exchange resin and semi-prep purification HPLC (Phenomenex Jupiter C18, 10 μm), (gradient elution (ACN: $H_2O + 0.1$% formic acid), 5 mL min^{-1}) as necessary was employed depending on the substrate. Analysis was carried out by HPLC equipped with a chiral column (Chiralpak IA, OJ–H) or Waters Symmetry C18 after GITC derivitisation. The products were confirmed and product yields calculated by matching HPLC retention times to characterised synthesised standards (run in triplicate), standard curves and by LC-MS profiles. HPLC of single enantiomer acids and derivatives was used in all cases to assign configuration of products.

GITC Derivatisation Method for HPLC Analysis of Free Amine Substrates[29]

Sequentially triethylamine (1.5 mmol) and GITC (1.5 mmol) were added to a solution of the amine in acetonitrile (1 mL). The resulting mixture was stirred at 40 °C for 1 h. The mixture was injected directly onto the chiral HPLC fitted with a C18 symmetry column, with mobile phase MeOH: $H_2O + 0.1$% TFA and flow rate of 1.0 mL min^{-1}.

Studies on the Biotransformation of Phenylglycinonitrile

The activity of resting cells of SET1 with phenylglycinonitrile was determined in reaction mixtures (1 mL) containing potassium phosphate buffer (100 mM) buffered to pH 7, 8, 9 and 10 containing 2-phenylglycinonitrile (0.01 M). The reaction mixture was incubated with resting cells of SET1 ($OD = 1$) at 25 °C for 96 hours and quenched by the addition of HCl (200 μL) with biomass removed via centrifugation. The reaction mixture was analysed immediately to prevent further decomposition of the substrate.

A Chiralcel CR+ column was used for the resolution of both 2-phenylglycinonitrile and 2-phenylglycine. Analytical conditions applied: Mobile phase of perchloric acid (16.3 g in 1 L) adjusted to pH 1, with a flow rate of 1.0 mL/min and a detection wavelength of 215 nm.% activity was calculated from the combined peak area of the enantiomers and the equation of the line generated from a series of standards for the nitrile and acid. Standard curves are provided in the supplementary information.

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Conflict of Interest

The authors declare no conflict of interest.

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