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A high throughput screening strategy for the assessment of nitrile-hydrolyzing activity towards the production of enantiopure β-hydroxy acids

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ABSTRACT

Nitrile hydrolysing enzymes have found wide use in the pharmaceutical industry for the production of fine chemicals. This work presents a strategy that facilitates the rapid identification of bacterial isolates demonstrating nitrile hydrolysing activity. The strategy incorporates toxicity, starvation and induction studies along with subsequent colorimetric screening for activity, further focusing the assessment towards the substrates of interest. This high-throughput strategy uses a 96 well plate system, and has enabled the rapid biocatalytic screening of 256 novel bacterial isolates towards β-hydroxynitriles. Results demonstrate the strategy's potential to rapidly assess a variety of β-hydroxynitriles including aliphatic, aromatic and dinitriles. A whole cell catalyst *Rhodococcus erythropolis* SET1 was identified and found to catalyse the hydrolysis of 3-hydroxybutyronitrile with remarkably high enantioselectivity under mild conditions, to afford (*S*)-3-hydroxybutyric acid in 42% yield and >99.9% ee. The biocatalytic capability of this strain including the variation of parameters such as temperature and time were further investigated and all results indicate the presence of a highly enantioselective if not enantiospecific nitrilase enzyme within the microbial whole cell.

1. Introduction

The awareness of the importance of chirality in conjunction with biological activity has led to an increasing demand for efficient methods for the industrial synthesis of enantiomerically pure compounds [1]. Many chiral drugs now employ chiral synthons in their synthesis typically utilising the chiral pool, kinetic resolution techniques, or asymmetric synthesis [2] to afford single enantiomer compounds. Drawbacks associated with these approaches can be the use of expensive substrates and/or chiral metal complex catalysts/reagents which also in the latter case may require specialised conditions, can be toxic and not easily recovered/reused [3]. The combination of chemical procedures with biocatalytic methods can offer an excellent alternative strategy for the production of such compounds [4,5].

The distinct features of enzymatic transformations of nitriles in particular are the formation of enantiopure carboxylic acids and the generation of enantiopure amides, which are valuable compounds in synthetic chemistry [6]. β-Hydroxy nitriles such as 3-hydroxybutyronitrile, 3-hydroxyglutaronitrile and 3-hydroxy-3-phenylpropionitriles can act as sources of β-hydroxy carboxylic acids *via* hydrolysis reactions however can be prone to elimination reactions under classical acid/base conditions [7]. Alternatively, nitrile biocatalysis can selectively facilitate this hydrolysis without affecting other acid- or alkali-labile functional groups present [8,9]. This potential has resulted in significant work to identify bacteria and fungi capable of hydrolysing such nitriles [10,11], whose products can be widely used as chiral precursors for pharmaceutical compounds. For example, 3-hydroxy-3-phenylpropionic acid and its derivatives, have been used as precursors to chiral drugs such as nisoxetine [12], fluoxetine [13] and tomoxetine [12]. Additionally, of particular commercial interest, is the nitrilase catalysed hydrolysis of 3-hydroxyglutaronitrile, the ethyl-ester of which is an intermediate to the cholesterol lowering drug (atorvastatin) Lipitor [14,10]. However the biotransformations of substrates having such a chiral centre remote from the cyano functional group have been reported to proceed with, in many cases, disappointingly low enantioselectivity and chemical yield [6,15–18].

A major issue in the development of a specific biotransformation is to find the appropriate biocatalyst. If there are no commercially available enzyme preparations the desired activities must be found either by screening for enzymatic activity in strains from culture collections or using microbial selection methods [19,20].

Selective enrichment is a method of choice for the isolation of nitrile hydrolysing micro-organisms, where nitrile substrates are used as a nitrogen source [19]. It is subsequently important

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to efficiently screen libraries of isolated strains to identify those possessing the desired reactivity and selectivity. Developing conventional methods such as high performance liquid chromatography (HPLC) or gas chromatography can be tedious and time consuming for assaying a large number of isolates.

Various high-throughput screening methods have been developed for nitrile hydrolysing enzymes, which have been reviewed by Martinkova [20] and Gong [21] recently. For example He et al. reported a simple, rapid and high throughput screening method for assaying nitrile hydrolysing enzymes based on ferric hydroxymate spectrophotometry [22]. Lin et al. found that a combination of ferrous and ferric ions could be used to distinguish α-amino nitriles, α-amino amides and α-amino acids in aqueous solution [23]. Zhang et al. recently developed a solid screen selective culture medium plate with bromcresol purple to screen isolates capable of converting iminodiacetonitrile to iminodiacetic acid [24]. However, colorimetric determination of ammonia as a common product of nitrilases and NHases/amidase activity provides a promising method for rapid screening [25]. For example the Berthelot method which quantifies ammonia formation colorimetrically at 640 nm after reaction with a phenol hypochlorite reagent [26]. Alternatively addition of Nesslers reagent to a solution containing ammonia produces an orange brown colour after development for a certain amount of time [27].

As mentioned previously highly effective and enantioselective biotransformations of β-hydroxynitriles would provide a convenient approach to producing optically active β-hydroxy carboxylic acids [28]. The objective of this study was to screen and identify bacteria with activity towards the β-hydroxynitriles from a novel bacterial isolate bank (previously collected from soil and seaweed) in our laboratory [29,30]. From the soil enrichment cultures a total of 187 isolates were purified, and 67 isolates were obtained from the seaweed enrichments [30]. We now report a high throughput screening strategy for the rapid identification of bacterial isolates possessing nitrile hydrolysing activity towards a variety β-hydroxynitriles including 3-hydroxybutyronitrile, 3-hydroxy-3-phenylpropionitrile and 3-hydroxyglutaronitrile, which represent an aliphatic, aromatic nitrile and dinitrile respectively. We also demonstrate the application of this strategy to a successful enantioselectivity screening study, resulting in the identification of an isolate demonstrating >99.9% ee for 3-hydroxybutyronitrile specifically.

2. Materials and methods

2.1. Materials

Racemic 3-hydroxybutyronitrile and 3-hydroxy-3-phenylpropionitrile were purchased from Sigma–Aldrich. All other chemicals were of analytical grade and obtained from various commercial sources.

2.2. Bacterial isolates

The 254 bacterial isolates used in this study are those described in Coffey et al. [30].

2.3. Nitrile toxicity studies

96 well Megablock® plates (Sarstedt Ltd.) containing 250 μl LB broth (Merck) and 10 mM β-hydroxy nitrile were inoculated from glycerol stocks of the isolate library and incubated at 25 °C with shaking at 250 rpm for 72 h. 5 μL of these cultures then transferred in a 96 well format to M9 [31] agar with modifications [32], (Merck agar-agar ultrapure (15% w/v) prepared in M9-media), containing β-hydroxy nitrile (10 mM) and incubated at 25 °C for 72 h.

2.4. Nitrogen starvation and induction of isolates

2.4.1. Nitrogen starvation

Successful isolates from Section 2.3 were inoculated to 250 μl M9 minimal media broth [31] with modifications [32], containing 10 mM β-hydroxynitrile as the sole source of N. Each well was then inoculated from M9-agar containing β-hydroxynitrile (10 mM) from Section 2.3 before incubation at 25 °C with orbital shaking at 250 rpm for 24 h.

2.4.2. Induction of nitrile metabolising enzyme activity

96-well Megablocks® containing 500 μL of M9 minimal media broth [31] with modifications [32] and β-hydroxy nitrile (10 mM) were inoculated with nitrogen starved isolates (20 μL) from Section 2.4.1 and incubated with shaking for 72 h at 25 °C and 250 rpm. 500 μL of 50% glycerol solution was then added to each culture before storage at −80 °C. These cultures of induced isolates served as inoculation and activity screening stocks for subsequent analyses.

2.5. An investigation of temperature on the growth of various isolates

96-well Megablocks® containing 500 μL of M9 broth and β-hydroxy nitrile (10 mM) were inoculated with nitrile induced isolates (20 μL) from Section 2.4.2 and incubated with shaking for 72 h at 15 °C, 20 °C, 25 °C and 30 °C at 250 rpm. The OD_{600nm} measured and recorded for future studies.

2.6. Standard curve for Nesslers colorimetric assay

Ammonium chloride (100 mM, 5.35 g) was dissolved in deionised water (1000 mL), this solution was used as a stock solution to prepare standards with the following concentration 1 mM, 2 mM, 3 mM, 4 mM and 5 mM. The absorbance was then measured at 425 nm. At NH_3 concentrations up to 10 mmol/L, the $A_{425\,nm}$ was directly proportional to NH_3 concentration $R^2 = 0.9992$.

2.7. Nesslers microscale ammonia assay

Induced isolates from Section 2.4.2 were initially screened for activity towards β-hydroxynitriles using the Nesslers colorimetric assay [27] modified and outlined in [33], in 96-well microtitre plates (Sarstedt Ltd.). Fresh cultures of each isolate were grown in M9-minimal media containing 10 mM nitrile as in Section 2.4.2 before washing 3 times with 500 μL of phosphate buffer). Each 150 μl reaction contained 10 mM nitrile and cells ($OD_{600\,nm} = 0.1$) in potassium phosphate buffer (100 mM, pH 7.0), Microtitre plates were sealed using adhesive film (Sarstedt) and incubated at 25 °C at 250 rpm for 24 h. The reaction was then quenched by adding 37.5 μl of 250 mM HCl. Plates were centrifuged at 500 g for 10 min to pellet the cells/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 min and then the absorbance 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.

2.8. General procedure for enantioselectivity screening towards 3-hydroxybutyronitrile

Racemic nitrile (5.1 mg, 5.9 μL, 10 mM) was added in one portion to a solution of potassium phosphate buffer (0.1 M, pH = 7.2, 6 mL) containing induced cells ($OD_{600\,nm}$ = 1), and activated at 25° C for 30 min with orbital shaking (250 rpm). The reaction was quenched after 24 h by removal of the biomass by centrifugation at 3000 × *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. 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 h. 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) was added before the solution was injected on the Chiral HPLC system. 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.

2.9. General procedure for large scale biotransformation towards 3-hydroxybutyronitrile

The procedure for large scale biotransformation of racemic 3-hydroxybutyronitrile was similar to the general procedure, with the exception of the use of potassium phosphate buffer (0.1 M, pH 7.0, 100 mL) containing 3-hydroxybutyronitrile (85.1 mg, 10 mM). The resulting aqueous solution was basified to pH 12 with aqueous NaOH (2 M) and extracted with ethyl acetate (3 × 100 mL). The aqueous solution was acidified using aqueous HCl (2 M) to pH 2 and extracted with ethyl acetate (3 × 100 mL), dried over $MgSO_4$ and the solvent removed under vacuum. The crude product was subjected to silica gel column chromatography eluted with a mixture of hexane and ethyl acetate (1:1) to give 3-hydroxybutyric acid in 42% yield (44 mg, 4.23 mmol) as clear oil. The configuration of the corresponding acid was determined by comparing the direction of specific rotation with that of an authentic sample. Enantiomeric excess values were obtained from HPLC analysis using a column of chiral stationary phase and correlated with literature. (*R*)-enantiomer elutes at 11.94 min, (*S*)-enantiomer elutes at 12.34 min [34]. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 4.19–4.27 (1H, m), δ = 2.45–2.58 (2H, m), δ = 1.23 (3H, d, J = 6.3 Hz). ^{13}C NMR (400 MHz, $CDCl_3$) δ = 117, 76, 64, 42, 22 $[\alpha]^d_{25}$ = +4.0 (c = 2.5, MeOH), and compared with that in the literature $[\alpha]^d_{25}$ = +4.1 (c = 2.7, MeOH) [35]. This experiment was performed in triplicate.

2.10. Chiral HPLC separations

Chiralcel AD-H and OJ-H columns (all from Daicel Chemical Industries) were used for chiral analysis. Chiralcel AD-H 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. Chiralcel OJ-H was used for the resolution of β-hydroxyamides and nitriles using the same mobile phase conditions with the exception of TFA. The biotransformation products of 3-hydroxybutyronitrile were first derivatised to their corresponding β-benzyloxyethers before analysis.

2.11. Conventional PCR screening and DNA Sequencing

Bacterial genomic DNA was purified using the DNeasy Tissue Kit (Qiagen, Germany) as per the manufacturer's instructions. The 16S ribosomal DNA was amplified using primers 63f and 1387r [36]. The 15 μl reaction mix contained 7.5 μl GoTaq® Green Master Mix (Promega UK), 15 pmoles each primer and 30 ng DNA. The PCR conditions used for amplification were; 1 cycle of 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min, followed by 1 cycle of 72 °C for 5 min. PCR products were purified using the DNA Clean and Concentrator – 5 kit (Zymo Research, CA, USA), as per the manufacturer's instructions. DNA sequencing of PCR products was performed using the BigDye 3.1 kit (Applied Biosystems) as per the manufacturer's instructions and analysed using an ABI Prism 310 Genetic Analyser (Applied Biosystems, CA, USA).

Nucleotide sequences were analysed using the BLASTn or BLASTx software [37] (http://www.ncbi.nlm.nih.gov/BLAST/) from the GenBank (NCBI) database The sequence of isolate SET1 was deposited in GenBank with accession number KF156942.

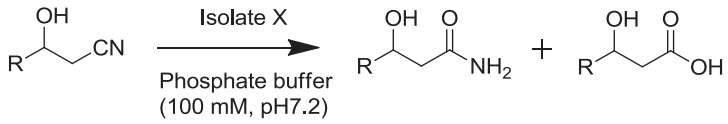
Fig. 1. Nesslers colorimetric activity assay towards 3-hydroxybutyronitrile, an example of library screening for nitrile-hydrolyzing enzyme activity in a 96-well plate format. The biotransformation was carried out at 25 °C with 10 mM nitrile. The reaction was then quenched by the addition of HCl. The biomass was removed by centrifugation. 20 μl of the supernatant was transferred to a microtitre plate, 181 μL of assay mastermix was added. The reaction was allowed to stand for 10 min and then the absorbance read at 425 nm.

3. Results and discussion

3.1. High throughput activity screening

The bacterial isolates were first subjected to toxicity studies with the nitriles of interest. By attempting to grow the different isolates in rich medium in the presence of β-hydroxynitriles, the isolates that were sensitive to the nitrile were identified and excluded from further screening. 145 of the initial 256 isolates could grow in the presence of 3-hydroxybutyronitrile, with 77 demonstrating growth after a 48 h period and the remaining 68 after 144 h. A total of 65 of the isolates grew in the presence of 3-hydroxy-3-phenylpropionitrile and 107 in the presence of 3-hydroxyglutaronitrile. The toxicity study demonstrated that some isolates show no growth in the presence of the nitriles, where as 16 isolates showed growth against two or more nitriles such as isolate SS1-12. The tolerant isolates then underwent a starvation process in order to ensure that the β-hydroxynitrile would be utilised as the nitrogen source during the induction stage, which involved attempting to grow the isolates in minimal media using the β-hydroxynitrile as the sole nitrogen source. As bacterial isolates utilising β-hydroxynitriles as a sole nitrogen source result in the release of ammonia, enzyme activity was monitored using the technique of Nesslerisation. A high-throughput technique was developed using the Nesslers microscale colorimetric assay to screen for activity in the hydrolysis of β-hydroxynitriles to β-hydroxycarboxylic acids using the Nesslers colorimetric assay [27] modified and outlined in [33], in 96 well microtitre plates (Sarstedt Ltd.) as shown in Fig. 1. Nitrile hydrolyzing activity was calculated by measuring the absorbance of the sample at 425 nm, and relating the absorbance to the standard curve in order to determine the concentration of NH_3 in the sample.

Table 1
Activity screening towards β-hydroxynitriles.



Isolate	R	Temperature for optimum growth (°C)	Optimum activity temperature (°C)	Activity[a] (mmol/L)
3-Hydroxyglutaronitrile				
F41	CH_2CN	15	25	6.29
F30	CH_2CN	15	25	2.150
NN30	CH_2CN	20	25	1.790
F32	CH_2CN	15	25	1.726
SS1-4	CH_2CN	15	25	1.468
SS1-24	CH_2CN	20	20	1.433
SET-1	CH_2CN	15	25	1.144
F37	CH_2CN	15	25	1.140
SS1-18	CH_2CN	25	25	0.910
SS1-12	CH_2CN	15	25	0.772
3-Hydroxybutyronitrile				
NN32	CH_3	15	25	9.91
F71	CH_3	15	25	4.389
NN30	CH_3	15	20	3.120
SS1-31	CH_3	20	30	2.42
LC3c	CH_3	30	20	1.521
SS1-12	CH_3	15	25	1.430
F69	CH_3	20	20	1.206
SET-1	CH_3	15	25	1.021
Sw2-33	CH_3	30	20	1.00
F36	CH_3	20	25	0.515
3-Hydroxy-3-phenylpropionitrile				
SS1-14	Ph	15	25	8.07
F62	Ph	15	25	7.64
SS1-12	Ph	15	25	1.61
SS1-24	Ph	15	25	0.21

[a] Activity determined using Nessler's colorimetric activity (24 h) assay expressed in terms of concentration (mmol/L) of NH_3 produced @ reported optimum temperature (column 4).

Reaction temperature is a key parameter in biotransformations which can significantly influence the activity, enantioselectivity and stability of a biocatalyst [38]. The isolate library was first screened for the hydrolysis of β-hydroxynitriles to β-hydroxycarboxylic acids at 25 °C and subsequently at various temperatures (Table 1). Based on the activity assay results the isolates were then divided into three subcategories based on the concentration of NH_3 produced (0.08–0.6 mmol/L = low activity, 0.6–5.8 mmol/L = good activity and >5.8 mmol/L = high activity). Preliminary results revealed that 47 isolates demonstrated activity towards 3-hydroxybutyronitrile, 34 isolates towards 3-hydroxyglutaronitrile while only 4 of the initial 256 isolates catalysed the hydrolysis of 3-hydroxy-3-phenylpropionitrile. This indicated sensitivity towards the phenyl ring at the b-position on the enzymatic reaction, and this is demonstrated in the ability of isolate to SS1-24 to show good activity towards 3-hydroxyglutaronitrile (1.43 mmol/L) where the same isolate demonstrates low activity (0.21 mmol/L) towards 3-hydroxy-3-phenylpropionitrile. This enabled the 10 isolates demonstrating the highest activity towards the target substrates to be selected for subsequent screening and optimisation.

As the bacterial strains have been isolated from various locations such as soil and sea weed, these isolates may show different growth patterns at various temperatures. It was decided also to monitor the growth of the 24 chosen isolates in the presence of the key nitriles at various temperatures ranging from 15 to 30 °C, and in each case an optimum temperature for induction was also determined for all future studies.

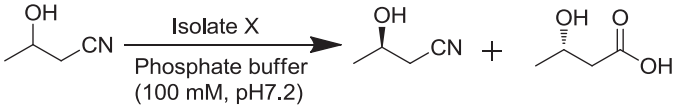
No significant influence of reaction temperatures on the activity of the chosen isolates was observed when the isolates were assayed at various temperatures ranging from 15 °C to 30 °C. The majority of isolates demonstrated their highest activity at 25 °C however in contrast optimum growth was observed at lower temperatures in many cases. Table 1 indicates the temperature for optimum growth and also the temperature which resulted in the optimum activity of the isolate.

This high throughput screening strategy allowed for the rapid identification of isolates demonstrating activity towards β-hydroxynitriles. The toxicity testing, nitrogen starvation and activity screening serve as a preliminary study where active isolates can be identified. The ultimate aim of this work was to identify novel enantioselective strains. In previous studies strains were isolated by enrichment with nitriles such as acetonitrile, benzonitrile, adiponitrile and acrylonitrile [29]. Our work attempted to induce strains directly with the target substrates to determine if enantioselective enzyme systems could be produced. Following the preliminary studies reported above the 10 isolates demonstrating the highest activity towards 3-hydroxybutyronitrile were chosen for further enantioselectivity screening towards 3-hydroxybutyronitrile (Table 1) to identify a link between our activity screening and enantioselectivity, results are presented in Table 2. This work serves as a demonstration of the application of this high-throughput screening strategy for the identification of both active and enantioselective nitrile hydrolysing isolates.

3.2. *Screening for enantioselective biotransformations of 3-hydroxybutyronitrile*

Having reduced the bacterial isolates from 256 to 24 having good to high activity towards β-hydroxynitriles, attention was focused on determining the enantioselectivity of the isolates. Initial enantioselectivity screening involved the biotransformation of racemic

Table 2
Enantioselective hydrolysis of 3-hydroxybutyronitrile catalysed by novel bacterial isolates.



Entry	Isolate	Optimum temperature[b] (°C)	Nitrile ee[c] (%)	Acid ee[c] (%)	Activity[d]	Refs.
1[a]	F36	15	13.9	31.6	Low	
2[a]	F69	15	1.4	27.6	Good	
3[a]	F71	15	12.5	2.0	Good	
4[a]	SS1-12	25	26.2	14.0	Good	
5[a]	SET1	15	ND	99.9	High	
6[a]	Paenibacillus sp. NN32	15	1.3	25.5	High	[1]
7[a]	*Burkholderia sp. LC3c*	15	5.2	13.1	High	[2]
8[a]	SW2 33, Serratia sp.	15	2.3	ND	Low	
9[a]	*Bacillus sp. NN30*	15	22.3	58.5	Good	[1]
10[a]	*SS1-31*	25	16.2	18.7	Good	

[a] Biotransformation was carried out by incubating 3-hydroxybutyronitrile (10 mmol/L) in a suspension of the named isolate (OD = 1) in phosphate buffer (pH 7.0) for 24 h.
[b] Temperature for optimumenantioselectivity.
[c] Determined by HPLC analysis using a chiral column (see Section 2.8).
[d] Activity determined using Nessler's colorimetric activity assay (at optimum temperature).

3-hydroxybutyronitrile. The reaction was carried out conveniently using whole cells of the isolates at 25° C in aqueous phosphate buffer (pH 7.0). The hydrolysis products were transformed to the corresponding benzyloxy derivatives *in situ* for ease of detection using HPLC. As the specific nitrile metabolising enzyme content of the cells was unknown the isolates were assayed for activity to produce both amide and the corresponding carboxylic acid. It was assumed that the cells possessed a nitrilase system as during the hydrolysis of nitriles with resting cells, no amide could be detected and, furthermore the amide was not used as a substrate. In all cases the nitrile was partially consumed during the incubation period, which is in agreement with the results obtained using the high throughput screening strategy.

This chiral screening study identified a single isolate SET1 which demonstrated exceptional enantioselectivity towards 3-hydroxybutyronitrile (>99.9% ee), (entry 5, Table 2). The effect of temperature on the enantioselectivity of the biotransformation was examined at 15 °C and 25 °C. Several isolates demonstrated higher enantioselectivity at lower temperatures, for example an increase in enantioselectivity was observed in the case of F36 from 21.3% to 31.6% ee, when the temperature was varied from 25 °C to 15 °C (entry 1, Table 2). In the case of isolate SET1 at both temperatures the corresponding acid was produced in >99.9% ee. In this case only a slight decrease in activity from 1.02 mmol/L to 0.98 mmol/L was observed when the temperature was reduced from 25 °C to 15 °C respectively.

Isolate SET1 (entry 5, Table 2), fell within the 'high' activity range of the screening scale however it did not have the highest activity as determined using the Nesslers colorimetric assay. In comparison, isolate NN32 (entry 6, Table 2) showed the highest activity (Table 1) but demonstrated much lower enantiocontrol. Isolate SET1 also only generated the corresponding (*S*)-carboxylic acid, and no amide was detected; thus it is believed that this isolate possesses a highly enantioselective nitrilase. It is also surprising to note that in the case of SET-1 the corresponding unreacted (*R*)-nitrile could not be detected in the reaction mixture after the 24 h reaction time.

To determine yield and configuration of the acid product formed using isolate SET1in the hydrolysis of racemic 3-hydroxybutyronitrile, a large scale biotransformation was carried out over 24 h. The *ee* value of 3-hydroxybutyric acid was measured by chiral HPLC analysis, and the absolute configuration was determined as (*S*) by comparison of optical rotation with an authentic sample [35]. The product 3-hydroxybutyric acid was isolated from the reaction mixture using column chromatography in 41% yield and >99.9% *ee*. The unreacted (*R*)-nitrile could not be recovered from the reaction mixture.

Having identified this very promising isolate SET1 we decided to further focus our efforts on this isolate and explore the high enantioselectivity isolate SET1 demonstrated towards 3-hydroxybutyronitrile. An enantioselectivity *vs.* time study was carried out where the biotransformation was monitored after 3, 6, 9, 24 and 36 h; results indicate that isolate SET1 is highly enantioselective and possibly enantiospecific with >99.9% ee of (*S*)-acid at each sample point. It is believed the hydrolysis of the (*S*)-nitrile proceeds rapidly, as only the remaining (*R*)-nitrile is detected after 3 h. This was further demonstrated by the formation of only *(S)*-3-hydroxybutyric acid after a 10 day incubation period. While the reaction afforded the corresponding (*S*)-3-hydroxybutyric acid with excellent enantioselectivity, neither amide nor the starting nitrile could be recovered after this time. In addition the reduction in intensity of the remaining (*R*)-nitrile is also evident after various timepoints, with complete consumption after 24 h, indicating the potential metabolism of the unreacted (*R*)-nitrile along with the desired biotransformation.

The exponential decay of the unreacted nitrile may be due to other enzyme systems present within the microbial whole cells. A possibility may be hydrolysis of the nitrile to the corresponding acid followed by isomerisation. An investigation into the deracemisation of the racemic 3-hydroxybutyric acid was carried out by incubation with the isolate over a 24 h period; however unchanged racemic acid was recovered. Other options include the possibility of an isomerase converting the remaining (*R*)-nitrile to (*S*)-nitrile which may then be converted to the corresponding (*S*)-acid, however the isolated yield never exceeds 50%. Other enzymes such as an aldoxime or alcohol dehydrogenase may also account for the loss of mass balance.

It has been reported that the enzyme catalysed kinetic resolution of β-substituted nitriles can demonstrate low enantioselectivity in some cases [39,15,16,17,40,18]. For example in the biotransformation of 3-hydroxybutyronitrile using whole cells of *Rhodococcus erythropolis* AJ270 containing a nitrile hydratase/amidase system, low enantiocontrol was observed with the formation of *(S)*-3-hydroxybutyric acid (17.8% ee) [34]. The enantioselectivity of the reaction was dramatically improved using a benzyl protection/docking strategy which resulted in the formation of the acid with 86.2% enantiomeric excess. In the case

of SET1 >99.9% ee may be achieved using 3-hydroxybutyronitrile with the free unprotected hydroxy group. The high enantioselectivity demonstrated by isolate SET1 may be as a result of the position of the chiral recognition site in the enzyme which is possibly located remote to the catalytic centre [35], this has also been observed by previous authors [6,34]. While the biocatalytic hydrolysis of β-hydroxynitriles to corresponding β-hydroxy acids and amides using microorganisms possessing nitrile hydratase/amidase have been well reported [41,18,39], in contrast microorganisms with nitrilase activity often demonstrate low or extremely low activity towards the hydrolysis of β-hydroxynitriles [7,9,18,39]. To the best of our knowledge this is the first isolate potentially demonstrating enantiospecificity towards 3-hydroxybutyronitrile.

DNA sequencing of a 432 bp region of the 16s rDNA of isolate SET1 indicates that it is a strain of *Rhodococcus erythropolis*. It is 100% identical over the sequenced region to *Rhodococcus* sp. TMS1-19 (GenBank acession JX949804) a strain isolated from a glacier in China.

4. Conclusion

In summary, this work describes the development of a high-throughput screening strategy for the identification of isolates with nitrile hydrolysing activity towards β-hydroxynitriles. This high-throughput method was used to identify a range of isolates demonstrating activity towards several β-hydroxynitriles. In a subsequent enantioselectiveity screening study of isolates demonstrating the desired activity towards 3-hydroxybutronitrile, an isolate demonstrating a highly enantioselective if not enantiospecific activity towards 3-hydroxybutyronitrile was identified. Hence a biosynthetic pathway for the production of (*S*)-3-hydroxybutyric acid from racemic 3-hydroxybutyronitrile was established using this novel *Rhodococcus erythropolis* strain SET1. This cost effective screening strategy developed in this study can be used to screen a large number of microorganisms for their ability to transform aliphatic, aromatic nitriles and dinitriles within a short time period, in particular the addition of a toxicity screen towards the various nitriles, further focuses the strategy. Further screening techniques can be applied to candidates to determine their enantioselectivity; of note in this particular study isolates with higher enantioselectivity generally did not display the highest activity, for example SET1 demonstrated moderate activity towards 3-hydroxybutyronitrile, however it had the highest enantioselectivity. This highlights the importance of carefully selecting the activity specifications of isolates chosen for enantioselectivity screening. Further studies of *R. erythropolis* SET1 towards the understanding of its remarkable enantioselectivity and the substrate scope towards various β-hydroxynitriles, including the effect of various substituents on the enantioselectivity of the biotransformations are actively being investigated in our laboratory.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2013.08.001.

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