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Expanding the repertoire of nitrilases with broad substrate specificity and high substrate tolerance for biocatalytic applications

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ABSTRACT

Enzymatic conversion of nitriles to carboxylic acids by nitrilases has gained significance in the green synthesis of several pharmaceutical precursors and fine chemicals. Although nitrilases from several sources have been characterized, there exists a scope for identifying broad spectrum nitrilases exhibiting higher substrate tolerance and better thermostability to develop industrially relevant biocatalytic processes. Through genome mining, we have identified nine novel nitrilase sequences from bacteria and evaluated their activity on a broad spectrum of 23 industrially relevant nitrile substrates. Nitrilases from *Zobellia galactanivorans*, *Achromobacter insolitus* and *Cupriavidus necator* were highly active on varying classes of nitriles and applied as whole cell biocatalysts in lab scale processes. *Z. galactanivorans* nitrilase could convert 4-cyanopyridine to achieve yields of 1.79 M isonicotinic acid within 3 h via fed-batch substrate addition. The nitrilase from *A. insolitus* could hydrolyze 630 mM iminodiacetonitrile at a fast rate, effecting 86 % conversion to iminodiacetic acid within 1 h. The arylaliphatic nitrilase from *C. necator* catalysed enantioselective hydrolysis of 740 mM mandelonitrile to (*R*)-mandelic acid in 4 h. Significantly high product yields suggest that these enzymes would be promising additions to the suite of nitrilases for upscale biocatalytic application.

1. Introduction

Nitrile compounds are ubiquitous in the environment [1] and can also be prepared from chemical synthesis using several routes, such as ammoxidation [2]. Nitrile-converting enzymes are therefore much sought after for biocatalytic applications in the synthesis of several carboxylic acids and amides as pharmaceutical precursors and bulk chemicals. Nitrilases (E.C. 3.5.5.1) can catalyze the single step hydrolysis of nitriles to carboxylic acids [3] and ammonia, via the conserved catalytic tetrad (Glu-Lys-Glu-Cys) in their active site [4]. The use of nitrilases enables eco-friendly nitrile hydrolysis under mild reaction conditions, in comparison to traditional chemical processes that tend to operate at extreme pH. Nitrilases further offer the added advantages of excellent enantio- and regioselectivity, which are helpful in the synthesis of enantiopure carboxylic acids and cyanocarboxylic acids that are very difficult to achieve using chemical hydrolysis [5,6]. In recent years, nitrilase biocatalysts have been successfully developed to facilitate the industrial production of fine chemicals such as nicotinic acid, acrylic acid, mandelic acid and D-amino acids [3]. Other products of interest include amino or hydroxy carboxylic acids and cyanocarboxylic acids, which are often employed as active intermediates in the synthesis of high value pharmaceuticals such as atorvastatin, (*S*)-pregabalin, (*R*)-baclofen and gabapentin [7]. Moreover, nitrilases have also found application in the surface modification of acrylic fibres in the textile industry [8] and in the bioremediation of toxic nitriles in waste water [9].

Intensive research efforts have been made in the last decade to identify novel nitrilases through genome mining or metagenomic analysis [10], and engineer them to develop bioprocesses for the efficient synthesis of such carboxylic acids. A large number of nitrilases from bacteria [10] belonging to genera such as *Alcaligenes*, *Bradyrhizobium*, *Acidovorax, Pseudomonas* etc., plants (*Arabidopsis*, *Brassica*) [11] and fungal sources including *Fusarium* sp., *Aspergillus niger*, *Giberella* and *Rhodococcus ruber* [12,13] have been identified with specificity for different nitriles. However, there still exists a relevant need for robust nitrilases that can efficiently hydrolyze a broad substrate spectrum of chemically diverse nitriles. A major obstacle in the economically viable synthesis of carboxylic acids using nitrilase biocatalysts is inhibition by the nitrile substrate or accumulated product. High substrate/product tolerant nitrilase biocatalysts are therefore important for the development of industrial applications [14]. Further, the low aqueous solubility

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of several nitriles requires the identification of nitrilase enzymes that possess high operational stability in biphasic media [15].

With a wealth of increasing sequence data, it has been possible in recent years to mine for enzymes from less explored sources and environments using bioinformatics analyses [10,16,17]. In this study, we have mined an evolutionarily diverse set of nine nitrilases from sequence databases that exhibit significant activity over a fairly broad spectrum of industrially relevant nitrile substrates. The nitrilase genes were cloned and overexpressed in *E. coli* BL21 (DE3) and screened for their ability to hydrolyze a panel of chemically diverse nitriles. Selected nitrilases were further evaluated with respect to their nitrile tolerance and subsequently their applicability in biotransformation. Using three nitrilases mined from this study with different substrate specificities, lab scale processes were established for the production of nicotinic and isonicotinic acids, (*R*)-mandelic acid and iminodiacetic acid with improved space-time yields.

2. Materials and methods

2.1. Chemicals and media

Lysogeny broth (LB), yeast extract and carbenicillin used for cultivation were procured from HiMedia (Mumbai, India). Enzymes, kits and reagents for genetic manipulation were purchased from New England Biolabs Inc. (Ipswitch, MA, USA). Nitrile substrates and carboxylic acid products were purchased from Sigma Aldrich (St. Louis, MO, USA) or Tokyo Chemical Industry Ltd. (Tokyo, Japan). Nitrilase genes were codon optimized for expression in *E. coli* and custom-synthesized by ThermoFisher Scientific (Ipswitch, MA, USA). Primers were procured from Eurofins Genomics Pvt. Ltd. (Bangalore, India). HPLC grade solvents were obtained from Fisher Scientific (Ipswitch, MA, USA). All other chemicals used were of analytical grade.

2.2. Mining for candidate enzymes, cloning and expression

Sequences of nitrilases from *Syntrophobacter fumaroxidans* (UniProt ID A0LKP2), *Sphingomonas witichii* (A5VE02), *Paraburkholderia graminis* (B1G3B9), and bll6402 from *Bradyrhizobium japonicum* (Q89GE3) were chosen as query sequences based on their broad substrate spectrum and used as query for mining new nitrilase sequences. Sequences with identity between 30–70 % to the query sequences were retrieved from NCBI database using the blastp search tool. The identified sequences were further processed to remove redundancy and filtered based on their similarity to reported nitrilases and environmental habitat of the source organism. Nine putative nitrilase sequences (Table S1, Fig. 1) were selected as representatives from phylogenetic analysis. The sequences were codon-optimized for expression in *E. coli* (Supplementary information 2) and custom synthesized. The putative nitrilase genes were first PCR-amplified using a high-fidelity Q5 polymerase (NEB) employing specific primers (Table S1). The PCR products and pET21a vector were digested with NdeI and XhoI restriction enzymes, and following gel extraction, ligated using T4 DNA ligase. For three nitrilases (*Cupriavidus necator*, *Beijerinckia* sp. and *Achromobacter insolitus*) cloning was performed using a ligation-independent polymerase incomplete primer extension (PIPE) cloning approach [18]. The recombinant plasmids were transformed into *E. coli* TOP10 cells, and following sequence verification further introduced into *E. coli* BL21 (DE3) cells for protein expression. All nitrilase genes were cloned without a His-tag.

2.3. Cultivation of recombinant strains for nitrilase production

E.coli BL21 cells expressing recombinant nitrilase were cultivated using a shake flask fed-batch protocol [19]. The seed culture grown overnight in LB was inoculated (3% v/v) into 500 mL baffled Erlenmeyer flask containing 100 mL LB medium supplemented with 150 µg/ml carbenicillin. Cultivation of recombinant *E. coli* was done at 32 °C and 180 rpm. Nutrient doses comprising 25 % glycerol (1 mL) and 50 % yeast extract (2 mL) were fed 6 h and 9 h after inoculation, respectively. Protein expression was induced 8 h after inoculation by the addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG), and the temperature was reduced to 25 °C. Cells were harvested 16 h after induction and lysed through sonication in buffer containing 50 mM Tris Cl (pH 7.5), 0.1 M NaCl, 1 mM DTT and 0.5 mM EDTA. The crude lysate was clarified using centrifugation at 12,000 rpm for 10 min. Protein was quantified using Bradford [20] method and nitrilase expression was checked by electrophoresis on 12 % polyacrylamide gel (SDS-PAGE [21]).

2.4. Nitrilase screening and enzyme assay

Conversion of nitrile substrates by recombinant nitrilases was screened using a microplate assay employing the phenol-hypochlorite method [22]. Whole cells (~ 0.1 mg dry cell weight) were incubated with 10 mM nitrile substrate in a 200 µl reaction on a 96-well microplate. Nitrile substrates were prepared in methanol as 100 mM stocks, ensuring an effective concentration of 10 % methanol in the assay mixture to aid solubility of the nitrile substrates. The reaction mixture contained 100 mM phosphate buffer pH 7.4 and was incubated at 37 °C; and the amount of ammonia formed as the by-product was estimated after 1 h or 4 h.

For selected enzymes, nitrilase activity was assayed using appropriate amount of cells in 200 µl reaction. After 10 min incubation at the optimum pH and temperature conditions (described in Results section), 20 µl of 1 M HCl was added to quench the reaction. The reaction mixture was centrifuged (12,000 rpm for 10 min) and filtered using a 0.45 µm syringe filter, and the product formed was monitored through high performance liquid chromatography (HPLC) analysis. One unit (U) of nitrilase was defined as amount of enzyme that catalysed the conversion of 1 µmol nitrile substrate per min.

Dry cell weight (DCW) measurements were made in triplicates from washed cell pellets of 1 mL culture (OD_{600} = 25) dried for 14 h at 65 °C. For the nitrilase assay, cells harvested from fresh culture were washed and resuspended in 0.1 M phosphate buffer pH 7 and an appropriate volume corresponding to the given dry weight was added to the enzyme reaction.

2.5. Biocatalytic nitrile conversion by selected nitrilases

A typical reaction mixture (5 mL in 25 mL shake flask) contained the nitrile substrate and appropriate amount of *E. coli* cell mass expressing the selected nitrilase suspended in 100 mM phosphate buffer pH 7. In the case of mandelonitrile, conversion experiments were performed in aqueous buffer containing 10 % methanol to aid substrate solubility. The catalytic reaction was performed at 37 °C with agitation at 150 rpm in an incubator shaker. Aliquots (100 µl) were withdrawn at regular predetermined time intervals and quenched with 10 µl of 1 M HCl. The reaction mixture was centrifuged and filtered using a 0.45 µm syringe filter, and the product formed was monitored through HPLC analysis.

2.6. Analytical methods

The residual nitrile substrates and acid products were quantified using analytical HPLC on a Shimadzu Nexera X2 system (Kyoto, Japan) equipped with a photodiode array (PDA) detector. Standard HPLC analyses were performed on a reverse phase Luna C18 column (150 mm x 4.6 mm, 5 µm, Phenomenex) with 10 µl sample injections. For cyanopyridines, the sample was eluted using a mobile phase of trifluoroacetic acid (TFA, 0.1 % in water) and acetonitrile at 65:35 v/v at a flow rate of 0.7 mL/min. The concentration of mandelonitrile and mandelic acid was determined in a 50:50 mobile phase of TFA (0.1 % in water): methanol at a flow rate of 0.8 mL/min. For the detection of

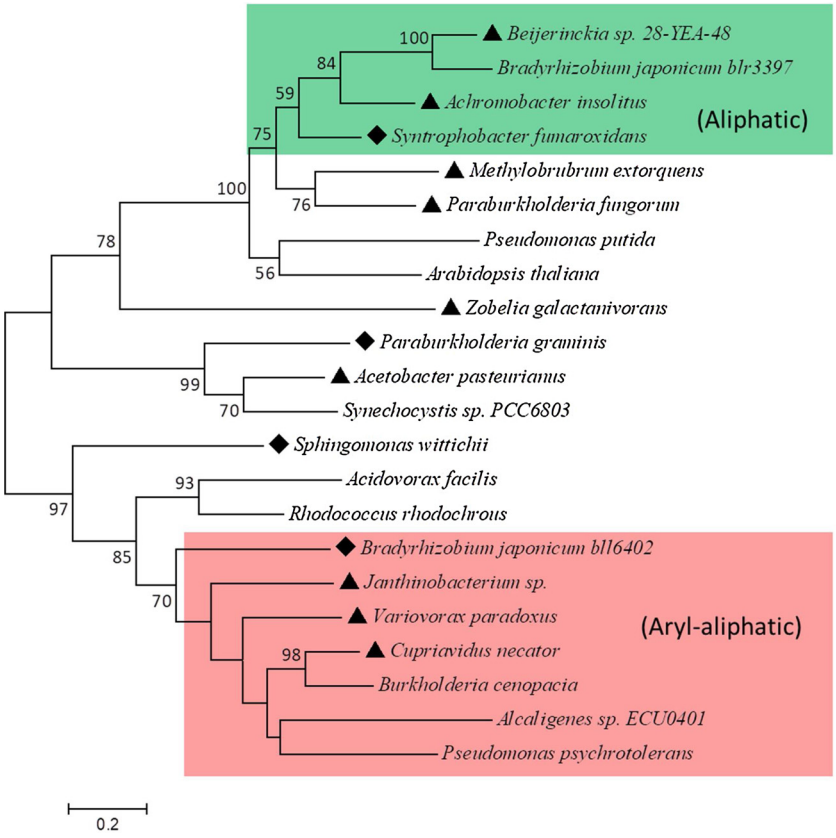

Fig. 1. Phylogenetic tree of nitrilases. Nitrilase sequences were aligned using MUSCLE, and phylogenetic analysis was performed using a Maximum Likelihood method. Numbers denote bootstrap values (% of 500 replications). (♦) denotes query sequences, (▲) denotes novel sequences mined in this study.

iminodiacetic acid, 5 µl of the filtered sample (diluted to < 50 mM IDA concentration) was mixed with 100 µl of 250 mM $NaHCO_3$ and then 20 µl of 1-fluoro 2,4-dinitrobenzene (FDNB, 1% v/v in acetonitrile). The solution was incubated for 1 h at 60 °C and quenched by adding 375 µl water before injecting on the HPLC column [23]. A mobile phase of 20 mM sodium acetate buffer pH 5.0 and methanol in the ratio 45:55 was used for elution of the product derivative. Peak areas at 210 nm (for mandelonitrile), 264 nm (cyanopyridines) and 365 nm (for derivatized iminodiacetic acid) and retention times were compared to authentic chemical standards.

The optical purity of mandelic acid formed was analysed by chiral HPLC on a Lux-cellulose 1 column (150 mm x 4.6 mm, 5 µm, Phenomenex) using an isocratic elution with Hexane: Isopropanol: TFA (95:5:0.1, v/v) at 0.8 mL/min. Enantiomer peaks detected at 228 nm and compared to authentic chemical standards.

2.7. Data collection and analysis

Experimental data were collected from three independent experiments for nitrilase screening and activity measurements, and results are expressed as mean ± standard deviation. Time-course data for biocatalytic conversion are from duplicate experiments.

3. Results and discussion

3.1. Selection of nitrilases

We adopted a genome mining approach for the identification of novel nitrilase sequences with potential for biocatalytic application. Nitrilases reported in literature as having broad substrate specificity were chosen as query sequences. The nitrilase bll6402 from *Bradyrhizobium japonicum* [24] has been documented for the conversion of mandelonitrile, hydroxynitriles and synthesis of cyanocarboxylic acids [25], while *Paraburkholderia graminivorans* BCG4 nitrilase [26] displayed wide-spectrum substrate specificity for aliphatic as well as aromatic nitriles. In addition, nitrilases from *Sphingomonas wittichii* and *Syntrophobacter fumaroxidans* were identified as acting on a broad spectrum of structurally diverse nitriles in a mining study by Vergne-Vaxelaire et al. [27]. Protein sequence hits were derived from a blastp search with 30–70 % similarity with these template nitrilase sequences and filtered to remove redundancy. Sequence alignment was performed using MUSCLE algorithm on MEGA7 software tool, to check the conservation of active site residues specific to nitrilases. Following phylogenetic analysis, nine candidate nitrilase sequences were selected as representatives from different clusters to potentially accommodate a broad substrate spectrum and diverse environmental habitat of the source organism (Table S1, Fig. 1). The nitrilase sequences from *Achromobacter insolitus*, *Methylobacterium extorquens* and *Beijerinckia* sp. showed 55–65 % homology with the *S. fumaroxidans* query sequence and blr3397 from *Bradyrhizobium japonicum* [28], indicating a potential aliphatic substrate preference. The nitrilases from *Cupriavidus necator*, *Janthinobacterium* sp. and *Variovorax paradoxus* clustered along with enzymes from *Burkholderia cenacepacia, Alcaligenes* sp. and bll6402 from *B. japonicum* that have been identified as acting on arylaliphatic nitriles such as mandelonitrile derivatives (Fig. 1). The nitrilase from *Z. galactanivorans* was chosen as a representative of marine bacteria and shared a low level of sequence identity (31 %) with the query sequences (Table S2).

3.2. Nitrilase expression and activity screening

The development of an efficient biocatalyst necessitates screening of activity and tolerance to high concentrations to substrates of industrial relevance. For analysis of activity and substrate preference, the nine candidate nitrilase genes were cloned and over-expressed in *E. coli* BL21. Nitrilase expression was achieved using a modified shake-flask protocol with added doses of glycerol and yeast extract [19] that allowed for enhanced cell growth and protein synthesis. While nitrilases

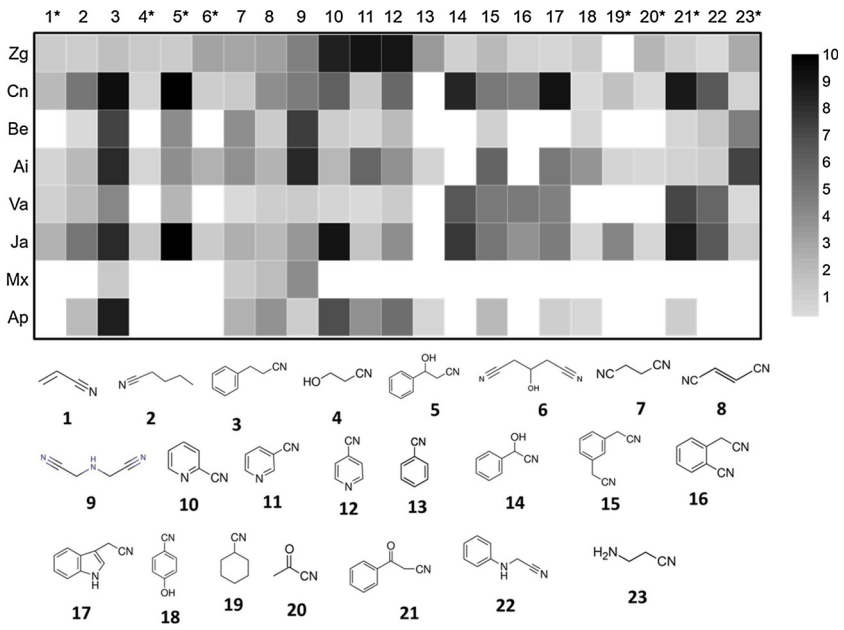

Fig. 2. Nitrilase activity screening (a) Heat map representing catalytic conversion of 23 nitrile substrates by nitrilases mined in the study. Whole cells (OD_{600} = 0.1) expressing the recombinant nitrilase were incubated with 10 mM nitriles in 100 mM phosphate buffer pH 7.0 and 37 °C for 1 h or 4 h (substrates denoted *). Gradation denotes mM substrate converted during the reaction. Blank cells denote the absence of nitrilase activity. (b) Structures of nitriles used in the substrate panel.

from *A. insolitus*, *Acetobacter pasteurianus*, *C. necator* and *Z. galactanivorans* were expressed well in soluble form compared to empty pET21a control, enzymes from *Beijerinckia* sp., *Janthinobacterium* sp. and *V. paradoxus* exhibited relatively lower levels of protein expression with a significant inclusion body fraction (Figure S1). Nevertheless, all the nitrilases yielded bands at 35 − 40 kDa in SDS-PAGE analysis, in agreement with the predicted subunit molecular weight [3].

Nitrilases are generally used as whole cell biocatalysts, where the catalytic efficiency is determined both by specific activity as well as expression level of the enzyme. Screening of nitrilase activity was therefore performed using whole cell suspensions, by quantifying the ammonia produced during the reaction [22]. The nitrilases were screened against a panel of 23 structurally diverse nitrile substrates including aliphatic and aromatic mono/dinitriles, hydroxylated, carbonyl and aminonitriles (Fig. 2). All substrates were commercially available and were used at a concentration of 10 mM in the assay. *E. coli* cells with empty pET21a used as negative control did not show detectable nitrilase activity with any of the substrates studied under the conditions used for screening. The nitrilases from *C. necator, A. insolitus*, *Z. galactanivorans* and *Janthinobacterium* sp. were observed to be the most promiscuous, with activity on more than 75 % of the substrates tested. As expected from their sequence similarity to *S. fumaroxidans* [27], nitrilases from *A. insolitus* and *Beijerinckia* sp. exhibited significant preference for iminodiacetonitrile and 3-aminopropionitrile. The enzymes were also able to convert some arylaliphatic nitriles like 3-phenylpropionitrile, and aliphatic dinitriles succino- and fumaronitrile. On the other hand, the nitrilases from *C. necator*, *Janthinobacterium* sp. and *V. paradoxus* preferred arylaliphatic nitriles with high activity on mandelonitrile, indole acetonitrile and 3-phenylpropionitrile (Fig. 2). In addition, the enzymes were able to hydrolyze β-hydroxy, keto and amino- substituted arylaliphatic nitriles. Their substrate spectrum also included aromatic dinitriles such as 1,3-phenylenediacetonitrile (PDA) and 2-cyanophenylacetonitrile (CPA). Cyanocarboxylic acids synthesized by the regioselective conversion of these dinitriles might be used as building blocks for pharmaceuticals and agrochemicals [7,29]. The nitrilase from marine bacterium *Z. galactanivorans* exhibited excellent substrate promiscuity with a preference for aromatic nitriles such as cyanopyridines and benzonitrile. Of the nine enzymes tested, the nitrilase from *M. extorquens* showed narrow substrate specificity for aliphatic dinitriles; while the *P. fungorum* nitrilase did not show significant activity on any of the nitriles in the substrate panel.

Based on the evaluation of nitrilase activity and expression levels, the nitrilases from *Z. galactanivorans*, *C. necator* and *A. insolitus* were selected for preparative scale conversions to demonstrate their suitability in potential biocatalytic applications for the production of isonicotinic acid, (*R*)-mandelic acid and iminodiacetic acid, respectively. *E. coli* cells expressing the appropriate recombinant nitrilase were used for preparative biocatalytic reactions. The biocatalytic reactions were generally performed in operating conditions of pH 7.0–7.5 and 37 °C, in order to ensure long-term stability of the whole cell biocatalysts.

3.3. Conversion of cyanopyridines

Nicotinic acid is an important food additive used as a vitamin supplement, and is also an intermediate in the production of inositol, hexanicotinate and *N,N*-diethylnicotinate [30]. The biocatalytic conversion of 3-cyanopyridine to nicotinic acid has gained considerable attention as a mild and efficient alternative to chemical methods involving the use of metal catalysts [31], enabling a global production of nicotinic acid up to 40,000 tons [32]. Nitrilases or nitrile hydratase-amidase systems from *Rhodococcus rhodochrous* [33], *Acidovorax facilis* 72W [34], *Alcaligenes fecalis* [35], *Ralstonia eutropha* [36], *Pseudomonas putida* [37], *Nocardia globerula* [31] and *Gibberella intermedia* CA3 − 1 [38] have been identified for nicotinic acid production. On the other hand, the production of isonicotinic acid from 4-cyanopyridine is relatively less explored. Isonicotinic acid is a commercially important intermediate in the production of anti-tuberculotic preparations such as isoniazid, anti-depressants (nialamide) and the anti-histamine terfenadine [39]. In our study, the nitrilase from the marine bacterium *Z. galactanivorans* was found to convert both cyanopyridine isomers to the respective carboxylic acids with negligible amide formation (< 1%) during the reaction. Therefore the enzyme was further evaluated for cyanopyridine conversion.

As a whole cell biocatalyst, *Z. galactanivorans* nitrilase was observed to exhibit higher activity on 4-cyanopyridine (9.5 U/mg DCW) over 3-cyanopyridine (4.1 U/mg DCW) when assayed at 50 mM substrate concentration. While maximum nitrilase activity was achieved at 40 °C, the enzyme could exhibit at least 80 % of maximum activity in the range 35 − 50 °C (Figure S2). The nitrilase was also highly active in the pH range 5–9, with the optimum pH at 6.5 − 7.0. The activity decreased drastically when the pH dropped below 5.0. A similar biochemical profile on cyanopyridine has been reported for the nitrilase from *Ralstonia eutropha* [36].

The *Z. galactanivorans* nitrilase also exhibited good tolerance for

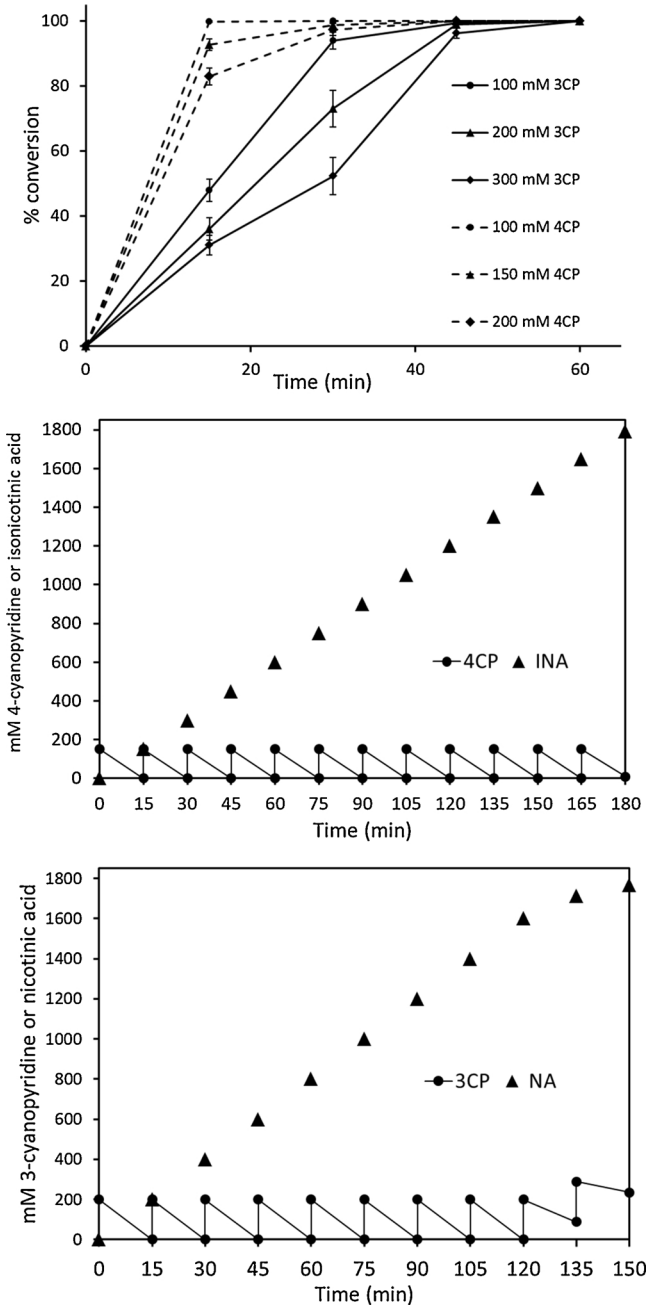

Fig. 3. Conversion of cyanopyridines by *Z. galactanivorans* nitrilase. (a) Effect of substrate concentration on the production of nicotinic and isonicotinic acid. (b) Time course of 3-cyanopyridine conversion to nicotinic acid in fed-batch mode. (c) Time course of 4-cyanopyridine conversion to isonicotinic acid in fed-batch mode.

both 3- and 4-cyanopyridine as substrates (Fig. 3a). Overall, while the reaction was faster with 4-cyanopyridine, a higher substrate tolerance was observed with 3-cyanopyridine. A resting cell concentration at 1.3 g/l DCW was able to completely convert up to 200 mM 4-cyanopyridine in 30 min, with 85 % conversion occurring within 15 min (Fig. 3a). In comparison, the nitrilase from *N. globerula* [40] converted 200 mM 4-cyanopyridine with 10 U/mL nitrilase loading in 40 min, while the *P. putida* nitrilase [41] at 3 g/l DCW could completely convert only up to 100 mM 4-cyanopyridine in 1 h. In the case of 3-cyanopyridine, *Z. galactanivorans* nitrilase was able to achieve complete conversion up to 200 mM substrate concentration after 30 min. However, the rate of the reaction was slower than for 4-cyanopyridine with 40 % conversion in 15 min. When the 3-cyanopyridine concentration was increased to 300 mM, the rate of conversion slowed down probably as a consequence of substrate toxicity with 98 % conversion achieved after 45 min (Fig. 3a).

Subsequently, fed-batch processes were designed using whole cell biocatalyst expressing *Z. galactanivorans* nitrilase with the objective to relieve the substrate inhibition and enhance the product yield. Based on preliminary experiments, the enzyme loading was kept at 6.5 g/l DCW of the biocatalyst while the initial substrate concentration was 150 mM 4-cyanopyridine or 200 mM 3-cyanopyridine. The process involved feeding of the respective substrate at 15 min intervals. Complete conversion of 4-cyanopyridine was achieved for 11 cycles of substrate feeding with the extent of conversion declining to 95 % in the 12th cycle (Fig. 3c). Thus, at the end of 12 cycles of substrate feeding, 1.79 M (220.4 g/l) isonicotinic acid product accumulated in the reaction mixture in 3 h using *Z. galactanivorans* nitrilase. In comparison, the nitrilase from *P. putida* CGMCC3830 [41] was reported to convert 1 M 4-cyanopyridine to isonicotinic acid (123 g/l) in 200 min before being subject to product inhibition. With subsequent substrate feeding, a final yield of 172 g/l isonicotinic acid was achieved in 380 min with *P. putida* nitrilase. Sharma et al. [40] reported a product yield of 958 mM (117.9 g/l) isonicotinic acid in 400 min using the *N. globerula* nitrilase, with product inhibition observed after the 7th feeding cycle. The volumetric productivity obtained in the present work using *Z. galactanivorans* biocatalyst was 73.2 g/l/h, which is a 2-fold improvement over the values (36 – 37 g/l/h) reported for nitrilases from *P. putida* [41] and *N. globerula* [40]. Increasing the concentration of 4-cyanopyridine to 200 mM in our study led to a sharp reduction in the rate of hydrolysis after the 6th cycle (data not shown), coupled with precipitation in the reaction mixture.

The fed-batch process for conversion of 3-cyanopyridine to nicotinic acid by *Z. galactanivorans* nitrilase involved a comparatively higher dosing of 200 mM substrate every 15 min, but complete conversion was achievable only up to 8 cycles of feeding. The extent of conversion dropped significantly to 55 % in the 9th cycle and to 18 % in the 10th cycle (Fig. 3b), possibly due to product inhibition. Nevertheless, a final product yield of 1.76 M (216.7 g/l) nicotinic acid could be achieved in 2.5 h with a productivity of 86.7 g/l/h. In comparison, Pai et al. [35] were able to achieve complete conversion of 1 M (104 g/l) 3-cyanopyridine in 6 h employing immobilized *E. coli* expressing the *A. fecalis* nitrilase, albeit with a high biocatalyst loading of 500 g/l wet cell weight. *P. putida* CGMCC3830 nitrilase has been utilized for the production of nicotinic acid by Dong et al. [42], accumulating 189 g/l nicotinic acid in 450 min. Using high cell density cultivation, Gong et al. [43] were able to scale up the process and achieved 541 g/l nicotinic acid in 290 min. The *Z. galactanivorans* enzyme identified in the present work performed better than many reported nitrilases at the lab scale, and has excellent potential to be developed for subsequent application in production of isonicotinic acid.

3.4. Conversion of iminodiacetonitrile

Iminodiacetic acid (IDA) is extensively applied as an intermediate in the manufacture of glyphosate herbicide [44] and in the synthesis of surfactants and chelating resins [23]. Current chemical methods for the industrial production of IDA and glyphosate require the use of strong acid and base, consequently generating large amounts of waste water and NaCl [44]. Hence there is strong interest in employing nitrilases to develop an alternative eco-friendly process. Nitrilases from a few organisms including *Alcaligenes fecalis* [23], *Arthrobacter aurescens* [45], *Lysinibacillus boronitolerans* [46] and *Acidovorax facilis* [47] have been reported to hydrolyze iminodiacetonitrile (IDAN) to IDA. However, slow conversion rates and inhibition at high substrate concentration has hindered their potential for scale up. In the present work, the nitrilase from *Achromobacter insolitus* exhibited soluble expression and high activity toward iminodiacetonitrile, and was further evaluated for IDA

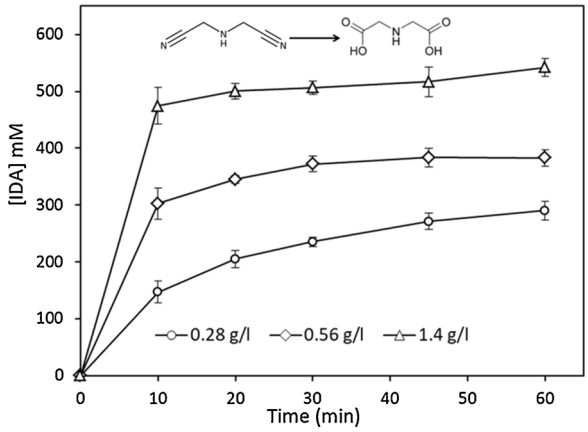

Fig. 4. Time course of IDAN biotransformation by *A. insolitus* nitrilase with increasing cell loading (g/l DCW).

production.

A. insolitus nitrilase exhibited maximum activity at pH 6 and 45 °C (Figure S2); it was able to retain 70 % of its maximum activity at the process operating conditions of pH 7 and 37 °C. The enzyme showed an activity of 15.4 U/mg DCW with 630 mM IDAN under optimal reaction conditions, which is a significant improvement over reported nitrilases. In comparison, nitrilases from *L. boronitolerans* [46] and *A. fecalis* [23] exhibit activities of 50.2 and 109.7 U/g DCW respectively with 100 mM IDAN, with lower tolerance for high substrate concentrations. During the course of IDAN hydrolysis by *A. insolitus* nitrilase, the substrate was rapidly converted to IDA. The whole cell biocatalyst achieved 75 % conversion with production of 472 mM IDA within 10 min, at a cell loading of only 1.4 g/l DCW (Fig. 4). The reaction rate significantly slowed after 10 min, probably due to inhibition by the IDA product. A final product yield of 542 mM IDA (72.1 g/l, 86 % conversion) was achieved after 1 h (Fig. 4). Increased biocatalyst load up to 6 g/l DCW or prolonged reaction times up to 5 h could only further the conversion till 95 % (data not shown). Although a high conversion rate was also observed at lower cell loading (0.28 g/l DCW), the final IDA yield was limited to 253 mM after 1 h (Fig. 4). Nevertheless, the biocatalytic process offers a considerable increase in conversion rate and productivity compared to the engineered *Acidovorax facilis* nitrilase variant [48], where 453 mM (60.3 g/l) IDA was achieved after 5 h reaction using enzyme loading of 100 g/l wet cell weight. The nitrilase from *A. aurescens* [45] could achieve 60 % conversion with 500 mM IDAN in 12 h; while the *A. fecalis* WBX11-MD271 mutant [49] could achieve 72 % conversion of up to 250 mM IDAN after 6.5 h. In another report, *L. boronitolerans* nitrilase [46] was able to tolerate only till a substrate concentration of 200 mM IDAN. In comparison, the *A. insolitus* nitrilase from the present study showed significantly improved substrate tolerance and achieved a biocatalyst productivity of 51.5 g IDA/g DCW. IDAN appears better suited as a primary substrate for nitrilases from *S. fumaroxidans*, *A. insolitus* and *Beijerinckia* sp. that exhibit preference for aliphatic nitriles (Fig. 1). Immobilization and recycling of *A. insolitus* nitrilase whole cells could further enhance the IDA yield and aid in the development of a commercially viable biocatalytic process for the industrial production of IDA.

3.5. Stereoselective conversion of mandelonitrile

(*R*)-mandelic acid is an important 2-hydroxy carboxylic acid intermediate employed in the production of semi-synthetic penicillins and cephalosporins, anti-tumour agents and anti-obsesity drugs [50]. It is also used in the chiral resolution of racemic alcohols or amines [51]. Chemical synthesis of mandelic acid from benzaldehyde, although fairly straightforward, is plagued by low yields and lack of enantioselectivity [52]. A biocatalytic approach using nitrilases offers the

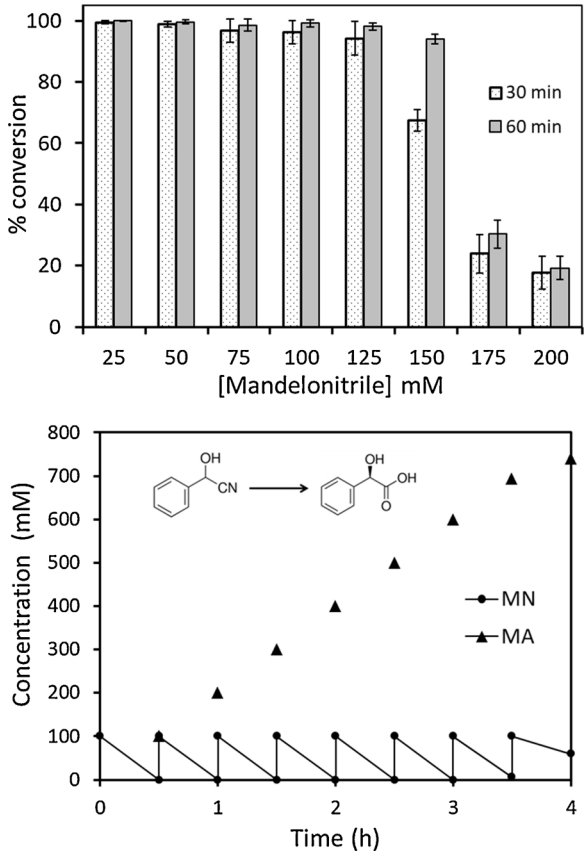

Fig. 5. (a) Effect of mandelonitrile substrate concentration on nitrilase conversion. (b) Enantioselective conversion of mandelonitrile to (*R*)-mandelic acid in fed-batch mode. Rac-Mandelonitrile at 100 mM (final concentration) was added to the reaction mixture after 30 min intervals.

advantage of high enantioselectivity with the use of inexpensive mandelonitrile as starting material, and also the opportunity to carry out dynamic kinetic resolution [50], thus providing a 100 % theoretical yield.

Three nitrilases from *Janthinobacterium* sp., *V. paradoxus* and *C. necator* identified in this study showed specificity for aryl aliphatic nitriles (Fig. 2). All three enzymes were active on 50 mM mandelonitrile, generating (*R*)-mandelic acid at 99.9 % ee. Of these, the *C. necator* nitrilase exhibited significantly higher tolerance for mandelonitrile (Table S3), and was chosen for further study. The enzyme exhibited 90–99 % conversion till 125 mM mandelonitrile within 30 min (Fig. 5a). At 150 mM mandelonitrile, the reaction required 1 h to reach 93 % conversion and higher concentrations led to inhibition and drastic reduction in nitrilase activity (Fig. 5). Nevertheless, *C. necator* nitrilase displayed excellent enantioselectivity (99.9 % ee) at all substrate concentrations tested, and the amount of amide formed was negligible (< 1%). It is also possible that the apparent low substrate tolerance of nitrilases from *Janthinobacterium* sp. and *V. paradoxus* could be a reflection of their reduced level of expression or due to non-optimal reaction conditions. The *C. necator* nitrilase was highly active on mandelonitrile in the pH range 6.5–9, with maximum activity at pH 8. It is known [53] that spontaneous decomposition of mandelonitrile to benzaldehyde and HCN is favoured at slightly alkaline pH, leading to racemization of the substrate and resulting in enhanced activity. The enzyme was also most active at 50 °C, but it exhibited 68 % of its maximum activity at the process temperature of 37 °C (Figure S2).

To circumvent the toxicity of mandelonitrile and enhance product yield, a fed-batch reaction was attempted using aqueous monophasic system with 10 % methanol as co-solvent. Mandelonitrile was dosed every 30 min to bring its concentration to 100 mM. Complete

conversion of mandelonitrile to (*R*)-mandelic acid was effected for 7 cycles of substrate feeding using 7 g/l DCW whole cell biocatalyst, following which accumulation of mandelonitrile and benzaldehyde was observed with a marked reduction in the rate of hydrolysis. The cumulative concentration of (*R*)-mandelic acid in the reaction mixture reached 740 mM (112.6 g/l) after 4 h (Fig. 5b), displaying a volumetric productivity of 28.2 g/l/h. In recent studies, Zhang et al. [54] used immobilized *E. coli* cells expressing the *A. fecalis* nitrilase in a stirred-tank reactor to achieve a cumulative (*R*)-mandelic acid yield of 805.3 mM (122.18 g/l) after 15 h fed-batch conversion. The nitrilase from *Burkholderia cepacia* [50] could convert 1.1 M mandelonitrile giving final isolated product yields of 0.9 – 0.96 M; the process was also upscaled to 10 L using continuous feeding with a product yield of 2.3 M (350 g/l) after 23 h [50]. Although the final product yields using *C. necator* nitrilase are lower than these studies, the volumetric (28.2 g/l/h) and catalyst productivity (185 g/g DCW) achieved in our study fall in the range of values reported for bench-scale process involving monophasic systems [55]. This demonstrates its potential for scale-up and application in biocatalytic production of (*R*)-mandelic acid, and it should be possible to enhance the process efficiency via high cell density cultivation or employing a biphasic solvent system involving ethyl acetate or toluene [55].

4. Conclusion

The present study demonstrates the potential of novel nitrilases identified through genome mining for biocatalytic application in the hydrolysis of with broad spectrum of nitriles. Three nitrilases evaluated in this study exhibited good substrate tolerance and performed well in preparative scale reactions for the synthesis of commercially relevant carboxylic acid products. The nitrilase from *A. insolitus* could convert 542 mM iminodiacetonitrile within 1 h, while the *Z. galactanivorans* nitrilase was able to produce 1.8 M isonicotinic acid from 4-cyanopyridine. The nitrilases were tolerant to high substrate loading, resulting in better volumetric and catalyst productivity over other reported nitrilases (Table S4). Employing strategies like high cell density cultivation and enzyme immobilization could be applied in scaling up the processes. Although the nitrilases mined in this study hydrolysed dinitriles to dicarboxylic acids, it should be possible via rational mutagenesis [56] to engineer their regioselectivity and enable the synthesis of cyanocarboxylic acids at a comparable rate. Further biochemical and structural characterization of these nitrilases is underway and could provide more information for protein engineering and design of efficient sustainable processes for nitrile hydrolysis.

Author statement

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Declaration of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Avinash Vellore Sunder: Conceptualization, Methodology, Investigation, Validation, Writing - original draft. **Shikha Shah:** Investigation, Visualization, Writing - original draft. **Pratima Rayavarapu:** Investigation, Formal analysis. **Pramod P. Wangikar:** Conceptualization, Supervision, Funding acquisition, Writing - review & editing.

Appendix A. Supplementary data

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