Screening and characterization of a nitrilase with significant nitrile hydratase activity

Ke Zhang · Tingze Pan · Liuzhu Wang · Hualei Wang · Yuhong Ren · Dongzhi Wei

Received: 22 March 2022 / Accepted: 9 August 2022 / Published online: 1 September 2022
© The Author(s), under exclusive licence to Springer Nature B.V. 2022

Abstract
Purpose We screened nitrilases with significant nitrile hydratase activity to exploit their potential in benzylic amide biosynthesis. We also investigated the factors affecting their hydration activity to support further research on benzylic amide production by nitrilase.
Methods A sequence-based screening method using previously reported crucial positions identified to be essential for amide-forming capacity of nitrilase (referred to as “amide-formation hotspots”) as molecular probes to identify putative amide-forming nitrilases.

Results Based on the previously reported “amide-formation hotspots,” we identified a nitrilase NitPG from Paraburkholderia graminis DSM 17151 that could produce a significant amount of mandelamide toward mandelonitrile and exhibited general hydration activity toward various benzylic nitriles. The time-course experiment with NitPG demonstrated that amide was also a true reaction product of nitrilase, suggesting that the nitrile catalysis by amide-forming nitrilase could be a post-transition state bifurcation-mediated enzymatic reaction. Further research demonstrated that low temperature, metal ion addition, and specific substrate structure could profoundly improve the amide formation capability of nitrilase.

Conclusions NitPG with broad hydration activity is a potential candidate for the enzymatic synthesis of benzylic amides for biotechnological applications. Studying the effect of nitrilase hydration activity could promote our understanding of the factors that influence amide and acid distribution.

Keywords Nitrile · Hydration activity · Nitrilase · Amide formation · Mandelamide
Introduction

Amides are important intermediates in the synthesis of fine chemicals and pharmaceuticals (Pitzer and Steiner 2016). In 2005, amide formation was identified as one of the most problematic synthesis processes in the pharmaceutical industry by the American Chemical Society Green Chemistry Institute Pharmaceutical Roundtable (ACS GCIPR) (Constable et al. 2007). Enzymatic synthesis has been reported to be a promising alternative to traditional chemical synthesis and the most atom-economical reaction for amide preparation is nitrile hydration (García-Álvarez et al. 2013; Gong et al. 2017). Nitrile hydratase (NHase, EC4.2.1.84) is generally used to catalyze nitrile hydration to produce the corresponding amide and has been successfully used in the industrial production of acrylamide, 5-cyanovaleramide, and nicotinamide (Yamada and Kobayashi 1996; Chen et al. 2009; García-Álvarez et al. 2013; Bhalla et al. 2018). Despite the successful utilization of NHase in industrial amide production, it has been reported that NHases generally have low thermostability and low stereoselectivity and require additional “activator proteins” and metal iron (Prasad and Bhalla 2010; Gong et al. 2017). Moreover, the narrow substrate scope (limited to aliphatic nitriles) of NHases also indicates that they are not ideal catalysts for the industrial production of benzylic amides (Cheng et al. 2020). Owing to the limitations of existing NHases, developing novel suitable biocatalysts that catalyze benzylic nitriles into corresponding amides is an important pursuit.

Nitrilases (NLases, EC 3.5.1.4) are another important class of nitrile-converting enzymes that can hydrolyze nitriles to corresponding acids and ammonia (Bhalla et al. 2018). An in-depth study of NLase has demonstrated that detectable amounts of amides are formed in the presence of NLase, indicating additional hydration activity (Martinkova and Kren 2010). Owing to its broad substrate scope and strict enantioselective properties, NLase with hydration activity is a potential alternative to NHase and offers excellent possibilities for industrial amide production, especially benzylic amides, which are less acceptable by NHase (Martinkova and Kren 2010; Gong et al. 2017).

However, NLase-mediated amide formation has largely been neglected, and there are few reports on amide formation in NLase-catalyzed hydrolysis of benzylic nitrile substrates (Kaplan et al. 2006; Winkler et al. 2007; Arfi and Nigam 2020). Most reported NLases exhibit limited hydration activity toward specific nitrile substrates and produced small amounts of amide as a by-product (<5%) (Martinkova and Kren 2010; Günther et al. 2018). Mandelonitrile is one of the most representative valuable benzylic nitriles, whereas mandelamide production has only been observed in some fungal arylacetonitrilases and a bacterial NLase from Pseudomonas fluorescens EBC191; the percentage of mandelamide in the total product decreased in the order P. fluorescens (19%) > Neurospora crassa (15%) > N. haematococca (6.2%) (Kiziak et al. 2005; Petrickova et al. 2012; Vesela et al. 2013). Thus, it is necessary to explore novel NLases with significant and general hydration activities to produce amide products from various benzylic nitriles. Moreover, systematic research has been conducted on amide-producing NLases to better understand the factors that influence the product distribution of NLases.

In this study, under the guidance of two reported key residues that were identified to play important roles in the amide formation of NLase, sequence analysis-based rational screening was performed to identify novel NLases with additional hydration activity toward mandelonitrile. Furthermore, the effects of different factors, such as pH, temperature, metal ions, organic solvents, and substrate structure, on the amide formation capability of NLase were systematically investigated to lay the foundation for applying NLases to amide synthesis.

Materials and methods

Materials

Plasmid pET28a (Tsingke, China) was used to clone NLase and determine its expression. Escherichia coli strains DH5α and BL21 (DE3) (Tsingke, China) were used as the hosts for cloning and expression, respectively. All nitriles, amides, acids, and other chemicals used in this study were of analytical grade and purchased from Aladdin Co. (Shanghai, China) or Sigma-Aldrich Co. LLC. (USA).
Sequence alignment

The NLase sequences used in this study were obtained from the strain library maintained by our laboratory. Multiple sequence alignment of the protein sequences was performed using MEGA ver. 11 software (Tamura et al. 2021). All parameters were set to their default values. The sequence alignments were edited and analyzed by a multiple sequence alignment editor and then modified using ESPript 3.0 (espript.ibcp.fr/ESPript/ESPript/) (Robert and Gouet 2014).

Cloning and expression of NLase genes in E. coli

The NLase primers used for amplification are listed in Table S1 (Online Resource 1). Recombinant DNA techniques and plasmid transformation were performed according to standard protocols. The E. coli strains for NLase protein expression were cultivated in Luria–Bertani broth containing 50 μg/mL kanamycin in a flask at 37 °C. To initialize the expression, isopropyl-β-d-1-thiogalactopyranoside was added as an inducer at a final concentration of 0.1 mM when the OD_{600} of the medium reached 0.6–0.8. The induction process was carried out at 20 °C for 16–18 h with shaking. After expression, cells were collected and washed twice with 30 mL physiological saline, and cell pellets were harvested by centrifugation at 8000×g at 4 °C for 10 min. NLase cell pellets were resuspended in sodium phosphate solution (20 mM, pH 8.0), and the resuspended cells were disrupted via sonication. The sonicated solution was centrifuged at 10,000 rpm for 20 min to remove cell debris, and the soluble fractions were obtained and stored at 4 °C for further study. Then, 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed to assess protein expression.

Enzyme assays

NLase hydration activity was assayed using mandelonitrile and phenylacetonitrile as substrates. Standard enzymatic reactions were performed at 30 °C in a reaction mixture (1 mL, pH 8.0) containing 50 mM sodium phosphate buffer, 10 mM nitrile substrate (100 mM stored in methanol), and 2 mg of dry cell enzyme or 100 μL crude enzyme solution. After incubation for 3 h, 10% (v/v) 2 M HCl was added to terminate the reaction. One unit (U) of enzyme activity was defined as the amount of enzyme required to converted 1 μmol per min under standard assay condition.

Effect of biochemical properties on amide formation of NLase

The effects of temperature, pH, metal ions, and organic solvents on the hydration activity of nitrilase from Paraburkholderia graminis DSM 17151 (named as NitPG) toward mandelonitrile and phenylacetonitrile were investigated. To determine the effect of temperature, reactions were performed at a range of temperatures (0–60 °C) and pH 8.0. The reaction system was shaken at different temperatures for 6 h, and 10% (v/v) 2 M HCl was added to the solution to quench the reactions. The effect of pH was studied at pH values ranging from pH 4.0 to 10.0 using sodium citrate-citric acid buffer (pH 4.0–6.0), sodium phosphate buffer (pH 6.0–8.0), and glycine-sodium hydroxide buffer (pH 8.0–10.0). All pH-dependent assays were carried out at 30 °C with shaking for 6 h. The effect of metal ions was investigated by adding metal ions (Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Li^+, K^+, Mg^{2+}, Mn^{2+}, Na^+, Ni^+, Zn^{2+}, Ag^+) to the reaction solution (50 mM sodium phosphate buffer, pH 8.0) at a final concentration of 1 mM at 30 °C for 6 h. To investigate the effect of organic solvents, different types of organic solvents (methanol, ethanol, isopropanol, dimethyl sulfoxide (DMSO), acetonitrile, and acetone) were added to the enzymatic reactions at a final concentration of 10% (v/v). The control was a sample without metal iron or organic solvent under the same experimental conditions. All experiments were conducted in triplicate.

Effect of substrate structure on amide formation of NLase

The effect of the substrate structure was studied using different nitriles as substrates. Enzymatic hydrolysis of different nitriles was carried out at 1 mL scale and immersed in a shaker at 30 °C for 12 h. Stock solutions of the substrate (in methanol), enzyme, and buffer (in water) were used. The final concentrations were 1 mM substrate, 10% methanol, and 50 mM sodium phosphate buffer (pH 8.0). The reaction was...
initiated by adding crude enzyme solution and diluted with 10% (v/v) 2 M HCl.

Analytical methods

The decrease in nitrile substrates and formation of acid and amide products were quantified by reversed-phase high-performance liquid chromatography (HPLC) analysis. For benzylic nitriles, a column of Zorbax SB-Aq (250 mm × 4.6 mm, 5 μm; Agilent Technologies, Ltd., USA) was used, and the eluting solvent system was methanol: water: TFA, 30: 70: 0.01 (flow rate: 0.9 mL/min; 30 °C). For heterocyclic nitriles, a Zorbax XDB-C18 column (4.6 mm × 250 mm, 5 μm; Agilent Technologies, Ltd., USA) was used with a mobile phase consisting of phosphoric acid (0.1%, v/v) and acetonitrile (75:25, v/v) at a flow rate of 1 mL/min. The eluate was monitored at a UV wavelength of 210 nm (230 nm for benzonitrile substrate).

Results and discussion

Identification of amide-producing NLases

To identify potential NLases with hydration activity, a well-described amide-producing NLase from P. fluorescens EBC 191 (NitPF) was selected as a reference enzyme (Fernandes et al. 2006). The influence of different amino acid substitutions in the sequence space of NitPF on its hydration activity has been well investigated, and two crucial positions (A165 and W188, referred to as “amide-formation hotspots”) were identified to be essential for its amide-forming capacity (Kiziak and Stolz 2009; Stolz et al. 2019). In the sequence of most characteristic NLases without additional hydration activity, a converted tryptophan (W) residue was generally present at positions 165 and 188 (Wang et al. 2013; Fan et al. 2017). Thus, the atypical alanine (A) residue at position 165 of NitPF was responsible for its additional hydration activity (Kiziak and Stolz 2009). Conversely, although a conserved W residue was present at position 188 of NitPF, the exchange of other proteinogenic amino acids at this position resulted in enhanced amide formation compared to the wild-type (Sosedov and Stolz 2015; Stolz et al. 2019). These results indicate that a W residue at these two positions is necessary for the natural hydrolytic activity of NLase. We hypothesized that NLase with a non-conserved W residue at either one or both of the relevant positions 165 or 188 (in NitPF) was most likely to exhibit additional hydration activity.

Thus, these two reported key residues were utilized as molecular probes to guide our screening for NLases with potential hydration activity. Multiple sequence alignment was performed among 34 uncharacterized NLases from the strain library of our lab. Part of the multiple sequence alignment result is presented in Fig. 1 with the “amide-formation hotspots” 165 and 188, marked with NitPF. As shown in Fig. 1, most candidate sequences carried a conventional W residue at homologous positions 165 and 188. Seven sequences (entries 1–7) with either one or both unconventional residues at relevant positions were selected as candidate sequences and are listed in Table S2 (Online Resource 1). These candidate NLases were cloned into the pET28a- (+) expression vector, and recombinant plasmids were successfully expressed independently in BL21(DE3) as soluble proteins (data not shown). Mandelonitrile and phenylacetonitrile were used as test substrates to confirm the proposed hydration activity of these NLases, and the results are summarized in Table 1. The ratio of amide in the total product (%; mole ratio) simplified as “amide ratio” was used to describe the hydration activity degree of NLase.

The results showed that these selected NLases, which were enzymatically active toward the tested substrates, exhibited additional hydration activity. Among these NLases, NitPG forms a significant amount (approximately 40%) of this by-product from mandelonitrile with a very high relative activity. The NitPG sequence was not conserved at the homologous positions of A165 and W188, which had F and Y at these two positions, respectively. Thus, the highest hydration activity toward both phenylacetonitrile and mandelonitrile was consistent with our hypothesis and indicated the cumulative effect of these two hotspots. NitRS showed secondary amide production (approximately 30%) with a relatively low relative activity (47.43%). Based on the evaluation of NLase amide formation and relative activity, NitPG was selected for further analysis.
**Fig. 1** Multiple sequence alignment of nitrilases in our strain library with amide-forming nitrilase from *P. fluorescens* EBC 191; the “amide-formation hotspots” were marked and identical residues were enclosed in a black box.

**Table 1** Hydrolysis phenylacetonitrile and mandelonitrile in the presence of candidate nitrilases

| Enzyme | Phenylacetonitrile | Mandelonitrile |
|--------|--------------------|----------------|
|        | Amide ratio %     | Relative activity % | Amide ratio %     | Relative activity % |
| Nit YK | 2.65               | 72.46           | 7.07                | 118.58             |
| Nit RS | 13.25              | 48.31           | 30.95               | 47.43              |
| Nit PG | 16.31              | 100b            | 38.88               | 100c               |
| Nit BP | 0.47               | 36.23           | 2.95                | 15.81              |
| Nit MP | –                  | N. D            | –                   | N. D               |
| Nit AM | 2.23               | 55.56           | –                   | N. D               |
| Nit RL | 1.50               | 9.66            | –                   | N. D               |

*a* No activity detected  
*b* The specific activity of NitPG toward phenylacetonitrile corresponding to 4.1 U/mg was defined as 100%  
*c* The specific activity of NitPG toward mandelonitrile corresponding to 2.6 U/mg was defined as 100%
Effect of reaction time on amide formation of NLase

To study the effect of reaction time on amide formation by NLase, the conversion of mandelonitrile and phenylacetonitrile by NitPG was analyzed in a time-course experiment. The reaction mixtures were withdrawn at different time intervals, and amide and acid production was analyzed by HPLC, as stated in the materials and methods section. As shown in Fig. 2, the amide ratios in the hydrolysis of both phenylacetonitrile and mandelonitrile remained constant as the reaction proceeded, indicating that the reaction time did not affect the amide formation of NLase. The time-independence of the amide ratio suggested that acid and amide were formed simultaneously throughout the experiment and not in an ordered manner. Hence, for NLase, amide was not a reaction intermediate in the hydrolysis of nitrile into acid, but a true reaction product formed directly from the nitrile. This result was consistent with the reported independence of the amide formation on reaction time of nitrilase from *Pseudomonas fluorescens* EBC191 and was further supported by the observation that mandelamide and phenylacetamide were not substrates of NitPG (Fernandes et al. 2006). It has been postulated that the hydrolysis of nitrile by amide-producing NLase could be a post-transition state bifurcation (PTSB)-mediated enzymatic reaction with PTSB, which refers to a phenomenon in which a single transition state leads to the formation of multiple products (Hare and Tantillo 2017). As the distribution of products of PTSB reactions seen in simulations has been found to be extremely sensitive to the local environment of the reaction system, studies have been conducted to identify factors determining the PTSB process of NLase to efficiently generate the desired amide products (Sheppard and Acevedo 2009).

Effect of pH and temperature on amide formation of NLase

The effect of pH and temperature on the amide formation of NLase was further investigated to better understand the factors that influence the product distribution of amides and acids. The reactions were performed in a standard assay with a longer reaction time to ensure that the amide product was detectable under all conditions. The effect of reaction pH on amide formation by NLase was tested using a series of buffers with pH values ranging from 4.0 to 10.0. As the catalytic activity of NitPG was fully inhibited at extreme pH values (4.0, 10.0), the amide ratio was only detected in the pH range of 5.0–9.0; the results are presented in Fig. 3a. No fluctuation was observed in the amide ratio among the tested pH ranges, indicating that pH did not influence the amide formation of NitPG.

Reactions at temperatures ranging from 0 to 60 °C were conducted to determine the effect of temperature on the hydration activity of NLase, and the results are shown in Fig. 3b. In contrast to pH, the effect of temperature on amide formation by NitPG was

---

**Fig. 2** Time course of NitPG toward phenylacetonitrile (a) and mandelonitrile (b); the gray lines with down-triangle represented for amide ratios of total product and the black lines with square represented for the conversions in both a and b; error bars represented the standard deviation of three measurements.
pronounced. The amide ratio toward mandelonitrile significantly decreased from 58% at 0 °C to 30% at 60 °C, and temperature also caused significant changes in the amide ratio of the reaction products of phenylacetonitrile (from 35 to 12%). These results suggest that temperature is a crucial factor in determining the hydration activity of NLase. Thus, a low temperature was the most favorable condition for amide production by NLase, which could influence the chemoselectivity with hydration activity towards the amide product. Enhanced amide formation with decreasing temperature was also observed during the hydrolysis of phenylacetonitrile by NitPF (Fernandes et al. 2006).

Effect of different chemicals on amide formation of NLase

NHase is a metalloenzyme, and tightly bound metal ions (Fe²⁺ or Co²⁺) are necessary for its hydration activity to produce amides (Prasad and Bhalla 2010). Generally, NLase activity is independent of metal cofactors. To determine whether the additional hydration activity of NLase also depends on specific metal ions, the effects of different metal ions (Ca²⁺, Co²⁺, Fe²⁺, Li⁺, K⁺, Mg²⁺, Na⁺, Ni⁺) and chelating reagent EDTA on the amide formation capability of NitPG were tested. As the thiol group of the catalytically active cysteine was identified as essential for the catalytic activity of NLase, several thiol-binding metal ions (Cu²⁺, Zn²⁺, Mn²⁺, and Ag⁺) were also included, and the results are shown in Fig. 4a.

According to the catalytic results, there was no negative effect of the metal-chelating agent EDTA on the amide formation of NitPG, indicating that the hydration activity of NLase has no dependence on divalent metal cofactors, which is different from that of NHase. This result was further supported by the lack of effect when divalent ions such as Mg²⁺, Fe²⁺, Ca²⁺, and Co²⁺ were added to the reaction mixtures. Most other tested ions, including thiol-binding metal ions, also did not affect the amide formation of NitPG, except for Cu²⁺. Studies have shown that thiol-binding metal ions usually inhibit the ability of NLase to hydrolyze nitriles (Zhang et al. 2011; Duca et al. 2014; Badoei-Dalfard et al. 2016). Interestingly, we first reported that the addition of Cu²⁺ improved the amide ratio of NitPG to a certain extent (5%). We further studied the impact of Cu²⁺ concentration (Fig. 4b) and found that the improvement in the amide ratio by Cu²⁺ addition was not related to the concentration of Cu²⁺ in the reaction system. The formation of amides by NLase is not yet fully understood. However, there is a well-founded hypothesis that the atypical cleavage of the tetrahedral intermediate formed during the NLase-catalyzed intermediate results in the formation of amide plus enzyme instead of acyl-enzyme and ammonia (Jiang et al. 2017).
Cu²⁺ may have improved the amide ratio by binding to the thiol group of catalytically-active cysteine and changing the active site conformation, causing a less-typical breakdown of the tetrahedral intermediate, which in turn releases more amide products.

The effect of organic solvents on amide formation by NLase was also studied because of the poor solubility of nitriles in the reaction process. The amide ratios of mandelonitrile and phenylacetonitrile were determined in 10% (v/v) ethanol, methanol, isopropanol, DMSO, acetone, and acetonitrile. As presented in Figure S1 (Online Resource 1), the introduction of organic solvents tested in this study did not affect the amide ratio of NitPG, with the observed amide ratio toward phenylacetonitrile and mandelonitrile in the presence of these supplements being 16% and 38%, respectively. These levels were similar to those of the control.

Effect of substrate structure on amide formation of NLase

To simultaneously evaluate the effect of substrate structure on amide formation by NLase and explore the application potential of NitPG in benzylic amide synthesis, a panel of heterocyclic nitriles (entries 1–3), benzylic nitriles (entries 4–6) and phenylacetonitrile derivatives with structural diversity (entries 7–14) were chosen as substrates, and the results are summarized in Fig. 5. According to the catalytic results, NitPG exhibited general hydration activity; notably, amide formation was observed for all tested substrates. The highest proportions of amides were obtained using 4-nitrophénylacetonitrile and 2-methylphenylacetonitrile as substrates (58% and 52%, respectively). Surprisingly, NLase NitPG efficiently catalyzed the hydrolysis of these two phenylacetonitrile derivatives to yield amides as the major product, although only low hydration activity was observed for phenylacetonitrile (16%). These results demonstrate that NLase might be utilized as a nitrile hydratase with specific substrates to form stoichiometric amounts of the corresponding amides.

The radar chart illustrates differences in the amide formation of NitPG in relation to different structural substrates. In the case of the heterocyclic nitriles, only small amounts of amides (<5%) were recorded, and there was no significant difference between the amide ratios of these cyanopyridine isomers. For benzylic nitriles, the amide ratio of NitPG decreased in the order of phenylacetonitrile > 3-phenylpropionitrile > benzonitrile, indicating no obvious correlation between the size of the substituents and the hydration activity of NLase. A much higher hydration activity was recorded for phenylacetonitrile than for 3-phenylpropionitrile and benzonitrile, as only...
the phenylacetonitrile has an acetonitrile-like residue attached to the aryl structure, the importance of an acetonitrile-like residue attached to the aryl structure for amide formation was demonstrated. For phenylacetonitrile derivatives, NitPG produced various amounts of amides, ranging from 5 to 58%. Large amounts of amides were formed from 4-nitrophenylacetonitrile, 2-methylphenylacetonitrile, and mandelonitrile (58, 52, and 38%, respectively). In contrast, significantly lower relative amounts of amides were formed from 4-methylphenylacetonitrile, alpha-methylphenylacetonitrile, and 2-nitrophenylacetonitrile (11, 9, and 5%, respectively). The amide ratio increased in the order alpha-methylphenylacetonitrile < phenylacetonitrile < mandelonitrile, the electronegativity of their α-substituents also increased in the order CH₃ < H < OH, suggesting that the synthesis of amide products increased as the electronegativity of the substituents at the α-position increased. This result strongly indicated that the amide ratio of NitPG was highly sensitive to electronic effects, the α-substituent with high electronegativity might influence the electron distribution of the tetrahedral intermediates and lead to the release of more amide product. A similar effect was recently described for the enzymatic hydrolysis of 2-fluoroacetonitriles using NLase AtNit1 from Arabidopsis thaliana and the hydrolysis of 2-chloro-2-phenylacetonitrile using NitPF (Wajant and Effenberger 2002; Fernandes et al. 2006).

Surprisingly, benzene ring substitution also profoundly influenced the amide formation of NLase, except at the 3-position (entries 10 and 13), which led to an amide ratio similar to that of phenylacetonitrile. 4-nitrophenylacetonitrile and 2-methylphenylacetonitrile were suitable substrates for producing large amounts of amide products, whereas amides were only produced at very low degrees toward 2-nitrophenylacetonitrile and 4-methylphenylacetonitrile. The high hydration activity is probably due to the presence of an electron-withdrawing group at the 4-position and electron-donating group at the 2-position. These results indicate that the substituent position of the benzene ring and the electronic effect of the substituents were relevant factors that strongly affected the amide formation of NLase. Thus, NitPG demonstrated a clear correlation between the structure of phenylacetonitrile derivatives and the relative proportion of the respective amides formed. However, the mechanism of the correlation remains unclear.

| Entry | Nitrile Substrate                   |
|-------|------------------------------------|
| 1     | 2-Cyanopyridine                    |
| 2     | 3-Cyanopyridine                    |
| 3     | 4-Cyanopyridine                    |
| 4     | Benzonitrile                       |
| 5     | 3-Phenylpropionitrile              |
| 6     | Phenylacetonitrile                 |
| 7     | alpha-Methylphenylacetonitrile     |
| 8     | (R,S)-Mandelonitrile               |
| 9     | 2-Nitropheny lacetonitrile         |
| 10    | 3-Nitropheny lacetonitrile         |
| 11    | 4-Nitropheny lacetonitrile         |
| 12    | 2-Methylphenylacetonitrile         |
| 13    | 3-Methylphenylacetonitrile         |
| 14    | 4-Methylphenylacetonitrile         |

Fig. 5 The amide formation of NitPG toward a series of nitriles; table on the left listed the tested nitrile substrates; in the radar chart on the right, the black numbers represented for the ratio of amides and the gray numbers represented for the substrate serial number.
Conclusions

NLase NitPG, which produces significant amounts of corresponding amide toward mandelonitrile, 4-nitrophenylacetonitrile, and 2-methylphenylacetonitrile was identified in this study. Additional tests showed that the relative formation of acids and amides was only subject to temperature, Cu$^{2+}$, and substrate structure, which also provided a deeper insight into the chemoselectivity of NLase. In summary, these amide-producing NLases were demonstrated to be promising alternatives to nitrile hydratases with application potential for the industrial synthesis of benzylid amides and provide a new route for amide production from nitriles, thereby expanding our biocatalysis toolbox.

Author contributions KZ and TZP: conceived and designed the study. KZ: conducted the literature search and performed the experiments. KZ and LZW: were involved in the analysis and interpretation of data. KZ: drafted the manuscript. HLW, YHR and DZW: The study was supervised and tutored. All authors read and approved the final manuscript.

Funding This work is supported by the grant from the National Key Research and Development Program of China (Grant No. 2021YFC2102100).

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

References

Arfa T, Nigam VK (2020) Studies on a thermostable nitrilase from Staphylococcus Sp and its In-silico characterisation. Biologia 75(12):2421–2432. https://doi.org/10.2478/s11756-020-00554-3

Badoei-Dalfard A, Ramezani-Pour N, Karami Z (2016) *Production and characterization of a nitrilase from pseudomonas aeruginosa RZ44 and its potential for nitrile biotransformation*. Iran J Biotechnol 14(3):142–153. https://doi.org/10.15171/ijb.1179

Bhalla TC, Kumar V, Kumar V, Thakur N (2018) Nitrile metabolizing enzymes in biocatalysis and biotransformation. Appl Biochem Biotechnol 185(4):925–946. https://doi.org/10.1007/s12010-018-2705-7

Chen J, Zheng RC, Zheng YG, Shen YC (2009) Microbial transformation of nitriles to high-value acids or amides. Adv Biochem Eng Biotechnol 113:33–77. https://doi.org/10.1007/10_2008_25

Cheng Z, Xia Y, Zhou Z (2020) Recent advances and promises in nitrile hydratase: from mechanism to industrial applications. Front Bioeng Biotechnol 8:352. https://doi.org/10.3389/fbioe.2020.00352

Constable DJC et al (2007) Key green chemistry research areas—a perspective from pharmaceutical manufacturers. Green Chem 9(5):411–420. https://doi.org/10.1039/b703488c

Duca D, Rose DR, Glick BR (2014) Characterization of a nitrilase and a nitrile hydratase from Pseudomonas sp. strain UW4 that converts indole-3-acetonitrile to indole-3-acetic acid. Appl Environ Microbiol 80(15):4640–4649. https://doi.org/10.1128/AEM.00649-14

Fan HY et al (2017) A novel nitrilase from Ralstonia eutrophica H16 and its application to nicotinic acid production. Bioprocess Biosyst Eng 40(8):1271–1281. https://doi.org/10.1007/s00449-017-1787-x

Fernandes BCM et al (2006) Nitrile hydratase activity of a recombinant nitrilase. Adv Synth Catal 348(18):2597–2603. https://doi.org/10.1002/adsc.200600269

García-Alvarez R, Crochet P, Cadierno V (2013) Metal-catalyzed amide bond forming reactions in an environmentally friendly aqueous medium: nitrile hydrations and beyond. Green Chem 15(1):46–66. https://doi.org/10.1039/c2gc36534k

Gong JS et al (2017) Nitrile-converting enzymes as a tool to improve biocatalysis in organic synthesis: recent insights and promises. Crit Rev Biotechnol 37(1):69–81. https://doi.org/10.3109/07388551.2015.1120704

Günther J et al (2018) The nitrilase PtNIT1 catalyzes herbivore-induced nitriles in *Populus trichocarpa*. BMC Plant Biol 18(1):251. https://doi.org/10.1186/s12870-018-1478-z

Hare SR, Tantillo DJ (2017) Post-transition state bifurcations gain momentum – current state of the field. Pure Appl Chem 89(6):679–698. https://doi.org/10.1515/pac-2017-0104

Jiang S et al (2017) Switching a nitrilase from Synechocystis sp. PCC6803 to a nitrile hydratase by rationally regulating reaction pathways. Catal Sci Technol 7(5):1122–1128. https://doi.org/10.1039/c7cy00060j

Kaplan O et al (2006) Purification and characterization of the recombinant enzyme. Microbiol 112:1122–1128. https://doi.org/10.1039/c6cy00060j

Kaplan O et al (2019) Purification and characterization of a nitrilase from *Aspergillus niger* K10. Appl Microbiol Biotechnol 73(3):567–575. https://doi.org/10.1007/s00253-006-0503-6

Kiziac K, Conradt D, Stolz A, Mattes R, Klein J (2005) Nitrilase from Pseudomonas fluorescens EBC191: cloning and heterologous expression of the gene and biochemical characterization of the recombinant enzyme. Microbiology (reading) 151(Pt 11):3639–3648. https://doi.org/10.1099/mic.0.28246-0

Kiziac K, Stolz A (2009) Identification of Amino Acid Residues Responsible for the Enantioselectivity and Amide Formation Capacity of the Arylacetoni nitrilase from Pseudomonas fluorescens EBC191. Appl Environ Microbiol 75(17):5592–5599. https://doi.org/10.1128/AEM.00301-09

Martinova L, Kren V (2010) Biotransformations with nitrilases. Curr Opin Chem Biol 14(2):130–137. https://doi.org/10.1016/j.cbpa.2009.11.018

Petrickova A, Sosedov O, Baum S, Stolz A, Martinova L (2012) Influence of point mutations near the active site on...
the catalytic properties of fungal arylacetonitrilases from *Aspergillus niger* and *Neurospora crassa*. J Mol Catal B 77:74–80. https://doi.org/10.1016/j.molcatb.2012.01.005

Pitzer J, Steiner K (2016) Amides in nature and biocatalysis. J Biotechnol 235:32–46. https://doi.org/10.1016/j.jbiotec.2016.03.023

Prasad S, Bhalla TC (2010) Nitrile hydratases (NHases): at the interface of academia and industry. Biotechnol Adv 28(6):725–741. https://doi.org/10.1016/j.biotechadv.2010.05.020

Robert X, Gouet P (2014) Deciphering key features in protein structures with the new ENDscript server. Nucleic Acids Res. https://doi.org/10.1093/nar/gku316

Sheppard AN, Acevedo O (2009) Multidimensional exploration of valley-ridge inflection points on potential-energy surfaces. J Am Chem Soc 131(7):2530–2540. https://doi.org/10.1021/ja803879k

Sosedov O, Stolz A (2015) Improvement of the amides forming capacity of the arylacetonitrilase from *Pseudomonas fluorescens* EBC191 by site-directed mutagenesis. Appl Microbiol Biotechnol 99(6):2623–2635. https://doi.org/10.1007/s00253-014-6061-4

Stolz A, Eppinger E, Sosedov O, Kizia C (2019) “Comparative Analysis of the Conversion of Mandelonitrilire and 2-Phenylpropionitrilire by a Large Set of Variants Generated from a Nitrilase Originating from *Pseudomonas fluorescens* EBC191.” Molecules. https://doi.org/10.3390/molecules24234232

Tamura K, Stecher G, Kumar S (2021) MEGA11: molecular evolutionary genetics analysis version 11. Mol Biol Evol 38(7):3022–3027. https://doi.org/10.1093/molbev/msab120

Vesela AB, Petrickova A, Weyrauch P, Martinkova L (2013) Heterologous expression, purification and characterization of arylacetonitrilases from *Nectria haematococca* and *Arthrobotrya benhamiae*. Biocatal Biotransform 31(1):49–56. https://doi.org/10.3109/10242422.2012.758117

Wajant H, Effenberger F (2002) Characterization and synthetic applications of recombinant AtNIT1 from Arabidopsis thaliana. Eur J Biochem 269(2):680–687. https://doi.org/10.1046/j.0014-2956.2001.02702.x

Wang HL, Sun HH, Wei DZ (2013) Discovery and characterization of a highly efficient enantioselective mandelonitril hydrolase from *Burkholderia cenocepacia* J2315 by phylogeny-based enzymatic substrate specificity prediction. BMC Biotechnol. https://doi.org/10.1186/1472-6750-13-14

Winkler M, Meischler D, Klemplier N (2007) Nitrilase-catalyzed enantioselective synthesis of pyrrolidine- and piperidinecarboxylic acids. Adv Synth Catal 349(8–9):1475–1480. https://doi.org/10.1002/adsc.200700040

Yamada H, Kobayashi M (1996) Nitrile hydratase and its application to industrial production of acrylamide. Biosci Biotechnol Biochem 60(9):1391–1400. https://doi.org/10.1271/bbb.60.1391

Zhang ZJ, Xu JH, He YC, Ouyang LM, Liu YY (2011) Cloning and biochemical properties of a highly thermostable and enantioselective nitrilase from *Alcaligenes* sp. ECU0401 and its potential for (R)-(−)-mandelic acid production. Bioprocess Biosyst Eng 34(3):315–322. https://doi.org/10.1007/s00449-010-0473-z

**Publisher’s Note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.