

Microbial Transformation of Nitriles to High-Value Acids or Amides

Jing Chen, Ren-Chao Zheng, Yu-Guo Zheng, and Yin-Chu Shen

Abstract Biotransformation of nitriles mediated by nitrile-amide converting enzymes has attracted considerable attention and developed tremendously in the recent years in China since it offers a valuable alternative to traditional chemical reaction which requires harsh conditions. As a result, an upsurge of these promising enzymes (including nitrile hydratase, nitrilase and amidase) has been taking place. This review aims at describing these enzymes in detail. A variety of microorganisms harboring nitrile-amide converting activities have been isolated and identified in China, some of which have already applied with moderate success. Currently, a wide range of high-value compounds such as aliphatic, alicyclic, aromatic and heterocyclic amides and their corresponding acids were provided by these nitrile-amide degrading organisms. Simultaneously, with the increasing demand of chiral substances, the enantioselectivity of the nitrilase superfamily is widely investigated and exploited in China, especially the bioconversion of optically active α -substituted phenylacetamides, acids and 2,2-dimethylcyclopropanecarboxamide and 2,2-dimethylcyclopropanecarboxylic acid by means of the catalysts exhibiting excellent stereoselectivity. Besides their synthetic value, the nitrile-amide converting enzymes also play an important role in environmental protection. In this context, cloning of the genes and expression of these enzymes are presented. In the near future in China, an increasing number of novel nitrile-amide converting organisms will be screened and their potential in the synthesis of useful acids and amides will be further exploited.

Keywords Amidase, Application, Nitrilase, Nitrile, Nitrile hydratase

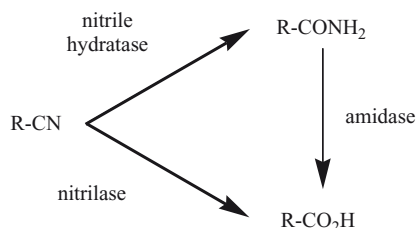
J. Chen, R.-C. Zheng, Y.-G. Zheng (✉), and Y.-C. Shen
Institute of Bioengineering, Zhejiang University of Technology, Hangzhou 310014,
People's Republic of China
e-mail: zhengyg@zjut.edu.cn

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1 Introduction

Biotransformation and biocatalysis have gained increasing interest in recent years due to their mild conditions (physiological pH and ambient temperature), environmentally attractive catalysts, high activities and inherent excellent selectivities including chemo-, regio- and enantio- selectivities [1, 2]. Biocatalysis is now an established method in the synthesis of organic compounds and is especially useful for the production of chiral substances. By virtue of these obvious advantages, biotransformation is becoming a promising alternative to the traditional acid- or base-catalysed reactions, so is the case of nitrile hydrolysis. Nitriles, as the substrates, are widespread in the environment and they are produced by plants in various forms, such as cyanoglycosides, cyanolipids, ricinine, phenylacetonitrile, etc. [3]. Despite the fact that a majority of nitriles are highly toxic, mutagenic and carcinogenic in nature [4, 5], they are an important class of compounds for their ability to afford significant intermediates in the synthesis of acids, amides, amines, amidine, esters, aldehydes, ketones and so on by chemical and enzymatic hydrolysis. Chemical hydrolysis of nitriles was extensively applied to synthesize amides and acids previously; however, these applications may not be suitable for the hydrolysis of nitriles in the presence of sensitive groups. In sharp contrast, enzymatic hydrolysis of nitriles could alleviate this problem ascribed to the mild reaction conditions. Besides, three enzymes, namely nitrile hydratase (EC 4.2.1.84), nitrilase (EC 3.5.5.1) and amidase (EC 3.5.1.4) involved in the transformation of nitriles or amides exhibit great potential of chemo-, enantio- and regioselective synthesis [6].



Scheme 1 The pathways of nitrile compounds by nitrile-amide converting enzymes

As a result, these enzymes have evoked substantial attention and they are becoming more and more demanding. These enzymes operate either by direct hydrolysis of nitrile to the corresponding acid (by a nitrilase enzyme) or by sequential action of an enzyme that hydrates the nitrile to the amide and the latter is transformed to the acid (by an amidase enzyme) (Scheme 1) [7, 8]. To date, various nitrile-amide converting organisms isolated from bacteria, fungi and plant have been described [9, 10]. Among them, most of them have been derived from bacterial species by enrichment strategies with nitriles as the sole nitrogen source [11]. Some reactions mediated by nitrile-converting enzymes have been applied on a large scale in industry. Productions of acrylamide [12] and nicotinic acid [13] on an industrial scale have proved the commercial value of these enzymes. With the fast development of these enzymes, an upsurge of biotransformation of nitriles has been taking place in China as well. According to the statistical data, an increasing number of reports have appeared and several institutes and universities have taken part in this research in recent years. As a result, a variety of microorganisms harboring nitrile-amide converting activities have been isolated, identified and characterized in various places, some of which have already applied with moderate success. Moreover, much work has focused on the organization and regulation of the genes encoding for nitrile metabolism. For example, research on the expressions of nitrile-degradation enzymatic system in recombinant strains has been carried out in China in recent years.

2 Description of Three Classes of Nitrile-Amide Converting Enzymes

2.1 Nitrile Hydratase

Nitrile hydratase, known as metalloenzyme, is a vital enzyme in the bienzymatic hydrolysis of nitriles to acids, which transforms nitriles to the corresponding amides. Asano et al. first reported the occurrence of nitrile hydratase from *Rhodococcus rhodochromus* J-1 (formerly identified as *Arthrobacter* sp. J-1) to degrade acetonitrile, which was later applied with excellent success to the production of acrylamide from

acrylonitrile on an industrial scale [14]. These findings promoted the intensive investigation of nitrile hydratase including physiochemical properties, substrate specificities and the reaction mechanism. According to the presence of metal co-factor, nitrile hydratase can be classified into two kinds: ferric nitrile hydratase and cobalt nitrile hydratase. The existence of metal ions in the active site of the enzyme is presumably effective in enhancing the folding or stabilization of the subunit that is dominantly consisted of α and β subunits. NHase can also be classified into high and low molecular weight (H- and L-NHases) on the basis of molecular weight of the enzyme. So far, a considerable number of microorganisms were successfully screened (Table 1).

Additionally, two new bacterial strains, *Pseudomonas marginales* MA32 and *Pseudomonas putida* MA113, containing nitrile hydratase resistant to cyanide were isolated from soil samples by an enrichment procedure [40]. In contrast to known nitrile hydratases, which rapidly lose activity at low to moderate cyanide concentrations, the enzymes tolerated up to 50 mM cyanide. Cyanide-resistant nitrile hydratase will find great application in the hydration of α -hydroxynitriles for the production of α -hydroxyamide because cyanide is always present in aqueous solutions of α -hydroxynitriles due to their tendency to decompose to the respective carbonyl compound and prussic acid.

Table 1 Some previously reported microorganisms with nitrile hydratase activity

Microorganisms	Substrates specificity
<i>Agrobacterium tumefaciens</i> d3 [15]	Arylnitriles, arylalkylnitriles, acrylonitrile
<i>Arthrobacter</i> sp. J-1 [16]	Alipatic nitriles
<i>Bacillus cereus</i> [17]	Acrylonitrile
<i>Bacillus</i> sp. BR 449 [18]	Acrylonitrile
<i>Bacillus smithii</i> SC-J05-1 [19]	Arylnitriles
<i>Brevibacterium imperialis</i> CBS 489-74 [20]	Acrylonitrile
<i>Pseudomonas chlororaphis</i> B23 [21]	Alkylnitrile
<i>Pseudomonas putida</i> [22]	Acetonitrile
<i>Pseudonocardia thermophila</i> JCM 3095 [23]	Acrylonitrile
<i>Rhodococcus rhodochrous</i> J-1 [24, 25]	Alkylnitrile, heterocyclic nitriles, arylnitriles
<i>Rhodococcus rhodochrous</i> R312 [26]	Alkylnitrile, benzonitrile
<i>Rhodococcus rhodochrous</i> LL 100-21 [27, 28]	Alkylnitriles, acrylonitrile, arylalkylnitriles, 3-cyanopyridine
<i>Rhodococcus erythropolis</i> BL1 [29]	Alkylnitriles, arylalkylnitriles
<i>Rhodococcus rhodochrous</i> A4 [30, 31]	Alkylnitriles, arylnitriles, cycloalkylnitriles, arylnitriles, heterocyclic nitriles, arylalkylnitriles
<i>Rhodococcus</i> sp. AJ270 [32]	Wide spectrum nitrile hydratase
<i>Rhodococcus</i> sp. SHZ-1 [33]	Acrylonitrile
<i>Nocardia</i> sp. 108 [34]	Acrylonitrile
<i>Rhodococcus</i> sp. ZJUT-N595 [35]	Acrylonitrile, glycolonitrile, 2,2-dimethylcyclopropanecarbonitrile
<i>Rhodococcus</i> sp. N 774 [36]	Aliphatic nitriles
<i>Candida guilliermondii</i> CCT 7207 [37]	Cycloalkylnitriles, arylnitriles, heterocyclic nitriles
<i>Candida famata</i> [38]	Alkylnitriles
<i>Cryptococcus flavus</i> UFMG-Y61 [39]	Isobutyronitrile

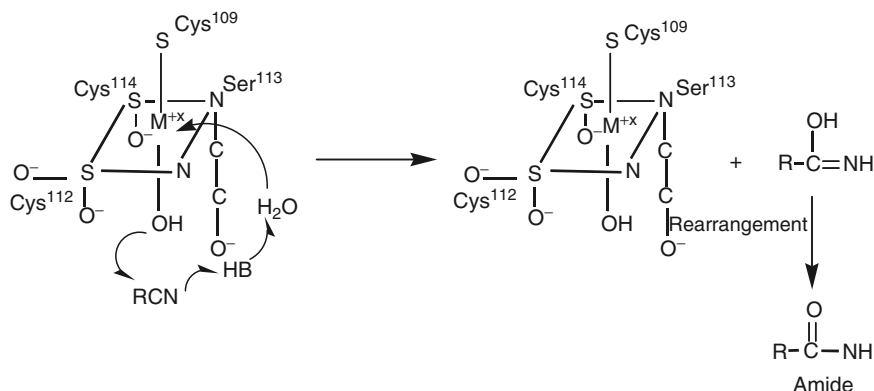


Fig. 1 Catalytic reaction mechanism of nitrile hydratase

To date, many studies focused on the mechanism of NHase mediated catalysis. Kobayashi et al. put forward a possible mechanism as follows. A water molecule was activated by the metal-bound hydroxide ion after the combination of the metal ion and water molecule. Imidate as an intermediate was initially formed via attacking on nitrile carbon by the activated water molecule. The imidate were then tauomerized to form the amide form (Fig. 1) [41].

2.2 Nitrilase

Nitrilase, the first nitrile-converting enzyme, was discovered in barley approximately 40 years ago and famous for the ability to convert indole-3-acetonitrile to the auxin indole-3-acetic acid [42]. From then on, several microorganisms harboring nitrilase activity have been screened, purified and characterized. Bacteria, fungi as well as plants provide excellent source of nitrilase, with bacterial species as the main source (Table 2), which are generally derived using enrichments from environmental samples. In the case of plants, numerous studies were carried out with *Arabidopsis thaliana*, from which four kinds of nitrilase were separated numbered NIT1, NIT2, NIT3 and NIT4. Although it was found that these enzymes were capable of transforming indole-3-acetonitrile to indole-3-acetic acid, recent results have indicated that NIT1, NIT2, NIT3 showed significant preference for 3-phenylpropionitrile, whose product, phenylacetic acid, is found in nasturtium. NIT4, however, was effective in hydrolyzing β -cyano-L-alanine [45].

Depending on the substrate specificity, nitrilase is differentiated into three subclasses: aliphatic nitrilase, aromatic nitrilase that shows preference for aromatic and heterocyclic nitriles and arylacetoneitrilase which is highly specific for arylacetoneitriles [61].

2.3 Amidase

Amidase, amide bond-cleaving enzymes, exists ubiquitously in nature in both prokaryotic and eukaryotic forms. To the best of our knowledge, amidase-mediated processes have been extensively investigated, especially, the hydrolysis of amides to the corresponding carboxylic acid and ammonia. Additionally, hydroxamic acids were formed owing to the acyl transfer activity of amidase in the presence of hydroxylamine. Two reactions involved are shown in Scheme 2.

Besides, amidase is also capable of catalyzing diverse reactions such as ester hydrolysis, hydroxamic acid hydrolysis, acid hydrazide hydrolysis, amide transfer on hydrazine, ester transfer on hydroxylamine, ester transfer on hydrazine and so on [69].

Therefore, amidase turns out to be efficient and promising tools for the synthesis of various compounds. In addition, with regard to nitrile hydratase in Gram-positive bacterium, its enantioselectivity was always combined with the amidase's. Namely, their cooperation gave rise to the excellently pure products. However, the former usually displayed almost no stereoselectivity with the amidase being a major contributor. Consequently, significant attention has been paid to the isolation and discovery of amidase-producing organisms including bacteria, yeasts, fungi, plants and animals (Table 3).

Although amidase catalyzes many reactions, some of them proceed at a comparative low rate employing esters or carboxylic acids as acyl donors. In sharp contrast, high amidase activity is achieved in the presence of water (H_2O) and hydroxylamine (NH_2OH) as the cosubstrates when amide is used as the substrate, indicating that these two compounds functions as efficient acyl acceptors [69].

Both the amidase-catalyzed hydrolytic reaction and the acyl transfer reaction share the same reaction mechanism. In view of this, study on the mechanism of the acyl transfer reaction shed light on that of hydrolytic reaction, in which case, there is difficulty in investigating the mechanism where water is the cosubstrate. A possible mechanism suggested the reaction belonged to Ping Pong Bi Bi type: The carbonyl group of amide undergoes a nucleophilic attack by the enzyme, leading to the formation of a tetrahedral intermediate, which is consequently converted to an acyl-enzyme intermediate with the release of

Amide hydrolysis:



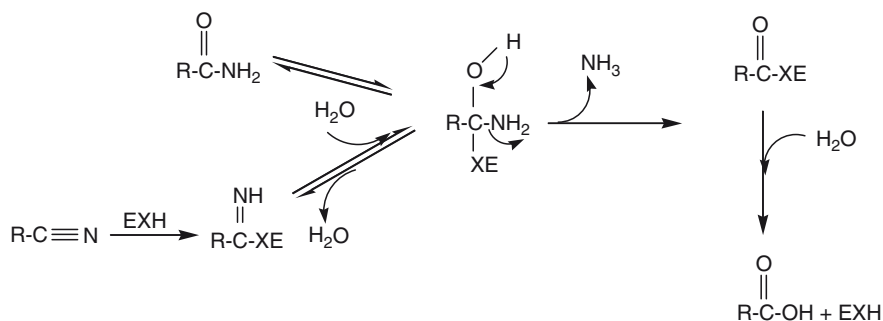
Amide acyl transfer reaction:



Scheme 2 The pathways of hydrolysis and transfer of amide Amide hydrolysis: $\text{RCONH}_2 + \text{H}_2\text{O} \rightarrow \text{RCOOH} + \text{NH}_3$ Amide acyl transfer reaction: $\text{RCONH}_2 + \text{NH}_2\text{OH} \rightarrow \text{RCONHOH} + \text{NH}_3$

Table 3 Some previously reported microorganisms with amidase activity

Microorganisms	Substrate specificity
<i>Rhodococcus erythropolis</i> MP50 [70]	Aromatic amide
<i>Geobacillus pallidus</i> RAPc8 [71]	Aliphatic amide
<i>Sulfolobus tokodaii</i> strain 7 [72]	Aromatic amide
<i>Delftia acidovorans</i> [73]	d-Amino acid amide
<i>Arthrobacter</i> sp. J-1 [74]	Aliphatic amide
<i>Rhodococcus rhodochrous</i> M8 [75]	Aliphatic amide
<i>Xanthobacter flavus</i> NR303 [76]	l-Amino acid amide
<i>Brevibacterium</i> sp Strain R312 [77]	Aryloxypropionamides
<i>Variovorax paradoxus</i> [78]	d-Amino acid amide
<i>Pseudonocardia thermophila</i> [79]	Aliphatic, aromatic and amino acid amide
<i>Pseudomonas</i> sp. MCI3434 [80]	Heterocyclic amide
<i>Klebsiella oxytoca</i> [81]	Aliphatic amide
<i>Brevibacillus borstelensis</i> BCS-1 [82]	Aromatic and aliphatic amide
<i>Ochrobactrum anthropi</i> SV3 [83]	Amino acid amide
<i>Stenotrophomonas maltophilia</i> [84]	Peptide amide
<i>Agrobacterium tumefaciens</i> strain d3 [85]	Aromatic amide
<i>Sulfolobus solfataricus</i> MT4 [86]	Aliphatic and aromatic amide
<i>Klebsiella pneumoniae</i> NCTR1 [87]	Aliphatic amide
<i>Bacillus stearothermophilus</i> BR388 [88]	Wide spectrum amidase
<i>Delftia tsuruhatensis</i> ZJB-05174 [89]	2,2-Dimethylcyclopropanecarboxamide

**Fig. 3** Mechanism of amidase-catalysed reaction

ammonia. The acyl–enzyme complex in turn is subjected to attack by water or hydroxylamine (Fig. 3) [9, 90].

Importantly, a majority of amidases bear enantioselectivity, which contributed to the synthesis of chiral carboxylic acid via nitrile hydratase and amidase. However, these nitrile-hydratase-associated amidases are, surprisingly, mostly *S*-stereospecific. *R*-Enantioselective amidases are gaining more and more interest because of their potential application in the production of d-amino acids and other optically active compounds. Hydroxamic acids, products of the acyl transfer reactions, can be detected easily and fairly specifically by the addition of acidic ferric chloride solution, which results in the production of a deep magenta color [91]. Therefore, this

acyl transfer reaction can be employed in a colorimetric screening procedure for active and enantioselective amidases. By employing the amidase-catalyzed acyl transfer reaction, *Delftia tsuruhatensis* producing *R*-enantioselective amidase was screened in our lab [89, 92].

3 The Isolation and Identification of the Nitrile-Amide Converting Organisms in China

Biotransformation of nitriles is of great potential in organic synthesis and it provides green access to various carboxylic acids and amides; thus nitrile-amide converting enzymes are of broad use and commercial interest. Recently, biotransformation of carboxylic acids and amides via these enzymes has been a hot issue in China. The scarcity of appropriate nitrile-amide converting biocatalysts and the difficulty in commercial availability of these enzymes promoted the screening and discovery of the novel nitrile-amide converting organisms in China. So far, a series of organisms producing nitrile-amide degrading enzymes were isolated, identified and characterized, some of which were purified. Among the obtained organisms, it was observed that some harbored nitrile hydratase, some produced nitrilase and others could form amidase. It was also found that the existences of nitrile hydratases were always accompanied by amidases, so amides and acids were formed in different proportions in such kinds of microbes mediated reactions. A prominent example is *Rhodococcus* sp. AJ270, which is a powerful and robust nitrile hydratase/amidase-containing microorganism isolated by Wang et al. Later, its broad applications in transforming various nitriles were substantially explored [32]. *Rhodococcus* sp. AJ270 as well as other nitrile-amide converting organisms was dominantly obtained by enrichment strategies where nitriles were employed as the sole nitrogen source ascribed to the highly toxic nature. Owing to the fact that screening a desired organism for a particular biocatalytic process is always a time-consuming and tedious job, some direct and sensitive readouts of the nitrile-amide converting enzyme activity have to be considered and developed. Conventional routes employ high-performance liquid chromatography, liquid chromatography-mass spectrometry, capillary electrophoresis, or gas chromatography to determine the enzyme activity, where determinations have to carry out one by one. Furthermore, those traditional enrichment strategies usually result in the isolation of a rather restricted group of microorganisms. A successful instance of application of high throughput screening method in our research was the isolation of *Delftia tsuruhatensis*, a *R*-stereospecific amidase producing bacterium. *R*-Enantioselective amidases are of considerable industrial interest due to potential applications in the production of optically active compounds [92]. More recently, Zhu et al., driven by the attempt to find a fast, convenient and sensitive method, reported a more accurate and innovative high throughput route. In their paper, a novel time-resolved luminescent probe: *o*-hydroxybenzonitrile derivatives could be applied to detect the activity of the nitrilases. By the action of nitrilases, *o*-hydroxybenzonitrile derivatives could be

transformed to the corresponding salicylic acid derivatives, which, upon binding Tb^{3+} , served as a photon antenna and sensitized Tb^{3+} luminescence. Because of the time-resolved property of the luminescence, the background from the other proteins (especially in the fermentation system) in the assay could be reduced and, therefore, the sensitivity was increased. Moreover, because the detection was performed on a 96- or 384-well plate, the activity of the nitrilases from microorganisms could be determined quickly [93].

Moreover, some other high throughput methods have been reported as alternatives to conventional screening methods. A critical review on selection and screening strategy for enzymes of nitrile metabolism based on spectrophotometric and fluorimetric methods has been published [94]. Recently, convenient screening methods have been developed on the basis of the color variation of indicators which are added to the mixture in advance. Once the acid formed, the color would have a dramatic change [95]. Additionally, a new method for nitrilase screening has been developed to detect nitrilase activity. The ammonia product of nitrilase mediated conversion of nitriles forms a complex with the cobalt ion results in a color change, which can readily be quantified using a spectrophotometer at 375 nm. The assay has the potential to be used for the real-time monitoring of nitrilase-catalyzed reactions [96]. More noticeably, Hu et al. introduced a simple and rapid high-throughput screening method based on a colorimetric reaction of glycolic acid with β -naphthol in sulfuric acid solution to isolate glycolonitrile-hydrolyzing microorganisms. Four strains able to convert glycolonitrile to glycolic acid were isolated from soil samples using this screening method, among which *Rhodococcus* sp. ZJUT-N595 displayed the highest hydrolytic activity [35].

These soil-derived nitrile-amide hydrolyzing organisms have been currently under active development and some have even achieved with small to moderate success. The advantage of applying whole cell biocatalysts lies in that they can be relatively easily and cheaply prepared and the whole cell catalyzed reactions can be operated much more easily. Nevertheless, some small aliphatic nitriles, hydroxyl- and amino-substituted nitriles give lower yields and appear to be alternatively metabolized when whole cell biocatalysts were applied [32]. Hence, on one hand, careful monitoring of the reaction is strongly recommended to achieve the maximal desired product. On the other hand, the use of purified enzymes is of substantial significance and benefit in case that substrate or product utilization by whole cells exists. Due to this, the purification and characterization of nitrile-amide converting are under progress in China.

4 Factors Affecting the Activity and Enantioselectivity of Nitrile-Amide Converting Enzyme

Numerous factors, such as some culture conditions like carbon source, nitrogen source, inducer and conversion conditions like temperature, pH, reaction time, cosolvent and so on, turn out to affect the activity and enantioselectivity, and consequently the biomass production.

4.1 Inducer

Nitrile hydratase and nitrilase are generally inducible, with a paucity of them being constitutive. Namely, the activity could be detected only in the presence of suitable inducers. Substrate, product, or their analogs are usually functioned as inducers, with the exception of some extremely toxic nitriles, such as mandelonitrile, in which case, the growth of the microorganism was completely inhibited. Urea and ϵ -caprolactam, potential nitrile hydratase inducers, play important roles in the induction of nitrile hydratase activity. Moreover, with the addition of different inducers, microorganism harboring versatile nitrile-converting enzyme activities exhibited various activities. A distinguished instance was *R. rhodochrous* J-1, a currently utilized organism in commercial synthesis of acrylamide in Japan, which was found to contain two inducible nitrile hydratases, one of which was specific for aliphatic nitriles induced by urea and the other for aromatic nitriles with cyclohexanecarboxamide and crotonamide as the inducers [24, 97]. Similar phenomena were found in *Nocardia Rhodochrous* LL100–21 [98], *R. rhodochrous* NCIMB 11216 [99, 100], *Bacillus pallidus* DAC521 [46, 101] and *Nocardia globerula* NHB-2 [102]. *Bacillus subtilis* ZJB-063, a newly isolated strain in our research, exhibited nitrilase activity without addition of inducers, indicating that the nitrilase in *B. subtilis* ZJB-063 is constitutive. Interestingly, the strain exhibited nitrile hydratase and amidase activity with the addition of ϵ -caprolactam. The versatility of this strain in the hydrolysis of various nitriles and amides makes it a potential biocatalyst in organic synthesis [67]. In a word, selecting a suitable inducer is of great significance in the formation of nitrile-amide converting enzymes, and in enhancing the activity as well.

4.2 Metal Ions

As far as nitrile hydratases are concerned, metal ions predominantly including Fe^{3+} and Co^{2+} are essential in the exhibition of its activity. In case of Fe^{3+} type nitrile hydratase, only with the addition of Fe^{3+} in the nutrient broth acted as co-factor could nitrile hydratase activity be observed, so is the Co^{2+} type nitrile hydratase [24]. Nitrile hydratase from the fungus *Myrothecium verrucaria* even has Zn^{2+} in the active site and in such case Zn^{2+} is necessary for the formation of the enzyme [103].

Unlike nitrile hydratases, nitrilases show no requirement of any metal co-factor. Instead, they are proved to have catalytically essential cysteine residues. Effect of metal ions on the nitrilase activity in many studies indicated that thiol binding reagents like CuCl_2 and AgNO_3 were strong inhibitors of the nitrilase activity [61, 63, 67]. The high sensitivity to these metal ions suggested that one or more thiol residues were necessary for this enzyme and these ions should not be involved in the nutrient broth or reaction mixture.

4.3 Effect of Light on Nitrile Hydratase

As mentioned earlier, the activity of nitrile hydratase displayed unique features with the exposure to light. A nitrile hydratase producing strain *Rhodococcus* sp. N-771 exhibited extremely low activities when the cultivation was carried out in the dark. However, recovery of activity occurred with the irradiation of light [104, 105].

4.4 Amidase Inhibitor

As for amidase, it is a generally accepted fact that urea would have a negative effect on the activity, which is an important feature, especially in the production of some valuable amides via organisms with nitrile hydratase and amidase activity. Undesired acid would form by the cleavage on the amide by accompanying amidase. However, addition of urea could not only protect the amide from serious acid contamination, but also keep the amount of amide constant. Previous research also demonstrated the inhibitory effect derived from the competitive inhibition for active site of amidase between urea and the reactive amide [74, 106]. With that exception, no significant inhibition effect of urea on amidase catalyzed acyl transfer activity and hydrolytic activity from *D. tsuruhatensis* ZJB-05174 was observed, indicating that the inhibition effect of urea was not exclusive [89]. Bauer et al. indicated that diethyl phosphoramidate was also an excellent inhibitor of the amidase from *Agrobacterium tumefaciens* strain d3 [15]. In the presence of urea or chloroacetone, amidase activity in *Bacillus* spp. was inhibited and the amide intermediate was accumulated [106]. Bearing this in mind, we can procure amides in high yields and with excellent purity.

4.5 Temperature and pH

Temperature and pH significantly affect the enzyme activity, and sometimes enantioselectivity. Nitrile-converting biocatalysts mediated reactions are often operated in a narrow pH range, neutral or slightly alkaline. These enzymes mostly exhibit comparatively low activity in too acid or alkaline environments. Therefore, addition of HCl is employed to stop the reaction. With the exception, a nitrile hydrolyzing acidotolerant black yeast *Exophiala oligosperma* R1 was isolated in order to convert α -amino- and α -hydroxynitriles whose enzymatic conversion was hampered by their low stability under neutral conditions [107].

Besides, pH could sometimes affect enantioselectivity to a certain extent. Attempts were made by Wang et al. to improve the enantioselectivity of nitrile of the biotransformation of phenylglycine nitrile by altering the buffer pH from 7.62 to 7.13.

Results indicated that the enantioselectivity of the transformation was enhanced and both the amide and acid form were obtained with ee values over 99% at a pH of 7.13 within 8 h though slower rate was observed [108].

The effect of temperature on the enzyme activity is duple. On one hand, according to Arrhenius's equation ($k = Ae^{-\frac{E_a}{RT}}$), enhancement of the activity occurs with an increase of temperature. On the other hand, enzyme inactivation accompanies an increase of temperature. A large portion of nitrile-amide converting enzymes are not thermal stable and they are usually inactive above 50 °C. A small alteration in temperature may lead to substantial change in enzyme activity. *B. subtilis* ZJB-063, for example, displayed an abrupt decrease at 40 °C and only 13.36% of the activity at 32 °C was inspected, indicating that enzyme activity are sensitive to temperature [109]. Enzymes of good thermostability are of significant importance in pharmaceutical industry. The discovery of thermophilic nitrile-metabolizing microorganisms enables nitrile conversion at high temperature above 50 °C. Zheng et al. succeeded in screening an amidase producing bacterium with excellent thermostability [89].

Along with enzyme activity, enantioselectivity is also influenced by temperature. Wang and coworker observed that lowering the reaction temperature from 30 to 20 °C would lead to increased enantioselectivity of *Rhodococcus* sp AJ270 mediated synthesis of optically active 2, 2-dimethylcyclopropanecarboxylic acid [110].

More interestingly, temperature played an unexpected role in the stereoselectivity of amidase in *D. tsuruhatensis* ZJB-05174. It was detected that enzyme underwent an unusual temperature-dependent reversal of stereospecificity. With the increase in the reaction temperature, the E-value dropped from 27 (30 °C) to 5 (46 °C). When the reaction temperature reached 56 °C, it exhibited reversal stereospecificity, though with a comparatively low E value of 0.03 [89]. This phenomenon can be interpreted by temperature variation caused change on the difference in activation free energy $\Delta\Delta G^\ddagger$, which can be divided into its enthalpic $\Delta\Delta H^\ddagger$ and entropic $\Delta\Delta S^\ddagger$ components [111].

4.6 Organic Solvents

As is generally accepted, organic solvents are widely applied in the lipase or esterase mediated reactions because of the hydrophobic nature of esters. Most nitriles also exhibit low solubility in aqueous solutions. Supplementing organic cosolvents to the reaction mixture containing a nitrile-amide converting biocatalyst is considered to be a useful way to increase the availability of the substrate. However, addition of organic solvents might lead to the inactivation of enzyme. Hence, the amount and suitable kind of the solvents should be carefully evaluated in terms of activity and the availability of soluble substrates. There is only a limited portion of *p*-methoxyphenylacetone nitrile available to the enzyme in aqueous solution when fed to *B. subtilis* ZJB-063 due to its low solubility, which in turn resulted in low activity.

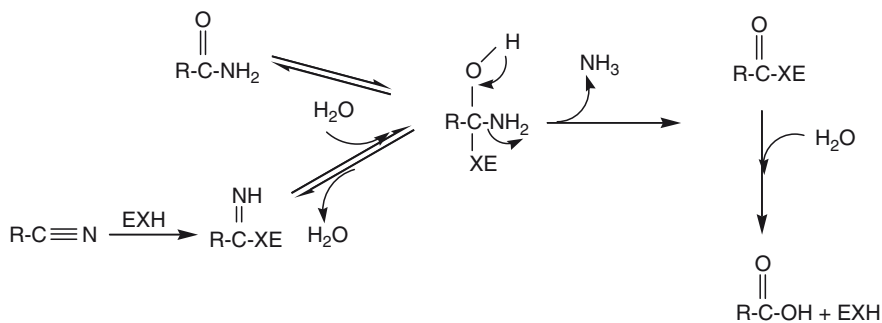
DMSO and methanol of 5 vol.% in the reaction mixture, as the cosolvents, resulted in approximately 62.7% and 15.2% enhancement of activity, respectively, compared to the control where there was no cosolvent. Further increase in concentration of the two solvents, on the contrary, caused a loss of activity, implying that protein denaturation occurred at concentrations above 5 vol.% [109].

Besides, some foreign researches indicated that high percentages of organic solvents were also acceptable by some nitrile-amide converting enzymes. A purified nitrile hydratase from *Rhodococcus equi* A4 showed high resistance to organic solvents and it could tolerate up to 90 vol.% isooctane or pristine [112].

Organic cosolvent showed an effect not only on the activity but also the enantioselectivity of nitrile-amide converting enzymes. Hydrocarbons and methanol (5 vol.%) increased the enantioselectivity of the nitrile hydratase from *Rhodococcus equi* for the conversion of 2-(6-methoxynaphthyl)propionitrile from moderate to good ($E = 14.8\text{--}41$) [112].

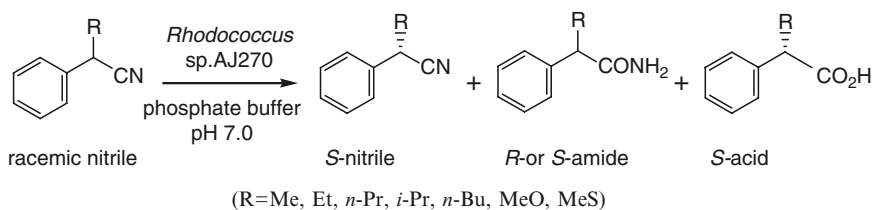
4.7 Steric and Electronic Factors

Both steric and electronic factors dramatically affected the reactivity and more importantly, the enantioselectivity. Considerable investigations concerning the effect of substituents have been carried out. Both the kind and position of substituents have much to do with enzyme activity. Wu and Li conducted a successful asymmetric hydrolysis of α,α -disubstituted malonamides to afford enantiopure (*R*)- α,α -disubstituted malonamic acids employing the strain *Rhodococcus* sp. CGMCC 0497 (Scheme 3). In their study, it seemed that the results were not only influenced by steric hindrance but by an electronic effect of the substrates as well. Among the substrates bearing aromatic ring substituents in the *ortho*-, *meta*-, and *para*-positions, all *para*-substituted ones gave products with excellent ee values, slightly higher



Scheme 3 Asymmetric hydrolysis of α,α -disubstituted malonamides by *Rhodococcus* sp. CGMCC 0497

than *ortho*- and *meta*-substituted ones (Table 4) [113]. More noticeably, when α -substituted phenylacetone nitriles and phenylacetamides were subjected to the *Rhodococcus* sp. AJ270 catalyzed hydrolysis, the reaction outcome was remarkably affected by the nature of α -substituent (Scheme 4). As shown in Table 5, small groups appeared to have no obvious effect on enzyme activity, whereas introduction



Scheme 4 Enantioselective biotransformations of racemic α -substituted phenylacetone nitriles and phenylacetamides by *Rhodococcus* sp. AJ270

Table 4 Enantioselective hydrolysis of various α -substituted- α -methylmalonamides by *Rhodococcus* sp. CGMCC 0497

Substrate (R)	Yield (%)	<i>e.e.</i> (%)
C ₆ H ₅	94	97
<i>o</i> -ClC ₆ H ₄	94	97
<i>m</i> -ClC ₆ H ₄	94	95
<i>p</i> -ClC ₆ H ₄	92	>99
<i>p</i> -CH ₃ C ₆ H ₄	97	>99
<i>p</i> -MeOC ₆ H ₄	95	>99
<i>p</i> -FC ₆ H ₄	95	>99
<i>p</i> -BrC ₆ H ₄	97	>99
C ₆ H ₅ CH ₂	94	>99
C ₃ H ₇	94	91

Table 5 Enantioselective hydrolysis of α -substituted phenylacetone nitriles by *Rhodococcus* sp. AJ270

Substrate (R)	Time (h)	nNtrile (%)	Configuration <i>e.e.</i> (%)	Amide 2 (%)	Configuration <i>e.e.</i> (%)	Acid 3 (%)	Configuration <i>e.e.</i> (%)
Me	10	-	-	42	<i>R</i> , >99	48	<i>S</i> , 90
Me	13.5	-	-	36	<i>R</i> , >99	58	<i>S</i> , 67
Et	70	-	-	58	<i>R</i> , 35	39	<i>S</i> , >99
Et	96	-	-	34	<i>R</i> , 96	40	<i>S</i> , >99
<i>n</i> -Pr	150	55	<i>S</i> , 24	27	<i>S</i> , 41	8	<i>S</i> , >99
<i>n</i> -Pr	214	33	<i>S</i> , 28	40	<i>S</i> , 13	13	<i>S</i> , >99
<i>i</i> -Pr	120	-	-	47	<i>R</i> , >99	46	<i>S</i> , >99
<i>n</i> -Bu	300	36	<i>S</i> , 36	34	<i>S</i> , 20	23	<i>S</i> , 98
MeO	46	-	-	78	0	-	-
MeO	72	-	-	56	0	-	-
MeS	120	-	-	64	<i>R</i> , 15	10	<i>S</i> , 96

of bulky and conformationally flexible groups such as *n*-propyl and *n*-butyl resulted in a much slower rate. Surprisingly, polar group like methoxy and methylthio exhibited no effect on the rate of hydration step. In sharp contrast, both electronic and steric factors displayed detrimental effects on amidase in *Rhodococcus* sp. AJ270. Enantioselectivity of the strain, determined by the combination of selectivities of nitrile hydratase and of amidase, with the latter being a major contributor, seemed to be extremely sensitive to electronic factor too [114].

Rhodococcus sp. AJ270 also showed highly preference for aromatic and heteroaromatic nitriles, in which case the first step, hydration of the linear nitrile substituent, is not significantly hindered by steric or electronic factors, while the second amidase-mediated step showed high sensitivity to steric factors. It was observed that *para*- and *meta*-substituted benzonitriles were converted to the corresponding acids at a comparatively rapid rate in high yield irrespective of the electronic nature of the substituent. Nevertheless, benzonitriles with substituent at the *ortho*-position were rapidly and efficiently converted to amides, while conversion of amides to acids proceeded slowly, suggesting that the first step, is not significantly hindered by steric or electronic factors, while the second amidase-mediated step showed high sensitivity to steric factors [32].

It was evident that electronic nature showed a great effect on initial rate of nitrilase mediated hydrolysis of *para*-substituted phenylacetoneitriles. The reaction was accelerated by electron-withdrawing substituents while slowed down by electron-donating substituents [67]. Similar results were achieved by Geresh et al. in whose study a Hammett-type linear free energy correlation with a ρ value (reaction constant) of 0.96 well described the relationship between the initial rates of the nitrilase catalyzed hydrolysis and the *para*-substituents [115].

5 Applications of Nitrile-Amide Converting Enzymes

Recently, a considerable number of products serving as the intermediates of pharmaceutical, fine chemical and food additives have been derived from enzyme-dependent reactions [116]. Biotransformation of amides or acids provides a feasible and valuable route. In fact, some have been successfully applied to industrial production, meanwhile, some are under active development.

5.1 Bioconversion of Various High-Value Amides and Acids

5.1.1 Industrial Production of Acrylamide and Enzymatic Manufacture of Acrylic Acid

Acrylamide, an important fine chemical with broad uses, is mainly applied in the synthesis of polyacrylamide. Achievements in bioconversion of acrylonitrile made it

possible that enzyme-based manufacture would substitute its traditional production mode. *R. rhodochrous* J-1 nitrile hydratase has been well investigated as a powerful biocatalyst for the production of acrylamide, and was currently utilized in commercial synthesis of acrylamide in Japan [97]. In China, *Nocardia* sp. 163, a soil derived organism from Taishan Mountain in the 1980s, harbors nitrile hydratase activity active on acrylonitrile. Later it became the isolate with the highest NHase activity after optimization of culture conditions by Shanghai Pesticide Research Institute. After that, a tens of thousand tons per year scale acrylamide production facility was set up [117].

As mentioned earlier, nitrile hydratase and amidase always coexist in the organism which leads to the formation of amides and acids in a certain portion. Due to this, a comparatively high ratio of nitrile hydratase/amidase activity is of great necessity to avoid contamination of the acrylamide by acrylic acid. In other words, effective measures should be taken to inhibit the amidase activity to a significant extent. Addition of urea, chloroacetone and phosphoramidate could sometimes efficiently inhibit the amidase activity, which in turn resulted in the accumulation of acrylamide [15, 74, 106].

As far as acrylic acid is concerned, it is a commodity chemical with an estimated annual production capacity of 4.2 million metric tons. Acrylic acid and its esters can be used in paints, coatings, polymeric flocculants, paper and so on. It is conventionally produced from petrochemicals. Currently, most commercial acrylic acid is produced by partial oxidation of propene which produces unwanted by-products and large amount of inorganic wasters [118]. Nowadays, there has occurred an innovative manufacture method using nitrile-amide converting enzymes. Being different from manufacture of acrylamide, its manufacture requires microorganisms with an excess of amidase over nitrile hydratase, namely the rate of amidase-mediated step markedly exceeds the first step. Alternatively, nitrilase possessing microorganisms affords acids as well. In our research, a nitrilase-producing strain *Arthrobacter nitroguajacolicu* ZJUTB06-99 was newly isolated from soil sample in order to develop a production process of acrylic acid by biotransformation of acrylonitrile. According to previous reports, ϵ -caprolactam induced *R. rhodochrous* J-1 cells containing abundant nitrilase were used in the manufacture of acrylic acid, 390 g L⁻¹ of which were obtained under a periodic substrate feeding system [119].

5.1.2 Biotransformation of Aliphatic and Arylaliphatic Amides and Acids

Aliphatic and arylaliphatic amides and acids are readily available from the corresponding nitriles by the nitrile hydratase/amidase systems or nitrilase. A series of aliphatic nitriles, whether saturated or unsaturated, could be efficiently hydrolysed to the corresponding acids by the use of whole cells of *Rhodococcus* sp. AJ270. However, it was unsuitable for the preparation of amides, since the rate of the amide hydrolysis by amidase was greater than that of nitrile hydration. With one exception, unlike acrylonitrile and cinnamonitrile, methacrylamide was isolated after short activity due to the effect of adjacent substituent on the amidase-catalysed step [32].

In our study, a nitrilase from *B. subtilis* ZJB-063 was predominantly active on arylacetoneitriles. It was observed that electronic nature had a great effect on the initial rate of nitrilase mediated hydrolysis of *para*-substituted phenylacetoneitriles. The reaction was accelerated by electron-withdrawing substituents (Cl, NO₂) while slowed down by electron-donating substituents (OH, CH₃, OCH₃) [67]. Moreover, aliphatic and arylaliphatic amides could be provided by purified nitrile hydratase to avoid contaminated acids [19, 25].

5.1.3 Biosynthesis of Aromatic Acids and Amides

In previous studies there existed a hypothesis that aromatic nitriles were acceptable substrates for nitrilases while aliphatic nitriles were the same for nitrile hydratases. This hypothesis, however, was proved to be contradictory to many later observations which provided the evidence for the existence of nitrilases effective for aliphatic nitriles [120] and nitrile hydratases active on benzonitrile, its mono- or disubstituted derivatives [30, 32, 121, 122].

R. rhodocrous AJ270, a nitrile hydratase and amidase-containing microorganism, efficiently hydrolyzed all the selected aromatic nitriles including *para*-, *meta*- and *ortho*-substituted ones. Among these compounds, those with *para*-, *meta*-substituents gave acids at a fast rate, whereas conversion of aromatic nitriles bearing adjacent substituents almost ceased at the step of amides, and subsequent conversion of amides to acids proceeded rather slowly. The above finding clearly indicated that amidase was more sensitive to the electronic nature of the substituents. Disubstitution of benzonitrile with methoxy at positions 2 and 6 displayed a significantly adverse effect on nitrile hydratase. Displacement of methoxy by fluorine decreased the effect on nitrile hydratase but the step of hydrolysis of amides to acids still proceeded extremely slowly. This adjacent disubstitution phenomenon further confirmed that amide hydrolysis is stringently dependent on adjacent steric factors while nitrile hydration has a slight steric limitation [32].

5.1.4 Bioconversion of Heterocyclic Nitriles

In the past decades, commercial productions of acrylamide, nicotinamide and nicotinic acid have witnessed the significant use of nitrile-converting enzymes. Nicotinamide and nicotinic acid belong to heteroaromatic compounds. Nicotinamide and its acid are water-soluble B-complex vitamins (Vitamin B₃ or PP) used in pharmaceutical formulations, and as additives in food and animal feed; furthermore, their deficiency leads to pellagra. Moreover, nicotinic acid and its ester and amide derivatives have medical applications as antihyperlipidemic agents and peripheral vasodilators [123]. Currently, they are produced by chemical synthesis at high temperatures and pressures. Alternatively, they can be prepared under mild conditions by the bioconversion of 3-cyanopyridine with nitrile-converting containing microorganisms or enzymes.

R. rhodochrous J-1, a versatile nitrile-converting enzymes containing micro-organisms, had high activity toward 3-cyanopyridine in the presence of crotonamide as an inducer (Fig. 4). This process straightforwardly transferred to industrial level and was soon developed into an industrial production scale of nicotinamide for the following reasons. First, 3-cyanopyridine could be converted to nicotinamide without formation of nicotinic acid. Namely, the strain displayed considerably high nitrile hydratase activity and it is noteworthy that nicotinamide is not contaminated by nicotinic acid, since nicotinamide is almost inert to the low amidase activity in this strain. Second, there seemed no occurrence of substrate inhibition as compared to conversion of acrylonitrile. Finally, the fermentation mode turned out to be pseudocrystal fermentation (namely, crystalline substrate, solution of substrate, solution of product, crystalline product). From the synthetic point of view, various useful amides other than nicotinamide and acrylamide can be obtained by using the

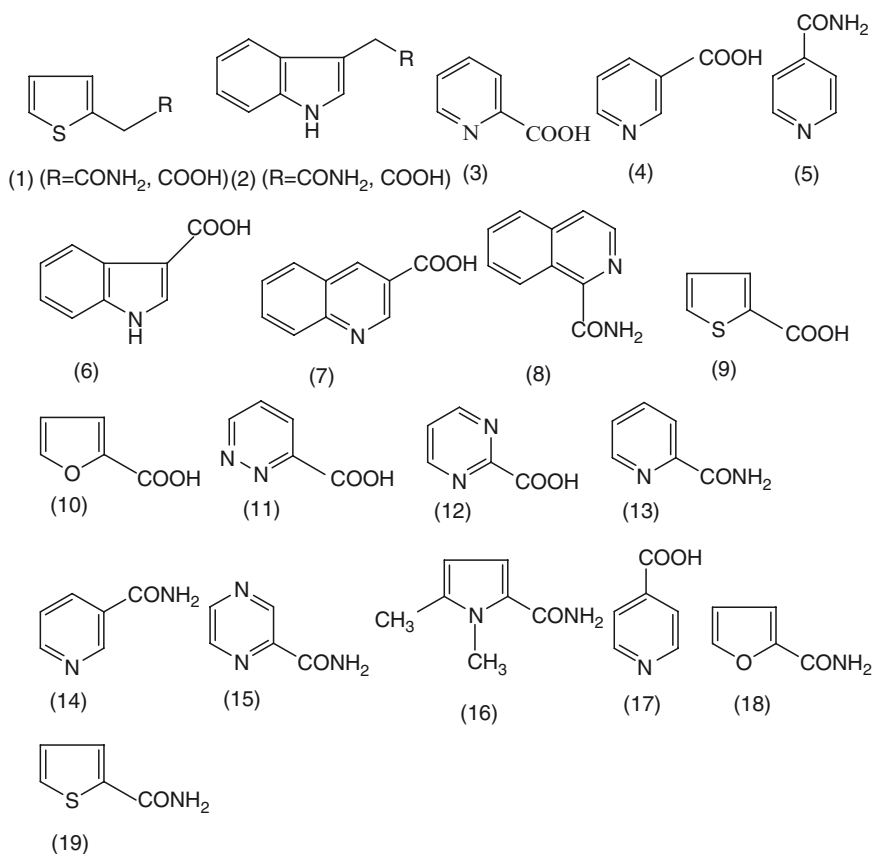


Fig. 4 Biotransformation of heterocyclic nitriles by the whole cells of *A. niger* K10 (1, 2, 6–8, 16, 17, 20), *Rhodococcus* sp. AJ270 (3–12, 16–19), *R. rhodochrous* NCIMB 11216 (6, 11, 12), *R. rhodochrous* J-1 (5, 13–15), *Rhodococcus* sp. strain YH3-3 (18, 19) and *R. equi* A4 (5, 13–15)

R. rhodochrous J-1 cells cultured in the presence of crotonamide. Isonicotinamide and pyrazinamide, useful tuberculostatics, can be produced in this way as well [124]. As for nicotinic acid, a thermostable nitrilase produced by the thermophilic bacterium *B. pallidus* DAC521 catalyzed the direct hydrolysis of 3-cyanopyridine to nicotinic acid without detectable formation of nicotinamide [125].

In recent years, investigation was carried out on the preparation of nicotinamide with *Corynebacterium glutamicum* in China [126, 127]. As a robust bacterium, *Rhodococcus* sp. AJ270 showed high nitrile hydratase activity against heterocyclic nitriles. The adjacent substituent impact existing in the hydrolysis of aromatic nitriles was encountered in the cases of heterocyclic nitriles. Those bearing an adjacent C=O/C=N remained intact after a long conversion time (Fig. 4) [32].

Our lab also provided an enzymatic route for the manufacture of nicotinamide. In addition, biosynthesis of 2-chloronicotinic acid is currently in active progress, which is a useful agricultural and pharmaceutical intermediate. Various other heteroaromatic amides and carboxylic acids procured currently via chemical synthesis could be produced biocatalytically from their nitriles. A cobalt-containing nitrile hydratase purified from *Rhodococcus* sp. strain YH3-3 was able to convert 2-cyanothiophene and 2-cyanofuran along with cyanopyrazine [128]. The nitrile hydratase from *R. equi* A4 also showed capacity for some heterocyclic nitriles, and similar results were observed that 2-cyanopyridine was transformed with a lower rate than 3- and 4-cyanopyridine (Fig. 4) [30]. In addition, bioconversion of some heterocyclic amides and acids could also be accomplished by *R. rhodochrous* J-1, *R. rhodochrous* NCIMB 11216 and *Aspergillus niger* K10 (Fig. 4) [25, 129].

5.1.5 Bioconversion of Alicyclic Nitriles

In the past few decades, a considerable amount of research concerning biotransformation of amides and acids has been carried out, the majority of which focused on the bioconversion of aromatic nitriles to its equivalent amides and acids and a paucity of which was concerned with the hydrolysis of alicyclic nitriles. Matoishi et al. conducted the hydrolysis of alicyclic mono- and dinitriles and amides mediated by *R. rhodochrous* IFO 15564, from which a variety of six-membered alicyclic cyano carboxylates, amido carboxylates, dicarboxylates from the corresponding nitriles were obtained (Fig. 5). The formation of these compounds was presumably ascribed to the stereochemistry of the substrate, the nature of substituents and presence of double bonds in alicyclic rings. These factors resulted in the rate difference of nitrile hydratase and amidase between enantiomers or enantiotopic groups, which in turn enabled kinetic resolution or asymmetrization [130].

Much attention has been paid in recent years to the preparation of enantiopure cyclopropyl compounds owing to the fact that enantiomers of these compounds often exhibit different biological activities. Hence, Wang and his coworker undertook the study of *Rhodococcus* sp. AJ270 mediated biotransformation of *trans*-2-arylcylopropanecarbonitriles, by which not only optically active acids but also the amides were obtained (Scheme 5). In addition, bioconversions of 2-arylcylopropanecarbonitriles

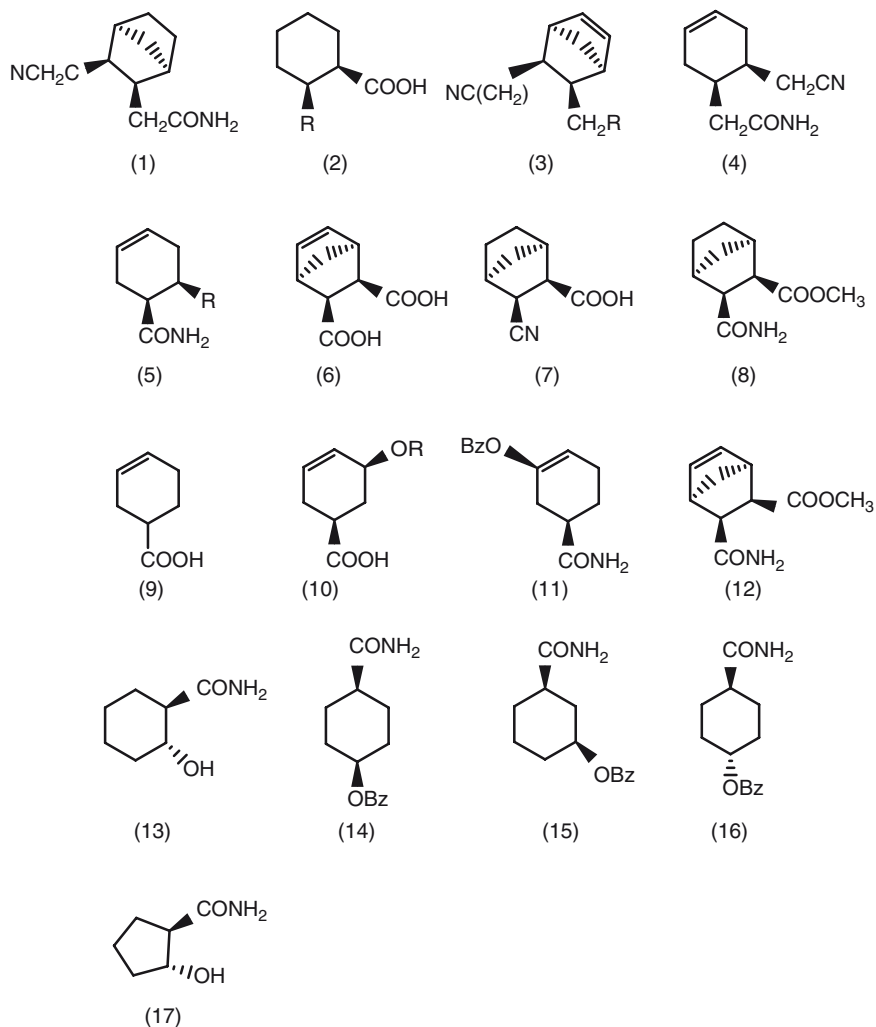
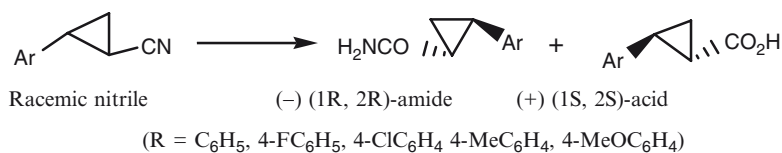


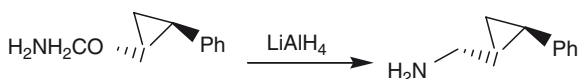
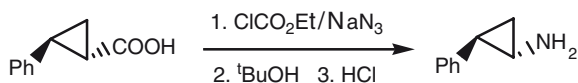
Fig. 5 Alicyclic mono-, di- and cyanocarboxylic acids and their amides prepared by the use of *Rhodococcus rhodochrous* IFO 15564 (R=CONH₂, COOH) (**1 – 12**) and *R. equi* A4 (**13 – 17**)



Scheme 5 Enantioselective biotransformations of racemic *trans*-2-arylcyclopropanecarbonitriles by *Rhodococcus* sp. AJ270

Table 6 Bioconversions of 2-arylcyclopropanecarbonitriles and its analogs by *Rhodococcus* sp. AJ270

Ar	Time (h)	Amide (%)	Amide (<i>e.e.</i>)	Acid (%)	Acid (<i>e.e.</i> %)
C ₆ H ₅	0.5	37	78	37	73(81)
C ₆ H ₅	1	50	>99	39	55
C ₆ H ₅	3	25	>99	61	19
4-FC ₆ H ₄	2	44	89	48	75(91)
4-FC ₆ H ₄	5	33	>99	66	30
4-ClC ₆ H ₄	4	25	>99	43	52(78)
4-ClC ₆ H ₄	5.5	29	>99	62	41
4-MeC ₆ H ₄	2	46	70	32	75
4-MeC ₆ H ₄	5	32	>99	53	71(82)
4-MeOC ₆ H ₄	7	31	44	36	17
4-MeOC ₆ H ₄	10	39	21	41	12

**Scheme 6** Reduction of (–)-(1*R*,2*R*)-2-phenylcyclopropanecarboxamide using LiAlH₄**Scheme 7** Preparation of (+)-(1*S*,2*R*)-2-phenylcyclopropylmethylamine by a modified Curtius rearrangement

and its analogs with *para* substituents, a methyl, chlorine and fluorine, led to good to excellent enantioselectivity of the corresponding amides and acids, especially the amides. Exceptionally, comparatively low enantioselectivity of both amides and acids was detected in the reaction of 2-(4-methoxyphenyl)cyclopropanecarbonitrile, indicating that methoxy group caused a more dramatic effect than the above sterically smaller ones (Table 6). Moreover, (–)-(1*R*, 2*R*)-2-phenylcyclopropylmethylamine, a potential candidate for antihypertensive agent, could be provided by the reduction of (–)-(1*R*, 2*R*)-arylcyclopropanecarboxamide (Scheme 6). (+)-(1*S*, 2*R*)-2-Phenylcyclopropylmethylamine, antidepressant tranylcypromine, was obtained from (+)-2-arylcyclopropanecarboxylic acid via a modified Curtius rearrangement (Scheme 7) [131].

In addition, a recombinant nitrilase AtNITI from *A. thaliana* bore the capability of regio- and stereoselective hydrolysis of 3-(2-cyanocyclohex-3-enyl)propenenitriles, which consisted of four isomers numbered A–D prepared by Diels–Alder reaction of 1-cyano-1, 3-butadiene. Consequently, isomer D was exclusively hydrolyzed among the four dinitriles and thus (*E*)-*cis*-3-(2-cyanocyclohex-3-enyl)-propenoic acid was dominantly accumulated. Besides, isomer C was hydrolyzed to a small extent as well,

which only occurred after complete conversion of D and when the enzyme concentration was high enough, while isomers A and B remained intact, in spite of higher enzyme concentration and prolonged conversion time. This outcome was considered to be explained by three factors: on one hand, the high regioselectivity, on the other hand, the high selectivity, including both (*E*)- and *cis* selectivity (Fig. 5) [132]. Similar discrimination between geometric isomers of substituted alicyclic nitriles was also accessed with the nitrile hydratase from *R. equi* A4. It was noted that *trans*-4-benzoyloxycyclohexanecarbonitrile, *cis*-3-benzoyloxy cyclohexanecarbonitrile, *trans*-2-hydroxycyclohexanecarbonitrile and *trans*-2-hydroxycyclopentanecarbonitrile were converted to the equivalent amides. In contrast, *cis*-2-hydroxycyclohexanecarbonitrile and *cis*-2-hydroxycyclopentanecarbonitrile gave a majority recovery of them. Although *cis*-4-benzoyl-oxy-cyclohexanecarbonitrile was an acceptable substrate for the enzyme, the reaction proceeded at a rather low rate (Fig. 6) [133].

Moreover, an impressive example of the application of nitrile-amide converting enzymes was enzymatic synthesis of optically active 2-methyl- and 2,2-dimethylcyclopropanecarboxylic acid and their derivatives. 2-Methyl- and 2,2-dimethylcyclopropanecarboxylic acid derivatives are key intermediates of curacin A, a potent antimetabolic agent, and of cilastatin, a renal dehydropeptidase inhibitor commonly administered with penem and carbapenem antibiotics to prevent their degradation in the kidney, respectively. In the past, no direct asymmetric synthesis of these compounds has been reported. Optically active 2-methylcyclopropanecarboxylic acid and its amide derivatives have been prepared from the optical resolution or through the multistep transformations. Recently, nitrile-hydratase-associated amidases participated in the synthesis of optically active acids [110]. *S*-(+)-2,2-

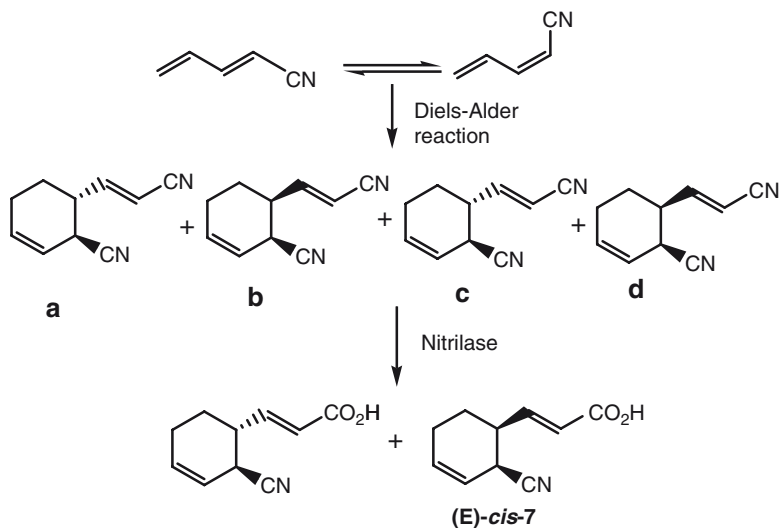
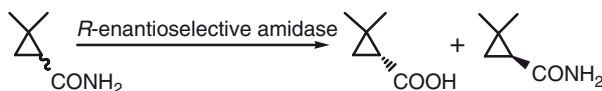


Fig. 6 Regioselective hydrolysis of 3-(2-cyanocyclohex-3-en-1-yl)-propanenitriles by a recombinant nitrilase AtNITI from *A. thaliana*



Scheme 8 Preparation of (*S*)-2,2-dimethylcyclopropanecarboxamide by (*R*)-enantioselective amidase in *D. tsuruhatensis* ZJB-05174

Dimethylcyclo-propanecarboxamide has been obtained from kinetic resolution of racemic 2,2-dimethylcyclopropanecarboxamide employing amidase-containing microbes or the amidases, which was a process adopted in Lonza for industrial operation. The above preparation involved in amidases exhibiting *R*-enantioselective. However, *Comamonas acidovorans* A18 was the only strain reported abroad, containing *R*-enantioselective amidase and has gained success in kinetic resolution of the racemic mixture [134]. Recently, *D. tsuruhatensis* ZJB-05174, capable of *R*-enantioselective degradation of 2,2-dimethylcyclopropanecarboxamide, was isolated employing a newly established colorimetric screening method. This isolate may be a suitable candidate for the production of (*S*)-2,2-dimethylcyclopropanecarboxamide from its racemic form after optimization of culture and biotransformation conditions (Scheme 8) [89].

5.1.6 Enantioselective Hydrolysis of Racemic Nitriles and Amides

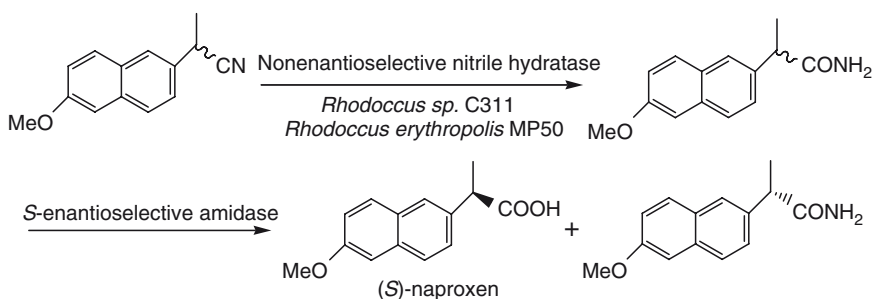
Enantioselective Hydrolysis of Racemic Nitriles by Nitrile Hydratase

Although a significant number of nitrilases and amidases exhibited different degrees of enantioselectivity, nitrile hydratases were previously assumed to be relatively non-stereospecific. However, later on reports concerning enantioselective hydration of α -arylpropionitriles appeared. Unfortunately, an extreme paucity of nitrile hydratases exhibited enantioselectivity that gave rise to products with the ee value higher than 75%. *Pseudomonas putida* NRRL 18668, a soil derived Gram-negative bacterium, contained a nitrile hydratase capable of stereoselective hydrolysis of 2-(4-chlorophenyl)-3-methylbutyronitrile with more than 90% enantiomeric excess (ee) to the (*S*)-amide, which appeared to be the first description of a stereoselective nitrile hydratase from a Gram-negative organism. This strain is also capable of a two-step hydrolysis of 2-(4-isobutylphenyl)-propionitrile and 2-(6-methoxy-2-naphthyl)-propionitrile to the (*S*)-acids (ibuprofen and naproxen respectively) with stereoselectivity residing primarily in the aliphatic amidase [135]. Subsequently, another Gram-negative isolate *Agrobacterium tumefaciens* d3 appeared harboring enantioselective nitrile hydratase, which hydrated racemic 2-phenylpropionitrile, 2-phenylbutyronitrile, 2-(4-chlorophenyl)propionitrile, 2-(4-methoxy)propionitrile or ketoprofen nitrile, and the corresponding (*S*)-amides were formed with the highest enantiomeric excesses (ee >90% until about 30% of the respective substrates were converted) [136].

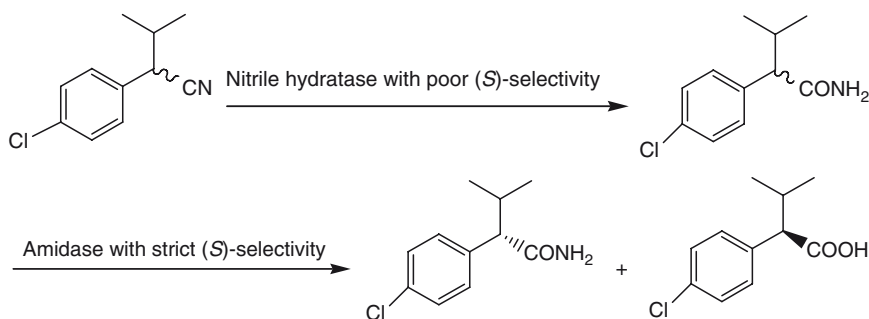
Former studies showed that in the nitrile hydratase-amidase system enantiomeric discrimination occurred during amide hydrolysis. As an exception, *Rhodococcus* sp. 270, known as a nitrile hydratase and amidase containing organism, was effective for converting (*R*)-2-phenyl- butyronitrile to the corresponding amide with an *ee* value of 83%, suggesting that the amidase turned to be inactive for the amide and the formation of the chiral amide was due to the *R*-enantioselectivity of the nitrile hydratase [137].

In general, nitrile hydratase are coupled with amidase in organisms, and in most cases, formations of optically pure amides and acids are owing to the higher enantioselectivity of amidase compared with that of nitrile hydratase. A number of amides and acids were obtained via this method, for example, enzymatic synthesis of (*S*)-naproxen by *Rhodococcus* sp. C3II and *Rhodococcus erythropolis* MP50. From racemic naproxen nitrile, *Rhodococcus* sp. C3II formed *S*-naproxen amide and subsequently *S*-naproxen. Racemic naproxen amide was hydrolysed to *S*-naproxen. *Rhodococcus* sp. MP50 converted racemic naproxen nitrile predominantly to *R*-naproxen amide and racemic naproxen amide to *S*-naproxen. With both strains racemic naproxen amide was converted to *S*-naproxen with an enantiomeric excess >99% at a conversion rate up to 80% of the theoretical value (Scheme 9) [138]. By the hydrolysis of (*R,S*)-isopropyl-4 -chlorophenylacetoneitrile using cells of *Pseudomonas* sp. B21C9, enantiopure (*S*)-2-isopropyl-4 -chlorophenylacetic acid, an intermediate of pyrethrins could be obtained. It appeared that the process proceeded via stepwise reactions by a nitrile hydratase exhibiting poor (*S*)-selectivity and an amidase exhibiting strict (*S*)-selectivity (Scheme 10). Heating resulted in the racemization of (*R*)-amide, which in turn caused enhanced yield of (*S*)-acid [139].

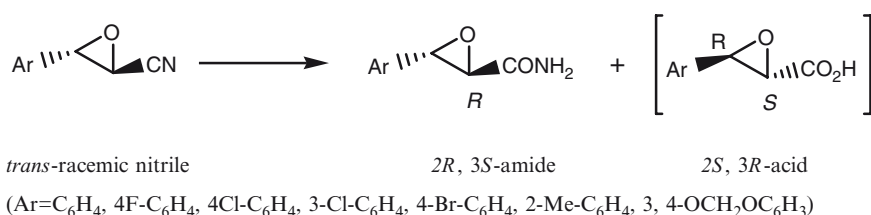
It was also described in China that *Rhodococcus* sp. AJ270 could be utilized for the preparation of enantiopure carboxylic acids and derivatives via stereoselective hydrolysis of a series of racemic α -substituted phenylacetoneitriles and amides. The overall enantioselectivity of the process is mainly determined by the combination of selectivities of nitrile hydratase and amidase, with the latter being a major contributor, which was consistent with the above findings. Enantioselective synthesis



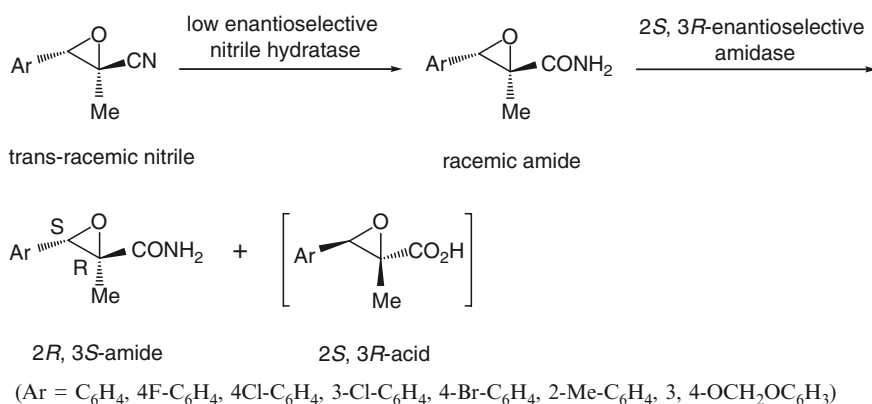
Scheme 9 Preparation of *S*-naproxen by *Rhodococcus* sp. C3II and *R. erythropolis* MP50



Scheme 10 Preparation of (*S*)-2-isopropyl-4'-chlorophenylacetic acid by *Pseudomonas* sp B21C9

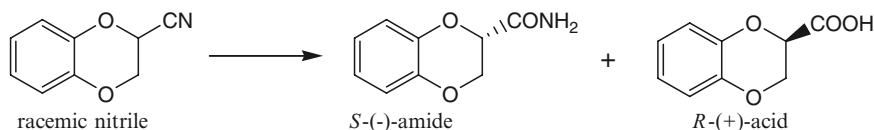


Scheme 11 Biotransformations of racemic *trans*-2,3-epoxy-3-arylpropanenitriles by *Rhodococcus* sp. AJ270



Scheme 12 Biotransformations of racemic *trans*-3-aryl-2-methyloxiranecarbonitrile by *Rhodococcus* sp. AJ270

of optically active 2-methyl-2,2-dimethylcyclopropanecarboxylic acids (see Sect. 5.1.5) and chiral cyclopropane compounds were successfully performed by the same strain (see Sect. 5.1.5). Very recently, it was observed that *Rhodococcus* sp. AJ270 are able to catalyze the hydrolysis of oxiranecarbonitrile to synthesize



Scheme 13 Enantioselective biotransformations of racemic **1**, 4-benzodioxane-2-carbonitrile by *Rhodococcus* sp. AJ270

optically active *2R,3S*-3-aryloxiranecarboxamides (Scheme 11) [140] and *2R,3S*-3-aryl-2-methyloxiranecarboxamides (Scheme 12) [141]. As a useful microorganism, *Rhodococcus* sp. AJ270 is also able to rapidly catalyze enantioselective hydrolysis of racemic 1,4-benzodioxane-2-carbonitrile under very mild conditions, yielding *2S*-1,4-benzodioxane-2-carboxamide and *2R*-1,4-benzodioxane-2-carboxylic acid in high yields with excellent enantioselectivity, which are very important entities in medicinal chemistry for they are chiral building blocks in the design and synthesis of chiral therapeutic agents (Scheme 13) [142].

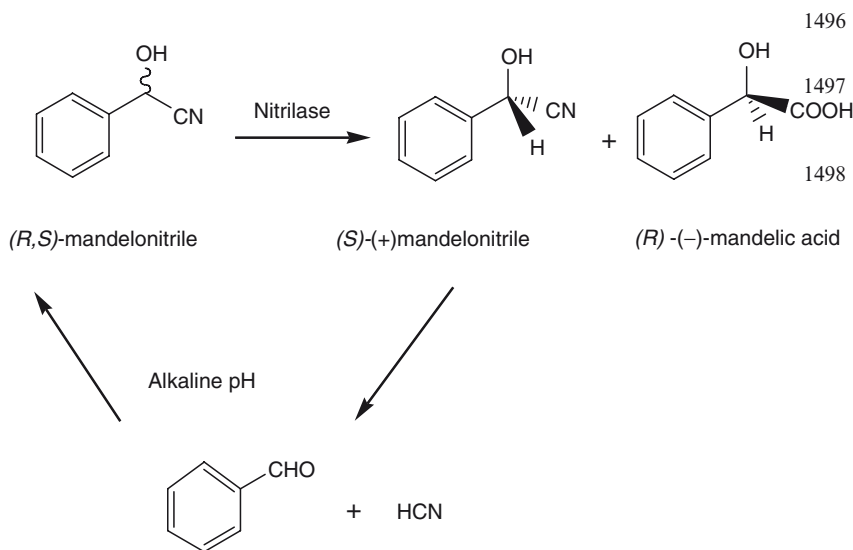
Enantioselective Hydrolysis of Racemic Nitriles by Nitrilase

The previously limited evidence for stereoselective nitrilases has been extended recently [143, 144] and their functions have been widely applied in the manufacture of various optically active carboxylic acids.

Amino acids are widely used as building blocks for the pharmaceutical industry, feed additives and human nutrition. Nitrilase participated manufacture of amino acids represents an attractive approach. In previous studies, formation of l-amino acids from α -aminonitriles was achieved using alginate immobilized *Acinetobacter* sp. APN containing nitrilase [145]. Later on, several optically active amino acids were produced from α -aminonitriles by *R. rhodochrous* PA-34 [146].

An intelligent screening method gave access to enantioselective nitrilases that are highly adapted to the production of high-value hydroxyl carboxylic acid derivatives, such as (*R*)-(-)-mandelic acid, which was described as a key intermediate of semi-synthetic cephalosporins and penillins, a chiral resolving agent, chiral synthon for the synthesis of anti-tumor agents and anti-obesity agents. Three isolates including *P. putida*, *Microbacterium paraoxydans* and *Microbacterium liquefaciens* gave rise to the desired product, (*R*)-(-)-mandelic acid. In terms of growth rate, enzyme activity, enantioselectivity and thermostability, *P. putida* was more suitable compared to the other two organisms. Thus it was selected for further use [95].

Noticeably, *Alcaligenes faecalis* ATCC 8750 was famous for the effective hydrolysis of mandelonitrile to (*R*)-(-)-mandelic acid. Interestingly, enantiomeric excess of (*R*)-(-)-mandelic acid formed from mandelonitrile was 100% and the yield attained was approximately 91%. Spontaneous racemization of *S*-mandelonitrile because of the chemical equilibrium accounted for the high yield. The observation was inconsistent

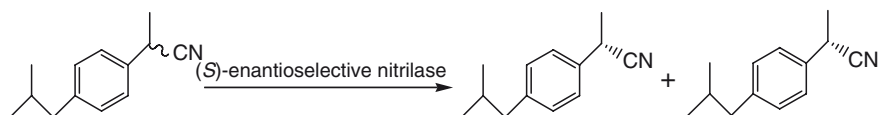


Scheme 14 Preparation of (R)-(-)-mandelic acid by *A. faecalis* ATCC 8750

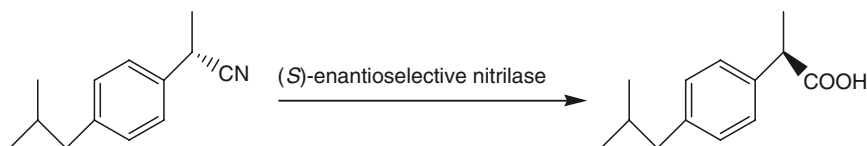
with other enantioselective bioprocess where the yield attained was 50% at most. Later studies successfully completed the purification and characterization of the nitrilase, meanwhile, immobilization of the nitrilase (Scheme 14) [147, 148]. In particular, the immobilized nitrilase is useful for the production of hydroxy analogues of methionine derivatives that could have an interest in cattle feeding and in the transformation of compounds bearing other acid- or base-sensitive groups [149].

Researchers in China conducted the nitrilase-mediated manufacture of (R)-(-)-mandelic acid as well. He et al. screened a microbial strain identified as *Alcaligenes* sp. ECU0401 harboring a stereoselective nitrilase for the kinetic resolution of racemic mandelonitrile to (R)-(-)-mandelic acid with an enantiomeric excess of >99.9% [150]. Aiming at obtaining microorganisms with high enzyme activity and excellent selectivity, our lab has been undertaking bioconversion of (R)-(-)-mandelic acid and have made much progress. This convenient and practical approach to producing (R)-(-)-mandelic acid was developed into commercial application by Mitsubishi Rayon [151].

As mentioned earlier, optically active 2-arylpropionic acids like (S)-naproxen, (S)-ibuprofen and (S)-ketoprofen could be produced from the respective 2-arylpropionitrile by the aid of the sequential action of nitrile hydratase and amidase, besides, these acids could also be procured via (S)-enantioselective nitrilases. It was evident that interaction of racemic 2-(4'-isobutylphenyl) propionitrile with *Acinetobacter* sp. strain AK226 yielded S-(+)-2-(4'-Isobutylphenyl) propionic acid (S-(+)-ibuprofen) with a 95% enantiomeric excess (Scheme 15). The observation that R-enantiomer of the nitrile was inert to the organism and no detection of the



Scheme 15 Production of *S*-(+)-ibuprofen by (*S*)-enantioselective nitrilase in *Acinetobacter* sp. strain AK226



Scheme 16 Production of (*S*)-naproxen by (*S*)-enantioselective nitrilase in *R. rhodochrous* ATCC 21197

amide throughout the reaction suggested *Acinetobacter* sp. strain AK226 was a highly enantioselective nitrilase possessing bacterium [152]. Moreover, nitrilase in *R. rhodochrous* ATCC 21197 also appeared to be able to convert racemic 2-arylpropionitrile to optically active 2-arylpropionic acids. (*S*)-Naproxen could be obtained by this organism and excellent enantioselectivity could be achieved at the cost of conversion (Scheme 16) [153].

Enantioselective Hydrolysis of Racemic Amides by Amidase

Amidases with enantioselectivity are universal and these amidases catalyzed reactions offered optically pure compounds of pharmaceutical importance such as amino acids or 2-arylpropionic acids. Thus, they have attracted substantial interest and much progress has been made. A typical example of an enantioselective amidase catalyzed industrial biotransformation is the enzymatic chiral resolution of (*R*, *S*)-2,2-dimethylcyclopropane carboxamide providing the optically pure *S*-isomer. The process was developed by Lonza AG (see Sect. 5.1.5) (Lonza AG, CH). Similarly, using an *R*-enantioselective amidase producer, *D. tsuruhatensis* ZJB-05174, to produce (*S*)-2,2-dimethylcyclopropane carboxamide was investigated in our lab (See Sect. 5.1.5). The route, furthermore, is under progressive development and its potential of industrial application has been developed by Hisun Pharmaceutical Co., Ltd (Zhejiang).

As previously exemplified, efficient kinetic resolution of racemic piperazine-2-carboxamide and racemic piperidine-2-carboxamide to the corresponding enantiomerically pure carboxylic acids by the aid of whole cells of wild-type microorganisms harboring stereospecific amidases. The attained acids, (*S*)- and (*R*)-piperazine-2-carboxylic acid, and (*S*)-piperidine-2-carboxylic acid belong to non-proteinogenic amino acids and are used as precursors of numerous bio-active

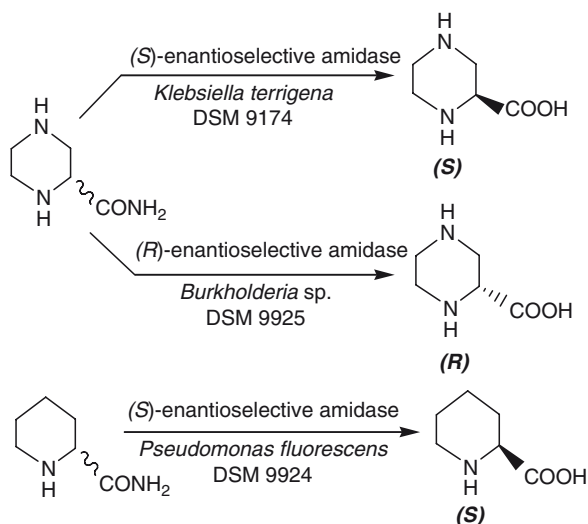


Fig. 7 Preparation of chiral non-proteinogenic amino acids by enantioselective hydrolysis of racemic amides

compounds. Due to the value of (S)-piperazine-2-carboxylic acid, which can be used for the synthesis of the HIV protease inhibitor Crixivan, its biosynthesis route employing *Klebsiella terrigena* DSM 9174 was developed into commercial application by Lonza AG [154] (Fig. 7).

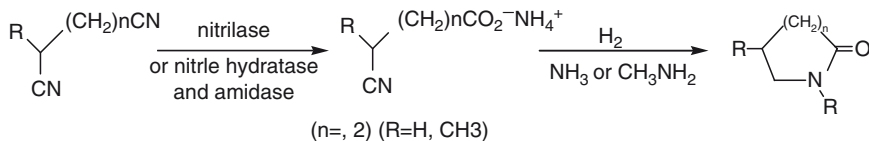
Besides, amidase often coexists with the nitrile hydratase and formation of optically active acids is always ascribed to the combination of the poor selectivity of nitrile hydratase and excellent selectivity of amidase, which was discussed above (see “Enantioselective Hydrolysis of Racemic Nitriles by Nitrile Hydratase” above). As described above, in spite of the various applicability of the amidase, their application on a large or commercial scale has remained unexplored.

5.1.7 Regioselective Biotransformation of Di- and Trinitriles

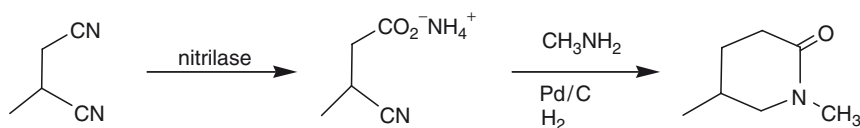
It is rather difficult to conduct regioselective hydrolysis of dinitriles by chemical methods in a single step. An enzymatic approach, however, offers significant advantages since ammonium cyanocarboxylates from dinitriles could be obtained in nearly quantitative yields in one step. *Acidovorax facilis* 72W that possessed a regioselective nitrilase was successfully utilized for the conversion of an aliphatic α,ω -dinitrile to an ammonium salt of ω -cyanocarboxylic acid, which was then directly converted to the corresponding lactam by hydrogenation, without detection of the intermediate ω -cyanocarboxylic acid or ω -aminocarboxylic acid (Scheme 17) [155]. The process could also be achieved by the action of a combined nitrile hydratase and amidase present in *Comamonas testosteroni* 5-MGAM-4D, where an

aliphatic α,ω -dinitrile is initially converted to an ω -cyanoalkylamide by the nitrile hydratase, and the ω -cyanoalkylamide is subsequently attacked by the amidase to the corresponding ω -cyanocarboxylic acid ammonium salt, implying that the regioselectivity of the system originated from nitrile hydratase (Scheme 17). Typically, a chemoenzymatic process for the preparation of 1,5-dimethyl-2-piperidone from 2-methylglutaronitrile with greater than 98% regioselectivity at 100% conversion has been demonstrated using immobilized *A. facilis* 72W cells [156] (Scheme 18). Besides, purification and characterization of the regioselective aliphatic nitrilase from this strain was carried out; furthermore, the nitrilase gene was cloned, sequenced and over-expressed in *Escherichia coli*, yielding a recombinant microorganism which more efficiently hydrolyzed aliphatic dinitriles to cyanocarboxylic acids in comparison with the wild type [157]. Thus, five- or six-membered ring lactams could be obtained from the sole product of regioselective conversion of dinitriles by means of nitrilase in *Acidovorax facilis* 72W.

As exemplified, a biocatalytically commercial scale process for the highly regioselective hydration of adiponitrile to 5-cyanovaleramide, which is required in the first step of manufacture of a new herbicide, has been demonstrated using *Pseudomonas chlororaphis* B23 cells immobilized in calcium alginate beads [158]. In terms of enzyme stability and productivity of 5-cyanovaleramide, it was proved that it was superior to all other whole-cell microbial catalysts which were examined (Scheme 19)



Scheme 17 Regioselective hydrolysis of aliphatic α,ω -dinitriles by nitrilase in *A. facilis* 72W or nitrile hydratase/amidase in *C. testosteroni* 5-MGAM-4D



Scheme 18 Regioselective hydrolysis of 1,5-dimethyl-2-piperidone from 2-methylglutaronitrile by immobilized *A. facilis* 72W



Scheme 19 Regioselective hydrolysis of adiponitrile by *P. chlororaphis* B23

[21]. Desymmetrization of prochiral dinitriles was also investigated by Crosby, Turner and their co-workers, in whose study, *O*-substituted-3-hydroxyglutaronitriles were converted using different *Rhodococcus* whole-cell catalysts and obtained the corresponding monocyanocarboxylic acids in good enantiomeric excess [159]. It is also interesting to note that biotransformation of a disubstituted malononitrile catalyzed by *R. rhodochrous* 21197 yielded an amido-acid with excellent enantioselectivity [160].

Regarding di- and tri-nitrile degradation, Asano et al. described it in the early 1980s [161–163]. Besides, *Rhodococcus* sp. AJ270 efficiently hydrolyzed a variety of dinitriles with excellent regioselectivity. Aliphatic dinitriles $\text{NC}[\text{CH}_2]_n\text{CN}$ underwent regioselective hydrolysis to give the mono acids with up to four methylenes between the nitrile groups, whereas those bearing $n > 4$ gave the diacids with good yield (Table 7). Significant regioselectivity was discovered using a range of α,ω -dinitriles $\text{NC}[\text{CH}_2]_n\text{X}[\text{CH}_2]_n\text{CN}$ with an ether or sulfide linkage as substrates. These compounds were efficiently transformed into the mono acids when an oxygen is placed β , γ or δ to the nitrile or β or γ -sulfur substituent is present (Table 8). As an efficient and robust

Table 7 Conversion of $\text{NC}[\text{CH}_2]_n\text{CN}$ **1** into $\text{NC}[\text{CH}_2]_a\text{CO}_2\text{H}$ **2** and/or $\text{HO}_2\text{C}[\text{CH}_2]_n\text{CO}_2\text{H}$ **3** by *Rhodococcus* sp. AJ270

Substrate (n)		Reaction condition		Product yield (%)	
1		Amount (mmol)	Time (h)	2	3
2		5	3	30	-
2		5	24	17	-
3		5	3	41	-
3		5	16	35	-
4		5	3	41	4
4		5	24	28	23
4		5	48	-	26
5		5	3	-	46
5		5	48	-	74
6		5	48	-	78
7		5	48	-	88
8		5	48	-	89

Table 8 Conversion of $\text{NC}[\text{CH}_2]_n\text{X}[\text{CH}_2]_n\text{CN}$ **4** into $\text{NC}[\text{CH}_2]_n\text{X}[\text{CH}_2]_a\text{CO}_2\text{H}$ **5** and/or $\text{HO}_2\text{C}[\text{CH}_2]_n\text{X}[\text{CH}_2]_n\text{CO}_2\text{H}$ **6** by *Rhodococcus* sp. AJ270

Substract		Reaction conditions		Product yield (%)		
n	X	Amount (mmol)	Time (h)	5	6	4
2	O	3	48	61	-	-
3	O	5	1	35	Trace	35
3	O	3	72	-	70	-
4	O	3	2	73	-	-

(continued)

Table 8 (continued)

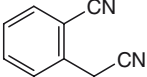
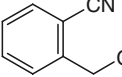
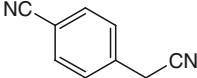
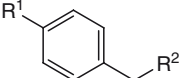
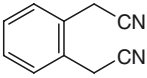
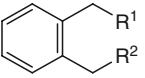
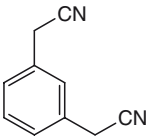
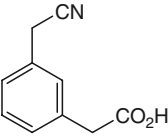
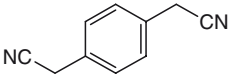
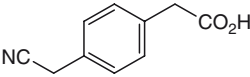
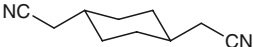
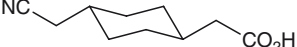
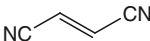
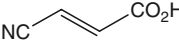
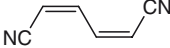
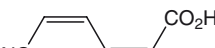
Substrate		Reaction conditions		Product yield (%)		
n	X	Amount (mmol)	Time (h)	5	6	4
4	O	3	120	75	5	-
5	O	3	2	-	25	60
5	O	3	48	-	97.5	-
2	S	3	1	45	-	10
2	S	3	48	-	60	-
3	S	3	2	52	-	29
3	S	3	96	83	-	-
4	S	3	2	-	83	Trace
4	S	3	96	-	86	-
2	NPh	2	64	93	-	-
2	NC ₆ H ₄ Cl- <i>p</i>	2	168	71	-	26
2	NC ₆ H ₄ OMe- <i>p</i>	2	72	91	-	-
3	NPh	1.5	168	-	-	95
3	NC ₆ H ₄ OMe- <i>p</i>	1.5	168	-	-	92
4	NPh	1.5	168	-	-	94
5	NPh	1.5	168	-	-	99

nitrile-amide converting organism, the strain also effectively catalyzed the hydrolysis of a variety of miscellaneous aliphatic dinitriles into mono acids with the exception of *o*-phenylenediacetonitrile where *o*-phenylenediacetamide was derived as the major product (Table 9). Finally, efficient regiocontrol was also accessible when *m*- and *p*-dicyanobenzenes were used as substrates [164, 165] (Table 10).

Other nitrile-converting enzyme from *Rhodococcus* genus also exhibited regioselectivity. Nitrilase of *R. rhodochrous* NCIMB 11216 showed regioselectivity to some extent as well. The extent of conversion with saturated aliphatic dinitriles was very low, whereas hydrolysis of unsaturated aliphatic dinitriles occurred at similar rates to that of aromatic mononitriles. Greater structural rigidity of these compounds caused by the presence of a double or the aromatic ring could possibly accounted for the above observed phenomenon. In case of the hydrolysis of dicyanobenzenes, it proceeded also at a relatively low rate, among which 1, 4-dicyanobenzene bearing most spatially separated nitrile groups were converted to mononitrile at the highest rate. However, biotransformation of 1,3-dicyanobenzene turned out to be somewhat different with the addition of different inducers. The propionitriles-induced cells hydrolyzed not only 3-cyanobenzoate but also both the nitrile groups in 1,3-dicyanobenzene, yielding diacids. In sharp contrast, benzonitrile-induced cells were incapable of hydrolyzing 3-cyanobenzoate, so the process halted at the formation of mononitrile. Besides, 2-cyanophenylacetic acid could be prepared from α -cyano-*o*-tolunitrile by both of the nitrilases [99].

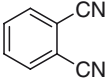
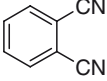
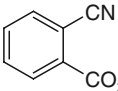
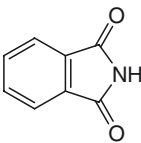
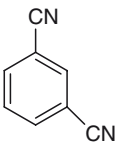
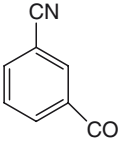
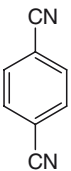
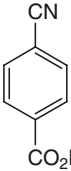
Dadd et al. assessed the biotransformation of 2-, 3- and 4-(cyanomethyl) benzonitriles employing *R. rhodochrous* LL100-21. As a result, 2-(cyanophenyl) acetic

Table 9 Conversions of miscellaneous dinitriles into acids by *Rhodococcus* sp. AJ270

Substrate	Condition		Product and yield
	Concentration (mM)	T(h)	
	2	24	 80%
	2	24	 $R^1 = \text{CN}, R^2 = \text{COOH}$ 65% $R^1 = \text{COOH}, R^2 = \text{CN}$ 16%
	3	24	 $R^1 = R^2 = \text{CN}$ 13% $R^1 = \text{CN}, R^2 = \text{COOH}$ 11% $R^1 = R^2 = \text{CONH}_2$ 65%
	3	30	 67%
	3	30	 69%
	1.5	39	 99%
	5	14	 67%
	5	14	 86%

acid was formed as the sole product by propionitrile or benzonitrile induced cells which may be explained by the hypothesis that aliphatic side chain of the compound prevented the hydrolysis of the aromatic cyano group owing to steric hindrance. Conversely, these cells led 3- and 4-(cyanomethyl) benzonitriles to 3- and 4-(cyanomethyl) benzoic acid as the major products with other products of relatively low concentrations. More interestingly, acetonitrile induced cells resulted in a mixture of

Table 10 Conversions of aromatic dinitriles into acids *Rhodococcus* sp. AJ270

Substrate	Conversion time (h)	Product and yield (%)
	70	 52%
		 12%
		 12%
	24	 71%
	20	 81%

different products from 2-, 3- and 4-(cyanomethyl) benzonitriles indicating less regioselectivity in such case [28].

5.1.8 Conversions of Nitriles Bearing Labile Functional Groups

Biocatalysts have been applied in the hydrolysis of nitriles containing other labile groups which are unable to survive under harsh chemical conditions. Attempts were made by Klempier et al. to selectively hydrolyze nitriles with acetal and ester groups in the molecule using *R. rhodochrous* NCIMB 11216 (Fig. 8). While the former functional moieties were inert to the catalyst during the hydrolysis, cleavage of the latter ones (leading to cyano acids and diacids) reduced the product yields, which was caused by the ubiquitous esterase activity [166]. Hence, suppressing the esterase activity of this strain was of significant importance, such as by modification of the growth media or employing RNAi technology. Alternatively, purified enzymes turned out to be more preferable.

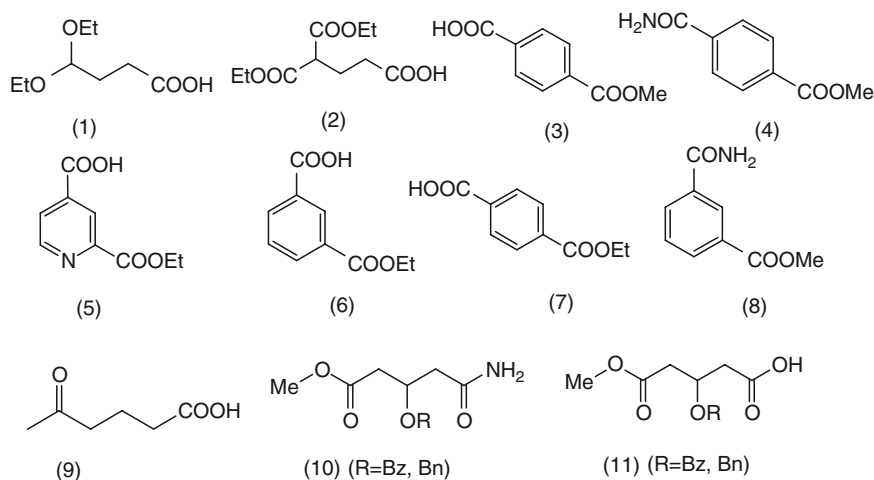


Fig. 8 Production of amides and acids from nitriles bearing labile functional groups by whole cells of *R. rhodochrous* NCIMB 11216 (1–3, 5, 6, 9), *R. equi* A4 (3, 4, 7, 8, 10, 11) and *Rhodococcus* sp. AJ270 (3)

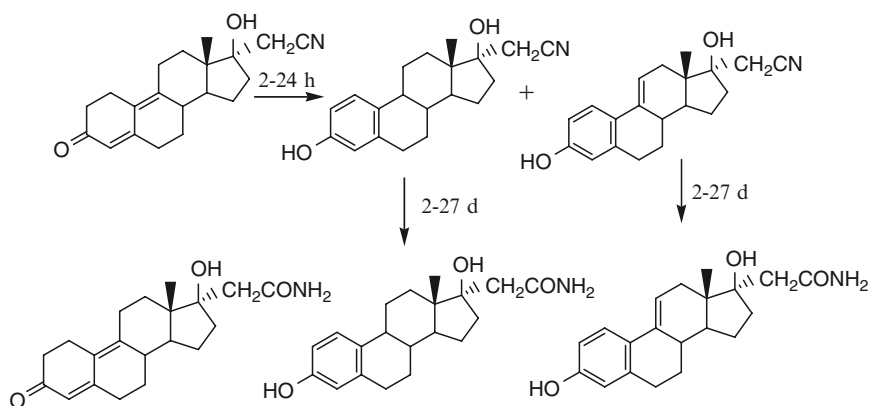


Fig. 9 Bioconversion of 17α-cyanomethyl-17β-hydroxy-estra-4,9-dien-3-one by whole cells of *R. erythropolis*

Excellent chemoselectivity was also observed for the hydrolysis of cyanobenzoates catalyzed by whole cells of *R. equi* A4. Monomethyl isophthalate and monomethyl terephthalate could be obtained from methyl-3-cyanobenzoate and methyl-4-cyanobenzoate, respectively, via resting cells of the strain [167]. Moreover, this strain is also capable of chemoselectively hydrolysing methyl (*R,S*)-3-benzoyloxy-4-cyanobutanoate and methyl (*R,S*)-3-benzoyloxy-4-cyano-

butanoate into monomethyl (*R,S*)-3-benzoyloxyglutarate and monomethyl (*R,S*)-3-benzyloxyglutarate, respectively [168] (Fig. 8). Hydrolysis of the progestin dienogest (17 α -cyanomethyl-17 β -hydroxy-estra-4, 9-dien-3-one) was performed by the nitrile hydratase-containing microorganism *R. erythropolis*. Along with the slow hydrolysis of cyano group, aromatization of ring A and hydroxylation occurred as well. After prolonged fermentation, the 17 α -acetamido derivatives of estradiol and of 9(11)-dehydroestradiol were formed. Three of the metabolites were also prepared synthetically [169]. *Rhodococcus* sp. AJ270 also made it possible to synthesize some carboxylic acids and amides from the nitrile bearing sensitive groups (Fig. 9) [164].

5.2 Biodegradation and Bioremediation

Due to the intrinsic nature of nitriles as highly toxic, carcinogenic and mutagenic, it is necessary to control and monitor the discharge of these organonitriles into the environment. Typical examples of these compounds include acetonitrile, acrylonitrile and benzonitrile that are widely used in laboratories and industries as solvents and extractants, or used as an ingredient in pharmaceuticals, plastics, synthetic rubbers, drug intermediates (chiral synthons), herbicides and pesticides (e.g., dichlobenil, bromoxynil, ioxynil, buctril), etc. Recent awareness of environmental pollution caused by chemical-based industries has necessitated the development of enzyme-based process as alternatives to currently employed chemical processes. Moreover, bioconversion and biotransformation becomes partial or total replacement of currently employed toxic chemical process due to the distinct advantages of biotransformation [9]. Hence, their decomposition and detoxification by convenient and efficient methods is fairly urgent and challenging. More importantly, biodegradation and bioremediation, a convenient and cost-effective method, has the capability of eliminating these compounds by degrading them into harmless intermediates or, to a more desired form, carbon dioxide and water [170].

To the best of our knowledge, the huge potential of nitrile-converting enzymes has been explored in biodegradation of nitriles by many researchers. A variety of microorganisms, were demonstrated to be effective on metabolism of some organonitriles. As previously reported, a considerable number of nitrile-converting whole-cell biocatalysts have been applied to the removal of acrylonitrile waste effluents from the manufacture of acrylamide. A mixed culture of bacteria including different nitrile hydrolyzing enzymes that degrade effluent from the manufacture of acrylonitrile containing acrylonitrile, fumaronitrile, succinonitrile, etc. are grown in batch and continuous culture on these components of waste completely degraded all of the components [171].

Very recently, researchers from Singapore successfully enriched a whole consortium from the activated sludge of a pharmaceutical wastewater treatment plant and investigated its capability of biodegradation of saturated (acetonitrile), unsaturated (acrylonitrile), and aromatic (benzonitrile) organonitrile compounds [172].

Due to the potency and efficiency of biodegradation, similar studies were also conducted in China, especially in Taiwan, and moreover, some achievements were obtained [173–175]. Nitrile-converting enzymes have also participated in the cyano group-containing herbicide decomposition. A bromoxynil-degrading soil microorganism *Agrobacterium radiobacter* was used for degradation of the herbicide under nonsterile batch and continuous conditions. The efficacy of degradation was enhanced by addition of ferrous, cobaltous or cupric ions [176].

6 Cloning and Expression of Nitrile-Amide Converting Enzymes

Cloning and overexpression stands out as a promising method for the production of the desirable enzymes in sufficient quantities. So are the nitrile-amide converting enzymes, and there appeared numerous reports with respect to cloning and expression of these enzymes which are now accessible in sufficient quantities. As for nitrile hydratase, both the H- and L-nitrile hydratases genes, composed of two subunits of different sizes, have been cloned from *R. rhodochrous* J-1. The amino acid sequences of each subunit of the H- and L-NHases from *R. rhodochrous* J-1 showed generally significant similarities to those from *Rhodococcus* sp. N-774, but the arrangement of the coding sequences for two subunits is reverse. Each of the NHase genes was expressed in *E. coli* cells under the control of lac promoter only when they were cultured in the medium supplemented with CoCl_2 [177]. The stereoselective nitrile hydratase from *P. putida* 5B has been over-produced in *E. coli*. A clearly enhanced enzyme activity six times higher than the native strain and same stereoselectivity was observed [178].

In China, researchers have carried out similar work and some progress has been made. To obtain a recombinant *Rhodococcus* or *Nocardia* with not only higher enzymatic activity but also better operational stability and product tolerance for bioconversion of acrylamide from acrylonitrile, an active and stable expression system of nitrile hydratase (NHase) was tried to construct as the technical platform of genetic manipulations. Two NHase genes, NHBA and NHBAX, from *Nocardia* YS-2002 were successfully cloned into *E. coli* and *Pichia pastoris* system, however, expression level remained extremely low and the protein was unstable. To solve this problem, a possible genetic strategy, site-directed mutagenesis of the α -subunit of the NHase was carried out. After the successful mutagenesis, *E. coli* XL1-Blue (pUC18-NHBAM) was screened and the NHase activity was much higher than that of the prototype [179].

Along with the nitrile hydratase, some amidases have undergone the cloning and expression. Recently, Cheong et al. have undertaken the research concerning cloning of a wide-spectrum amidase from *Bacillus stearothermophilus* BR388 in *E. coli* and improving amidase expression using directed evolution. As a desired result, this mutant, prepared by PCR-based random mutagenesis which resulted in the substitution of arginine for histidine at position 26, demonstrated a 23-fold increase in amidase activity compared to the wild-type [88]. The amidase gene

from the hyperthermophilic archaeon *Sulfolobus solfataricus* has been cloned, sequenced, and overexpressed in *E. coli* and the recombinant thermophilic protein was expressed as a fusion protein with an N-terminus six-histidine-residue affinity tag [86].

In the case of nitrilase, the cloning of this enzyme first occurred in *E. coli* and encoded a bromoxynil-degrading activity from *Pneumoniae* subsp. *ozanae* [180]. In the previous studies, four nitrilases have been cloned from *A. thaliana* [181]. The corresponding gene of a regioselective aliphatic nitrilase from *A. facilis* 72W was cloned and over-expressed in *E. coli*, yielding a microorganism that efficiently and regioselectively catalyzes the conversion of aliphatic dinitriles to cyanocarboxylic acids. However, it was observed that, though a markedly increased quantity of nitrilase protein was obtained, the majority is present as inclusion bodies and is inactive. The phenomenon was consistent with the outcome obtained when nitrilase from *C. testosterone* was expressed in *E. coli*, although this was significantly improved with the co-expression of groESL chaperones [60]. *R. rhodochrous* J-1, appeared promising as a versatile nitrile-amide producing bacterium. Hence, it was investigated extensively, recent studies focusing on the molecular level. Komeda et al. demonstrated that the 1.4-kb downstream region from a nitrilase gene (*nitA*) was found to be required for the isovaleronitrile-dependent induction of nitrilase synthesis. Sequence analysis of the 1.4-kb region revealed the existence of an open reading frame (*nitR*) of 957 codes for a transcriptional regulator in *nitA* expression [182]. In China, researchers from Tongji University introduced a series of work concerning cloning and sequencing of nitrilase from an efficient degrader *Nocardia* sp. C-14-1. Southern blotting showed that there was a single nitrilase gene in the genome of C-14-1. Meanwhile, DNA sequencing and analysis suggested that there was a fragment of 1,143 bp DNA sequence encoded the nitrilase [183]. Besides, it was found that the expression of the *Vitreoscilla hemoglobin* (*vgb*) gene in vivo could improve the fermentation density and then contribute the extracellular secretion of the product of bromoxynil-specific nitrilase (*bxn*) gene. The recombination plasmid pPIC9K-*vgb-bxn* was constructed and transformed into *P. pastoris* GS115. The results of PCR and SDS-PAGE indicated that the *vgb* gene and *bxn* gene had integrated into the genome of *P. pastoris* GS115 and expressed in efficient level [184]. Subsequently, the *bxn* gene encoding bromoxynil-specific nitrilase was cloned from genomic DNA of *Klebsiella ozaenae* by PCR and over-expressed in *E. coli* DE3. The recombinant accessibility made it a promising candidate for eliminating bromoxynil to non noxious substances [185]. It could be concluded that the cloning and over-expression of the encoding genes resulted in a better understanding of enzyme function and the reaction mechanism, which in turn would lead to improvements in biotechnological applications.

Protein engineering of nitrilase have also been practiced to improve the substrate and product tolerance and specific activity. A high-activity biocatalyst based on an *A. facilis* 72W nitrilase was developed, where protein engineering and optimized protein expression in an *E. coli* transformant host were used to improve microbial nitrilase specific activity for glycolonitrile by 33-fold compared to the wild-type strain [186]. Gene site saturation mutagenesis (GSSM) evolution technology was employed to improve enantioselectivity of nitrilase-catalyzed desymmetrization of

3-hydroxyglutaryl nitrile to afford (*R*)-4-cyano-3-hydroxybutyric acid, an intermediate to the cholesterol-lowering drug Lipitor [187]. Changing Ala to His in position 190 provided a 10% increase in the enantiomeric excess at the commercially relevant 3 M substrate reaction concentration.

7 Conclusions and Future Prospects

The past few decades have witnessed the fast development of nitrile-amide converting enzymes, both their reaction mechanisms and applications in manufacture of a series of pharmaceuticals and chemicals. Formerly, great contribution was made by hydrolases such as esterases and lipases in the production of enantiopure synthons. Nowadays, with the discovery of numerous nitrile-amide converting microorganisms and their extremely fast development, these enzymes are becoming more and more appreciated by organic chemists and are showing competency to compete with esterases and lipases. Besides their synthetic value, these enzymes also play an important role in protecting the environment due to the capability of removal highly toxic nitrile compounds which are rather detrimental to human beings, animals and plants. In order to exploit fully their biotechnological potential, researches concerning the following aspects should be carried out in the following ways: (1) Overcoming some disadvantages of the nitrile-converting biocatalysts, such as narrow substrate specificity, low thermostability and pH stability, low tolerance of substrate and product, undesired and unsatisfied enantioselectivity. (2) Screening and discovery of new nitrile-amide converting enzymes with promising and attractive properties. As previously demonstrated, microorganisms producing nitrile-amide converting enzymes turned out to be dominantly from prokaryotic ones and eukaryotic organisms constitute only a small part. The latter ones, however, were always neglected as an excellent source of nitrilase, nitrile hydratase and amidase. Moreover, these organisms may provide some different properties like excellent thermostability, regio-, enantio- selectivity, and improved stability in some acidic and alkaline media, which are favored by some process. (3) Employing genetic engineering to alter some undesired properties of wild type strain. Bearing these in mind, researchers would make great progress in techniques related to screening, cultivation, protein and genetic engineering and hence it is possible to isolate novel enzymes with extremophilic characteristics.

Despite the advantages of nitrile-amide converting enzymes catalyzed synthesis of a range of industrially useful acids and amides, a paucity of them have achieved success in industrial application, with the commercial production of acrylamide and nicotinamide being the most successful. Manufacture of many other substances has been proved to be accomplished by these enzymes, especially the processes involve the regioselective and enantioselective hydrolysis of some prochiral compounds and racemic nitriles. These compounds, nevertheless, are difficult if not impossible to convert by means of traditional chemical methods. Chemical hydrolysis of many nitriles with labile substituents catalyzed by acid or base is also virtually impossible

because of the drastic reaction conditions required. Thus, their powerful potential in commercial application is being developed. In China, bioconversion of the high-value acids and amides has also been a hot issue. It is believed that an increasing number of novel nitrile-amide converting organisms will be screened and their potential in the synthesis of useful acids and amides will be further exploited in the near future. Furthermore, though cloning of the genes and expression of these versatile biocatalysts are presented in detail in many literatures and some of the recombinants exhibited rather high activity, it need to be largely exploited for the nitrile-converting enzymes. In a word, there is enough space for the development of these biocatalysts and there will turn on a bright and promising future of these enzymes.

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