BIOREMEDIATION OF NITRILES

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INTRODUCTION

Nitriles are cyano (—C≡N) group-bearing compounds that are produced naturally or synthetically for numerous industrial purposes mainly in the production process of various chemicals, pesticides, and polymers (Ramteke et al. 2013). It is also produced as the waste products and byproducts of pharmaceuticals, agriculture, and chemical industries (Martínková et al. 2009). The presence of nitriles was detected in the samples collected from marine areas, coastal areas, and the sewage sludge of the water treatment plants (Fang et al. 2015). Nitriles are widely used in pharmaceuticals as it is more water-soluble or reduce susceptibility towards oxidative metabolism in the liver. Currently, over 30 nitrile-containing products are marketed for various medicinal indications and about 20 leads are in the developmental stage (Fleming et al. 2010). In the field of agriculture, a variety of nitriles including benzonitrile and its derivatives like dichlobenil (2, 6 dichlorobenzonitrile), chloroxynil (3, 5-dichloro-4-hydroxybenzonitrile) and bromoxynil (3,5-dibromo- 4 hydroxybenzonitrile) were the active constituents of various herbicides mainly used for the crop varieties like corn, wheat, barley, rice, and berries (Amrutha and Nampoothiri 2022). Acetonitrile is another nitrile substrate employed as a solvent in industries although acrylonitrile is used for the production of plastics and precious metals.

Nitriles are toxic effluents that are lethal, carcinogenic, and mutagenic. They are capable of causing serious health issues among humans which includes gastric issues, nausea, bronchial irritation, coma, skeletal deformities, convulsions, and respiratory distress (Amrutha and Nampoothiri 2022). The nitriles used in the herbicides can persist in soil for a long period and can cause pollution to the ecosphere.

Therefore significant attention is necessary to remove these toxic substances from the biosphere. Biological methods are mostly preferred for the detoxification of nitriles over chemical methods, as it is more sustainable, efficient, and low-cost(Tao and Xu 2009). Predominantly, microorganisms are capable enough to convert environmental contaminants to less toxic forms. Microbes degrade the nitrile compounds mainly via two pathways (Fang et al. 2015). The first pathway involves the conversion of nitrile directly into a carboxylic acid and ammonia using the enzyme nitrilase and the second pathway employs two enzymes; nitrile hydratase and amidase. Nitrile hydratase will convert nitrile to its amide form, then amidase acts on it and converts it into its corresponding acid (Prasad and Bhalla 2010). This paper will discuss the currently reported studies for the bioremediation of nitriles with the help of microorganisms.

NITRILASE SUPERFAMILY

The nitrilase superfamily, also referred to as CN-hydrolases, is a group of enzymes that can catalyse the hydrolysis of non-peptide carbon-nitrogen bonds. It consists of 13 different branches based on catalytic activity and sequence identity including biotinidase, nitrilase branches, N-terminal amidase, carbamylase, etc. (Pace and Brenner 2001). Nitrilase branch is closely related to cyanide hydratase and cyanide dehydratase enzymes in which Cyanide hydratase, hydrolyze cyanide to formamide whereas, cyanide dehydratase hydrolyzes cyanide to formic acid and ammonia. All the members of the nitrilase superfamily consist of a catalytic triad of amino

acids including glutamic acid, lysine, and cysteine. Other members of the nitrilase superfamily are differentiated from the nitrilase branch by a conserved cysteine-tryptophan-glutamic acid motif situated at the cysteine residue of the catalytic triad(Novo et al. 2002). By altering the amino acid residue cysteine, may lead to the complete loss of nitrilase activity in Arabidopsis thaliana and Alcaligenes faecalis JM3 which was reported in many studies (Vorwerk et al. 2001). Moreover, nitrilases consist of a sulfhydryl group which is necessary for the catalytic activity and therefore it is considered as thiol enzymes(O'Reilly and Turner 2003).

NITRILASES

Nitralases are nitrile-degrading enzyme which was discovered by Mahadevan and Thiman in 1964, they can metabolize nitrile substituent compounds and have various industrial applications including drug synthesis (Ramteke et al. 2013). Nowadays the insistence on nitrilase enzymes were high due to their capability to act as effective biocatalysts in various synthetic applications. It was extensively distributed in nature and their existences are reported in plants, fungi, and mostly in bacteria including Acinetobacter, Nocardio, Corynebacterium, Arthrobacter, Klebsiella, Rhodococcus, etc were reported to use nitriles as primary sources of carbon and nitrogen(Kaul, Banerjee, and Banerjee 2007). The role of nitrilases in the microbes is still unknown but certain studies hypothesized their role in the recycling of nitrogen and detoxification of nitriles (Sharma et al. 2018). Usually, microbial nitrilases are classified into aromatic, aliphatic, and aryl aceto nitrilases based on their respective substrates such as aromatic nitriles, aliphatic nitriles, and aryl acetonitriles(Kobayashi and Shimizu 1994)(Thuku et al. 2009)(Gong et al. 2012).

Additionally, nitrilases are also employed in the bioremediation of toxic nitrile from contaminated land and water. Pseudomonas stutzeri is a bacterium identified from the sewage of a metal-plating factory having good cyanide degrading properties and can tolerate higher concentrations of Potassium cyanide, due to the presence of the enzyme cyanide dehydratase (Sewell et al., 2003). The nitrilase from the bacterial species Klebsiella pneumoniae subsp.

Ozaenae is found to be highly active against the herbicide bromoxynil (McBride et al., 1986). This enzyme has also been expressed in plants which provide herbicidal resistance to transgenic lines(Stalker et al., 1988b). The hydrolysis of 3-cyanopyridone to nicotinic acid, a vitamin used in medicines and animal feed, which can be carried out using the nitrilase of Rhodococcus Rhodochrous J1 was reported (Mathew et al., 1988). Nitrilases are also used in the industrial production process of carboxylic acid otherwise the manufacturing process of carboxylic acids demands various chemical methods which require extreme conditions of pH and temperature. (Osswald et al., 2002)

Scientists put forward various high-throughput methods for the identification of bacterial nitrilases. They screened the nitrilase activity by incorporating the DNA samples from the environment to a bacterial expression system. DeSantis and colleagues (2002) developed around 200 nitrilase sequences using this method and Robertson and colleagues (2004) introduced 137 novel nitrilases, which comprise cysteine catalytic triad, glutamic acid, and lysine. Scientists are also working in the isolation process of nitrilases from the organisms growing naturally in harsh conditions to find out stable enzymes even in high temperatures and pH. For example, Studies reported the nitrilase activity of thermophilic bacterium B. pallidus strain Dac521 (Almatawah et al., 1999) and hyperthermophilic archeon Pyrococcus abyssi (Mueller et al., 2006) were found to be stable at high temperatures. Additionally, the accessibility of genome sequence data paved the way for discovering new novel nitrilase genes from various microorganisms.

Even though the commercial application of nitrilases is less in progress due to difficulties in the isolation process of its active form and often found to be unstable. The stability can be increased considerably by the addition of reducing agents like 2-mercaptoethanol and dithiothreitol which will help in the prevention of oxidation of thiol residues in the enzymes which plays a significant

role in the hydrolysis reaction. Other than these reagents, glycerol and ammonium sulfate has been used to stabilize nitrilase, which prevents the dissociation of enzyme subunit (Kiziak et al., 2005).

Aromatic nitrilases

In 1977, Harper isolated aromatic nitrilases from the fungal nitrilases of Fusarium Solani but the amino acid composition of the aromatic nitrilases was undetermined. Research studies were done in the isolation of aromatic nitrilases from Fusarium solani which can degrade benzo-nitrile as a prominent source of nitrogen and carbon using the method (NH4)2SO4 precipitation, DEAE-cellulose chromatography, and gel filtration on Sephadex G-200 (Harper 1977). Later the partial amino acid sequence of this species was determined with the help of the inducers 2-cyanopyridine and Valero nitrile(Sharma et al. 2018). A high amount of Aromatic nitrilases was isolated from the fungus Aspergillus Niger k10 which was cultured on 2- cyanopyridine and its preferential substrates include 4-chlorobenzonitrile, 3-cyanopyridine, 3-chlorobenzonitrile, thiophen-2-acetonitrile, 1,4-dicyanobenzene, benzonitrile, and 4-cyanopyridine. Furthermore, it releases amides as by-products for the substrates 1,4-dicyanobenzene, 4-cyanopyridine, 4-chlorobenzonitrile, and 2-cyanopyridine(Kaplan et al. 2006). Many Studies have reported the nitrilase activity of filamentous fungi such as Fusarium oxysporum f. sp. melonis (Fom) (Martínková et al. 2009), Fusarium solani O1(Vejvoda et al. 2006), Fusarium i IMI 196840 (Vejvoda et al. 2010).

The isolation of aromatic nitrilases from Nocardia species (bacteria), which can utilize benzonitriles as the source of carbon and nitrogen was reported (Harper 1977). Studies on the bacterial species Rhodococcus rhodochrous JI revealed the abundance of aromatic nitrilase compounds and its capability to act as a catalyst in the direct hydrolysis of the compound Nicotinonitrile to nicotinic acid without the formation of nicotinamide (Mathew et al. 1988). Propionitrile-utilizing microorganisms like Rhodococcus rhodochrous NCIMB 11216 (Hoyle, Bunch, and Knowles 1998) and Rhodococcus rhodochrous PA-34 also showed nitrilase activity.

Rhodococcus rhodochrous PA-34 isolated from the soil can hydrolyze various α-aminonitriles to optically active amino acids (Bhalla et al. 1992). Nitralase activity was induced in the strain Dac521 of the thermophilic bacterium Bacillus pallidus strain in a minimal medium supplemented with benzonitrile(Almatawah, Cramp, and Cowan 1999). Geobacillus pallidus RAPc8 is another moderate thermophilic gram-positive bacterium from the sediments of Australian lakes capable of performing nitrilase activity(Williamson et al. 2010). An organism showing higher specificity towards the substrates including 4-cyanopyridine, 3-cyanoopyridine, and benzonitrile known as Rhodococcus sp. NDB 1165 was isolated from the soil of Himalayan temperate forests(Prasad et al. 2007). Studies also highlight the nitrilases activity of certain bacteria, Pseudomonas aeruginosa 10145 (Alonso, Oestreicher, and Antunes 2008), Alcaligenes sp. ECU0401(Zhang et al. 2011), Arthrobacter nitroguajacolicus ZJUTB06-99 (Shen, Zheng, and Shen 2009) and Alcaligenes faecalis MTCC 10757 against the specific substrate of aromatic nitrilases(Nageshwar et al. 2011).

Aliphatic nitrilases

Aliphatic nitrilases from various microbes were identified and purified with different substrate specificities. Nitrilases from isovaleronitrile-induced cells of the bacterium Rhodococcus rhodochrous J1 possess higher activity with 2-thiophenecarbonitrile, 4-tolunitrile, 2-furonitrile, 3-chlorobenzonitrile and a benzonitrile (KOBAYASHI, NAGASAWA, and YAMADA 1989). The aliphatic nitrilase was detected and isolated from the bacterium Comamonas testosteroni sp. (Ct) which shows better activity on cyanovaleric acid and adiponitrile(Lévy-Schil et al. 1995). Similarly, aliphatic nitrilases were also isolated from the microbial species Nocardia globerula which was found in the forest of Manali, Himachal Pradesh(Chauhan et al. 2003). Nigam et al. reported bioremediation of acrylonitrile with the help of thermophilic bacteria in Streptomyces sp. MTCC 7546 in both the immobilized and free state (Nigam et al. 2009). The activity of nitrilase enzyme from Acinetobacter sp. AK226 showed maximum activity with acrylonitrile and benzonitrile (Lévy-Schil et al. 1995). Nitrilase enzyme from the bacteria Synechocystis sp. strain PCC6803

(Yamamoto, Fujimatsu, and Komatsu 1992), Acidovorax facilis 72W (Ambler, Auffret, and Clarke 1987), and Cyanobacterium Synechocystis sp. strain PCC6803 were able to hydrolyze fumarodinitrile (trans-1,2-dicyanoethene), where Cyanobacterium Synechocystis sp. strain PCC6803 shows 120 times higher activity with fumarodinitrile when compared with its activity in benzonitrile. Studies noticed that the recombinant AtNIT1 nitrilase of Arabidopsis thaliana had higher activity in the hydrolysis of 3-phenylpropionitrile, benzonitrile, and 3-nitroacrylonitrile. They also analyzed the activity in AtNIT1, AtNIT2, AtNIT3, and AtNIT4 nitrilases in indole acetonitrile (IAN) and found that it is not preferred for the degradation of indole acetonitrile (IAN). Nitrilase from Rhodococcus sp. NDB1165 (Prasad et al. 2007), R. rhodochrous K22(Kobayashi et al. 1990), Alcaligenes sp. ECU0401(Zhang et al. 2011) was also reported to have higher activity on aliphatic nitriles.

Arylacetonitrilases

These nitrilases belong to the fourth class that does not interact with benzonitrile (Ramteke et al. 2013). Arylacetonitrilase has been isolated from the isovaleronitrile-induced cells of bacterium Alcaligenes faecalis JM3, which was confirmed using SDS/polyacrylamide gel electrophoresis, ampholyte electrofocusing, and double immunodiffusion in agarose gel. They found that these enzymes are particularly for arylacetonitrile substrates such as 3-pyridylacetonitrile, p-chlorobenzylcyanide, p-fluorobenzylcyanide, p-tolylacetonitrile, and 2-thiophenacetonitrile. Its activity is restricted to nitrile groups attached to heteroaromatic and aromatic rings. To have maximum activity, the enzyme requires thiole aggregate such as 2-mercaptoethanol and dithiothreitol(NAGASAWA et.al 1990). The nitrilase enzymes of bacterial strain Acinetobacter sp. AK 226, benzonitrilase or acrylonitrilase was able to hydrolyze aliphatic, aromatic, dinitrile, and heterocyclic nitriles (Yamamoto and Komatsu 1991). The growth of Pseudomonas fluorescens DSM 7155 on phenyl acetonitrile catalyzed the hydrolysis of the substrate arylacetonitrile into a carboxylic acid and ammonia as products (Layh, Parratt, and Willetts 1998). The arylacetonitrilase of Pseudomonas putida was homogenously purified by employing a mixture of (NH4)2SO4

fractionation and several chromatographic methods. This nitrilase required a lowering environment for proper functioning and it was highly sensitive to metal ions and thiol reagents. These showed the essential requirement of the thiol group for the action of enzymes concerning the proposed mechanism for the catalytic reaction nitrilases (Banerjee, Kaul, and Banerjee 2006). A research group isolated a recombinant enantioselective nitrilase from the bacterium strain Pseudomonas fluorescens EBC191. These enzymes are found to be highly active towards hydrolyzed heterocyclic, bicyclic, arylacetonitriles, and para-substituted phenyl-acetonitriles and possess 47% sequence identity with arylacetonitrilase of A.faecalis JM3 and both are less reactive with aliphatic nitriles and benzo nitriles (Kiziak et al. 2005). The 16s rDNA sequence of the strain ZJB-063 of Bacillus subtilis shows nitrilase activity without the inclusion of any inducers. It is highly specific to arylacetonitriles and less active towards also aliphatic nitriles and heterocyclic nitriles. Other than its nitrilase activity it also exhibits amidase and nitrile hydratase activity in the addition of ε-caprolactam(Zheng et al. 2008).

Conclusion

Many studies are highlighting the potential of nitrilases in the biodegradation of nitriles isolated from different microbes. The catalysis activity and thermostability of nitrilases can be improved by making advancements in the field of isolation, screening, and genetic engineering. The availability of nitrilase-producing organisms is beneficial for the cell manufacturing industries in the production process of carboxylic acids, in textile industries, and in waste treatment due to its bioremediation potential and surface modification property. High throughput screening is commonly employed to select good biocatalysts. Novel approaches such as rational protein design and the various combination of rational design and directed evolution should be considered for a powerful and flexible biocatalyst with efficient catalytic activity, wide substrate spectrum, and superior operational reliability. The applicability of nitrilase can be increased by

extending the research on microorganisms living in extreme conditions. In the future, novel new nitrilases and their wide application can be expected.

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