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Revisión

Understanding nitrile-degrading enzymes: classification, biocatalytic nature and current applications

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Abstract

Nitrile-degrading enzymes commonly known as nitrilase enzymes are able to metabolize nitrile-substituent compounds and they have several industrial applications, for example: in drugs synthesis. It is also common to observe their exploitation for obtaining chemical compounds with commercial interests related to cosmetics production, paints and additives. In addition, these are frequently used in the active metabolites synthesis of pesticides. Due to the catalytic nature of such proteins, it is possible to take advantage of their biotechnological potential to be applied in various scientific fields including synthetic biocatalysis and environmental remediation, since they have been successfully used for soils nitrile-wastes decontamination such as cyanide, bromoxynil and benzonitrile. On the other hand, these enzymes are considered very important intermediaries of metabolic pathways related to indolic compounds that are produced by bacteria, plants and superior fungi, acting in most cases as vegetal growth hormones. Given the fact that indole-derivative molecules play an important role in physiological responses in superior organisms, nitrilase enzymes may be considered as important part of unknown multi-enzymatic secondary metabolites pathways. In light of the above considerations, this review attempts to summarize the current status of nitrilase research and describing in detail the main characteristics of nitrile-converting enzymes with emphasis on fungal proteins, including their function and catalytic selectivity. Likewise, their relationship with plant metabolism and biotechnological importance in bioremediation processes is discussed.

Keywords: *nitrile-degrading enzymes, indolic compounds, indole-3-acetic acid, cyanide, environmental remediation.*

Resumen

Las enzimas degradadoras de compuestos nitrilo conocidas comúnmente como enzimas nitrilasas, son capaces de metabolizar compuestos nitrilo-sustituyentes, y tienen diversas aplicaciones industriales como por ejemplo: en la síntesis de fármacos. Asimismo, es común observar su explotación para la obtención de compuestos químicos de interés comercial relacionados con la elaboración de cosméticos, pinturas y aditivos. Además, éstas suelen emplearse también de manera frecuente en la síntesis de metabolitos activos de plaguicidas. Debido a la naturaleza catalítica de dichas proteínas, actualmente es posible explotar su potencial biotecnológico para ser aplicado en diversos campos, incluyendo la biocatálisis sintética y remediación ambiental, ya que han sido utilizadas con éxito para la descontaminación de suelos impactados con cianuro, bromoxinil y benzonitrilo. Por otra parte, dichas enzimas son importantes intermediarias de rutas metabólicas de compuestos indólicos producidos por bacterias, plantas y hongos superiores, actuando en la mayoría de los

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casos como hormonas de crecimiento vegetal. Debido al hecho de que las moléculas derivadas de compuestos indólicos juegan un papel importante en las respuestas fisiológicas de organismos superiores, las enzimas nitrilasas podrían ser consideradas parte importante de rutas metabólicas multienzimáticas desconocidas en la síntesis de metabolitos secundarios. La presente revisión crítica tiene la finalidad de describir a detalle las principales características de las enzimas nitrilasas haciendo énfasis en aquellas de origen fúngico, incluyendo su clasificación, función y selectividad catalítica. Igualmente, se discute su relación con el metabolismo de las plantas y la importancia biotecnológica que tienen en procesos de biorremediación.

Palabras clave: *enzimas nitrilasas, compuestos indólicos, ácido indol-3-acético, cianuro, remediación ambiental*.

1. Introduction: historical outline of nitrilase enzymes

The nitrilase superfamily groups a wide range of thiol enzymes which are commonly involved in several metabolic pathways, such as product biosynthesis and post-translational modification in eukaryotic and certain prokaryotic species (Pace and Brenner, 2001). This group of enzymes can also refer to CN-hydrolases due to their capacity to catalyse the hydrolysis of non-peptide carbon-nitrogen bonds, being the substrate specificity a common way to classify their nature. The main biotechnological importance of this type of peptides lies in their high potential to degrade several nitrile compounds under different environmental conditions, which in some cases enantioselectivity may be observed (Martínková and Mylerová, 2003). Because of these properties, nitrilase enzymes are considered potential candidates for conducting bioremediation processes. The first studies regarding nitrilases were conducted at the end of 1950s when Thimann and Mahadevan (1958) indentified nitrilase activity in plants for the first time. Later, through several researches nitrilase activity was also observed in bacteria (Hook and Robinson, 1964) and fungi from genera *Aspergillus*, *Penicillium*, *Gibberella* and *Fusarium,* which showed the ability to convert 3-indolacetonitrile (IAN) into indole-3-acetic acid (IAA) (Thimann and Mahadevan, 1964). Notwithstanding, the nitrile-hydrolyzing ability of these organisms was not studied any further at the time. After these previous studies, Strobel (1966; 1967) examined with no detail earlier the enzymes hydrolyzing 2-aminopropionitrile and 4-amino-4-cyanobutyric acid in an unidentified basidiomycete. Until today, it is known that the first nitrilase purification and partially characterized was an enzyme from *Pseudomonas* sp. (Robinson and Hook, 1964). In the late 1970s, research into nitrilases was intensified in the context of increasing interest of their biotechnological exploitation. From this moment, new nitrilases were purified and characterized from both prokaryotic and eukaryotic organisms (for a review see Martínková *et al*. 2009). Since nitrilases from genus *Rhodococcus* were identified as promising nitrile-hydrolytic enzymes due to their substrate specificity toward aromatic and aliphatic nitriles (Kobayashi and Shimizu, 1994), many other enzymes belonging to different bacterial genera (i.e. *Alcaligenes*, *Bacillus*, *Pseudomonas*) have also been purified and characterized (for a review see O'Reilly and Turner, 2003). While knowledge of nature and structure of prokaryotic nitrilases have substantially improved in the last twenty years (for reviews see Banerjee *et al*. 2002; Zhou *et al*. 2005), almost no further work has been developed to a complete characterization to nitrilases from filamentous fungi until recently. In recent years, a great number of putative fungal nitrilases are available from protein databases, although just a few enzymes have been purified and

characterized. Nowadays, the purified nitrilases from *Fusarium solani* O1, *Fusarium oxysporum* f. sp *melonis*, *Aspergillus niger* K10, *Gibberella intermedia* CA3-1 are some of the most important complete purified enzymes due to the fact that have shown reliable experimental enzymatic properties related to nitrilase activity (Vejvoda *et al*. 2008; Kaplan *et al*., 2006a; Goldlust and Bohak, 1989; Wu *et al*. 2013). Despite the fact of the high potential for nitrile-hydrolyzing that this group of proteins possesses, the unsatisfactory level of knowledge for nitrilases prompts to screen for these enzymes, optimize their production and describe their biochemical and biocatalytic properties. In fact, there is little recent information related to nitrilases genome sequencing (Luque-Almagro *et al*. 2013; Kaplan *et al*. 2013). Due to the above, this review aims to summarize current knowledge of nitrilase enzymes including those that accept organic cyanides and substrates related to specificity for metal cyanides (cyanide hydratases and dihydratases). Its focus will be mainly in the practical aspects of this topic which include structure classification and molecular function, their role in metabolic pathways of nitrogen transformation in plants as well as biocatalytic applications and biodegradation of environmental nitrile-contaminants.

2. Nitrilases in nature

2.1 Classification, function and substrate specificity of nitrile-degrading enzymes

There are three categories of nitrilases according to substrate specificity: aliphatic nitrilases, which act on aliphatic nitriles such as acrylonitrile, glutaronitrile and β-cyano-L-alanine; aromatic or heterocyclic nitrilases that are able to hydrolyze benzonitrile and cyanopyridine; and arylacetonitrilases that prefer substrates like arylacetonitriles such as phenylacetonitrile, phenylpropionitrile and IAN, an important intermediary indolic-compound involved in auxins biosynthesis (Brenner, 2002). Nitrilase enzymes also catalyse metabolic reactions involving degradation and biotransformation of complex nitrogenous compounds such as, bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) and acetonitrile which are considered highly hazardous substances for human health. Such metabolization processes take place through reactions that involve the hydrolysis of nitriles into their corresponding carboxylic acid and ammonia as subproducts (Howden and Preston, 2009). Many organisms belonging to eukaryotic and prokaryotic domains such as plants, fungus, animals and bacteria, perform several biochemical reactions related to nonpeptide carbon-nitrogen hydrolysis through nitrilase enzymes biocatalysis. Nitrilase and amidase reactions produce indolic compounds as organic subproducts (i.e. IAA and IAN) as well as biotin and β-alanine among others, resulting in a deamination of protein and aminoacid substrates trough a nucleophilic substitution of a conserved cysteine that attacks a cyano or carbonyl carbon (Harper, 1985; Ambler *et al*. 1987; Novo *et al*. 1995; Stevenson *et al*. 1990; Bork and Koonin, 1994). The nitrilase superfamily contains a closely group of related cyanide hydratase and cyanide dihydratase enzymes which preferentially hydrolyse cyanide to formamide, while cyanide dihydratase enzymes hydrolyse this compound to formic acid and ammonia (Singh *et al*. 2006). Most branches of the nitrilase superfamily do not contain nitrilase enzymes precisely, due to the fact that several amide-hydrolyzing and amide-condensing enzymes among this group can be found. Hitherto, nitrilase enzymes can be classified in 13 branches and grouping of each depends on its enzyme activity and substrate specificity (Pace and Brener, 2001).

Branches 1-3

The first group of proteins related to nitrilase enzymes can be found in both

eukaryotic and prokaryotic organisms. The most representative evidence of nitrilase activity in plants can be seen in *Arabidopsis thaliana*, which *in vivo* effect evidences the conversion of IAN into IAA. This reaction was confirmed through an experiment related to a recessive mutation in a nitrilase gene resulting in reduced sensitivity to the auxin-like effects of IAN (Normanly *et al*. 1997). Aliphatic amidases and amino-terminal amidases can be found in two groups which are branches 2 and 3. These comprise a small compilation of nearly identical proteins commonly found in *Pseudomonas*, *Bacillus*, *Brevibacteria*, *Helicobacteria* and *Saccharomyces*. The enzymes hydrolyze substrates like glutamine and asparagine, specifically the amino carbonyl sidechains through the utilization of a conserved cysteine which forms part of a catalytic triad on the amino acid sequence of the protein. It is known that Nta1 protein from *Saccharomyces cerevisiae* is capable to perform an amino-terminal deamination of asparagine and glutamine residues, releasing aspartate and glutamate as subproducts (Baker and Varshavsky, 1995). The release of subproducts such as biotin-lysine, biotin peptide-conjugates and biotin methylester is also common in this kind of reactions (Cole *et al*. 1994). Previous studies have shown that biotinidases are able to recycle the vitamin biotin. Commonly, some persons show biotinidase deficiency as part of an autosomal disease with a recessively inherited neurocutaneous disorder (Wolf, 2012). Due to the fact that biotinidases posses a conserved carboxy-terminal domain, vanins and GPI-80 protein are grouped into the biotinidase branch (branch 4) which contain a similar carboxy-terminal domain and a GPI anchor (Aurrand-Lions *et al*. 1996; Suzuki *et al*. 1999).

Branches 4 and 5

Branch 4 of nitrilase superfamily is the only group of proteins with amidase reaction that prefers secondary amine substrates as opposed to simple amides. Granjeaud *et al.* (1999) developed an EST (Expressed Tag Sequence) description of the first vanin gene family conserved from fly to human. Branch 5 is related to β-ureidopropionase enzymes which are involved in the catabolism of pyrimidine bases and the production of β-alanine (Kvalnes-Krick and Traut, 1993). The reaction type of these proteins is the hydrolysis of linear amides whose chemical pathway is usually related to thymine degradation, resulting in the release of (R)-3-amino-2-methylpropanoate. On the other hand, a reductive reaction that involves uracil degradation may also be seen when pyrimidines are reduced to β-aminoacids, CO_2 and ammonia (Matsuda *et al*. 1996). Some nitrilase substitutes like pyrimidines can be catabolized through different pathways, but the best characterized one is a reductive pathway in which like uracil degradation, pyrimidines are reduced to CO_2 and ammonia. Examples like an oxidative pathway are only found in a few bacterial species and have not been characterized nearly as well (Kao and Hsu, 2003; Walsh *et al*. 2001).

Branch 6

Carbamylase enzymes comprise another type of nitrilases grouped in branch 6. A lot of bacteria are able to express hydrolase enzymes for the decarbamylation of D-aminoacids. These enzymes have been exploited in the production of β-lactam antibiotics and some other secondary metabolites with pharmacologic interest (Louwrier and Knowles, 1997). *Deinococcus radiophilus*, an extremophilic bacterium is capable to use the aspartate amino-N as nitrogen source for glutamine synthesis through a substrate-channelled pathway delivering glutamine to carbamoyl-phosphate synthetase (McPhail *et al*. 2009). In addition, Wang *et al.* (2008) found that the binding of carbamoyl phosphate to the enzymes aspartate and ornithine transcarbamoylase from *Escherichia coli* reduces the rate of thermal decomposition of carbamoyl

phosphate by a factor greater than 50000. Likewise, a preceding antecedent has shown that both of these transcarbamoylases use a binding mechanism were carbamoyl phosphate binds allowing the formation of enzyme-carbamoyl phosphate complex.

Branches 7-9

The finding of nitrilase-related domains in proteins allows correlating the ability of bacterial NAD Synthetase to convert glutamine into a lees complex nitrogen source such as ammonia. It has been observed that a nicotinamide mononucleotide synthetase from *Francisella tularensis* is related to an alternative rout of NAD synthesis were the amidation of NaMN (glycohydrolase) occurs before the adenynylation reaction that converts the intermediate to the NAD factor (Sorci *et al*. 2009). Spencer and Preiss (1967) discovered that NAD synthetase represents 20 % of the activity relative to ATP in *E. coli,* which may be involved in two possible substrates, ammonia or glutamine to perform the final step in the Press-Handler pathway for NAD + biosynthesis (Ozment *et al*. 1999). Braun's lipoprotein as well as known as BLP or Murein lipoprotein, is one of the most abundant membrane-like peptides found in some gram-negative cell walls. The protein is bound at its C-terminal end to a lysine by a covalent bold to the peptideglycan layer embedded outside the membrane (Seltmann and Holst, 2002). Braun's lipoprotein is a major component of the outer membrane of *E. coli* and has been studied for decades (Tokunaga *et al*. 1982).

Branch 10 (NIT proteins)

NIT proteins are perhaps the most important and studied nitrilase enzymes. NIT was originally identified as an approximately 300 aminoacid amino-terminal extension on fly and worm homologs (Pekarsky *et al*. 1998), of human (Ohta *et al*. 1996) and murine (Fong *et al*. 2000), a *Fhit* tumor suppressor protein. This group acts on a wide range of aromatic nitriles like acetonitrile and also in some aliphatic nitriles including the corresponding acid amines (Brady *et al*. 2004). In species like *A. thaliana*, NIT enzymes perform their catalytic activity through an essential Cys^{179} and Cys^{186} residues (Vorwerk *et al*. 2001). *Agrobacterium* sp. and *Alcaligenes faecalis* nitrilases perform a reaction type that involves a hydrolysis of amide bond (Wieser *et al*. 1997; Petersen and Kiener, 1999). Likewise, it has been demonstrated that *Nocardia globerula* NHB-2 nitrilase is capable to catalyse the biotransformation of 4-cyanopyridine (a complex nitrogen source) to isonicotinic acid (Sharma *et al*. 2012). It is worth mentioning that their industrial potential lies in the mild and often stereoselective hydrolysis of nitriles, due to the fact that nitrilases are heterogeneous in terms of their substrate specificity. Nowadays, the gene and protein databases contain a large number of sequences that encode putative and characterized nitrilases which enzymatic activities have not been studied at all. When searching for enzymes sequences with defined substrate specificity, a comparison of their homology and specific regions with those of known enzymes may be useful (Seffernick *et al*. 2009).

Branches 11-13

Branches 11 and 12 are grouped in a similar branch due to their distinctive similarity with no characterized members. Branch 12 may content Rosetta Stone proteins that are interactive peptides such as acetate CoA transferase in *E. coli* that fuse into a single chain in other organisms. In this case, a branch 12 protein may contain a Rosetta Stone in that a particular nitrilase-related domain is found to be fused into a terminal domain (amino) of an approximately 210 aminoacids (Marcotte *et al*. 1999). The branch 12 is also associated with a domain that groups the RimI superfamily of amino-terminal acetyltransferases (Yoshikawa *et al*. 1987). The enzyme reaction type is an acyl group

transfer that typically involves acetyl-CoA plus a ribosomal protein and L-alanine. In *E. coli* is very common to find a group of enzymes that acetylate the N-terminal alanine residues of specific ribosomal proteins (EC 2.3.1.88). Crystal structure of RimI has been determined in complex with CoA, AcCoA and CoA-S-acetyl-ARYFRR bisubstrate inhibitor. The structures are consistent with a direct nuclophilic addition-elimination mechanism with Glu^{103} and Tyr^{115} acting as the catalytic base and acid, respectively. This is way the RimI-bisubstrate complex suggests that several residues change conformation upon interacting with the N terminus of S^{18}, including Glu^{103}, the proposed active site base, therefore facilitating proton exchange and catalysis (Hirosama *et al*. 2008). Figure 1 shows a strict consensus tree grouping some of the most important characterized nitrilases to date, including some nitriles accepted as substrates by each. Table 1 shows some of the nitriles accepted as substrates by nitrilases from fungus like *Aspergillus* and *Fusarium*.

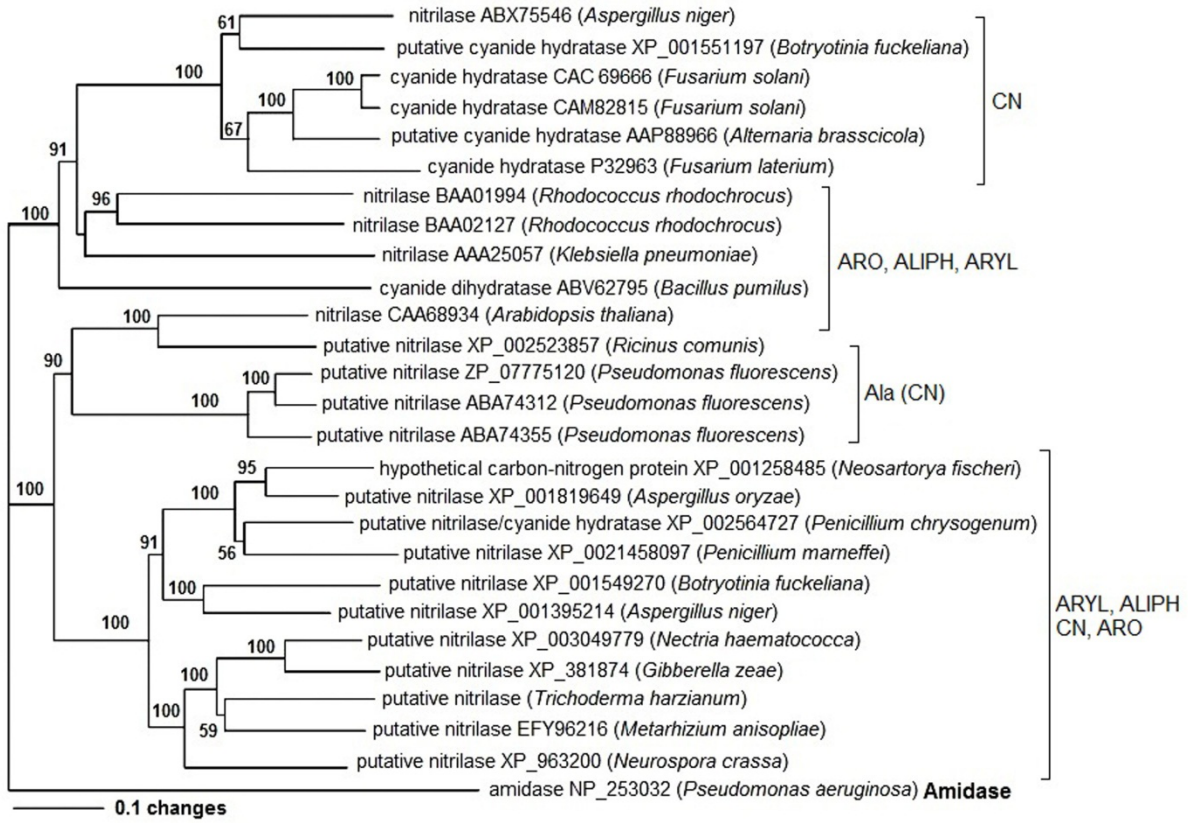

Figure 1. Neighbor-joining (NJ) tree of ten nitrilase/cyanide hydratase and sixteen putative nitrilase/cyanide hydratase proteins from fungi, bacteria and plants. Proteins, indicated as accession numbers (database available in http://ncbi.nlm.nih.gov) were aligned using Clustal X program (Thompson *et al*. 1997). The NJ tree was generated with PAUP program using the neighbour-joining method. Numbers on branches indicate bootstrap values from an analysis of 1,000 replicates. Amidase protein from *Pseudomonas aeruginosa* was used as the outgroup. The tree is annotated with the most active substrate for each protein; ARO, aromatic; ALIPH, aliphatic; ARYL, arylacetonitriles; Ala (CN); CN, cyanide).

3. Nitrile degrading enzymes and their role in plant metabolism

Nitrilases are also present in plants in cyanide metabolism, a nitrogenous compound synthesized by these during defense response and that originated as a co-product of ethylene biosynthesis. IAN can be hydrolyzed by nitrilase activity into auxins phenyl acetic acid and IAA. The hydrolysis of IAN into IAA by nitrilase activity is an extremely well characterized reaction in plants and bacteria (Bartel, 1997; Spaepen *et al*. 2007). However, the IAN pathway is one of the several that have been identified for IAA biosynthesis

in plants (Howden *et al*. 2009a; Lehmann *et al*. 2010). The importance of the production of auxins in plant development and physiology is recognized, as well as in the biosynthesis of IAA in plant-microorganism interactions (Kobayashi *et al*. 1993). While the biological role of plant nitrilases is well understood, very little is known about the importance of these enzymes in fungi. Some filamentous fungi like *Trichoderma* species are able to colonize the entire root of plants and their effects can be seen as positive promoting lateral root development, leading to enhanced plant growth (Harman *et al*. 2004; Shoresh *et al*. 2010; Yedidia *et al*. 2001). Recently, it has been observed that using L-tryptophan (Trp) as an IAA precursor increased the production of IAA in *Trichoderma virens* cultured, and was linked to the promotion of lateral root development in *A. thaliana*-*T. virens* fungal interactions (Contreras-Cornejo *et al*. 2009). Several studies have related nitrilase genes and enzymes as mentioned, with the conversion of IAN into the plant growth factor IAA (Bartling *et al*. 1992; 1994; Howden *et al*. 2009a). Since the 1980s, bacteria have been mined as a source of nitrilases which have been exploited for biochemical synthesis and for environmental remediation (Kobayashi *et al*. 1993; Thuku *et al*. 2007). Fungal nitrilases have been less exploited than bacterial nitrilases and although several reports suggest that the occurrence of aromatic nitrilases in filamentous fungi is common (Kaplan *et al*. 2006b; Martínková *et al*. 2009), only some fungal aromatic nitrilases have been purified and characterized to date, these include those from *F. solani* IMI196840, *F. oxysporum* f. sp. *melonis*, *F. solani* O1, *A. niger* K10, *Nectria haematococca* and *Arthroderma benhamiae* (Goldlust and Bohak, 1989; Harper, 1977b; Kaplan *et al*. 2006b; Vejvoda *et al*. 2008; Veselá, *et al*. 2013). According to GenBank research, a large number of putative nitrilase genes from filamentous fungi have been deposited in the database. However, only recently the nitrilase gene of *A. niger* K10 has been functionally analyzed (Kaplan *et al*. 2011), which was found to be highly homologous with putative nitrilases from *Aspergillus* and *Penicillium*. IAN is an intermediate for IAA production and biosynthesis of this phytohormone is not limited to higher plants (Lehmann *et al*. 2010). Various pathways operate in IAA biosynthesis among bacteria that inhabit the rhizosphere of plants: the indole-3-pyruvic pathway (IPyA), the indole-3-acetamide (IAM) pathway, the tryptamine (TAM) pathway and the IAN pathway (Spaepen *et al*. 2007). According to Zhao (2012), auxin biosynthesis regulated through transcriptional and protein level may remain unknown. IAA, tryptophol, IAM, IPyA and indole-lactic-acid were indentified from *Colletotrichum acutatum* cultures supplemented with Trp, showing that this fungal pathogen may also synthesize IAA using various pathways (Chung *et al*. 2003). It has been also documented that biosynthesis of IAA from Trp proceeds through IPyA and IAAld in *Ustilago esculenta* (Chung and Tzeng, 2004). The ability of some bacteria to produce auxins may be associated with pathogenicity, symbiosis or plant growth promotion. It is known that the nitrilase of *Pseudomonas syringae* pv. *syringae* B7286, an arylacetonitrilase, is capable of hydrolyzing IAN into IAA, and allows it to use IAN as nitrogen source. This enzyme may represent an additional mechanism for IAA biosynthesis by *P. syringae*, or may be used to degrade and assimilate aldoximes and nitriles produced by the plant secondary metabolism (Howden *et al*. 2009b). Little information is available about the role of IAN into IAA conversion in fungal-plant interactions. This pathway has been also found in plant pathogenic fungal species such as *Taphrina wiesneri*, *Taphrina deformans* and *Taphrina pruni*, which cause hyperplastic diseases in plants like cherry, peach, and plum, respectively (Yamada *et al*. 1990). Understanding

indolic-pathways in plants, may lead to a bioprospecting when coupling the power of "omics" with biotechnology generating as a result new hypotheses in fundamental biology (Morath *et al*. 2012).

Table 1. Nitriles accepted as substrates by nitrilases from: *Aspergillus niger* K10; *Fusarium solani* O1; *Fusarium solani* IMI 196840; *Fusarium oxysporum* f. sp. *melonis*. According to Martínková *et al.* (2009) all nitriles shown have not been examined as substrates of all the four nitrilases.

Substrate	*Aspergillus niger* K10	*Fusarium solani* O1	*Fusarium solani* IMI 196840	*Fusarium oxysporum* f. sp. *melonis*
Benzonitrile	+	+	+	+
3-hydroxybenzonitrile	+	+	+	+
3-chlorobenzonitrile	+	+	+	
4-chlorobenzonitrile	+	+	+	
1,4-Benzodinitrile	+			
3-cyanopyridine	+	+	+	+
4-cyanopyridine	+	+	+	
Propionitrile	+	+	+	+
Butyronitrile	+	+	+	+
Valeronitrile	+	+	+	
Acrylonitrile				+

+ represents enzymatic activity observed.

4. Biotechnological importance of nitrile-degrading enzymes in environmental remediation

Nitrilase enzymes are becoming very important in the production and synthesis of different pharmaceutical and industrial chemicals. The importance and versatility as biocatalysts of these peptides lies in their potential applications in different scientific fields including synthetic biocatalysis and bioremediation (Banerjee *et al.* 2002). A bioremediation strategy refers to an application of a biological process for the cleaning of hazardous pollutants that are present in the environment, which is continuously contaminated by a large array of chemicals with different structures and toxicity levels that may be released from several anthropogenic activities (Gianfreda and Rao, 2004). The main sources of pollution can be classified in three different orders which comprise: industrial activities, munitions waste and agricultural practices. The recent development of chemical industries has produced a large variety of chemical compounds that include pesticides, colorants, fuel, solvents, polycyclic aromatic hydrocarbons (PAHs), explosive, dyes and nitrile-substituents. Although these chemicals have contributed to develop new technologies and a better lifestyle for the humanity, several of them may accumulate in soil, water and air (Iwamoto and Nasu, 2001). Because of their nature, these are highly persistent and clear examples are nitriles. The general toxicity of nitriles in humans are expressed as gastric system destabilization including vomiting and nausea, bronchial irritation, respiration problems, convulsions, non induced coma which leads to irreversible brain damage, lameness and skeletal deformities. The most severe symptoms of nitrile intoxication are due to their capacity to inactivate the respiratory system by tightly to citocrome-c-oxidase (inhibition of the electron transport chain) (Solomonson and Spehar, 1981). Some other nitrile compounds like dihalogenated benzonitrile analogues are active in a number of herbicides and their use, chemical and physical properties including environmental toxicity have been reviewed recently (Holtze *et al*. 2008). As reviewed

by Banerjee *et al*. (2002), some fungi and bacteria have enzymatic capabilities to metabolize natural and synthetic nitrile-complex. These enzymes may be of both constitutive and inducible nature and show similar features like optimum activity at alkaline pH with good stability in a large range of temperatures. In the mid 1990s, it was reported the containing of different nitrile hydrolyzing enzymes in mixed cultures of bacteria which were capable to biodegrade effluents from acrylonitrile manufacturing industries, concluding that 99 % of detectable toxic components measured decreased (Wyatt and Knowles, 1995). Likewise, previous studies have demonstrated that acrylonitrile-containing polymer emulsions direct decontamination may be treated by nitrile hydratase enzymes, as well as the engineering of transgenic plants resistant to the herbicide bromoxynil resulting from the introduction of microbial bromoxynil-specific nitrilase genes, confirming that this group of proteins are potential prospects for the bioremediation of polluted places (Battistel *et al*. 1997; Freyssinet *et al*. 1996). It is known that nitrile-degrading enzymes also act over nitrile herbicides like dichlobenil (2,6-dichlorobenzonitrle) and bromoxynil. The peptides degrade these cyano group-containing herbicides and prevent them from entering the food chain. Some microorganisms like *Agrobacterium radiobacter* are commonly used for the degradation of both herbicides when they are highly persistent in soils. Species like *Trichoderma spp*. and *Fusarium spp.* have previously been demonstrated to degrade metallocyanides through the release of extracellular cyanide hydratase and dihydratase enzymes (Muffadal and Lynch, 2005). Another clear example of this is the degradation of simple cyanide by fungi, for example; purified cyanide hydratase enzymes from *Fusarium* were found to be capable to catalyze the hydration of cyanide to formamide (Cluness *et al*. 1993). This metabolic pathway was clarified further in *F. solani* and it was demonstrated that fungal metabolism of cyanide is carried out by a mechanism consisting of a two-step hydrolytic pathway: conversion of cyanide into formamide by cyanide hydratase, followed by the conversion of formamide into formate by an amidase (Dumestre *et al*. 1997). Cyanide is a highly persistent chemical compound in the environment when released indiscriminately, in most cases, residues are found from industrial processes or as a by-product from mining exploitation, and today, the biota health repercussion from its contact is well known. Likewise, some bacteria like *Rhodococcus* UKMP-5M have been recently applied to degrade cyanide (Nallapan *et al*. 2013). A general metabolic pathway proposed by Prasad and Bhalla (2010) for nitrile synthesis and degradation in microorganisms can be seen in figure 2. Cyanide hydratases are primarily fungal enzymes which are considered the first responsible from cyanide conversion. The result of this chemical reaction is the formation of formamide that subsequently decomposes to carbon dioxide and ammonia by another formamide hydratase enzyme (FHL). This kind of peptides belongs to the leases family in which hydrolyases can be found. The systematic name of this type of enzyme is usually formamide hydro-lyase (cyanide-forming) (Gupta *et al*. 2010). Cyanide hydratases of several microorganisms may have similarities among them and represent a much more closely related group of enzymes. The first cyanide hydratase was purified by *Stemphylium loti*, a pathogenic fungus of a cyanogenic plant. According to its kinetic study, it was observed that the highest activity occurred in the pH range of 7.0-9.0. This means that one of the first reports of this peptide showed the neutral-alkaline nature of the family (Kunz *et al*. 1994). The common cause of nitrile entry into the environment may be effluent from industrial processes either engaged in nitrile production or processing (Wyatt and Knowles, 1995). On the other hand,

accidental spillage of nitrile from storing tank (Deshkar *et al*. 2003), use of nitrile compounds as chemical herbicides (Vosahlova *et al*. 1997) and processing of oil shale deposit for extraction of oil lead to significant contamination of air, soil and groundwater (Hawthorne *et al*. 1985; Aislabie and Atlas, 1988). Due to this fact, removal of nitrile in nature from industrial effluents and contaminated places should be mandatory. NHase in combination with some other kind of nitrilase enzymes such as amidases or nitrilases, have an important application in bioremediation processes of hazardous nitriles from the contaminated air, soil and water systems. Nitrile-degrading enzymes originated from microorganisms are widely distributed in soils that degrade a wide range of nitrile as carbon and nitrogen source. A consortium of microorganisms from the adapted sludge and acetonitrile-degrading organisms has been proved quite effective for biodegradation of organonitriles (i.e. saturated, unsaturated aliphatic and aromatic nitriles) in pharmaceutical waste-water treatment plants (Li *et al*. 2007). Baxter *et al*. (2006) explored the potential of a known acetonitrile-metabolizing organism (*Rhodococcus* sp. AJ270) for degradation of acetonitrile and investigated its effects on resident soil bacterial community, concluding that the use of such microorganism could play an important role in the detoxification of this toxic compound and thus, decreasing the risk of environmental contamination. Kobayashi and Shimizu (1998) proposed that the use of specialized consortia of microorganisms could be a viable alternative to activated sludge for the degradation and management of toxic chemical wastes. Likewise, Kohyama *et al*. (2006) developed a process for the treatment of acetonitrile-containing wastes by employing two nitrile-degrading microorganisms (viz. *Rhodococcus pyridinovorans* S85-2 and *Brevundimonas diminuta* AM10-C) as sources of NHase and amidase respectively. Bioremediation of nitrile contaminated soil with this strain was successful as the nitrile degrading organism became rapidly established within the microbial community of soil and it was also noted that the addition of acetonitrile significantly affected composition of bacterial community in the soil. In some cases, the biodegradation of nitriles by NHase also leads to hazardous metabolites, for example: 2,6-dichlorobenzamide from dichlobenil as dead-end product which is not hydrolyzed by amidase, and is more soluble and mobile than the parent compound in soil and groundwater (Holtze *et al*. 2008). However, nitriles of shale oil can be selectively degraded by mixed cultures of nitrile hydrolyzing organisms such as *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* (Aislabie and Atlas, 1988). The soil microorganisms like *A. radiobacter* 8/4 (Vosahlova *et al*. 1997), *F. solani* (Harper, 1977a), *Klebsiella ozaenae* (McBride *et al*. 1986) and *Nocardia* sp. NCIMB 11215 (Harper, 1985) harbouring nitrile metabolizing enzymes efficiently degrade nitrile herbicides like bromoxynil, ioxynil (3,5-diiodo-4-hydroxybenzonitrile), and dichlobenil (2,6-dichlorobenzonitrile).

5. Conclusions and future perspectives

The exploitation of the catalytic properties of nitrile-degrading enzymes is constantly increasing due to the recognition of their applicability in the production of several synthetic compounds with industrial and pharmaceutical interest. In addition, because of their role in plant metabolism as well as plant-microbe interactions, the understanding of their structure and enzymatic properties allow better advances in genetic manipulation and biosynthetic regulation. Further structural studies of this type of proteins including application of site-directed mutagenesis or by directed evolution, genome-mining and molecular characterization, may enable more stable engineered structures. A major

understanding of the physical properties of such enzymes including elucidation of reaction mechanisms, may lead to improved properties like enhanced enzyme activity and higher thermostability. With an adequate manipulation of these characteristics, bioremediation processes would be easiest and successful. Despite recent discovers made in the last decade, further application-oriented studies may required to better exploit their biotechnological applications. In addition, deeper substrate specificity studies may help to understand their catalytic properties, which are considered one of the most critical steps in a manipulated enzymatic reaction. The discovery of new nitrilase/cyanide hydratase enzymes that have the ability to hydrolyze nitrile-substituent compounds whether fungal, plant or bacterial nature, increases prospects to engineer proteins with directed-catalytic properties that assist in detoxification processes of cyanide-containing industrial wastewater. Through better elucidation of structure and reaction mechanisms of nitrilase enzymes it is possible to increase the probability of better progress in biosynthetic regulation including higher enzyme activity, stereospecificity and a wide range of applicability over a range of pH and temperature. Including these properties in a multi-enzyme complex could be considered for future environmental application-oriented studies that require fully exploit their biotechnological potential. Despite the above considerations, further work is required to optimize the technological application of biological systems both in wastewater and soils, with emphasis on the development of microorganism processes that face extreme environmental conditions, including high-level toxicity. In addition, new technology may be required to ensure an effective chemical and physical remediation strategy to combat nitrile pollution.

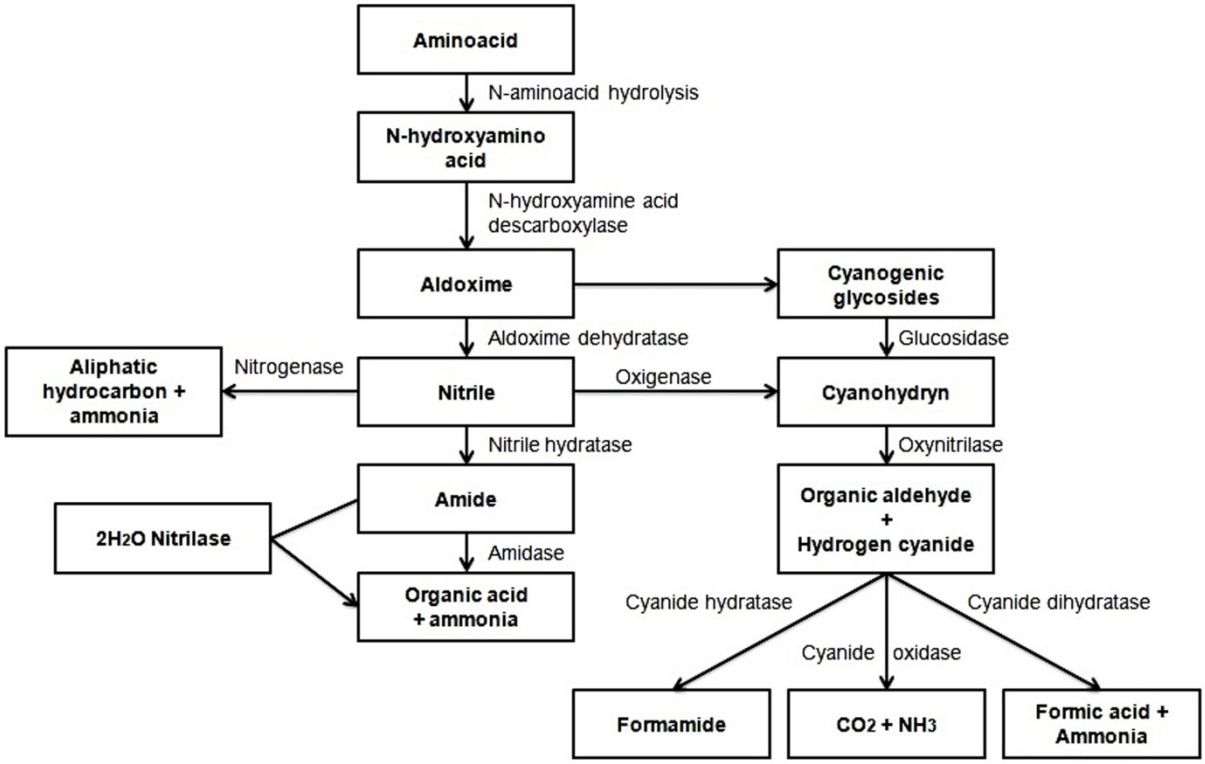

Figure 2. General metabolic pathway for nitrile synthesis and degradation in microorganisms. Prasad and Bhalla (2010).

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