



# A review on remediation of cyanide containing industrial wastes using biological systems with special reference to enzymatic degradation

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## Abstract

Cyanide is a nitrile which is used extensively in many industries like jewelry, mining, electroplating, plastics, dyes, paints, pharmaceuticals, food processing, and coal coking. Cyanides pose a serious health hazard due to their high affinity towards metals and cause malfunction of cellular respiration by inhibition of cytochrome *c* oxidase. This inhibition ultimately leads to histotoxic hypoxia, increased acidosis, reduced the functioning of the central nervous system and myocardial activity. Different physicochemical processes including oxidation by hydrogen peroxide, alkaline chlorination, and ozonization have been used to reduce cyanide waste from the environment. Microbial cyanide degradation which is considered as one the most successful techniques is used to take place through different biochemical/metabolic pathways involving reductive, oxidative, hydrolytic or substitution/transfer reactions. Groups of enzymes involved in microbial degradation are cyanidase, cyanide hydratase, formamidase, nitrilase, nitrile hydratase, cyanide dioxygenase, cyanide monooxygenase, cyanase and nitrogenase. In the future, more advancement of *omics* technologies and protein engineering will help us to recoup the environment from cyanide effluent. In this review, we have discussed the origin and environmental distribution of cyanide waste along with different bioremediation pathways and enzymes involved therein.

**Keywords** Cyanide waste · Remediation techniques · Cyanidases · Nitrilases · Enzymatic degradation pathway

## Introduction

Cyanides are C≡N group containing compounds available in the form of nitriles, carbonitriles and cyanides. These compounds are extensively used in precious metal extraction industries and in agrochemical industries. These compounds

are not only highly toxic to living beings but also recalcitrance to biodegradation (Luque-Almagro et al. 2011). Apart from being generated as industrial waste, cyanide wastes are also generated by degradation of cyanoglycosides and cyanolipids present in plants during raw plant processing. They are also released by microorganisms during their metabolic activity (Mekuto et al. 2016). Plants, fungi as well as bacteria are also synthesized cyanide through the process of cyanogenesis (Raquel et al. 2008; Bhalla et al. 2018). Physical and chemical methods of cyanide treatment are relied on uses of hazardous reagents and these processes are very expensive. Despite cyanide toxicity, few microorganisms are able to detoxify cyanides by using various enzymes present in their system. During the last few decades bioremediation is considered as one of the best alternatives to chemical treatment processes. Although bioremediation is an ecologically viable technique for cyanide degradation, their use at the industrial scale is still skeptical and under investigation at bench and pilot scale. The recent development of “omics” technology has contributed significantly in cyanide remediation process by gathering information in genome level from different microorganisms and converted

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them in genomic-proteomic-metabolomic techniques, called “cyanomics” (Luque-Almagro et al. 2016). In this review, we have discussed the origin and distribution of cyanide waste in the environment along with different bioremediation techniques adopted in the last few decades for curbing down the cyanide pollution. A detail enzymatic degradation pathway is also discussed.

### Sources and distribution of cyanide in the environment

Cyanogenic compounds are ubiquitous in nature and produced by bacteria, fungi, plants and some insects during their various activities such as, at the time when they need to control mating behavior and need to fight against predators (Kumar et al. 2013; Frapolli et al. 2012; Baxter and Cummings 2006).

Around 2500 plant species were reported to have this kind of secondary metabolites in their system (Panter 2018). Cyanic/cyanide compounds are synthesized by many plants in the form of cyanolipids and cyanoglycosides which act as a deterrent against pests like insects and animals (Møller 2010). Ingestion of these plants causes acute or chronic plant poisoning in animals and humans. Pits and seeds of some plants (bitter almonds, apples, apricot, peaches, barley, lima beans, sorghum, flax seed) have a substantial amount of cyanoglycoside which can be further metabolized to produce cyanide; however, edible portion of plants have a low concentration of such compounds. Some species of bamboo shoots are bitter in taste and contains a cyanoglycoside known as taxiphyllin (Young 1954). Plants naturally synthesize these cyanogenic compounds using enzyme hydroxynitrile lyase. These compounds are in inactive forms and stored in one part of the cell and the activating enzyme is present in another part of the cell. Upon tissue damage due to pest/herbivore chewing activity or mechanical injury, the cyanogenic compounds and activating enzymes come into contact with each other and cyanide is then cleaved from the sugar, hence acting as a defense agent against pest and herbivore (Sharma et al. 2005).

To defy plants cyanide defense system, insects feeding on them slowly developed tolerance towards these chemicals by the process of co-evolution. These insects such as burnet moths e.g. *Zygaena filipendulae* not only became tolerant to cyanoglycosides but also started forming similar compounds like their host and also sequestered them from the host and utilized these cyanoglycosides against their predators. Zagrobelny et al. (2009) have identified the genes for cyanoglycoside synthesis in the six-spotted burnet moth using pyrosequencing. These cyanoglycosides not only act as a defense agent but also act as a pheromone. Female burnet discharges a trail of hydrogen cyanide which acts as an attractant for the male burnet moth. The male having a

higher concentration of cyanide is preferred over others and during mating these cyanoglycosides are exchanged from male to female (Zagrobelny et al. 2007). These compounds provide an extra defense to eggs. They also act as a stored source of reduced sugar and nitrogen for the burnet moth.

Other than natural sources, cyanide compounds are also added to the environment by many anthropogenic activities. Cyanide compounds have many applications in the pharmaceutical industry, polymer manufacturing, mining and steel manufacturing, electroplating and agrochemical production (Kumar et al. 2013). The effluents from such industries are recalcitrant to treatment and pose many environmental problems. Agricultural practices (while using nitrile herbicides) also significantly contribute to increased cyanide level in soil and environment. Dichlobenil, ioxynil, bromoxynil and chlorothalonil are few nitrile based herbicides which are used as pest control agents in a variety of crops e.g. wheat, rice, corn, and barley (Lovecka et al. 2015). Cyanide is used as a laxative in precious metal mining as they form tight complexes with gold, silver, iron, heavy metals and also help them to solubilize from the ore. Cyanide is used in relatively dilute concentration for gold metal extraction too, known as the gold cyanidation process (He and Kappler 2017). Other than waste, road salt additives also contribute cyanide in the soil. During road construction, sodium ferrocyanide and ferric ferrocyanide (road deicing salt) are used as anti caking compounds. Due to sunlight exposure and microbial activity, ferrocyanide chemically degrade and cyanide ions are released into the soil (Paschka et al. 1999). In soil, the cyanide compounds are fairly mobile and move from soil to water and air. However, in the soil, the concentration of cyanide does not go up, mainly due to volatilization and microbial action (Paschka et al. 1999; ATSDR 2016).

### Different strategies adopted in the last few decades for cyanide removal

Owing to their potential environmental toxicity, different cyanide detoxification strategies were adopted and modified during the last few decades. Many chemical and physical methods have been found useful in this detoxification process but numerous factors such as the chemical composition of the waste, its volume, the effluent quality, reagents availability are negatively affecting the feasibility of these methods (Akcil and Mudder 2003; Botz et al. 2015). In the last two decades, many conventional methods including natural, physical, chemical and biological methods (phytoremediation and microbial remediation) have been followed to remove cyanide containing waste from different environmental compartments. In the following section, we have discussed the cyanide bioremediation methods followed by the enzymatic degradation pathways.

**Table 1** List of plant species able to remove cyanide (excluding the list published by Yu et al. 2004)

Plant species	Concentration of cyanide removed by phytoremediation	References
<i>Eichhornia crassipes</i>	10 mg CN <sup>-</sup> L <sup>-1</sup> ; 35 mg CN <sup>-</sup> kg <sup>-1</sup> h <sup>-1</sup> (leaf cuttings)	Ebel et al. (2007)
<i>Hordeum vulgare</i> , <i>Avena sativa</i> , <i>Sorghum bicolor</i>	delta 15N per thousand = 1000–1500 (roots) delta 15N per thousand = 500 (shoots)	Samiotakis and Ebbs (2004)
<i>Sambucus chinensis</i>	12.6 mg CN <sup>-</sup> kg <sup>-1</sup> h <sup>-1</sup> at 30 °C	Yu et al. (2005b)
<i>Salix babylonica</i>	9.72 mg CN <sup>-</sup> kg <sup>-1</sup> h <sup>-1</sup> at 32 °C	Yu et al. (2005b)
<i>Linum usatassimum</i> , <i>Sorghum bicolor</i>	± 140 mg CN <sup>-</sup> kg <sup>-1</sup> plant	Kang et al. (2007)
<i>S. babylonica</i>	< 9.3 mg CN <sup>-</sup> L <sup>-1</sup>	Yu et al. (2005a)
<i>Salix viminalis</i>	< 8 mg CN <sup>-</sup> L <sup>-1</sup>	Larsen et al. (2004)
<i>Arabidopsis thaliana</i> (transgenic)	Up to 1.2 mM (in growth medium) and 2.5 mM (applied via foliar spray)	Kebeish and Al-Zoubi (2017)

### Biological methods of cyanide removal

It is well evidenced that phytoremediation of thiocyanate and metal–cyanide complexes and microbial biodegradation of cyanide from ore mining wastewaters are the most successful methods of cyanide removal (Dash et al. 2009). In bacterial bioremediation process, free cyanide and metal-complexed cyanides are transformed to ammonia and bicarbonate; the free metals thus formed are either precipitated out from the solution or get adsorbed within the biofilm. In this context, it is worth mentioning that iron cyanides are less readily biodegraded and bioadsorbed than cyanide complexes of zinc, nickel, and copper (Akcil 2003; Botz 2001). Both aerobic and anaerobic biological treatments are followed to remove cyanide and thiocyanate after standardizing the environmental factors (temperature, pH, oxygen levels, and nutrition). For aerobic biological treatment of cyanide and thiocyanate many processes have been reported which include packed beds, rotating biological contactors (Campos et al. 2006), sequencing batch reactors, biological filters (White and Schnabel 1998), facultative lagoons, and activated sludge (Kaewkannetra et al. 2009).

### Phytoremediation

Phytoremediation is one of the successful bioremediation strategies for cyanide detoxification. In this context Yu et al. (2004) have done substantial work on this method whereas a concise review on this topic was published by Kumar et al. (2017). Other plant species which were able to remove cyanide from soil and solutions are also listed in Table 1 (excluding the list published by Yu et al. 2004). Most of the plant species reported for phytoremediation were shown to have low tolerance and slow cyanide degradation rate. Phytoremediation is an extremely slow process which requires huge land area and cyanide concentration < 10 mg CN<sup>-</sup> L<sup>-1</sup>. This process is therefore practically not feasible to implement in arid regions. While cyanogenic plants were reported

to synthesize cyanoglycosides and cyanolipids, some non cyanogenic species (maize and some Chinese vegetation) were also reported to have cyanide degrading activity (Yu et al. 2004; Yu and Gu 2007). In contrast to microbial degradation, phytoremediation process was found superior in case of metal cyanide complexes (e.g. iron cyanide) (Aronstein et al. 1994). In this case, the plant species were not only tolerant but also showed positive growth pattern in the presence of cyanides. For example, *Salix babylonica* exhibited modified enzyme activities (superoxide dismutase, catalase, peroxidase), high rate of transpiration, increased chlorophyll content and soluble protein while engaged in phytoremediation of cyanide (Yu and Gu 2009). However, the fundamental biological mechanisms involved in this process are still unknown. A simplistic mechanism is discussed in the following section to understand the process.

The plant–microbe interactions (in symbiotic association) in rhizosphere emit many secondary metabolites and in some cases exuded biosurfactants (e.g., rhamnolipids). Biosurfactants help to increase the solubility as well as bioavailability of the hydrophobic organic nitriles (Volkering et al. 1998; Siciliano and Germida 1998). Beside this, phytosiderophores (present in roots) have also influenced sequestration and translocation of the metalocyanide in the plant (Taiz and Zeiger 2002). Sometimes fungi, when engaged in symbiotic association with plants, are able to increase root surface area and can detoxify toxic cyanide through stabilization, extraction or by degradation (Ebbs 2004). The root uptake of the organic pollutant is not easy as there is no transporter in the plasma membrane owing to the xenobiotic nature of the pollutant. The suberine layer of plant root endodermis acts as impermeable membrane and resists the toxic material flow directly into xylem by root apoplast (Taiz and Zeiger 2002). Translocation of the pollutants into the plant roots and within plant tissue is governed by simple diffusion mechanism which further depends on the hydrophobicity of the compounds (Trapp and McFarlane 1995). Once entered inside the tissue, these

compounds are bound to chelators (GSH ( $\gamma$ -glu-cys-gly)/PCs- phytochelatins) and subsequently transported actively to the vacuole by ABC-type transporter (Pickering et al. 2000; Cobbett and Goldsbrough 2000; Mäser et al. 2001). In vacuoles, these conjugated compounds form more complex structure after reacting with sulfide molecules; after that these complex molecules can either stay in accumulated form within the plant tissue or may be metabolized. These stored complexes are further catabolized partially into stable intermediates (McCutcheon and Schnoor 2003) by the action of enzymes coming from endophytic microorganisms (Barac et al. 2004). These enzymes may modify these complexes by adding side groups which further increases solubility of these organic effluents and hence can initiate its degradation by phytoenzymes. The major enzymes involved in this process are  $\beta$ -cyanoalanine synthase, rhodanese, formamide hydrolyase, cyanide dihydratase (Miller and Conn 1980).

Among these enzymes,  $\beta$ -cyanoalanine synthase (CAS) is a ubiquitous enzyme and is able to catalyze the reaction between cysteine and cyanide which ultimately results in  $\beta$ -cyanoalanine and sulfide formation (Maruyama et al. 2001).  $\beta$ -cyanoalanine is a wide spread amino acid found in the plant kingdom. They are neurotoxic in nature and acted as a defense agent against herbivory (Piotrowski et al. 2001). It was also found that asparagines (nitrogen storage form) could be formed from  $\beta$ -cyanoalanine by the action of  $\beta$ -cyanoalanine hydrolase (Castric et al. 1972). In plants, rhodanese and formamide hydrolase are not very common enzymes (Miller and Conn 1980). Rhodanese has been reported in a few plants e.g. *Brassica oleracea* (Tomati et al. 1972), cassava (Emmanuel and Emmanuel 1981), *Sorghum* sp. (Myers and Fry 1978). Formamide hydrolase has been reported in Japanese apricot and loquat (Shirai 1978; Miller and Conn 1980). Inorganic cyanide is first transformed into formamide by formamide hydrolase which further can form formaldoxime and finally converted into formic acid and ammonia (Shirai 1978). The role of cyanide hydratases and cyanide dihydratases is discussed in a later section.

### Microbial remediation

In microbial remediation process bacteria, fungi, algae and yeasts are involved in cyanide remediation. Temperature and pH are important parameters for determining the biodegradation rate and it was found that 20–40 °C temperature and 6–9 pH are optimum for microbial bioremediation process. Many fungal species such as *Fusarium solani*, *Fusarium oxysporum*, *Penicillin miczynski*, *Scytalidium thermophilum*, *Trichoderma polysporum* (either individually or in consortia) as well as many bacterial species belonging to the genera *Pseudomonas*, *Bacillus*, *Rhodococcus* and *Serratia* have been reported for their cyanide degrading ability (Barclay et al. 1998; Acera et al. 2017; Singh et al. 2018;

Manso Cobos et al. 2015; Ibáñez et al. 2017; Maniyam et al. 2013; Bhalla et al. 2018). Varieties of enzymatic pathways are involved in the biotransformation and biodegradation processes which include degradative pathways (e.g. hydrolytic, oxidative, and reductive pathway forming  $\text{NH}_3$ ,  $\text{HCO}_2\text{H}$ ,  $\text{CO}_2$ ,  $\text{CH}_4$  and  $\text{RCO}_2\text{H}$ ) and assimilative pathways (substitution/transfer/synthesis). Most of the cyanide degrading microbes are aerobes; however anaerobic degradation of cyanide has also been reported and recently reviewed by Luque-Almagro et al. (2018). It was found that among all the degradative pathways, only reductive/hydrolytic pathways are feasible under anaerobic conditions (Fallon 1992). Enzymatic cyanide detoxification and cyanide assimilation by different microorganisms have been reviewed extensively and documented well in a number of reviews (Akcil and Mudder 2003; Samiotakis and Ebbs 2004; Baxter and Cummings 2006; Huertas et al. 2006; Dash et al. 2009; Gupta et al. 2010; Kumar et al. 2017; Luque-Almagro et al. 2016; Mekuto et al. 2016; Park et al. 2017). The following section has elaborately described the enzymatic degradation pathways of cyanide bioremediation.

### Enzymatic degradation pathways of cyanides

Microbes employ different pathways to deal with toxic chemicals that are present in their habitat. The cyanide metabolizing microbes have followed five different pathways to mineralize the cyano compounds (Dash et al. 2009; Ebbs 2004). Table 2 described the various enzymes (along with their sources) involved in the enzymatic degradation pathway of cyanide biodegradation. In the following sections, the enzyme and their mechanism of action are discussed in detail.

#### Oxidative pathway

In the oxidative pathway the cyanide compounds are converted into ammonia and carbon dioxide using three different enzymes, viz., cyanase, cyanide monooxygenase, cyanide dioxygenase enzymes (Dash et al. 2009; Ebbs 2004).

#### Cyanase (EC 4.2.1.104)

Cyanase belongs to the lyase family of enzymes and generally inducible in nature. These enzymes have been reported in plants (Aichi et al. 1998), in cyanobacteria (Harano et al. 1997) and in many microorganisms. It is used by many organisms for cyanate detoxification when the concentration of cyanate is low in the system (Luque-Almagro et al. 2011). Cyanate and isocyanate (i.e. present in equilibrium) irreversibly carbomylated lysine into homocitrulline, modified the protein structure and further rendering them in the inactive or dysfunctional state (Koeth et al. 2013). Cyanate

**Table 2** Enzymes involved in enzymatic degradation pathway of cyanide biodegradation and sources of enzymes (Gupta et al. 2010; Bhalla et al. 2018)

Enzymes	Pathway involved	Reaction catalyzed	Cofactor requirement	Source of enzyme
Cyanase	Oxidative pathway	$\text{HCN} \xrightarrow{\text{NADPH}} \text{OCN}^+$ $\text{OCN}^+ + \text{HCO}_3^- + 2\text{H}^+ \rightarrow \text{CO}_2 + \text{NH}_3$	Bicarbonate and NADPH dependent	<i>Escherichia coli</i> , <i>Serratia proteamaculans</i> , <i>Sordaria macrospora</i> , <i>Arabidopsis thaliana</i> , <i>Pseudomonas pseudocataligenes</i> , <i>Oryza sativa</i> , <i>Tetranychus urticae</i> , <i>Bemisia tabaci</i> <i>P. putida</i>
Cyanide dioxygenase	Oxidative pathway	$\text{HCN} + \text{O}_2 + 2\text{H} + 2\text{e}^- \xrightarrow{\text{Cyanide dioxygenase}} \text{CO}_2 + \text{NH}_3$	NADPH	<i>Pseudomonas fluorescens</i> , <i>Trametes versicolor</i> , <i>Phanerochaete chrysosporium</i> , <i>Pleurotus sajor-caju</i>
Cyanide monooxygenases	Oxidative pathway	$\text{HCN}^- + \text{O}_2 + 2\text{H} + 2\text{e}^- \xrightarrow{\text{Cyanide monooxygenase}} \text{OCN}^- + \text{H}_2\text{O}$	NADPH and reduced Pterin	<i>Azotobacter chroococcum</i> , <i>Klebsiella tropicus</i> , <i>Herbaspirillum thermoautotrophicum</i> , <i>Streptomyces thermoautotrophicum</i> spp., <i>Rhodospirillum rubrum</i>
Nitrogenase	Reductive pathway	$\text{HCN} + 6\text{H} + 6\text{e}^- \xrightarrow{\text{Nitrogenase}} \text{CH}_4 + \text{NH}_3$	Fe, Mo cofactor	<i>E. coli</i> , <i>Ferrobacillus ferrooxidans</i> , <i>Thermobacillus denitrificans</i> , <i>Desulforomaculum nitrificans</i> , <i>Azotobacter vinelandii</i> , <i>Thiobacillus</i> sp., <i>Bacillus brevis</i> , <i>Fusarium</i> strains, <i>Trichoderma</i> strains, <i>Pseudomonas aeruginosa</i>
Rhodanase	Substitution pathway	$\text{S}_2\text{O}_3^{2-} + \text{HSCH}_2\text{CH}(\text{NH}_2)\text{CO}_2\text{H} \xrightarrow{\text{Rhodanase 1}} \text{HS}_2\text{CH}_2\text{CH}(\text{NH}_2)\text{CO}_2$ $\text{HS}_2\text{CH}_2\text{CH}(\text{NH}_2)\text{CO}_2\text{H} + \text{HCN} \xrightarrow{\text{Rhodanase 2}} \text{SCN}^- + \text{HSCH}_2\text{CH}(\text{NH}_2)\text{CO}_2\text{H}$		Wide distribution in living organisms. <i>Leishmania major</i>
Mercaptopyruvate sulfurtransferase	Substitution pathway	$\text{HSCH}_2\text{C}(\text{O})\text{CO}_2^- + \text{RSH} \xrightarrow{\text{MPST}} \text{RSSH} + \text{CH}_2\text{C}(\text{O})\text{CO}_2^-$ $\text{RSSH} + \text{HCN} \rightarrow \text{SCN}^- + \text{RSH}$ $\text{R-CH}_2\text{CH}(\text{NH}_2)\text{CO}_2\text{H}$		
Cyanide hydratase (formamide as intermediate)	Hydrolytic pathway	$\text{MPST-3-mercaptopyruvate sulfurtransferase}$ $\text{HCN} \xrightarrow[\text{H}_2\text{O}]{\text{Cyanide hydratase}} \text{HCONH}_2 \xrightarrow{\text{Amidase}} \text{HCOOH} + \text{NH}_3$		<i>Stenophyllum loti</i> <i>Fusarium solani</i> IHEM 8026 <i>Fusarium oxysporum</i> <i>Gloeocercospora sorghi</i> <i>Fusarium lateritium</i> <i>Helminthosporium turcicum</i>
Cyanide dihydratase/cyanidase	Hydrolytic pathway	$\text{HCN} \xrightarrow[\text{2H}_2\text{O}]{\text{Cyanide dihydratase}} \text{HCOOH} + \text{NH}_3$		<i>Alcaligenes xylosoxidans</i> subsp. <i>denitrificans</i> , <i>Bacillus pumilus</i> C1, <i>Pseudomonas stutzeri</i> AK61
Nitrilase	Hydrolytic pathway	$\text{RCN} \xrightarrow[\text{H}_2\text{O}]{\text{Nitrilase}} \text{RCOOH} + \text{NH}_3$		<i>Fusarium oxysporum</i> , <i>R. rhodococcus</i> J1, <i>Klebsiella oxytoca</i> , <i>Rhodococcus rhodochrous</i> PA-34, <i>Nocardia</i> sp. <i>Alcaligenes faecalis</i> JM3 <i>Acinetobacter</i> sp. AK226 <i>Pseudomonas thermophila</i> JCM3095

Table 2 (continued)

Enzymes	Pathway involved	Reaction catalyzed	Cofactor requirement	Source of enzyme
Nitrile hydratase (amide as product)	Hydrolytic pathway	$\text{RCN} \xrightarrow[\text{H}_2\text{O}]{\text{Nitrile hydratase}} \text{RCONH}_2 \xrightarrow{\text{Amidase}} \text{RCOOH} + \text{NH}_3$	Metal cofactor	<i>Pseudonocardia thermophila</i> , <i>Corynebacterium</i> , <i>Pseudomonas</i> , <i>Rhodococcus rhodochrous</i> , <i>Rhizobium Klebsiella pneumoniae</i> , <i>Pseudomonas putida</i> MA113
Thiocyanate hydrolase	Hydrolytic pathway	$\text{SCN} + 2\text{H}_2\text{O} \xrightarrow{\text{Thiocyanate hydrolase}} \text{S}=\text{C}=\text{O} + \text{NH}_3 + \text{OH}^-$	Cobalt	<i>Thiobacillus thiooparus</i> TH1115
Cyanoalanine synthase	Synthesis pathway	$\text{HSCH}_2\text{CH}(\text{NH}_2)\text{CO}_2\text{H} + \text{HCN} \xrightarrow{\text{Cyanoalanine synthase}} \text{NCCH}_2\text{CH}(\text{NH}_2)\text{CO}_2\text{H} + \text{H}_2\text{S}$	Pyridoxal-Phosphate	Higher plants and fungi, <i>E. coli</i> , <i>Chromobacterium violaceum</i> , <i>Bacillus magisterium</i>
$\gamma$ -cyano- $\alpha$ -aminobutyric acid synthase	Synthesis pathway	$\text{CH}_3\text{COOCH}_2\text{CH}(\text{NH}_2)\text{CO}_2\text{H} \xrightarrow{\text{CN}^-} \text{NCCH}_2\text{CH}(\text{NH}_2)\text{CO}_2\text{H} + \text{CH}_3\text{COO}^-$ $\text{NCCH}_2\text{CH}(\text{NH}_2)\text{CO}_2\text{H} \xrightarrow{\text{COCl}_2 + \text{NaBH}_4} \text{NH}_2\text{CH}_2\text{CH}_2\text{CH}(\text{NH}_2)\text{CO}_2\text{H}$ <p style="text-align: center;">L-2,4- diaminobutyric acid</p>	Pyridoxal-Phosphate	<i>Bacillus stearothermophilus</i> , <i>Chromobacterium violaceum</i>



acts as a competitive inhibitor of nitrate and also oxidizes the reductively inactive enzymes and reactivates them. Cyanase catalyses the conversion of cyanate into carbon dioxide and ammonia in two steps processes. In the first reaction, the bicarbonate which acts as nucleophile reacts with cyanate (which is produced by its oxidation in soil) and forms an unstable carbamate as an intermediate which further spontaneously breaks down to form ammonia and carbon dioxide by decarboxylation reaction (Luque-Almagro et al. 2011). Earlier in their study, Luque-Almagro et al. (2008) reported an alkaliphilic bacterium *Pseudomonas pseudoalcaligenes* CECT5344 which showed good growth in different cyano compounds by using these compounds as a sole source of nitrogen. The microbe harbored two independent pathways for cyanide degradation. One pathway was cyanase mediated, consisting of *cynFABDS* gene cluster and another one was Nitrilase (*NitC* gene) enzyme mediated pathway. A recent article published by Cabello et al. (2018), reviewed the application of transcriptomics and proteomics techniques for getting insight into cyanide degradation pathway of *Pseudomonas pseudoalcaligenes*.

An earlier report published by Sung and Fuchs 1992, demonstrated that *E. coli* strain K-12 possessed cyanide degrading genes in the form of *cyn* operon. The regulatory protein known as CynR was usually present in the upstream of *cyn* operon. The molecular weight of CynR was 32-kDa and it belongs to LysR regulatory proteins (Sung and Fuchs 1992). The *cynR* promoter shared the overlapping region with *cyn* operon promoter on the opposite strand (Lamblin and Fuchs 1994). *Cyn* operon consists of three genes; *cynT* (cyanate permease), *cynS* (cyanase), and *cynX* (unknown hydrophobic protein) (Anderson et al. 1990). Guilloton et al. (1992) illustrated that *cynT* gene product was actually coded for carbonic anhydrase whose molecular weight was 24 kD. The enzyme was found to be in the oligomeric form in the solution and in the presence of bicarbonate, it partially dissociated from that structure. In *cyn* operon, the perceptible function of inducible carbonic anhydrase is to catalyze rapid hydration of carbon dioxide (formed due to decomposition of cyanate ion) to diffuse it out from the cell. In this way it helps the cell to replenish bicarbonate which is required by the cyanase enzyme to degrade cyanate ion. The second gene of *cyn* operon is the *cynS* gene which encodes for cyanase enzyme. *CynS* gene is usually activated only in the presence of high concentration of cyanate ion whereas arginine in excess acts as down regulator of this gene (Elleuche and Pöggeler 2008; Anderson et al. 1990).

The CynX is an unknown hydrophobic protein and not characterized till date. It is supposed to be a transporter protein for cyanate ion. Pao et al. (1998) included CynX as a member of major facilitator superfamily (MFS) of transporters. The size of the protein in *E. coli* was found 393 amino acids size range which putatively might consist of 11 or 12

transmembrane structures. The protein has also exhibited considerable homology with the tartrate permease of *Agrobacterium vitis* and *Bacillus subtilis* at the sequence level (Pao et al. 1998). However, they were expressed at a much lower rate as compared to *cynT* and *cynS* gene.

At enzyme level, cyanase activity is regulated by the concentration of bicarbonate which acts as an inhibitor of cyanase enzyme. At low concentration, it shows uncompetitive inhibition. In that case, bicarbonate ions are bound to the anionic site of the active site and are interfered with cyanate ion binding. Whereas, at higher concentration of bicarbonate ions, the inactive complex formed at the active site and then degradation occurred to restart the binding action. This type of inactive rapid equilibrium formation between bicarbonate and cyanate ion is known as ping pong inhibition (Anderson and Little 1986).

### Cyanide monooxygenase

Besides cyanases, two other enzymes are utilized by microbes for detoxification of cyanide i.e. cyanide monooxygenase and cyanide dioxygenase. Kunz et al. (1992) reported the presence of cyanide monooxygenase (also known as CNO) in *Pseudomonas fluorescens* NCIMB 11764 when the strain grew in cyanide solution (300 mM). In this case cyanide was degraded in two steps; first, it was converted to an intermediate formamide which then oxidatively cleaved to form formate and ammonia. The ammonia was then assimilated by conventional pathways (Kunz et al. 1992). The enzyme required pterin as cofactors (Fernandez et al. 2004). The formate produced in the aforesaid reaction was further oxidized to formic acid by formate dehydrogenase enzyme (FDH).

### Cyanide dioxygenase

Cyanide dioxygenase enzyme converted cyanide into ammonia and carbon dioxide in a single step reaction. This enzyme also depends on pterin cofactor for its activity like cyanide monooxygenase. Cyanohydrins are also formed in cyanide dioxygenase mediated cyanide degradation pathways (Ebbs 2004).

### Reductive pathway

The reductive pathway of cyanide degradation utilizes a well known enzyme called nitrogenase. This enzyme generally converts atmospheric nitrogen into ammonia and also be able to reduce cyanide into methane and ammonia. The first report of nitrogenase mediated reductive transformation of cyanide was reported by Hardy and Knight Jr (1967). An inducible nitrogenase from the strain *Klebsiella oxytoca* was isolated by Liu et al. (1997) after growing the strain in

KCN. They transformed the strain with a *nif* containing plasmid and compared it with wild strain. The wild strain and transformed strain both exhibited diauxic growth in medium containing ammonium chloride and potassium cyanide. In culture broth, ammonium chloride was a preferred substrate over potassium cyanide and methane was detected as one of the main compounds in the growth medium (Liu et al. 1997). Another  $N_2$ -fixing bacterium *Azotobacter chroococcum* NCIMB 8003 which contains nitrogenase enzyme was reported by Kelly (1968). He found that the strain was successful to reduce cyanide into ammonia by the action of the nitrogenase enzyme. Recently its genome was sequenced by Robson et al. (2015); however, no other cyanide metabolizing enzymes were found in this strain. In this context, a nitrogen fixing and strict aerobic strain of *Azotobacter vinelandii* was reported by Koksunan et al. 2013 and was extensively investigated for bioremediation of cyanide-contaminated wastewater. The strain *A. vinelandii* exhibited high nitrogenase expression as well as fast growth kinetics. The strain was first grown on nitrogen-free sucrose medium and its cyanide reduction ability was investigated. After that, the process was applied to remediate cyanide containing wastewater collected from cassava mill. A rapid cyanide reduction was observed in the early stationary growth phase rather than in the exponential phase and it was found that the cyanide concentration (4 mM-NaCN) was reduced by 69.7% within 66 h in 3L bioreactor (Koksunan et al. 2013).

### Hydrolytic pathway

The hydrolytic pathway is the most widely exploited pathway for the degradation of cyanide by the microbes. Many hydrolases which are responsible for the degradation of inorganic as well as organic cyanides and nitriles have been studied extensively and reported in many literatures (Martínková et al. 2017; Dent et al. 2009; Jandhyala et al. 2005; Basile et al. 2008; Crum et al. 2016; O'Reilly and Turner 2003). Cyanidase, cyanide hydratase, formamidase, nitrilase, nitrile hydratase, amidase and thiocyanate hydrolase are the enzymes taking part in this pathway. In the hydrolytic pathway, direct cleavage of carbon nitrogen triple bond by the enzyme results in the formation of corresponding amide or acid and ammonia (Bhalla et al. 2012; Gupta et al. 2010).

Cyanidase and cyanide hydratase enzymes are highly specific in terms of their substrate specificity (cyanide). The former enzyme is mainly found in bacteria while later one is of fungal origin. Formamidase enzyme takes formamide as substrate and converts it into formic acid and ammonia. Nitrilase and amidase belong to Nitrilase superfamily of enzymes and convert organic nitriles and amide into their corresponding acids. Whereas, nitrile hydratase, a metallo-enzyme help bacteria to use nitriles as nitrogen and carbon source. It catalyses the conversion of organic cyanide into an

amide by adding water molecule to the cyano group which is further utilized as a substrate for amidase. Generally, in bacteria, the nitrile hydratase and amidase genes are cotranscribed and cotranslated. The enzyme thiocyanate hydrolase is responsible for catalyzing the reaction of thiocyanate to sulfate, ammonia and carbon dioxide (Gupta et al. 2010).

### Cyanidase/cyanide dihydratase (EC 3.5.5.1)

Cyanidases (CDH) are cyanide degrading nitrilases which do not require any cofactor/co-substrate for their catalytic activity. *Alcaligenes xylosoxidans* subsp. *denitrificans* DF3 was reported to have this enzyme which hydrolyzed inorganic cyanide directly into formate without formation of any intermediate (Ingvorsen et al. 1991). Few other organisms, viz., *Bacillus pumilus* C1 (Meyers et al. 1991, 1993), *Pseudomonas fluorescens* NCIMB 11764 (Kunz et al. 1992) and *P. stutzeri* AK61 (Watanabe et al. 1998) were also reported which showed cyanidase activity when subjected to cyanide degradation. *P. fluorescens* NCIMB 11764 in this regard was quite unique because it has devised a different pathway for cyanide degradation. The strain possessed cyanide hydratase as well as cyanide dihydratase activities. The authors proposed that there may be a possibility that both the activities may be present in a single enzyme, but no detailed mechanism was proposed (Kunz et al. 1992). In another study, Martínková et al. (2015) investigated the bioremediation potentiality of *Bacillus pumilus* in the presence of cyanide waste. They found that cyanide degradation was improved when the process was carried out in alkaline pH. From that finding, they have engineered the strain by directed evolution combined with site-directed mutagenesis and got ample activity of these enzymes at pH 10. In an earlier report by Wang et al. (2012), demonstrated that mutants of *Bacillus pumilus* C1 cyanide dihydratase (CynD<sub>pum</sub>) showed improved activity at higher pH too (at pH 10).

The structural information of CDH enzyme was derived from *P. stutzeri* AK 61 homologue and it was reported that the enzyme was a homo-oligomeric spiral structured and comprising of 14 subunits with two fold symmetry (Sewell et al. 2003). In the case of *Bacillus pumilus* C1, it was found that cyanide dihydratase was consisting of 18 subunits and the fundamental structure was a dimer of molecular weight 37 kDa. It exhibited reversible, pH-dependent structural switching; at pH 8.0 the enzyme has a spiral structure and at pH 5.4 the enzyme subunit was reorganised and formed left handed helix. The putative amino acid sequence deduced from ORFs revealed that they shared high similarity with the cyanide dihydratase of Gram negative bacterium *P. stutzeri* AK61 (Jandhyala et al. 2003). The enzyme also exhibited similarity with nitrilases of fungal and bacterial origin (Thuku et al. 2007). It was also observed that C-terminal domain of cyanide dihydratase of *Bacillus pumilus*



significantly influenced the thermostability and pH tolerance of the CDH enzyme because of its involvement in oligomerization at the interface of C surface (Crum et al. 2015).

An earlier report by Watanabe et al. (1998) described the cloning and expression of cyanidase gene from *P. stutzeri* AK61 to *E. Coli*. From the sequence data, the ORFs were searched and a protein consisting of 334 amino acids having a molecular weight of 37.5 kDa was predicted. Translated protein was shown to share 35.1% homology with nitrilase of *Rhodococcus rhodochrous* K22. Cyanide hydratase of *Fusarium lateritium* also exhibited homology up to 26.4%. The proposed active site had conserved cysteine residue like other nitrilase which played a pivotal role in catalysis. However the mechanism of action of cyanidase was not elucidated in detail; few other studies suggested that cysteine present in the active site might act as a nucleophile and could attack HCN, forming thioimidate as intermediate. Thioimidate was further metabolized to ammonia and an acyl product.

### Cyanide hydratase (EC 4.2.1.66)

Cyanide hydratases belong to lyase superfamily and participated in cyanoamino acid metabolism. This enzyme was first reported in *Stemphylium loti*, a fungus which infected the common cyanogenic plant bird's-foot trefoil (which is a low growing perennial legume) (Fry and Millar 1972). The cyanide hydratase mediated cyanide metabolism was restricted to fungi only and was not reported much in bacteria/plant/animals (Rinágelová et al. 2014). Most of the reported cyanide hydratases showed substrate specificity only for HCN, whereas nitrilases exhibited wide substrate specificity. Few fungi were also reported which could degrade metalocyanide; *Fusarium solani* and *Fusarium oxysporum* N-10 have been reported to degrade tetracyanonickelate (II) (Barclay et al. 1998; Yanase et al. 2000). Similarly, *Fusarium lateritium* also exhibited low cyanide hydratase activity with small aliphatic nitriles (acetonitrile and propionitrile) as well as with aromatic ones (benzonitrile) (Nolan et al. 2003). Other cyanide hydratase harboring microbes are *Micromonospora braunnam* (actinomycetes), *Gloeocercospora sorghi*, *Leptosphaeria maculans*, *Aspergillus niger* K10 (Wang and Van-Etten 1992; Cluness et al. 1993). These cyanide hydratases have shown significant homology within these fungi at the amino acid level (Bhalla et al. 2012).

Recently we have proposed parts of the mechanism of cyanide hydratase enzyme from *Serratia marcerans* based on computational modeling and structural analysis (Kushwaha et al. 2018). The modeled structure exhibited a characteristic nitrilase superfamily catalytic triad consisting of E (at positions 45), K (at position 120) and C (at position 151). This modeled structure could reveal an active site within the range of 45 to 176 amino acid residues containing

catalytic triad cys-lys-glu (Fig. 1a). In the catalytic mechanism of cyanide degradation, cysteine was involved in the nucleophilic reaction occurred on the nitrile, resulting in the formation of a thioimidate. Lysine acted as a proton donor and caused hydrolysis; leading to the formation of tetrahedral intermediate. It results in C–S bond breakage and formamide was released. Glutamate acted as a proton acceptor which further caused secondary hydrolysis; leading to the formation of a second tetrahedral intermediate, which decomposed to form formic acid (Fig. 1b).

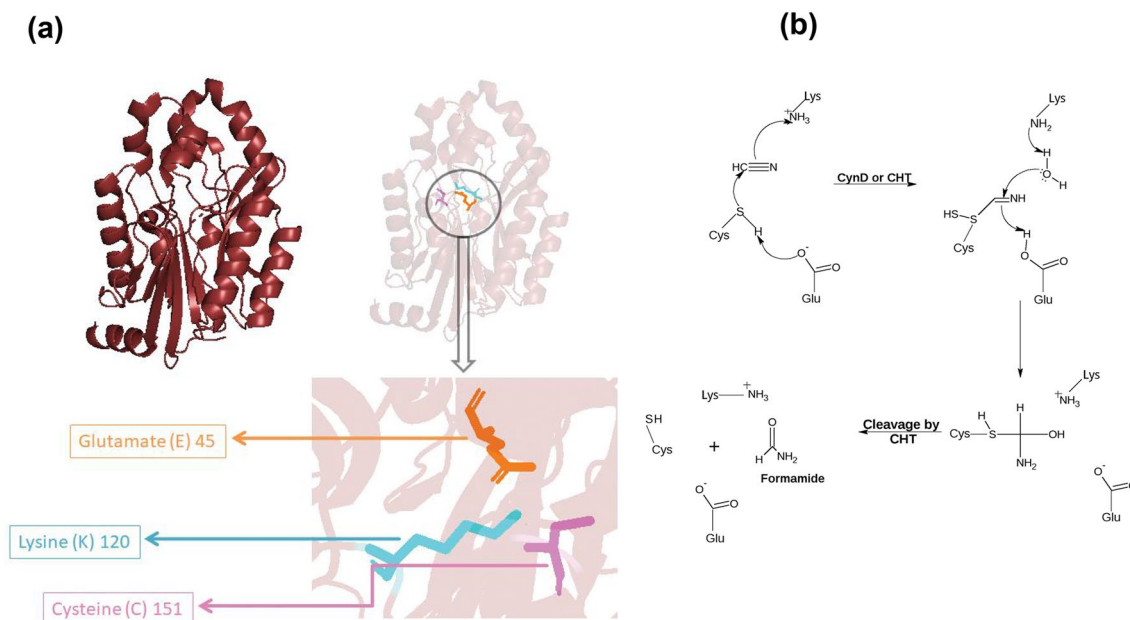
### Substitution pathway

This pathway involves the transfer of a sulfur group from thiosulfate to cyanide and forming thiocyanate which is further assimilated subsequently as an alternate nitrogen source during microbial growth. The enzyme involved in such reactions belongs to sulfurtransferase family. The two important members of this family which take part in cyanide metabolism are rhodanese and mercaptopyruvate sulfurtransferase (Dash et al. 2009; Gupta et al. 2010; Bhalla et al. 2012).

### Rhodanese (EC 2.8.1.1)

The rhodanese enzymes are ubiquitous enzymes and play a key role in sulfur metabolism. Rhodanese is also termed as thiosulfate sulfurtransferase. The enzyme transfers sulfur atom from donor to acceptor molecule. In cyanide metabolism, rhodanese enzymes help to transfer sulfur from thiosulfate (donor) to cyanide and less toxic thiocyanate molecule is formed (called ping pong mechanism) which is then assimilated in the cytoplasm of the organism. This enzyme has been reported in many eucaryotes and prokaryotes. *Escherichia coli*, *Azotobacter vinelandii*, *Bacillus brevis*, *Fusarium* sp., and many *Thiobacillus* sp. were reported to have rhodanese enzyme. *Pseudomonas aeruginosa* was reported to have constitutive rhodanese activity (Alexander and Volini 1987; Bordo and Bork 2002; Cipollone et al. 2004, 2006, 2008; Ezzi et al. 2003; Oyedeji et al. 2013; Wang and Volini 1968). Rhodanese enzymes are also used by many animals for cyanide detoxification in their metabolic pathway; e.g. Giant pandas absorbed more than 65% of cyanide by eating bamboo shoots (1 kg bamboo shoots contain 3.2 mg cyanide) and approximately 80% cyanide was converted to thiocyanate by rhodanese enzyme, which ultimately discharged through their urine (Huang et al. 2016).

Rhodanese activity and expression levels are dependent on the cyanide exposure to the tissue. Chaudhary and Gupta (2012) elucidated the catalytic activity of mitochondrial rhodanese of bovine. It was found that the enzyme consisted of 293 amino acids and possessed two equal sized domains, viz. N-terminal domain (inactive) and C-terminal domain (catalytic active). In the active site of rhodanese the



**Fig. 1** Mechanism of the cyanide hydratase activity. **a** Cyanide hydratase modelled structure of *S. marcescens* strain WW4 (Kushwaha et al. 2018). The catalytic site within the modelled structure consisted of a triad containing amino acid residues namely, a cysteine at position 151, lysine at position 120 and a glutamate at position 45. **b** Catalytic mechanism of cyanide hydratase: cysteine is involved in the nucleophilic attack on the nitrile which results in the

formation of a thioimidate. Lysine acts as a proton donor. It causes breaking of the C–S bond leading to the release of formamide. Glutamate acts as a proton acceptor which then activates second hydrolysis leading to second tetrahedral intermediate, which decomposes to release formic acid (*CHT* cyanide hydratase, *CynD* cyanidedihydratase)

sulfhydryl groups and aromatic groups were present in close proximity to each other. These aromatic groups have protected the catalytic sulfhydryl groups. The enzyme showed competitive inhibition in the presence of aromatic ions (aromatic sulfonates) but no inhibition was found in the presence of aliphatic ions which concluded that tryptophan was present in the active site. Gliubich et al. (1996) reported that the sulfhydryl group present in active site got oxidized and formed a sulfenyl group (–S–OH) when the rhodanase enzyme treated with hydrogen peroxide. This process has led to the inactivation of the enzyme. Other sulfhydryl group modifying agents like alkylating agents, aliphatic mercaptans, aromatic nitro compounds are also could eliminate the rhodanase activity. The alkylating agents are responsible for the substitution reaction with cysteinyl sulfur, while aromatic nitro compounds are responsible for intermolecular oxidation between two enzyme monomers. The aromatic mercaptans formed mixed disulfides with enzyme monomer (Wang and Volini 1968).

### Mercaptopyruvate sulfurtransferase

Mercaptopyruvate sulfurtransferase enzymes belong to transferase enzyme family and are involved in many processes like iron sulfur clusters maintenance in protein (Alphey et al. 2003), molybdopterin cofactor biosynthesis for xanthine

oxidase (Sörbo 1957), metabolism of selenium (Hannestad et al. 1981), thiamine and 4-thiouridine biosynthesis and cyanide detoxification (Ogata and Volini 1990). These enzymes are up-regulated when the organisms undergo stress condition, e.g. exposure to peroxide, hyposulphur conditions and infection by bacteriophage (Pagani et al. 1984).

These enzymes are widely distributed in a living system like rhodanase enzyme. They are also known as 3-mercaptopyruvate cyanide sulfurtransferase/ $\beta$ -mercaptopyruvate sulfurtransferase. This enzyme was first reported in trypanosomatid parasite *Leishmania major* (Williams et al. 2003) and found to be comprised of three domains; N-terminal domain, central domains and C-terminal domain. The active site of the enzyme contained conserved three residues Asp-61, His-75, and Ser-255 just like a serine protease triad and a cys residue in proximity at the Cys-253 position. The C terminal domain of enzyme was unique; consisting of 80-amino acids and involved in protein folding as well as in the interaction of sulfurtransferase with proteins (Nandi et al. 2000).

### Conclusions

Cyanide containing toxins are usually generated from industrial plants. Many environmental and human health problems are caused by cyanide and its derivatives. Due to their high

affinity towards metals, different metalloproteins get dysfunctional. Different strategies based on enzymatic cyanide degradation have been adopted and many useful enzymes (originated from different microbes and plants) such as cyanidase, cyanide hydratase, formamidase, nitrilase, nitrile hydratase, cyanide dioxygenase, cyanide monooxygenase, cyanase and nitrogenase and their mechanism of action have been studied extensively in this context. In order to understand the molecular mechanism of cyanide toxicity and to achieve efficient methods of their biodegradation, the next generation 'omics' technologies have been applied successfully during the last couple of decades. In the future, more advancement of omics tools and protein engineering based methods may help us to further strategize more efficient technologies to depollute the environment from the cyanide containing waste.

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