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Phenol Toxicity and Phytoremediation

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Abstract – Widespread applications of phenol in manufacturing industries and oil refineries had resulted in unprecedented leakage of phenol into the environment, which can cause serious health effects such as tissue necrosis and cardiac arrhythmia upon contact or ingestion. Plants exposed to phenol had reduced seed germination index, inhibited growth or even fatality. There are many technologies currently practised to remediate phenol pollution such as physiochemical methods (adsorption to activated carbon and chemical oxidation), biological methods (biodegradation by bacteria or fungus, and soil bioaugmentation), and phytoremediation method (using hairy roots of plants). As physiochemical and microbial phenol degradation are destructive and costly, phytoremediation is widely studied as an alternative phenol remediator which is environmental friendly and cost effective. Microorganisms can detoxify the aromatic xenobiotic through the aerobic or anaerobic pathway. Aerobic degradation of phenol is through either the *meta*- or *ortho*-pathway of catechol cleavage while anaerobic degradation occurs through the benzoate pathway. In plants, degradation of phenol is also through catechol cleavage as in microorganisms. However, different enzyme systems were utilised in the different pathways involved.

Keywords: Biodegradation, Pathways, Phenol, Physiochemical remediation and Phytoremediation.

Introduction

Phenol, also referred as benzenol or hydroxybenzene, is a weak acidic mono-substituted aromatic alcohol with empirical formula C_6H_6O. At room temperature, it is a hygroscopic crystalline solid which is clear or light pink in colour (Balachandran, Murugan, Karpagam, Karnan & Ilango, 2014). In the past century, phenol was applied to create aseptic condition for surgery as it has alcoholic antiseptic properties (Ibanez, 2012). At present, the principal use of phenol is as a precursor to produce phenolic resins (El-Naas, 2010a; Golbaz, 2014). Phenol is broadly applied in coal or oil refineries, and to manufacture pharmaceuticals, plastics, pulps, papers and dyes (Abdelkreem, 2013; El-Naas, 2010b; Luo, 2015; Paisio, 2013; Sihem, 2012). After these industrial processes, phenolic compounds were discharged and accumulated in the environment as pollutant of high priority (Abdelkreem, 2013; Ibanez, 2012; Liu, 2010). Society has a raising concern on phenol contamination as it causes water to be non-potable and is one of the most hazardous organic pollutants even in low concentrations (Abdelkreem, 2013; El-Naas, 2010a; Ibanez, 2012; Liu, 2010; Vasudevan, 2014). In industrial effluents, content of phenolic compounds (around 50 - 2000 mg/L) is usually over the established limits (below 0.5 mg/L) and too dangerous to be exposed to living organisms (Paisio, 2009; Sihem et al., 2012).

Health effects

Due to the poor biodegradability and high water affinity of phenol, this brings greater risk when contaminating water (Liu, 2010). Phenol can cause health effects both directly and indirectly (Sihem et al., 2012). Although not strongly acidic, direct contact with high phenol concentration will cause skin irritation, chemical burns and tissue necrosis (Basha et al., 2010; Nair et al., 2008; Wang, 2011). Acute ingestion of phenol will damage mucosal membranes in mouth, oesophagus and stomach,

leading to symptoms such as gastrointestinal discomfort, vomiting, diarrhoea and dark urine (Baker et al., 1978; Gosselin et al., 1984; McCall et al., 2009; Wang, 2011). This would occur as phenol impairs the tight junction proteins that regulate the epithelial barrier as observed in colonic epithelial cells (Pedersen, Brynskov and Saermark, 2002; McCall, 2009). Similar damages were reported by Luo (2015) and Vasudevan (2014) in which phenol altered the permeability of cellular membranes and caused cytoplasmic coagulation.

Other than the above mentioned effects, phenol exposure also caused many other sicknesses such as profused sweating, haemolytic anaemia, hypotension and cardiac arrhythmia or irregular heartbeat (Basha et al., 2010). Furthermore, phenol could also bioaccumulate and biomagnify in the food chain causing carcinogenicity, mutagenicity, neurotoxicity, reproductive and developmental damages (Huang et al., 2014; Paisio, 2013). Many other researchers also reported the damages of phenol on human kidney and liver (Shi, 2013; Tootian, 2012; Wang, 2011). Shi (2013) found that phenol damaged the kidney more severely than the liver while Tootian (2012) described nephrotoxicity in mice. Deichmann and Keplinger (1981) also reported degenerative impairment in epithelium of the renal tubules), mitochondrial distortion resulting in inefficiency of renal function and dilation of urinary space of renal corpuscles.

Phytotoxicity

According to Ibanez (2012), phenol contamination resulted in build-up of reactive oxygen species (ROS) in plants leading to oxidative stress, which inhibited growth. These ROS could also react with DNA, proteins, lipids and other cellular molecules causing deleterious effects (Ashraf, 2009). Hence, plants prevent accumulation of ROS by scavenging and detoxifying it using antioxidants such as peroxidase, catalase and carotenoids (Seckin, 2010). Ibanez et al. (2012) reported that peroxidase and ascorbate peroxidase activities in *Vicia sativa* were significantly elevated upon exposure to 100 mg/L phenol as a xenobiotic respond against ROS. Jha et al. (2013) demonstrated phenol phytotoxicity on germination index with seeds of *Triticum aestivum* and *Phaseolus mungo*. Their results showed that phenol at 100 mg/L reduced seed germinations and growth. Ibanez et al. (2012) also reported similar results where germination index of *V. sativa* decreased significantly upon exposure to over 250 mg/L phenol.

Phenol was reported to affect the sensitive photosynthetic apparatus first, and then followed with other enzymatic systems (Flocco et al., 2002; Ibanez, 2012). Reduction of chlorophyll content in alfalfa plants was demonstrated by Flocco et al. (2002) upon acute treatment of 500 mg/L phenol besides causing severe inhibition of growth, yellowing of leaves and loss of roots turgidity. Coniglio (2008) reported that phenol concentration of 100 mg/L had induced growth inhibition and root browning in *Brassica napus* by causing cell deaths and membrane disruptions. Brown colouration of roots was also observed in *Helianthus annuus* L. (Jha et al., 2013). Ucisik et al. (2006) demonstrated that transpiration in willow tree was inhibited by 500 mg/L phenol, with eventual tree death.

Physiochemical remediation

There are many phenol removing technologies being utilised and many on-going researches were done. For examples, physical treatments such as ion exchange, ultrafiltration and reverse osmosis (Jha et al., 2013; Vasudevan, 2014) or chemical treatments such as oxidation using ozone, manganese oxides and hydrogen peroxide (Sihem et al., 2012). However, these physiochemical treatments are destructive, expensive, require high energy and chemical input, and will generate toxic effluents (Jha, 2013; Li, 2010; Mrozik, 2010; Vasudevan, 2014). Wetlands had been engineered to remediate wastewater (Gottschall et al., 2007). Wetland plants function to prevent erosion and create microbial habitats for pollutants removal (Ho et al., 2012). Although wetlands can remediate wide range of pollutants including phenol, this method requires wide area and takes a long time to establish a stable and balanced microbial environment.

Another common wastewater treatment method is the adsorption method that uses solid phases made of activated carbon (Abdelkreem, 2013). This adsorption treatment is simple and able to remove variant contaminants from the sites (Bhatnagar, 2010; Liu, 2010). However, this method is expensive

because activated carbons require a complex activation process, leading to cost problem in regenerating absorbents (Caetano, 2009; El-Naas, 2010a; Sihem, 2013). Hence, there are growing interests in the production of low cost polymeric adsorbents that have larger surface area, mechanically more rigid, and having adjustable pores size. Examples of such polymeric adsorbents are polymethyl methacrylate (Al-Muhtaseb et al., 2011), anionic resins (Caetano et al., 2009) and magnetic polymer beads (Ho et al., 2010). Several researches had focused on converting agricultural residues into alternative solid adsorbents such as sawdust (Larous and Meniai, 2012) and cereal by-products (Sihem et al., 2012), which were said to be more natural, vegetal and low cost. Besides reducing cost, these alternative adsorbents can add value to agricultural wastes and help solve biomass disposal problem (Rodrigues, 2011). Unfortunately, this adsorption treatment of wastewater still resulted in secondary pollution because the phenol was not degraded but rather transferred to another phase (El-Naas, 2010a).

Microbial remediation

A huge interest had been gearing towards biological remediation of pollution, which was said to be more environmental-friendly (Basha et al., 2010; Wang, 2011). For example, Chatterjee et al., (2010) reported the application of fungus, *Rhizopus oryzae*, to remediate organophosphorus pesticide while Lika and Papadakis (2009) demonstrated the ability of algae to biotransform phenol.

Among microorganisms, biodegradation of phenol using bacteria and fungus are most extensively researched both theoretically and experimentally. Basha et al. (2010) mentioned that microbial degradation of phenol begun long time ago using fungi (*Fusarium flocciferum*), yeast (*Trichosporon cutaneum*) and bacteria (*Pseudomonas putida*). According to El-Naas (2010b), bacterial remediation of phenol is mainly studied using *Pseudomonas* spp. For instances, *Pseudomonas aeruginosa* (Song et al., 2009), *Pseudomonas pseudoalcaligenes* (Kurzbaum et al., 2010) and *P. putida* (Muftah et al., 2009). On the other hand, fungus such as *Phanerochaete chrysosporium* was also known for its remarkable ability to degrade phenol (Lu et al., 2009). As compared to bacteria, fungi can extend their growth areas through hyphal formation (Basha et al., 2010). Moreover, they can grow under stressed conditions such as low pH, low water activity or nutrients deficiency where bacteria would be killed or inactivated (Davis and Westlake, 1978). Overall, these methods have disadvantages such as high production cost, restricted mobility and metabolic inhibition (Husain, 2010). Also, acclimatisation of the microorganisms prior to the remediation is essential to achieve efficient microbial degradation (El-Naas, 2010b)

Bioaugmentation of contaminated sites was well discussed by Mrozik and Piotrowska-Seget (2010). This method involved introducing single strain or microorganism consortia, which are often genetically engineered, into polluted soil. One example is bioaugmentation of 4-chloronitrobenzene polluted soil with *P. putida* (Niu et al., 2009). However, there were some issues regarding this method, which were poor dispersion of inoculants into the subsurface environment and low survival of microorganisms in the soil (Mrozik and Piotrowska-Seget, 2010). To overcome these constraints, researchers suggested application of surfactants (Franzetti et al., 2009) or transfer packaged catabolic genes to indigenous microflora (Massa et al., 2009).

Other than these direct applications of microorganisms, extracted enzymes such as peroxidases were also commonly used as phenol biocatalysts (Deva et al., 2014). However, they were expensive to extract, were easily inactivated and had narrow scope of remediation as enzymes work specifically based on type of substrate. Besides remediating phenol, peroxidase has the ability to perform oxidoreduction and free radical polymerisation (Hamid and Khalil-ur-Rehman, 2009). Therefore, they were often extracted, immobilised and utilised in industries of dyes decolourisation, pharmaceuticals and compounds biotransformation diagnostics (Rusdi et al., 2014).

Phytoremediation

Considering the high cost, environmentally destructiveness and formation of toxic by-products by current remediation technologies, an emergent technology that uses vegetation abilities to remove contaminants or pollutants from the soil, water and sediments arises (Afzal et al., 2014). This

technology is known as phytoremediation, which can act solely by plants or cooperatively with a microbial ecosystem formed at the rhizosphere with the microorganisms such as fungi, bacteria and actinomycetes (Gonzalez et al., 2013). Phytoremediation is comparably lower cost and environmental friendlier (Eapen & D'Souza, 2005). To achieve successful phytoremediation, one of the most important steps is selecting the right plant that can survive the contaminated environment and has a contaminant-removing ability (Suchkova et al., 2014). Hence, researchers are often on the lookout for native plants as potential phytoremediator candidates. For example, Alifragkis et al. (2013) isolated *Verbascum undulatum* Lam. from mining areas as heavy metals hyperaccumulator while Testiati et al. (2013) isolated *Globularia alypum* L. and *Rosmarinus officinalis* L. from former industrial site as metals stabiliser.

Instead of growing plants in soil or hydroponically (Flocco et al., 2002), most studies of phytoremediation were conducted with *in vitro* plant cells or tissue cultures (Jha et al., 2013; Singh et al., 2006). For the study of xenobiotic detoxification, hairy roots cultures are commonly used (Nepovim et al., 2004) as its organised nature allows large scale cultivation in bioreactors (Suresh et al., 2005) and capable of unlimited propagate in culture media (Pletsch et al., 1999). Previous researches using hairy roots cultures were done on the phytoremediation of polychlorinated biphenyls (Mackova et al., 2001), aminotoluenes (Nepovim et al., 2004) and pesticide (Suresh et al., 2005). The successful applications of hairy roots to phytoremediate phenol had been implemented on *H. annuus* L. (Jha et al., 2013), *B. napus* (Gonzales, 2013) and tobacco plant (Talano, 2010).

Generally, phytoremediation can be divided into several methods, such as phytodegradation, phytotransformation, phytoextraction, phytostabilisation, rhizofiltration and phytovolatilisation (Ibanez et al., 2016). Phytodegradation is a process whereby the plants degrade or break down contaminants into harmless substances (Park et al., 2011). Phytotransformation occurs when plant modifies, degrades, immobilizes or inactivates contaminants through some metabolism pathways (Vamerali et al., 2010). Phytoextraction only aims to remove contaminants from contaminated sites and might store the contaminants in harvestable biomass (Bhargava et al., 2012). Plants possessing the ability of phytostabilisation do not uptake pollutants but only reduce the mobility and phytoavailability of the pollutants (Bolan et al., 2011). Aquatic plants rhizofiltrate pollutants by filtering water and absorbing pollutants from the aquatic systems (Zhang et al., 2009). Through phytovolatilisation, some plants removed contaminants from the soil or water, translocate them to the aerial parts and finally volatilise or release the pollutants into the air (Vamerali et al., 2010).

Rhizoremediation is a type of phytoremediation where the symbiosis relationship between plants and rhizospheric microorganisms promotes phenol degradation, providing an efficient and sustainable remediation technology (Ontanon et al., 2014; Yamaga, Washio & Morikawa, 2010). An example of rhizoremediation was demonstrated by Gonzalez et al. (2013) using hairy root culture of *B. napus* inoculated with *Burkholderia kururiensis* KP 23 and *Agrobacterium rhizogenes* LBA 9402. In another study, *P. aeruginosa* strain SZH16 was observed to have the ability of reducing phenol phytotoxicity through *in situ* phenol biodegradation, and hence, facilitated *Zea mays* growth (Wang et al., 2011). Besides detoxifying contaminants, rhizospheric microorganisms can protect plants against plant pathogens, reduce plant stress hormone level and provide essential nutrients to the plants (Gerhardt et al., 2009). For instances, *Pseudomonas fluorescens* had been considered an biocontrol candidate against phytopathogens and insect pests as it produced antibiotics such as pyrrolnitrin and 2,4-diacetlphloroglucinol (Fenton et al., 1992; Kamilova, Lamers & Lugtenberg, 2008). In a project conducted by Lu et al. (2010), *P. fluorescens* was genetically engineered to express an active scorpion neurotoxin which enabled dual protection of crops against both pathogenic fungi and insects.

Enzymatic degradation pathways

Biodegradation pathways of phenol are very complicated involving different enzymes and substrates. Hence, they are easily inhibited by many factors such as alternative carbon sources available, initial phenol concentration, pH and temperature (Shourian et al., 2009). Researches on optimisation of phenol degradation conditions were done on microorganism such as *Ochrobactrum* sp. (Kilic, 2009) and *Corynebacterium glutamicum* (Lee et al., 2010). These researches are very important in

contributing useful information for optimal design and operation of bioremediation or treatment of wastewater containing phenol.

Degradation pathways in bacteria

Bacterial degradation of phenol can occur aerobically and anaerobically (Wang et al., 2011). Compared to anaerobic pathway, aerobic pathway is faster and more productive as it involves less energy consumption (Nair et al., 2008). This was practised by *Acientobacter calcoceticus, Candida tropicalis* and *Pseudomonas* spp (Basha et al., 2010).

According to Kwon and Yeom (2009), the aerobic phenol biodegradation pathway starts with the incorporation of a molecular oxygen into phenol by means of adding a second hydroxyl group at the *ortho*-position of the existing hydroxyl group by phenol hydroxylase to form a dihydroxybenzene (catechol) as shown in Figure 1. According to Basha et al. (2010), this oxygen incorporation consumes a reduced pyridine nucleotide ($NADH_2$). The resulting catechol will then undergo ring fission or cleavage pathway, which could be either the *meta*- or *ortho*-pathway. In the *meta*-pathway, the enzyme catechol-2,3-dioxygenase cleaves the aromatic ring adjacent to the two hydroxyl groups (extradiol fission) producing 2-hydroxymuconic semialdehyde.

In the *ortho*- or β-ketoadipate pathway, the enzyme catechol-1,2-dioxygenase cleaves the aromatic ring between the two hydroxyl groups (intradiol fission) producing a cis, cis-muconate (Banerjee and Ghoshal, 2010). The products from both the *meta*- and *ortho*-pathways are intermediates which will be channelled to the Krebs cycle to be further metabolised to produce useful metabolites such as pyruvic acid, acetic acid and acetyl-CoA (Basha et al., 2010; Nair et al., 2008). From previous researches, *Pseudomonas* sp. SA01 (Shourian et al., 2009) and *Bacillus cereus* (Banerjee and Ghoshal, 2010) metabolised phenol via the *meta*-pathway while *Aureobasidium pullulans* (Suenaga et al., 2009) and *Streptomyces setonii* (An et al., 2001) cleaved the phenol via the *ortho*-pathway.

Anaerobic degradation of phenol is less advanced (Basha et al., 2010). Williams and Evans (1975) proposed that the anaerobic phenol degradation in *Paracoccus denitrificans* occurs through the benzoate pathway. In this pathway as shown in Figure 2, phenol is first carboxylated at the *para* position by 4-hydroxybenezoate carboxylase, followed by dehydroxylation as observed in *Thauera aromatica* and *Desulphobacterium phenolicum* (Basha et al., 2010).

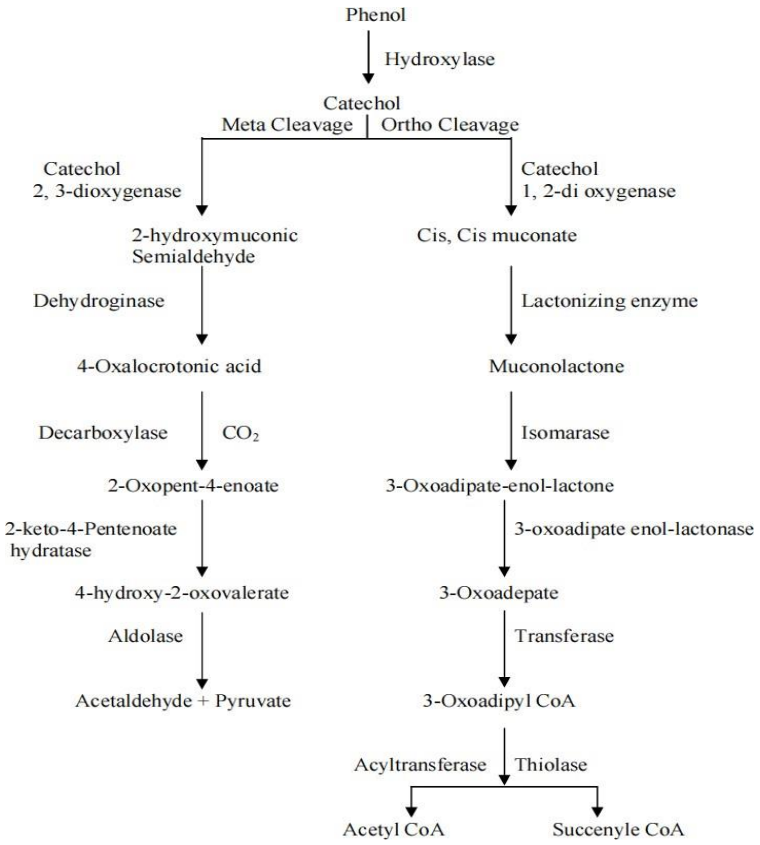

Figure 1: Flow chart of aerobic phenol degradation pathway (Adapted from Basha et al., 2010)

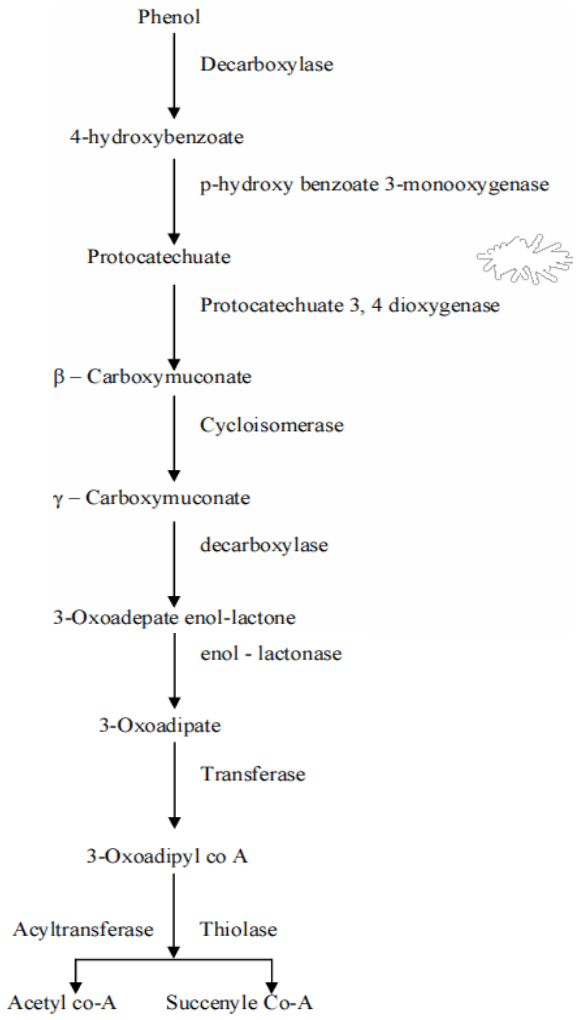

Figure 2: Flow chart of anaerobic phenol degradation pathway (Adapted from Basha et al., 2010)

Degradation pathways in plants

Similarly to microbial phenol degradation, phytodegradation of phenol occurs through the catechol-cleavage pathways as shown in Figure 3. According to Jha et al. (2013), phenol was initially hydroxylated into catechol and then oxidised into quinone. This aromatic intermediate is then cleaved into muconic acid, which then undergoes β-oxidation to generate fumaric acid and enters the tricarboxylic acid cycle (Durmishidze et al., 1979). The carbon skeleton of xenobiotic phenol can be degraded completely as phenol cleavage leads to formation of fumaric acid or other intermediates, which enter the Krebs cycle for energy production (Mithaishvilia et al., 2005). Besides the Krebs cycle, fumaric acid can also be used in other general metabolisms. The enzymatic system used in plants is different from the microorganisms' in a way that peroxidase is utilised to generate catechol instead of phenol hydroxylase (Jha et al., 2013).

Peroxidases are haem proteins containing ferriprotoporphyrin IX as prosthetic group (Hamid and Khalill-ur-Rehman, 2009). These peroxidases are oxidoreductases, which catalyse the reduction of peroxides. Therefore, most of the earlier researches regarding phenol degradation by peroxidase added hydrogen peroxide into the medium to react stoichiometrically with phenol (Jha et al., 2013). This is because phenol and hydrogen peroxide are co-substrates in the peroxidase catalysed reactions. Although increase in H_2O_2 concentration will increase efficiency of peroxidases, further increase in its concentration above optimum level will irreversibly deactivate the enzymes (Arnao et al., 1990).

Hence, polyethylene glycol (PEG) was often added to act as a protective layer (Alemzadeh and Nejati, 2009). Application of PEG increased phenol removal efficiency of rapeseed hairy root (Paisio et al., 2010) but had no effects on *H. annuus* L. (Jha et al., 2013) and soybean peroxidase (Caza et al., 1999).

Due to increasing interests on peroxidases' activity of oxidoreduction and free radicals polymerisation (Hamid and Khalil-ur-Rehman, 2009), peroxidases were often extracted, immobilised and utilised in industries for dyes decolourisations, pharmaceuticals and compounds biotransformation diagnostics (Rusdi et al., 2014). Researches of peroxidase from plants includes kiwi (Rusdi et al., 2014), tomatoes (Paisio et al., 2010), soy bean (Saboora and Hejri, 2009), horseradish (Iran and Siamak, 2009) and tobacco (Alderete et al., 2009). Although this enzyme can be found in several parts of plants such as leaf or stem, concerns are mainly on plants' roots (Jha et al., 2013; Saboora and Hejri, 2009). This is because roots have large surface area enhancing phenol removal and were in contact with phenol-contaminated soil or water.

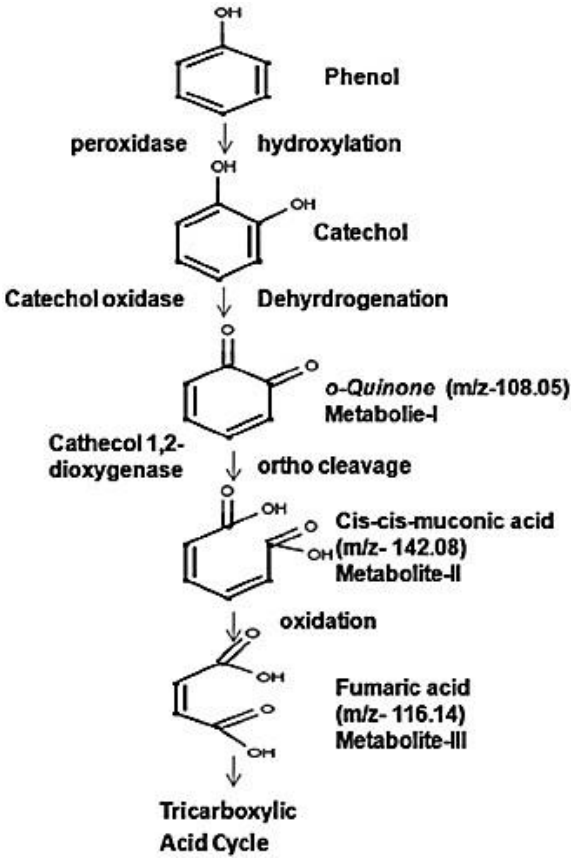

Figure 3: Flow chart of phenol phytodegradation pathway
(Adapted from Jha et al., 2013)

Conclusion

Phenol pollution is a threat to environment and living organisms as it is corrosive and toxic even in low concentration. Even though phenol toxicology is well studied since last century, industrial and domestic discharges of phenol into the environment causing soil and water pollution is still very common today. To overcome this problem, researchers are looking at all possible technologies and methods to remove and degrade phenol. Among all the technologies available, common treatments applied including adsorption, rhizoremediation and bioaugmentation. However, all these methods have their own drawbacks. Hence, continuing researches on modifying and improvising phenol treatments are crucial. Phytoremediation has a bright future considering its better advantages than other existing methods in phenol removal.

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