RECENT ADVANCEMENTS IN THETOXICOLOGICAL ASSESSMENT ANDBACTERIAL - MEDIATED BIODEGRADATION PATHWAYS OF PARACETAMOL

Abstract

Paracetamol or acetaminophen (N-acetyl-para-aminophenol) (APAP) ($C_8H_9NO_2$) is the most widely used over the counter (OTC) non-opioid analgesic and antipyretic agent used to treat mild to moderate pain and fever. Being an OTC drug, it is freely available, resulting in its rampant overuse, especially during Covid and in the post Covid period.Ingestion of Paracetamol at doses above the recommended levels has been found to causehistopathological and neurotoxic effects. Thus, this global issue of improper use and environmental discharge of paracetamol and the associated risks raise concerns and demand for a systematic approach towards its monitoring and remediation. Bioremediation of paracetamol from environmental samples using bacteria such as *Pseudomonas* sp., *Bacillus* sp., *Enterococcus* sp. has been shown to be aneffective, environment-friendly and low-cost method. The present chapter highlights the recent advancements in the toxicity studies on unnatural exposure to paracetamol and its bacterial biodegradation pathways.

Keywords: Acetaminophen, Wastewater, Biodegradation, Bacteria, Toxicity.

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I. INTRODUCTION

Paracetamol or Acetaminophen is an antipyretic, non-opioid analgesic drug that is used to treat fever and moderate pain. It is the most commonly used OTC analgesic drug worldwide and is also recommended by the World Health Organization (WHO) as a first-line treatment drug for pain conditions. Initially approved by the U.S. FDA, 1951 (Swann 1994), paracetamol is currently available in various forms such as regular and effervescent tablets, syrup, and injection, and is also used in combination with other drugs in many other medications for allergy, cold, pain, sedative, among others (Jóźwiak-Bebenista and Nowak 2014).

Among all the pharmaceutical compounds contaminating the surface and wastewaters worldwide, acetaminophen prevalence and concentrations have been found to be among the highest (more than 100 ng/L in rivers and 100 μg/L in sewage treatment plant influents) Rios-Miguel et al. (2022). The exposure with paracetamol at undesirable concentration shows various pathological conditions. Mossa et al. (2012) showed that liver of rats has various biochemical and histopathological changes when exposed to the paracetamol at concentration 66mg/kg. Blecharz Klin et al. (2015a) has studied the neurotoxicity associated with the exposure of acetaminophen at 5 and 15mg/kg concentration and found that it causes defect in the neurotransmission process associated with medulla oblongata in early developmental stages.

Direct effects on spinal cord were also observed by Blecharz Klin et al. (2015b). Hepatotoxicity has also been observed in alcoholic people and it can be fatal at over dosage (Artnak and Wilkinson 1998; Bridger et al. 1998; Denison et al. 1987; Himmelstein et al. 1984; Johnson et al. 1981; Yasunaga et al. 1985). Although the effect of paracetamol on pregnant ladies and there unborns is still unclear but few studies interlinked the paracetamol exposure in offsprings with ADHD (attention deficit hyperactivity disorder) and ASD (autism spectrum disorder) later in life (Endesfelder et al. 2021).The exposure with paracetamol whether therapeutic or environmental, causes ill effects in the human beings and other organisms.

Recent knowledge about the accumulation of medicinal products in the environment matrices and its possible effect on aquatic speciesshows the necessity of conducting environmental risk assessment (ERA)studyof medicinal product before marketing it for human use as per the European Council Directive 2001/83/EC, 2001 (Valverde 2006).The environmental exposure with any pharmaceutical compound can be calculated as the ratio of the predicted environmental concentration (PEC) and measured environmental concentration (MEC) to the predicted no-effect concentrations (PNEC) of the pharmaceuticals (Ashfaq et al.2017a).

Thus, the easy access and widespread use of paracetamol and the increased use of its combination medications have become one of the main causes of the intentional or non-intentional overdose, resulting in the commonly observed drug induced liver injury (DILI) (Rotundo and Pyrsopoulos 2020). Also, the discharge of excess drug into the environment through patients' urine and hospital wastewater has caused environmental pollution and toxicity. Several physical and chemical processes have been used for removal of Paracetamol from environmental samples. Nonetheless, the degradation of paracetamol by microbes is

considered as an environmentally friendly and cost-effective process,and results in the complete mineralization of the contaminant. The biodegradation of paracetamol in the environment is reported for bacteria, fungi, algae. Bacterial degradation of paracetamol has been shown to give promising results by several researchers. In the light of the above, the present chapter focuses on the recent advances in the toxicology of paracetamol and its bacterial mediated degradation pathway analysis.

II. CHARACTERISTICS OF PARACETAMOL

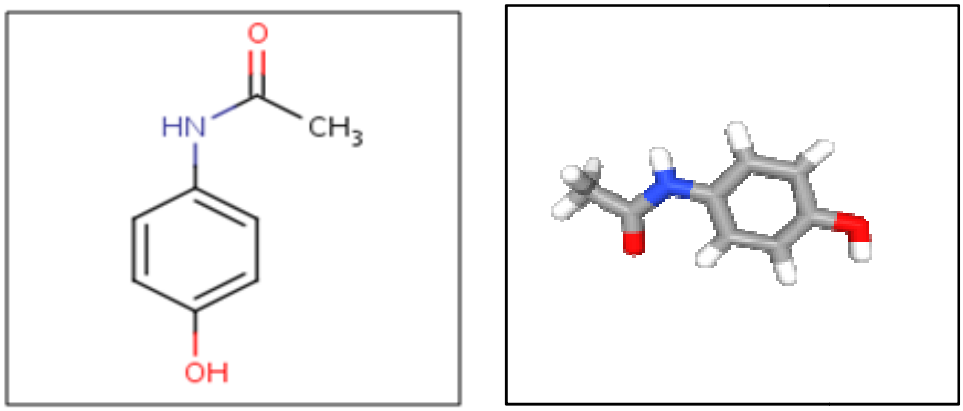
Paracetamol or para-hydroxyacetanilide is a derivative of phenol that is chemically named N-acetyl-para-aminophenol, in which one of the hydrogen atoms of amino group is replaced by an acetyl group. Known as Acetaminophen in the Americas and Paracetamol in the rest of the world, it is sold under different brands like Crocin, Tylenol, Excedrin, Calpol, Panadol, etc). Chemically, paracetamol or 4-hydroxyacetanilide is a white, odorless crystalline solid (Di Martino et al. (1997). Some of the important physical properties of paracetamol are described in Table 1. The 2D and 3D structures of paracetamol has been depicted in Figure 1.

Table 1: Physical Properties of Paracetamol

Parameter	Physical Property	Reference
Appearance	white crystalline powder	Di Martino et al., (1997)
Colour/Form	White colourless crystalline powder	IARC (1990)
Molecular Weight	151.16 g/mol	IARC (1990)
Molecular formula	$C_8H_9NO_2$ or $HOC_6H_4NHCOCH_3$	IARC (1990)
Taste	Slightly Bitter	Lewis (2007)
Odour	Odorless	Lewis (2007)
pH	about 6 (saturated aqueous solution)	NTP (1992)
Melting point	169-170.5 °C	Di Martino et al., (1997)
Boiling point	>500 °C	ILO (2008)
Solubility	Soluble in water (1:70, 1:20 at 100°C)	NTP (1992)
Density	1.293 g/cu cm at 21 °C	Haynes, (2014)
Environmental risk	Toxic to aquatic life.	ILO (2008)

In the 1980s, acetanilide was found to be effective in treating fever, but due to its high toxic effects, it was later replaced by one of its safer chemical derivatives, that is, *N*-acetyl-*para*-aminophenol, or paracetamol. The mechanism of action of paracetamol is complex and it has an effect on the central and peripheral nervous system. It is a selective cyclooxygenase (COX-1 and COX-2) inhibitor, suppresses prostaglandin production and is involved in the endocannabinoid system and serotonergic pathways, thus resulting in analgesic and antipyretic effects (Ayoub 2021; Przybyła et al. 2021). However, paracetamol is known for its hepatotoxic effects at doses above the recommended levels. A dose of 4000 mg within 24 h has been recommended by the US FDA, and for the European countries, a maximum dose of

a 3000 mg of paracetamol in older adults has been recommended (Rotundo and Pyrsopoulos 2020).



(a) (b)

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Figure 1: Structure of Paracetamol (a) 2D structure, (b) 3D structure

III. TOXICITY ASSESSMENT OF PARACETAMOL

During COVID -19 prevalence as a pandemic the usage of paracetamol for the treatment of fever and pain increased a lot. Due to its excessive usage, the degradation products of paracetamol alongwith the unused parent compound has accumulated in environmental matrices specially the water bodies. In the last decade, the increased concentration of pharmaceuticals in wastewater has been observed and paracetamol is one of them.Paracetamol has multidirectional mechanism of action (Figure 2) which is different from other drugs belonging to this group of non-steroidal anti-inflammatory drug (NSAID). It causes inhibition of cyclooxygenase (COX), a constitutive enzyme that is involved in the synthesis of prostaglandin-an inflammatory pain mediator. Prostaglandin is further transformed into other active compounds having different effects on human body(Ghanem et al. 2016).

The exposure risk associated with the paracetamol in the environmental matrices has been studied by several researchers. They have done toxicity testing both *in vitro* and *in vivo.* It has been observed that toxicity mechanism of paracetamol is highly conserved and it is same in both vertebrates and invertebrates. The neurotoxicity which is an important parameter in ecotoxicity testing has been widely studied by Oliveira et al. (2015).It has been observed that acetylcholinesterase (AChE) and Se-GPx activity is inhibited in *Daphnia magna* when exposed to 10ug/L of APAP (Oliveira et al.2015). Seawater mussel *Mytilus galloprovincialis* also showed similar effect when exposed to paracetamol at concentration ranging from 0.023 to 0.403 mg/L (Solé et al. 2010). Nunes et al. (2015) and Ramos et al. (2014) postulated that the inactivation of enzymes in the presence of paracetamol is due to the oxidative stress that is the most common effect of paracetamol exposure.*Torpedo californica* (fish) (Weiner et al. 1994), Rodents (Delwing-de Lima et al.2010), *Brachionus koreanus* (rotifer) (Rhee et al.2013), and *Anguilla anguilla* (European eel) (Nunes et al.2015) have shown similar effect because of the oxidative stress from paracetamol exposure.

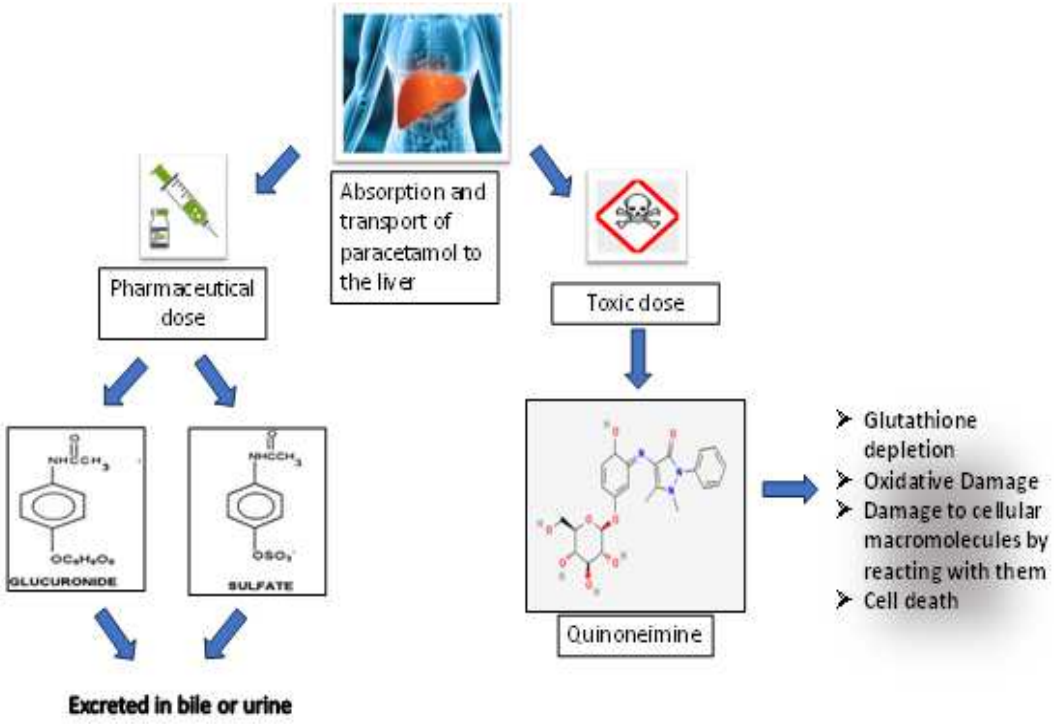

Figure 2: The multidirectional mechanism of action of Paracetamol (Irwin et al. 2004).

The oxidative changes in the sample can be assessed by measuring changes in the activity of glutathione peroxidase and catalase which are the most common biomarkers of oxidative stress.Different authors have reported contradictory results on the activity of oxidative stress biomarkers. Studies done by Oliveira et al. (2015) and Monteiro et al. (2006) have reported the decrease in activity of biomarkers related to oxidative stress whereas studies on *Orcheochromis mossambicus* (Kavitha et al. 2011), *Cyprinus carpio* (Nava-Álvarez et al. 2014), or *Dreissena polymorpha* (Parolini and Binelli 2012) showed opposite results i.e. increase in oxidative stress biomarkers were observed when exposed to paracetamol. The various studies on paracetamol toxicity have been summarized in Table 2.

Table 2: Toxicity assessment of paracetamol on different organisms

Organism	Assays	Exposure time	Tested conc.	Toxicity	Reference
Cyprinus carpio	Biomarkers tests were done: lipid peroxidation, superoxide dismutase (SOD), catalase (CAT), and total and selenium-dependent glutathione-peroxidase (GPx),	96 h	100 µg/L	• Oxidative stress • Increase in GPx activity	Nava-Álvarez et al. (2014)

Rhamdia quelen	Piscine micronucleus test, of steroid hormone dosage and comet assay assessed	21 days	0.25, 2.5 µg/L	• Oxidative stress • Neuronal disruption • Reduction inhemoglobin • Decrease in leukocytes and thrombocytes • Increase in estradiol levels hepatotoxicity	Guiloski et al. (2017)
Diopatra neapolitana	Tissue regenerative capacity as a biomarker	39 days	5, 25, 125, 625, 3125 µg/L	• Diminished regenerative capacity	Freitas et al. (2015)
Daphnia magna	Total cholinesterases, CAT, glutathione-S-transferases (GSTs), GPx	48 h	0.00001, 0.001, 0.25 mg/L	• Inhibition of enzymes like AChE and GPx	Oliveira et al. (2015)
Mytilus galloprovincialis	Different biochemical assays were done like acetylcholinesterase (AChE), GSTs, CAT, Lipid peroxidation levels (LPL)	10 days	20, 200 µg/L	• Hepatic lipid peroxidation levels increased • inhibition of AChE activity	Solé et al. (2010)
Anguilla anguilla	Battery of antioxidant biomarkers: CAT, AChE and GSTs, thiobarbituric acid reactive substances (TBARS) assay, lactate dehydrogenase, (LDH)	96h	5, 25, 125, 625, 3125 µg/L	• Inhibition of AChE activity	Nunes et al. (2015)
Brachionus koreanus	AChE activity assessed	24h	0.1, 1, 10, 100, 1000, 10,000,	• Inhibition of AChE activity	Rhee et al. (2013)

			100,000 µg/L		
Oncorhynchus mykiss	Biochemical biomarkers: CAT, GPx, glutathione reductase (GRed) and GSTs activity and also LPL, TBARS assessed	96 h (acute exposure), 28 days (chronic exposure)	0.05, 0.5, 5 mg/L (acute exposure), 12.5, 25, 50 µg/L (chronic exposure)	• Increase in catalase activity • Increase in GPx activity	Ramos et al. (2014)
Tisbebatta gliai.	Evaluation was done by calculating risk quotients (RQs) based on MEC (the highest exposure concentration of the compound in the medium)/PNEC (predicted no effect concentration) ratios	48 h	1.7, 2.2 ,3.4, 4.5 ,6.7, 8.4, 11.2, 16.8, 17.0, 22.6, 25.5, 34.0, 33.9, 51.0 mg/L (in mixture with other pharmaceuticals)	• At environmental conc., low or no risk.	Trombini et al. (2016)
Hediste diversicolor	Biomarkers such as CAT, GSTs, AChE, and cyclooxygenase (COX) activities, LPL, TBARS	28 days	0, 30, 60, and 120 µg/L	• No changes are observed in COX activity • alteration are observed in CAT activity • decrease in GSTs Activity	Daniel et al. 2022

The other effects related to paracetamol exposure as observed in male fish *Rhamdia quelen* are severe changes of hematological parameters, i.e., reduction of hemoglobin and hematocrit, mild blood congestion and leukocyte infiltration, and disruption of hypothalamic-pituitary-gonadal axis (Guiloski et al. 2017). The regenerative tissue capacity of a polychaete

Diopatraneapolitana has found to be decreased when exposed to environmentally relevant concentration of paracetamol (25 μg/L) (Freitas et al. 2015). Different plant species such as *L. culinaris* Med. and *P. sativum* L have shown cytotoxic effect in the roots when exposed to paracetamol. The paracetamol exerted effect on these plants species at each concentration tested. It hampered the mitotic processes, inhibits root growth and showed the presence of micronucleus (Mercado and Galvis 2023).

Other than *in vivo* and *in vitro* toxicological studies, the ratio between predicted or measured environmental concentration to the predicted or measured no effect concentration of the compound is also commonly used in ecological risk assessment of pharmaceutical compounds (Ashfaq et al. 2017a, b) which is generally termed as risk quotient (RQ). It gives a direct insight on the harmful dosages of various pollutants. The value of RQhigher than 1 indicates high risk to the Aquatic community as shown by Bouissou-Schurtz et al. (2014). It has been observed that the values of RQ for paracetamol as calculated in *Streptocephalusproboscideus*, *Daphnia*,green algae and *D. magna* were, 9.2, 64, 0.11 and 5.0 respectively. The values clearly indicate that except green algae all the other three under study are in high risk to paracetamol exposure.

IV. MECHANISM OF PARACETAMOL DEGRADATION BY BACTERIA

The microorganisms such as Bacteria, Algae and Fungi utilize paracetamol as energy and carbon source. The degradation depends on environmental factors such as pH, temperature, and cellular structure such as cell structure and physiology of cell membrane. At high and low temperature, the porosity and rigidity of cell membrane hinders the efficient uptake of molecules or enzyme activity thereby the reaction becomes slow. pH regulated the toxicity and uptake of molecules through the cell membrane. At acidic pH, the paracetamol is present in protonated form whereas at alkaline pH the phenolate form occurs.

The degradation of paracetamol occurs at optimum temperature and pH (Żur et al. 2018). The degradation and mineralization of paracetamol is done with the help of a diverse array of enzymes and pathways. The degradation of paracetamol is reported under aerobic as well as anaerobic environments (Figure 3 and 4).

There are several pathways reported for paracetamol degradation under aerobic conditions, and it occurs mainly through hydroquinone pathway and pyrocatechol pathway (Figure 3). In hydroquinone pathway, the paracetamol is converted to hydroquinone through amidohydroxylation in which the paracetamol is first converted to 4-aminophenol by releasing acetate. Thereafter, amino groups are replaced by hydroxyl group to form hydroquinone. In the case of hydroxylation, the paracetamol is converted to hydroquinone releasing acetamide. The hydroquinone is converted to 4-hydroxymuconic semialdehyde by hydroquinone 1,2-dioxygenase for further metabolism through tricarboxylic cycle. The enzymes involved in amidohydroxylation are amidohydroxylase and amino acid oxidase. The enzymes involved in initial hydroxylation are hydrolytic enzymes.

In another pathway i.e., pyrocatechol pathway, the paracetamol is converted to N-(4-hydroxyphenyl)-acetamide by hydroxylase. The N-(4-hydroxyphenyl)-acetamide can be degraded through the following routes (a) N-(4-hydroxyphenyl)-acetamide oxidation to benzoquinone and hydroquinone, (b) N-(4-hydroxyphenyl)-acetamide conversion to

pyrocatechol by oxygenase and formamide CGTase. These two routes finally generate intermediates (p-phenol) which can undergo ring fission for further degradation through TCA cycle (Wu et al. 2012; Żur et al. 2018).

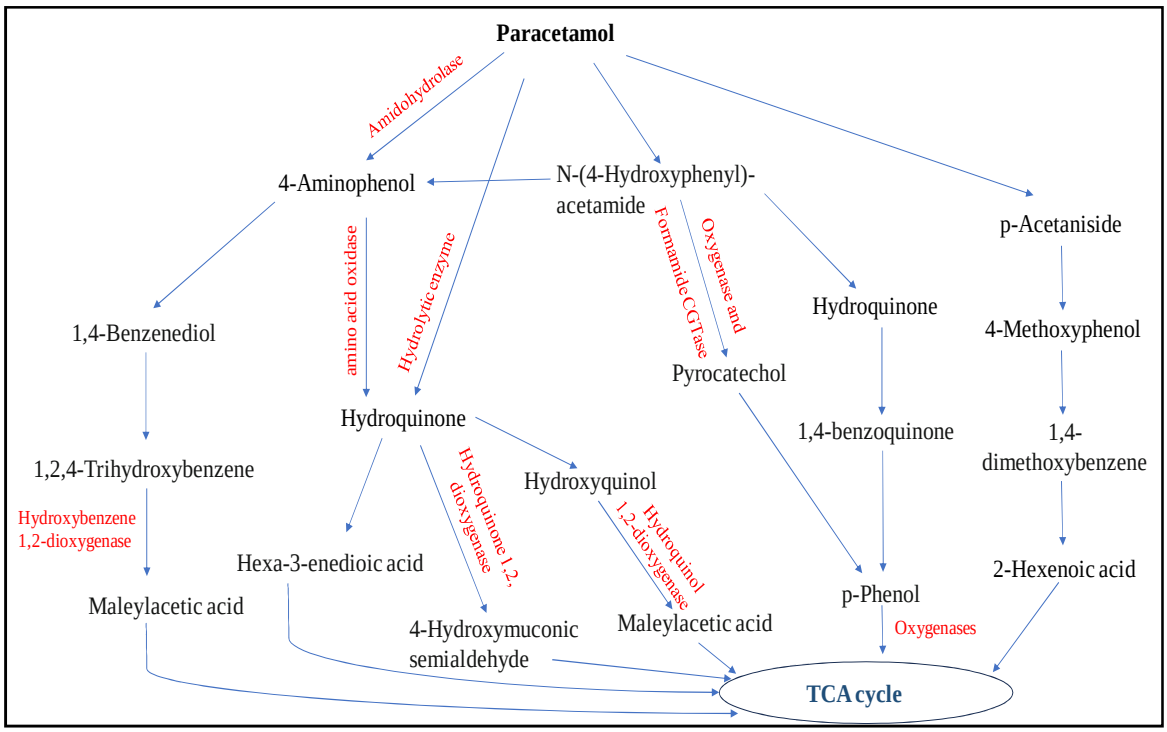

Figure 3: Pathways of aerobic degradation of Paracetamol degradation by bacteria (Li et al. 2014; Takenaka et al. 2003; Wu et al. 2012; Żur et al. 2018),

A different pathway of paracetamol degradation was observed in *Burkholderia* sp. AK-5, in which the p-aminophenol is transformed to 1,2,4-trihydroxybenzene by two successive hydroxylation generation 1,4-benzenediol as intermediate. Thereafter, 1,2,4-trihydroxybenzene undergo conversion to maleylacetic acid by 1,2,4-trihydroxybenzene 1,2-dioxygenase (Takenaka et al. 2003). Another pathway for paracetamol degradation was proposed while studying its microbial degradation in soil, where the paracetamol is converted to 3-hydroxyacetaminophen. The enzymes involved in the process are cytochrome P-450 hydroxylase. This 3-hydroxyacetaminophen can be further degraded via two routes (a) methylation to p-acetaniside which further gets degraded via intermediates such as 4-methoxy phenol, 1,4, dimethoxybenzene and 2-hexenoic acid, (b) oxygenation to N-acetylp-benzoquinone imine which further degraded via., para-benzoquinone (Li et al. 2014).

When the genetic studies were performed to identify the genes involved in paracetamol degradation, it was observed that plasmid plays an important role in paracetamol degradation. The *Pseudomonas* sp. ST-4 has been shown to degrade 4-aminophenol through plasmid (Khan et al. 2006). The genes responsible for hydroquinone degradation and their regulation have been elucidated in *Pseudomonasputida* DLL-E4 (Hu et al. 2014).There are several reports available where the *Pseudomonas* sp. has been studied for paracetamol degradation in the concentration range 50-4000 mg/L and the pathway have been suggested based on the detection of intermediated or enzymes (Poddar et al. 2022; Zur et al. 2018;

Zhang et al. 2013; Hu et al. 2013). In a similar study, microbes such as *Brevibacterium, Corynebacterium, Enterococcus* and *Bacillus* sp., isolated from activated sludge sample were studied for paracetamol degradation. Cultures of *Brevibacterium, Corynebacterium* and *Enterococcus* resulted in 86-97% degradation of paracetmol at 200mg/L concentration but in case *Bacillus* complete mineralization of paracetamol was observed along with detection of degradation intermediates viz., 4-aminophenol, hydroquinone and 2-hexenoic acid (Palma et al. 2021).

The degradation of paracetamol under anaerobic condition was also attempted by the researchers. Recently a study on paracetamol degradation by *Bacillus drentensis* strain S1 was performed under anaerobic conditions and the pathway was also deciphered based on the detection of degradation intermediates viz., phenothiazine and 2-isopropyl-5-methylcyclohexanone (Figure 4). This strain was reported to degrade 94.5% (initial concentration 300mg/ L) at pilot scale under anaerobic conditions (Chopra and Kumar 2020).

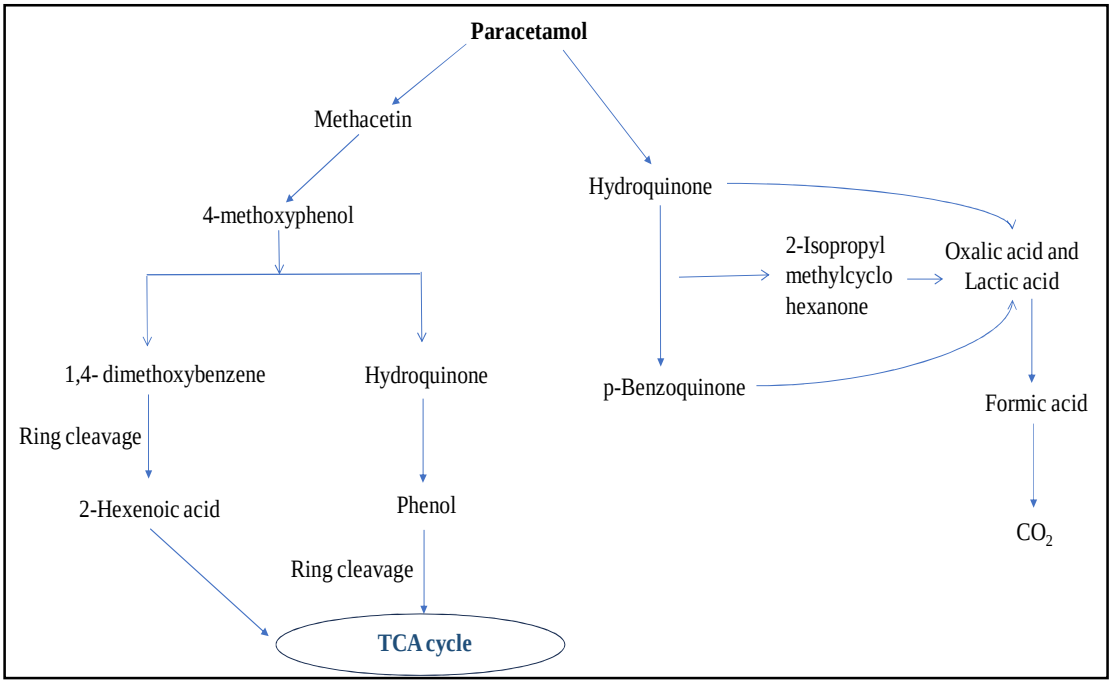

Figure 4: Pathways of anaerobic degradation of Paracetamol degradation by bacteria (Chacón et al. 2022; Chopra and Kumar 2020).

In another study, the degradation of paracetamol was reported in mangrove sediments. A total of 16 microbial genera (*Bacillus*, *Enterobacter and Arthrobacter* could be identified) were found to be associated with paracetamol degradation. Furthermore, they have studied the role of electron acceptor viz., $NaNO_3$, Na_2SO_4 and $NaHCO_3$ addition for enhanced paracetamol degradation under anaerobic conditions and $NaNO_3$ showed the best degradation in case of mangrove sediments (Yang et al., 2020). Chacón et al (2022) studied the role of raw biochar and designer redox-active biochars in paracetamol degradation. They observed that under anaerobic conditions, the degradation of paracetamol occurs via methylation route as 4-methoxyphenol was predominantly present during the degradation.

V. CONCLUSION

Paracetamol or acetaminophen is one of the most commonly used over-the-counter pharmaceutical drug worldwide. It is the standard first-line treatment for fever and pain. Its use has further increased post-Covid. Paracetamol's easy availability has resulted in excessive use leading to an increase in its concentration in the environment. Acetaminophen levels have been found to be among the highest of all the pharmaceuticals in surface and river waters. Several researchers have tested the risks associated with exposure to paracetamol in the environment in several organisms and have reported hepatotoxic and neurotoxic effects. It has been observed that the mechanism of toxicity of paracetamol is highly conserved and same in both vertebrates and invertebrates. For removal of paracetamol from the environment, biological route through bacterial biodegradation has been shown to be quiet effective and eco-friendly. Various pathways of paracetamol degradation and mineralization like hydroquinone and pyrocatechol pathways among others have been reported in different bacterial strains. Bacterial biodegradation of paracetamol under anaerobic conditions have also been studied. Further research into more effective and widely applicable routes of removal of paracetamol from the environment along with its rational and efficient use is much needed for better management of this most commonly used medicine that is here to stay for many more years to come.

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