CHAPTER 2

REVIEW OF LITERATURE

Currently about 95% of world’s total energy demands (residential, industrial, transportation etc.) are fulfilled by use of fossil fuels (oil, coal and natural gases). Oil play a preeminent role in world’s total energy consumption, as it accounts for over 33.06% followed by 23.67% and 30.34% of natural gas and coal respectively [1], [33]. Among all fractions of crude oil, middle distillate fraction i.e. diesel is the highly consumed fraction. Diesel is the major transportation fuel worldwide and according to the projection of IEA (International Energy Agency, France) its consumption is expected to increase by 85% through 2040 [1]. Crude oil reserves are being exhausted much faster than new ones are being discovered. Consumption to production ratio of light crude oil is increasing day by day and after few years peak oil (point in time when highest petroleum extraction rate is achieved) condition will attain. Afterward, the rate of conventional oil (light crude oil) production will enter terminal decline phase and we will be left with unconventional oil i.e. heavy or extra heavy crude oil. However, to meet present demand, heavier fractions are being used as blending components to produce diesel oil. Heavy distillates are rich in poly-homocyclic and heterocyclic aromatic compounds. Use of diesel, containing higher amount of polycyclic and heterocyclic (mainly nitrogen and sulfur as heterocycles) aromatics, is directly correlated with the various environmental and health related issues. Thus, much emphasis is laid on improving the quality of diesel. Nitrogen and sulfur are the major impurities present in diesel. Diesel fuel, with high nitrogen and sulfur content, on combustion releases oxides of nitrogen (NO, NO₂, N₂O) and sulfur (SO₂) which are the major contributors of environmental problems like acid rain, smog formation, greenhouse effect, destruction of the ozone layer and particulate matter formation [2], [3], [34]-[38]. Particulate matters are well known to cause adverse
effect on cardiovascular system, respiratory track illness and also responsible for lung cancer mortality [39]-[42]. Due to such adverse effects of these emissions, diesel fuel specifications have increasingly been tightened. The combination of increasing demand of transportation fuels and newer stringent regulations on their specifications presents a technological challenge for the global refining industry.

Sulfur is the most abundant heteroatom in the crude oil and has adverse impact on the environment. A conventional hydrotreating process known as hydrodesulfurization (HDS) is used to produce low sulfur diesel [4]-[8]. Biodesulfurization (BDS) has been very well studied [43]. It led to the development of the process up to pilot scale for direct sulfur removal using the cells of *Rhodococcus erythropolis* [30]. Recently, denitrogenation of fuel oil has drawn much attention as the presence of nitrogen heterocycles affect the quality of diesel fuel. These compounds inactivate the chemical catalysts used in the conventional hydrotreating process, hydrodesulfurization (HDS), to produce clean diesel fuel with low sulfur. Several nitrogen-containing heterocyclic aromatic hydrocarbons are known to poison the refinery catalysts [44]-[46]. As catalysts used in hydro-refining are costly, inactivation adds cost to these processes.

The nitrogen heterocycles found in crude oils are characterized into two classes, basic molecules and non-basic molecules. The overall nitrogen content of crude oils average typically around 0.3%, out of which a major fraction, about 70-75%, is comprise of non-basic nitrogen heterocycles [47]. The basic nitrogen heterocycles found in diesel oil are acridine, phenanthidine, quinoline and isoquinoline while non-basic fraction contains carbazole and indole (Figure 2.1). Basic nitrogen heterocycles are easy to denitrogenate using hydrotreatment process while non-basic compounds get converted into basic compounds. These resulting basic nitrogen heterocycles deactivate the chemical catalyst during downstream operations in the oil refinery [24]. CAR is the major representative of non-basic nitrogen heterocycle present in diesel. Due to low reactivity, CAR is difficult to denitrogenate using physicochemical method and thus chosen as a model compound in this study. It consists of two benzene rings fused on either side of a five membered pyrrole system (Figure 2.1). CAR is considered as a contaminant present in petroleum for
many reasons. Firstly, the incomplete combustion of fossil fuel leads to the emission of oxides of nitrogen and thus contributes to major environmental problems like acid rain, greenhouse effects, destruction of the ozone layer and particulate matter formation [20], [34]-[41]. Secondly, presence of N-compounds affects the stability of fuels and also interfere with the conventional sulfur removal process i.e. hydrodesulfurization. During hydrotreatment process, carbazole get converted into its basic derivatives which are then adsorbed to the active sites of the catalyst and leads to its deactivation [9], [10]. Denitrogenation of CAR will thus result in the improvement of hydrodesulfurization efficiency. This represents a major economic improvement. Thirdly, the presence of CAR promotes the corrosion of refining equipments, which adds to the refining cost [20]. Lastly, CAR is a known carcinogenic and mutagenic compound [42].

![Figure 2.1: Nitrogen heterocycles present in diesel.](image)

### 2.1 Degradation of carbazole

Owing to the adverse effect on fuel quality and hydrolefining, presence of nitrogen in diesel is an extremely serious issue. Nitrogen should be removed from CAR prior to HDS
Various methods, both physicochemical and biological approaches, have been reported to degrade or transform CAR.

### 2.1.1 Physicochemical degradation

Clean fuels research has now become an important issue of environmental catalysis studies, globally. Currently, nitrogen removal from CAR is mainly based on the use of conventional catalytic hydroprocessing (hydrotreatment followed by distillation) method known as hydrodenitrogenation (HDN). Highly used catalysts for HDN are γ-alumina (γ-Al₂O₃) supported active metals (Mo, W) sulfides with sulfides of promoters (Ni, Co) [50-53]. Recently, Tominaga and Nagai [17] proposed a reaction scheme of HDN of CAR on the γ-Mo₂N(1 1 0) slab surface which results in the hydrogenation of the aromatic rings followed by C—N bond cleavage (Figure 2.2). Though, it removes nitrogen from

![Figure 2.2](image-url): Proposed reaction scheme of hydrodenitrogenation of carbazole [17].
CAR but the process is energy and capital intensive, as it consumes more hydrogen per mole of heteroatom and the reaction is carried out at high temperature and pressure. However, there is a demand of alternative technology to achieve complete denitrogenation as HDN does not work below a minimum CAR concentration. Furthermore, catalysts used in this process are known to influence the composition of treated products [20].

2.1.2 Microbial metabolism of carbazole

Microorganisms as biogeochemical agents play an important role in the conversion of complex organic compounds into simple inorganic compounds. Due to rapid industrialization, microorganisms have been exposed to various toxic compounds which in the course of evolution, resulted in the development of defense mechanism against these compounds. Microorganisms convert toxic compounds either in to carbon dioxide or transform them to less toxic metabolites [54]. The ability of microorganisms to catalyze degradation of various hazardous environmental pollutants such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls (PCBs), heterocyclic aromatic hydrocarbons and pesticides, has generated considerable interest for using either whole cell microorganisms or purified enzymes as biocatalyst for bioremediation of wide range of toxic compounds from contaminated soils and water [55]-[70]. Moreover, microorganisms can operate efficiently at a wide range of conditions and are highly selective which results in low energetic costs and maintenance. The alternative approach to remove CAR is the use of bacterial cells or enzymes as biological catalysts. The microorganisms during its growth at ambient temperature can utilize CAR as a substrate for its growth. Biodegradation or biotransformation studies of CAR have two main focuses, with different motives. First approach is the microbial degradation of CAR by the breakage of C—N and C—C bonds leading to the complete mineralization of nitrogen heterocycle, which targets the complete degradation of CAR from contaminated sites. The second approach which targets the petroleum biorefining is, to develop a microbial process for the specific removal of nitrogen from CAR and its derivatives, by the selective cleavage of both C—N bonds without affecting the C—C bonds [30]. Various microorganisms, both bacteria and fungus, are known to degrade CAR.
2.1.2.1 Bacterial metabolism of carbazole

A variety of CAR degrading bacteria have been reported during the last two decades. Various Gram-negative and Gram-positive bacteria are able to utilize CAR as a sole source of carbon, nitrogen and energy like Arthrobacter, Burkholderia, Dietzia, Erythrobacter, Gordonia, Janthinobacterium, Klebsiella, Kordiimonas, Mycobacterium, Novosphingobium, Pseudomonas, Ralstonia, Sphingomonas, Xanthomonas, Nocardioides etc. (Table 2.1).

In 1993, a group of researchers for the first time reported the isolation of CAR degrading bacteria, Pseudomonas sp. CA10 [26] and on the basis of metabolite analysis, proposed the biodegradation pathway (Figure 2.3). Pseudomonas resinovorans strain CA10 converts CAR to anthranilic acid by angular dioxygenation, meta-cleavage and hydrolysis [27], [28]. Degradation of CAR starts through the action of angular dioxygenase enzyme which converts CAR in to a dihydroxylated intermediate (1-hydro-1,9a-dihydroxycarbazole) by dioxygenation at angular position (C9a—C1), adjacent to the nitrogen atom (See Figure 2.3). Dihydroxylated intermediate is an unstable compound and considered to be converted in to 2’-aminobiphenyl-2,3-diol. The resulting compound then converted in to 2-hydroxy-6-oxo-6-(2’-aminophenyl)-hexa-2,4-dienoic acid (HOADA) by the action of a meta-cleavage enzyme. Metacleavage compound is then converted into anthranilic acid by the action of hydrolase enzyme. An enzyme, anthranilate-1,2-dioxygenase, then converts anthranilic acid into catechol, which is further degraded to cis,cis-muconic acid. This compound then converts into an intermediate of tricarboxylic acid cycle via the β-ketoadipate pathway.
Table 2.1: List of known carbazole degrading bacteria

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Strain</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Arthrobacter</em> sp.</td>
<td>P1-1</td>
<td>[71]</td>
</tr>
<tr>
<td><em>Burkholderia</em> sp.</td>
<td>CB1, IC049, IC129, IC138, IMP5GC</td>
<td>[72]-[74]</td>
</tr>
<tr>
<td><em>Janthinobacterium</em> sp.</td>
<td>J3, J4, IC161</td>
<td>[73], [75]</td>
</tr>
<tr>
<td><em>Klebsiella</em> sp.</td>
<td>LSSE-H2</td>
<td>[76]</td>
</tr>
<tr>
<td><em>Nocardioides</em> sp.</td>
<td>IC177</td>
<td>[77]</td>
</tr>
<tr>
<td><em>Novosphingobium</em> sp.</td>
<td>KA1, J30, NIY3</td>
<td>[75], [78]-[80]</td>
</tr>
<tr>
<td>(KA1 formerly known as <em>Sphingomonas</em> sp. KA1 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp.</td>
<td>CA06, CA10, BC039, BC040, BC041, BC043, BC044, BC045, BC046, K23, K22, K15, J11, IC017, LD2, ATCC 31258 / INCQS 00520, F297, OM1, ATCC 17484, KK1, C3211, XLDN4-9, XLDN-R</td>
<td>[23], [25], [26], [73], [75], [81]-[90]</td>
</tr>
<tr>
<td><em>Ralstonia</em> sp.</td>
<td>RJGII.123</td>
<td>[92]</td>
</tr>
<tr>
<td>(formerly known as <em>Xanthomonas</em> <em>ampelea</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Sphingomonas</em> sp.</td>
<td>CB3, CDH7, GTIN11, CP19, J40, M2, IC033, IC075, IC081, IC097, IC145, XLDN2-5, VKMB-2434, JS1</td>
<td>[72], [73], [75], [80], [93]-[99]</td>
</tr>
<tr>
<td><em>Neptuniibacter</em> sp.</td>
<td>CAR-SF</td>
<td>[100], [101]</td>
</tr>
<tr>
<td><em>Bacillus</em> sp.</td>
<td>KUKK-4.5, T2.3, T2.6</td>
<td>[102], [103]</td>
</tr>
<tr>
<td><em>Kordiimonas</em> sp.</td>
<td>OC3, OC65, OC9, OC115</td>
<td>[104]-[106]</td>
</tr>
<tr>
<td><em>Erythrobacter</em> sp.</td>
<td>IC114, OC4, OC85</td>
<td>[73], [104]-[106]</td>
</tr>
<tr>
<td><em>Janibacter</em> sp.</td>
<td>YY1</td>
<td>[107], [108]</td>
</tr>
<tr>
<td><em>Gordonia</em> sp.</td>
<td>F.5.25.8</td>
<td>[109]</td>
</tr>
<tr>
<td><em>Dietzia</em> sp.</td>
<td>P4</td>
<td>[110]</td>
</tr>
<tr>
<td><em>Chryseobacterium</em> sp.*</td>
<td>*NCY and **NCW</td>
<td>[111]</td>
</tr>
<tr>
<td>and <em>Achromobacter</em> sp.**</td>
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<tr>
<td><em>Achromobacter</em> sp.</td>
<td>CAR1389</td>
<td>[112]</td>
</tr>
<tr>
<td><em>Methylobacterium</em> sp.</td>
<td>GPE1</td>
<td>[113]</td>
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</table>
The initial angular dioxygenation, at the position of C9a—C1, is unusual and the need for the oxidation at this position itself explains carbazole’s resistance to microbial attack. Although, complete mineralization of CAR is good for bioremediation but it is not suitable for the biorefining of crude oil because of the loss of carbon value during complete degradation of CAR. A microorganism *Pseudomonas ayucida* IGTN9m able to remove nitrogen from petroleum by selective cleave of C—N bonds in quinoline, without affecting C—C bonds, has been isolated and characterized [91]. Though, denitrogenation of CAR and indole has not been reported by this microorganism, these compounds are found to be non-toxic growth substrates when used simultaneously with the quinoline as an alternate nitrogen source in mineral salt medium. The denitrogenation study when performed in biphasic medium (mineral salt medium: petroleum) resulted in only 5% nitrogen removal from petroleum while 68% of quinoline removal was observed.
Being a major representative of organonitrogen compounds in petroleum, denitrogenation of CAR is desirable. Though, several carbazole biodegradation pathways have been proposed, none of the pathway is reported for the selective removal of nitrogen from CAR. However, a directed evolution method can be used for the development of a modified enzyme for the denitrogenation of CAR from *Pseudomonas auxicida* IGTN9m.

### 2.1.2.2 Fungal metabolism of carbazole

Diverse fungi including filamentous fungi, white-rot fungi, basidiomycetes and deuteromycetes have been reported to degrade or detoxify various PAHs [114], [115]. There are two known and extensively reported mechanisms of fungal PAHs metabolism, one involving cytochrome p450 system [116], [117] and other utilizing some soluble extracellular enzymes, like lignin peroxidases (LiPs), manganese peroxidases (MnP) [118]-[121] and laccases (Lac) [122]-[125]. Owing to nonspecific characteristics, these enzymes can oxidize a wide range of organic pollutants.

In year 2000, Bressler has reported fungal oxidation of CAR and its alkylated derivative (N-ethylcarbazole) by purified extracellular ligninolytic enzyme, laccase, produced by white-rot fungus *Coriolopsis gallica* [126]. Complete oxidation of CAR and N-ethylcarbazole was observed in 1 h when 5 laccase units were used. Unfortunately no oxidized products were detected after the completion of the experiment. In a separate study published, biotransformation of CAR in to a non toxic monohydroxylated product was reported by *Aspergillus flavus* VKM F-1024 [127]. A typical laccase catalyzed redox reactions are depicted in Figure 2.4.

As much as the pathway of bacterial CAR degradation has been well studied, data regarding similar activities in fungi is limited till date. However, fungi may be equally important as bacteria in the bioremediation of soil and water contaminated with CAR and its derivatives because of several reasons. The ligninolytic fungus produces soluble extracellular enzymes that directly attack the CAR and other PAHs where as bacterial CAR degrading enzymes are intracellular in nature. Moreover, ligninolytic fungi can also incorporate hydroxyl group in the aromatic ring of CAR, similar to the bacteria, and
hence lead to the detoxification [54]. But as far as the petroleum refining is concern, fungal laccase are not suitable due to its nonspecific nature of substrate degradation.

Figure 2.4: Schematic representation of laccase catalyzed redox cycles for substrates oxidation in the absence (A) or presence (B) of chemical mediators.

2.2 Bacterial genes involved in carbazole degradation

The primary genes (car) involved in degradation of CAR have been cloned and well characterized in both Gram-negative as well as Gram-positive bacteria. Among all, *Pseudomonas resinovorans* strain CA10 is the most studied bacterium having CAR-catabolic genes (*car*<sub>CA10</sub> genes). These genes are located on a 199 kb circular plasmid, pCAR1, and clustered in the form of an operon as *car*<sub>AaAaBaBbC</sub>AcAd [128]-[130]. Analysis of the *car* gene cluster in *Pseudomonas resinovorans* CA10 also revealed that the *car*<sub>Aa</sub> gene present in a 1,263-bp DNA region is tandemly duplicated except for one base. Furthermore, these *car*<sub>Aa</sub> genes are separated from the *car*<sub>AcAd</sub> gene cluster by the 2-kb DNA fragment of *car*<sub>BaBb</sub> and *carC* genes. Gene cluster *car*<sub>AaAcAd</sub>, encodes for carbazole-1,9a-dioxygenase which catalyzes the first step of CAR degradation pathway [27]. *Meta*-cleavage enzyme, the second enzyme of CAR degradation pathway, is collectively encoded by *car*<sub>Ba</sub> and *car*<sub>Bb</sub>, and the gene *carC* encodes for hydrolase enzyme [28]. It has been hypothesized that the *car* gene cluster in *Pseudomonas* has been
recruited from other microorganisms primarily because of two reasons; firstly, genes are encoded on the plasmid pCAR1, having conjugation potential [85], [86]. Secondly, because of the fact that the G+C content of car gene cluster is lesser (approximately 48.0 to 53.3%) than that of total DNA of *Pseudomonas resinovorans* CA10 i.e. 61.9% [131].

Although, the CAR catabolic operons are nearly similar in both Gram-positive and Gram-negative organisms [73], [75], [79], [131], *car* operon of Gram-positive *Nocardioides aromaticivorans* IC177 is organized more orderly than others [77]. Firstly, the gene arrangement of the *carC* and *carBaBb* genes in the strain IC177 is reversed relative to that in well described *car* gene clusters of gram negative bacteria. Secondly, in Gram-negative organisms there is an undefined open reading frame (ORF) in between genes which is not there in Gram-positive organisms. Lastly, in IC177 all *car* genes overlap with each other, while such overlapping in not present in *car* genes of Gram-negative bacteria. These points suggest that CAR degrading gene has evolutionary history and these genes in Gram-positive organism are more evolved. The arrangement of *car* genes in some of the known CAR degrading microorganisms is depicted in Figure 2.5.

Nojiri *et al.* [131] studied the genes flanking the *car* operon in CA10 and identified certain genes upstream of *car* operon, which are also involved in CAR degradation. Southern hybridization revealed the presence of one copy of *antABC* genes which encode for anthranilate-1,2-dioxygenase. The *antABC* gene cluster is located 21 kb upstream region of the *car* operon [132]. In 2003, the complete nucleotide sequence of pCAR1 revealed that both *ant* and *car* gene clusters were present within the 73 kb transposon Tn4676 [129]. Car and Ant proteins together degrade CAR completely to catechol (See Figure 2.3). Northern hybridization and RT-PCR analyses, carried out by Urata *et al.* [132] revealed that an extra copy of *Pant* (promoter of *ant* operon which is thought to be translocated due to transposition of ISPre1) is located about 2.1 kb upstream of *carAa* gene. They also described that *Pant*, induced with anthranilate, also drives the transcription of *carAa* gene. Genes encoding for proteins involved in transport of organic compound into the cell were also identified upstream of *car* operon [131].
Figure 2.5: Arrangement of car genes in carbazole degrading bacteria. Arrows indicate the 5' → 3' direction and the respective size of the car genes. CA10: *Pseudomonas resinovorans* CA10 [131] (Accession No. AB047548; Length of car gene cluster: 6136 bp), OM1: *Pseudomonas stutzeri* OM1 [84] (Accession No. AB001723; Length of car gene cluster: 6136 bp), XLDN4-9: *Pseudomonas* sp. XLDN4-9 [133] (Accession No. DQ060076; Length of car gene cluster: 4870 bp), J3: *Janthinobacterium* sp. J3 [75] (Accession No. AB095952; Length of car gene cluster: 4867 bp), IC177: *Nocardioides aromaticivorans* IC177 [77] (Accession No. AB244528; Length of car gene cluster: 4609 bp), GTIN11: *Sphingomonas* sp. GTIN11 [95] (Accession No. AF442494; Length of car gene cluster: 3452 bp), KA1 car-I: *Sphingomonas* sp. KA1 [75] (Accession No. AB095953; Length of car gene cluster: 3452 bp), KA1 car-II: *Sphingomonas* sp. KA1 [134] (Accession No. AB220949; Length of car gene cluster: 3486), JS1: *Sphingomonas* sp. JS1 [99] (Accession No. EU854302; Length of car gene cluster: 3455 bp).
2.3 Bacterial enzymes for carbazole degradation

The conversion of CAR to anthranilic acid is catalyzed by angular dioxygenase, extradiol dioxygenase and hydrolase enzymes encoded by carA, carB and carC genes, respectively.

2.3.1 Carbazole dioxygenase (CarA)

The enzyme carbazole-1,9a-dioxygenase (CARDO), encoded by carA gene, was originally isolated from Pseudomonas resinovorans strain CA10 [27]. It is an aromatic-ring-hydroxylating dioxygenase system having ability to catalyze the dioxygenation of CAR at the carbon positions of 9aC and 1C. Functional and structural analysis of CARDO revealed that it is a member of Rieske non-heme iron oxygenase systems (ROSs). ROSs are considered as the initial catalyst in the catabolic pathway of various toxic environmental pollutants such as PAHs, heterocyclic aromatic hydrocarbons, dioxins, polychlorinated biphenyls etc.[135]-[145]. They are characterized as multicomponent enzymes having two or three protein components comprising an electron transport chain (ETC) and a catalytic oxygenase component. ETC mobilizes electrons from reduced nicotinamide adenine dinucleotide (NADH) via flavin and Rieske-type [2Fe-2S] redox centers to the terminal dioxygenase, which then transfers it to the oxygen molecules.

CARDO is a multicomponent enzyme consisting of homotrimeric terminal oxygenases (44-kDa monomer, CarAa), a ferredoxin (13-kDa monomer, CarAc) and a ferredoxin reductase (37-kDa monomer, CarAd) units, encoded by the carAa, carAc and carAd genes, respectively [146]. As shown in Figure 2.6, ETC components of CARDO_{CA10} (CarAc and CarAd) transfer electron from NADH to the catalytic unit, CarAa. In the CARDO_{CA10} system, terminal oxygenase component contains a Rieske-type [2Fe–2S] cluster and a mononuclear iron binding site. Ferredoxin component of CARDO_{CA10} contains a Rieske-type [2Fe–2S] cluster while ferredoxin reductase component consist of a plant-type [2Fe–2S] cluster and a FAD as the prosthetic group.
2.3.1.1 Classification of carbazole dioxygenase

Based on the number of ETC components and nature of their redox centers, ring-hydroxylating oxygenases (RHOs) have been classified into five classes viz. IA, IB, IIA, IIB and III [147] (Table 2.2). RHOs belonging to class IA and IB are two-component systems composed of terminal oxygenase and an electron transfer unit (ferredoxin reductase), while other classes IIA, IIB, and III are three-component system consisting of a terminal oxygenase and two electron transfer components (ferredoxin, and ferredoxin reductase). The reductase component of class I contains flavin (flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) in class IA and IB, respectively) and a plant type [2Fe–2S] cluster binding domain. In class IIA and IIB the flavin (FAD) and the [2Fe-2S] cluster (IIA, plant type; IIB, Rieske type) binding domains are located separately on reductase and ferredoxin components. RHOs grouped under class III contain a Rieske-type [2Fe–2S] ferredoxin, while their reductases consist of both FAD and a chloroplast-type [2Fe–2S] cluster. Terminal oxygenase unit of all classes of Banie system contains mononuclear (Fe$^{2+}$) and Rieske-type [2Fe–2S] cluster binding domains. A homo-multimeric terminal oxygenase component made up of α subunits, is a key characteristic of class IA RHOs while other classes (IB, IIA, IIB and III) possess a
Table 2.2: Batie classification system of ring-hydroxylating oxygenases

<table>
<thead>
<tr>
<th>Class</th>
<th>Electron transport unit</th>
<th>Catalytic unit</th>
<th>Enzymes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reductase</td>
<td>Ferredoxin</td>
<td>Oxygenase</td>
<td></td>
</tr>
<tr>
<td>IA</td>
<td>FMN/ [2Fe-2S]_P</td>
<td>[2Fe-2S]_R/ Fe^{2+}</td>
<td>3-Chlorobenzoate-3,4-dioxygenase, Phenoxybenzoate dioxygenase, Pthalate dioxygenase</td>
<td>[148]</td>
</tr>
<tr>
<td>IB</td>
<td>FAD/ [2Fe-2S]_P</td>
<td>[2Fe-2S]_R/ Fe^{2+}</td>
<td>Anthranilate dioxygenase, Benzoate-1,2-dioxygenase, Toluene-1,2-dioxygenase (Pseudomonas putida), 2-Oxo-1,2-dihydroquinoline-8-monoxygenase</td>
<td>[151]</td>
</tr>
<tr>
<td></td>
<td>FAD</td>
<td>[2Fe-2S]_P</td>
<td>[2Fe-2S]_R/ Fe^{2+}</td>
<td>Dibenzofuran dioxygenase, Pyrazon dioxygenase</td>
</tr>
<tr>
<td>IIIB</td>
<td>FAD</td>
<td>[2Fe-2S]_R</td>
<td>[2Fe-2S]_R/ Fe^{2+}</td>
<td>Benzene-1,2-dioxygenase, Biphenyl dioxygenase, Carbazole dioxygenase (Sphingomonas sp. CB3), Cumene dioxygenase, Toluene dioxygenase (Pseudomonas putida F1)</td>
</tr>
<tr>
<td></td>
<td>FAD/ [2Fe-2S]_R</td>
<td>[2Fe-2S]_R/ Fe^{2+}</td>
<td>Carbazole-1,9a-dioxygenase (Pseudomonas sp. CA10), Dibenzothiophene dioxygenase, Naphthalene dioxygenase, 2-Nitrotoluene dioxygenase, Nitrobenzene dioxygenase.</td>
<td>[27]</td>
</tr>
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</table>

FMN; flavin mononucleotide, FAD; flavin adenine dinucleotide, [2Fe-2S]_R; Rieske-type iron-sulfur cluster binding domain, [2Fe-2S]_P; Plant-type iron-sulfur cluster binding domain, Fe^{2+}; mononuclear iron-binding domain.
hetero-multimeric oxygenase composed of α (large) and β (small) subunits with some exceptions.

Although, the above mentioned properties of the ETC proteins suggest that according to Batie’s classification CARDOCA10 system should be classified as a class III dioxygenase, but CARDOCA10 has however been shown to be a novel Rieske non-heme iron oxygenase system because of several important distinguishing characteristics [164]. Firstly, while typical class III ROSs contain the terminal oxygenase component that consists of both α and β catalytic subunits with the α3β3 (or α2β2) configuration, CarAa consists of only one catalytic (α) subunit with the α3 (trimeric) configuration [142]. This homomultimeric structure is typical characteristics of class IA ROSs, whose terminal oxygenases have been reported to have α2, α3, α4, or α6 configurations. Secondly, according to the phylogenetic analysis, CarAd was found to be more closely related to the reductases which are involved in multicomponent monooxygenase systems, compared to those in ROSs [165]. Thirdly, CarAa is a unique type of oxygenase as it is reported to show low amino acid sequence homology (<19%) with other well known catalytic subunits of terminal oxygenases of ROSs and also showed the highest homology of 35% with the monooxygenase [166]. Lastly, the reductase component of CARDOCA10 consists of a plant-type [2Fe–2S] cluster which is the key characteristics of class I RHOs.

At present, several bacteria possessing CARDOs have been isolated. The CARDOs from Pseudomonas resinovorans CA10, Janthinobacterium sp. J3, Pseudomonas stutzeri OM1, and Pseudomonas sp. XLDN4-9 belong to class III in Batie’s classification [75], [84], [89], [146], [167]. Other CARDOs isolated from Sphingomonas sp. KA1 and GTIN11 belong to class IIA [79], [95], while the CARDO from Nocardioides aromaticivorans IC177 belongs to class IIB [77].

2.3.1.2 Oxygenation reactions catalyzed by carbazole dioxygenase

CARDO, as unique ROSs, has a broad substrate range and have the ability to catalyze diverse oxygenations like angular dioxygenation, lateral dioxygenation and monooxygenation of various PAHs [31]. ROSs generally functions by adding two
oxygen atoms (in the form of hydroxyl groups) to the benzene ring to form cis-dihydriodols, known as lateral dioxygenation. In contrast, in the CARDO system a carbon (9aC) bonded to the nitrogen atom in the substrate (CAR) and its adjacent carbon (1C) in the aromatic ring are hydroxylated (Figure 2.7 A). This type of reaction is called angular dioxygenation, and is catalyzed by very few ROSs. Molecular mechanism of angular dioxygenation catalyzed by CARDO was studied based on the crystal structure of terminal oxygenase component of CARDO (CARDO-O) from Janthinobacterium sp. J3 and substrate bound ferredoxin component of CARDO (CARDO-F) from Pseudomonas sp. CA10 (CARDO-F CA10: CARDO-O J3 binary complex crystals) [164]. It was observed that upon binding to the active site, the conformation of the amino acids near the active site changed resulting in the closure of the lid over the substrate binding pocket. CAR was trapped inside the pocket in such a manner that C1 and C9a were located approximately 4.3 Å from the iron at the active site while the hydroxyl ligand of the non-heme iron was located about 2.8-2.9 Å from these two carbon atoms [168]. This binding exposed the imino nitrogen of the carbazole within the hydrogen binding distance of carbonyl oxygen of glycine (Gly178).

CARDO can also catalyze angular dioxygenation of dibenzo-p-dioxin and dibenzofuran (Figure 2.7 A) [60]. Compared to other degradation reactions for dioxin, angular dioxygenation is an attractive and ideal degradation reaction because this reaction forms an unstable hemiacetal intermediate that spontaneously converts the hetero ring-cleaved compound. Thus, this single step oxygenation leads to the destruction of the planar structure of dioxin that is responsible for dioxin’s toxicity [165]. Therefore, the bacteria having the CARDO activity or CARDO itself could be a vital tool in the bioremediation of dioxin contamination.

The CARDO also has ability to catalyze the lateral dioxygenation of biphenyl, anthracene and naphthalene by incorporating both oxygen atoms of molecular dioxygen to the tandemly linked carbon atoms of an aromatic ring (cis-configuration) (Figure 2.7 B). Owing to its diverse oxygenation property, CARDO is known to catalyze monooxygenation of dibenzothiophene and fluorene (Figure 2.7 C).
Figure 2.7: Diverse oxygenation catalyzed by carbazole dioxygenase: (A) Angular dioxygenation, (B) Lateral dioxygenation.
2.3.2 Meta-cleavage enzyme (CarB)

The enzyme 2’-aminobiphenyl-2,3-diol-1,2-dioxygenase (CarB), having meta-cleavage activity against hydroxylated aromatic ring, catalyzes the second step of CAR degradation pathway. Enzyme CarB attacks on the meta-position of catechol ring of 2’-aminobiphenyl-2,3-diol to produce 2-hydroxy-6-oxo-6-(2’-aminophenyl)-hexa-2,4-dienoic acid (HOADA) (Figure 2.8). The ring cleavage of catecholic compounds is catalyzed by the enzymes from one of two distinct classes: intradiol dioxygenase or extradiol dioxygenase (Figure 2.9) [169]. Intradiol dioxygenases use non-heme Fe (III) to cleave the aromatic ring ortho to (between) the hydroxyl groups, while extradiol dioxygenases utilize non-heme Fe (II) to cleave the aromatic ring meta (adjacent) to the hydroxyl groups. Interestingly, a few Mn(II)-dependent extradiol dioxygenases with strong sequence similarity to Fe(II) counterparts have also been reported [170]-[173]. Although, the difference between intradiol dioxygenase and extradiol dioxygenase may appear to be minor, their structures are completely different and also, they follow different catalytic mechanisms of action. Another important difference between these enzymes is that the intradiol dioxygenases generally cleave catechols having mildly electron-withdrawing groups while extradiol dioxygenases cleave catechols possessing electron-donating substituents. CarB is characterized as an extradiol dioxygenase.
containing non-heme Fe(II) in their active site and cleaves C—C bond next to the vicinal hydroxyl functional groups, that is the *meta* position (*meta*-cleavage).

![Chemical structure](image)

**Figure 2.8:** Enzymatic reaction catalyzed by *meta*-cleavage enzyme (CarB)

![Chemical structure](image)

**Figure 2.9:** Representation of ring cleavage of catecholic compound by extradiol and intradiol dioxygenases.

Amino acid sequence comparison indicates that extradiol dioxygenases can be classified into two primary divisions: class II (or I) and class III. The class II (or I) enzymes all contain the pro site consensus sequence, whereas the class III includes enzymes whose sequences could not be aligned with those of class I or II enzymes. While the class I members are comprised of smaller subunits (162-190 amino acids), the class II enzymes have a ~300 amino acid subunit. Based on alignment studies, the class II enzyme is
considered to have evolved from a class I enzyme through gene duplication [174]. However, common features around the active sites of class II and III extradiol dioxygenases suggest that they share the same catalytic mechanism despite their completely different 3D structures. In the proposed mechanism, the two hydroxyl groups of the catechol directly coordinate to the Fe(II) active site in a trigonal bipyramidal geometry [175]. Then the O\textsubscript{2} molecule binds to the ferrous ion and the iron-mediated charge transfers from the catechol to O\textsubscript{2}, resulting in the O\textsubscript{2} activation, which attacks the C–C bond of the substrate catechol ring.

Though most of the extradiol dioxygenases reported are homo-multimeric, CarB is an \(\alpha_2\beta_2\) hetero-tetramer made up of two different proteins \textit{viz.} carBa and CarBb similar to the Class III protocatechuate 4,5-dioxygenase LigAB of \textit{Pseudomonas paucimobilis} SYK6 [176]. Amino acid sequence of CarBb is 31\% homologous with LigB while there is no considerable sequence similarity between CarBa and LigA. Catalytic site of CarB is composed of ferrous ion containing conserved LigB domain in the CarBb subunit.

Larentis \textit{et al.} in 2006 [177], proposed the \(\alpha_2\beta_2\)-heterotetrameric 3D model of CarB dioxygenase. The \(\alpha_2\beta_2\)-heterotetrameric model was comprised of dimeric CarBb subunits (\(\beta\)-chain), and two monomeric subunit of CarBa (\(\alpha\)-chain). Each subunit of CarBb is directly attached to the single CarBb subunit, while there is no direct interaction between two \(\alpha\)-subunits of CarBa. Characterization of enzymatic catalytic site indicated that Fe(II) in the catalytic site of the enzymatic complex was coordinated by three amino acid residues \textit{viz.} His12, His53, and Glu230 (CarBb subunit) which is a typical characteristic of class III extradiol dioxygenase [28], [178]. The 3D model of CarB dioxygenase also indicated that His182 residue was responsible for deprotonation of one of the hydroxyl group of the substrate (2,3-dihydroxybiphenyl) by a hydrogen bond. According to the proposed mechanism, the two hydroxyl groups of the catechol aromatic ring directly coordinate to the Fe(II) active site in a trigonal bipyramidal geometry. Then the O\textsubscript{2} molecule binds to the ferrous ion and the iron-mediated charge transfers from the catechol to O\textsubscript{2}, resulting in the O\textsubscript{2} activation, which attacks the C–C bond of the substrate catechol ring [178], [179].
Though, enzyme CarB was originally isolated from *Pseudomonas resinovorans* strain CA10 as an enzyme catalyzing cleavage of 2′-aminobiphenyl-2,3-diol in *meta* fission, its high catalytic activity \( (k_{cat}/K_m) \) towards the substrates 2,3-dihydroxybiphenyl (13.2 fold increased) and 2,2′,3-trihydroxybiphenyl (5.6 fold increased) was also reported [180]. However, affinity of 2′-aminobiphenyl-2,3-diol for CarB, calculated in terms of the Michaelis-Menten constant \( (K_m) \) was 7.37 and 2.64 fold greater than 2,3-dihydroxybiphenyl and 2,2′,3-trihydroxybiphenyl, respectively.

### 2.3.3 Hydrolase (CarC)

CarC (2-Hydroxy-6-oxo-6-(2′-aminophenyl)-hexa-2,4-dienoate hydrolase) enzyme, catalyzes the third step of CAR degradation pathway and hydrolytically converts 2-hydroxy-6-oxo-6-(2′-aminophenyl)-hexa-2,4-dienoate into anthranilic acid and 2-hydroxypenta-2,4-dienoate (HPD) (Figure 2.10) [28]. Purification, characterization, and steady state kinetic study of CarC enzyme have been carried out and CarC was also found to convert the *meta*-cleavage products (MCPs) from dibenzofuran and biphenyl into salicylate (and HPD) and benzoate (and HPD), respectively [181]. The MCP hydrolases belong to the α/β hydrolase family and most of them are classified into two major groups, groups I and III [182]. Group I is mainly involved in the degradation of bicyclic molecules like biphenyl, whereas group III is involved in the degradation of monocyclic compounds having short alkyl side chains such as toluene, xylene, and isopropylbenzene. The CarC enzyme seems to be classified into the group I MCP-hydrolases, because it preferentially hydrolyzes the MCPs derived from biphenyl-type (two aromatic rings) compounds [181]. However, according to phylogenetic relatedness CarC belongs to the group III MCP-hydrolases.

Similar to BphD<sub>RHA1</sub> (biphenyl dioxygenase of *Rhodococcus* sp. strain RHA1) and CumD<sub>IP01</sub> (cumene dioxygenase of *Pseudomonas fluorescens* strain IP01) the subunit structure of CarC formed typical α/β –hydrolase folds [136], [138]. As conserved in the other MCP-hydrolases, the catalytic-site of CarC is composed of Ser114, Asp233 and His261 residues.
2.4. Process development for carbazole removal

Process development and scale-up are essential to obtain a competitive commercial product. The main limitations of biotreatment process when apply in refining industries are associated with its low stability and the cost of biocatalysts. At the industrial level biological process, the reusability of the biocatalysts could also be a key factor that decides the effectiveness of biotransformation over time. In order to solve these problems immobilization is the most promising approach. Biotreatment of fuel oils by immobilized cells shows numbers of advantages compared to free cells, like reusability in continuous process, increased biocatalyst stability, low contamination risks and ease of biocatalyst separation from treated samples, hence, results in the reduction of the cost for bioprocessing. These advantages have encouraged researchers to explore the application of immobilized cells for the biodegradation of toxic aromatic pollutants like dibenzothiophene, CAR, phenol, pyrene, pyridine, quinoline etc. [183]-[191]. However, in case of oil biorefining, this may not work due to the use of two phase system (aqueous-oil) and passage of oil into immobilized biocatalyst which usually would have an aqueous microenvironment, would significantly limit transfer of carbazole to the biocatalyst. To

Figure 2.10: Enzymatic reaction catalyzed by 2-Hydroxy-6-oxo-6-(2'-aminophenyl)-hexa-2,4-dienoate hydrolase enzyme (CarC).
overcome this limitation, a new method was developed for the biodesulfurization of dibenzothiophene in which bacterial cells were coated with magnetite nanoparticles via adsorption [192]. The magnetic nanoparticles were robustly adsorbed on the bacterial (Pseudomonas delafieldii) cell surfaces because of their high surface energy surface energy and specific surface area. In this type of systems it is possible to concentrate the dispersed coated cells by applying external magnetic field and to reprocess them. This approach can also be used in case of biodenitrogenation studies.

2.5 Recombinant biocatalyst

Biotechnological upgradation of crude oil is of great interest due to increasing level of nitrogen-containing heteroaromatic compounds. CAR and its alkylated derivatives which are the representatives of nitrogen-containing heteroaromatics present in the greatest abundance in petroleum factions. Despite the fact that a number of CAR degrading microorganisms have been reported, none of them is principally appropriate for the BDN because nitrogen is only removed after extensive carbon degradation, which would result in the loss of fuel value. Thus, there is a need of recombinant biocatalysts which would able to remove nitrogen specifically from CAR, without affecting its carbon backbone. Scientists are trying to construct recombinant microorganisms that are suitable for biorefining industry.

Recombinant plasmids carrying car genes (carA, carB and carC) for CAR degradation have been examined in E. coli and they showed the ability to transform a wide range of PAHs other than CAR [28], [31]. Host engineering has also been conducted to tackle the issue of solvent tolerance when CAR degrading bacterial strains are exposed to fuel oil in a real biorefining process. Pseudomonas putida Idaho is a unique organic solvent tolerant bacteria and possess special solvent tolerant ability different from other bacterial strains. Though, it does not have the solvent active pump for the solvent tolerance, but can use toluene, 1,2,4-trimethylbenzene, m-xylene, p-xylene, and 3-ethyltoluene as sole carbon and energy source [193]. Pinkart and White in 1997 [194] investigated the role of cell envelop in the solvent tolerance mechanism of Pseudomonas putida Idaho and found that it has the ability to fix the damaged membranes by the efficient turnover and increased
phospholipid biosynthesis. The car genes from Pseudomonas sp. LD2 were cloned and expressed in the solvent-tolerant Pseudomonas putida Idaho and the non-solvent tolerant Pseudomonas putida KT2440. Biodegradation studies revealed that solvent-tolerant recombinant strain efficiently degraded carbazole mixed in an organic phase, while the non-solvent tolerant Pseudomonas putida KT2440 harboring the same car genes was not able to remove CAR [195].

Kilbane [30] proposed an approach to design a suitable biocatalyst (able to remove nitrogen specifically from CAR) for biorefining. According to that approach, recombinant microorganism can be tailored by expressing the carAaAcAd genes (encode for CARDO) together with the gene encoding an amidase in a single host. The CARDO catalyses the first step of known carbazole degradation pathway would convert carbazole into 2'-aminobiphenyl-2,3-diol, a product with free amine group. The resulting product would then be easy to denitrogenate using an amidase/deaminase.

To develop a bioprocess for simultaneous removal of nitrogen and sulfur atoms from CAR and dibenzothiophene, respectively, the CARDO encoding genes (carAaAcAd) from Pseudomonas sp. strain XLDN4-9 were cloned and expressed in the sulfur utilizing host, Rhodococcus erythropolis strain XP [29]. The resulting recombinant strain SN8 was able to degrade dibenzothiophene, CAR and its alkylated derivatives simultaneously. In order to detoxify nitrogen, sulfur and oxygen pollutants simultaneously, Yu et al. [133] cloned and expressed all three car genes i.e. carABC genes in the same sulfur utilizing host strain XP. The resultant recombinant showed simultaneous degradation of CAR, dibenzothiophene and dibenzofuran (oxygen-containing heterocyclic aromatic hydrocarbon).