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A Comprehensive Review of Aliphatic Hydrocarbon Biodegradation by Bacteria

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COVER LETTER FOR SUBMISSION OF MANUSCRIPT

Subject: **SUBMISSION OF A MANUSCRIPT FOR EVALUATION**

Dear Editor-in-Chief,
I am enclosing herewith a review manuscript entitled "**A Comprehensive Review of Aliphatic Hydrocarbon Biodegradation by Bacteria**" for publication in the journal of Applied Biochemistry and Biotechnology.

In this review we tried to discuss the latest findings regarding the metabolism of aliphatic hydrocarbons under both aerobic and anaerobic conditions. Included is data on newly studied catabolic systems and related enzymes involved in hydrocarbon degrading activities. Since oil and hydrocarbon contamination is a big concern for many environmentalists, such review papers can assist researchers to deal with this problem. This review is also useful for those scientists who work on improving the quality of crude oil in terms of both removal of sulfur and conversion of heavy oils to light oils.

With the submission of this manuscript, I undertake that the manuscript has not been submitted for publication elsewhere.

For the Editors, I would like to disclose the following information about the project:

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Summary

Hydrocarbons are relatively recalcitrant compounds and are classified as high priority pollutants. However, these compounds are slowly degraded by a large variety of microorganisms. Bacteria are able to degrade aliphatic saturated and unsaturated hydrocarbons via both aerobic and anaerobic pathways. Branched hydrocarbons and cyclic hydrocarbons are also degraded by bacteria. The aerobic bacteria use different types of oxygenases, including monooxygenase, cytochrome-dependent oxygenase and dioxygenase, to insert one or two atoms of oxygen into their targets. Anaerobic bacteria, on the other hand, employ a variety of simple organic and inorganic molecules, including sulphate, nitrate, carbonate and metals, for hydrocarbon oxidation.

Key words: Aliphatic hydrocarbons, Metabolism, Anaerobic bacteria, Aerobic bacteria

1) Introduction

Hydrocarbons originate both from the biosynthetic activity of microorganisms and plants (as a result of enzymatic reduction of fatty acid molecules) [1] and from slow geochemical processes acting on biological compounds under high temperature and pressure over prolonged geological periods [2]. However increased demands for oil products due to industrialization have greatly increased the background level of exposure of the environment to different hydrocarbons [3]. All the activities in the oil industries, including exploration, extraction, transportation, refining and oily waste management, are possible sources of environmental pollution [4]. The effluents of petroleum wells, oil refinery operations, industrial waste waters, fuel consumption, wood-processing activities, detergents, pesticides, paints and other chemicals release huge quantities of hydrocarbons into soil and air [5]. Crude oils contain a mixture of different low and high molecular weight aliphatic hydrocarbons and several types of monocyclic and polycyclic aromatic compounds, in which one or more of the carbon atoms in their backbone can also be substituted by nitrogen, sulphur or oxygen. A soap molecule is also a hydrocarbon, consisting of a long hydrocarbon chain containing a carboxylic acid group at the end , and is the potassium or sodium salt of such a fatty acid[6]. The length and nature of hydrocarbons varies in oil derived compounds from C1-C10, such as make up Gasoline [7], C9 – C16, such as are found in Jet fuel and Kerosene [8] and C10-C50, such as waste oil [9]. The categories of typical hydrocarbon chains will be discussed later in the text. Since the components of hydrocarbons are highly recalcitrant to degradation (due to their low water solubility), they remain for a long time. The US EPA has classified these compounds as priority contaminants of natural resources [10]. On the other hand, metabolism of hydrocarbons by microorganisms is a relatively complex process in which the organisms first take up the hydrocarbons and then convert these metabolically inactive molecules to more active forms for further catalysis [11]. Although each group of hydrocarbons is broken down through specific enzymatic cascades, their final product is frequently similar.

This review attempts to describe the microbial abilities and the pathways used for degradation of hydrocarbons in both aerobic and anaerobic conditions and to update previous data using that from the latest publications. The authors have paid more attention to the aliphatic hydrocarbons, although in some cases aromatic hydrocarbons were used as prototype examples.

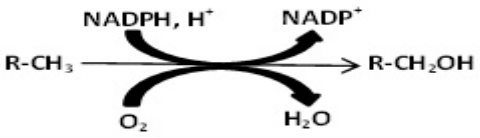
2) Uptake of hydrocarbons into microbial cells

Microorganisms are challenged by the hydrophobicity and insolubility of hydrocarbons which both causes toxic effects on the cells, and restricts their absorption into the cells [12]. Since hydrocarbons interrupt membrane fluidity, the cell membrane composition of the exposed microorganisms has to change in order to stabilize the structure of the phospholipid bilayer [13]. The intensification of these changes depends on the type of hydrocarbons and their carbon chain length, and include changes from cis-to-trans isomers and from saturated fatty acids to unsaturated form or the reverse [14]. For instance, while C2-C4 alcohols increase the ratio of unsaturated fatty acid in cell membrane, longer alkanols induce the production of saturated fatty acids [13, 14]. These limitations can be resolved using microdroplets, macrodroplets or dissolution of the hydrocarbon molecules into water [15]. Most of the hydrocarbon degrading bacteria produce surfactant compounds in order to emulsify hydrocarbon molecules to form droplets or micelles, which finally are taken up by microorganisms in various ways [15]. The extremely hydrophobic surfaces of many of the hydrocarbon degrading bacteria enable them to associate with hydrocarbon droplets or even to pass through into the organic phase of the droplets. Due to the difficulty of solubilisation and the slow dissolution of high molecular weight (HMW) hydrocarbons, their uptake in aquatic systems is slower than low molecular weight (LMW) hydrocarbons. Since the rate of metabolism of a hydrocarbon is dependent on its uptake, HMW hydrocarbons are metabolized slower than LMW hydrocarbons [16]. Both passive and active transportation systems for hydrocarbons have been found in microorganisms [17, 18]. For instance, at a concentration of higher than 4.54 μmol/L *Pseudomonas* sp. DG17 takes up *n*-octadecane by a facilitated passive transport systems, while at lower concentrations of this hydrocarbon the system is switched into an energy-dependent transportation [17].

3) Metabolic shunts of n-Alkanes

n-Alkanes are the most swiftly decomposing components of aliphatic hydrocarbons, and are divided into four molecular weight groups: the gaseous alkanes, the aliphatic hydrocarbons with lower molecular weight (C8–C16), the aliphatic hydrocarbons with medium molecular weight (C17– C28) and finally, the aliphatic hydrocarbons with high molecular weight (>C28) [19]. Although the smallest group of hydrocarbons are easily degraded by most hydrocarbon degrading microorganisms, degradation of aliphatic hydrocarbons up to C44 also occurs. However, these long chain alkanes are first enzymatically activated before degradation (Table 1) [20].

First and most commonly, n-alkanes are oxidized by an electron carrier dependent monooxygenase system, the alkane hydroxylases, to their corresponding alkan-1-ol (Figure 1) [21, 22]. Several bacteria, including toluene-oxidizing bacteria [23], ammonia-oxidizing bacteria [24], methane oxidizing bacteria [25] and propane-oxidizing bacteria [26] can use monooxygenases to oxidize hydrocarbons by a reaction in which an oxygen atom from O_2 is incorporated into the hydrocarbons (reaction 1). Based on the substrate, these enzymes are called toluene mono/dioxygenase, methane monooxygenase, ammonia monooxygenase, propane monooxygenase and alkane oxygenase [23-26]. In addition, many of these enzymes are broadly able to oxidise other types of hydrocarbons, such as halogenated aliphatics.



(Reaction 1)

The monooxygenases isolated in prokaryotes are classified into two categories based on their electron transport system and the microorganisms in which they are found: a) A rubredoxin dependent enzyme (containing 2FeO), encoded by the gene alkB in most of bacteria and alkM in *Acinetobacter* sp., b) an alkane hydroxylase containing cytochrome P450 monooxygenases in the CYP153 family of bacteria [20, 27, 28]. The first enzyme isolated was a non-heme diiron monooxygenase alkane hydroxylase located in the cell membrane of *Pseudomonas putida* GPo1 that is encoded by the gene alkB and requires the electron transporter subunit, rubredoxin reductase and rubredoxin [29]. Later, other homologous enzymes with a conserved sequence at the active site of the protein were isolated from different microorganisms, especially α-Proteobacteria, β-Proteobacteria and γ-Proteobacteria as well as the Actinomycetales [30]. Except for some enzymes isolated from pseudomonads and some γ-Proteobacteria such as *A. borkumensis*, capable of mineralizing C5–C12 alkanes, other kinds of enzymes are required to attack aliphatic hydrocarbons longer than 10 atom carbons [20]. LadA is a flavoprotein dependent monooxygenase isolated from a thermophilic microorganism (*Geobacillus thermodenitrificans* NG80-2) that activates the long-chain alkanes (C15 to C36) for degradation through breaking down an O_2 molecule and inserting an oxygen atom into its structure [31]. In this reaction, the flavin is reduced by NAD(P)H and then interact with O_2 to transfer one oxygen atom to the alkane [21].

The cytochrome P450 enzymes are a group of heme (iron protoporphyrin IX) containing monooxygenase enzymes found in both eukaryotes and prokaryotes that function in association with molecular oxygen, NAD(P)H and an electron-transfer system(s) to oxidize a large variety of compounds [32]. In contrast to eukaryotes, in which the enzyme is associated with cellular membranes, the bacterial P450 cytochromes are soluble in the cytoplasm [27, 32]. Based on the bacterial species, the structures of these enzymes are different and while in many of the bacteria these enzymes consist of three components (hydroxylase, P450cam and putidaredoxin), an analogous enzyme in *Bacillus megaterium (*P450BM3) contain only one large peptide-chain that performs both the electron transportation and substrate oxygenation [27]. The active site of all categories of Cyt-P450 consists of a cysteinate and an iron protoporphyrin IX, and this interacts with O_2 for the oxidation of substrates [33].

Overall, the electron obtained from NADPH is transferred through an electron transport system, consisting of a reductase and FES redoxin compound, to the Cyt-P450 where the electrons are used for the hydroxylation of a substrate, such as a hydrocarbon, at the expense of breaking down molecular oxygen (reaction 2) [34]. However, the cytochrome produced by *Bacillus megaterium* and *Sphingomonas paucimobilis* uses peroxides, instead of O_2 as the oxidant agent in the oxidative reaction [34]. Several bacteria, such as *Rhodococcus rhodochrous* ($P450_{oct}$) [35], *Acinetobacter* Sp. [36] and many others utilize their Cyt P-450 for the initial activation of hydrocarbon degradation, yielding the corresponding alcohols [37].

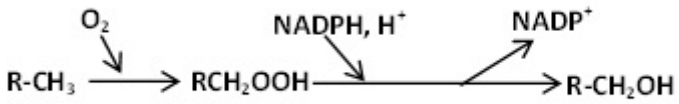
(Reaction 2) NADPH → Reductase (FAD and FMN) → Redoxin (FeS) → Cytochrome P450

$$RH + O_2 \xrightarrow{\text{Cyt-P450}} ROH + H_2O$$

Overall: $RH + NAD(P)H + O_2 + H^+ \rightarrow ROH + NAD(P)^+ + H_2O$

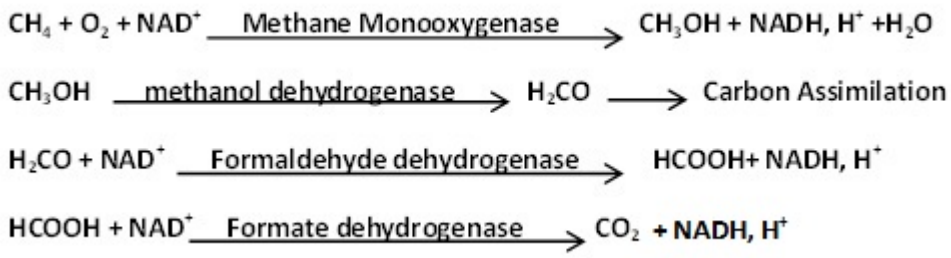
In eukaryotes, including fungi, endoplasmic nucleotide-dependent cytochrome P450 reductases (CPR) transfer two electrons from NADPH to cytochrome P450 which uses these electrons for a variety of molecular biotransformation reactions such as the detoxification or biosynthesis of secondary compounds [38]. For example this reaction, in *Saccharomyces cerevisiae* is used in the ergosterol biosynthesis pathway and for the metabolism of many polyaromatic hydrocarbons such as benzo[a]pyrene [38].

In addition to these two prototype reactions for the oxidation of alkanes, there are some other types of alkane metabolism which can be seen in special microorganisms. Through an alternative terminal pathway some yeasts such as *Mortierella* sp. utilize alcohol oxidases instead of alcohol dehydrogenases, producing either or both ω-hydroxic acids and α-ω,dioic acids (figure 1) [39, 40]. Also, some bacteria such as *Bacillus* sp. and *Rhodococcus* sp. and some fungi such as *Fusarium* sp. and *Aspergillus* sp. are able to oxidize sub-terminally the n-alkanes to the 4-, 5- or 6-ol products and to a lesser amount the 2- and 3-ols. These alcohols are further oxidized by (pyridine nucleotide-linked) dehydrogenases into their corresponding aldehyde and fatty acids. The resulting fatty acids may enter a β-Oxidation process or may be inserted into the cell membrane [41]. In addition to these oxidative reaction in which only one atom oxygen is used, the Finnerty pathway is a process in which dioxygenase systems are able to transform n-alkanes first into their corresponding hydroperoxides and then into the corresponding alkan-1-ol (reaction 3) [42]. In this pathway, microorganisms oxidise initially n-Alkanes to the corresponding hydroperoxide and later reduce the intermediate by NADH, H^+ to alcohol [42]. In addition to alcohol, some microorganisms convert the hydroperoxide intermediate into the corresponding aldehyde. For instance, *Acinetobacter* sp. M-l is able grow rapidly on high molecular weight alkanes (Cl3 to C44) through oxidation via a n-alkane dioxygenase [43].



(Reaction 3)

Methanotrophic bacteria oxidize CH_4 at the expense of O_2, producing CO_2 molecules that are excreted from the cells to the environment [44]. Methane monooxygenases (MMOs) are three-component enzymes, which are classified into membrane-associated particulate copper-dependent (pMMO) and soluble non-heme di-iron monooxygenases (sMMO) based on their solubility, sensitivity to cyanide and dependency on copper, [45]. Due to the intensive oxidizing potential of the active site of the enzyme, in addition to methane, this enzyme is able to catabolize several different (linear and cyclic) substrates [46]. The reductive potential of NAD(P)H assists the enzyme to split the covalent bond in O_2, incorporating it into methane structure to produce methanol. The other oxygen atom is combined with two hydrogen atoms to make a H_2O molecule [46].

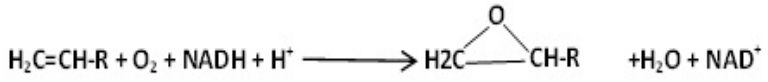


It is an important fact that most of the alkane degrading enzymes have a wide substrate range. This wide spectrum of substrate degradation ability is not just restricted to the common alkane monooxygenases as other monooxygenases, including the cytochrome containing monooxygenases and dioxygenases are able to degrade a large variety of substrates, too [30, 47]. Furthermore, each microorganism may express more than one type of monooxygenases. *Pseudomonas putida* GPo1, for instance, produces a complex of a non-heme membrane associated monooxygenase (alkB), a soluble rubredoxin protein (alkG) and a rubredoxin reductase (alkT) that can catalize the initial step for the degradation of C5 to C12 *n*-alkanes [48].

4) Metabolism of alkenes

Unsaturated hydrocarbons (alkenes) are more sensitive than alkanes and aromatic hydrocarbons to degradation due to the higher reactivity of their double bonds [49]. Biodegradation of unsaturated aliphatic hydrocarbons (C2-C6) has been seen in a large variety of bacterial divisions [50-53]. In the same way as for alkane monooxygenase, several microorganisms can insert an oxygen atom into an alkene substrate via a monooxygenase activity, producing an epoxide molecule (figure 2) [54].

Epoxidation is a process in which O_2-/NADH-dependent monooxygenases, namely alkene monooxygenases (AMOs), add an oxygen atom to the double bonds of the unsaturated aliphatic hydrocarbons (reaction 4) [55]. Biochemically, based on a hypothesis by Weijers and colleagues [56], a nucleophilic attack to the C1 or C2 of epoxides (1,2-epoxyalkanes and 2,3-epoxyalkanes, respectively) by the sulfhydryl group of an enzyme opens the epoxide ring and yields a β-hydroxythioether intermediate. Then, transfer of hydrogen from C-2 to NAD^+ converts the β-hydroxythioether to its corresponding β-ketothioether, which further undergoes a carboxylation attack to the terminal carbon by an epoxide carboxylase.

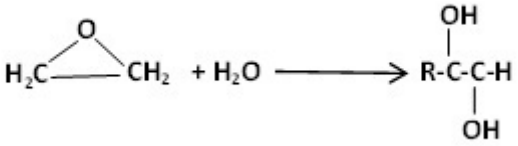


(Reaction 4)

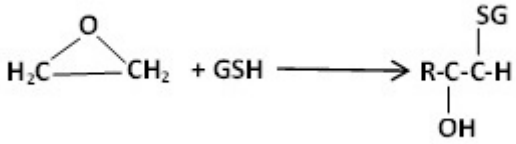
AMOs, like MMO, are multi-component enzymes, three in *R. rhodochrous* B-276 and four in *Xanthobacter* Py2, belonging to the diiron oxygenase family with a highly conserved (binuclear or four nuclear, respectively)-iron core that interacts with Glutamate and Histidine residues [55, 57]. This core binds the O_2 molecule necessary for the hydroxylation and epoxidation of a broad range of unsaturated alkenes as well as a variety of chlorinated alkenes, such as tri-chloro-ethene, vinyl chloride and 1,3-dichloropropene [55, 58].

Some other alkene-utilizing bacteria produce flavin active-centre monooxygenases which are used especially for the production of chiral epoxides, particularly those compounds with aromatic substituents [57]. *Rhodococcus* sp. ST-5 and ST-10 and several *Pseudomonas* strains use a styrene monooxygenase (SMO) to epoxide the vinyl side-chain of styrene. This system is a two-component FAD-associated enzyme comprising of a flavin-oxidoreductase unit (SMOB) and a monooxygenase unit (SMOA) [59]. In addition, some bacteria use cytochrome P450 system to insert an oxygen atom, obtained from molecular oxygen) into alkenes to form epoxides [60, 61]. *Olaromonas* sp. strain JS666 for instance, uses cytochrome P450 monooxygenase to trigger the oxidation of *cis*-1,2-dichoroethene (*c*DCE) in aerobic conditions [62].

Although the broad substrate activity of alkene degrading bacteria is beneficial for remediation purposes, there are some limitations for their application. Sometimes the products of these enzymes are xenobiotic for themselves, requiring other enzymes to mineralize these resulting compounds [63]. A bioremediation process will be most promising if the substrates mineralise completely to simple molecules such as CO_2 and H_2O [64, 65]. Furthermore, in some cases, the product of these enzymes, especially epoxides, are toxic for the enzyme itself and other cellular proteins [66]. These products are able to inactivate irreversibly the enzyme involved in the initial reaction of the biodegradation process and to interact with the other biological contents of cells (such as DNA, lipids and proteins, leading to their inactivation [66, 67]. Oxidation of trichloroethylene (TCE) by an alkane monooxygenase, produced by *Rhodococcus corallines*, yields a trichloroethylene epoxide, which is able to bind covalently both to the enzyme and to other cellular proteins [66]. Therefore, those hydrocarbon degrading organisms that utilise epoxidation mechanisms have to neutralize these toxic compounds. In a manner similar to mammalian systems, several microorganisms use an oxide hydrolases or glutathione-S-transferases (GSTs) to neutralize epoxides via the addition of a water molecule or a glutathione originated hydrogen, respectively (reactions 5 and 6) [68, 69].



(Reaction 5)



Reaction 6)

In a completely different pathway, some bacteria use carboxylases for CO_2 assimilation through degradation of the epoxides, yielding corresponding β-keto acids. Growth of *Xanthobacter* Py2 in a media containing propene as the sole source of carbon, for instance, leads to production of epoxypropane, which is subsequently converted to acetoacetate via a CoM-dependent pathway [70]. In this pathway the resulting (S or R) epoxypropane is activated by a CoA and after assimilation of one CO_2 molecule through two reactions catabolized by a NAD dependent

dehydrogenase and a NADP-dependent oxydoreductase is converted first to 2-ketopropyl-CoM and finally to acetoacetate, respectively (reaction 7) [71].

(Reaction 7)

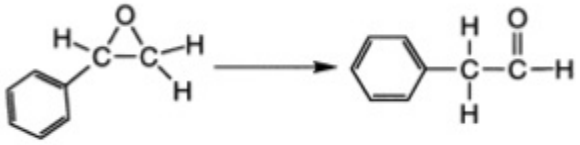
Propene + NADH, H^+ + O_2 → (R or S)epoxypropane + NAD^+

(R or S)epoxypropane + CoM → 2-(R or S)epoxypropyl-CoM

2-(R or S)epoxypropyl-CoM + NAD^+ → 2-Ketopropyl-CoM + NADH,H^+

2-Ketopropyl-CoM + CO_2 + NADPH → Acetoacetate + NADP

Since this reaction is regulated by CO_2, in the absence of this key molecule the catabolising bacteria use another strategy in which isomerases convert the epoxides to nontoxic compounds such as ketones and aldehydes [70]. In the case of the degradation of styrene by *Rhodococcus opacus* 1CP, for instance, Styrene oxide isomerase (SOI) converts the emerged styrene oxide to phenyl-acetaldehyde (reaction 8) [72]. Furthermore, growth of *Xanthobacter* strain Py2 in a media containing butylene-1,2-oxide or propylene oxide leads to production of methylethyl-ketone and acetone, respectively (reaction 9) [56]. Also, spontaneous and slow degradation of the produced epoxides can sometimes yield their corresponding diol [73].



(Reaction 8)



(Reaction 9)

5) Branched chain alkanes

Biodegradation of branched chain alkanes, especially l3-branched (anteiso-) hydrocarbons, quaternary compounds and isoterpenoids is more difficult than for simple alkanes and alkenes, and most bio-degraders resort to complete degradation of these molecules [74, 75]. Although most investigators reported the branched chain alkanes to be very recalcitrant compounds, microorganisms are able to degrade these compounds [76].

Many microorganisms express an inducible enzymatic system that hydroxylates the terminals of different kinds of branched hydrocarbons to produce their corresponding acids or ketones [76-78]. For instance, isoprene (2-methyl-1,3-butadiene) is initially oxidised by a monooxygenase expressed by isoprene degrading bacteria, such as *Rhodococcus* AD45, in which the methyl-substituted double bond is epoxidised to produce 1,2-epoxy-2-methyl-3-butene [76, 79]. The epoxide ring of this intermediate molecule is opened by glutathione-*S*-transferase to produce 1-hydroxy-2-glutathionyl-2-methyl-3-butene (HGMB). Further oxidation of the terminal hydroxyl group by dehydrogenases yield an organic acid referred to as 2-glutathionyl-2-methyl-3-butenoic acid (GMBA) [77, 79].

Many highly branched hydrocarbons, such as pristane (2,6,10,14-tetramethylpentadecane), farnesane (2,6,1O-trimethyldodecane), norpristane (2,6,1O-trimethylpentadecane) and phytane (2,6,10,14-Tetramethylhexadecane) are initially activated by a hydroxylation mechanism [80]. Pristane is a highly persistent isoprenoid alkane found in microorganisms and also higher organisms, which is derived from the degradation of tocopherols at high temperatures and also from the breakdown of methyl-trimethyl-tridecylchromans [81]. This compound is sensitive to enzymatic degradation by many microorganisms such as *Rhodococcus ruber* and *Mycobacterium neoaurum*, and is degraded through either or both of ω-hydroxic acids and α-ω-dioic acids, yielding terminal mono or di-carboxylicic acids (figure 3) [78].

6) Cycloaliphatic compounds

Several types of cycloaliphatic hydrocarbons such as cyclopentane, methylcyclopentane and cyclohexane are found extensively in crude oils and some petroleum products [82]. In addition to these oil originated compounds, alicyclic hydrocarbons are released into the environment via different natural activities, including through microbial or plant production of terpenoids and lipids[83, 84], and artificial manmade activities, such as production of nylons, flavours, herbicides, insecticides and solvents [85]. Although these compounds are hardly degraded by microbial activities, due to the low solubility of these hydrocarbons and their toxic effects on biological systems, many bacteria are able to metabolise these hydrocarbons through an oxidative pathway, converting these compounds to adipic acid [82, 86].

Biochemical and genetic studies on the degradation of cyclohexane by *Acinetobacter* sp. showed involvement of five enzymes encoded by two operons (chn BER ORF and chnADC ORF). These two operons are located in the chromosome in an opposite direction to each other and are regulated by a regulatory protein encoded by *chn*R). After hydroxylation of cyclohexane by cyclo-hexanone monooxygenase (encoded by *chn*B) at the expense of NAD(P)H and O_2, the produced cyclohexanol is converted by NAD(P)H-dependent aldehyde dehydrogenase (encoded by chnE) to cyclohexanone in which a NAD(P) is reduced to NAD(P)H (figure 4). Incorporation of one atom of oxygen into the ring of the resulting cyclohexanone by cyclohexanone monooxygenase (encoded by chnA) through oxidation of NADPH and reduction of O_2 to H_2O converts this intermediate to caprolactone. After breaking the ring through insertion of one molecule of H_2O into the ring via caprolactone hydrolase (encoded by chn C), the produced 6-hydroxy hexanoic acid is further oxidized by 6-hydroxyhexanoic acid dehydrogenase (encoded by chnD) to 6-oxohexanoic acid and then by NAD(P)+-dependent aldehyde dehydrogenases to adipic acid [82, 87].

Rhodococcus ruber SC1 oxidizes cyclododecane as its sole source of energy and carbon in a way analogous to the biodegradation of cyclohexane (figure 5) [88]. A monooxygenase initiates oxidation of the compound in which the bacterium converts cyclododecane to cyclododecanol at the expense of one NAD(P)H and one O_2. A similar process involving a NAD(P)-dependent cyclododecanol dehydrogenase oxidizes the resulting cyclododecanol into cyclododecanone. From this point, the products of a cluster of four genes (*cddABCD)* promote the oxidation process of cyclododecanone: a monooxygenase (encode by *cdd*A) inserts an atom oxygen into the ring and converts the emerged cyclododecanone to lauryl lactone, which in turn accepts one molecule of H_2O as a result of the action of lactone hydrolase (encoded by *cdd*B) to produce an acidic intermediate (12-hydroxy lauric acid).

Two NAD(P) dependent dehydrogenases, namely 12-hydroxylauric acid dehydrogenase (encoded by *cdd*C) and 12-oxolauric acid dehydrogenase (encoded by *cdd*D) convert this intermediate to 12-oxolauric acid and finally to DDDA (dodecanedioic acid) [89].

Although several microorganisms are able to independently degrade cycloaliphatic compounds to simpler molecules, these compounds can also be catabolised by a mixed community of microorganisms through a mechanism referred to as co-metabolism [90, 91]. A consortium of *Rhodococcus* spp., *Sphingomonas* sp. and *Stenotrophomonas* sp., for instance, use cyclohexane as their sole source of energy and carbon [90]. Also, a consortium of *Rhodococcus* sp. (80 to 90% of the population), *Pseudomonas cepacia* and *Flavobacterium* sp. (together, 10-20% of the population) has been reported with the ability to completely degrade cyclohexane to CO_2 and H_2O. In this last consortium hydrocarbons are initially oxidised by a monooxygenase to alcohols or ketones, which are then consumed by other members of the community [91].

7) Anaerobic hydrocarbon biodegradation

Microbial degradation of many different substrates, especially recalcitrant hydrocarbons, is very limited under anaerobic conditions, due to a requirement for molecular oxygen as the final electron acceptor, slowing down these biological activities [92]. However, based on a large variety of studies on microorganisms and the G_0 values of the anaerobic degradation reactions, anaerobic metabolism of hydrocarbons by microorganisms is energetically possible [93, 94]. Slow degradation of these molecules by anaerobic bacteria provides the required energy and carbon sources for the growth of other catabolizing microorganisms and methane that is used by many other aerobic organisms, including some animals, that are dependent on methane oxidising microorganisms [95]. A large diversity of facultative and obligatory anaerobic microorganisms (bacteria and archaea) have been isolated with the ability to anaerobically degrade hydrocarbon molecules. These microorganisms utilize anaerobic respiration (using nitrate, nitrite, nitrous oxide, sulphate, thiosulfate, carbonate and metal ions), or fermentation or anoxygenic phototrophic reactions, for the donation of electrons and hydrogen for substrate catabolism activities [52]. For anaerobic microorganisms, both sulphate and nitrate reducing bacteria, shorter chain length alkanes are more recalcitrant than mid- to long- chain length alkanes [96]. Due to the lack of evaporation of short length hydrocarbons (up to n-C17) in anaerobic conditions, these compounds can build up and exert a toxic effect on the cell membranes of microorganisms, inhibiting their degradation [96]. Furthermore, for sulphate reducing bacteria degradation of branched alkanes, such as pristane and phytane, is more efficient than for normal alkanes [96].

Anaerobic hydrocarbon decomposition is usually found in deep and anoxic conditions such as natural oil and gas leakages/seeps on land or the sea floors and those sites polluted with oil or its by-products [94]. Furthermore, this type of biodegradation can occur below the surface of areas exposed to oxygen where aerobic biological activity ceases as all the oxygen is utilised [97]. In most cases, degrading activity of aerobic microorganisms in a low gradient of oxygen limits oxygen availability, leading to a succession of anaerobic hydrocarbon degrading microorganisms [97]. After oxygen exhaustion, there is a sequential employment of the electron acceptors (nitrate, ferric iron, sulphate and H_2 subsequently) to supply the highest potential energy from the degradation of hydrocarbons [98, 99]. Indeed, in this microbial succession the changes are based on the available energy and

electron acceptor systems, beginning with nitrate and nitrite reducing microorganisms, followed by metal ($Mn^{+}5$ or $Fe^{+}3$, U^{+6}) reducing bacteria, sulphate-reducing bacteria and finally methanogenic microorganisms [99].

Denitrifying bacteria. These hydrocarbon degrading bacteria belong to many different microbial phyla, mainly to the Betaproteobacteria (order Rhodocyclales and genera *Azoarcus*, *Georgfuchsia*, *Thauera* and *Aromatoleum*), *Castellaniella defragrans* (a species belonging to the family Alcaligenaceae and the order Burkholderiales) and *Magnetospirillum* (belonging to the Alphaproteobacteria) [100-102], and degrade different aliphatic and aromatic hydrocarbons at the expense of nitrate reduction (reaction 10) [102].

(Reaction 10) $\mathbf{5\ Hexadecane\ (C_{16}H_{34}) + 98\ NO_3^- + 18H^+ \rightarrow 80\ HCO_3^- + 49\ N_2 + 54\ H_2O}$

Sulphate-reducing microorganisms. Many of these bacteria, including Deltaproteobacteria (like *Desulfobacula toluolica*, *Desulfococcus oleovorans*, *Desulfatibacillum alkenivorans*, *Desulfosarcina cetonicum*, *Desulfoglaeba alkenexedens*, *Desulfatibacillum aliphaticivorans*) [103], *Desulfotomaculum* [104] and *Archaeoglobus fulgidus* (an archeon) [105] are able to degrade long/short chain saturated/unsaturated aliphatic hydrocarbons, non-substituted aromatics (such as naphthalene and benzene) and alkyl-benzenes (such as like xylenes, toluene and ethylbenzene). *Desulfatiferula* strains BE2801 and LM2801T [52] and *D. aliphaticivorans* strain $CV2803_T$ [106] Sulfate reducing bacteria oxidise the double bond of 1-alkenes, converting them to the corresponding 1-alkanols and further to fatty acids and finally to CO_2 (Reaction 11). Hydratase, a reversible enzyme with the ability to catalyse the rearrangement of hydrogen and hydroxyl groups on double bonds, plays a crucial task in the production of secondary alkanols (substitution of -Ol on C2-C5) from 1-alkenes [107]. In addition to the oxidative attack, *D. aliphaticivorans* strain $CV2803_T$ [106] is able to add an organic residue (such as methyl or ethyl) to alkanes and alkenes, yielding the corresponding methyl/ethyl- fatty acids [49].

(Reaction 11) $\mathbf{4\ Hexadecane\ (C_{16}H_{34}) + 49\ SO_4^{2-} + 34\ H^+ \rightarrow 64\ HCOO^{3-} + 49\ H_2S + 4H_2O}$

Metal oxidizing microorganisms. In addition to nitrogen and sulphur containing inorganic compounds, some bacteria are able to use other inorganic compounds as electron recipients while metabolising hydrocarbons. For instance, *Geobacter* and *Georgfuchsia* reduce metal-ions (Fe(III), Mn(IV) or U(VI)) through anaerobic degradation of hydrocarbons [102, 108]. In addition to these genera, a toluene degrading bacterium (strain G5G6), belonging to the Betaproteobacteria is able to mineralize toluene completely to CO_2 while utilizing Fe^{3+}, Mn^{4+} and NO^{3-} as its terminal electron acceptors [102]. Furthermore, *Dechloromonas aromatica* RCB [109] and strain Y5 [110] oxidize toluene in reactions coupled to (per)chlorate and arsenate, respectively. Since sites contaminated with oil products may contain arsenic in addition to aromatic compounds, this ability of microorganisms to degrade aromatics in association to As(V) reduction is beneficiary in terms of environmental remediation [110].

Intra-aerobic anaerobes. This category of microorganisms are able to extract oxygen from chlorate, perchlorate, nitric oxide (NO) or nitrate in order for it to be utilized as an electron acceptor [111, 112]. *Alicycliphilus denitrificans* and *Pseudomonas chloritidismutans*, for instance, possess two periplasmic molybdenum-containing enzymes, chlorate reductase and perchlorate reductase, which enable them to reduce chlorate and perchlorate to

chlorite. Chlorite, in turn, is immediately reduced by a heme-containing enzyme (chlorite dismutase) into Cl_2 and O_2 (reaction 12) [112, 113].

(Reaction 12) $\mathbf{ClO^{2-} \rightarrow Cl^- + O_2}$ $\mathbf{(\Delta G^{o/} = -100\ kJ\ mol^{-1}\ O_2)}$

Furthermore, several intra-aerobic-anaerobe bacteria are able to employ a denitrification pathway for obtaining molecular oxygen by breaking nitric oxide or nitrate down to N_2 and O_2 (reaction 13). The extraction of O_2 by denitrifying bacteria emerged through investigations on the oxidation of methane by a pure culture of a methane oxidizing microorganism (*Methylomirabilis Oxyfera*) in which the catabolism of methane to CO_2 is associated with nitrate reduction (reaction 13 and 14) [114].

(Reaction 13) $\mathbf{2NO_2\text{-} \rightarrow N_2 + O_2}$ $\mathbf{(\Delta G^{0/} = -173kJ\ mol{-}1\ O_2)}$

(Reaction 14) $\mathbf{3\ CH_4 + 8\ NO^{-2} + 8\ H^+ \rightarrow 3\ CO_2 + 4\ N_2 + 10\ H_2O}$ $\mathbf{(\Delta G^{o/} = -928\ kJ\ mol{-}1\ CH4)}$

Methanogenesis. The production of methane and CO_2 from the anaerobic degradation of squalene (a methyl-branched alkene with formula $C_{30}H_{50}$) and 1-hexadecene was first described by Schink [115]. Alkenes are initially activated by addition of a H_2O to the double bond or by addition of a hydroxyl to the carbon atom adjacent to the unsaturated bond (reaction 15) [52]. In the case of hexadecane, this compound is first converted to hexadecan-1-ol and after oxidation to hexadecanoic and palmitate, which are broken down to acetate units and are finally catabolised to methane (reaction 16) [91].

(Reaction 15) **Palmitate:** $\mathbf{C16H32 + 12\ H20 \rightarrow 12\ CH4 + 4HCO^{3-} + 4H^+}$

(Reaction 16) **Hexadecan:** $\mathbf{4(C_{16}H_{34}) + 45\ H_2O \rightarrow 49\ CH_4 + 15HCO^{3-} + 15H^+}$

Several anaerobic microorganisms are able to mineralize hydrocarbons to methane as part of microbial syntrophic consortia. Such a consortium can be seen in the syntrophic relationship between methanogenic archaea and hydrogen-producing bacteria for the degradation of aromatic hydrocarbons to methane and CO_2. In this cooperation, the H_2 generated by special hydrocarbon degrading microorganisms, such as *Smithella* sp., *Syntrophus* sp. and *Desulfotomaculum* sp. is used for reduction of CO_2 by methanogenic archaea to produce methane (reactions 17 and 18) [116, 117].

(Reaction 17) $\mathbf{Toluene + 14\ H_2O \rightarrow 7CO_2 + 18\ H_2}$

(Reaction 18) $\mathbf{4H_2 + CO_2 \rightarrow CH_4 + 2H_2O}$

Anoxygenic sulphate dependent phototrophic bacteria. In addition to the anaerobic respiratory microorganisms, some anoxygenic sulphate dependent phototrophic bacteria (such as *Blastochloris sulfoviridis)* [108] and fermentative prototrophs (such as *Pelobacter acetylenicus* belonging to Deltaproteobacterium) [118] have been isolated that are able to degrade hydrocarbons anaerobically. These bacteria comprise only a small population of a microbial community; for instance, toluene degrading phototrophs comprise only 1% of the

cultivable lithotrophic microorganisms. Because phototrophic microorganisms obtain their energy from light, the whole organic substrate is probably converted into cell components ($C_4H_8O_2N$) (reaction 19) [108].

(Reaction 19) $(17C_7H_8 + 25HCO^{3-} + 36\ NH_4^+ \rightarrow 36C_4H_8O_2N + 3H_2O + 11H^+)$

8) The Biochemistry of Anaerobic Hydrocarbon biodegradation

Anaerobic biodegradation pathways are more diverse than aerobic catabolism and can be divided into 5 groups, including addition of a fumarate to methylene or methyl groups of hydrocarbons [119], Oxygen-independent hydroxylation on 2nd or 3rd terminal C-atoms (to make secondary or tertiary alcohols) [120], Carboxylation of unsubstituted carbon atoms of aromatics [121], Hydration of the double and triple bond of alkenes and alkynes [122] and Reverse methanogenesis [123].

8.1) Fumarate Addition Reactions

A variety of anaerobic hydrocarbon degraders, such as sulphate reducing bacteria, denitrifying microorganisms, iron-reducing bacteria, methanogenic consortia and anaerobic phototrophic microorganisms add a carboxyl group to a hydrocarbon in order to achieve greater degradation [124-126]. The target carbon atom for the addition of the carboxyl group is different depending on the microbial species; C1 in SRBs, C1, C2 and C3 in *Desulfatibacillum aliphaticivorans* [106].

The anaerobic degradation of toluene via fumarate is an additional system that has been widely studied in SRBs and denitrifying bacteria, which is used as a prototype example of this kind of metabolic pathway (figure 6) [127]. First, the addition of a fumarate by oxygen-sensitive benzylsuccinate synthase (BSS) enzyme converts toluene to (R)-benzylsuccinate. In this reaction, removing one atom of hydrogen from the methyl group of toluene converts the molecule to a benzyl radical intermediate. This radical is very unstable and attacks the unsaturated bond of a fumarate molecule regaining the hydrogen atom (associated with the enzyme) to form a benzylsuccinate. The benzylsuccinate is then substituted with succinyl-CoA in preparation for further oxidative processes. Throughout this reaction, a succinate is released, which is converted (by succinate dehydrogenase) to fumarate in order to regenerate the fumarate source. Succinyl-CoA functions as CoA-SH donor and is added to the complex via a CoA transferase enzyme (BbsEF) in order to activate benzylsuccinate. Finally, through some β-Oxidation processes, a benzyl-CoA is produced and the succinyl-CoA is released for continuing the cycle. Benzoyl-CoA is the critical intermediate in the anaerobic catabolism of aromatic hydrocarbons that is further oxidized via breaking the aromatic ring (by benzoyl-CoA reductase) thereby converting it to aliphatic compounds that in turn are attacked by further hydrolytic and oxidative reactions, such as β-oxidation. A similar reaction has been seen in the degradation of xylene, o-toluidine and the cresol isomers by SRBs [127, 128].

Also, many SRBs and denitrifying bacteria are able to use this system for the degradation of a broad spectra of alkanes and cycloalkanes, from three to twenty carbons, depending on the bacterial species (figure 7) [129, 130]. This reaction is initiated by substitution of a fumarate to the sub-terminal methylene carbon of the alkanes, and thereby converts the straight chain hydrocarbon to a branched compound (1-methylalkyl- succinate). After substitution of a CoA-SH to the terminal methyl group of the branched methylalkyl-succinate by succinate-CoA,

the terminal methyl CoA is transferred to one carbon before the branch to generate a 4-methyl-branched fatty-acyl-CoA molecule. This intermediate is easily catabolized to one propionyl-CoA and acetyl-CoA units via the β-oxidation reaction. The produced propionyl-CoA is used to recharge the fumarate supply of the pathway [131, 132].

The similarities between the small subunit of the enzyme involved in the addition of the carbon group to the alkane with the corresponding enzyme involved in the anaerobic activation of toluene indicated a similar process for the activation of aliphatic and aromatic compounds in anaerobic conditions [133]. Furthermore, based on sequencing studies, the genes involved in this process for both denitrifying bacteria and SRB (ass-1 and ass-2 operons) are the same as for other fumarate-adding enzymes [103].

8.2) Oxygen-Independent Hydroxylation

Although ethylbenzene is mainly degraded through the fumarate addition pathway, several denitrifying bacteria use an anoxic hydroxylation reaction in which a periplasmic ethylbenzene dehydrogenase (EBDH) hydroxylates the terminal carbon of the side chain of this molecule to produce S-1-phenylethanol (figure 8) [134]. This enzyme is a soluble periplasmic molybdenum-containing enzyme belonging to the DMSO reductase family [134] with three different subunits (α, β and γ). Two of the subunits (αand β) are homologous to the *Escherichia coli* nitrate reductase) in terms of protein structure and amino acid sequences [135]. In addition, the active site of this enzyme contains five iron-sulphur compounds (αand β in subunits) and a heme b cofactor (placed in γ subunit)[135]. After the movement of the resulting (S)-1- phenylethanol through the cell membrane via passive diffusion [136], the molecule is firstly oxidized to acetophenone (by NAD-dependent alcohol dehydrogenase) and then the product is carboxylated by acetophenone carboxylase (APC) to benzoyl-acetate [137, 138]. At the next step, a thioesteration process by benzoylacetate-CoA ligase (BAL) transfers a CoA to the molecule and the benzoyl-acetyl-CoA is finally is cleaved to benzoyl-CoA and acetyl-CoA units. *Aromatoleum aromaticum* PbN1 is a denitrifying bacterium with an ability to catabolize n-propylbenzene through the same biochemical shunt as is used in the degradation of ethylbenzene, leading to the production of benzoyl-CoA and propionyl-CoA [136].

8.3) Carboxylation

Many anaerobic bacteria use carboxylase enzymes at the first step of anaerobic hydrocarbon degradation to add a carboxyl group directly to both aliphatic, such as propylene, and aromatic hydrocarbons, such as benzene, phenanthrene, naphthalene and biphenyl, converting them to their corresponding carboxylate forms [138, 139]. Growth of *Xanthobacter* strain Py2 on propylene oxide is a clear example in which the bacterium assimilates CO_2 to utilize the substrate. In this reaction, *Xanthobacter* first isomerises the propylene to an enol or keto form of acetone and then binds the assimilated CO_2 to the acetone to produce acetoacetate, which in turn is broken down to two acetates [140]. Also, growth of *R. rhodochrous* B-276 on 2-propanol and acetone induce expression of an acetone carboxylase that adds an inorganic CO2 to acetone to yield an acetoacetate [141].

8.4) Unsaturated bond (Alkene and Alkyne) Hydration

Anaerobic degradation of alkene via a hydration reaction is a recently identified reaction in which microorganisms add a H_2O molecule to the hydrocarbon, converting it to a primary alcohol [106]. This mechanism was firstly

found in *Castellaniella defragrans* in which the bacterium produces a periplasmic enzyme, namely linalool dehydratase/isomerase (LDI), capable of catalysing reversibly the hydration of linalool hydrocarbon to an unsaturated hydrocarbon (myrcene) and of isomerization of myrcene to geraniol (figure 9). This geraniol is then transferred to the cytoplasm where it is oxidized to granate [142].

Some bacteria such as *N. rhodochrous and Pelobacter acetylenicus* are able to use hydrating reactions to mineralize several unsaturated alkenes and alkynes such as ethine and acetylene [92, 143]. This bacterium adds a water molecule to acetylene via an enzymatic reaction catalysed by acetylene hydratase and produces a C_2-enol intermediate that is immediately converts to acetaldehyde (reaction 20). Acetylene hydratase is an enzyme containing molybdenum, tungsten, Fe_4S_4 as well as guanine dinucleotide, belonging to the DMSO-reductase family [122]. The acetaldehyde is further oxidised to acetate, which is activated by CoA to be consumed in cellular metabolism [92, 143].

(Reaction 20) $\mathbf{C_2H_2 + H_2O \rightarrow CH_3CHO}$

$\mathbf{2\ CH_3CHO + 3H_2O \rightarrow H_3C{-}COO^- + C_2H_5OH + 5\ H^+}$

$\mathbf{H_3C{-}COO^- + ATP + CoA \rightarrow Acetyl\text{-}CoA}$

7.7.5) Reverse Methanogenesis

Anaerobic marine environments provide suitable conditions for a group of Mn(IV) and Fe(III) reducing bacteria that use a reverse methanogenic reaction as the initial step for the degradation of methane [144]. The final methanogenic reaction in all methanogens is catalysed by methyl-coenzyme M reductase (Mcr) that binds to coenzyme B (CoBSH) via methyl-coenzyme M (CoMSH) to produce a complex of CoM-S-S-CoB-heterodisulfide and methane. Several methanotrophic microorganisms use this enzyme for the initial activation of methane and take up the molecule in the form of Methyl-Co-M [123].

In addition to independent microorganisms, several consortia of SRB containing specialised archaea belonging to the methanosarcinales are able to use the reverse methanogenesis process [145]. The H^+ and electrons produced through oxidation of CH_4 by methanotrophs are transported to the SRBs in order to reduce sulphate (reaction 21). Although the mechanism in which the archaea are able to transfer the redox equivalents to their SRB partners is not fully understood, existence of an electron transport shuttle [146] or some end-products such as methyl-sulphides [147], formate [148] and acetate [149, 150] are hypothesised.

Reaction 21: $\mathbf{CH_4 + 3\ H_2O \rightarrow HCO_3^- + 9\ H^+ + 8\ e^-}$ *E in situ* = –0.289 V ($E^{o\prime}$= –0.239 V)

$\mathbf{SO4^{2-} + 9\ H^+ + 8\ e^- \rightarrow HS^- + 4\ H_2O}$ *E in situ* = –0.248 V ($E^{0\prime}$= –0.217 V).

Overall: $\mathbf{CH_4 + SO4^{2-} \rightarrow HCO_3^- + HS^- + H_2O}$

9) Application of hydrocarbon degrading enzymes in biotechnology

While some bacteria are killed directly by hydrocarbons and/or its products, some microbial populations are able to degrade these components as their nutritional source. These microorganisms may be commercially useful for

applications in several different processes such as the production of special enzymes and chemicals and for cleaning up hydrocarbon contaminants (crude oil and/or its products) from different environments [151].

1) Chemicals and enzymes

Several microorganisms are used for the production of solvent chemicals from the degradation of hydrocarbons. *Arthrobacter* sp., for instance, is able to produce a large quantity of routine solvents through the degradation of naphthalene and *n*-hexadecane [152]. Also, Methanotrophs have been abundantly used for the biotransformation of several organic chemicals (such as propylene to epoxypropane) and for the production of several biological compounds such as bioplastics, single cell protein (SCPs) and astaxanthin [153].

Furthermore, some of the hydrocarbon degrading enzymes expressed by microorganisms are potentially of use in biotechnology. Naphthalene dioxygenase (NDO), for instance, is the initial enzyme for the degradation of naphthalene and is used in several different industries for the production of some medicines such as Crixivan (a HIV protease inhibitor) [154], production of enantiopure arene *cis*-diols (prostaglandin E2 and polyphenylene) [155] and the conversion of indoles into indoxyls and glucose to inositols [156]. Furthermore, NDO is a crucial enzyme involved in the degradation of many aromatic hydrocarbons from soil and aquatic habitats and thus is considered as a useful enzyme in Biodegradation [157].

Cytochrome P450 monooxygenase is an enzyme utilised in several industries for both bioremediation and the synthesis and improvement of specific chemicals, pharmaceutical compounds and fragrances [27]. The p450 monooxygenase isolated from *Curvularia* sp, for instance, is currently used for biotransformation of Reichstein S to hydrocortisone [158, 159]. Further, the oxidation of Compactin by a p450 monooxygenase from *Actinomadura macra* leads to the production of an effective drug (Pravastatin) for the reduction of blood cholesterol level [160]. The production of erythromycin by *Saccharopolyspora erythraea* also requires a hydroxylation step performed by P450eryF (CYP107A1) [161].

2) Bioremediation

Environmental contamination resulting from the release of crude oil and its products is one of the more common global environmental issues. The increasing demands for oil products in this century have led to an intensification of environmental pollution [162]. Crude oil consists of a mix of various hydrocarbons, including alkanes and aromatics, which are categorized as serious and hazardous environmental pollutants [163]. The degree of toxicity of crude oil and its products depends on the chemical compounds present and their concentration [163, 164]. However, many microorganisms are able to use these molecules as their source of carbon and thus are able to decrease their toxicity [165]. Since oil contamination is a critical challenge for the environment, such studies are necessary for understanding the natural pathways involved in degradation of oil components and for the application of natural processes in oil bioremediation.

3) Biomarkers in oil biotechnology

Biomarkers are defined as those measurable activities or substances that represent a specific biological trait [166]. In terms of ecological investigations, biomarkers are used for tracking any biological activity of interest such as

the effects of toxic compounds or their assessment, source and distribution in an environment [166]. Since biomarkers are able to measure the biological activity of interest before any tangible changes occur, abundant research has been performed focussing on the discovery of new biomarkers. For this purpose, several new and emergent technologies, such as mass spectrometry, metabolomics, metagenomics, genomics and proteomics have been applied to find new markers or a combination of agents that may be useful biomarkers. In terms of oil biotechnology, biomarkers are used for identification of petroleum deposits, determining the quality of oil, the efficiency of oil bioremediation and to identify the source of oil spills in the environment [167]. Although oils are highly complex mixtures, varying from volatile molecules to very heavy aliphatic and aromatic hydrocarbons, their combination is unique from oil to oil (known as oil fingerprints) [168]. After the release of the mixture into an environment, its composition will change due to physical factors (dissolution, evaporation, degradation, photooxydation, oil–mineral aggregation (OMA), water–oil emulsification) and biological activities (biodegradation) [169, 170]. A few days after oil spilling, the rate of loss through evaporation reaches 5-10% for heavy oils, 40% for petroleum products and 70% for light crudes [171]. The biodegradation rate is mainly dependent on the nature of oil, the quantity of spilled oil, physical factors (such as temperature, pH and salinity) and the accessibility of nutrients, water and oxygen [172]. An aged degraded oil sample is almost exhausted of branched and shorter straight chain n-alkanes and the concentration of PAHs is also much reduced [173]. Some of the highly degradation-resistant molecules can be used for the analysis of the molecular changes that have occurred in the samples and for determining the fingerprints of sources [173]. The selection of a suitable biomarker for oil analysis is dependent primarily on the nature of oil, conditions of the environment and purpose of the study [170]. In general, these compounds can be categorized into Volatile hydrocarbons (especially BTEX , benzene, toluene, ethyl methylbenzene, and Xylene), saturated aliphatic alkanes (C8 through C40), isoprenoids (such as pristane, phytane, trimethyl-C13, norpristane and farnesane), volatile paraffins and isoparaffins, cycloalkanes (mainly cyclopentane and cyclo-hexane compounds), PAHs and their alkylated (C1–C4) homologues, total petroleum hydrocarbons, total unresolved complex mixtures (UCM), stable carbon isotope ratio ($d^{13}C$) and oxygen/nitrogen-containing heterocyclic hydrocarbons [167, 174]. In addition to measurement of individual molecules, the ratios of some special compounds such as n-Cls/phytane and n-C17/pristane are used for tracking the effects of weathering on the mixture through times. Although, due to a similar volatility, physical factors do not change the ratios of pristane/phytane, n-Cas/phytane, and n-C17/pristane, biodegradation decrease the n-C1s/phytane, and n-C17/pristane ratios since microorganisms preferentially use short chain alkanes [174]. Furthermore, since PAHs are biologically converted into their corresponding alkylated homologues compounds, comparison of these compounds with their parents is used for determining the extent of oil weathering in a polluted site [175]. Finally, since the amount of triterpanes and steranes are unique from oil to oil and because triaromatic steranes such as phenanthrene, dibenzothiophene and terpanes are more recalcitrant than other aromatic compounds they are used as effective biomarkers to investigate weathering events in oil and to track the origin of spilled oil [176, 177].

10) Conclusion

Hydrocarbons are a group of chemical compounds consisting only of carbon and hydrogen atoms linked with covalent bonds that make them highly stable. Due to a lack of functional groups, these compounds show a weak solubility in water. Since the cyclic aromatic hydrocarbons are considered to be resistant to degradation due to

their low reactivity, US EPA has classified these compounds as priority contaminants of natural resources. Because these molecules pose serious threat to the human and environmental health, understanding the catabolic pathways involved in the degradation of such molecules is of great research interest and also aids in designing efficient strategies for the bioremediation of hydrocarbon impacted environments. These persistent molecules affect niches of the habitat, thereby leading to the loss of biodiversity at both microscopic and macroscopic levels.

Although degradation pathways for many of both the aliphatic and aromatic hydrocarbons and several hydrocarbon degrading microorganisms from diverse phylogenetic groups have been identified, environmental contamination due to these compounds is still a big issue for environmentalists. Indeed, application of microorganisms or consortiums of different microorganisms with special abilities to degrade these compounds and even biotechnological approaches, have often not been successful in giving a significant reduction of these contaminants in a short time. This issue is worse when we have to clean promptly pollution in soils and aquatic environments caused by oil tank spills. In addition to the type of habitat (aquatic or terrestrial) and climate, this failure can be ascribed to nutrient deficiency and the insolubility of hydrocarbons in water. Therefore, the microbial inoculum used for bioremediation of the polluted sites has to be supported by a strategy to resolve the problems. The inoculum can be supplied with additional nutrients, inorganic nitrogen and sulphur for instance, to support microbial growth. Furthermore the addition of a surfactant producing microorganism or chemical biosurfactants can effectively resolve the unavailability of hydrocarbons due to their lack of solubility. The main problem in terms of unavailability is the presence of long chain hydrocarbons or their derivatives in the crude oil. Therefore, the microbial inoculum must contain those microorganisms with the ability to degrade these high molecular weight hydrocarbons. Although it is possible to construct genetically engineered organisms by combining the biosurfactant producing genes and all hydrocarbon degrading genes, including the genes responsible for degradation of short and long chain aliphatic hydrocarbons, branched hydrocarbons, cycloaliphatics and aromatics, the current rules for using genetic engineered microorganisms (GEM) prohibit commercial utilization of these microorganisms at least in near future. As a result, application of a consortium of microorganisms will be more effective and rational if each microorganism in this consortium is chosen based on its ability for the degradation of a special type of hydrocarbon or for facilitating this degrading activity. We hope this review about the existing knowledge on degradation of different types of hydrocarbons by microorganisms will aid the researchers to further design and improve the technologies for bioremediation of the contaminants.

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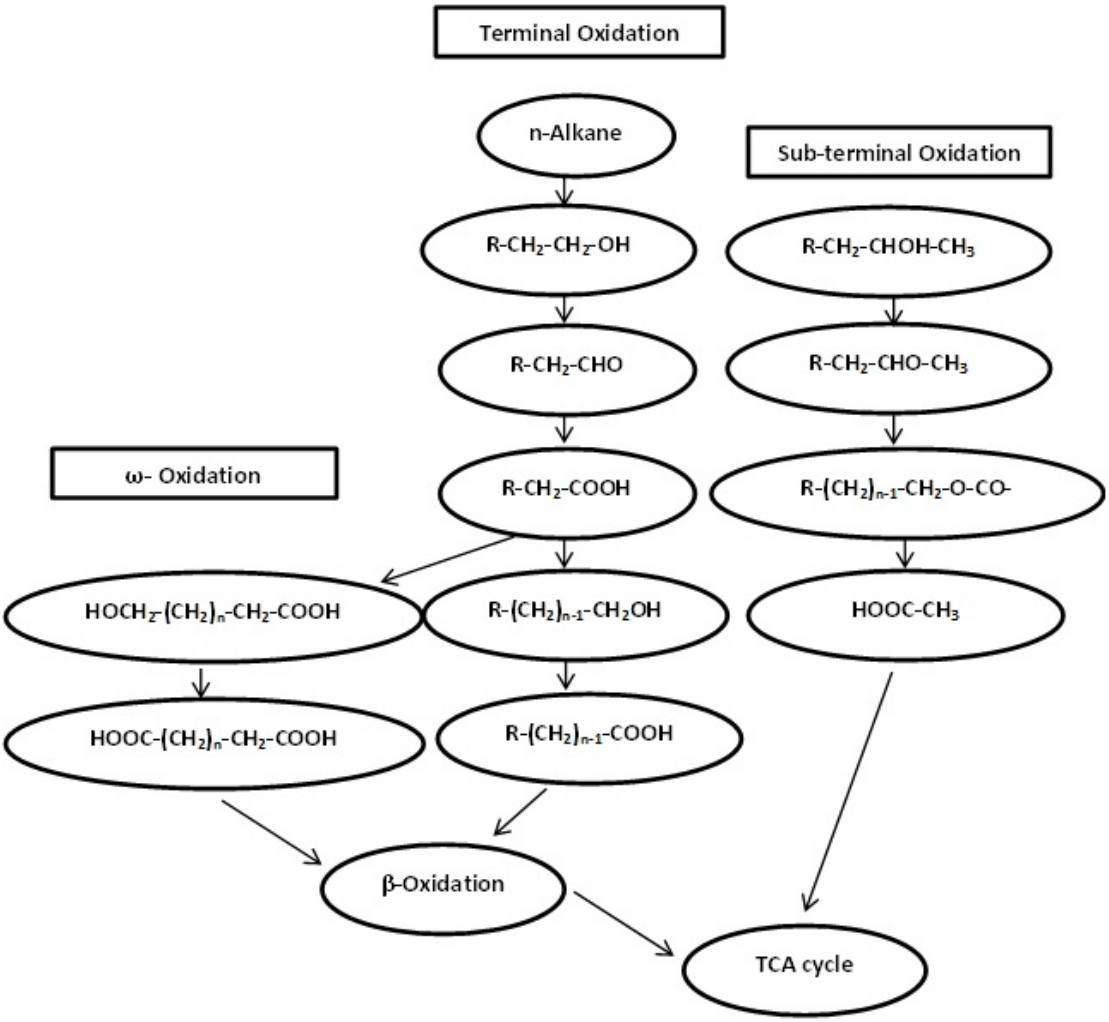


Figure 1. Three possible n-Alkane degradation pathways

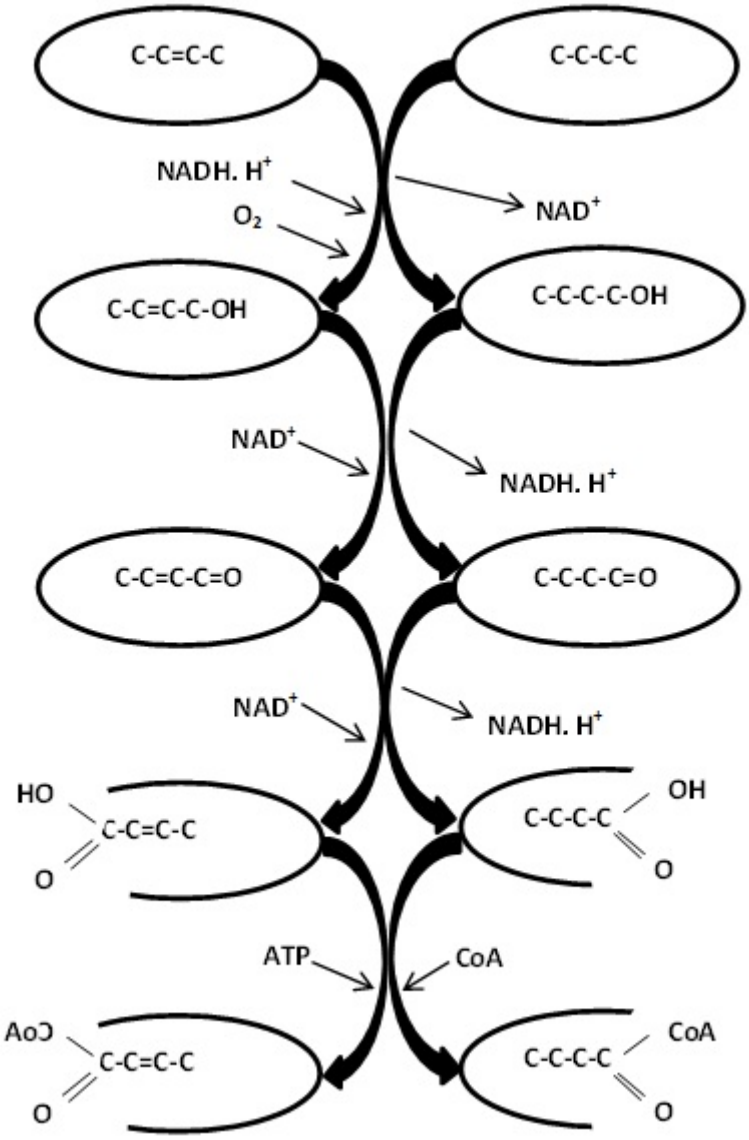


Figure 2. Biodegradation of butane and butene. The figure shows that both saturated and un-saturated C4 molecules are activated by a same pathway.

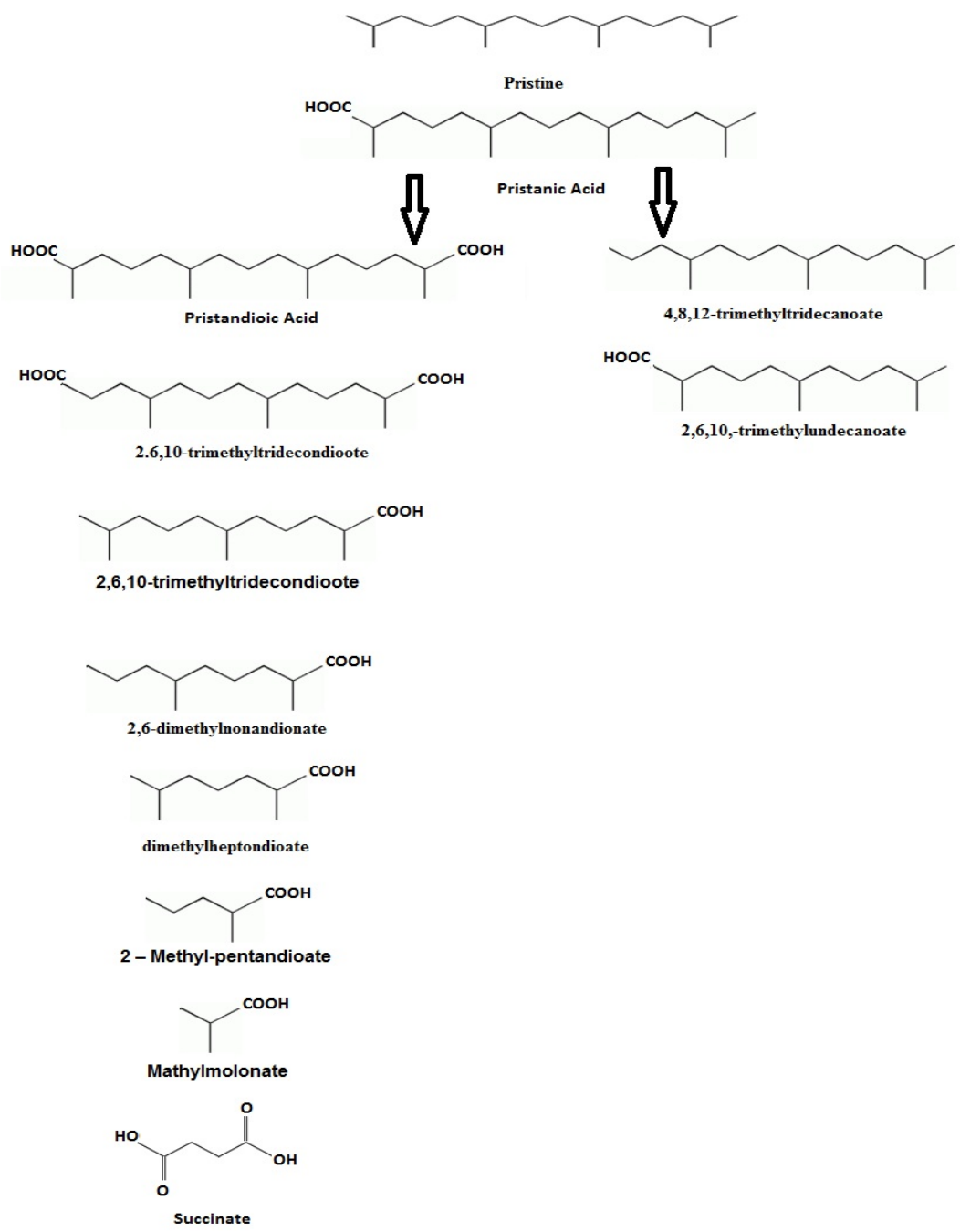


Figure 3. Biodegradation of Pristane. The resulting pristanic acid can pass through two different pathways. Pristandioic acid is degraded to succinate, which is one of critical intermediates in microbial metabolism. The fate of 2,6,10-trimethylundecanoate is not clear yet.

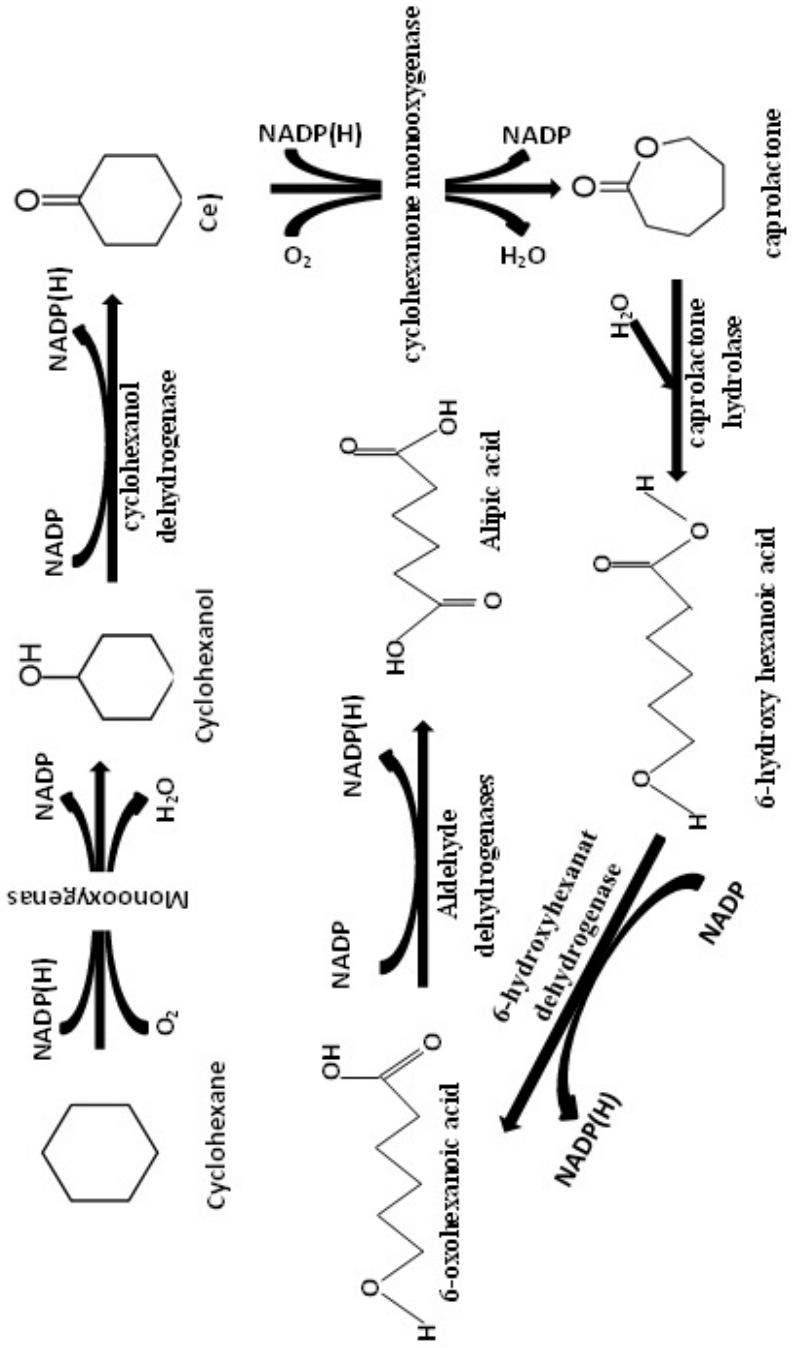


Figure 4. Biodegradation of Cyclohexane. The molecule is linearized through insertion of one atom of oxygen into the ring. The emerged 6-hydroxy hexanoic acid is oxidized through two NADP dependent oxidation to Alipic Acid.

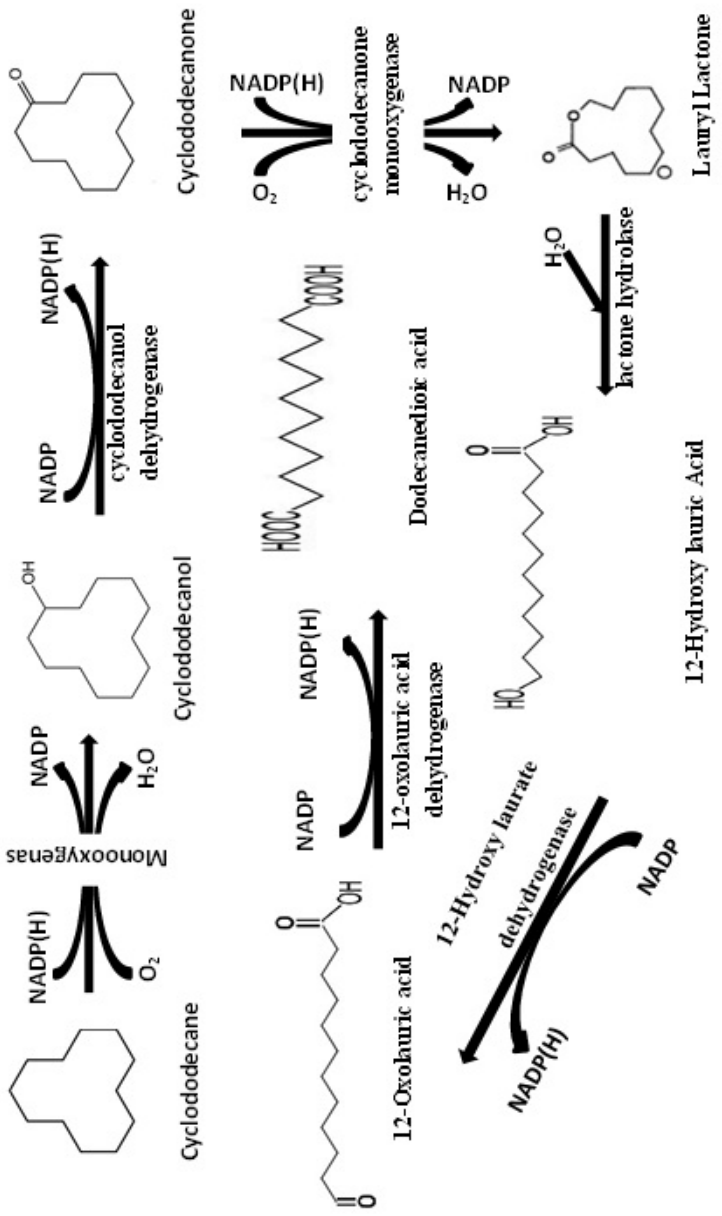


Figure 5. Conversion of a Cyclododecane to dodecandioic acid (DDDA) in *Rhodococcus* ruber CD4

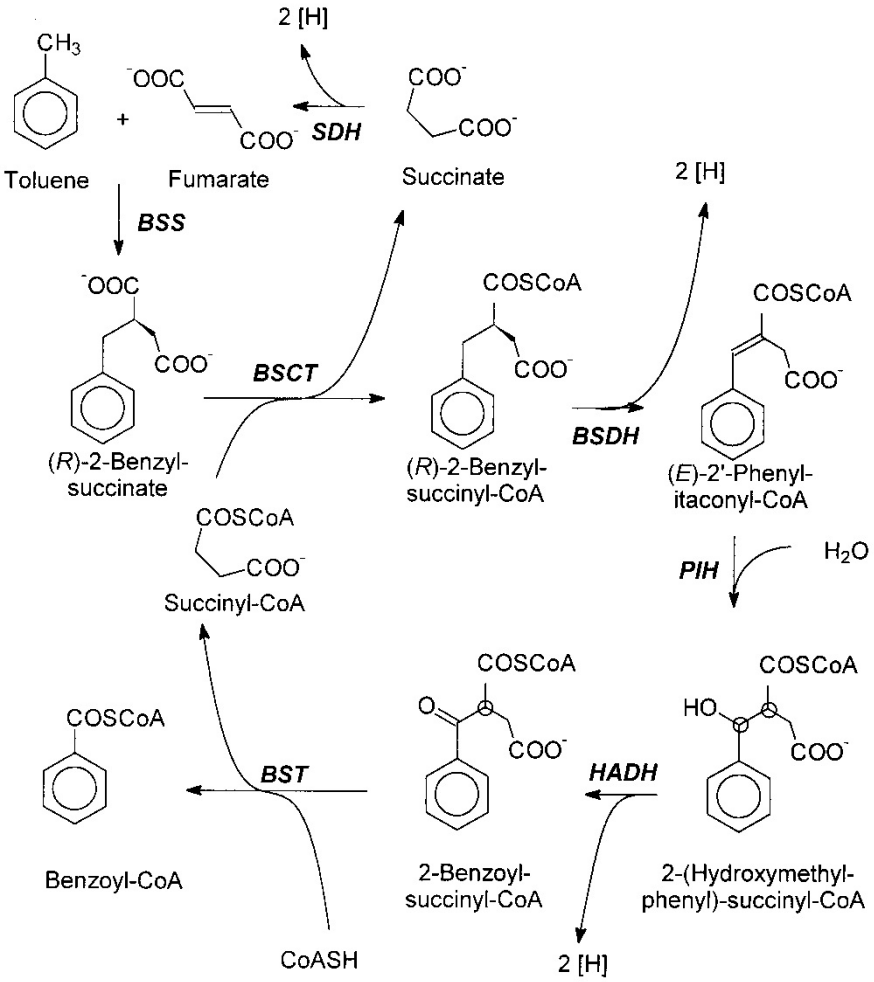


Figure 6. Catabolism of toluene via addition of fumarate to the methyl residue. In this route, toluene is activated to benzyl-CoA that is further oxidized to simplest metabolites. The release succinyl-CoA is oxidized to succinate and further to fumarate to supply the fumarate pool. BSS: benzylsuccinate synthase; BS-CT: succinyl-CoA:(R)-benzylsuccinate CoA-transferase; SDH: Succinate dehydrogenase**;** BS-DH benzyl succinyl-dehydrogenase; PIH: (*E*)-Phenylitaconyl-CoA hydratase; HADH, 3-hydroxyacyl-CoA dehydrogenase **;** BST, benzoylsuccinyl-CoA thiolase;

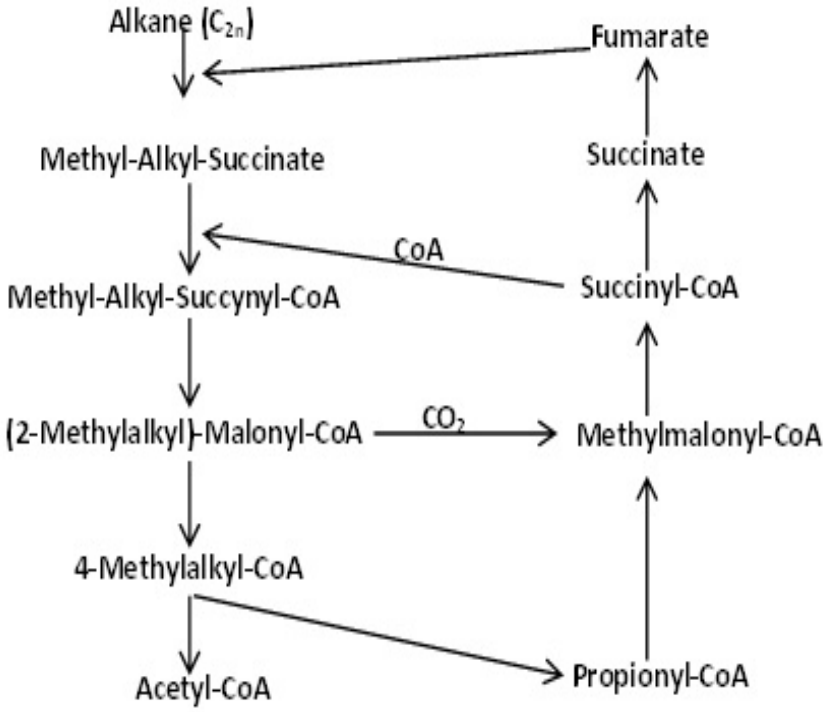


Figure 7. Addition of fumarate to alkanes. The emerged propionyl-CoA is converted to fumarate in order to continue this reaction.

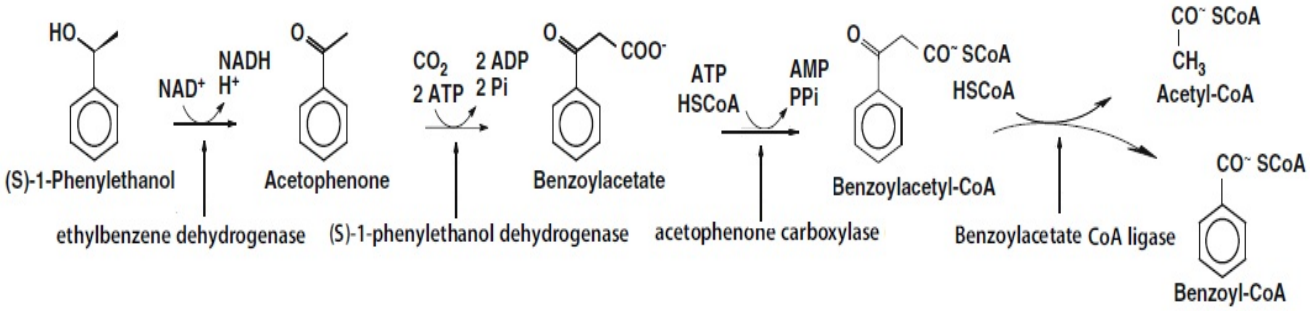


Figure 8. Oxygen-Independent Hydroxylation of phenylethanol. The first enzyme (ethylbenzene dehydrogenase) is found in the periplasmic space while other enzymes are present in the cytosol.

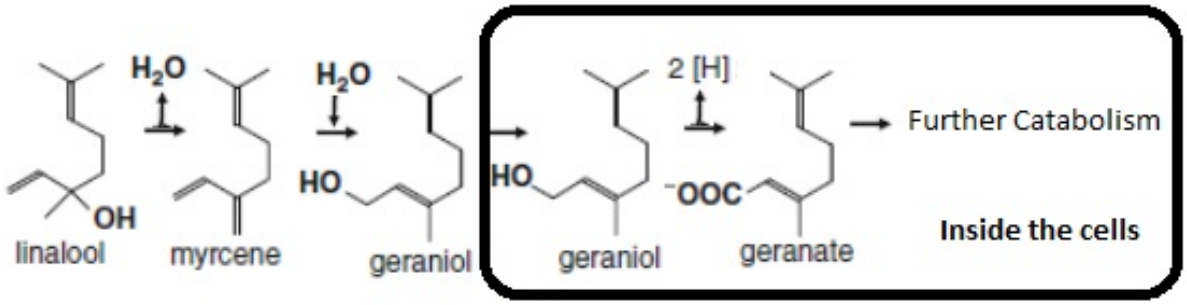


Figure 9. Hydration of Linalool. The molecule is first dehydrated and then re-hydrated in the extracellular environment. The resulting geraniol is transferred into the cell and is oxidized to geranate.

Enzymes	Methane monooxygenases	Alkane monooxygenases	Bacterial P450 (CY153, Class I)	Eukaryotic P450 (CYP52, Class II)	Dioxygenases
Chain length	C1-C4	C5-C16	C5-C16	C10-C16	C10-C30

Table 1. The known enzymes involved in degradation of aliphatic hydrocarbons