Chapter 17
Microbial Degradation of Alkanes

S. N. Singh, B. Kumari and Shweta Mishra

17.1 Introduction

Petroleum hydrocarbons are introduced into the environment due to their extensive use as fuels and chemicals. Besides, leaks and accidental spills occur often during exploration, production, refining, transport and storage of petroleum and petroleum products which used to add an additional burden of hydrocarbons to soils and water systems. The technologies commonly used for soil remediation of petroleum hydrocarbons include mechanical burying, evaporation, dispersion and washing. These remedial measures are not only cost intensive and time consuming, but also not very effective. On the other hand, bioremediation leads to complete mineralization of organic compounds into CO_2 and water by indigenous micro-organisms and hence a preferred choice also being eco-friendly and cost-effective.

Anthropogenic hydrocarbon contamination of soil is a global issue throughout the industrialised world (Macleod et al. 2001; Brassington et al. 2007). In England and Wales alone, 12% of all serious contamination incidents in 2007 were hydrocarbon related. Soil acts as a repository for many hydrocarbons, which is a serious concern due to their adverse impact on human health and environmental persistence for a long time (Jones et al. 1996; Semple et al. 2001).

Alkanes are a major fraction (>50%) of the crude oil depending upon the oil source. Alkanes are saturated hydrocarbons and chemically very inert as apolar molecules (Labinger and Bercaw 2002). They may be classified as linear (*n*-alkanes), cyclic (cyclo-alkanes) or branched (iso-alkanes) and found in three states: gaseous (C1–C4), liquid (C5–C16) and solid (>C17) (Fig. 17.1). Although

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S. N. Singh (ed.), *Microbial Degradation of Xenobiotics*, Environmental Science and Engineering, DOI: 10.1007/978-3-642-23789-8_17,

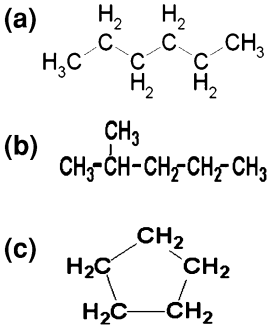

Fig. 17.1 Examples of linear; *n*-Hexane (**a**) branched; Iso-hexane (**b**) and cyclic alkanes; Cyclopentane (**c**)

highly inflammable, alkanes are less reactive as organic compounds. They are highly essential for modern life, but their inertness poses serious ecological problems when released to the environment. However, microbes have developed effective strategies involving specific enzymes and metabolic pathways to use *n*-alkanes as a carbon source. Thus, microbes have the capability to degrade alkanes and convert them to easily metabolizable substrates.

17.2 Microbial Degradation of Alkanes

Due to lack of functional groups as well as very low water solubility, aliphatic hydrocarbons exhibit both, low chemical reactivity and bioavailability for microorganisms. However, some microorganisms possess the metabolic capacity to use these compounds as carbon and energy sources for their growth (Berthe-Corti and Fetzner 2002).

A number of microbes including bacteria, fungi and yeasts have been reported to degrade alkanes using them as the source of carbon and energy (van Beilen et al. 2003; Wentzel et al. 2007). Bacteria with alkane degradation ability have also versatile metabolism to use other compounds in addition to alkanes as source of carbon (Margesin et al. 2003; Haryama et al. 2004). Use of bacteria in the degradation of alkane compounds has been extensively studied by Haryama et al. (2004). Many microbes have been reported for the degradation of aliphatic compounds, such as *Arthrobacter* sp., *Acinetobacter* sp., *Candida* sp., *Pseudomonas* sp., *Rhodococcus* sp., *Streptomyces* sp., *Bacillus* sp., *Aspergillus japonicus*, *Arthrobacter* sp., *Acinetobacter* sp., etc. In addition, some bacterial species are reported as highly specialized in degrading hydrocarbons and hence called hydrocarbonoclastic bacteria. They play a key role in the removal of hydrocarbons from the polluted environments (Head et al. 2006; Yakimov et al. 2007). Schneiker et al. (2006) found a marine bacterium (*Alcanivorax borkumensis*) capable of assimilating both linear or branched alkanes, but unable to metabolize aromatic hydrocarbons. *Alcanivorax dieselolei*, a *g*-proteobacterium, is also a member of the hydrocarbonoclastic bacteria and cannot assimilate sugars

or amino acids as sources of energy and carbon. But it can utilize some organic acids and alkanes. Notably, the spectrum of alkanes utilized by *A. dieselolei* (C5–C36) (Liu and Shao 2005) is substantially broader than those of most other previously described alkane degraders (van Beilen and Funhoff 2007). Other alkane degrading bacterial genera are *Thalassolitus* (Yakimov et al. 2004), *Oleiphilus* (Golyshin et al. 2002), *Bacillus*, *Geobacillus* (Marchant et al. 2006), *Thermus* (Meintanis et al. 2006) and *Oleispira* (Yakimov et al. 2003).

Acinetobacter sp. was found to be capable of utilizing *n*-alkanes of chain length C10–C40 as a sole source of carbon (Throne-Holst et al. 2007). Other bacterial genera, namely, *Gordonia*, *Brevibacterium*, *Aeromicrobium*, *Dietzia*, *Burkholderia* and *Mycobacterium* isolated from petroleum contaminated soil were proven to be potential degraders of hydrocarbons (Chaillan et al. 2004). Hexadecane degradation was observed by the bacteria, such as *Pseudomonas putida*, *Rhodococcus erythroplotis* and *Bacillus thermoleovorans* (Abdel-Megeed et al. 2010) and two bacterial strains; *Flavobacterium* sp. ATCC39723 and *Arthrobacter* sp. (Steiert et al. 1987). Hexadecane (HXD) is present in the aliphatic fraction of crude oil and is one of the major components of diesel (Chenier et al. 2003). Volke-Sepulveda et al. (2003) demonstrated that HXD biodegradation by *Aspergillus niger* was considerably higher in SSF (Solid state fermentation) than in submerged fermentation. Complete HXD conversion was achieved at a C/N ratio of 29 under SSF conditions (Stroud et al. 2008)**.** *Desulfatibacillum alkenivorans* AK-01 is a mesophilic sulfate-reducer isolated from estuarine sediment which utilizes C13–C18 alkanes, 1-alkenes (C15 and C16) and 1-alkanols (C15 and C16) as growth substrates.

Thermophilic alkane degrading bacterium, *Goebacillus thermoleovorans* (previously *Bacillus thermoleovorans*) B23 was reported from a deep-subsurface oil reservoir in Japan (Kato et al. 2001). This strain effectively degraded alkanes at 70°C with the carbon chain longer than dodecane (C12). Since tetradecanoate and hexadecanoate or pentadecanoate and heptadecanoate were accumulated as degradation intermediates of hexadecane or heptadecane degradation, respectively, it indicated that the strain B23 degraded alkanes by a terminal oxidation pathway, followed by *β*-oxidation pathway. Recently, another long chain alkane degrading *Geobacillus thermodenitrificans* NG80-2 was also isolated from a deep sub -surface oil reservoir and its complete genome sequence was determined (Feng et al. 2007).

Some organisms adapted to cold environment are capable of degrading high molecular weight petroleum hydrocarbons. Whyte et al. (1998) reported that *Rhodococcus* sp. strain Q15 was able to degrade alkanes up to *n*-C21 as well as some branched alkanes in diesel, and could also grow on dotriacontane (*n*-C32). *Rhodococcus* strains capable of growing on eicosane (*n*-C20) have been reported by Bej et al. (2000). Studies on petroleum biodegradation in soils from cold regions have reported that lower-molecular weight *n*-alkanes and unsubstituted aromatic hydrocarbons are biodegraded preferentially over the relatively higher-molecular weight *n*-alkane compounds, isoalkanes, alkylated aromatic hydrocarbons, isoprenoids and the branched and cyclic hydrocarbons (Sanscartier et al. 2009).

Besides, many yeasts and fungi, are also known to thrive on alkanes (van Beilen et al. 2003). Among fungal genera, *Amorphoteca*, *Neosartorya*, *Talaromyces* and *Graphium* and yeast genera, *Candida*, *Yarrowia* and *Pichia*, isolated from oil-contaminated soil were found potential degraders of petroleum of petroleum hydrocarbons (Chaillan et al. 2004). Singh (2006) has reported a group of fungi, namely *Aspergillus*, *Cephalosporium* and *Pencillium* to be high degraders of crude oil hydrocarbons. Among yeast species, *Candida lipolytica*, *Rhodotrula mucilaginosa*, *Geotrichum* sp. and *Trichosporam mucoides* isolated from contaminated water were capable to degrade petroleum compounds effectively (Boguslawska-Was and Dabrowski 2001). New genera containing alkane degraders are constantly being identified, leading to a better understanding of ecosystems.

17.2.1 Uptake of n-Alkanes

Alkanes are insoluble in water. The solubility of alkanes depends largely on the molecular weight. With the increase in molecular weight, the solubility decreases in water (Eastcott et al. 1988). Hydrocarbons with a chain length C12 and above are virtually water insoluble. It is still not very clear how alkanes enter the cells of bacteria. The uptake mechanism depends on the bacterial species, the molecular weight of alkane and physico-chemical environment (Wentzel et al. 2007). Low molecular weight alkanes are sparingily soluble in water to ensure a sufficient mass transfer to bacterial cell, while high molecular weight (medium and long chain *n*-alkanes) alkanes find their accessibility to cell either by adherence or by a surfactant-mediated process. This is the reason that alkane degrading bacteria produce diverse surfactants which facilitate the emulsification of hydrocarbons (Ron and Rosenberg 2002). Noordman and Janssen (2002) have reported an increase in the uptake of alkanes in presence of biosurfactants, such as hexadecane in cultures, however, their role in soils and other environments is still not very evident (Holden et al. 2002).

In addition, biosurfactants may also facilitate cell mobility and adhesion to surfaces or biofilms (Boles et al. 2005). They also shield bacterial cells from direct exposure to toxic substances (Kang and Park 2009). Depending on the solubility, the alkanes may be arranged as follow: linear alkanes > branched alkanes > cyclic alkanes with regard to their susceptibility to microbial degradation.

17.2.2 Aerobic Degradation of Alkanes

Aerobic alkane degraders activate alkane molecules using O_2 as a reactant. The alkane-activating monooxygenase overcomes the low reactivity of the hydrocarbon by producing reactive oxygen species. Oxidation of methane leads to formation of

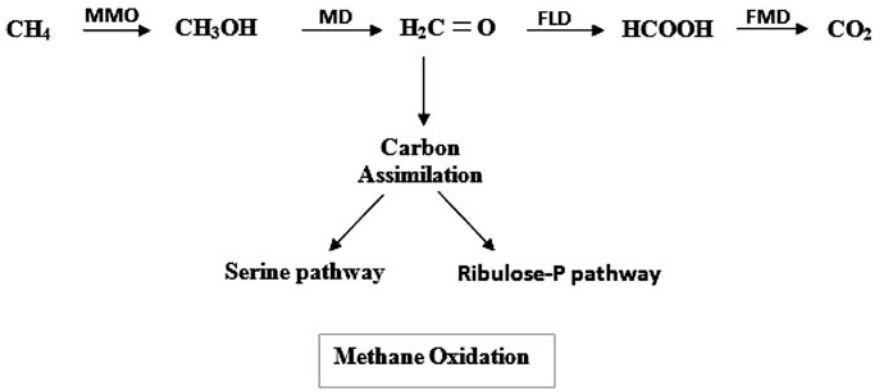

Fig. 17.2 Aerobic pathways of methane oxidation (after Rojo 2009)

methanol which is subsequently transformed to formaldehyde and then to formic acid (Fig. 17.2). This compound either gets converted to CO_2 or assimilated for biosynthesis of other organic compounds either by the ribulose monophosphate pathway or by the serine pathway depending upon the organism (Lieberman and Rosenzweig 2004). The complete degradation of hydrocarbons mainly occurs under aerobic conditions (Riser-Robert 1998). This process involves several steps as illustrated in Fig. 17.3: (1) Accessibility of chemicals to microbes having degradation ability. Since hydrocarbons are insoluble in water, their degradation essentially requires biosurfactants which are produced by bacteria. (2) Activation and incorporation of oxygen is the vital reaction catalysed by oxygenase and peroxidase. (3) Peripheral degradation pathways which convert hydrocarbons into intermediates of the tricarboxylic acid cycle (TCA) and (4) Biosynthesis of cell biomass from the central precursor metabolites i.e. acetyl-CoA, succinate and pyruvate, sugars are required for various biosynthesis and gluconeogenesis for growth.

Degradation of *n*-alkanes is initiated by the oxidation of a terminal methyl group to render a primary alcohol, which gets further oxidized to the corresponding aldehyde, and finally converted into a fatty acid. Fatty acids are conjugated to CoA and further processed by β-oxidation to generate acetyl-CoA (Wentzel et al. 2007) (Fig. 17.4). However, in some cases, both ends of the alkane molecule are oxidized through ω-hydroxylation of fatty acids at the terminal methyl group (ω position), rendering an ω-hydroxy fatty acid that is further converted into a dicarboxylic acid and processed by β oxidation (Coon 2005). Sub-terminal oxidation of *n*-alkanes has also been reported (Kotani et al. 2007). The product generated a secondary alcohol which is converted to the corresponding ketone, and then oxidized by a Baeyer–Villiger monooxygenase to render an ester. The ester is hydrolysed by an esterase, generating an alcohol and a fatty acid. Both terminal and sub-terminal oxidation can co-exist in some microorganisms.

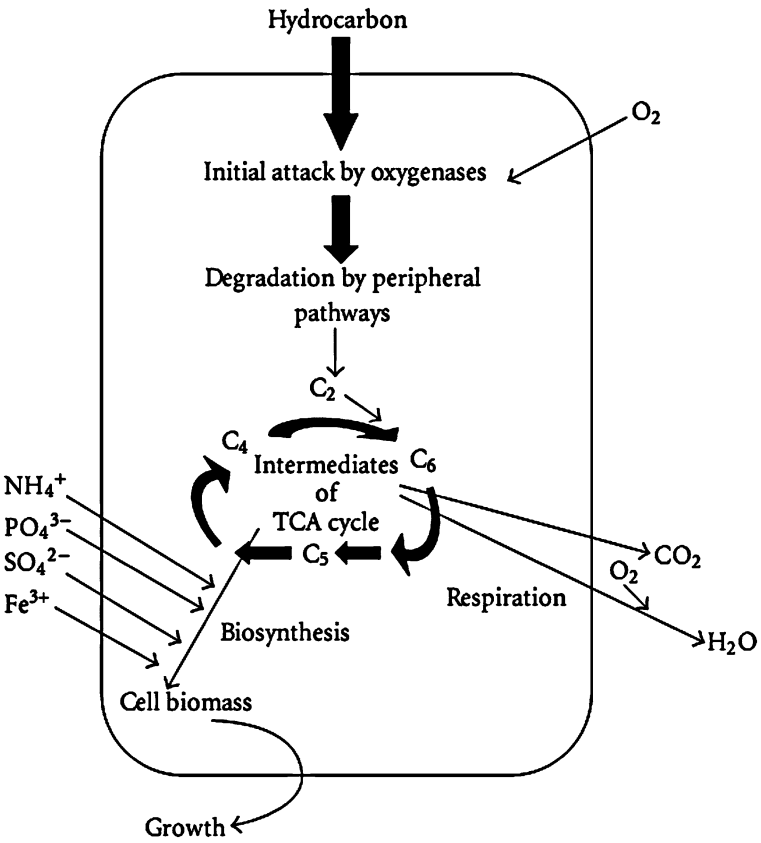

Fig. 17.3 Process of microbial aerobic degradation of hydrocarbons associated with growth process (after Fritsche and Hofrichter 2000)

Some strains of *Pseudomonas* are able to utilize alkanes as the sole carbon and energy source (Stanier et al. 1966). The initial pathway of alkane oxidation is the following:

$$R{-}CH_3 \rightarrow R{-}CH_2OH \rightarrow R{-}CHO \rightarrow R{-}COOH$$

This pathway has been established by simultaneous adaptation experiments (Heringa et al. 1961) and chromatographic analysis of the products of alkane oxidation (Thijsse and van der Linden 1963). *Acinetobacter* spp. can split a hydrocarbon at the number of ten position, forming hydroxyl acids. The initial steps appear to involve terminal attack to form carboxylic acid, sub-terminal dehydrogenation at the number ten position to form an unsaturated acid, and splitting of carbon chain to form a hydroxyl acid and alcohol. Highly branched isoprenoid alkanes, such as Pristane, have been found to undergo ω-oxidation with the formation of dicarboxylic acids as the major degradative pathway.

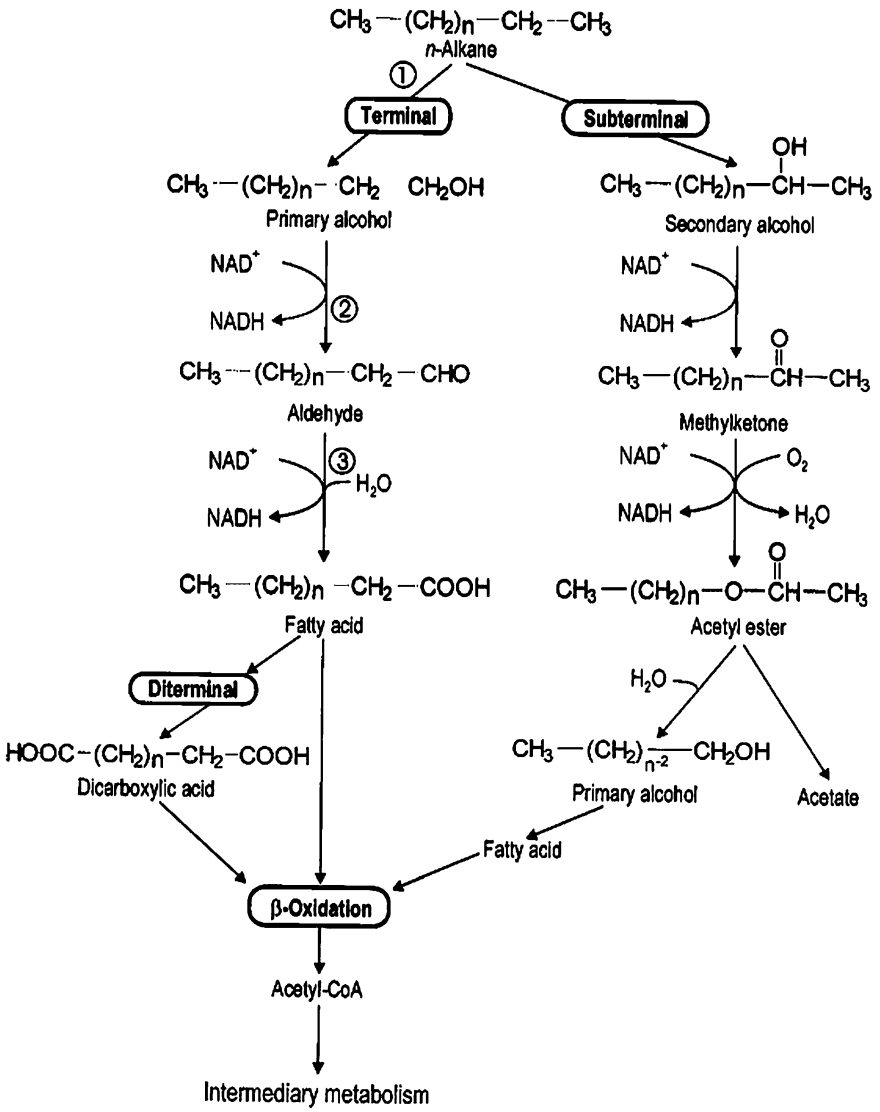

Fig. 17.4 Aerobic pathways of *n*-alkane degradation (after Fritsche and Hofrichter 2000)

Methyl branching increases the resistance of hydrocarbons to microbial attack. Methyl branching at β-oxidation requires an additional strategy, such as α-oxidation, ω-oxidation or β alkyl group removal (Atlas 1981). *Acremonium* spp. oxidize ethane to ethanol by NADPH dependent monooxygenase, which is subsequently oxidized to acetaldehyde and acetic acid. Acetate, thus formed, is assimilated into cellular carbon via reverse tricarboxylic acid cycle and glyoxalate

bypass. Similarly, a number of propane and butane utilizers have been reported that are also capable of growth on long chain alkanes, such as *n*-dodecane and *n*-hexadecane.

Long chain hydrocarbons (C10–C18) can be used rapidly by many high G + C Gram-positive bacteria, but only a few bacteria can oxidize C2–C8 hydrocarbons. Degradation of *n*-alkanes requires activation of the inert substrates by molecular oxygen with the help of oxygenases by three possible ways that are associated with membranes:

1. Monooxygenase attacks at the end producing alkan-1-ol:

$$R{-}CH_3 + O_2 + NAD(P)H + H^+ \rightarrow R{-}CH_2OH + NAD(P)^+ + H_2$$

2. Dioxygenase attack produces hydroperoxides, which are reduced to yield also alkan-1-ol:

$$\begin{aligned} R{-}CH_3 + O_2 &\rightarrow R{-}CH_2OOH + NAD(P)H + H^+ \\ &\rightarrow R{-}CH_2OH + NAD(P)^+ + H_2O \end{aligned}$$

3. Rarely, subterminal oxidation at C_2 by monooxygenase yields secondary alcohols.

Brevibacterium erythrogenes can use 2-methylundecane as substrate for growth by a combination of ω- and β-oxidation. *Arthrobacter* sp. has been reported to metabolize squalene (C30-multiple, methyl branched compound) to geranylacetone, which is accumulated in the medium as it cannot be further metabolized. Similarly, *Corynebacterium* sp. and *B. erythrogenes* have been shown to degrade pristane (2,6,10,14-tetramethyl pentadecane) involving ω-oxidation, followed by β-oxidation, yielding propionyl-CoA and acetyl-CoA units alternately.

17.2.3 Anaerobic Degradation of n-Alkanes

Apart from aerobic oxidation, anaerobic degradation also plays an important role in the recycling of hydrocarbons in the environment. Alkanes are also degraded through anaerobic process as reported by various workers (Callaghan et al. 2009; Higashioka et al. 2009). There are two known pathways of anaerobic *n*-alkanes degradation (Fig. 17.5). First pathway is the alkane addition to fumarate, and second is through putative pathways (So et al. 2003). Fumarate addition proceeds via terminal or sub-terminal addition of the alkanes to the double bond of fumarate, resulting in the formation of alkyl succinate which is further degraded via carbon skeleton rearrangement and β-oxidation. Alkane addition to fumarate has been documented for denitrifying bacteria (Wilkes et al. 2002), sulphate reducing consortia (Kniemeyer et al. 2007) and sulphate reducing bacteria (Callaghan et al. 2006; Kniemeyer et al. 2007). *Azoarcus* sp. HxN1, a denitrifying bacterium, uses C6–C8 alkanes, while *Desulfobacterium* Hdx3 metabolizes C12–C20 alkanes (reviewed in Widdel and Rabus 2001).

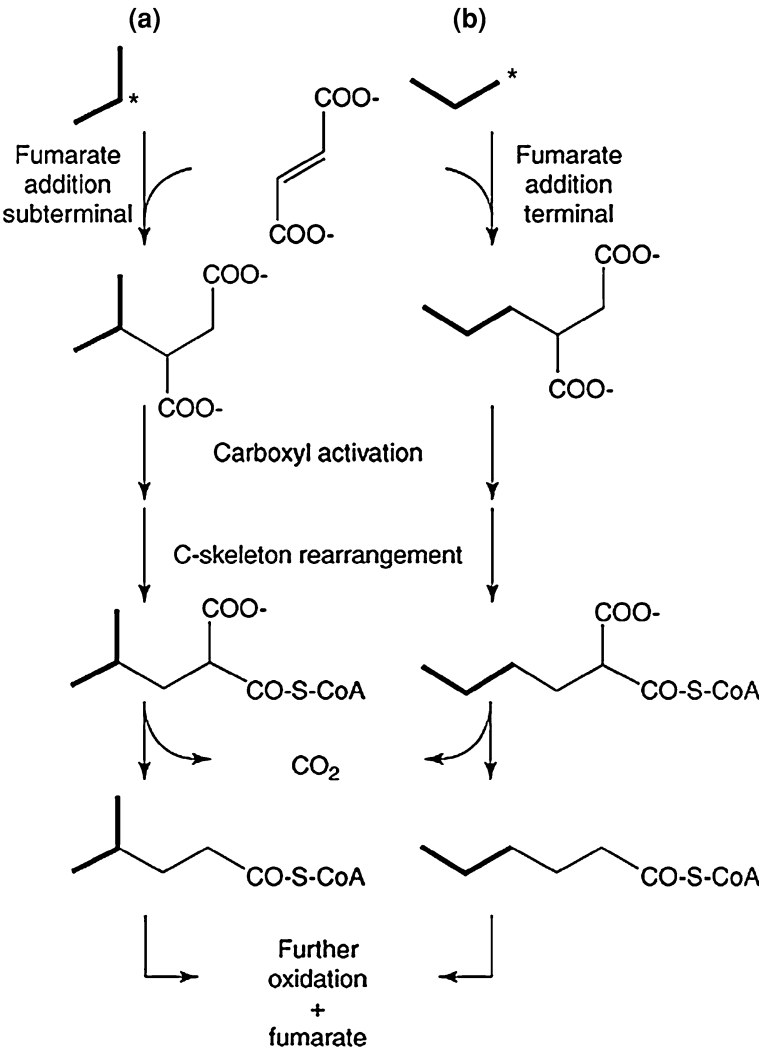

Fig. 17.5 Anaerobic activation of short chain alkanes by furarate addition. The formed methylalkylsuccinates are activated by binding with acetyl-coenzyme A (CoA), which yields a thioester that undergoes C-skeleton rearrangement, followed by decarboxylation and β-oxidation. **a** Activation of the secondary carbon in propane. **b** Activation of the primary carbon in propane, which requires more energy. * indicates the position of the radical carbon (after Kniemeyer et al. 2007)

Zedelius et al. (2011) studied alkane degradation under anaerobic conditions by a nitrate reducing bacterium to find out involvement of electron acceptor in substrate activation. Three bacterial isolates (HXN1, OcN1, HdN1) which were able to grow under aerobic conditions by coupling alkane oxidation to CO_2 with NO_3^- reduction to N_2, were compared for alkane metabolism (Fig. 17.6). Out of which,

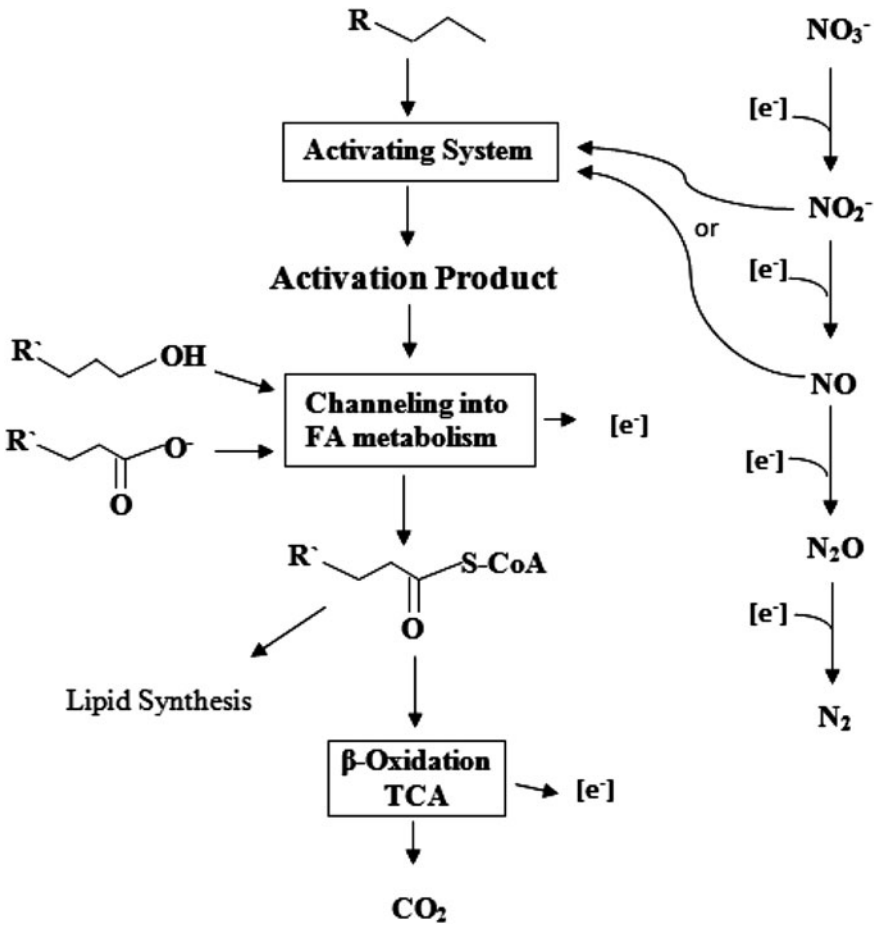

Fig. 17.6 Hypothetical involvement of denitrification intermediates in alkane activation. A small proportion of NO_2^- or NO is deviated from the respiratory chain for alkane activation. They may be used for activation indirectly (by yielding O_2 that is used by alkane monooxygenase; or by giving rise to another reactive factor or enzyme centre) or directly (as co-reactants introducing a polar group). The alkyl residue R′ may or may not be identical with the original residue R (depending on the activation mechanism and alkane C-atom being attacked). FA, fatty acid; TCA, tricarboxylic acid cycle (after Zedelius et al. 2011)

two strains HXN1 and OcN1 (both Betaproteobacteria) metabolized C6–C8 and C8–C12 alkanes, respectively. Both of them activated alkanes anaerobically in a fumarate-dependent reaction yielding alkylsuccinates as evidenced by metabolite and gene analyses. However, strain HdN1 was unique. It belonged to Gammaproteobacteria and utilized alkanes in the range of C6–C30. It also did not indicate fumarate-dependent alkane activation. While HXN1 and OcN1 grew on alkanes and NO_3^-, NO_2^- or N_2O added to medium, strain HDN1 oxidized alkanes only with NO_3^- or NO_2^- but not with N_2O. Since N–O species are the strong oxidants, these

strains may not activate alkane under the conditions of sulphate reduction or methanogenesis and allow a special mode of alkane activation.

Squalane (2,6,10,15,19,23-hexamethyltetracosane) is susceptible to microbial degradation and *Actinomycetes*, in particular, and those belonging to the genus *Mycobacterium*, are potent degraders of this multibranched saturated hydrocarbons. The putative pathway demonstrated that after the conversion of squalane to a dioic acid as one of the first intermediates, two propionyl- coA and acetyl-CoA molecules are oxidatively removed by β-oxidation route to form 3,7,11-trimethyldodecandioic acid as intermediate by a pathway analogous to that for degradation of the multiple branched alkane pristane (2,3,10,14-tetramethylpentadecane) (Berekaa and Steinbüchel 2000).

17.2.4 Non-Conventional Dissimilation Pathway

Sakai et al. (1996) observed a non-conventional dissimilation pathway in *Acinetobacter* sp. M1 in which *n*-alkanes are postulated to be converted to acid:

$$\mathrm{R{-}CH_3} \rightarrow \mathrm{RCH_2OOH} \rightarrow \mathrm{RCO(O)OH} \rightarrow \mathrm{RCHO} \rightarrow \mathrm{RCOOH} \quad \text{(Finnerty 1988)}.$$

However, there is little information available on the enzymes involved in the postulated pathway, particularly at the first step. They identified an enzyme—a flavoprotein which needed O_2 and Cu^{2+} for expression of its activity, but did not require NAD(P)H as a coenzyme. The enzyme reaction yielded hydroperoxide and the enzyme involved in *n*-alkane oxidation is likely to be a dioxygenase. Further, the postulated pathway is supported by the following observations: (1) *n*-alkane monooxygenase activity not detected, (2) low activity of fatty alcohol dehydrogenase, (3) induction of NAD(P)H-dependent long chain fatty aldehyde dehydrogenase in *n*-alkane grown cells.

Meng et al. (1996) isolated three kinds of enzymes designated A, B and C found in the cytoplasm of *n*-alkane grown *Acinetobacter* sp. M1, that catalyzed dioxygenation of *n*-alkanes to the corresponding *n*-alkyl hydroperoxides. Purified enzyme A consisted of four identical subunits having a molecular mass and strongly inhibited by several iron-chelating agents. Enzymes B and C were more active towards relatively short *n*-alkanes (C12–C16) where as enzyme A oxidized solid *n*-alkanes with the most preferable substrate being Tetracosane C24.

17.3 Oil Alkanes

Alkanes are the most important fraction of crude oil. The anaerobic degradation of alkanes is today of great significance for the oil industry. It is well established that microbial activities associated with oil reservoirs led to the decrease of oil quality, making refining more costly and recovery more difficult (Head et al. 2003).

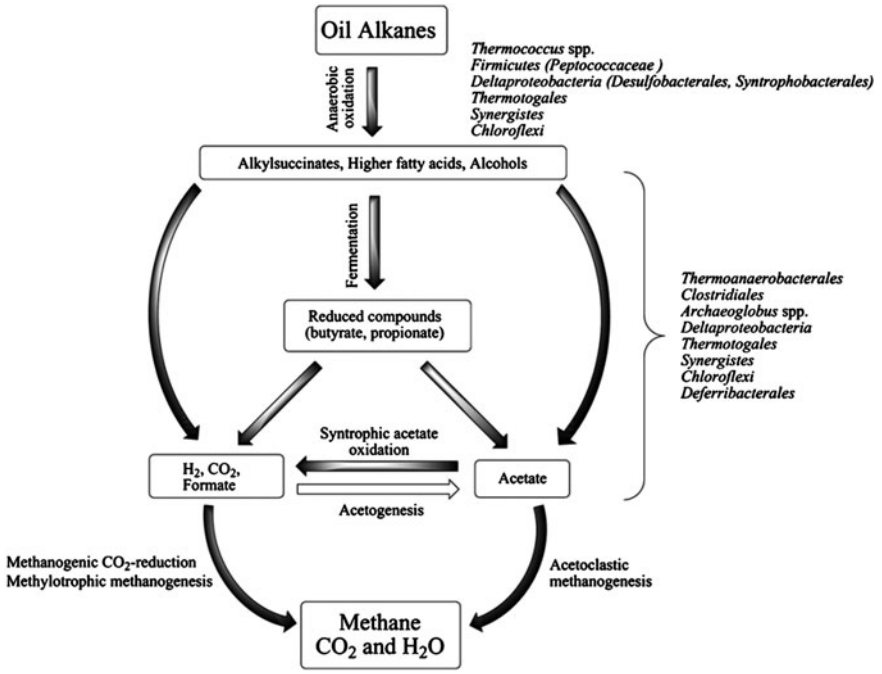

Fig. 17.7 Presumptive methanogenic degradation of oil alkanes (after Mbadinga et al. 2011)

Because of presence of microbial communities mainly dominated by anaerobes, the oil reserves are referred as 'geo bioreactors', in which fermentative, syntrophic, suthdogenic and methanogens are responsible for removal of alkanes from the saturated hydrocarbon fraction (Jones et al. 2008; Wang et al. 2010). Moreover, biogenic CH_4 production is the result of microbial degradation of oil alkanes. Since world demand for methane is likely to increase many folds in coming decades, the methanogenic conversion of oil alkanes to CH_4 is seen as a future solution for world increasing demand of energy (Fig. 17.7).

17.4 Enzymes Involved in Alkane Degradation

Ayala and Torres (2004) have indicated the involvement of three major enzymes in the degradation of alkanes; Methane monooxygenase (MMO), Alkane hydroxylase (Alk) and Cytochrome P450 monooxygenase (Fig. 17.8).

17.4.1 Methane Monooxygenase

Methane monooxygenase is expressed in microorganisms to use CH_4 as energy source and found in methanotrophs in two forms pMMO (particulate Methane

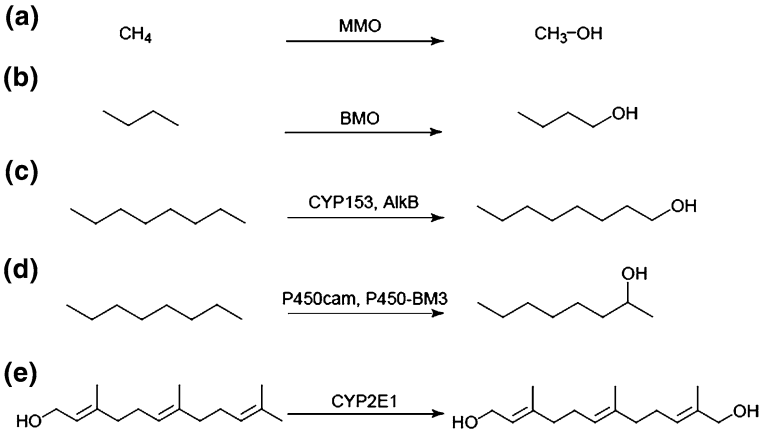

Fig. 17.8 **a** Methane to methanol by Methane Monooxygenase (MMO), **b** Butane to 1- butanol by Butane Monooxygenase (BMO), **c** Octane to 1-Octanol, **d** Octane to 2-Octanol, **e** Farnesol to 1-hydroxyfarnel (after van Beilen and Funhoff 2005)

monooxygenase) and sMMO (soluble Methane monooxygenase). While pMMO is a membrane-bound protein produced by all methanotrophs, sMMO is expressed by a subset of methanotrophs. pMMO is an iron–copper protein, produced under conditions of copper sufficiency (Nguyen et al. 1994) where as sMMO is an iron-containing enzyme produced only under Cu-depleted sites (Murrell et al. 2000b). sMMO is comprised of three components; an oxygenase, a reductase and a coupling protein (Fox et al. 1989). The NADH-dependent oxidation reaction catalysed by sMMO is reflected in Fig. 17.9. Both sMMO and Alk are characterized by the presence of diiron cluster in the hydroxylase component. The metallic center activates dioxygen during the oxidation of substrates. However, in sMMO, the diiron cluster is bridged by carboxylic residues, similar to the diiron centers of proteins, such as ribonucleotide reductase R2, stearoyl-ACP-9 desaturase and other monooxygenases, such as alkene monooxygenases, phenol monooxygenases and toluene monooxygenases (Leahy et al. 2003). sMMO shows a wide range of substrate specificity, including alkenes, aromatic, alicyclic and hetrocyclic compounds where as pMMO mediates the oxidation of a small group of alkanes (Murell et al. 2000a). Four different reaction mechanisms of sMMO for hydrocarbon hydroxylation have been suggested: (1) hydrogen atom abstraction from the substrate followed by radical recombination (Fox et al. 1990), (2) cation formation by electron abstraction from the substrate radical intermediate generated in first step followed by reaction with metal bound hydroxide (Jin et al. 2001), (3) direct insertion of the oxygen atom into the C–H bond (Valentine et al. 1997) and (4) cation formation on the substrate by transfer of a protonated oxygen from a hydroperoxy intermediate (derived from O_2), followed by loss of water (Choi et al. 1999).

Similar to sMMO, butane monooxygenase (BMO) is a non-heme iron monooxygenase and it can hydroxylate C2–C9 alkanes (Dubbels et al. 2007).

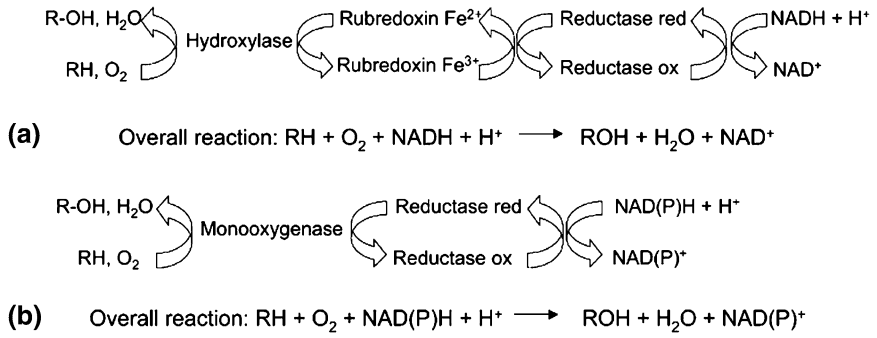

Fig. 17.9 Steps involved in the oxidation reaction catalysed by alkane hydroxylase (**a**) and methane monooxygenase and cytochrome P450 monooxygenase (**b**) (after Ayala and Torres 2004)

Chaperonin-like protein, BmoG is required for proper assembly of BMO (Kurth et al. 2008). However, its specificity is towards producing the terminal alcohols, unlike sMMO that produces sub-terminal alcohols.

Recently, a unique alkane monooxygenase that belongs to luciferase family was reported for *G. thermodenitrificans* (Li et al. 2008). Kato et al. (2009) reported that three novel membrane proteins, superoxide dismutase, catalase, and acyl-CoA oxidase in *G. thermoleovorans* B23 which were previously reported only in yeast, such as *C. tropicalis* (Shimizu et al. 1979), Activities of these enzymes were dramatically increased in the cells of *G. thermoleovorans* B23 when they were grown on alkanes.

17.4.2 Alkane Hydroxylase

This enzyme is three component monooxygenase, comprising a hydroxylase, a rubredoxin and rubredoxin reductase (Shanklin et al. 1997). The hydroxylase component is membrane-bound, while both rubredoxin and rubredoxin reductase components are soluble and cytoplasmic proteins. This enzymatic complex is able to oxidize medium and long chain linear alkanes using reducing equivalents from NADH or NADPH.

AlkB, an integral membrane protein, carries out a terminal hydroxylation of *n*-alkane (Kok et al. 1989). The electrons needed to carry out this step are delivered to AlkB via a rubredoxin reductase (AlkT) and two rubredoxins (AlkF and AlkG) (van Beilen et al. 2002). The resulting alcohol is further converted to a fatty acid via a pathway involving an alcohol dehydrogenase (AlkJ), an aldehyde dehydrogenase (AlkH) and an acyl-CoA synthetase (AlkK), that enters the β oxidation pathway (van Beilen et al. 2001). The histidine residues are required for activity in the members of this family (Shanklin et al. 1994). There is a

conserved NYXEHYG(L/M) motif in all identified alkane hydroxylases (Smits et al. 2002). This motif has been proposed as a signature for membrane-bound alkane hydroxylases (Smits et al. 2002).

Although crystal structure of Alk is not known, it is believed to have six transmembrane segments and a catalytic site that faces the cytoplasm. The active site includes four His-containing sequence motives that are conserved in other hydrocarbon monooxygenases which chelate two iron atoms (Shanklin et al. 1994). The diiron cluster allows the O_2-dependent activation of the alkane through a substrate radical intermediate (Shanklin et al. 1997; Bertrand et al. 2005). One of the O_2 atoms is transferred to the terminal methyl group of the alkane, rendering an alcohol, while the other one is reduced to H_2O by electrons transferred by the rubredoxin. Oxidation is regio- and stereospecific (van Beilen et al. 1995).

Baptist et al. (1963) have identified an enzyme system from *Pseudomonas putida* PpG6 grown on alkanes which is capable of oxidizing octane to octanoic acid, and the properties of the enzyme complex, which catalyzes the initial hydroxylation reaction, have been extensively studied (Mckenna and Coon 1970). In vitro, this hydroxylase complex is also capable of omega-oxidizing fatty acids (Mckenna and Coon 1970). This suggests that the oxidation of alkane and fatty acid chains might occur from both ends in strains with a functional hydroxylase.

The AlkB protein from *Pseudomonas putida* GPo1 is presently the best characterized Alk (van Beilen et al. 1994). It catalyses the first step of alkane degradation with the help of two electron transfer proteins, rubredoxin (AlkG) and rubredoxin reductase (AlkT) (van Beilen et al. 1994). Over the past decade, alkB-like hydroxylase genes have been detected in a wide range of alkane degrading bacteria, including *a*-, *b*- and *g*-proteobacteria; as well as in some high G + C content Gram-positive bacteria (Smits et al. 2002). Many of these contain more than one alkB homologue, such as *Pseudomonas aeruginosa* PAO1 (alkB1 and alkB2), *Rhodococcus erythropolis* Q15 (alkB1-4) and *Acinetobacter* sp. M1 (alkMa and alkMb).

The enzymes, that oxidize alkanes larger than C20, seem to be totally different. For example, *Acinetobacter* sp. M1, which can grow on C13–C44 alkanes, contains a soluble, Cu^{2+} -dependent Alk that is active on C10–C30 alkanes. It has been proposed to be a dioxygenase that generates *n*-alkyl hydroperoxides to render the corresponding aldehydes (Tani et al. 2001). A different *Acinetobacter* strain, DSM 17874, has been found to contain a flavin-binding monooxygenase, named AlmA, which oxidizes C20 to >C32 alkanes (Throne-Holst et al. 2007). Genes homologous to almA have been identified in several other long chain *n*-alkane degrading strains, including *Acinetobacter* sp. M1 and *A. borkumensis* SK2. A different long chain alkane hydroxylase, named LadA, has been characterized in *Geobacillus thermodenitrificans* NG80-2 (Feng et al. 2007). It oxidizes C15–C36 alkanes, generating primary alcohols. Its crystal structure has shown that it is a two-component flavin-dependent oxygenase belonging to the bacterial luciferase family of proteins (Li et al. 2008).

17.4.3 Cytochrome P450 Monooxygenase

These enzymes are heme proteins and catalyze the hydrocarbons using NAD(P)H as cofactor. They usually consist of two components; hydroxylase and reductase (Sono et al. 1996). These enzymes are usually membrane-bound and have a multi-component nature (Ayala and Torres 2004).

The molecular mechanisms of oxygen activation for some metalloenzymes are well investigated. Heme-oxygenases, such as CYP, hydroxylate inert hydrocarbon substrates by using a high-valent oxoiron(IV) porphyrin π-cation-radical intermediate similar to peroxidase compound I (Groves 2005). The consensus mechanism for oxygen activation and transfer involves a hydrogen atom abstraction-oxygen rebound pathway (Groves 2003, 2005). Hydroxylation of the very unreactive C–H bond of methane by non-heme diiron enzyme sMMO has many similarities to the P450 mechanism (Kopp and Lippard 2002; Newcomb et al. 2002).

Cytochrome P450 monooxygenase (CYP), present in certain strains of yeast *Candida*, is able to convert >C12 alkane by α, ω-oxidation to the corresponding dicarboxylic acids. The ω-oxidation of the alkane to alcohol is first reaction to be catalyzed by a hydroxylase complex composed of a CYP monooxygenase and NADPH and CYP oxireductase. Further oxidation to the acid is catalysed by fatty alcohol oxidase and a fatty aldehyde dehydrogenase (Gallo et al. 1973). Vatsyayan et al. (2008) studied the cytochrome P450 monooxygenase activity in the cells of *Aspergillus terreus* MTCC6324 and found that CYP catalysis of *n*-Hexadecane had followed both terminal and sub-terminal oxidations. The activity was localized in cytosol of *n*-hexadecane grown cells. CYP activity was obtained only when NADH was used as co-factor. No other compounds checked, such as NAD, NADP, NADPH, FMN, FAD and $FADH_2$, could serve as co-factor of the enzyme. Size of isolated enzyme was closer to that reported for *Fusamarium oxysporum* i.e. 118 kDa (Nakayama et al. 1996). The presence of secondary alcohol oxidase in mitochondrial fraction indirectly supports the existence of *n*-alkanes sub-terminal oxidation. van Beilin and Funhoff (2005) reported the sub-terminal oxidation of long chain alkane by bacteria and yeast.

In addition to these enzymes, other catabolic enzymes are also reported from the different microorganism as shown in Table 17.1.

17.5 Recombinant Bacteria for Alkane Degradation

Due to multi-component nature, recombinant production of CYP450 is difficult, but CYP BM-3 is readily expressed in *E. coli* (Peter et al. 2003).

Rothen et al. (1998) constructed a plasmid with gene coding for the three enzymes; alkane hydroxylase, alcohol dehydrogenase and aldehyde dehydrogenase simultaneously. The plasmid was inserted into an *E. coli* strain unable to

Table 17.1 Different enzymes involved in alkane degradation (van Beilen et al. 2003)

Enzymes	Microrganism	Substrate	Reference
sMMO	*Methylococcus capsulatus* *Methylisinus trichosporum* OB3b	C1–C10	Baik et al. (2003)
pMMO	All methanotrophs	C1–C5	Leieberman and Rosenzweig (2004)
Propane monooxygenase	*Pseudomonas butanovora* (ATCC 43655)	C2–C8	Kotani et al. (2003)
Butane monooxygenase	*Gordonia* sp. TYP	C3 and C13–C22	Sluis et al. (2002)
AlkB	*Acinetobacter*, *Alcanivorax*, *Burkholderia*, *Mycobacterium*, *Pseudomonas*, *Rhodococcus* etc.	C5–C16	Smits et al. (2002)
Cytochrome P450 (CYP153) monooxygenase	*Sphingomonas* sp. HXN-200, *Mycobacterium* sp. HXN1500 *Acinetobacter* sp. EB104	C4–C16	Maier et al. (2001)
Cytochrome P450 (CYP52) monooxygenase	*Candida maltosa*, *Candida tropicalls*, *Yarrowia lipolytica*	C10–C16	Craft et al. (2003)

metabolize fatty acids. The recombinant bacteria were able to oxidize octane to its corresponding carboxylic acid.

Glieder et al. (2002) produced a mutant 139-3 that was capable to catalyze the oxidation of medium chain alkanes. This mutant has the fastest known enzyme for alkane hydroxylation (more than 17 times faster than the MMO or Alk enzymatic systems).

A plasmid having three components of Alk system was introduced to a *Pseudomonas* lacking the alcohol dehydrogenase. Now the recombinant bacteria were able to transform C7–C11 alkanes to their corresponding alcohols (Bosetti et al. 1992).

Throne-Holst et al. (2007) constructed alkMa, alkMb and alkMa/alkMb disruption mutants of *Acinetobacter venetianus* 642. Single and double mutants were able to grow on *n*-alkanes (>C20).

17.6 Genes Involved in Alkane Degradation

The organization of the genes involved in alkane oxidation differs significantly among alkane degrading bacteria (van Beilen et al. 2003). The alkane degradation genes encoded by the OCT plasmid of *P. putida* GPo1 are clustered in two operons, and this pathway has clearly been transferred horizontally to many bacteria (van Beilen et al. 2001). When several alkane hydroxylases coexist in a single strain, they are normally located at different sites in the chromosome. Moreover, the regulators that control the expression of alkane degradation genes may or may not map adjacent to the genes they regulate. Therefore, the degree of clustering of alkane degradation genes is variable among bacterial strains.

Expression of the genes involved in the initial oxidation of alkanes is tightly controlled. A specific regulator assures that the pathway genes are expressed only in the presence of the appropriate alkanes. In addition, superimposed to this specific regulation, there are several mechanisms that modulate the induction of the pathway genes according to cell needs. The known specific regulators, that induce alkane degradation genes in response to alkanes, belong to different families, such as the LuxR/MalT, the AraC/XylS, the GntR or other non-related families of regulators. The *A. borkumensis* AlkS transcriptional regulator is believed to activate expression of the gene coding for the AlkB1 Alk and of downstream genes in response to alkanes (van Beilen et al. 2004; Schneiker et al. 2006). In a proteomic study, this regulator appeared associated to the membrane fraction, rather than to the cytoplasmic fraction (Sabirova et al. 2006). Some bacterial strains contain only one alkane hydroxylase, as is the case for the well-characterized alkane degrader *P. putida* GPo1. However, many other strains have several alkane degradation systems, each one being active on alkanes of a certain chain length or being expressed under specific physiological conditions. For example, *Acinetobacter* sp. strain M1 contains two AlkB related alkane hydroxylases, named AlkMa and AlkMb, which are differentially regulated depending on the alkane present in the medium. Expression of AlkMa, which is controlled by the AlkRa regulator, is induced by alkanes having a very long chain length (>C22), while that of AlkMb is induced by AlkRb in the presence of C16–C22 alkanes (Tani et al. 2001). *A. borkumensis* has two AlkB like alkane hydroxylases and three genes coding for cytochromes P450 believed to be involved in alkane oxidation (van Beilen et al. 2004; Schneiker et al. 2006). In addition, *A. borkumensis* seems to have other uncharacterized genes involved in the oxidation of branched alkanes and phytane (Schneiker et al. 2006). Finally, a gene similar to *Acinetobacter* sp. DSM 17874 almA, which oxidizes alkanes of very long chain length, has been predicted in *A. borkumensis* SK2 (Throne-Holst et al. 2007). Expression of all these alkane oxidation genes should be differentially induced according to the substrate present under each circumstance. The three *A. borkumensis* genes coding for similar cytochromes P450 of the CYP153 family are believed to participate in alkane degradation (Schneiker et al. 2006). Cytochrome P450-1 maps adjacent to other genes involved in the oxidation of alkanes. Cytochrome P450-2 is identical to P450-1,

and highly homologous to P450-3. Proteomic profiling analyses revealed that P450-1 and/or P450-2, which cannot be differentiated with this technique, are expressed in cells grown with either pyruvate or hexadecane as the carbon source, although expression was higher in alkane-grown cells (Sabirova et al. 2006). As P450-1 is probably co-transcribed with other adjacent genes that are upregulated by hexadecane, it is likely that expression of P450-1 is induced by hexadecane but, not that of P450-2 and P450-3. A gene coding for a transcriptional regulator of the AraC family maps close to P450-1.

Certain plasmids play an important role in adaptation of natural microbial populations to oil and other hydrocarbons. Some of the microbial catabolic pathways responsible for the degradation, including the alk (C5–C12 *n*-alkanes), nah (naphthalene) and xyl (toluene) pathways, have been extensively characterized and are generally located on large catabolic plasmids (Gary et al. 1990), but many reports describe and characterize microorganisms that can catabolize both aliphatic and aromatic hydrocarbons (Foght et al. 1990). Several environmental isolates of *Acinetobacter* sp. and *Alcaligenes* sp. (Lal and Khanna 1996), *Arthrobacter* sp. (Efroymson and Alexander 1991) and two *Rhodococcus* strains (Malachowsky et al. 1994) have been found to degrade both alkanes and naphthalene.

Vomber and Klinner (2000) used gene probe derived from alkB gene of *Pseudomonas aleovorans* ATCC 29347 to test the ability to assimilate short/medium chain of 54 bacetrial strains belonging to 37 species. The derived amino acid sequence of the alkB-amplificate of *Pseudomonas aureofaciens* showed high homology (95%) with AlkB from *P. oleovorans*. AlkB gene disruptants were not able to grow on decane.

17.7 Environmental Factors Regulating Biodegradation of Alkanes

Additional factors that influence the degradation process included soil pH, moisture and organic matter content and hydrocarbon aqueous solubility, octanol water partitioning coefficient and structure (Leahy and Colwell 1990; Ramírez et al. 2008). Effective biodegradation is dependent upon optimal biological (microbial functionality and biomass size), chemical (bioavailability and nutrients) and physical (water holding capacity) parameters (Towell et al. 2011).

17.7.1 Structure and Physical State

n-alkanes of intermediate chain length (C10–C24) are degraded most rapidly. Short chain alkanes (<C9) are toxic to many microorganisms, but being volatile, they are generally lost rapidly to the atmosphere. Higher chain length alkanes are generally resistant to biodegradation. Branching in alkanes generally reduces the rate of biodegradation. The bioavailability of hydrocarbons, which is largely a

function of concentration and physical state, hydrophobicity, sorption onto soil particles, volatilization and solubility of hydrocarbons, greatly affects the extent of biodegradation.

Water solubility of decane d10 is 0.052 mg/l, but the solubility of octadecane is almost tenfold less (0.006 mg/l). The water solubility of butane (C4) is 61.4 mg/l, but very toxic to cells. Short chain alkanes are toxic to microorganisms, because their increased water solubility results in increased uptake of the alkanes.

17.7.2 Temperature

Merin and Bucala (2007) reported that increase in temperature made the biological membranes to have more fluid due to increased vibrational activity to the fatty acid chains in the phospholipids bilayer. The increase in the rate of fluidity helps in increasing the rate of substance uptake from a cell's surrounding medium. Biodegradation of hydrocarbon has been shown to occur over a wide range of temperature from 0°C to as high as 70°C, though, in general optimum degradation occurs in the mesophilic temperature range. It also affects the solubility of hydrocarbon and enzyme activity. The stability of the enzyme CYP P450 monooxygenase in *Aspergillus terreus* MTCC6324 ranges between 25–40°C, maximum being at 25°C (Vatsayayan et al. 2008).

17.7.3 Nutrients

van Hamme et al. (2003) reported that nitrogen and phosphorus contents greatly affect the microbial degradation of hydrocarbons. They further stated that adjustment of the ratios of N and P by their addition in the form of slow releasing fertilizers stimulated the biodegradation of hydrocarbons. Österberg et al. (2006) found accelerated biodegradation of *n*-alkanes in aqueous solution by the addition of fermented whey. Bulking agents, such as compost, will enhance metabolism of organic contaminants because they provide extra nutrients, additional carbon sources and assist in retaining moisture content of the pile.

The increase in C/N ratios reduced the hexadecane (HXD) biodegradation. Limitation of microbial growth and metabolism in polluted soils can be related to the low concentration of inorganic nutrients, such as nitrogen, phosphorous and potassium, producing high C/N, C/P and C/K ratios (Volke-Sepulveda et al. 2006).

17.7.4 Oxygen

Hydrocarbons being highly reduced substrates, require an electron acceptor, with molecular oxygen being the most common. Though most studies have shown biodegradation of hydrocarbon to be an aerobic process, anaerobic biodegradation

of hydrocarbons has also been reported. In the absence of molecular oxygen, nitrate, iron, bicarbonate, nitrous oxide and sulfate, have been shown to act as an alternate electron acceptor during anaerobic hydrocarbon degradation.

17.7.5 pH

pH is not of much significance in marine environments since it is well buffered at about pH 8.5, but soil pH varies widely and pH between 7 and 8 has been found to support optimum degradation of alkanes in soils/sediments.

17.7.6 Surfactants

Surfactants are amphiphilic compounds, that reduce surface and interfacial tensions by accumulating at the interface of immiscible fluids or of a fluid and a solid, increase the contact surface areas of insoluble compounds, leading to increased mobility, bioavailability and subsequent biodegradation.

17.7.6.1 Synthetic Surfactants

The use of chemical surfactants has been extensively studied by various authors (Suchanek et al. 2000; Stortini et al. 2009). Chrzanowski et al. (2006) performed biodegradation studies of a model mixture of hydrocarbonyl dodecane and hexadecane (1:1 w/w) by applying different surfactants like: lecithin extracted from soyabeans, rhamnolipids from *Pseudomonas aeruginosa*, saponin, lutensol GD 70, Triton X-100 and Tween 20 with different concentration 150, 300 and 600 mg l^{-1} for 7 days. *Candida maltosa* was found capable to degrade hydrocarbons by a maximum of 92.7% in case of saponin (300 mg l^{-1}), followed by 90.3% in case of saponin (150 mg l^{-1}) and 80.9% with rhamnolipid (150 mg l^{-1}).

Surfactants have been also reported to increase the uptake and assimilation of alkanes such as hexadecane in liquid cultures (Beal and Betts 2000; Noordman and Janssen 2002), but their usefulness in soils and other situations is less evident (Holden et al. 2002). Surfactants produced by microorganisms probably have other roles as well, such as facilitating cell motility on solid surfaces (Caiazza et al. 2005), or the adhesion/detachment to surfaces or biofilms (Boles et al. 2005).

17.7.6.2 Biosurfactant

Microorganisms are grouped to endo- and exo-type ones, based on biosurfactant accumulation. Endo-type biosurfactants are bound up with the wall surface of

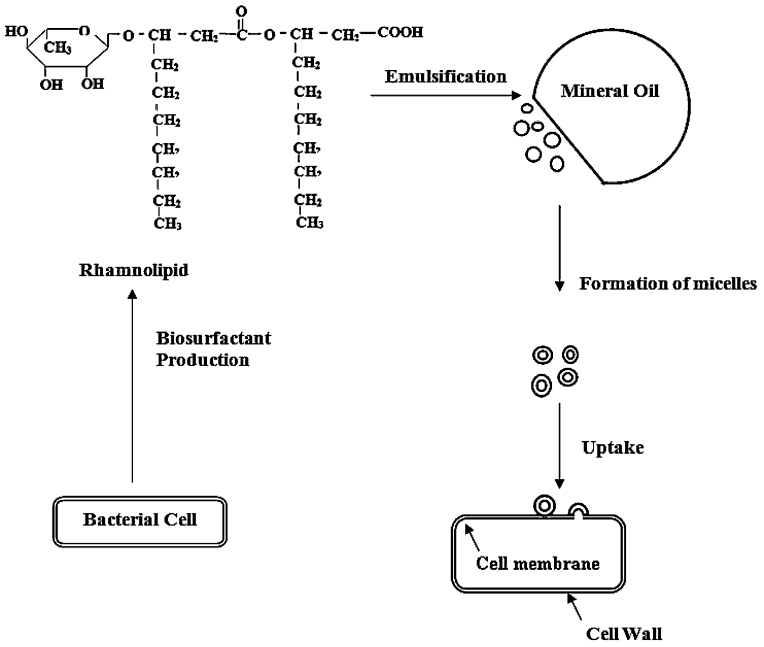

Fig. 17.10 Involvement of biosurfactants in the uptake of hydrocarbons and the emulsifying effect of a rhamnolipid produced by *Pseudomonas* spp. within the oil–water interphase and the formation of micelles (after Fritsche and Hofrichter 2000)

the microorganism cell and, as a rule, constitute components liposomally active (Al Tahhan et al. 2000). Exo-type biosurfactants are excreted into medium by cell to provide substrate access to cell surface, due to emulsion or suspension production in liquid medium (Deziel et al. 1996). Biosurfactants are very diverse in their chemical composition. They include glycolipids, lipopeptides, lipoproteins, phospholipids, fatty acids and polymeric surfactants (Rosenberg and Ron 1999).

Biosurfactants are organic molecules consisting of a hydrophilic moiety and act as emulsifying agents by decreasing the surface tension and forming micelles. The uptake mechanism of hydrocarbons and emulsification by rhamnolipid produced by the bacteria has been demonstrated in Fig. 17.10.

Microbial surfactants have advantages over synthetic surfactants due to lower toxicity, higher biodegradability and environmental compatibility (Cameotra and Makkar 2004). It may be produced cost effectively under *ex-situ* conditions and *in-situ* production may be stimulated at the site of contamination and can be recovered and recycled (Moran et al. 2000).

Bushnell and Haas (1941) were among the first to demonstrate bacterial production of biosurfactants. Based on molecular weight, microbial surfactants are classified in two groups (Hua et al. 2010). Glycolipids and lipopeptides are counted under low molecular-weight surfactants, whereas emulsa, alasan, biodispersan

and extracellular or cell membrane-bound bioemulsifiers (exopolysaccharide; EPS) are high molecular weight compounds. Maximum study was done with rhamnolipids produced by *Pseudomonas aeruginosa* (Rahman et al. 2002). It was shown that rhamnolipid extracts lipopolysaccharides (LPS) from cells of *Pseudomonas*, thereby increasing the hydrophobicity of the cell surface and promoting attachment of the cells to hydrocarbon droplets (Al-Tahhan et al. 2000). It is suggested that greater attachment stimulates hexadecane degradation (Al-Tahhan et al. 2000) while it was found that it inhibits octadecane degradation due to flocculation of the cells. Christova et al. (2004) reported that *Renibacterium salomininarum* 27BN also produced biosurfactant of glycolipid. It secretes two rhamnolipids RLL and RRLL from *Pseudomonas aeruginosa* when grown on hexadecane (2%) as sole source. At the end of 192 h, only $9.3 \pm 2.1\%$ residual hexadecane was obtained in cultures incubated with the whole cell.

Biosurfactant activities can be determined by measuring the changes in surface and interfacial tensions, stabilization or destabilization of emulsions and hydrophilic–lipophilic balance. The hydrophilic–lipophilic balance is directly related to the length of the hydrocarbon chain of fatty acids (Desai and Banat 1997). They are often good emulsifiers; the emulsions they form are more stable than the emulsions obtained by synthetic surfactants (Desai and Desai 1993).

In addition to solubility enhancement, EPS shields bacterial cells from direct exposure to toxic substances (Gutierrez et al. 2009). Ron and Rosenberg (2001) found that EPS alters the hydrophobicity by exposure of hydrophobic phospholipids tails of cells (Al-Tehhan et al. 2000). EPS, in case of biodegradation of petroleum hydrocarbon, was first reported by Watanabe and Takahashi (1997) in *Pseudomonas* sp. SLI and SLK. *Halomonas* spp. (Martinez-Checa et al. 2007) and marine *Enterobacter cloacae* (Iyer et al. 2006) have also been reported for the production of EPS. Iyer et al. (2006) found that emulsion of EPS produced by *Enterobacter cloacae* (EPS 71a) with hexane was stable up to 10 days between pH 2 and 10 in presence of NaCl in the range of 5–50 mg ml^{-1} at 35–37°C. Hua et al. (2010) found that EPS, secreted by *Enterobacter cloacae* strain TU during growing on *n*-hexadecane as the sole carbon source, composed of glucose and galactose with molecular weight of 12.4 ± 0.4 kDa. Kumar et al. (2007) observed the reduction of interfacial tension by EPS produced by *Planococcus maitriensis* Anita I for hexane and found that this EPS contained carbohydrate (12.06%), protein (24.4%), uronic acid (11%) and sulphate (3.03%).

17.8 Conclusion

Researches carried out on microbial degradation have provided new insights into the mechanism of alkane degradation. However, many aspects of degradation are still not very clear, particularly incorporation of alkanes into the microbial cell. A few new enzymes have been recently found which metabolize long chain

alkanes. Although there are indications for existence of new alkane hydroxylases, but they have been not yet characterized. We are still curious to know why there are several alkane hydroxylases with similar substrate specificities. Regulation and expression of genes coding for alkane degradation pathways are still not very clear. Elucidation of these pathways is very important to design bioremediation strategies for enhancing degradation of alkanes in the contaminated sites. Besides, recombinant and functionally improved strains have to be developed to enhance the process of biodegradation of oil hydrocarbons at contaminated sites.

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