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Catabolic Pathway of Decane and Eicosane by *P. frederiksbergensis*

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Alkane degradation is a widespread phenomenon in nature. Alkanes are major constituents of crude oil. They are also present at low concentrations in diverse non-contaminated because many living organisms produce them as chemo-attractants or as protecting agents against water loss. Many microorganismscapable of utilizing alkanes as a carbon and energy source have been isolated and characterized. This workdetected the metabolic of decane ($C_{10}H_{22}$), eicosane ($C_{20}H_{42}$). The secondary metabolites stimulated the knowledge of how bacteria metabolize alkanes aerobically, with a particular emphasis on the oxidation of long-chain alkanes, including factors that are responsible for chemotaxis to alkanes, transport across cell membrane of alkanes.

Key words: Alkane degradation, *P. frederiksbergensis,* Catabolic pathway.

Bioremediation of hydrocarbon-contaminated soils, which exploits the ability of microorganisms to degrade and/or detoxifyorganic contamination, has been established as an efficient, economic,versatile, and environmentally sound treatment (Austin and Groves, 2011; Liu, and Shao, 2005). Petroleum is a complex mixture of many thousands of compounds. These can be divided into four major groups: the alkanes, the aromatics, the resins, and the asphaltenes. In general, the alkane fraction is the most biodegradable, whereas the polar fraction (i.e., the resins and asphaltenes) is resistant to biological degradation. Both crude and refined oil consist of a large number of different compounds (Liu *et al.*, 2011). Petroleum hydrocarbons are the major constituents of crude oil (50 – 98 %) and *n*-alkanes represent 20 to 50 % of oil, depending on the source of the oil (Beal and Betts, 2000). Alkanes are major components in crude oil and are also present in many organisms, such as green plants (Dinamarca *et al*., 2003; Golyshin *et al*., 2002).Alkanes, ranging from methane to compounds with chain lengths of 40 or more carbon atoms, have generally been found to be degraded in both laboratory cultures and the natural environment (Hernández-Arranz *et al*., 2013). Some *n*-alkanes are recognized as carbon sources for microbial production, such as biodegradable polymers and biosurfactants (McKew *et al*., 2007; Canosa *et al*., 2000; Duran, 2010; Lai *et al*., 2012).

It has long been recognized that many microorganisms can use medium or long chain n-alkanes as sources of carbon and energy. This has stimulated many studies based on the usefulness of these organisms in the bioremediation of oil spills and contaminated sites (Sabirova *et al*., 2011; Hara *et al*., 2003). In general, n-alkanes are readily biodegradable and in almost any environment, there are microorganisms able to degrade these substances (Rojo, 2009; Head *et al*., 2006; Marchant *et al*., 2006).

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Bacterial oxidation of n-alkanes is a very common phenomenon in soil and water (Moreno *et al*., 2007; Parales, and Ditty, 2010). and is one of the major processes in geochemical terms. The estimated of amount of alkanes that are recycled per year amounts to several million tons from nature seepage and oil spills alone (Noordman and Janssen, 2002; Li *et al*., 2008). Even more relevant alkanes (mainly waxes or paraffin) are produced by plants, algae and other organisms because they are available to bacteria throughout the biosphere (Harayama *et al*., 2004). Plenty of strains which are able to degrade hydrocarbon contaminants under psychrophilic conditions have been isolated (Hearn *et al*., 2008). The following genera of microorganisms have been shown to be able to degrade alkanes: *Achromobacter, Acinetbacter, Bacillus, Actinomyces, Corynebacterium, Aeromonas, Alcaligenes, Benecha, Brevibacterium, Nocardia, Pseudomonas, Flavobacterium, Micromonospora, Vibrio* and 14 genera of Yeasts (Hearn *et al*., 2009; Parales and Harwood, 2002). One of these organisms is *Pseudomonas oleovorans*, which can grow on intermediate chain length *n*-alkanes by virtue of the *OCT* plasmid encoded alkane hydroxylase (Canosa *et al*., 2000).

Microorganisms that act in degradation like (yeast and bacteria) easily survive a period of inactivity due to missing nutrients and were reactivated within hours to degrade newly added crude oil (Hazen *et al*., 2010). Under substrate limiting conditions selectivity of degradation was found, destroying medium chain *n*-alkanes at a faster rate than long chain n-alkanes (C30 - C31). During degradation, the physical parameters of the crude oils (e.g., density, viscosity, average molecular weight) were altered and shifted into the direction of heavy oil. In vitro degraded oil is very similar to oil degraded in nature (Dinamarca *et al*., 2002; Coulon *et al*., 2007; Head *et al*., 2006).

Therefore, the degradation of aliphatic hydrocarbons in contaminated areas stimulated this study on the usefulness of biological treatments which are considered cheaper than alternative methods such as incineration, storage, or soil washing. Also study and the examination of the catabolic pathways of such compounds is the best key for such process.

MATERIALS AND METHODS

Source of microorganisms and Enrichment and cultivation of organisms

Microorganisms used in this study were isolated and characterized in Botany and Microbiology Dept.Using an enrichment culture technique, the pure cultures were aerobically cultivated with sterilized mineral salts media. The enrichment and propagation were carried out in sterilized Erlenmeyer flasks containing M_9 and MII media (without yeast extract). The cultivation was carried out in sterilized 100 ml flask containing 18 ml media, 1 ml *P. frederiksbergensis*supplemented with 1m*M* decane ($C_{10}H_{22}$), eicosane ($C_{20}H_{42}$). The pH value of the culture solution was adjusted to 7.0 with NaOH. Decane and Eicosanewere used in the first experiments as initial carbon sources. The purpose of this was to activate the culture and confirm the growth capability on *n*-alkanes before using for further experiments. The flasks were tightly sealed with screw caps and the control flasks without bacteria were incubated in parallel. After the incubation period of 10 days on a rotary water bath shaker at 4˚C and 200 rpm, growth was observed in M_9 media (Golyshin *et al*., 2002).

Hydrocarbon degrading bacteria and substrate selectivity

The following alkanes compounds were used as sole of carbon source and energy sources: octadecane ($C_{18}H_{38}$), docosane ($C_{22}H_{46}$), tetracosane($C_{24}H_{50}$), dotricontane ($C_{32}H_{66}$) and tetracontane ($C_{40}H_{82}$). The liquid compounds < C_{16} were directly added to the media after sterilization by filtration through a 0.2 µm filter. The *n*-alkanes > C_{16} were dissolved in hexane and then added to the media under sterilized hood. After the evaporation of hexane, for 18 ml sterilized M_9 media (without yeast extract), 1m*M* from each of the previous mentioned compounds was added. The culture was centrifuged to 13,000 rpm for 10 min and the pellet was washed three times, then it suspended in the mineral salt media. Then, the fresh medium was inoculated with 1 ml of the *P. frederiksbergensis*. The flasks were tightly sealed with screw caps and the control flasks without bacteria were incubated in parallel. The flasks were incubated on a rotary water bath shaker at 4°C and 200 rpm.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis*determination of n-alkanes*

In order to determine the biodegradation rate the following procedure was used. 7 ml vials containing 2 ml MSM supplemented with 10 µl pure culture and 1 m*M*of each compounds (Decane and Eicosane) were tightly sealed with Teflon rubber. The vials were incubated at optimal pH and temperature values on a rotary water bath shaker at 200 rpm for 15 days. The control vial without the bacteria was incubated in parallel. In time intervals of 2 days each vial was subjected to GC-analysis by centrifugation of whole vial for 10 min at 13,000 rpm. The supernatant was collected and the eicosane was extracted with hexane as an organic solvent with a ratio of 1:1 from each supernatant and centrifuged again for 10 min at 13,000 rpm three times. The eicosane containing apolar phase was subjected to GC-MS for quantitative and qualitative analysis. The GC-MS parameterswereThesamples were analyzed and quantified by GC-MS (HP-G1800 AGCD system). System parameters: inlet and detection temperatures 280°C, a temperature program with an initial oven temperature 150°C, final temperature 220°C and heating rate 15°C/min to 280°C with Helium as carrier gas at $P = 0.37$ atm.

Determination of the alkane catabolic pathway of *P. frederiksbergensis* by GC- MS

To examine the organism's ability to grow via terminal and/or subterminal pathway, two compounds of alkanes were used in order to identify the metabolic intermediates resulting from the growth of *P. frederiksbergensis*. For this purpose, *P. frederiksbergensis* cells were grown at optimal conditions using 20 ml of MSM supplemented with 1 m*M* of Decane and Eicosane. In time interval of 2 days, the culture supernatant was obtained by centrifugation at 13,000 for 20 min. For extraction of the intermediates, an equal amount from the sample and 1 *M* of methanolicNaOH were mixed well in glass vial and incubated in water bath at 60°C for 20 min. Equal amount of BF_3/methanol was then added after cooling in ice for 1 min in order to create methyl easters. After the incubation again at 60°C for 10 min, 200 µl of saturated NaCl was added and mixed well. The sample was transferred to 1.5 ml Eppendorf vial, then 300 µl hexane was added and finally centrifuged at 13,000 rpm for 10 min. The hexane layer was separated, dried with anhydrous Na_2SO_4, then the supernatant was transferred to a fresh vial. The water layer was extracted for second time and the hexane layer was combined with the previous extract, and then subjected to GC-MS. To ensure that detected metabolic intermediates were a result of *P. frederiksbergensis* biotic activity, sterile controls were supplemented with Decane and Eicosaneand run in parallel with the inoculated media and were analyzed by GC-MS.System parameters: inlet temperature was 250°C and detection temperatures was 280°C, a temperature program with an initial oven temperature 160°C for 1 min, final temperature 240°C and heating rate 10°C/ min to 240°C with Helium as carrier gas at $P = 0.37$ atm.

RESULTS AND DISCUSSION

Determination of the alkane catabolic pathway of *P. frederiksbergensis*

After 2 days of incubation on Decane and Eicosane, no metabolites were detected by GC-MS. After 5 days metabolites of both compounds were detectable. Decanol, decanal, decanoic acid, eicosanol, eicosanal, eicosanoic acid were identified by comparison of their retention times and mass spectra (Fig. 1-2).

The ability of *P. frederiksbergensis*to oxidize various *n*-alkanes may indicate that this strain might possess more than one alkane hydroxylase, one with specificity for short chain, the other for long chain alkane. Different alkane hydroxylasesin one organism may be responsible for oxidation of different range of alkanes with overlapping substrates ranges (Feng *et al*., 2007). Considering the broad substrates range of *P. frederiksbergensis*, an interesting question at these point was whether this isolate contained an additional *alk*B homologues that was not amplified or detected in southern hybridization. It was clear that *P. frederiksbergensis* degraded short, medium and long chain alkanes but not very longer chain alkanes such as C_{32} - C_{40}. This is a common feature of many other alkanes degradative microorganisms (Johnson and Hyman, 2006; Kasai *et al*., 2002). The inability of growing on very long chain alkanes was mainly due to the decrease of bioavailability of these compounds at low temperature and this was responsible for their increased recalcitrance.

Enhanced long chain alkanes recalcitrance at low temperature may have an importance in application for in *situ* bioremediation attempts in cold climate environment. Thus, successful bioremediation strategies would require the application of cold active solublizing agents to increase the bioavailability of very long chain alkanes (Qiao, and Shao, 2010; Pirnik *et al*., 1974).

Although the ability to grow exclusively with decane, but not hexadecane, was found to be specific for Gram-negative bacteria, *P. frederiksbergensis*represented an exception from this fact (Petruschka *et al*., 2001). To ensure that the detected metabolic intermediates were a result of *P. frederksbergensis*biotic activity, sterile controls supplemented with eicosane and decane were run in parallel with the inoculated media and

Table 1. Main fragments in the spectra of decane and eicosane

Metabolites	m/z	Relative Abundance Intensity (%)	Metabolites	m/z	Relative Abundance Intensity (%)
decanol	55	34	eicosanol	55	99
	57	100		57	100
	71	66		71	75
	85	45			
decanal	55	34	eicosanal	55	47
	57	100		57	100
	71	66		71	78
	69	34		69	48
	85	34			
decanoic acid	55	33	eicosanoic acid	55	99
	57	100		57	85
	71	16		69	100
	69	66			
	83	13			
	85	42			
	99	10			

Table 2. Growth of *P. frederiksbergensis*on various substrates

Compounds	Chemical structure	*P. frederiksbergensis*
2,4-Dichlorophenol	$C_6H_4Cl_2O$	-
Phenol	C_6H_5OH	-
2-Chlorophenol	C_6H_5ClO	-
Toluene	$C_6H_5CH_3$	-
Decane	$C_{10}H_{22}$	++
Naphthalene	$C_{10}H_8$	-
Dodecane	$C_{12}H_{26}$	+
n-Hexadecane	$C_{16}H_{34}$	++
n.Heptadecane	$C_{17}H_{36}$	+
n-Octadecanol	$C_{18}H_{38}O$	+
Octadecane	$C_{18}H_{38}$	+
Eicosane	$C_{20}H_{42}$	++
Docosane	$C_{22}H_{46}$	+
n-Docosanol	$C_{22}H_{40}O$	+
Tetracosane	$C_{24}H_{50}$	-
Dotricontane	$C_{32}H_{66}$	-

+ +: growth well +: growth -: no growth

were analyzed by GC-MS. After 8 days, both the eicosanol and decanol peaks had decreased and only traces of eicosane and decane (parent compounds) could be detected. Table 1 shows the most intense fragments found in the mass spectra of the metabolites of eicosane and decane.

Substrate spectra of *P. frederiksbergensis*

Aliphatic and aromatic compounds were tested as sole of carbon and energy source to find out the growth capability and substrate spectrum for *P. frederiksbergensis*. Table (2) describes the range of substrates utilized as carbon and energy source by P. frederiksbergensis. The isolates assimilated a wide range of n-alkanes such as short, medium (C10 - C16) and long chain alkanes (C20 - C22). The isolates did not degrade the aromatic compounds and longer chain alkanes such as (C32 - C40).

Biodegradative capabilities of *P. frederiksbergensis*

During the growth of *P. frederiksbergensis* on mixtures of *n*-alkanes, the uptake proceeded sequentially in order of increasing molecular weight. The biodegradative capability of utilizing mixture of decane ($C_{10}H_{22}$), hexadecane ($C_{16}H_{34}$) and eicosane ($C_{20}H_{42}$) as a sole of carbon and energy sources was examined (Fig. 3).

The degradation experiments were carried out using 1 m*M* of each compound. 100 % of decane was degraded after 9 days and hexadecane after 12 days.When about 90 % of decane and 60 % of hexadecane were consumed, the degradation of eicosane started. It was observed that only 5 % of eicosane was degraded after 6 days. The biodegradation of eicosane was slower compared to the biodegradation without mixing with decane and hexadecane. The biodegradation increased slowly even though the metabolism was high enough to maintain the bacterial activity stable. It was also observed that the first 6 days of incubation were the most important and critical stage for the biodegradation of the mixture.

PH dependence of growth by *P. frederiksbergensis*

Another interesting observation during

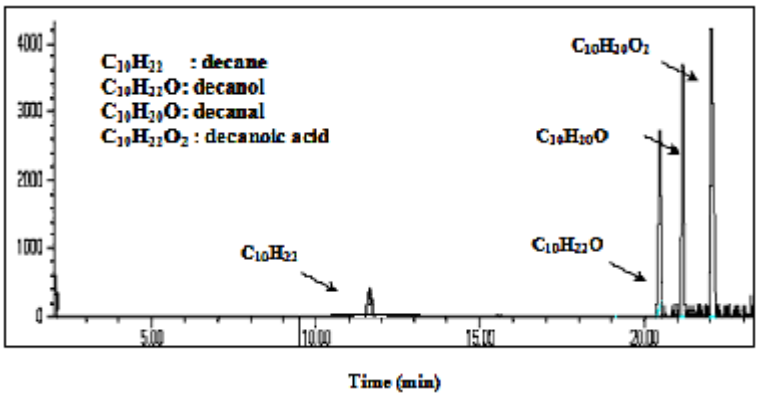


Fig. 1. GC-MS profile of intermediate metabolites of decane degradation by *P. frederksbergensis*

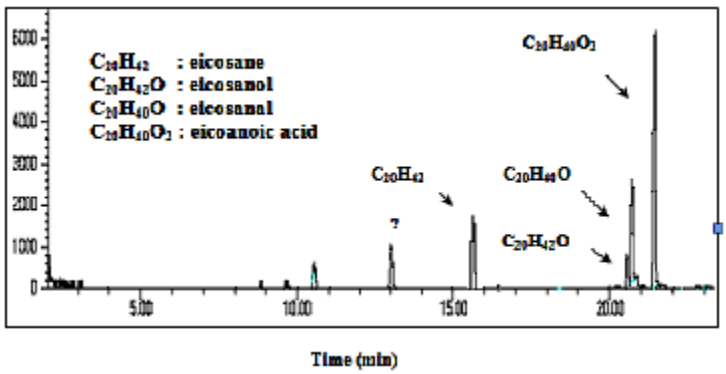


Fig. 2. GC-MS profile of intermediate metabolites of eicosane degradation by *P. frederksbergensis*

the growth of both *P.frederiksbergensis*, was that pH value dropped. This contributed substantially the medium acidification, as shown in Figure (4). This could explained that the isolated strains had the capability of oxidizing the eicosane to the corresponding fatty acid. It was clear that the increase in cell number specially in the exponential growth phase took place after the 5th till the 7th day and the bacteria reached the stationary growth phase due to the media acidity.

The ability to grow on medium or long chain n-alkane is a very common property of Gram negative as well as Gram positive bacteria. Only the Pseudomonas putida GPo1 alkane hydroxylase system has been studied in details with respect to enzymology, genetics, and potential applications (McKew *et al*., 2007; Meintanis *et al*., 2006). This study focused on *P. frederiksbergensis* and this is the first study focused on the molecular biology and biochemistry of this strain (Parales *et al*., 2008). The major challenge to meet was the activity assay for the oxidation of alkanes.

In the crude extract the activity was determined by the decrease of the substrate by GC-MS. Another challenge to meet the oxidation of alkanes in the crude extract was the insolubility and hydrophbicity of the substrates. It was possible to detect the resulting acid as a product

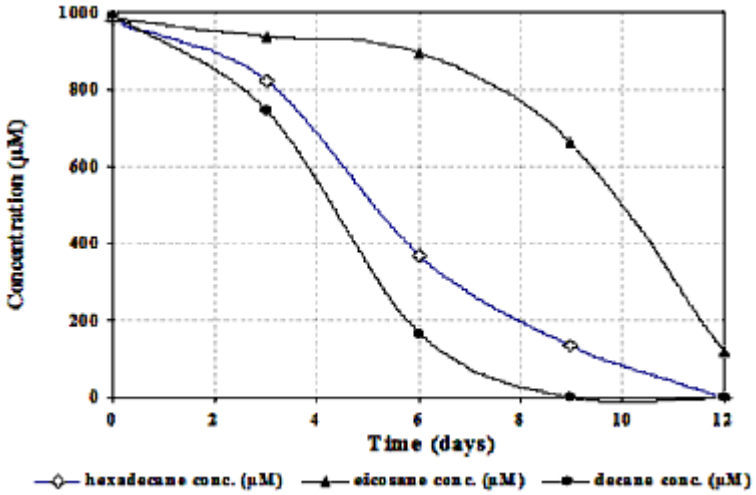


Fig. 3. Time course of the degradation of a mixture decane, hexadecane and eicosane by *P. frederiksbergensis*

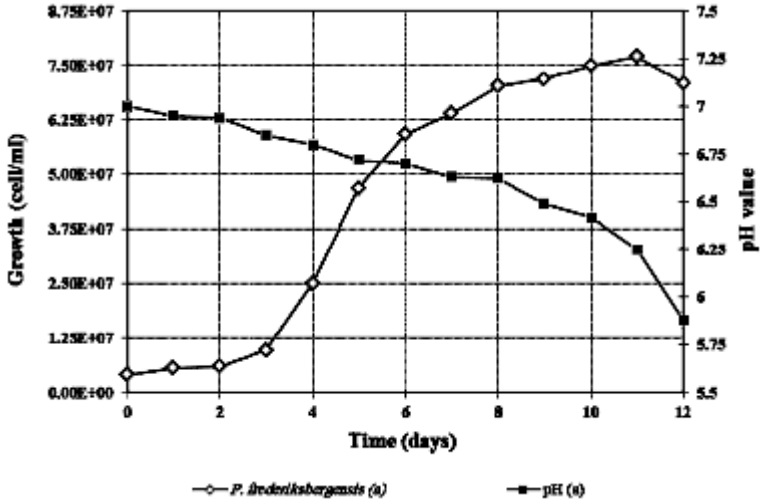


Fig. 4. PH change of the medium during growth of *P. frederiksbergensis* and *R. erythropolis*on eicosane

for the alcohol oxidation in the metabolic pathway. The fragmentation patterns observed in the mass spectra of the metabolites detected from eicosane and decane support the conclusion described by Briton, (1984). In GC-MS analysis the trouble was that the M+ peak can be quite weak, especially as the carbon number increases, Saturated hydrocarbons as a class exhibit quite distinctive mass spectra. The fact that saturated hydrocarbons are present was obvious, but their identification was difficult. This also applies to long chain alcohols, acids, ethers and some esters (Davis *et al*. 1987). Based on the facts known from alkane oxidation, it was assumed that the reaction catalyzed by alkane monooxygenase resulted in an alcohol which is further oxidized to the corresponding fatty acid via aldehyde by an alcohol and an aldehyde dehydrogenase, respectively (Figure 5). The presence of primary alcohols and aliphatic fatty acids of the same chain length in cultures grown on n-alkanes indicated that the oxidative attack was on one of the methyl groups of the hydrocarbon molecule. This mode of attack is similar to what has been observed in other bacterial systems metabolizing n-alkanes (Peng *et al*., 2007). The fundamental details of the metabolic pathways involving qualitative determination of intermediates in supernatants are well documented (Peng *et al*., 2008). In most cases the initial metabolic attack on n-alkanes is done by a hydroxylase (monooxygenase) enzyme producing the corresponding alkanol.

Such hydroxylation reactions may be linked to a number of electron carrier system. The fatty acid produced by all the pathways are then channeled into the ß-oxidation pathway (Petruschka *et al*., 2001). Secondary alcohols produced by sub-terminal oxidation are further oxidized to corresponding esters and hydrolytically cleaved to produce an acid and alcohol, the alcohol is then oxidized and the fatty acid is subjected to ß-oxidation. In many cases, the fatty acids may be

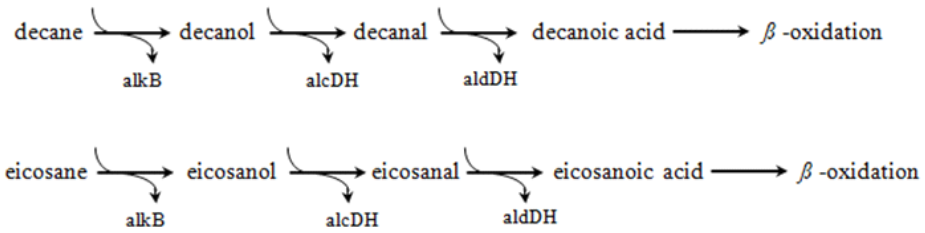
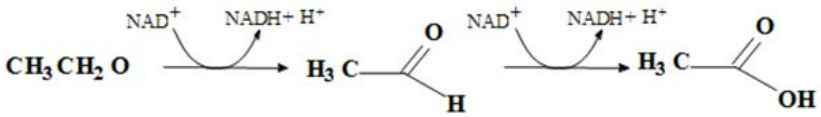


Fig. 5. Metabolic pathway of decane and eicosane by *P. frederiksbergensis*

incorporated directly into cell membranes (Kok *et al*., 1998). It was remarkable that oxygen limitation and medium acidity were very problematic during incubation in the small vials. The catabolism of compound like eicosane released some of the fatty acids containing H+ in the growth medium and this caused medium acidification (eq. 1).



Furthermore, the detected metabolites of decane, hexadecane and eicosane were all known as intermediates of the proposed degradation pathway via C2-cleavage. Moreover, the resulting alkanoic acid from the metabolic pathway has surfactant like properties (Kurth *et al*., 2008). It could be concluded, that the initial reaction of alkanes oxidation by this organism was terminal oxidation (Morales *et al*., 2006). The behavior of *P. frederiksbergensis* in utilizing decane, hexadecane and eicosane was unique. Such behavior suggested that some of decane and hexadecane metabolites may help in the initial process of eicosane degradation. The biodegradation

increased slowly even though the metabolism was high (Coon, 2005). During the growth on mixtures of n-alkanes, the uptake proceeded sequentially in order of increasing molecular weight. This was found to be due to lower affinities for substrates of longer chain length: the Km approximately doubled for additional -CH2 -CH2- unit on the chain. The uptake of these compounds was limited by the rate of initial adsorption under poorly agitated conditions (Lanfrancon *et al*., 2003; Mooney *et al*., 2006). It was also proved that the first 6 days of incubation were the most important and critical stage for the biodegradation of the compounds mixtures. When the bacteria are confronted with mixture of carbon sources, many species preferentially utilize one carbon source until depletion and afterwards they begin to utilize the remaining carbon sources (Li *et al*., 2008). The genes encoding the carbon catabolic enzymes are often regulated in a similar manner during growth on preferred carbon source (Panke *et al*., 1999; Lanfranconi *et al*., 2003).

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