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Growth, Physiology and Application of Selected Alkane Degrading Microorganisms

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To Manuela
To my parents

It's 106 miles to Chicago, we have a full tank of gas, half a pack of cigarettes, it's dark and we're wearing sunglasses.

The Blues Brothers

DANK

Allen, die mir bei der Erarbeitung und Abfassung dieser Diss geholfen und mich unterstützt haben, möchte ich an dieser Stelle herzlichst danken.

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Zürich, im September 1998

Patrick Wicht

TABLE OF CONTENTS

ABBREVIATIONS

alkB	Gene for the alkane hydroxylase of *Pseudomonas oleovorans*
AlkS	Gene product of *alkS*
alkS	Gene for the transcriptional activator of *alkB*
D	Dilution rate (h^{-1})
luxAB	Genes for subunits A and B of the luciferase
luxCDABE	Luciferase operon
ν	Degree of reduction
n-C12	Dodecane
n-C16	Hexadecane
OD_{546}	Optical density at 546nm
OD_{600}	Optical density at 600nm
P_{alkB}	Promotor of *alkB*
q_{CO_2}	Specific carbon dioxide production rate (mmol h^{-1} g^{-1})
q_{O_2}	Specific oxygen uptake rate (mmol h^{-1} g^{-1})
RLU	Relative light unit
rpm	Rotations per mintute
RQ	Respiratory quotient
s	Substrate concentration
s_0	Substrate concentration in the medium
x	Biomass dry weight (g l^{-1})
$Y_{av\ e-}$	Growth yield for available electrons in the carbon source (g $mole^{-1}$)
$Y_{x/C}$	Growth yield for substrate carbon
$Y_{x/s}$	Growth yield for carbon source
μ	Specific growth rate
μ_{max}	Maximum specific growth rate (h^{-1})

SUMMARY

The influence of selected growth factors on alkane biodegradation was studied. Most experiments were performed with the alkane degrading microorganism *Acinetobacter* sp. H01-N, and the linear alkane dodecane was used as a model compound. First, methods for the measurement of alkane and biomass concentrations in hydrocarbon fermentations were developed. For the determination of biomass, the most accurate method was obtained by adding a diluted solution of Triton X-100 to the cell suspension prior to centrifugation. Centrifugation was followed by standard methods for biomass determination. With this pre-wash method, reproducible and accurate results were observed. Residual alkane concentrations were determined by extracting samples with a mixture of methanol and chloroform. Using this method, 100% recovery of alkane substrate from culture liquids was possible. In contrast, extraction of cell samples with pentane resulted in incomplete recovery of alkane substrate, possibly due to the accumulation of alkanes within the cell. From these results, it was possible to speculate on the accumulation of alkanes in cells of *Acinetobacter* sp. H01-N. The results obtained can be interpreted that an active transport mechanism may have been responsible for uptake of alkanes by this organism.

Substrate transfer and transport limitation are important factors that govern alkane biodegradation. Results from batch experiments of growth on dodecane clearly demonstrated that *Acinetobacter* sp. H01-N exhibited distinct mechanisms to increase mass transport of alkane substrates. This was evident from the observation that the size of dodecane droplets decreased during batch cultivation, which was either due to the formation of a biofilm on the surface of the droplet or by action of surface active agents. The results did not however allow distinction between these two mechanism. Despite these adaptations to growth on alkanes, a high power input (stirrer speed of 2100 rpm) was required for sufficient mass transport in carbon limited continuous culture.

In groundwater and soil systems, where power input is lower, bioavailability is an even more important factor that determines the biodegradation rate of hydrophobic compounds. Consequently, for the implementation of a particular bioremediation strategy, the bioavailable concentration of pollutants is an important parameter. This can be measured with suitable whole cell biosensors. Such a sensor for middle-chain linear alkanes was developed. The biosensor strain carried the regulator gene *alkS* from *Pseudomonas oleovorans* and a transcriptional fusion of P_{alkB} from the same strain and

the promoterless luciferase genes *luxAB* from *Vibrio harveyi*. The sensor was tested in various aqueous samples. Results showed that the biosensor cells were readily inducible with octane, and the light emission correlated well with the octane concentration. In well-defined aqueous standard samples, the lowest octane concentration tested (24.5 nM) resulted in a significant increase of the light output compared to a blank containing no octane and could be quantified by using the sensor. In heating oil contaminated groundwater samples, a total inducer concentration of 6 nM in octane equivalents was detectable.

The presence of easily biodegradable compounds is an important factor that regulates pollutant biodegradation. During batch cultivations of *Acinetobacter* sp. H01-N on a substrate mixture of acetate and dodecane, dodecane was not degraded before acetate had been depleted. In contrast, when these carbon sources were supplied in carbon-limited chemostat at dilution rates below 0.3 h^{-1}, simultaneous oxidation of both carbon sources was observed. Dodecane induced oxygen uptake rates (reflecting catabolic enzmye activity) and the cell surface hydrophobicity increased in accordance with increasing fractions of dodecane in the medium feed. Subsequent transient experiments in carbon-limited continuous culture at a dilution rate of 0.1 h^{-1} showed rapid metabolic adaptation to the presence of dodecane. Metabolic responses started after 0.5 to 6 h, and the response time was dependent on the carbon source that was used before the shift and decreased when an alternate carbon source was present during transient growth.

ZUSAMMENFASSUNG

Der Einfluss von ausgewählten Wachstumsparametern auf den Abbau von Alkanen wurde untersucht. Die meisten Experimente wurden mit dem Alkan abbauenden Bakterium *Acinetobacter* sp. H01-N durchgeführt, und als Modellsubstrat für lineare Alkane wurde Dodekan verwendet. Zuerst wurden Analysemethoden für Biomasse- und Alkankonzentrationen in Kohlenwasserstoff enthaltenden Kulturlösungen entwickelt. Die Biomassebestimmung gelang am besten mit einer Methode, bei der die Zellsuspension zuerst mit einer verdünnten Triton X-100 Lösung versehen wurde. Danach wurde die Suspension zentrifugiert und die Biomasse gemäss Standardmethode bestimmt. Mit dieser Methode waren reproduzierbare und genaue Messungen möglich. Alkane konnten quantitativ mit einem Gemisch aus Methanol und Chloroform von Zellsuspension extrahiert werden. Im Gegensatz dazu war die Substratwiederfindung unvollständig, wenn die Proben mit Pentan extrahiert wurden. Dies war vermutlich darauf zurückzuführen, dass Alkane in den Bakterien akkumulierten. Diese Resultate deuteten an, dass Alkane in *Acinetobacter* sp. H01-N möglicherweise durch einen aktiven Transportmechanismus aufgenommen wurden.

Substrattransfer und -transport limitieren häufig den Abbau von Alkanen. Batchversuche auf Dodekan bestätigten jedoch, dass Substrattransport in Kulturen von *Acinetobacter* H01-N erhöht war. Das wurde deutlich durch die Beobachtung, dass Alkantröpfchen während Batchexperimenten mit diesem Organismus kleiner wurden. Obwohl die Resultate keine schlüssigen Hinweise auf den eigentlichen Mechanismus gaben, war zu vermuten, dass dieser Effekt entweder durch einen Biofilm auf den Alkantröpfchen oder durch die Ausscheidung von oberflächenaktiven Substanzen verursacht wurde. Trotz dieser Anpassung des Bakteriums an hydrophobe Substrate war ein hoher Leistungseintrag (2100 rpm) nötig, um in kohlenstofflimitierten kontinuierlichen Kulturen Transportlimitation zu vermeiden und um zu gewährleisten, dass das Substrat vollständig umgesetzt wurde.

In Grundwasser- und Bodensystemen ist der Leistungseintrag deutlich geringer als in Bioreaktoren. In solchen Systemen spielt die Bioverfügbarkeit eine noch wichtigere Rolle. Für die Wahl einer bestimmten Bioremediations-Strategie ist es deshalb besonders wichtig, die bioverfügbaren Konzentrationen eines Schadstoffes zu kennen. Diese Konzentration kann mit einem Ganzzell-Biosensor gemessen werden. Wir entwickelten einen Biosensor für mittelkettige Alkane. Der Sensor enthielt das

Regulatorgen *alkS* aus *Pseudomonas oleovorans* und eine transkriptionelle Fusion aus P_{alkB} desselben Organismus und den promotorfreien *luxAB* Genen aus *Vibrio harveyi*. Der Sensor wurde dann in verschiedenen wässerigen Proben getestet. Die Biosensorzellen waren mit Oktan induzierbar, und die Lichtemission korrelierte mit der Oktankonzentration. In definierten Standardproben betrug die tiefste getestete Konzentration 24.5 nM Oktan. Diese Konzentration führte zu einer signifikanten Erhöhung der Lichtemission und konnte durch Messungen mit dem Sensor quantifiziert werden. In mit Heizöl verunreinigten Grundwasserproben war gar eine Gesamtinduktor-Konzentration von 6 nM (als Oktanäquivalente) nachweisbar.

Ein weiterer wichtiger Faktor, der den Abbau von Schadstoffen beeinflusst, ist das Vorhandensein von leicht-abbaubaren Kohlenstoffquellen. In Batchversuchen mit *Acinetobacter* sp. H01-N auf einer Mischung von Acetat und Dodekan wurde letzteres nicht abgebaut, solange Acetat noch vorhanden war. Wurde der Organismus jedoch in kohlenstofflimiterter kontinuierlicher Kultur bei Verdünnungsraten unter 0.3 h^{-1} gezüchtet, wurden beide Substrate simultan verwertet. Die Dodekan induzierten Substrataufnahmeraten (welche die Aktivität der katabolen Enzyme widerspiegeln) sowie die Zelloberflächenhydrophobizität nahmen zu, wenn der Dodekananteil im Medium erhöht wurde. Transientexperimente bei einer Verdünnungsrate zeigten dann, dass *Acinetobacter* sp. H01-N den Stoffwechsel schnell an das Vorhandensein von Dodekan anpassen konnte. Metabole Reaktionen waren messbar nach 0.5 bis 6 h und waren abhängig von der Kohlenstoffquelle vor dem Shiftexperiment und vom Vorhandensein einer zweiten Kohlenstoffquelle während des Transientwachstums.

CHAPTER 1 General Introduction

Both crude and refined oil consist of a large number of different compounds. Alkanes are major constituents of oil (15 to 60%). In general, alkanes are readily biodegradable and in almost any environment, there are microorganisms present that are able to degrade these compounds. A well-studied alkane degrader is Acinetobacter *sp. H01-N, which readily degrades long-chain linear alkanes. The prevailing growth conditions primarily govern biodegradation of alkanes, since alkane degrading micro-organisms are distributed ubiquitously. The importance of various physical-chemical factors (pH, temperature, oxygen availability) that regulate hydrocarbon biodegradation is widely recognised. Other important aspects which are less well understood include how microorganisms are able to cope with the simultaneous presence of a wide variety of easily degradable carbon sources, and how they adapt to the presence of alkanes under such multiple-substrate growth conditions. Due to the hydrophobic nature of alkanes, the bioavailability of alkanes also needs to be considered in assessing their environmental fate.*

1.1. OIL IN THE ENVIRONMENT

1.1.1. Composition of crude oil

Petroleum hydrocarbons are the major constituents of crude oil (50-98%) (Clark Jr. and Brown, 1977; Hunt, 1979; Tissot and Welte, 1984) and consist of alkanes (linear and branched), cycloalkanes as well as aromatic compounds (1-ring and polyaromatic compounds). An analysis of the gasoline fraction of 9 crude oils showed that alkanes make up 29 to 70%, cycloalkanes 20 to 67% and aromatic compounds 6 to 14% (Perry, 1984). Crude oil also contains non-hydrocarbons including NSO compounds (containing nitrogen, sulphur or oxygen), porphyrins, asphaltenes (high molecular weight compounds), and trace metals (mainly V and Ni) (Posthuma, 1977). Fuels and feedstocks from crude oil consist mostly of the same groups of hydrocarbons as crude oil itself, although over a narrower boiling range. Due to the cracking process, the contents of alkenes and cycloalkanes increase and during alkylation, additional branched compounds are generated (National Research Council, 1985).

1.1.2. Input,...

Although some petroleum hydrocarbons in ecosystems originate from natural sources, major contaminations occur due to antropogenic activities (Table 1.1). Accidents involving super tankers have attracted most public attention. Between 1960 and 1989, 50 major tanker accidents occurred with the release of more than 20000 tons each (National Research Council, 1985). After the Exxon Valdez grounding in 1989, for example, 35600 tons of oil (267000 barrels) contaminated several hundred miles of coast line in the Arctic region of Prince William Sound with devastating impact on the ecology. From such incidents, a lot of knowledge was gained on the impact and fate of oil in the environment and on remediation techniques. It should be noted, though, that the major route of contamination is primarily by continuous low-level input rather than accidental spills (Table 1.1).

TABLE 1.1. Input of petroleum hydrocarbons into marine environments

Source	Probable range (millions of tons per year)[a]
Natural Sources	0.02 - 2.0
Offshore production	0.04 - 0.06
Transportation	0.95 - 2.62
Atmosphere	0.05 - 0.5
Municipipal and industrial wastes and runoffs	0.59 - 3.12
Total	1.7 - 8.8

[a] Adapted from (National Research Council, 1985)

1.1.3. ...effects,...

Detrimental effects of various petroleum hydrocarbons on microorganisms, plants and animals are well documented (for overview see (National Research Council, 1985; Shales et al., 1989)). Many petroleum hydrocarbons exhibit acute toxicity. This is particularly the case for low molecular weight compounds since they are more water-soluble and consequently more bioavailable (Shales et al., 1989). The toxicity of short-chain hydrocarbons has, for example, been attributed to their relatively higher solubility compared to long-chain alkanes (Klug and Markovetz, 1971). Since hydrocarbons accumulate in biological membranes (Sikkema et al., 1994), perturbation of membrane structure and function has been recognized as a possible mode of toxic action (Sikkema et al., 1995). However, carcinogenic or mutagenic effects, particularly with aromatic hydrocarbons, have also been observed (Smith, 1990). Such long-term effects on ecosystems and organisms should not be neglected due to the long persistence of many hydrocarbons.

1.1.4. ...and fate of petroleum hydrocarbons

Physical processes such as dispersion, sorption, or evaporation are responsible for transport and distribution of hydrocarbons (Robotham and Gill, 1989) and influence the availability of hydrocarbon compounds for degradation. Hydrocarbons are degraded either by photooxidation or by biodegradation. The environmental relevance of photooxidation of hydrocarbons is not yet clear, and estimates of its efficiency vary (Floodgate, 1984). However, it is clear that, as a result of photooxidation, the composition of crude oil changes. This will have consequences for its toxicity and biodegradability.

Biodegradation is an important process for the removal of petroleum hydrocarbons from most environments. However, the rates at which oil is biodegraded in natural environments vary considerably. Generally, the degradation rates of non-adapted communities have been reported to be rather low. In marine environments, rates below 11 g m^{-3} of seawater y^{-1} were detected with non-adapted communities, whereas, following adaptation, much higher rates can be expected (up to 18 kg m^{-3} of seawater y^{-1}) (Atlas, 1995). The extent and rate of biodegradation is governed by several factors that can be grouped as follows:

- The hydrocarbon must be biodegradable
- The hydrocarbon must be bioavailable
- The prevailing growth conditions must permit biodegradation of the hydrocarbon

All three factors must be fullfilled should biodegradation be applied for remediation measures.

Biodegradability. The biodegradability of aliphatic (reviewed in (Britton, 1984; Klug and Markovetz, 1971; Lindley, 1995; Ratledge, 1978; Singer and Finnerty, 1984; Watkinson and Morgan, 1990)), alicyclic (reviewed in (Perry, 1984)) and aromatic hydrocarbons (reviewed in (Cerniglia, 1984; Smith, 1990)) have been the subject of numerous reviews. Typically, 0.1% of a population in uncontaminated environments are able to degrade hydrocarbons, whereas in contaminated environments up to 10 % of the bacterial population is able to do so (Atlas, 1995). The different constituents in oil products degrade to different extents. Generally, alkanes are the most easily degraded while degradation of asphaltenes is most difficult (Atlas, 1995). Some hydrocarbons appear to be very recalcitrant. The alicyclic hopanes, for example, are extremely

recalcitrant and are consequently being used as internal standards in order to monitor bioremediation (Prince et al., 1994). The poly-branched alkane pristane, that was also thought to be recalcitrant, was later found to be readily biodegradable under laboratory (Pirnik, 1977) and field conditions (Cooney et al., 1985).

Bioavailability. Bioavailability (or the accessibility of pollutants) has been recognised as an important factor that limits biodegradation of hydrophobic compounds (Alexander, 1991; Mihelic et al., 1993). In order to become accessible for microbial attack, hydrophobic compounds in soil, sediments and aqueous systems have to desorb from particulate material or to dissolve from a separate phase (Mihelic et al., 1993), which can limit biodegradation rates (Stucki and Alexander, 1987). Since pollutants and microorganisms are often heterogeneously distributed, substrate transport also controls the accessibility of pollutants and, consequently, their biodegradation (Harms, 1996).

Bioavailability also plays an important role in the control of alkane biodegradation. The apparent recalcitrance of solid octadecane (C18) and hexatriacontane (C36) in cultures of a *Pseudomonas* sp. could be overcome by liposome encapsulation of the alkanes (Miller and Bartha, 1989), which demonstrated that biodegradation was limited by substrate transport and solubilization. However, the spontaneous rate of dissolution is not the only factor that governs biodegradation and the microorganisms are often able to enhance the dissolution rate or to act directly on the insoluble compound (Thomas et al., 1986).

For both the definition of bioremediation strategies and the monitoring of bioremediation programmes, it is important to know the bioavailability of hydrophobic compounds. Unfortunately, classical chemical analysis by extraction fails to distinguish between total contaminant concentration and bioavailable concentration. In order to measure the bioavailable concentrations of various pollutants, whole cell biosensors have been shown to be a suitable tool. As an additional advantage, such biosensors can be made to be specific for a narrow range of compounds and measurements are easily performed. In this kind of biosensor, the regulatory promoter of relevant catabolic genes is coupled to reporter genes such as for example the genes for bacterial luciferase (King et al., 1990; Steinberg, 1995).

Growth conditions. Since hydrocarbon biodegradation is often governed by physical-chemical factors (Alexander, 1991; Atlas, 1981; Atlas, 1995; Bossert and Bratha, 1984; Floodgate, 1984; Leahy and Calwell, 1992), hydrocarbons may persist in natural environments although they are readily biodegradable. Several factors that influence

biodegradation have been identified including oxygen availability, salinity, pH, temperature, nutrient availability (Atlas, 1981), and the type and composition of the crude oil (Atlas, 1975; Sugiura et al., 1997; Westlake, 1974).

Another factor that influences biodegradation is the presence of easily degradable carbon sources (Harder, 1982; Schmidt and Alexander, 1985). In nature, a wide variety of potential growth substrates are typically present at very low concentrations (Münster, 1993). Therefore, specialist microbial strains will not feed on the contaminant only but will utilise several substrates concomitantly or sequentially. The strategy for mixed substrate utilisation will depend on the prevailing growth conditions. Often, it is found that simultaneous utilisation is typical at low carbon concentrations, whereas at higher concentrations of individual carbon sources, sequential utilisation is observed (Harder, 1982).

Pollutant biodegradation does not usually begin before the end of a lag period (Bradford et al., 1982). Lag periods between 24 h and 2 to 4 weeks have been reported for the mineralization of crude oils in marine environments (Atlas, 1995). This acclimation period is caused by the requirement to induce the relevant catabolic genes and/or the growth of specialist strains, and enviromental growth factors govern the rate at which this process proceeds (Wiggins et al., 1987). With respect to the utilization of substrate mixtures, it is therefore of particular interest, how the presence of other carbon sources influences the acclimation period for pollutant biodegradation (Bally and Egli, 1996).

Bioremediation. There are essentially two different approaches to bioremediation (Atlas, 1995; Morgan and Watkinson, 1989; Morgan and Watkinson, 1989). In one of them, specialist microorganisms are seeded to a polluted area (Finn, 1983) and in the other one, the activity of indigenous microorganisms is enhanced by addition of nutrients (Bruchon et al., 1996), electron acceptors (Hess et al., 1996), or biosurfactants (Churchill et al., 1995). Both approaches were tested after the Exxon Valdez oil spill. These investigations showed that the rate of biodegradation was fivefold enhanced when limiting nutrients (P, Ca, N) were appropriately supplied (Atlas, 1995). Within 10 days the black shoreline turned white on treated beach areas (Atlas, 1995). In contrast, the addition of specialist strains did not yield the expected success. In general, these field trials showed that bioremediation can be a cost-effective method for clean-up of contaminated shore-lines (Atlas, 1995). Physical washing of the coast line cost $ 1 million per mile, while hundreds of miles could be cleaned by bioremediation for under

$ 1 million plus an additional $ 10 million for research, development and safety tests (Atlas, 1995).

1.2. BIODEGRADATION OF LINEAR ALKANES BY *ACINETOBACTER* SP. H01-N

Most strains of *Acinetobacter* share the ability to assimilate aliphatic hydrocarbons (Asperger and Kleber, 1991). Of 106 strains of *Acinetobacter* tested, only 19 failed to use dodecane, tridecane and hexadecane as the sole source for carbon and energy (Baumann et al., 1968). Several of these alkane degrading strains including *Acinetobacter* sp. H01-N have been the subject of intense studies which resulted in valuable insights into alkane microbiology (Table 1.2).

1.2.1. Early work and substrate specificity

Nearly 40 years ago, the gram-negative, obligately aerobic and immotile bacterium *Acinetobacter* sp. H01-N, originally designated as *Micrococcus certificans*, was isolated from Iowa soils by using hexadecane enrichments (Stewart et al., 1959). Initial characterisation of this organism showed its capability to use several long-chain linear alkanes in the range of decane to eicosane (Finnerty et al., 1962; Stewart and Kallio, 1959). In addition to linear alkanes, *Acinetobacter* sp. H01-N grows on some phenyl-alkanes, and on a range of branched alkanes. However, it does not degrade the poly-branched alkane pristane (Finnerty et al., 1962).

TABLE 1.2. Physiology of *Acinetobacter* sp. H01-N during growth on non-hydrocarbon substrates and during growth on hexadecane

Physiological modification	Growth substrate		References
	non-hydrocarbon[a]	hexadecane	
Intracellular membranes	-[b]	+	(Scott et al., 1976)
Hydrocarbon inclusions	-	+	(Scott and Finnerty, 1976)
Extracellular membrane vesicles	-	+	(Käppeli and Finnerty, 1979; Käppeli and Finnerty, 1980)
Cellular phsopholipid content	low	high	(Makula and Finnerty, 1971)
De novo fatty acid synthesis	+	-	(Sampson and Finnerty, 1974)
Extracellular lipids	-	+	(Makula and Finnerty, 1972)
Alkane oxidation induced	-	+	(Kennedy and Finnerty, 1975)

[a] nutrient broth, acetate or succinate
[b] symbols: +, present; -, absent

1.2.2. Uptake of alkanes and ultrastructural modifications

Middle and long-chain linear alkanes exhibit extremely low water-solubilities. For example, only 29 nM of dodecane or 13 nM of hexadecane, which are typical growth substrates for *Acinetobacter* sp. H01-N, can be dissolved in water at 25°C (Schwarzenbach et al., 1993). Microbial growth on such compounds requires special uptake mechanisms (Erickson and Nakahara, 1975; Singh and Desai, 1986). Three mechanisms can be distinguished.

- Uptake of free alkanes (alkane molecules "truely" dissolved in water)
- Uptake of solubilized alkane substrate (emulsified alkanes and submicron droplets (< 1μm))
- Uptake via attachment of cells to hydrocarbon droplets (> 1μm)

Uptake of free hexadecane. Free hexadecane was taken up by *Acinetobacter* sp. H01-N by a process that was sensitive to metabolic inhibitors, uncouplers, sulfhydryl reagents, and pronase treatment (Ventullo, 1978), and hexadecane was concentrated 26-fold in the cell (Ventullo, 1978). Experiments also showed that this process was inducible, since non-hydrocarbon grown cells or chloramphenicol treated cells did not take up hexadecane (Ventullo, 1978). Therefore, uptake of free hexadecane was possibly a protein mediated active transport process. These findings are somewhat surprising, since it is generally assumed that alkanes diffuse freely through membranes due to their hydrophobic nature (Ratledge, 1978; Watkinson and Morgan, 1990). However, active transport of alkane substrates has been observed for example in the yeast *Yarrowia lipolytica* (Bassel and Mortimer, 1985). In *Acinetobacter* sp. A3, the induction of two proteins in the outer membrane was seen during growth on crude oil (Hanson et al., 1994). Their presence was attributed to the uptake of hydrocarbons, which would suggest an uptake mechanism other than simple diffusion.

It has been questioned whether uptake of free alkanes plays a role at all (Hommel, 1990; Thomas et al., 1986). The requirement for either emulsifying agents (Koch et al., 1991; Rosenberg and Rosenberg, 1981) or attachment of cells to the organic phase (Efroymson and Alexander, 1991; Rosenberg and Rosenberg, 1981) for growth on alkanes has been shown for several microorganisms. *Acinetobacter* sp. H01-N possesses an elaborate system to enhance transport and uptake of alkane substrates.

Uptake of solubilized alkanes. *Acinetobacter* sp. H01-N produces lipids that exhibit emulsifying properties (Makula et al., 1975). In addition, this strain excretes membrane vesicles into the medium (Käppeli and Finnerty, 1979; Käppeli and Finnerty, 1980), which resemble the outer membrane in chemical composition, however with a 360-fold enrichment in lipopolysaccharide (Käppeli and Finnerty, 1979). Similar induction of membrane vesicles has been observed in another *Acinetobacter* by growth on hexadecane (Borneleit et al., 1988; Claus et al., 1984). The vesicles of *Acinetobacter* sp. H01-N bind up to 1.1 µmole of hexadecane per mg of protein (Käppeli and Finnerty, 1979; Käppeli and Finnerty, 1980). Experiments with labelled hexadecane demonstrated an uptake of vesicle-solubilized substrate in an azide- and cyanide-sensitive process (Singer and Finnerty, 1984). The uptake model suggested that the membrane vesicles fuse with the outer membrane and release their content into the cell (Finnerty and Singer, 1985; Singer and Finnerty, 1984).

During growth of *Acinetobacter* sp. H01-N on alkanes, intracellular modifications can be observed (Finnerty and Singer, 1985; Kennedy et al., 1975; Scott and Finnerty,

1975). A striking observation was the presence of an intracellular membrane system (Scott et al., 1976), which correlated with an increased phospholipid content of hexadecane grown cells (Makula, 1970; Makula et al., 1975). These membranes are present only in alkane grown cells (Kennedy and Finnerty, 1975). During growth on other carbon sources such as acetate or nutrient broth they cannot be seen (Kennedy and Finnerty, 1975). In electron micrographs, they appear in contact with the cytoplasmic membrane and with hydrocarbon inclusions (Kennedy et al., 1975; Scott and Finnerty, 1975). Hydrocarbon inclusions are spherical, cytoplasmic bodies containing unmodified alkane substrate within a membrane monolayer (Scott and Finnerty, 1976). In non-hydrocarbon grown cells, these bodies are absent (Kennedy and Finnerty, 1975; Scott and Finnerty, 1976). A number of other alkane degrading microorganisms contain similar inclusions (Müller et al., 1983; Scott and Finnerty, 1975) In a *Pseudomonas* strain they have been observed during growth on naphthalene (Scott and Finnerty, 1975).

Based on all of these observations, a model for the uptake and utilisation of alkanes in *Acinetobacter* sp. H01-N has been proposed. According to the model, alkane substrate is transported in membrane vesicles to the cell (Finnerty and Singer, 1985). The content of the vesicle is released into the cell and transported within the intracellular membrane system to the site of oxidation. The intracellular membrane system thereby represents an invagination of the cytoplasmic membrane. Enzymes for alkane oxidation may be localised in the intracellular membrane system or associated with the hydrocarbon inclusions. Indeed, aldehyde dehydrogenase activity, for example, has been found in membrane fractions and in the membranes of hydrocarbon inclusions (Fox et al., 1992; Singer and Finnerty, 1985).

Direct contact between substrate and cells. *Acinetobacter* sp. H01-N is able to attach to hydrocarbon droplets (Scott and Finnerty, 1976). This ability may be related to an increase in cell surface hydrophobicity. It has been shown for *Acinetobacter* sp. 2CA2 that the cell surface hydrophobicity increased during batch growth on hexadecane (Neufeld et al., 1980) and in *Acinetobacter* sp. A3 during growth on crude oil (Hanson et al., 1994). Decreased hydrophobicity of *Acinetobacter calcoaceticus* RAG-1 due to the presence of capsular polysaccharides resulted in decreased ability to attach to hydrocarbons (Rosenberg et al., 1982). In addition to hydrophobicity, the presence of fimbriae may also play a role in adherence to hydrocarbon substrates (Rosenberg et al., 1982).

1.2.3. Formation of wax esters

Wax-esters were detected as a storage product of alkane oxidation during batch-growth of *Acinetobacter* sp. H01-N on several alkanes (Makula et al., 1975; Stewart and Kallio, 1959). In *Acinetobacter* spp., wax-esters are produced during carbon-excess growth conditions and serve as carbon and energy reserve materials (Fixter et al., 1986). Production of wax esters has been shown with other carbon substrates as well (Geigert et al., 1984). During growth on hexadecanol, for example, wax-ester inclusions were isolated from *Acinetobacter* sp. H01-N (Singer et al., 1985). The wax-ester was identified as hexadecyl-palmitate. Interestingly, this ester could not be used as a growth substrate (Singer et al., 1985), indicating that some of the wax-esters were dead-end products of alkane metabolism rather than intermediates.

1.2.4. Biochemistry of linear alkane biodegradation

Alkane mono-oxygenase and alkane dioxygenase. Two different routes have been proposed for the initial attack of alkanes in *Acinetobacter* sp. H01-N. In one possible pathway (a), alkanes are oxidized to the corresponding mono-terminal alcohols (Britton, 1984).

$$R\text{-}CH_3 + O_2 + NAD(P)H + H^+ \longrightarrow R\text{-}CH_2OH + NAD(P)^+ + H_2O \qquad (a)$$

This reaction is mediated by a mono-oxygenase where one atom of molecular oxygen is incorporated into the alkane molecule and the other is reduced to water. Two electrons originating from NAD(P)H are required, which are transferred to the terminal oxygenase by an electron-carrier system containing either rubredoxin or cytochrome P-450 (Britton, 1984).

In an alternative route (b), alkanes are oxidised to hydroperoxides, which requires the presence of a dioxygenase that incorporates two atoms of oxygen into the alkane substrate (Britton, 1984).

$$R\text{-}CH_3 + O_2 \longrightarrow R\text{-}CH_2OOH \qquad (b)$$

The resulting hydroperoxide is then reduced to the corresponding alcohol (c) (Britton, 1984), or is directly converted into the aldehyde without the occurrence of a primary alcohol (d) (Finnerty, 1977; Finnerty, 1988; Sakai et al., 1996).

$$R\text{-}CH_2OOH + NAD(P)H + H^+ \longrightarrow RCH_2OH + NAD(P)^+ + H_2O \quad (c)$$

$$R\text{-}CH_2OOH \longrightarrow R\text{-}CHO + H_2O \quad (d)$$

Since *Acinetobacter* sp. H01-N readily oxidises both long-chain alcohols (Singer et al., 1985) and n-alkyl hydroperoxides (Stewart et al., 1959), pathways a and b appear possible. To date, no molecular proof exists for either of the mechanisms. However, since hydroperoxides occur in cell-free extracts during alkane oxidation (Finnerty, 1988) and since no hydroxylase activity is detectable in this strain (Finnerty, 1990), it has been proposed that alkanes are oxidised via pathway b and d (Finnerty, 1988). Both alkane monooxygenase (Asperger et al., 1984; Claus et al., 1980) and dioxygenase activity (Maeng et al., 1996; Maeng et al., 1996; Sakai et al., 1996) have been shown in other strains of *Acinetobacter*.

Long-chain fatty-alcohol dehydrogenase. Although it seems rather unlikely that alcohols are intermediates in alkane metabolism of *Acinetobacter* sp. H01-N (Finnerty, 1988), this strain nevertheless possess several alcohol dehydrogenases for the oxidation of alcohols of different chain-lengths (Singer and Finnerty, 1985) that catalyze the following reaction.

$$R\text{-}CH_2OH + NAD(P)^+ \longrightarrow R\text{-}CHO + NAD(P)H + H^+$$

For middle and long-chain alcohols, Singer and Finnerty found two dehydrogenases (Singer and Finnerty, 1985). One enzyme (ADH-B) is a soluble, constitutive $NADP^+$-dependent dehydrogenase with specificity for middle-chain alcohols and octanol as the optimal substrate. The other enzyme (hexadecanol dehydrogenase, HDH) is assumed to be an inducible NAD^+-dependent dehydrogenase which is induced 5 to 11-fold by growth on hexadecane or hexadecanol. HDH activity can be measured in the membrane and the soluble fraction of cell extracts (Singer and Finnerty, 1985). In contrast to these results, subsequent investigations by Fox et al. indicated the presence of two $NADP^+$-dependent alcohol dehydrogenase activities with middle-chain length alkane specificity and of one soluble, constitutive NAD^+-dependent enzyme with specificity for long-chain alkanes (Fox et al., 1992). Although different results were obtained for the regulation of enzyme expression, the latter was assumed to be identical with HDH found by Singer and Finnerty (Singer and Finnerty, 1985). It remained unclear from

these results whether alcohol dehydrogenases play a role in the alkane metabolism of *Acinetobacter* sp. H01-N. If any, the enzyme designated HDH might have a function in alkane dissimilation due to its substrate specificity for long-chain alcohols which is in agreement with the substrate spectrum for this strain (Fox et al., 1992; Singer and Finnerty, 1985). The function of the $NADP^+$-dependent alcohol dehydrogenases remain completely unclear (Wales and Fewson, 1994).

Long-chain fatty aldehyde dehydrogenases. Similar to the presence of multiple alcohol dehydrogenases, *Acinetobacter* sp. H01-N possesses several aldehyde dehydrogenases (Singer and Finnerty, 1985) that catalyze the following reaction.

$$R\text{-}CHO + NAD(P)^+ \longrightarrow R\text{-}COO^- + NAD(P)H + H^+$$

One of these enzymes (FALDH-b) appears to be constitutive and NAD^+-dependent, whereas the other (FALDH-a) is induced by hexadecane and several aldehydes and is $NADP^+$-dependent (Fox et al., 1992; Singer and Finnerty, 1985). Although, the activities of these enzymes are found in both the soluble and membrane fraction (Singer and Finnerty, 1985), FALDH-a activity seems to be mainly associated with the membranes of hydrocarbon inclusion bodies (Fox et al., 1992). The inducibility of FALDH-a with alkanes suggests that aldehydes are intermediates of alkane metabolism. However, the individual functions of the different aldehyde dehydrogenases are not entirely clear. Possibly, the products of one of the enzymes are used for wax ester synthesis, while the products of the other are used for b-oxidation (Fox et al., 1992). In addition to FALDH-a and FALDH-b, a nucleotide-independent aldehyde dehydrogenase has recently been isolated (Fox et al., 1992), which is unique for alkane degrading bacteria. The enzyme accepts Würster's-Blue (a dye consisting of perchlorate, bromine and tetramethyl-*p*-phenylendiamine (Michaelis and Granick, 1943)) as an artificial electron acceptor. However, neither the natural electron acceptor, nor the physiological role of this dehydrogenase have yet been resolved.

Oxidation of fatty acids. Although there exist no explicit investigations in this strain (nor in other *Acinetobacter* spp.), it is most likely that the resulting fatty acids are further degraded to acetyl-CoA via b-oxidation (Asperger and Kleber, 1991; Ratledge, 1984), which then enters the tricarboxilic acid cycle and glyoxylate shunt. Studies concerning enzyme activity of glyoxylate and tricarboxilic acid cycle in *Acinetobacter* during growth on alkanes have been performed with *Acinetobacter* sp. 69-V (Kleber and Aurich, 1973; Kleber et al., 1983; Kleber and Göbel, 1975). They have confirmed

the induction of the enzymes of the glyoxylate cycle during alkane degradation in this strain.

1.2.5. Genetics and regulation of alkane biodegradation in *Acinetobacter* sp. H01-N

To date, not much is known on the genetics of alkane degradation in *Acinetobacter* sp. H01-N. Although a wide variety of plasmid isolation techniques has been applied, no plasmids have been observed in this strain, indicating that alkane catabolic genes are localized on the chromosome (Singer and Finnerty, 1984). By analysis of Alk^- mutants, two independent loci - *alkX* and *alkY* - have been defined, both of which are necessary for alkane degradation (Singer and Finnerty, 1984). However, their functions have not yet been resolved.

Alkane degradation is an inducible process in *Acinetobacter* sp. H01-N (Kennedy and Finnerty, 1975). Induction is required for alkane catabolic enzymes, as well as for the formation of intracytoplasmic membranes and of hydrocarbon inclusion bodies. Alkane oxidation is repressed in the presence of non-hydrocarbon substrates (Kennedy and Finnerty, 1975). Inducible alkane degradation is observed in many other alkane degrading bacteria (Rehm and Reiff, 1982) including some *Acinetobacter* spp. (Asperger and Aurich, 1977; Asperger et al., 1984; Aurich and Eitner, 1973; Kleber et al., 1983) as well. Repression of alkane degradation has been observed in *Acinetobacter* sp. 69-V (Haferburg et al., 1983) but not in *Acinetobacter* sp. EB104 (Asperger et al., 1984; Kleber et al., 1983).

SCOPE OF THIS THESIS

Many alkane degrading microorganisms including *Acinetobacter* sp. H01-N have been characterized with respect to their physiology, biochemistry and genetics of alkane biodegradation. The experiments leading to those findings have mostly been carried out in typical laboratory environments, i. e. with excess carbon and during growth on the pollutant as the only carbon source. However, different growth conditions may be typical in natural or technical environments. Since alkane biodegradation is primarily limited by the prevailing growth conditions, it is important to understand the functioning of alkane degrading microorganisms during growth under other conditions than those specified for typical laboratory experiments. One major difference between "nature" and the "laboratory" is the presence of multiple carbon sources. Therefore, one aim of this study was to investigate the physiology of a model organism (*Acinetobacter* sp. H01-N) during growth on substrate mixtures containing both an alkane and non-hydrocarbon substrates. We were interested in alkane utilization in the presence of other carbon substrates during excess carbon and carbon limiting growth conditions. Furthermore, we were interested how the model organism adapted to the presence of the alkane after substrate switches from non-hydrocarbon substrates (Chapter 3). Another important factor that governs the degradation rate of alkanes is their accessibility for microbial attack. In technical environments, substrate transport can be enhanced by vigorous mixing. Therefore, the influence of different power inputs on alkane utilization in a bioreactor was investigated. In addition, in the same reactor system, the effect of growing cultures of *Acinetobacter* sp. H01-N on the solubilization and emulsification of the alkane substrate was studied (Chapter 4).

Besides the understanding of alkane biodegradation under different growth conditions, the possible applications of alkane degrading microorganisms in environmental biotechnology are of particular interest. One possible application is the development of biosensors for the monitoring of bioavailable pollutant concentrations. The bioavailability of alkanes is especially important in natural environments where diffusion is the major transport mechanism. In order to choose a suitable bioremediation strategy, it is useful to know the bioavailable alkane concentration, which can be measured by using a biosensor. Therefore, another aim of this thesis was to develop a microbial whole-cell biosensor that is suitable for the measurement of bioavailable alkane concentrations in contaminated ground water (Chapter 5).

In addition to these results, methods for the measurement of analytical methods for biomass and substrate during microbial cultivations on hydrocarbon substrates have been developed (Chapter 2).

CHAPTER 2 Measurement of Alkane and Biomass Concentrations in Hydrocarbon Fermentations and Some Comments on the Accumulation of Pristane in *Acinetobacter* sp. H01-N

Methods were developed for the quantification of biomass and residual hydrocarbon concentrations in cultures of Acinetobacter *sp. H01-N during growth on long-chain alkanes. Biomass could be measured accurately by washing the cells with Triton X-100 prior to centrifugation and subsequent measurement of either dry weight of the resulting pellet or light scattering of the resuspended cells. Quantification of long-chain alkanes was tested with the long-chain alkane pristane, which did not serve as a substrate for* Acinetobacter *sp. H01-N. This compound could be extracted nearly quantitatively (>92%) from culture supernatants with a mixture of methanol and chloroform. Alkanes were then quantified using gas chromatography. When batch cultures were extracted with pentane, recovery of alkanes was below 70%. Improved recoveries (>90%) with pentane extraction were observed when cells were inactivated prior to the addition of pristane. These results indicated that pristane was possibly taken up actively by* Acinetobacter *sp. H01-N.*

2.1. INTRODUCTION

Compared to typical water-soluble carbon sources for the cultivation of microorganisms, alkanes present technical and analytical difficulties as a consequence of their physical-chemical properties. Since alkanes are very reduced compounds, high oxygen input is required and high heat production is observed during growth on these compounds (Erickson, 1981). Due to their hydrophobicity, a high power input is required for the production of fine emulsions that allow efficient mass transport ((Einsele, 1972), Chapter 2 of this work).

As a consequence of their high hydrophobicity, alkanes also tend to accumulate within biological membranes (Sikkema et al., 1995). This can impede their quantification and lead to underestimation of residual alkane concentrations (Brown and Cooper, 1992; Hug and Fiechter, 1973). *Acinetobacter* sp. H01-N not only accumulates alkanes in membranes, but also stores hexadecane intracellularly (Kennedy et al., 1975; Scott and Finnerty, 1976), and is known to produce extracellular membrane vesicles that bind hexadecane (Käppeli and Finnerty, 1979; Käppeli and Finnerty, 1980). Long-chain alkanes are typically extracted from bacterial cultures with solvents such as the short-chain alkanes pentane or hexane, or the chlorinated hydrocarbons methylene chloride or chloroform. In order to account for losses during extraction, internal standards are added (Brown and Cooper, 1992). However, since changes in growth physiology probably influence the accumulation of alkanes, the internal standard should be present during the entire growth experiment. An ideal internal standard for experiments with *Acinetobacter* sp. H01-N is the branched alkane pristane. This compound is not degraded by *Acinetobacter* sp. H01-N (Finnerty et al., 1962) and has similar properties as linear long-chain alkanes. The recalcitrance of pristane in combination with its alkane-like properties has also led to its use as an internal standard in field-evaluations of bioremediation after oil-spills (Madsen, 1991).

The water-insolubility of alkanes is also the reason why most standard methods for the quantification of biomass are inadequate for microbial cultivation on alkanes. In the presence of alkanes, centrifugation of culture liquids results in only partial sedimentation of cells and part of the cells are lost during decantation of the supernatant (Gutierrez and Erickson, 1978; Hug and Fiechter, 1973; Neufeld et al., 1980; Scott and Finnerty, 1975). Filtration of cell suspensions is equally difficult since filters are clogged immediately by alkanes (Hug and Fiechter, 1973). Similarly, measurements of the OD is hampered by the

presence of turbid alkane-in-water emulsions. Therefore, these standard methods have been adapted to hydrocarbon fermentations in the past. Often, a solvent is used to wash cells or to dissolve hydrocarbons (Gutierrez and Erickson, 1978; Hug and Fiechter, 1973; Miura et al., 1978). However, solvent treatment of cells results in cell breakage. Such effects have been considered on rare occasions only, and if so, it has been assumed that the susceptibility of cells for solvent treatment did not change with changing cell physiology (Hug and Fiechter, 1973). Since *Acinetobacter* sp. H01-N grows on various carbon sources other than linear alkanes including acetate or succinate, it is possible to easily test such effects with this strain during growth on acetate or succinate. For this purpose, biomass concentrations can be determined with both standard methods and methods that are designed for biomass determinations in the presence of alkanes, and then results can be compared.

In this chapter, methods for the measurement of both biomass and alkane concentrations were tested and optimized. Their accuracy and reliability under different growth conditions were tested. Additionally, by testing extraction methods for alkanes with both growing and inactivated cultures, it was shown that *Acinetobacter* sp. H01-N was possibly taking up pristane actively.

2.2. MATERIALS AND METHODS

2.2.1. Cultivation conditions

Acinetobacter sp. H01-N was maintained and cultivated as described elsewhere (see Materials and Methods of Chapter 4).

Some experiments were performed with inactivated cells. Cells were inactivated as follows. First, cells were pregrown in continuous culture on dodecane at a dilution rate of 0.1 h^{-1}. Cells were harvested, washed once in chemostat medium without carbon source (pH 6.8), and concentrated to an OD_{546} of approximately 1. Cells were inactivated either by addition of 0.2% sodium azide or by heat treatment during 3 min at 60°C.

2.2.2. Biomass concentration

Several methods for the quantification of biomass in hydrocarbon fermentations were tested. These methods are listed in Table 2.1.

TABLE 2.1. Methods tested for the measurement of biomass in hydrocarbon fermentations.

Microorganisms	Hydrocarbons	Measurement	References
M. japonica *P. oleovorans* *Acinetobacter*	n-C14, n-C12, pristane, phenol	OD Dissolve alkane with ethanol or propanol and diethylether	(Miura et al., 1978), This work
Candida spp.	n-C16	Dry weight (Filtration) Wash cells with solvents such as propanol, hexane etc.	(Hug and Fiechter, 1973)
Candida spp.	n-C16	Dry weight or OD (Centrifugation) Wash cells with mixture of ethanol, butanol and chloroform	(Gutierrez and Erickson, 1978; Hug and Fiechter, 1973)
Arthrobacter sp. *Acinetobacter* sp.	n-C12, heptamethylnonane, pristane	Dry weight or OD (Centrifugation) Wash cells with Triton X-100	This work, (Efroymson and Alexander, 1991)

OD_{546}. OD_{546} was determined in a spectrophotometer (Uvikon 860, Kontron Instruments, Switzerland) in 1 cm glass or polystyrene cuvettes. If alkanes were present, the following two methods were tested.

- The cell suspension was mixed with an equal amount of a solvent mixture of diethylether and propanol. The solvent was added to dissolve the alkane and, consequently, to remove the opacity that was caused by the alkane-water emulsion. This method was adapted from Miura and co-workers (Miura et al., 1978).
- In another method, 0.1 ml of Triton X-100 (1:10 in water) was added to 2 ml of cell suspension. The mixture was centrifuged at maximum speed in an Eppendorf centrifuge for 5 minutes. The supernatant with the alkanes was discarded. The pellet was resuspended in 1 ml of distilled water, and the OD_{546} was determined.

Gravimetric method by filtration. In cultures without alkanes, cells from 20 to 25 ml of culture liquid were collected on pre-tared Millipore filters (0.2 µm or 0.45µm pore diameter) by suction. The filters were washed twice with 10 ml of distilled water. Filters were then dried to constancy at 105°C. In the presence of alkanes, the same method was used except that filters with bacteria were washed once with 5 ml of propanol to remove alkanes before washing them with water. Subsequently, filters were treated in the same way as they were when samples contained no alkanes.

Gravimetric methods by centrifugation. In cultures without alkanes, 20 to 25 ml of cell suspension was centrifuged at 4500 g for 10 minutes, washed once with 5 ml of distilled water, and the resulting pellet was dried to constancy at 105°C. If alkanes were present, one of the following solvents or detergent was added to 20 ml of culture liquid prior to centrifugation in order to remove cells from alkanes.

- 2 ml of a mixture of chloroform, ethanol and butanol (10:10:1 vol/vol/vol) (Hug and Fiechter, 1973)
- 2 ml of propanol
- 1 ml of Triton X-100 (1:10 in distilled water)

Subsequently, cells were centrifuged and the biomass concentrations were measured in the same manner as described for samples without alkanes.

2.2.3. Extraction and quantification of residual hydrocarbons

Hydrocarbons were extracted from bacterial cultures in 40 ml culture tubes that were closed with Teflon lined caps. In order to avoid losses of hydrocarbons due to pipetting, samples were directly collected from the reactor into these tubes, and sample volume was determined by weighing. Cellular reactions were immediately stopped by filling 5 ml of solvent into the tubes prior to sampling. For alkane determination during shake flask experiments, cells were grown in 40 ml culture tubes, and solvents were added directly into these tubes for extraction. For extraction experiments of alkanes from samples with inactivated cells, pristane and dodecane were added to 5 ml of inactivated cell suspension ($OD_{546} \approx 1$) at final concentrations between 150 and 750 mg l^{-1} each. The mixture of alkanes and inactivated cells was incubated for at least one hour on a reciprocal shaker at 120 rpm at room temperature before extraction experiments were performed. Pentane and a mixture of chloroform and methanol (1:2 v/v) (Käppeli and Finnerty, 1979) were tested as solvents for the extraction of alkanes (Table 2.2).

TABLE 2.2. Methods tested for the extraction of residual alkane substrates.

Extraction with	Remarks	Reference
pentane	-	This work
pentane	Freezing/thawing of cells	This work
chloroform and methanol	-	(Käppeli and Finnerty, 1979) This work

For pentane extractions, 20 ml of pentane was added per 5 ml of culture liquid. Either cell suspensions were extracted immediately after sampling or the mixture of cells and pentane was frozen at -20°C and thawed at room temperature twice before extraction. In all cases, the mixture was extracted by vigorous shaking during 5 minutes at room temperature. The pentane phase was allowed to separate on ice for 15 minutes. Sometimes, a stable emulsion at the water-pentane interface was observed. This emulsion was destroyed by addition of a small aliquot of anhydrous Na_2SO_4.

With methanol and chloroform, a mixture composition of 0.8:1:2 sample: chloroform:methanol vol/vol/vol was prepared, which resulted in a monophasic system. This solution was shaken vigorously for 5 minutes. Potassium chloride was added at an assay concentration of 0.2 mM. The mixture was again extracted for 5 minutes, which caused the chloroform phase to separate at the bottom of the tube. The two phases were allowed to separate for at least 10 minutes. The top phase containing water and methanol was discarded, and the chloroform phase was kept for analysis of alkanes.

Alkane quantification was made by injecting 1.2 µl of the pentane or chloroform phase respectively into a GC (Carlo Erba Strumentazione, Milan, Italy). Operating conditions were as follows. Injection took place in splitless mode at an injector temperature of 260°C. The FID detector was operated with air (120 kPa) and hydrogen gas (60 kPa) and was maintained at a temperature of 280°C. The GC was equipped with a fused silica column PS 089 (5% phenyl, inner diameter 0.25 mm, length 15 m; kindly provided by C. Schaffner, EAWAG). The carrier gas was hydrogen (50 kPa). The column temperature was programmed as follows. 5 min isotherm at 60°C, temperature increase to 180°C at 40°C min^{-1}, 3 min isotherm at 180°C, temperature increase to 250 °C at maximum operable rate, 3 min isotherm at 250°C. Dodecane appeared after a retention time of 6.8 ± 0.1 minutes.

2.3. RESULTS

2.3.1. Measurement of the biomass concentration

The accuracy and reliability of the tested methods for the measurement of biomass concentrations in presence and absence of alkanes is summarized in Table 2.3.

Gravimetric methods by filtration. Biomass concentration was first determined in culture liquids without alkanes. For that purpose cells were grown on acetate to stationary phase. As indicated by the yield coefficient, biomass determination by filtration of such samples resulted in an underestimation of the biomass concentration (Table 2.3). Cells were also grown on acetate in the presence of dodecane, which did not serve as a growth substrate under such conditions. With exponentially growing cells, filtration of samples and subsequent washing of cells with propanol resulted in accurate measurements according to the expected yield coefficient (not shown). However, the apparent concentration of stationary phase cells was approximately 40% below the expected value, and measurements varied greatly (Table 2.3).

TABLE 2.3. Comparison of different methods to determine biomass concentration in hydrocarbon fermentations

Solvent	Growth substrate	Rel. $Y_{x/C}$ (%)[a]	Rel. error (%)	Remark
Method: Dry weight (Filtration)				
Water	acetate	84	6	20 min filtration time
Propanol	acetate/dodecane	variable	-	dependent on growth phase
Method: Dry weight (Centrifugation)				
Water	acetate	100	3	
Water	dodecane	variable	-	dependent on growth phase
$CHCl_3$, MeOH	acetate/dodecane	63	10 - 20	incomplete sedimentation
Propanol	acetate/dodecane	-	-	no pellet
Triton X-100	acetate/dodecane	97	2-5	firm pellet in all cases
Method: OD				
Propanol/Et_2O	dodecane/acetate	-	-	flocculation
Triton X-100	dodecane/acetate	95	5-10	

[a] The apparent growth yield on acetate is shown as relative values using the specified methods to determine biomass concentrations. The yield that was obtained from the centrifugation method and water as a solvent was set to 100%, since this value fitted literature data best (Bell, 1972; Payne, 1970; Roels, 1983). The yields that were obtained with other methods were related to this value.

For all samples tested and even with dilute samples (OD_{546} < 0.25) of culture fluids, clogging of filters was observed in the presence or without alkanes, which led to filtration times of 20 minutes and more for one sample. Possibly, underestimation of biomass concentration was therefore caused by membrane fouling and, consequently, irreversible loss of biomass during filtration. When stationary phase cells were washed with propanol, additional cell disruption was probably caused by the solvent.

Gravimetric methods by centrifugation. When cells were grown on acetate, the biomass concentration could be quantified by standard centrifugation methods including washing of cells with water (Table 2.3). However, if cells were grown on a mixture of acetate or dodecane only part of the total biomass sedimented during centrifugation and this fraction was dependent on the growth phase (see Chapter 4). The rest of the cells floated on top of the supernatant, resulting in subsequent loss of biomass during decantation. Therefore, several methods were tested to separate hydrocarbons from cells prior to centrifugation.

First, cells were washed with a mixture of butanol, methanol and chloroform. In the presence of this mixture, incomplete sedimentation was observed. Additionally, cells were most probably dissolved. These effects led to underestimation of the biomass concentration by almost 40% (Table 2.3). When propanol was added to cell samples, formation of a pellet during centrifugation was completely prevented.

In contrast, washing of cells with a dilute solution of Triton X-100 prior to centrifugation resulted in a firm pellet under all conditions tested. In order to control the method, the biomass concentration in cells from acetate batches (Table 2.3) and from carbon limited continuous cultures on dodecane (not shown) was determined with both addition of Triton X-100 and without Triton X-100. No significant difference was observed, which indicated that Triton X-100 did not cause cell lysis.

OD_{546}. Typically, the emulsion of dodecane in water that was formed in the bioreactor at a stirrer speed of 1000 rpm exhibited an OD_{546} of approximately 1. Therefore, direct measurement of the OD in order to determine the biomass concentration was not possible in the presence of alkanes. First, alkanes were dissolved by addition of a mixture of diethylether and propanol. The addition of this solvent mixture caused immediate flocculation of cells, and the OD could not be measured. Alternatively, Triton X-100 was used in a similar manner as for dry weight determinations. With this method, reproducible results were obtained (Table 2.3).

2.3.2. Residual hydrocarbon concentration

In order to test different extraction methods for alkanes from cell samples, *Acinetobacter* sp. H01-N was grown in batch on a mixture of dodecane and pristane. Alkanes were extracted with pentane or a mixture of methanol and chloroform.

When samples were extracted with pentane, the apparent pristane concentrations decreased during degradation of dodecane (Fig. 2.1). The pristane concentration was lowest after dodecane had been depleted (Figs. 2.1 and 2.2). Assuming that dodecane was the only growth substrate, a growth yield of 1.22 g (g carbon)$^{-1}$ was determined. This value was in agreement with the growth yield on dodecane alone (1.15 g (g carbon)$^{-1}$), which indicated that pristane was not incorporated into biomass. The observation that pristane concentrations slightly increased again after dodecane had been depleted (Fig. 2.1 and 2.2) also indicated that pristane was not degraded but accumulated in cells and some was released again during stationary phase.

This was confirmed during subsequent experiments by using a mixture of chloroform and methanol as the extraction solvent. However, with pentane only part of pristane could be recovered (Fig. 2.2). Additional samples were extracted with pentane after repeated freezing and thawing of cells (not shown). By using the mixture of methanol and chloroform, pristane was recovered almost at its initial concentration for all biomass concentrations tested (Table 2.4 and Fig. 2.3).

The amount of pristane that accumulated in cells of this organism was calculated from the observed difference of the total amount of pristane added and the amount of pristane that was extracted with pentane from untreated cells. At the beginning of the stationary growth phase during batch growth on dodecane, 910 nmoles of pristane were bound per mg of biomass, which corresponded to 23% of the biomass dry weight.

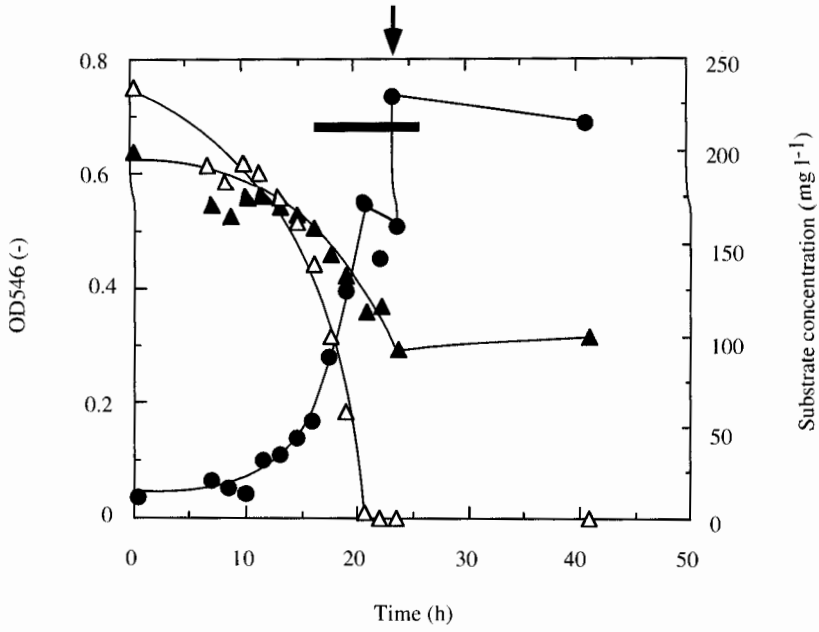

Figure 2.1. Batch growth of *Acinetobacter* sp. H01-N on a mixture of dodecane and pristane. Symbols: (●) OD546, (Δ) dodecane, and (▲) pristane concentration. The bar indicates occurrence of wall growth in the headspace of the bioreactor. The arrow indicates time when the biofilm was removed by short-time increase of stirrer speed to 3000 rpm.

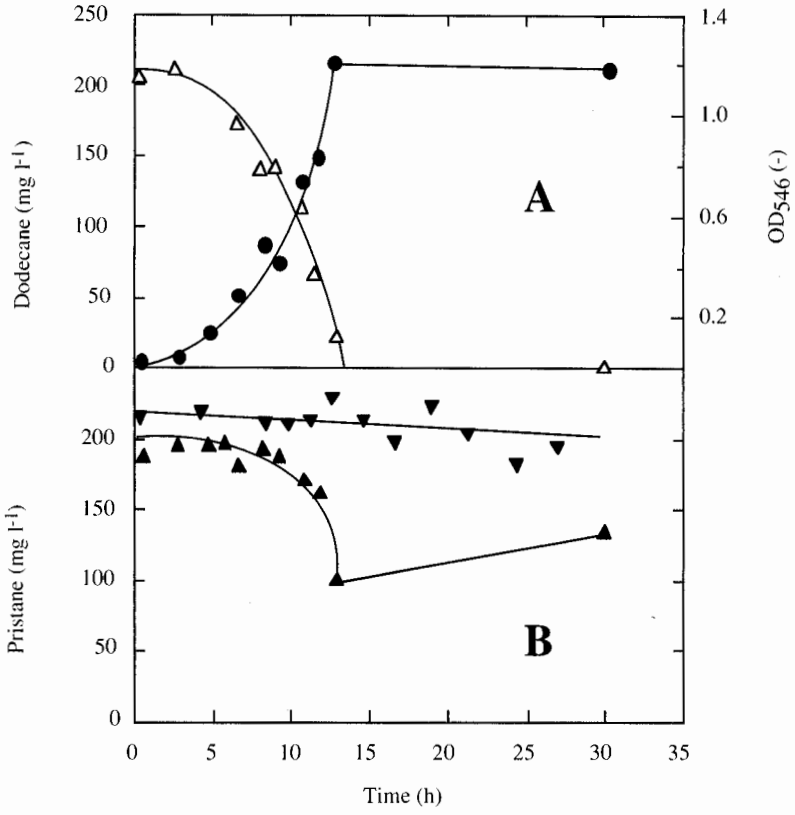

Figure 2.2. Effect on different solvents on apparent pristane concentration during batch growth of *Acinetobacter* sp. H01-N on a mixture of dodecane and pristane. A. (●) OD_{546} and (Δ) dodecane concentration. B. Pristane concentration after extraction with pentane (▲) and a mixture of methanol and chloroform (▼).

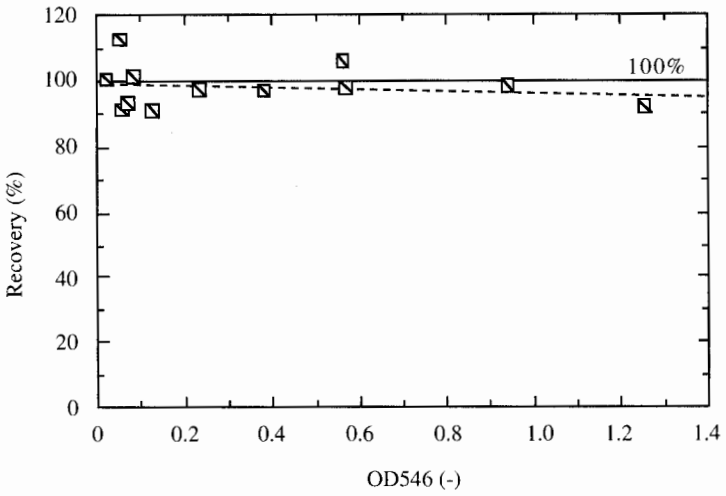

Figure 2.3. Recovery of pristane from cell suspensions of *Acinetobacter* sp. H01-N at different biomass concentrations with methanol:chloroform extraction. Dotted line shows linear regression of data, and solid line indicates 100% recovery.

TABLE 2.4. Recovery of pristane from an active culture of *Acinetobacter* sp. H01-N after extraction with different methods.

Method	Recovery (%)[a]
Pentane extraction	53 - 74
Pentane extraction / freezing-thawing	80.4 ± 8.6
Methanol-chloroform extraction	91.9 ± 3.8

[a] Alkanes were extracted from a culture with an OD546 of 1.2

In order to test whether pristane was taken up actively, pristane and dodecane were added to a suspension of inactivated cells of *Acinetobacter* sp. H01-N. After an incubation time of 3 h, alkanes were extracted from this cell suspension with pentane (Table 2.5). With inactivated cells, recovery was above 90% for all concentrations of alkanes tested, for both azide and heat treatment. These results indicated that pristane might be taken up actively.

TABLE 2.5. Recovery of pristane and dodecane from inactivated cultures of *Acinetobacter* sp. H01-N after extraction with pentane.

Alkane[a]	Recovery from inactivated cultures[b]	
	heat	azide
Pristane	106.3 ± 4.8	98.4 ± 2.4
Dodecane	95.6 ± 6.4	93.5 ± 0.9

[a] Alkane concentrations of between 150 and 750 mg l^{-1} were tested. Results were not different within this range of concentration.
[b] $OD_{546} = 1$

2.4. DISCUSSION

2.4.1. Measurement of biomass concentration in hydrocarbon fermentation

A reliable method for the determination of biomass in hydrocarbon fermentations was developed. This method included the washing of cells with Triton X-100. Without the addition of this compound, *Acinetobacter* sp. H01-N did not sediment during centrifugation in the presence of alkanes. This effect was possibly caused by accumulation of dodecane in cell membranes and by accumulation of cells on dodecane droplets. When Triton X-100 was added, sedimentation was observed. Triton X-100 probably prevented cells from accumulating on alkane droplets. Similarly, the addition of Triton X-100 has been shown to prevent the accumulation of an *Arthrobacter* sp. on heptamethylnonane droplets containing hexadecane or naphthalene as carbon substrates (Efroymson and Alexander, 1991). Since Triton X-100 interacts with cell membranes

and leads to permeabilization of cell membranes (Miozzari et al., 1978), it may well be that alkane molecules were redissolved from cell membranes, which allowed cells to sediment during centrifugation. The permeabilization of cells, if any due to the presence of Triton X-100, would result in the release of cellular compounds to the medium and, consequently, to an underestimation of the biomass concentration. However, such an effect was not observed. This was shown by control measurements of the biomass concentration of samples from acetate grown cells without the addition of Triton X-100.

When different solvents were added to cell suspensions, biomass dry weight could not be determined successfully. The cells did not sediment completely, and it appeared that the susceptibility of cells to solvent treatment was dependent on the physiological state of the cells. In contrast, a mixture of chloroform, butanol and ethanol was applied successfully for the determination of biomass concentrations in samples of the yeast *Candida lipolytica* in the presence of hexadecane (Gutierrez and Erickson, 1978; Hug and Fiechter, 1973). With this strain, complete sedimentation was observed in the presence of the solvent mixture even when equal amounts of solvent and sample were mixed (Hug and Fiechter, 1973). Cell breakage was nevertheless observed in the presence of the solvent mixture. Therefore, the method was calibrated by measuring the biomass concentrations of samples from stationary phase cells after washing the cells with water and subsequently comparing these results to the concentrations that were obtained after washing the cells with the solvent mixture (Hug and Fiechter, 1973). Although the method was used successfully, it remained unclear from those results, whether the susceptibility of the cells to solvent treatment was influenced by their physiological state.

2.4.2. Hydrocarbon concentration

A mixture of chloroform and methanol was used to quantitatively extract alkanes from cultures of *Acinetobacter* sp. H01-N. The problems of hydrocarbon analysis in cultures of *Acinetobacter* spp. have been pointed out before by Brown et al. (Brown and Cooper, 1992) and were attributed to the intracellular storage of hexadecane (Kennedy et al., 1975; Müller et al., 1983; Scott and Finnerty, 1975; Scott and Finnerty, 1976), and the binding of hexadecane in membrane vesicles (Borneleit et al., 1988; Käppeli and Finnerty, 1979). Our results showed that despite these effects, the mixture of methanol and chloroform was suitable for the complete recovery of pristane from growing cultures of *Acinetobacter* sp. H01-N. These results were further substantiated by chemostat experiments, where theoretical wash-in curves of dodecane could be verified experimentally with this method (see Chapter 4).

2.4.3. Accumulation of pristane in *Acinetobacter* sp. H01-N

When samples were extracted with pentane, pristane and dodecane were recovered at high percentages from suspensions of inactivated cells, whereas poor recoveries were observed with active cells. Alkanes accumulate in cell membranes due to their extremely different solubility in water and in membrane bilayers (Sikkema et al., 1994; Sikkema et al., 1995; Westermann, 1991). By using the equation by Sikkema (Sikkema et al., 1994) and assuming uptake of pristane by diffusion, one would expect approximately 15 nmoles (mg biomass)$^{-1}$ of pristane to accumulate in the cells with virtually all of it being present in the cell membranes. In contrast, results showed that 910 nmoles (mg biomass)$^{-1}$ were maximally bound to biomass. This suggested active transport and accumulation of pristane in the cell. Similar intracellular concentrations of the growth substrate hexadecane of between 130 and 360 nmoles (mg biomass)$^{-1}$ have been reported for the same microorganism (Finnerty and Singer, 1985; Scott and Finnerty, 1975). For this compound an active uptake mechanism has been postulated and confirmed by studies with inhibitors (Kennedy et al., 1975; Singer and Finnerty, 1984). Active uptake of alkanes has been reported for other microorganisms as well (Bassel and Mortimer, 1985), although, in general, alkanes are taken up by passive diffusion or by group translocation (see for example (Britton, 1984; Singh and Desai, 1986).

Results also showed that the pristane concentration in the medium increased again during the stationary phase. Due to the high intracellular pristane concentration at the beginning of the stationary phase, pristane would be expected to diffuse into the medium as soon as no source of energy was available to maintain active uptake of this compound. In similar batch experiments on a mixture of potential carbon sources including naphthalinedisulfonic acid, Beckmann (Beckmann, 1976) observed a decrease in naphthalinedisulfonic acid concentration of 40%. However, the naphthalinedisulfonic acid concentration increased again, and, at the end of the experiment, it returned to its initial level. Although no explanation for this observation was given by the author, it might well be that similar effects as described here were responsible for this observation.

Although the results indicated active uptake of pristane, the final proof for active uptake of pristane would require additional uptake experiments with labeled pristane and the analysis of intracellular pristane concentration (Scott and Finnerty, 1976).

CHAPTER 3 Growth of *Acinetobacter* sp. H01-N on dodecane: Drop size, alkane solubility and accumulation of bacteria at the water-dodecane interface

Alkane degrading microorganisms exhibit distinct properties to increase the availability of hydrophobic substrates. The alkane degrading strain Acinetobacter *sp. H01-N accumulated at the water-dodecane interface during batch growth on dodecane. Towards the end of the batch growth phase, 90% of the biomass was bound to the water-dodecane interface. As indicated by a rapid decrease in the Sauter mean diameter of dodecane drops, dodecane emulsions were stabilized during batch growth. As an additional effect, alkane solubility increased at least 10-fold in the presence of continuously growing cells of* Acinetobacter *sp. H01-N. Despite these adaptations to growth on dodecane, high power input was needed for sufficient mass transfer during carbon limited continuous culture on dodecane. A stirrer speed of 2100 rpm was required in order to achieve nearly complete substrate utilization. This stirrer speed corresponded to a power input of 9.5 kJ per mg of degraded alkane substrate or 116 W* l^{-1}.

3.1. INTRODUCTION

A wide variety of bacteria, yeasts and filamentous fungi are able to use linear alkanes as a sole source of carbon and energy (Watkinson and Morgan, 1990). Among these compounds, long-chain linear alkanes with more than 9 carbon atoms are the most readily metabolized (Klug and Markovetz, 1971). The water-solubility of long-chain alkanes is extremely low and decreases with increasing chain-length. For example, the solubility in water of dodecane, which was used as an alkane substrate in this study, is 29 nM at 25°C (Schwarzenbach et al., 1993). Such concentrations are too low to support growth and, consequently, sufficiently high transfer rates from the dispersed phase to the microbial cell are necessary in order to avoid mass transfer limitations (Bajpai and Prokop, 1975; Blanch and Einsele, 1973; Miller and Bartha, 1989; Stucki and Alexander, 1987).

Microorganisms take up alkane substrates in the form of solubilized and pseudo-solubilized molecules (e. g. submicron droplets) (Chakravarty et al., 1975) or are able to undergo direct contact with alkane droplets ((Moo-Young and Shimizu, 1971; Neufeld and Zajic, 1984), for review on hydrocarbon uptake see (Erickson and Nakahara, 1975; Singh and Desai, 1986)). These processes are enhanced by production of surfactants (Claus et al., 1984; Guerra Santos, 1985; Käppeli and Finnerty, 1979; Käppeli and Finnerty, 1980) and adaptation of the microbial cell surface to the presence of hydrophobic substrates (Neufeld et al., 1980; Rosenberg et al., 1982). The presence of both surfactants and alkane degrading microorganisms also stabilize hydrocarbon emulsions (Neufeld and Zajic, 1984). One aspect of this is a reduction in the size of the alkane droplets (Bajpai and Prokop, 1975). Reduction of drop size results in an increased specific interfacial area, which is especially important for hydrocarbon uptake, since the overall mass transfer rates increase and more bacteria can be in direct contact with the water-dodecane interface. Despite these microbial strategies for enhanced uptake of hydrophobic substrates, appropriate technical measures including optimization of reactor setup or the application of high power inputs are nevertheless required for high biomass productivities and efficient substrate turnover rates during growth on scarcely soluble carbon sources (Einsele, 1972).

During growth on hexadecane, *Acinetobacter* sp. H01-N appears to apply both strategies, increase in substrate solubility and direct contact to hexadecane droplets (Singer and Finnerty, 1984). Surface active compounds are produced that enhance

hexadecane solubility in the cell-free spent growth medium of this strain (Käppeli et al., 1980; Käppeli and Finnerty, 1979; Käppeli and Finnerty, 1980). Results also indicate that this strain accumulates on hexadecane droplets during batch growth although this effect has not been quantified (Scott and Finnerty, 1975).

In this chapter, alkane solubility in growing cultures of *Acinetobacter* sp. H01-N and the accumulation of this strain on dodecane droplets were further investigated. As a measure for emulsion stabilization, the effect of growth in batch culture on the Sauter mean diameter of dodecane droplets was investigated. Power input requirements were also optimized during carbon limited growth in continuous culture.

3.2 MATERIALS AND METHODS

3.2.1. Experimental

Cultivation conditions and analytical methods. A detailed description of the cultivation conditions and routinely used analytical methods have been given elsewhere (See Material and Methods Chapter 4). Selected parameters for the characterization of the bioreactor configuration and the applied water-dodecane emulsion are listed in Appendix 2.

Measurement of the Sauter mean diameter and quantification of the biomass fraction at the water-dodecane interface. The Sauter mean diameter, d_{32}, was measured as follows. A sample of the culture was withdrawn from the reactor. One droplet of the emulsion was immediately placed onto a microscope slide, covered with a cover-slip, and viewed at 1250-fold magnification under a light microscope (Olympus BH2, Japan). A picture was taken instantaneously and the drop diameter was determined on the picture. From this, the Sauter mean diameter was calculated (see below). With this method drop size was possibly overestimated due to coalescence. However, d_{32} was measured directly without prior stabilization of the emulsion for the following reasons. (i) low organic volume fractions (Φ) were used which decreased the occurrence of coalescence, (ii) bacterial cultures are known to stabilize water-hydrocarbon emulsions (Neufeld and Zajic, 1984), and (iii) the application of additional surface active compounds such as Triton X-100 in order to stabilize the emulsion led to complete dissolution of the dispersed phase at low values of Φ. An overview of other methods and comments on different methods to determine d_{32} are found in Bajpai and Prokop (1974).

The same pictures of dodecane droplets were used to determine the fraction of biomass that was bound to dodecane droplets. For that purpose, both cells at the surface of droplets and suspended cells were counted on each picture. From these numbers, the fraction of biomass at the water dodecane interface was calculated.

Dissolved alkane concentration in the culture broth of *Acinetobacter* sp. H01-N and estimation of the Monod constant for dodecane. Dissolved alkane concentrations were determined in a continuous culture of *Acinetobacter* sp. H01-N during growth on dodecane in the presence of pristane at a dilution rate of 0.1 h^{-1}. A sample of cell suspension (7 ml) was poured into a soft-wall ultracentrifugation tube. The sample was centrifuged at 20000 g for 30 minutes. After centrifugation, free hydrocarbon floated on top of the tube. The lower two-thirds of the supernatant were carefully transferred into a syringe by penetrating the wall of the tube with a syringe needle. The content of the syringe was filtered through hydrophilic poly-acetate filters with a pore size of 0.45 µm (Schleicher-Schüll, Germany) to remove residual hydrocarbon droplets and floating cells and cell debris. The filtrate was extracted with pentane and concentrated on ice using a gentle vacuum. The residue was dissolved in 0.5 ml of pentane and analyzed by GC.

3.2.2. Calculations

Characterization of dodecane dispersions. Dodecane emulsions were characterized with their Sauter mean diameter, d_{32}, and with the specific interfacial area, a. The Sauter mean diameter is defined as follows.

$$d_{32} = \frac{\sum n_i \cdot d_i^3}{\sum n_i \cdot d_i^2} \qquad (1)$$

where d_i, diameter of drops in class i, and n_i, number of drops with diameter d_i. The specific interfacial area was calculated from the Sauter mean diameter with equation 2.

$$a = \frac{6 \cdot \Phi}{d_{32}} \qquad (2)$$

where Φ is the dispersed organic phase fraction.

Many equations have been developed to correlate the Sauter mean diameter with other engineering parameters (see for example (Bajpai and Prokop, 1975; Calabrese et al., 1986)). To predict the average drop size in turbulent fluids, two equations were used (Table 3.1).

TABLE 3.1. Equations to predict Sauter mean diameter of dilute dispersions in turbulent fluids.

Equation	Applicable range	Dominant forces	Ref.
$d_{32}^0 = C_1 \cdot D_I \cdot We^{-0.6}$ (3)	$L >> d_{32} >> \eta$	inertial forces	(a), (b)
$d_{32}^0 = C_2 \cdot D_I \cdot We^{-1/7} \cdot Re^{-4/7}$ (4)	$L >> \eta >> d_{32}$	viscous forces	(c)

References: (a) Shinnar, 1961; (b) Shinnar and Church, 1960; (c) Chen and Middleman, 1967

Symbols: d_{32}^0, Sauter mean diameter in water; D_I, Impeller diameter; C_1, C_2, empirical constants; We, Weber number; Re, Reynolds number; L, width of impeller blade (approximates macroscale of turbulence); η, microscale of turbulence. For units and equations see Appendix 2.

These equations were derived analytically from dimensionless analysis of relevant forces by Shinnar (Shinnar, 1961) and Shinnar and Church (Shinnar and Church, 1960), and are valid for dilute dispersions ($\Phi < 0.01$) (Calabrese et al., 1986), which were used exclusively in this study. It should be noted from equations 3 and 4 that the Sauter mean diameter was independent of Φ under these conditions. The microscale of turbulence, η, that was required to decide on which of the two equations was applicable was calculated as follows.

$$\eta = \left(\frac{\mu_c^3}{\rho_c^3 \cdot \overline{\epsilon}} \right)^{0.25} \qquad (5)$$

where, μ_c, dynamic viscosity; $\overline{\epsilon}$, average energy dissipation, which was equal to the power input per unit mass (see below).

The Sauter mean diameter during batch growth. Reduction of the drop size during batch growth was attributed to increasing cell numbers that interacted with the drops and/or to the microbial production of emulsifying agents. Two different approaches were developed to predict the Sauter mean diameter of dodecane droplets during batch growth as a function of the biomass concentration (Table 3.2). Stepwise development of equations 6 and 7 and the detailed description of the underlying assumptions are listed in Appendix 1.

Calculations were made with Kaleidagraph software on a Macintosh computer. The Sauter mean diameters for x=0 g l^{-1} biomass were calculated by extrapolating equation 6 and 7 to zero biomass. The empirical constant C_4 in equation 7 was set to zero.

TABLE 3.2. Prediction of Sauter mean diameter during batch growth.

Equation		Assumed mechanism for drop reduction
$d_{32}(x) = d_{32}^0 - \underbrace{C_6 \cdot x^{1/3}}_{\Delta d_{32}}$	(6)	– Degradation of dodecane. – Prevention of coalescence and break-up of drops by biofilm formation.
$d_{32}(x) = d_{32}^0\left(e^{-xC_3} + C_4\right)$	(7)	– Emulsification by surfactants. – Exponential relation between biomass and extent of drop reduction.

Symbols: d_{32}^0, Sauter mean diameter in water; x, biomass; t, time, C_3, C_4, C_6, empirical constants.

Accumulation of bacteria at the water-dodecane interface. The maximal number of cells that could accumulate at the water-dodecane interface if all drops were covered with one layer of bacteria, N_{IF}, was calculated as follows.

$$N_{IF} = \frac{4a\left(2.5 + d_{32}/d_c\right)}{2\sqrt{3}\cdot d_{32}\cdot d_c} \tag{8}$$

where, d_C is the cell diameter (for *Acinetobacter* sp. H01-N, d_C=0.8 µm (Finnerty et al., 1962), and own microscopic observations). For the development of equation 8 by Moo-Young and Shimizu (Moo-Young and Shimizu, 1971), trigonometric principles were used to calculate the area that is occupied by a spherical cell on a spherical drop. From the calculated cell numbers, N_{IF}, biomass concentrations were calculated by using a calibration curve.

Power input. The power input, P, was approximated as follows (see for example (Nielsen and Willadsen, 1994)).

$$P/V = \underbrace{\left(\rho_c n_I N_P D_I^5/V\right)}_{\text{const.}}\cdot N^3 \tag{9}$$

where N_P is the power number; N, stirrer speed; n_I, number of impellers; ρ_C, density of the continuous phase. A constant value of 5.2 was used for N_P, which was valid when Rushton impellers were used and for Reynolds number, Re >10000 (Nielsen and Willadsen, 1994); Additional power input due to gas flow was neglected since low volumetric air flows in combination with high stirrer speeds were applied.

Steady state mass transfer rates and $k_W a$. The mass transfer rate, r_T, of dodecane from the organic to the water phase was calculated according to well-known principles based on the two-film model (see for example (Nielsen and Willadsen, 1994)). Calculations were performed as follows.

$$r_T = k_W a\left(s^* - s_c\right) \tag{10}$$

where k_W is the mass transfer coefficient related to concentrations on the water side, s* is the alkane concentration in water at equilibrium between the two phases, and s_C is the alkane concentration in the water phase. r_T was also calculated by solving mass balance equations for the organic phase substrate and the dissolved alkane substrate under steady state conditions, yielding

$$r_T = D\rho_d(\Phi_{in} - \Phi_{out}) \quad (11)$$

for the organic phase substrate balance, and

$$r_T = r_S = \frac{D \cdot x}{Y_{x/s}} \quad (12)$$

for the dissolved alkane balance. With D, dilution rate; ρ_d, density of the organic phase; r_S, substrate consumption rate; x, biomass concentration; $Y_{x/s}$, growth yield. k_Wa was calculated by combining equations 10 and 11 or 12.

$$k_Wa = \frac{r_T^{eq.11/12}}{(s^* - s_c)} \quad (13)$$

where $r_T^{eq.11/12}$ is the mass transfer rate according to equation 11 or 12. For calculation, an estimate for s* was made by adapting the data presented by Käppeli and Finnerty (Käppeli and Finnerty, 1980) for the apparent solubility of hexadecane in the spent growth medium of *Acinetobacter* sp. H01-N.

k_W was also calculated by applying the equation of Chakravarty et al. (Chakravarty et al., 1975).

$$k_W = 0.16 \cdot N^{0.6} \cdot D_I^{0.2} \cdot \rho_c^{0.1} \cdot D_m \cdot \mu_c \quad (14)$$

where D_m, diffusion coefficient of dodecane in water at 25°C; μ_c, dynamic viscosity of water at 25°C. Equation 14 is part of an equation that was originally developed to predict k_Wa. However, we used equations 2 and 6 in order to calculate a rather than using the original approach of Chakravarty (Chakravarty et al., 1975).

3.3. RESULTS

3.3.1. Accumulation of bacteria at the water-dodecane interface

Microscopic observations of samples from batch cultures of *Acinetobacter* sp. showed that this microorganism accumulated at the water-dodecane interface during growth on dodecane as the sole carbon source. The fraction of bound cells increased during batch growth. After 8 h of cultivation, when measurements were started, 20% of the total biomass was bound to the surface of dodecane droplets. After 20 h of cultivation, when two-thirds of the dodecane substrate was degraded the fraction of bound cells was approximately 90% (Fig. 3.1). At this time, most droplets were covered with a single layer of cells. Only few bacteria could be observed that accumulated within a second layer around dodecane drops. This observation was in agreement with the calculated maximal number of bacteria that could accumulate at the water-dodecane interface in a single layer (equation 8). According to this calculation, 2.2 g of biomass per liter could accumulate at the total water-dodecane interface area in a single layer of cells. This estimated concentration was more than twice the biomass concentration present.

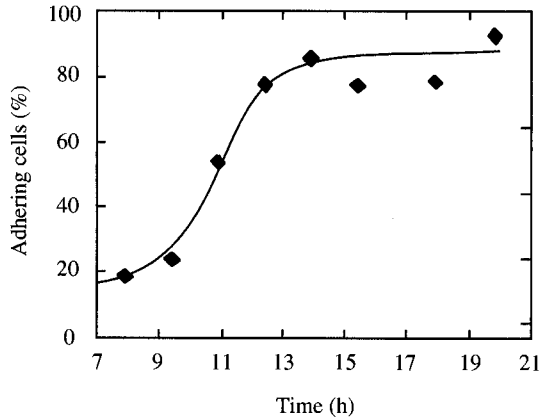

Figure 3.1. Fraction of total biomass that accumulated at the water-dodecane interface during batch growth of *Acinetobacter* sp. H01-N on dodecane.

3.3.2. Influence of biomass concentration on the Sauter mean diameter

The Sauter mean diameter decreased during batch growth (Fig. 3.2). After 8 h of cultivation, a Sauter mean diameter of 9.5 μm was observed. Subsequently, the mean Sauter diameter decreased, and after 20 h, a value of 2.1 μm was measured. After 23 h of batch growth, a residual dodecane concentration of 5.3 mg l^{-1} was measured, and no dodecane drops were detectable. In order to find out whether the Sauter mean diameter decreased primarily due to (i) the consumption of dodecane with subsequent inhibition of coalescence by the observed biofilm at the water-dodecane interface or (ii) the microbial production of surface active compounds, two equations were developed (equations 6 and 7, Table 3.2). These equations were used to fit experimental data of the Sauter mean diameter with the biomass concentration (Fig. 3.2). Curve fit with either of these equations did not reveal a difference in the goodness of the fit. The correlation coefficients were 0.93 in both cases (Table 3.3). On the basis of these calculations alone, it was therefore not possible to decide which of the two assumptions accounted primarily for the size reduction of the dodecane drops.

TABLE 3.3. Curve fits of the Sauter mean diameter as a function of the biomass and prediction and calculation of empirical constants.

Equation		Parameters
$d_{32}(x) = d_{32}^0 - C_6 \cdot x^{1/3}$	(eq. 6)	d_{32}^0 = 20.8±2.4 μm
		C_6 = 18.6 ± 3.0 μm $kg^{-1/3}$
		r^2 = 0.93
$d_{32}(x) = d_{32}^0\left(e^{-xC_3} + C_4\right)$	(eq. 7)	d_{32}^0 = 14.0±2.0 μm
		C_3 = 1.7±0.4 m^3 kg^{-1}
		r^2 = 0.93
$d_{32}^0/D_I = C_1 We^{-0.6}$	(eq. 3)	C_1 = 0.021-0.031
$d_{32}^0/D_I = C_2 We^{-0.14} Re^{-0.57}$	(eq. 4)	C_2 = 0.36-0.53

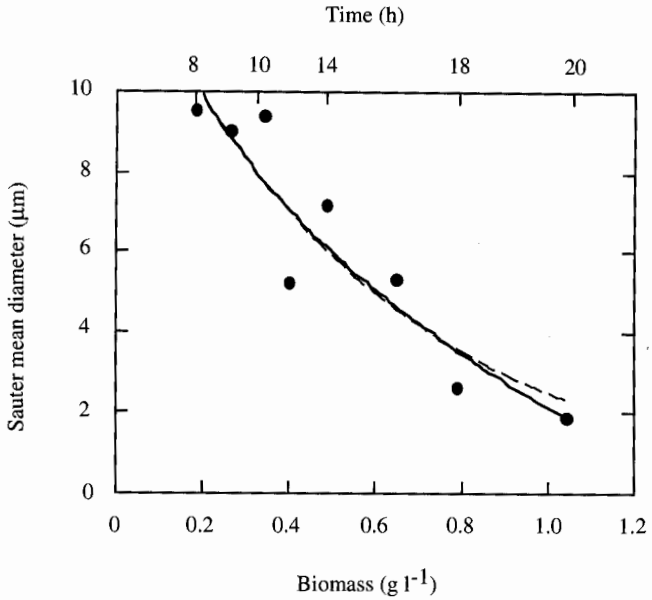

Figure 3.2. Sauter mean diameter versus biomass concentration during batch growth of *Acinetobacter* sp. H01-N on dodecane. (●) experimental data; (solid line) prediction of the Sauter mean diameter according to equation 6, (dashed line) prediction of the Sauter mean diameter according to equation 7.

3.3.3. Sauter mean diameter in pure water-dodecane emulsion and characterization of the reactor setup

Equations 6 and 7 were extrapolated to zero biomass concentration in order to estimate the Sauter mean diameter of dodecane drops in a cell-free dodecane-in-water emulsion for the used reactor. The extrapolated Sauter mean diameters were 14 or 21 µm (Table 3.3). From these calculations, the empirical constants for equation 3 and 4 were also determined (Table 3.3).

The microscale of turbulence, η, was 16 μm for a stirrer speed of 1000 rpm. Therefore, even in the absence of cells, Sauter mean diameters were in the range or smaller than η, and equation 4 was used for further calculations of the Sauter mean diameter at stirrer speeds ≥1000 rpm (see Table 3.1).

3.3.4. The specific interfacial area

During batch growth, the specific interfacial area increased from 7.9 cm^{-1} after 8h to 12.1 cm^{-1} after 20 h (Fig. 3.3). In subsequent samples, no dodecane droplets were detected. Therefore, this value of 12.1 cm^{-1} probably was the maximum specific interfacial area under the growth conditions applied. These experimentally determined values were then compared with the expected interfacial areas for the cell free system. For that purpose, the extrapolated values for the Sauter mean diameter of the water-dodecane emulsion and the measured dispersed volume fractions were used to calculate the interfacial area. In that case, the specific interfacial area would have decreased from 5.4 cm^{-1} to 1.1 cm^{-1} (equation 6) or from 3.6 cm^{-1} to 1.6 cm^{-1} (equation 7) respectively (Fig. 3.3). These data showed that the specific interfacial area increased 7 to 11-fold in the presence of bacteria compared to a similar pure water-dodecane emulsion.

3.3.5. Solubility of dodecane

Alkane solubility in growing cultures of *Acinetobacter* sp. H01-N was measured during carbon limited growth on a mixture of pristane and dodecane. Pristane was added since it was not degraded by this strain.

The "dissolved" residual dodecane concentration was 153 nM ± 58 nM which was approximately five times higher than its water solubility at 25°C (29 nM). Pristane solubility was 112 nM ± 9.3 nM, approximately 10-times the water-solubility of hexadecane. Since pristane was not degraded by *Acinetobacter* sp. H01-N, this value reflected the pristane solubility under the prevailing growth conditions. From residual dodecane concentrations, a Monod constant, K_S, of 260 nM ± 89 nM was calculated for *Acinetobacter* sp. H01-N on dodecane. This value was 9-times higher than the water solubility of dodecane at 25 °C.

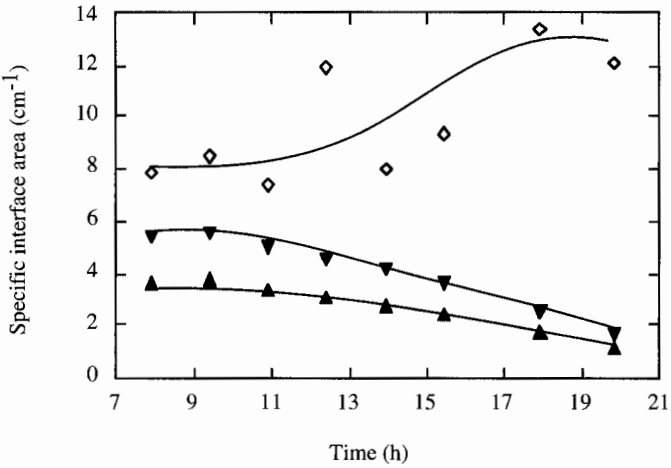

Figure 3.3. Specific interfacial area during batch growth of *Acinetobacter* sp. H01-N on dodecane. The specific interfacial area according to measured Sauter mean diameters (◇) was compared to values that were expected for the pure water-dodecane dispersion according to equation 6 (▲) and 7 (▼).

3.3.6. Influence of the power input on the biomass productivity and substrate turnover

The influence of the power input on the substrate turnover and on the biomass productivity was measured during carbon limited growth on dodecane by increasing the stirrer speed (Fig. 3.4). The biomass concentration increased linearly when the stirrer speed was increased from 1500 to 2100 rpm, corresponding to an increase in power input from 73 to 116 W l^{-1}. At a stirrer speed of 2100 rpm, a maximal biomass concentration of 410 mg l^{-1} was observed. In contrast, the residual dodecane concentration remained constant for stirrer speeds between 1500 and 1800 rpm. At lower stirrer speeds, dodecane concentrations were probably underestimated due to inhomogenous distribution of dodecane. When the stirrer speed was further increased to 2100 rpm, the residual dodecane concentration decreased clearly. At this stirrer speed, the alkane substrate was utilized almost completely.

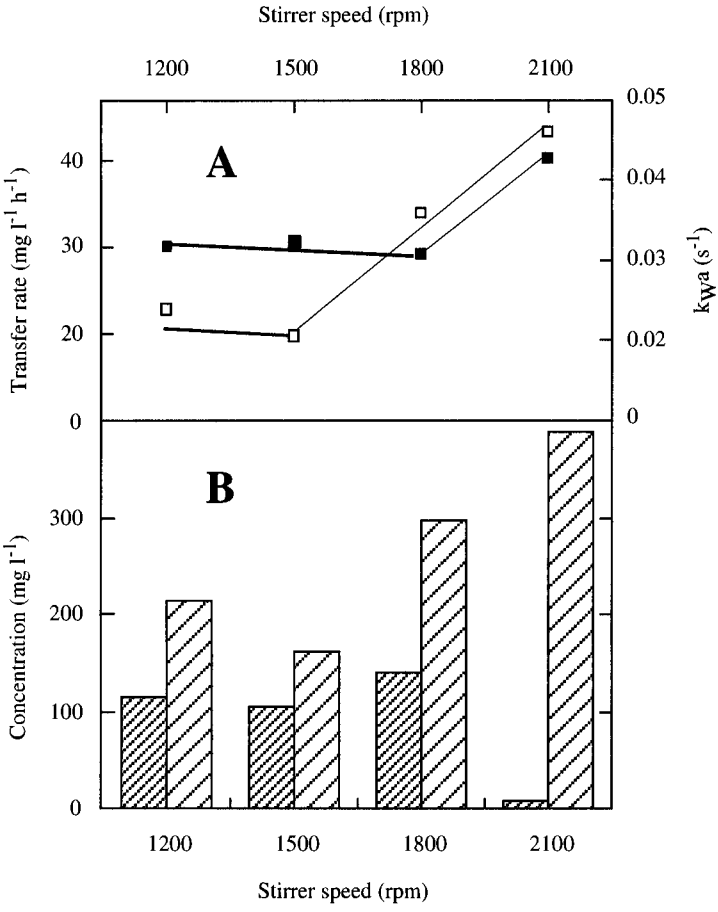

Figure 3.4. The effect of the stirrer speed on a continuous culture of *Acinetobacter* sp. H01-N during growth on dodecane at a dilution rate of 0.1 h^{-1}. A. Mass transfer rates and corresponding $k_W a$ values. Calculations were made according to the mass balance for the organic phase substrate (■), and the biomass productivity (□). B. Effect of the stirrer speed on biomass (▨), and residual substrate concentration (▨).

Mass transfer rates were calculated according to the mass balance for either substrate or biomass (Fig. 3.4.B). For stirrer speeds of 1800 and 2100 rpm, the resulting rates correlated well. At lower stirrer speeds, the mass transfer rates according to the substrate balance were higher than the corresponding rates according to biomass balance, which again may have been due to non-homogeneous mixing at lower stirrer speeds and under-estimation of dodecane concentrations.

The stirrer speeds between 1500 and 2100 rpm were tested in increasing order. For subsequent control of the observed effect, the stirrer speed was reduced again to 1200 rpm, which resulted in a power input of 42 W l^{-1}. After 16 hours, biomass productivity had decreased, and the residual dodecane concentration had increased. However, the culture had not yet re-established steady state conditions.

3.4. DISCUSSION

Microorganisms have developed different strategies to enhance transport and uptake rates of alkane substrates (Singh and Desai, 1986). Results showed that *Acinetobacter* sp. H01-N stabilized an emulsion of dodecane in water during batch growth. One mechanism by which dodecane droplets were stabilized was probably caused by accumulation of bacteria at the water-dodecane interface. However, alkane solubility was also increased in the presence of *Acinetobacter* sp. H01-N which indicated the presence of surface active compounds. Despite these effects, high power input was required to maintain carbon limited continuous cultures where almost complete utilization of the alkane substrate occurred.

3.4.1. Accumulation at the water-dodecane interface

Accumulation of cells at the water-dodecane interface was observed during late batch growth on dodecane. A similar behavior, although not quantified has been observed for the same organism growing on hexadecane (Scott and Finnerty, 1975), and has been quantified for another *Acinetobacter* sp. (Neufeld and Zajic, 1984). This behavior indicated that cell surface properties changed during batch growth. It has been observed before, that the cell surface hydrophobicity of an *Acinetobacter* sp. was highest at very early and late stages of batch growth on hexadecane (Neufeld et al., 1980). Alternatively, other adaptation to growth on alkanes such as the development of fimbriae (Rosenberg et al., 1982) or the occurence of capsules (Rosenberg et al., 1982) may play a role for the attachment of cells to dodecane droplets.

Since a major fraction of *Acinetobacter* sp. H01-N did not accumulate at the dodecane-water interface during early stages of batch growth, such behavior appeared not to be required for uptake of alkanes by this organism. In other cases, adhesion to the organic phase has been shown to be a requirement for growth on alkanes. For example, no growth of an *Arthrobacter* sp. on hexadecane was observed when adhesion of this strain to the organic phase was prevented with Triton X-100 (Efroymson and Alexander, 1991), whereas the same organism degraded the more soluble compound naphthalene under "non-adhering" conditions. In another study, Rosenberg and Rosenberg (Rosenberg and Rosenberg, 1981) isolated non-adherent mutants of *Acinetobacter calcoaceticus*. These mutants were not able to grow on hexadecane, unless hexadecane solubility was enhanced by addition of the emulsifier emulsan. Since *Acinetobacter* sp. H01-N did not accumulate at the water-dodecane interface during early batch growth, it seems therefore likely that this strain produced sufficient amounts of surfactants or emulsifiers at this stage.

3.4.2. Alkane solubility

In the experiments presented here, a moderate increase of alkane solubility was observed during carbon limited continuous cultures. The concentration of pristane, which did not serve as a growth substrate, was increased approximately 10 times compared to its water solubility. In the spent growth medium of the same organism, hexadecane solubility was increased 110-fold, which was accompanied by the production of membrane vesicles (Käppeli and Finnerty, 1979; Käppeli and Finnerty, 1980). From linear extrapolation of those data to biomass concentrations used here, a 50-fold increase of the pristane solubility would be expected. This was in fair agreement with the measured increase. However, alkane solubility may be as high as 2000 to 5000 times the water solubility in the presence of alkane degrading microorganisms (Goma et al., 1973; Gutierrez and Erickson, 1978). It should be noted though, that "solubility" includes truly solubilized molecules (real solubility), accommodated molecules (33) and submicron droplets (Gutierrez and Erickson, 1978; Moo-Young and Shimizu, 1971), and that the apparent solubility is highly dependent on the applied method (Bajpai and Prokop, 1975). Such an increase in solubility is due to the presence of surface active compounds (Hommel, 1990; Rosenberg et al., 1979; Singh and Desai, 1986), that have been observed for *Acinetobacter* sp. H01-N (Käppeli et al., 1980).

Another way of approaching the solubility of dodecane during continuous growth of *Acinetobacter* sp. H01-N was to estimate k_W, and to calculate the required dodecane solubility to explain the observed mass transfer rate (Table 3.4). k_W was calculated with the model by Chakravarty (Chakravarty et al., 1975), that fitted experimental data of several workers reasonably well (see for example (Lageveen, 1986)). The specific interfacial area was calculated according to equations 2 and 6. At lower stirrer speeds, the expected dodecane solubility was in the same range as measured by Käppeli and Finnerty (Käppeli and Finnerty, 1979). In contrast, at a stirrer speed of 2100 rpm the expected increase of dodecane solubility was more than 2000-fold. This indicated that at such a stirrer speed, the production of submicron droplets probably was the important process to produce susceptible forms of alkane substrate rather than simple mass transport of individual molecules from the organic to the water phase. This also implied that the approach used to predict the Sauter mean diameter failed to yield reasonable values under these conditions.

TABLE 3.4. Expected increase in dodecane solubility according to estimated values of k_Wa

Stirrer speed (rpm)	Sauter diameter[a] (μm)	k_Wa (s^{-1})[b]	expected increase in dodecane solubility
1500	7.4	1.7×10^{-2}	61
1800	5.0	1.2×10^{-2}	158
2100	3.8	1.1×10^{-3}	2240
2100	0.01[b]	4.0×10^{-1}	6

[a] The Sauter mean diameter was calculated according to equations 4 and 7. Empirical parameters were used according to Table 3.3.
[b] A mean drop diameter of 10 nm (submicron droplets) was assumed.
[c] k_Wa was calculated by using the equation of Chakravarty (Chakravarty et al., 1975) for the calculation of k_W, and equation 2 for the calculation of a.

3.4.3. Sauter mean diameter

Results confirmed that *Acinetobacter* sp. H01-N utilised both an increase in alkane solubility and direct contact to the organic phase in order to take up alkane substrates, as has been proposed for this strain during growth on hexadecane (Käppeli and Finnerty, 1979; Scott and Finnerty, 1975; Singer and Finnerty, 1984), and for other *Acinetobacter* sp. during growth on diesel oil (Marín et al., 1996). It is expected that both effects also cause reduction of the Sauter mean diameter (Ascón-Cabrera and Lebeault, 1995; Blanch and Einsele, 1973). Surfactants cause a reduction of the interfacial tension, σ, between the water and the dodecane phase which leads to stabilization of small droplets. A bacterial film on dodecane droplets stabilizes the emulsion in three ways. (i) The interface viscosity between the two phases increases which stabilizes the emulsion (Taylor and Hawking, 1992). (ii) A mechanical barrier to coalescence is evoked by a biofilm (Friberg and Yang, 1996). (iii) Bacterial net charge increases repulsive forces between oil droplets (Taylor and Hawking, 1992). In the oil industry, for example, particles with a net negative charge of around -35 mV are used to stabilize oil emulsions during oil recovery (Taylor and Hawking, 1992), which is identical with the net negative charge of *Acinetobacter* sp. H01-N (see Chapter 4).

Results showed that the mean diameter of dodecane droplets indeed decreased during batch growth. Two correlations (Equations 6 and 7) were developed to find out which of the two mechanisms (biofilm formation or production of surfactants) caused the major decrease in Sauter mean diameter. Unfortunately, both equations fitted the data equally well. Since dodecane drops were only loosely covered with cells of *Acinetobacter* sp. H01-N during early batch growth, it appeared that the presence of surface active compounds was primarly responsible for the size reduction of drops at this stage, whereas biofilm formation was more important for drop size reduction at late exponential batch growth.

By extrapolation of equation 6 and 7 to zero biomass, the Sauter mean diameter in the pure water-dodecane emulsion could be estimated. The estimated values according to these calculations were in agreement with data by Yoshida et al. (Yoshida and Yamada, 1971) for a similar reactor system as used here. By extrapolating equation 6 and 7 to zero biomass, it was also possible to calculate the empirical constants in equations 3 and 4 that were used to predict the Sauter mean diameter in the pure water-dodecane emulsion. For equation 3, the obtained value for constant C_3 was within the range of previously determined values (Chatzi et al., 1989). For equation 4, unfortunately, no

experimental data are available from the literature. Since typical drop sizes were in the range of the microscale of turbulence, η, equation 4 that includes the effect of viscous forces appears to be better for the description of the used system (Shinnar, 1961; Shinnar and Church, 1960). However, more data at different power inputs are required to test the applicability of this equation.

3.4.5. Power input

Although, *Acinetobacter* sp. H01-N exhibited distinct properties to enhance alkane availability, high power input was required to maintain carbon limited continuous cultures on dodecane as the sole carbon source. In the experiments presented here, a dodecane transfer rate of 0.23 mmol l^{-1} h^{-1} was observed at a stirrer speed of 2100 rpm and stable steady states could be maintained at least at a dilution rate of 0.1 h^{-1} (μ_{max} = 0.29 h^{-1}). Blanch and Einsele used an optimized reactor configuration and stirrer speeds of even 2500 to 3000 rpm in order to achieve a maximal hexadecane transfer rate of approximately 4.3 mmol l^{-1} h^{-1} (calculated from Fig. 9 in (Blanch and Einsele, 1973)). Alternative strategies to enhance mass transport are the addition of an inert solvent or the addition of surfactants (Köhler, 1992), or the application of carbon excess conditions (Lageveen et al., 1988). However, if a pure hydrocarbon substrate is supplied as in this study, high power input is required for high biomass productivities and substrate turnover rates (Blanch and Einsele, 1973; Einsele, 1972). Under the applied growth conditions, sufficiently high mass transport rates could be achieved at a stirrer speed of 2100 rpm in order to maintain steady state cultures of *Acinetobacter* sp. H01-N during carbon limited continuous growth on dodecane. This was important for further investigations of this strain's physiology during growth on dodecane.

APPENDIX 1. DEVELOPMENT OF EQUATIONS TO ESTIMATE SAUTER MEAN DIAMETER

Drop size reduction during batch growth at low Φ was attributed to increasing cell numbers that interact with the drops and/or to the microbial production of emulsifying agents. Two approaches were developed to predict the Sauter mean diameter of dodecane droplets during batch growth with the alkane substrate being present as a pure dispersed organic phase.

A.1.1. Drop stabilization due to biofilm formation

Assumptions. (i) The drop size decreased due to substrate consumption, i. e. the effect of emulsifying agents was neglected. (ii) Coalescence and drop breakage was prevented by cells that accumulate at the water-dodecane interface. (iii) The diameter of all drops was d_{32}, (iv) the drop number remains constant. (v) The dissolved alkane concentration is negligible.

Development of equation 6. A dodecane droplet with the mass m_i has the diameter d_i, that is

$$d_i = \left(\frac{6m_i}{\pi \rho_d} \right)^{1/3} \qquad \text{(A1)}$$

where ρ_d is the density of dodecane. Since all drops have the same diameter d_{32} (assumption iii) and since the drop number per unit volume, N_d, remains constant (assumption iv), equation A1 becomes

$$d_{32} = \left(\frac{6}{n_d \pi \rho_d} \right)^{1/3} s_d^{1/3} \qquad \text{(A2)}$$

where s_d is the concentration of the dispersed phase substrate, which is approximately equal to the total substrate concentration s (assumption v). If coalescence is prevented (assumption ii), drop diameter decreases due to substrate consumption. The difference in drop size in relation to substrate consumption is

$$\Delta d_{32} = \left(\frac{6}{n_d \pi \rho_d} \right)^{1/3} \left(s(t_1) - s(t_2) \right) \qquad \text{(A3)}$$

or, after replacing s by the biomass concentration, x, and setting $x(t_1=0)=0$

$$\Delta d_{32} = \left(\frac{6}{n_d \pi \rho_d Y_{x/s}} \right)^{1/3} x(t)^{1/3} \qquad \text{(A4)}$$

Hence, a final equation can be formulated that correlates the Sauter mean diameter with the biomass concentration during batch growth

$$d_{32}(x) = d_{32}^0 - \underbrace{C_6 \cdot x^{1/3}}_{\Delta d_{32}} \qquad \text{(A5)}$$

C_6 and d_{32}^0 are constant, the latter being the Sauter mean diameter in the absence of biomass (equations 3 or 4). This equation is identical with equation 6 (Table 3.2).

A.1.2. Drop stabilization due to production of emulsifiers

Assumptions. (i) Drop diameter at a certain emulsifier concentration is proportional to the diameter in a water-hydrocarbon emulsion without emulsifier. Drop diameter is much bigger in the latter case (Moo-Young and Shimizu, 1971). (ii) Emulsifier concentration is proportional to the biomass concentration, or cells themselves act as an emulsifying agent (iii) The effect of different emulsifier concentrations on the drop size is exponential in nature (2). (iv) All drops have the same diameter d_{32}.

Development of Equation 7. In the presence of biomass, $d_{32}(x)$ is reduced by a factor $\alpha << 1$ compared to d_{32}^0 (assumptions i and ii).

$$d_{32}(x) = \alpha \cdot d_{32}^0 \qquad \text{(A6)}$$

which has been proposed by Moo-Young and co-workers (Moo-Young and Shimizu, 1971). The results of Bajpai and Prokop showed that α is an exponential function of the biomass concentration for a limited range of biomass concentrations (Bajpai and Prokop, 1975). Equation A7 shows a possibility to fit their data

$$\alpha(x) = e^{-xC_3} + C_4 \qquad \text{(A7)}$$

C_3 and C_4 are empirical constants. Substituting α in equation A6 with equation A7 yielded equation A8, which is identical with equation 7 (Table 3.2).

$$d_{32}(x) = d_{32}^0\left(e^{-xC_3} + C_4\right) \qquad \text{(A8)}$$

For large x, the term between brackets will become constant at C_4, and consequently will $d_{32}(x)$. It is expected that $C_4 << 1$ (assumption i). On the other hand, since $C_4 << 1$, the term in brackets will become 1 for x=0, and $d_{32}(x=0) = d_{32}^0$, as it should be. For small biomass concentrations, d_{32} decreases exponentially as a function of the biomass concentration.

APPENDIX 2. LIST OF SYMBOLS USED IN CHAPTER 3

Symbol	Unit[a]	Definition	Equation	Value/Range
a	m^{-1}	Specific interfacial area	2	-
C_i	variable	Empirical constant i	-	-
D	h^{-1}	Dilution rate	-	0.1 h^{-1}
d_c	m	cell diameter	-	8×10^{-7}
d_i	m	Diameter of drop i	-	-
D_I	m	Impeller diameter	-	0.05
D_m	$m^2\ s^{-1}$	Diffusion coefficient for dodecane at 25°C[d]	-	7×10^{-10} [d]
$d_{32}(x)$	m	Sauter mean diameter in the presence of biomass	6, 7	$<9 \times 10^{-6}$
d_{32}^0	m	Sauter mean diameter in the absence of biomass	3, 4	-
k_W	$m^2\ s^{-1}$	mass transfer coefficient	13, 14	-
L	m	Macroscale of turbulence (≈width of impeller blade)	-	≈0.01
m_i	kg	Mass of drop i	-	-
N	s^{-1}	stirrer speed[a]	-	17-35
n_d	m^{-3}	Number of drops per unit volume	-	-
n_I	-	number of impellers (Rushton type impellers[c])	-	3
N_{IF}	-	Number of bacteria at drop surface	8	-
N_P	-	Power number	9	5.2[c]
P/V	W	Power input	9	13-116
Re	-	Reynolds number	$\rho_c N D_I^2/\mu_c$	>45000
r_T	$kg\ m^{-3}\ s^{-1}$	transfer rate of dodecane	10, 11	-
s	$kg\ m^{-3}$	Total substrate concentration	-	-
s_c	$kg\ m^{-3}$	dissolved dodecane concentration	-	-
s_d	$kg\ m^{-3}$	Dispersed phase substrate concentration	-	-

Symbol	Unit[a]	Definition	Equation	Value/Range
s*	kg m^{-3}	dissolved dodecane concentration at equilibrium	-	-
t	s	time	-	-
V	m^3	working reactor volume	-	1.6×10^{-3}
We	-	Weber number	$\rho_c N^2 D_I^3/\sigma$	>1300
x	g l^{-1}	Biomass	-	-
α	-	Proportionality factor	A6	<<1
Φ	-	dispersed organic volume fraction	-	<<0.01
η	m	Microscale of turbulence	5	
μ_c	kg m^{-1} s^{-1}	dynamic viscosity of continuous phase	-	9.2×10^{-4} [d]
ρ_c	kg m^{-3}	density of continuous phase	-	997[d]
ρ_d	kg m^{-3}	Density of dispersed phase	-	750
σ	N m^{-1}	Interface tension	-	0.026[b]
$\bar{\epsilon}$	W kg^{-1}	Average energy dissipitation	$P/(V\rho_c)$	0.013 - 0.12

[a] For calculations, SI-units were used. In the text or for figures, units were sometimes used according to common usage in microbiology.
[b] Value for water-alkane mixtures according to (Guerra Santos, 1985)
[c] See (Nielsen and Willadsen, 1994) for overview
[d] Values according to (Lide, 1995)

CHAPTER 4 Growth and Physiology of *Acinetobacter* sp. H01-N on Dodecane: Mixed-Substrate Utilization under Different Growth Conditions

Utilization of acetate and dodecane by the alkane degrading strain Acinetobacter sp. H01-N was investigated both during batch growth and during carbon limited growth in continuous culture. During batch growth on single substrates, μ_{max} was 0.24 h^{-1} on dodecane and 0.68 h^{-1} on acetate. The yield coefficients were 0.99 g biomass (g carbon)$^{-1}$ and 1.13 g biomass (g carbon)$^{-1}$ for acetate and dodecane respectively. During batch growth on the substrate mixture, acetate and dodecane were utilized sequentially with acetate being utilized first. During subsequent dodecane utilization, no biomass was produced indicating formation of a dead-end product. In carbon limited continuous culture, both substrates were utilized simultaneously at dilution rates below 0.3 h^{-1}. The formation of biomass from the two substrates was additive. Dodecane utilization was dependent on induction with dodecane. Dodecane induced oxygen uptake rates were reduced by approximately 50% during growth on the substrate mixture compared to growth on dodecane alone. With increasing dilution rates, dodecane induced oxygen uptake rates decreased. At a dilution rate of 0.3 h^{-1}, dodecane induced oxygen uptake rates were identical in cells that were grown on the substrate mixture and cells that were grown on acetate alone. A similar dependency on both dodecane flux and dilution rate was observed for the surface hydrophobicity indicating that physiological adaptations to the presence of dodecane were regulated in the same manner as the synthesis and activity of dodecane enzymes. Shift experiments at a dilution rate of 0.1 h^{-1} during carbon limited growth showed that Acinetobacter sp. H01-N

responded rapidly to the presence of dodecane. Dodecane induced oxygen uptake rates above basal activity were detected between 0.1 and 0.6 volume changes after the shift. The response time was dependent on the carbon source that was used before the shift and on the presence of acetate during transient growth.

4.1. INTRODUCTION

Acinetobacter sp. H01-N is able to metabolize linear alkanes in the range of C10 to C20 (Finnerty et al., 1962). Several of the enzymes for alkane metabolism are induced during growth on alkanes only (Fox et al., 1992; Kennedy and Finnerty, 1975; Singer and Finnerty, 1985; Singer and Finnerty, 1985). Inducibility of alkane degradation has also been shown in other *Acinetobacter* sp. (Asperger et al., 1984) and in *Pseudomonas oleovorans* (Eggink et al., 1988). *Acinetobacter* sp. H01-N responds with distinct ultrastructural modifications to growth on alkane substrates. Modifications include the formation of intracytoplasmic membranes, hydrocarbon inclusions (Kennedy et al., 1975; Scott and Finnerty, 1976), and the production of extracellular vesicles which solubilize hexadecane (Käppeli and Finnerty, 1979; Käppeli and Finnerty, 1980). As a response to growth on alkanes, ultrastructural modifications (Chen et al., 1996; Müller et al., 1983; Scott and Finnerty, 1975) and increased cell surface hydrophobicity (Neufeld et al., 1980) have also been observed in other alkane degrading microorganisms.

In contrast to the laboratory, in natural systems microorganisms are usually exposed to a wide variety of carbon sources, such as sugars, short-chain fatty acids etc. These substrates are usually present at very low concentrations of a few nanomoles to micromoles per liter (Münster, 1993). In batch culture grown on a mixture of hexadecane and either acetate (5 mM), ribose (130 mM), or nutrient broth (0.8%)-yeast extract (0.5%), acetate pregrown cells of *Acinetobacter* sp. H01-N showed diauxic growth. Hexadecane was always the second carbon source to be used (Kennedy and Finnerty, 1975). Morphological changes were not observed during growth on acetate.

Diauxic growth and morphological changes may be the result of growth in the presence of excess carbon source. In this study we investigate growth of *Acinetobacter* sp. H01-N under carbon source excess and limitation and its behaviour on substrate mixtures and responses to shifts from one substrate to the other.

4.2. MATERIALS AND METHODS

4.2.1. Microbial strain and cultivation

Microbial strain. *Acinetobacter* sp. H01-N (DSM 1139) described by Stewart et al. (Stewart and Kallio, 1959; Stewart et al., 1959) and by Finnerty et al. (Finnerty et al., 1962) was used in all experiments. The organism was stored in 15 % glycerol at -80°C.

Growth media. The strain was grown either in nutrient broth (Biolife, Milan, Italy) or in minimal media. Minimal medium A was used for shake flask experiments. It contained per liter of nanopure water: 2 g $(NH_4)_2SO_4$, 0.1 g NaCl, 0.62 g $MgCl_2 \cdot 6H_2O$, 0.01 g $CaCl \cdot 2HO$, and 2 - 5 ml trace element solution (Kemmler, 1992), which consisted of (mg l^{-1}): 1500 $FeCl_2 \cdot 4H_2O$, 60 H_3BO_3, 100 $MnCl_2 \cdot 4H_2O$, 120 $CoCl_2 \cdot 6H_2O$, 70 $ZnCl_2$, 25 $NiCl_2 \cdot 6H_2O$, 15 $CuCl_2 \cdot 2H_2O$, 25 $Na_2MoO_4 \cdot 2H_2O$, and 26000 EDTA·$Na_4 \cdot (H_2O)_4$. 100 ml per liter phosphate buffer from stock-solution (pH 6.8) was added to medium A after autoclaving. The stock solution contained (per liter) 20 g KH_2PO_4 , and 50 g $Na_2HPO_4 \cdot 2\ H_2O$. For cultivation in a bioreactor a modified medium B was used. It contained per liter of nanopure water: 2 g $(NH_4)_2SO_4$, 0.2 g NaCl, 0.5 g KCl, 0.62 g $MgCl_2 \cdot 6H_2O$, 0.01 g $CaCl \cdot 2HO$, 0.75 ml H_3PO_4, and 2 ml trace element solution as in medium A. The media were supplemented with either acetate (0.45 - 2.5 g l^{-1}), succinate (0.45 - 2.5 g l^{-1}), dodecane (0.14 - 0.59 g l^{-1}), or a mixture of dodecane and acetate. Acetate and succinate were prepared as stock solutions and were autoclaved separately. Dodecane was filter-sterilized through 0.2 µm pore size Teflon filters (Nucleopore) prior to its addition to the sterile medium. For some batch experiments, dodecane was mixed with an equal volume of pristane prior to its addition to the medium.

Continuous culture experiments. Stock cultures were first inoculated into nutrient broth for overnight growth. Subsequently they were transfered to medium B supplemented with 0.37 g l^{-1} acetate. After reaching the stationary phase, the culture was switched to continuous culture operation mode, with the appropriate carbon sources. Dodecane was fed directly to the reactor with a syringe pump (B. Braun, Melsungen, Germany) equipped with a glass syringe and Teflon plunger.

For cultivation, a bioreactor (MBR, Wetzikon, Switzerland) with a working volume of 1.8 l was used. Temperature was maintained at 25°C, pH was maintained at 6.8 by the addition of 1 N NaOH or 1 N H_3PO_4. Aeration rate was 0.04 l air (l culture broth)$^{-1}$

min^{-1} (vvm). During steady-state growth with acetate as the sole carbon source, the stirrer speed was set at 1000 rpm, otherwise at 2100 rpm.

Steady-state measurements were performed after at least 6 volume changes, when carbon dioxide production rate and biomass concentrations were constant. Steady state cultures served for inoculation of batch and transient growth experiments.

Batch growth experiments. Batch experiments on single carbon sources were inoculated with 3 - 5 % vol/vol of a steady-state culture of *Acinetobacter* sp. H01-N. Inocula were pre-grown at a dilution rate of 0.1 h^{-1}, and 0.3 h^{-1} for experiments with dodecane and acetate respectively. Acetate grown cultures were also used for experiments with succinate. Batch growth experiments were performed in a bioreactor (3 l working volume, stirrer speed 1000 rpm), baffled Erlenmeyer flasks or in test tubes. Erlenmeyer flasks were incubated on a gyratory shaker at 150 rpm. For reproducibly sampling dodecane, cultures were incubated in test tubes sealed with teflon lined caps. The test tubes were placed in a horizontal position on a reciprocal shaker at 150 rpm, and one tube was sacrificed for each sample point.

4.2.2. Analytical methods

Biomass. Details for the measurement of biomass concentrations are given in chapter 2. Biomass dry weight was determined by centrifugation using Triton X-100 to remove residual dodecane. Optical density measurements were performed on resuspended cell pellets after samples had been centrifuged in the presence of Triton X-100. The accuracy of these methods was checked with protein determination according to Lowry et al. (Dunn, 1989), and filtration of 20 ml of culture fluid through pre-tared 0.2 µm Teflon membrane filters (Millipore). The filters were washed once with 5 ml of propanol and then with 10 ml of distilled water, and were dried at 105°C to constancy.

Dodecane. Dodecane concentrations were determined with a GC (Carlo Erba, Italy). Samples were extracted with a mixture of chloroform and methanol according to the method of Käppeli and Finnerty (Käppeli and Finnerty, 1979) with some modifications. Details of the used extraction method and the analytical procedure are specified in chapter 2.

Acetate and succinate. Acetate and succinate were determined using an anion exchange chromatography system (Dionex AG, Olten, Switzerland). Anions were

separated with a high capacity analytical column AS10 that was used in combination with the guard column AG10 and an anion trap column (ATC-1). Anion concentrations were determined with a CD20 conductivity detector. The eluent signal was repressed by a self-regenerating suppressor. Three eluents with no NaOH (eluent 1 (E1)), or 100 mM NaOH (eluent 2 (E2)) or 400 mM NaOH (E3) were used. For the preparation of the eluents, 1 l of nanopure water was degassed by vacuum in an ultrasonic bath for 10 min and subsequently aerated with helium gas for 15 minutes. 4.7 ml (E2) or 18.8 ml (E3) of bicarbonate free 50% wt/vol NaOH (J. T. Baker, The Netherlands) were then added, and the eluents were stored under helium at excess pressure. The eluent flow was set to 1 ml min^{-1} and the step gradient methods listed in Table 4.1 and 4.2 were used. Retention times were 10.5 min for acetate, and 14.3 min for succinate. The detection limit was approximately 2 mg l^{-1}.

Gas analysis. 2 ml of air from the reactor gas outlet were injected into a Shimadzu GC 8APT (Shimadzu, Japan) for analysis of carbon dioxide and oxygen. The GC was equipped with two parallel stainless steel columns. Nitrogen and oxygen were separated in a molecular sieve 5A 80/100 column of 5.5 m length (Brechbühler, Switzerland); the other compounds were separated in a Poropak Q 80/100 column of 4.9 m length (Waters, USA). The carrier gas was helium (60 ml min^{-1}). Sample loop and column oven were maintained constantly at 80°C. A TCD detector was used to quantify oxygen and carbon dioxide. Calibration was performed with different gas mixtures obtained from Carbagas (Carbagas, Switzerland) and with air.

Substrate induced oxygen uptake rates. Oxygen uptake rates were recorded with a Clark type oxygen cell (Rank Brothers, England). The assay mixture consisted of 2.88 ml of basal medium B without carbon source (pH 6.8, adjusted with 1 N NaOH), 100 µl of concentrated cell suspension, and 20 µl of acetate or succinate stock solution (0.3 M each). The concentrated cell suspension was prepared by collecting 8 ml of culture broth from the bioreactor, washing it once in medium B (pH 6.8) and concentrating it 10 or 20-fold in the same medium. Oxygen uptake rates in the presence of a carbon substrate were then corrected for the endogenous oxygen uptake rates that were measured separately. Dodecane induced oxygen uptake rates were measured in a slightly modified assay. 2.9 ml of reactor medium B (pH 6.8) without carbon source, 100 µl of concentrated cell suspension, and 5 µl of dodecane were mixed in a glass tube and sealed with a Teflon-lined cap. The sample was shaken vigorously immediately before transferring it into the mixing chamber, where the oxygen uptake rates recorded. Endogenous uptake rates were determined separately in parallel experiments.

TABLE 4.1. Step gradient method for the measurement of acetate

Time (min)	Fraction of total eluent flow (%)			Remark
	E1[a]	E2[b]	E3[c]	
0.00	95.0	5.0	0.0	Resolution of acetate peak
1.00	95.0	5.0	0.0	
12.00	93.7	6.3	0.0	
12.01	80.0	20.0	0.0	Elution of inorganic anions
22.00	69.5	0.0	30.5	
22.01	55.0	0.0	45.0	
27.00	55.0	0.0	45.0	
27.50	95.0	5.0	0.0	Starting column reconditioning
36.00	95.0	5.0	0.0	

[a] E1: H_2O nanopure
[b] E2: 100 mM NaOH
[c] E3: 400 mM NaOH

TABLE 4.2. Step gradient method for the measurement of succinate

Time (min)	Fraction of total eluent flow (%)			Remark
	E1[a]	E2[b]	E3[c]	
0.00	85.0	0.0	15.0	Resolution of succinate peak
1.00	85.0	0.0	15.0	
20.00	75.0	0.0	25.0	
20.50	55.0	0.0	45.0	Elution of inorganic anions
30.00	55.0	0.0	45.0	
30.50	85.0	0.0	15.0	Starting column reconditioning
40.00	85.0	0.0	15.0	

[a] E1: H_2O nanopure
[b] E2: 100 mM NaOH
[c] E3: 400 mM NaOH

Cell surface hydrophobicity and ζ-potential. Cell surface hydrophobicity was inferred from contact angles of water drops on bacterial lawns, that were determined according to the method of van Loodsrecht et al. (van Loodsrecht et al., 1987). 40 ml of culture liquid were harvested from the bioreactor. The cells were washed once and resuspended in 20 ml of 0.1 M phosphate buffered saline (PBS) at pH 6.8 consisting of (per liter) 4.9 g NaCl, 0.29 g KH_2PO_4, 1.19 g K_2HPO_4. Approximately 3 ml of this suspension were collected on 0.45 μm pore-size poly-acetate membrane filters (Schleicher-Schüll, Germany). The filters were mounted on glass slides, and air-dried for 2 h at room temperature, and the contact angles were determined with a microscope equipped with a goniometric eye piece (Krüss GmbH, Hamburg, Germany).

The above cell suspension in PBS was diluted 10 times for the determination of the electrophoretic mobility with a Doppler electrophoretic light scattering analyzer (Zeta-master, Malvern Instruments Ltd., Worcestershire, UK) (Jucker et al., 1996). The zeta-potential was calculated from the electrophoretic mobility according to the method of Hemholtz-Smluchowski (Hiementz, 1986).

Dodecane bound cells and freely suspended cells. 2 ml of culture fluid were centrifuged with or without the addition of 0.1 ml of a Triton X-100 solution (1:10 vol/vol in water). The resulting pellets were resuspended in water, and the OD_{546} was determined. When Triton X-100 was added, the pellet consisted of the total biomass. When Triton X-100 was omitted, only loosely dodecane bound and freely suspended cells were pelleted. Therefore, the fraction of the total biomass bound to dodecane was calculated from the difference in OD_{546} observed with the two experimental procedures.

4.3. RESULTS

4.3.1. Batch growth on dodecane, acetate and succinate as sole carbon sources

Acinetobacter sp. H01-N was first cultivated on acetate, dodecane and succinate each as the sole carbon and energy source in order to determine the growth yields per substrate carbon and per available electron in the substrate ($Y_{X/C}$, and $Y_{av\ e^-}$ respectively) as well as the maximum specific growth rates (μ_{max}) of this strain on the three carbon substrates.

Acinetobacter sp. H01-N pregrown on acetate grew readily on both acetate and succinate without lag (Fig. 4.1A and B). It exhibited similar maximum specific growth rates, μ_{max}, and growth yield per substrate carbon, $Y_{X/C}$, on the two substrates (Table 4.3). A difference was observed for the yield per available electron, $Y_{av\ e^-}$ (Table 4.3).

During batch growth on dodecane, considerable lag phases of several hours were observed depending on the type of inoculum and on the cultivation conditions applied (Table 4.4, Fig. 4.1C). Although this effect was not studied in detail, results indicated that the lag phase was in general smaller with high inocula sizes, with batch derived inocula and in shake flask type experiments. When dodecane was supplied as a pure organic phase, an average μ_{max} of 0.24 $h^{-1} \pm 0.05\ h^{-1}$ was measured (Table 4.3). In the presence of pristane μ_{max} was slightly higher (Table 4.3). $Y_{X/C}$ on dodecane was 1.13 g biomass (g carbon)$^{-1}$, and $Y_{av\ e^-}$ was 2.2 g mole^{-1} (Table 4.3).

TABLE 4.3. Maximum specific growth rate, μ_{max}, of *Acinetobacter* sp. H01 on the carbon sources used in this study.

Carbon source	μ_{max} (h^{-1})	$Y_{X/C}$ (g g^{-1})[b]	$Y_{av\ e^-}$ (g mole^{-1})[c]
acetate	0.68 (±0.04)	0.99	2.9
succinate	0.71 (±0.05)	0.98	3.4
dodecane	0.24 (±0.05)	1.13	2.2
dodecane/pristane[a]	0.30 (±0.05)[a]	-	-
nutrient broth	1.05 (-)	-	-

[a] Growth rate on dodecane
[b] Growth yield for substrate carbon
[c] Growth yield for available electrons in the substrate

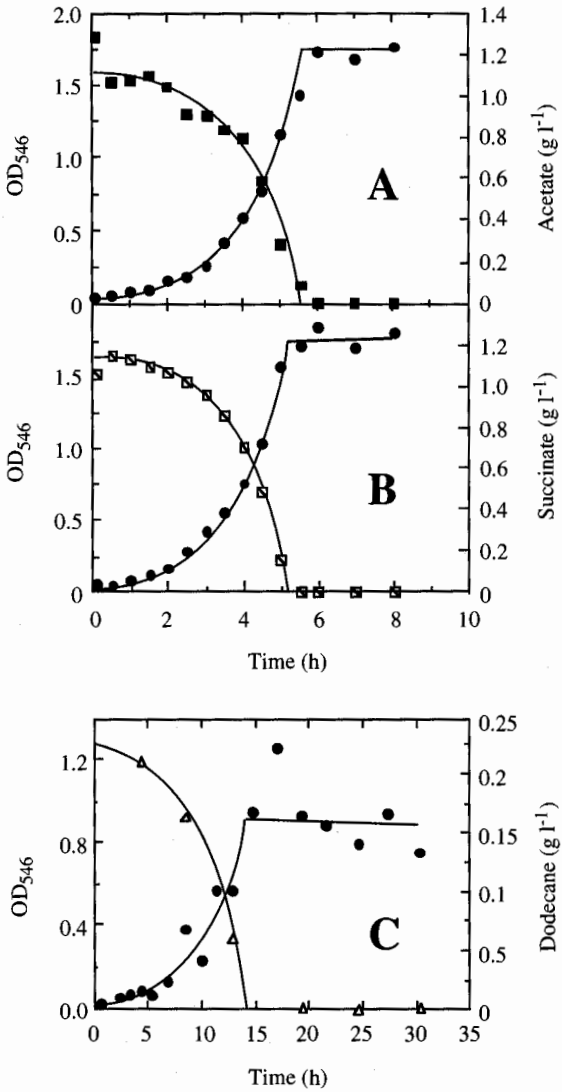

Figure 4.1. Batch growth of *Acinetobacter* sp. H01-N on acetate (A), succinate (B), and dodecane (C) as the sole carbon source. Cells were grown in shake flasks. Dodecane had been added as a pristane solution. (●) OD546, (■) acetate, (⧅) succinate, (Δ) dodecane.

TABLE 4.4. Lag phase in batch cultures of *Acinetobacter* sp. H01-N growing on dodecane.

Lag phase (h)	Inoculum	Initial OD_{546}	System
8	chemostat[a]	<0.01	bioreactor
7	chemostat[a]	0.03	bioreactor
10	chemostat[a]	0.04	bioreactor[d]
13	chemostat[a]	0.02	bioreactor[d]
<0.5	batch[b]	1.07	bioreactor[d]
<0.5	batch[c]	0.02	culture tubes[d,e]
<0.6	batch[c]	0.02	culture tubes[d,e]
3	batch[c]	0.05	shake flask[f]

[a] Chemostat culture, dilution rate D=0.1 h^{-1}, carbon source dodecane.
[b] Stationary phase cells (7 h) after batch growth on dodecane. The second growth phase was started by pulsing additional dodecane to the culture.
[c] Overnight batch cultures on dodecane as the sole carbon source.
[d] Batch growth experiments on dodecane in the presence of pristane.
[e] Incubated on a reciprocal shaker at 150 rpm
[f] Incubated in baffled shake flasks on a gyratory shaker at 160 rpm.

4.3.2. Batch growth on a mixture of acetate and dodecane

In subsequent batch experiments, *Acinetobacter* sp. H01-N was grown on a mixture of acetate and dodecane under excess carbon conditions. Batch experiments were inoculated with 5% of steady-state cultures of *Acinetobacter* sp. H01-N growing on dodecane as the sole carbon and energy source. This ensured that all of the required enzymes to degrade dodecane were induced at the beginning of each experiment. It can be noted from Fig. 4.2 that acetate was utilized preferentially during batch growth on a mixture of acetate and dodecane. During the degradation of acetate, the dodecane concentration remained nearly stable. In the experiment shown in Fig. 4.2A, for example, 522 mg l^{-1} of dodecane carbon were initially detected, and 457 mg l^{-1} of dodecane carbon were still present when acetate was depleted. Only after depletion of acetate did the dodecane concentration decrease at a more rapid rate (Fig. 4.2A). The

resulting two distinct growth phases - first on acetate and, subsequently, on dodecane - are described in more detail in the following sections.

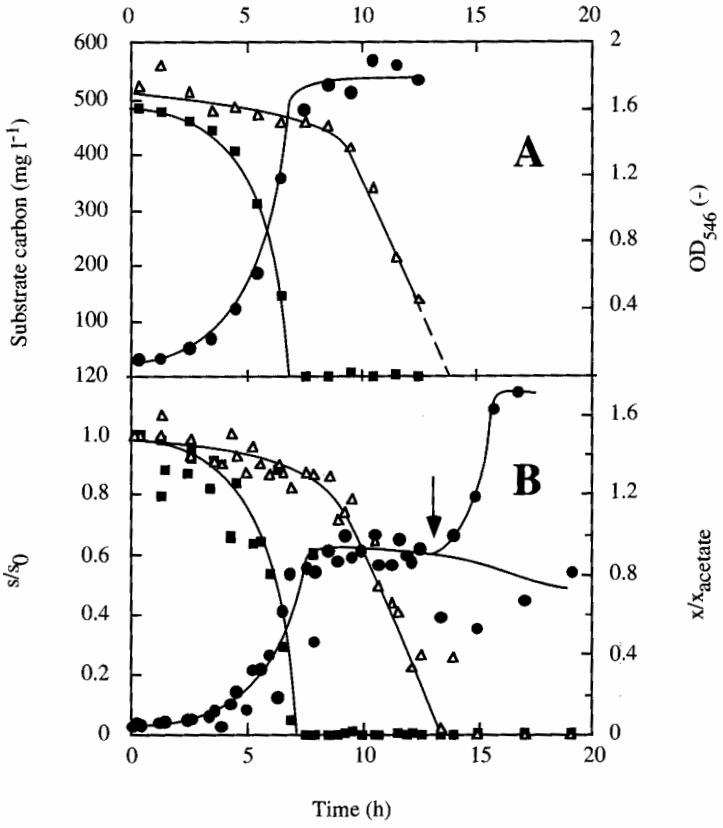

Figure 4.2. Batch growth of *Acinetobacter* sp. H01-N on a mixture of acetate and dodecane. A. Typical growth pattern. B. Overlay of three independent experiments at total initial substrate carbon concentrations of 1 g carbon per liter or 0.3 g carbon per liter respectively. (●) biomass, (Δ) dodecane, (■) acetate. The arrow indicates a pulse of additional acetate for control purposes (see text).

Batch growth of Acinetobacter sp. H01-N on acetate in the presence of dodecane. Dodecane pregrown cells utilized acetate as the preferential substrate without a lag phase (Fig. 4.2). However, during early exponential growth on acetate, these cells exhibited a specific growth rate of only approximately 0.2 h^{-1}. The specific growth rate increased during growth on acetate up to 0.8 h^{-1} (Fig. 4.3A). The specific growth rate of cells pre-grown on a mixture of acetate and dodecane remained nearly stable during the entire experiment (Fig. 4.3B), suggesting that dodecane pre-grown cells required adaption before acetate utilization functioned optimally.

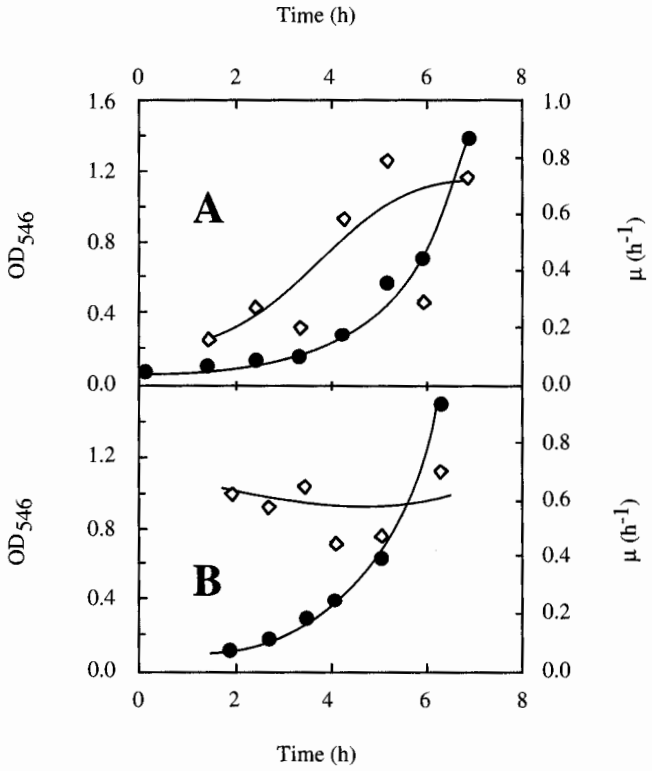

Figure 4.3. Adaptation to growth on acetate in the presence of dodecane by differently pregrown cells of *Acinetobacter* sp. H01-N: (A) chemostat, dilution rate 0.1 h^{-1}, dodecane as sole carbon source, (B) chemostat, dilution rate 0.1 h^{-1}, acetate and dodecane as carbon sources. (●) OD546, (◇) μ

During exponential growth on acetate, the amount of cells that were bound to dodecane decreased rapidly (Fig. 4.4). During the late exponential growth phase on acetate, only 7% of the total biomass was bound to dodecane. The amount of bound cells increased immediately in the subsequent stationary phase. Here up to 50% of the biomass appeared to be bound to dodecane. These results suggested that the ability of *Acinetobacter* sp. H01-N to accumulate at a hydrophobic interface was counteracted by the presence of acetate.

Utilization of dodecane after the depletion of acetate. Approximately 1.5 h after acetate had been depleted, the dodecane concentration started to decrease rapidly (Fig. 4.2). During the degradation of dodecane, no growth was observed. This result was reproducible (Fig. 4.2B). The constant biomass concentration was not an analytical artifact. The total protein concentration, and a gravimetric filter method gave the same result. In order to check whether the lack of biomass increase was due to non-carbon nutrient exhaustion, an acetate pulse was applied after 14 h (Fig. 4.2B). The result was an immediate increase in the biomass concentration (Fig. 4.2B). This confirmed carbon limitation of the medium and full activity of the culture towards acetate.

Despite the ability of the culture to immediately respond to an acetate pulse after 14 h, the acetate induced oxygen uptake rates decreased rapidly after the depletion of acetate (Fig. 4.5). In contrast, the dodecane induced oxygen uptake rates slightly increased after the depletion of acetate (Fig. 4.5). Due to the presence of high residual dodecane concentrations, this rate was difficult to determine. A clear induction was visible at least in one experiment (Fig. 4.5).

In the absence of growth, i. e. at constant biomass concentrations, a linear decrease in the dodecane concentration would be expected. This was indeed observed (Fig. 4.6), and in all experiments tested, a correlation coefficient of above 0.98 was calculated. The transformation rate was determined at biomass concentrations of 0.15 g l^{-1} and 0.5 g l^{-1}. From these data, a first-order reaction rate constant of 0.18 h^{-1} was estimated.

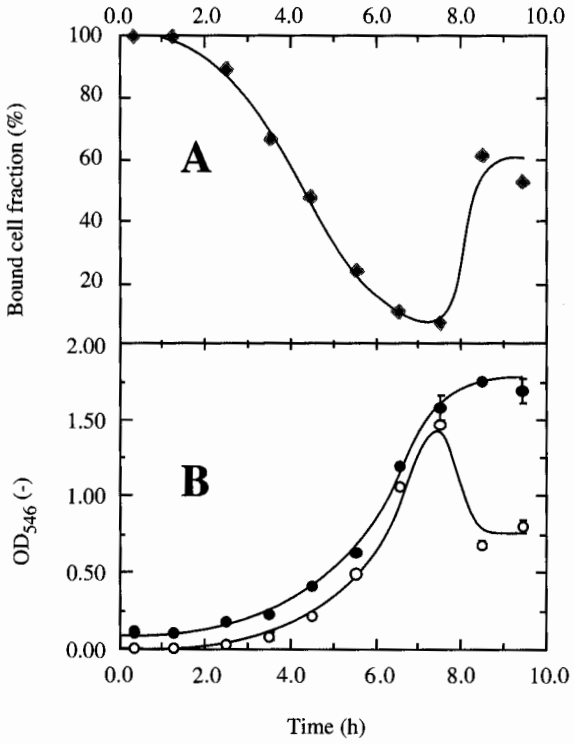

Figure 4.4. Adherence of *Acinetobacter* sp. H01-N to dodecane during batch growth on acetate in the presence of dodecane. A. (◆) fraction of adhering cells. B. (●) total biomass, (○) non-adhering fraction of biomass, error bars indicate the standard deviation.

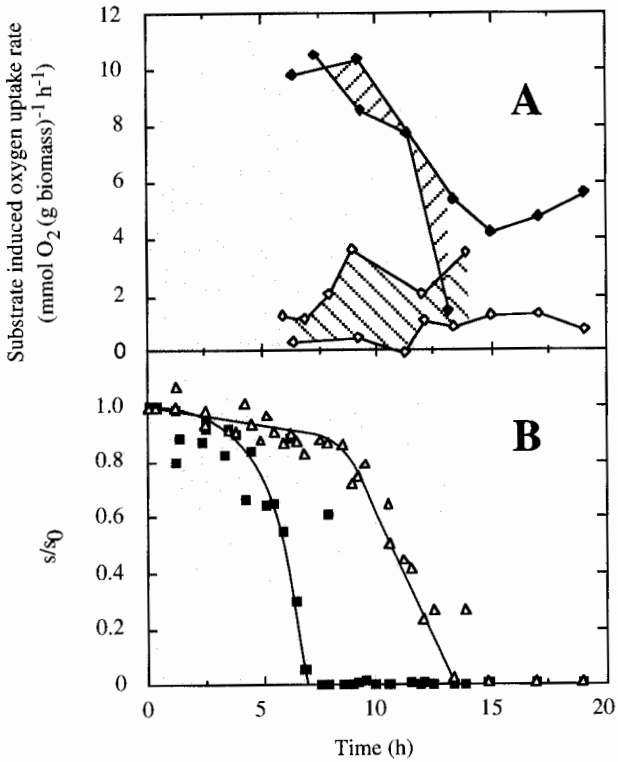

Figure 4.5. Substrate induced oxygen uptake rates during batch growth of *Acinetobacter* sp. H01-N on a mixture of acetate and dodecane. Grey areas indicate time periods when substrate consumption was observed. (A) Acetate (◆) and dodecane (◇) induced oxygen uptake rates. Data of two experiments are shown. Shaded areas indicate the range of measured data. (B) Corresponding residual acetate (■) and dodecane (△) concentrations.

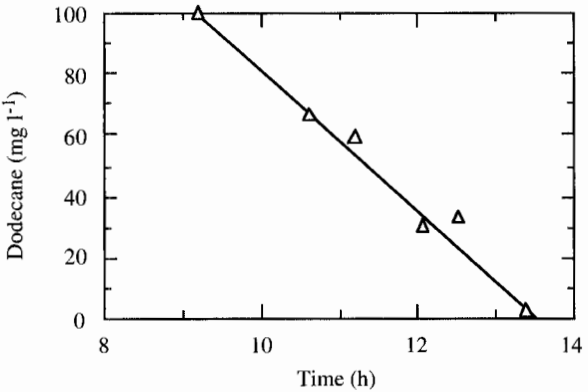

Figure 4.6. Dodecane concentration after the depletion of acetate during a typical batch experiment with *Acinetobacter* sp. H01-N growing on a mixture of acetate and dodecane. In the experiment shown, initial dodecane carbon was 0.12 g l^{-1}, and the average biomass concentration during degradation of dodecane was 0.15 g l^{-1}.

4.3.3. Growth of *Acinetobacter* sp. H01-N in carbon limited chemostat on acetate and dodecane

In order to more closely investigate the influence of different growth conditions on the utilization of acetate and dodecane by *Acinetobacter* sp. H01-N, growth experiments were performed in carbon limited chemostat, and steady-state cultures of this strain were analyzed.

Growth. During carbon-limited growth on a substrate mixture of dodecane and acetate (185 mg l^{-1} carbon each), low residual substrate concentrations of both substrates were observed at dilution rates below 0.3 h^{-1} which indicated simultaneous substrate utilization (Fig. 4.7). At a dilution rate of 0.3 h^{-1}, dodecane was not degraded. The residual acetate concentration remained below the detection limit at this dilution rate. Growth at higher dilution rates on a mixture of dodecane and acetate was not tested.

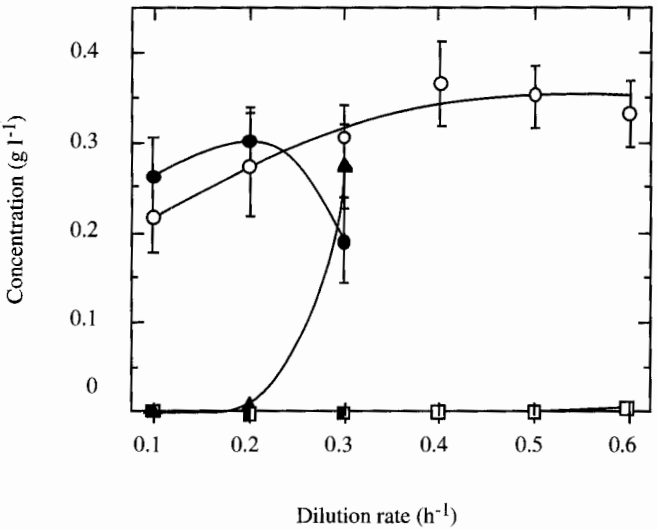

Figure 4.7. Growth of *Acinetobacter* sp. H01-N in carbon-limited continuous culture either on acetate or on a mixture of acetate and dodecane as a function of the dilution rate. Acetate (□) and dodecane (▲) concentrations. Biomass during growth on acetate (○) or on the substrate mixture (●).

During growth on the substrate mixture at a dilution rate of 0.1 h^{-1}, a biomass concentration of 0.27 g l^{-1} was measured (Table 4.5). If the experimentally determined growth yields during single substrate growth (Table 4.5) were additive during mixed substrate utilization, a value of 0.30 g l^{-1} would be expected. Within experimental uncertainties, this value was in agreement with the measured value.

Respiratory quotient. Since the degrees of reduction, ν,are different for acetate ($\nu_{Acetate} = 4.0$) and dodecane ($\nu_{dodecane} \approx 6.2$), decreasing respiratory quotients, RQ, would be expected in the order: growth on acetate, growth on the substrate mixture and growth on dodecane. This effect was indeed observed (Table 4.5). Measured RQ at a dilution rate of 0.1 h^{-1} were compared with a generalized equation for microbial growth (Table 4.6). Since carbon recovery from residual dodecane, carbon dioxide and biomass

was only 91% ± 2.2%, product formation was assumed in calculation of theoretical RQ for the missing carbon. For dodecane, measured and predicted values were in fair agreement (Table 4.6) Similar results were found for the substrate mixture (Table 4.6). Significant differences between predicted and measured RQ values were observed for acetate ($P>0.01$, n=6) at a dilution rate of 0.1 h^{-1} (Table 4.6). However, at increased dilution rates measured and predicted RQ were in agreement (approximately 1.05).

TABLE 4.5. Steady state growth of *Acinetobacter* sp. H01-N in carbon-limited chemostat at a dilution rate of 0.1 h^{-1}

Parameter	Unit	Carbon source[a]		
		Acetate	Dodecane	Mixture
acetate	g l^{-1}	b.d.[b]	-	b.d.[b]
dodecane	g l^{-1}	-	0.01	<0.01
biomass	g l^{-1}	0.22	0.39	0.27
$Y_{x/C}$	g g^{-1}	0.59	1.05	0.73
RQ	-	1.3	0.4	0.7
Max. O_2 uptake rate				
Acetate	mmol g^{-1} h^{-1}	12	4.7	7.8
Dodecane	mmol g^{-1} h^{-1}	1.7	8.0	4.7
Contact angle	°	36	64	56
ζ-potential	mV	-18	-24	-20

[a] total carbon concentration was 0.37 g l^{-1}, the substrate mixture consisted of 0.185 g l^{-1} acetate carbon and 0.185 g l^{-1} dodecane carbon.
[b] below detection limit

TABLE 4.6. Comparison of calculated and measured RQ values at a dilution rate of 0.1 h^{-1}

General equation[a]	$\cdot\ \gamma^{c}CH_{1.8}O_{0.5}N_{0.2}^{d} + \delta CO_2 + \varepsilon H_2O + \phi CH_cO_d^{e}$
Carbon balance	$1 = \gamma + \delta + \phi^{f}$
Electron balance[g]	$\nu substrate - 4\beta = \gamma\nu biomass + \phi\nu product$

Substrate	Relevant stochiometric coefficients		Respiratory quotient, RQ	
	β	δ	theor. ($=\delta/\beta$)	measured
Acetate	0.67	0.68	1.02	1.36 ± 0.30
Dodecane	0.83	0.34	0.41	0.38 ± 0.05
Mixture	0.69	0.47	0.68	0.72 ± 0.13

[a] General growth equation with product formation.
[b] acetate CH_2O; dodecane $CH_{2.17}$; mixture 0.5 CH_2O + 0.5 $CH_{2.17}$
[c] γ was determined experimentally
[d] biomass composition was assumed according to (Roels, 1983)
[e] dodecanoic acid ($C_{12}H_{24}O_2$) was assumed to be the organic product in the presence of dodecane, no product formation was assumed during growth on acetate (see General Introduction).
[f] Φ was assumed to be 0.1 in the presence of dodecane, and 0 during growth on acetate alone.
[g] $\nu_{acetate} = 4$, $\nu_{dodecane} \approx 6.12$, $\nu_{mixture} \approx 5.10$, $\nu_{biomass} \approx 4.2$, $\nu_{product} \approx 5.7$

Substrate induced oxygen uptake rates. At a dilution rate of 0.1 h^{-1}, the highest dodecane induced oxygen uptake rates were measured during growth on dodecane (Table 4.5). During growth on the substrate mixture, these rates were approximately 40% lower. In both of these cases, dodecane induced oxygen uptake rates were significantly ($P>0.05$, $n=3$) higher than in acetate grown cells. However, a small basal activity was still detectable in the absence of dodecane as a growth substrate.

At dilution rates between 0.1 h^{-1} and 0.2 h^{-1}, dodecane induced oxygen uptake rates were significantly ($P>0.05$, $n=3$) higher during growth on the substrate mixture compared to the basal level rate in acetate grown cells (Fig. 4.8B). At a dilution rate of 0.3 h^{-1}, the dodecane induced oxygen uptake rates were equal during growth on acetate alone and on the substrate mixture.

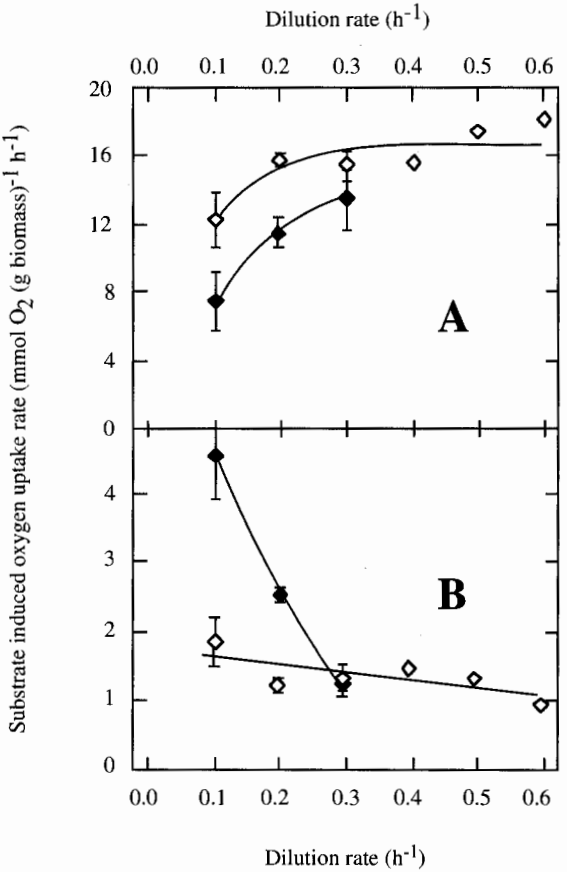

Figure 4.8. Substrate induced oxygen uptake rates of *Acinetobacter* sp. H01-N in continuous culture at different dilution rates. The strain was cultivated either with acetate (◇) or a mixture of acetate and dodecane (◆) as the carbon source. Substrate induced oxygen rates were measured with acetate (A), and dodecane (B).

Acetate induced oxygen uptake rates were also influenced by the composition of the carbon substrate mixture (Table 4.5). Acetate induced oxygen uptake rates increased with increasing dilution rates between 0.1 h^{-1} and 0.3 h^{-1} (Fig. 4.8A) with both acetate as the sole carbon source or in the mixture with dodecane. However, at dilution rates below 0.3 h^{-1}, acetate induced oxygen uptake rates were approximately 30% higher during growth on acetate alone. At a dilution rate of 0.3 h^{-1}, similar rates were observed in both cases ($P \leq 0.1$, n=3) (Fig. 4.8A). During growth on acetate, acetate induced oxygen uptake rates remained constant at an average of 17 (±1.3) mmol (g biomass)$^{-1}$ h^{-1} at higher growth rates.

Surface properties. Batch results indicted that *Acinetobacter* sp. H01-N exhibited different surface properties during growth on dodecane and on acetate. Therefore, the cell surface hydrophobicity and the net surface charge of this strain in relation to the used growth substrate were determined as a possible physiological response to growth on acetate and/or dodecane.

During growth on acetate alone, almost no influence of the dilution rate on either the contact angle nor on the ζ-potential was observed (Fig. 4.9A and B). The average contact angle was 36° for dilution rates between 0.1 h^{-1} and 0.5 h^{-1} (Fig. 4.9A). Only at a dilution rate of 0.6 h^{-1}, a slight increase of the contact angle was noted (40.8°), which coincided with the occurrence of wall growth. The ζ-potential of acetate grown cells was constant for all dilution rates tested at an average value of -17 mV (Fig. 4.9B). During growth on the substrate mixture, the contact angles decreased notably with increasing dilution rates (Fig. 4.9A). At a dilution rate of 0.1 h^{-1}, a contact angle of 56° was observed. A similar value was found with dodecane alone (64°). At a dilution rate of 0.3 h^{-1}, the contact angle was 36° which was equal to the contact angle on acetate as the sole carbon source. During growth on the substrate mixture, the ζ-potential remained constant for all dilution rates tested. The average ζ-potential of -20 mV was slightly below the value for growth on acetate alone.

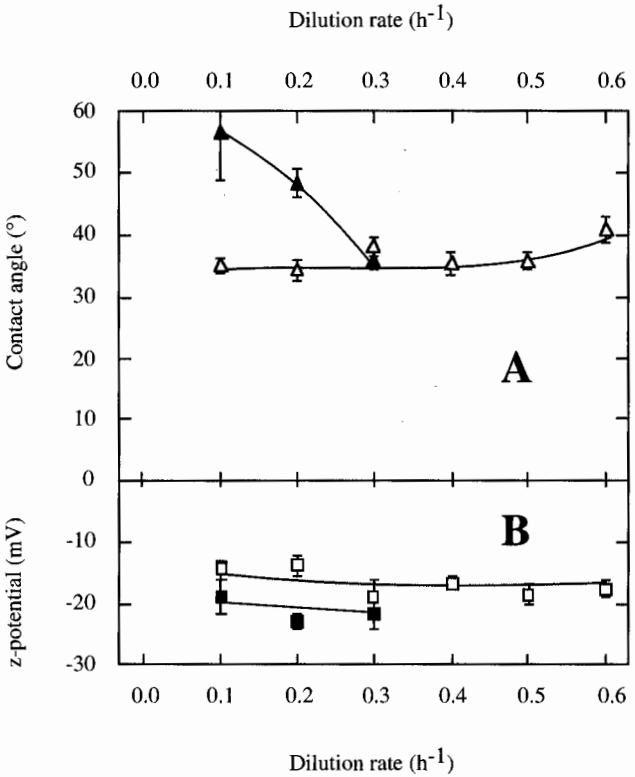

Figure 4.9. Surface properties of *Acinetobacter* sp. H01-N in continuous culture at different dilution rates. The strain was cultivated either with acetate (open symbols) or a mixture of acetate and dodecane (closed symbols) as the carbon source. Contact angles (A) and ζ-potential (B) are shown.

4.3.4. Dynamic behavior of *Acinetobacter* sp. in carbon limited continuous culture during growth on acetate, succinate and dodecane

In both natural and technical environments, steady state growth conditions are rare, and microorganisms constantly have to adapt to changing growth conditions. Therefore, the capacity of *Acinetbacter* sp. H01-N to switch growth from a non-hydrocarbon substrate to dodecane was investigated in continuous culture.

Transient growth of *Acinetobacter* sp. H01-N after a shift in carbon source from acetate to dodecane. Transient growth in carbon limited continuous culture was first investigated by replacing acetate with dodecane as the sole carbon source. The total carbon concentration in the medium feed was kept constant at 0.185 g l^{-1} during the entire experiment, and the dilution rate was kept at 0.1 h^{-1}.

During the first 2 h after the shift dodecane was washed in indicating that no degradation of dodecane occurred during this time (Fig. 4.10A). After 4 h, dodecane concentration peaked at 47 mg l^{-1} and decreased thereafter. The OD_{546} decreased slightly in the first two hours before biomass increased to a new steady state of 0.51 (OD_{546}) after 12 h. The observed increase in OD_{546} was in agreement with the growth yield for dodecane at a growth rate of 0.1 h^{-1} (Table 4.5).

Metabolic adaptation to the presence of dodecane as the new carbon source was followed by measuring substrate induced oxygen uptake rates with dodecane and acetate during transient growth (Fig. 4.10B). After an initial low rate, the dodecane induced oxygen uptake rate started to increase after 2 h, reaching 81% of the maximum of 7.8 mmol O_2 (g biomass)$^{-1}$ h^{-1} after 6 h. The acetate induced oxygen uptake rate decreased immediately after the shift. Initially, the decrease was faster than predicted by wash-out, reaching however a constant level after 12 h (Fig. 4.10B). Adaptation to the new substrate was also reflected by a change in respiratory quotient and the specific gas exchange rates for oxygen and carbon dioxide (not shown).

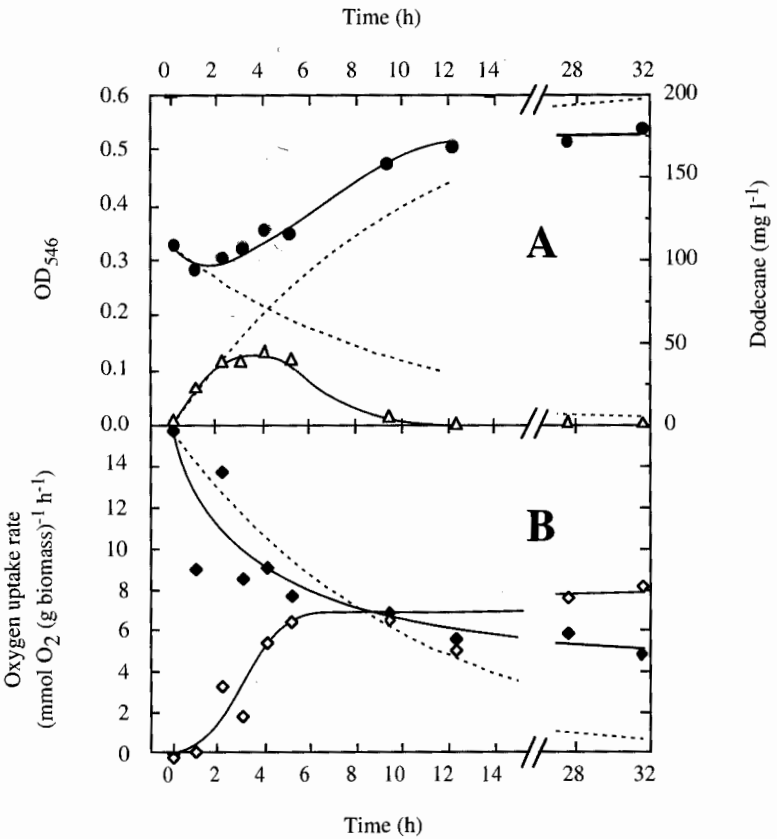

Figure 4.10. Transient growth *Acinetobacter* sp. H01-N after switching the carbon source from acetate as the sole carbon source to dodecane during carbon limited continuous culture at a dilution rate of 0.1 h^{-1}. A. OD546 (●) and residual dodecane concentration (Δ); (dotted lines) theoretical wash-in of dodecane and wash-out of biomass respectively. B. Acetate (◆) and dodecane (◇) induced oxygen uptake rates; (dotted line) theoretical wash-out curve of the actate induced oxygen uptake activity.

Transient growth of *Acinetobacter* sp. H01-N after a shift in carbon source from acetate to a mixture of acetate and dodecane. Changes in cell activitiy as a result of the addition of a second substrate were investigated by adding dodecane to a steady-state culture growing on acetate (acetate and dodecane concentrations both at 0.185 (g carbon) l^{-1}). With acetate still present, the residual dodecane concentrations remained below the theoretical wash-in curve during the first 4 h and decreased again to 2 mg l^{-1} after 72 h (Fig. 4.11). The acetate concentrations remained below detection limit of approximately 2 mg l^{-1} during the entire experiment (not shown).

After a short lag of 2 h, the biomass concentrations (measured as OD_{546}) increased exponentially with a specific growth rate of 0.25 h^{-1}. This rate was identical with the maximum specific growth rate, μ_{max}, for *Acinetobacter* sp. H01-N on dodecane. After 72 h, 92 % of the expected biomass concentration according to the yield on the substrate mixture was reached.

After addition of dodecane as an additional carbon substrate, dodecane induced oxygen uptake rates increased immediately (Fig. 4.11B). A plateau of 4.4 (± 0.9) mmol O_2 (g biomass)$^{-1}$ h^{-1} was reached after 4 h. This plateau coincided with the expected steady state value of 4.6 (± 0.6) mmol O_2 (g biomass)$^{-1}$ h^{-1} (Fig. 4.8). The acetate induced oxygen uptake rates decreased only very little from 12.2 mmol O_2 (g biomass)$^{-1}$ h^{-1} before the shift to 10 mmol O_2 (g biomass)$^{-1}$ h^{-1} after 72 h (Fig. 4.11B). This value was higher than the steady state value of 7.6 (± 1.5) mmol O_2 (g biomass)$^{-1}$ h^{-1} determined during carbon limited growth on a mixture of acetate and dodecane (Fig. 4.8A).

Transient growth of *Acinetobacter* sp. H01-N after a shift in carbon source from succinate to dodecane. Because the presence or absence of acetate had an influence on the adaptation of a continuous culture to dodecane utilization, experiments were carried out with succinate in place of acetate to see whether the non-hydrocarbon substrate played a role in this adaptive process.

After replacing succinate by dodecane in carbon limited steady state culture, the hydrocarbon concentration increased steadily but remained below the wash-in curve (Fig. 4.12A). Some wash-out of the culture was apparent for the first 2 h after the shift. Then biomass stabilized. The value measured after 32 h represented approximately 80 % of the expected OD_{546} according to the growth yield on dodecane at a dilution rate of 0.1 h^{-1}.

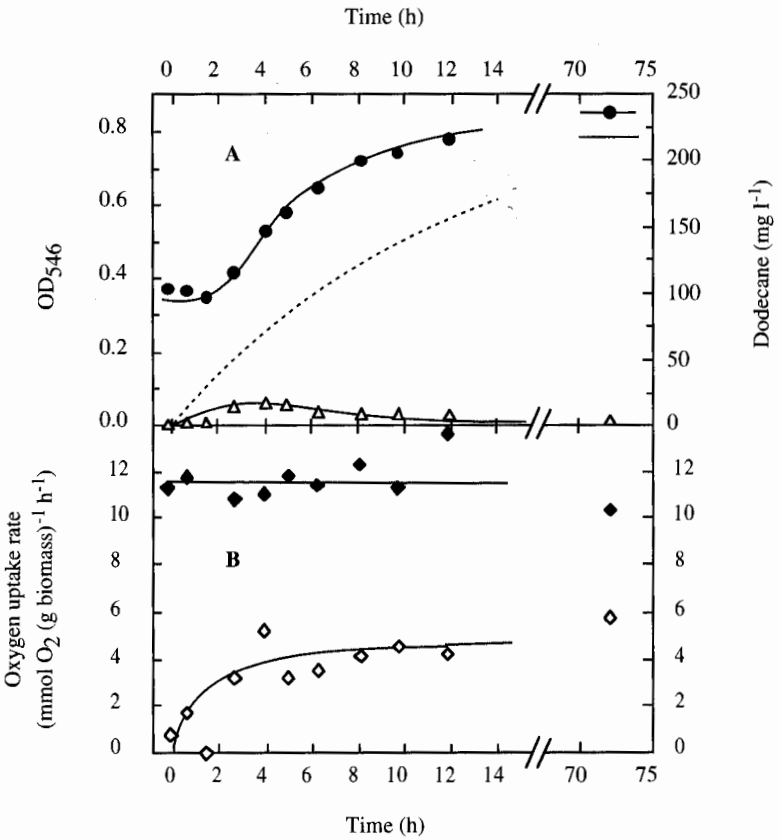

Figure 4.11. Transient growth *Acinetobacter* sp. H01-N after a shift in carbon source from acetate as the sole carbon source to a mixture of acetate and dodecane during carbon limited continuous culture at a dilution rate of 0.1 h^{-1}. A. OD_{546} (●) and residual dodecane concentration (Δ); (dotted line) theoretical wash-in curve for dodecane. B. Acetate (◆) and dodecane (◇) induced oxygen uptake rates.

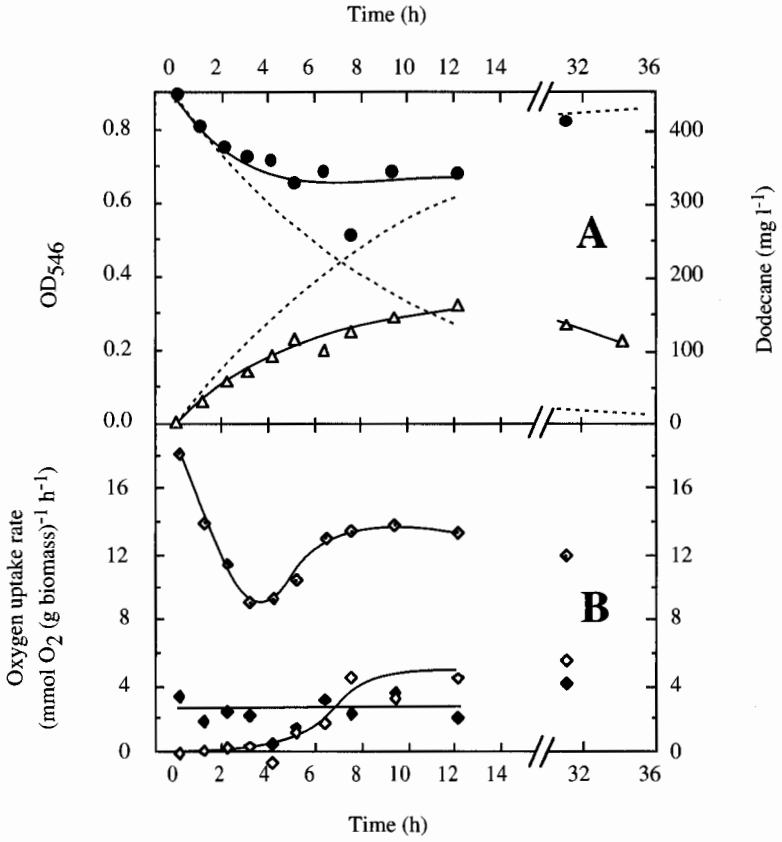

Figure 4.12. Transient growth *Acinetobacter* sp. H01-N after a shift in carbon source from succinate to dodecane as the sole carbon source during carbon limited continuous culture at a dilution rate of 0.1 h^{-1}. A. OD_{546} (●) and dodecane concentration (Δ); (dotted lines) theoretical wash-in of dodecane and wash-out of biomass respectively. B. Succinate (◈), acetate (◆) and dodecane (◇) induced oxygen uptake rates.

The dodecane induced oxygen uptake rate increased only after about 5 h and reached a steady-state of 5.8 mmol O_2 (g biomass)$^{-1}$ h^{-1} after 8 h (Fig. 4.12B). This value is below typical steady state uptake rates for dodecane grown cells at a dilution rate of 0.1 h^{-1} (Table 4.5). The acetate induced oxygen uptake rate remained throughout the entire experiment below typical values for growth on acetate (15 mmol O_2 (g biomass)$^{-1}$ h^{-1}) or dodecane (4.7 mmol O_2 (g biomass)$^{-1}$ h^{-1}) (Table 4.5 and Fig. 4.12B). The succinate induced oxygen uptake rate transiently decreased and became nearly stable after 8 to 10 h (Fig. 4.12B). After 30 h, a final value of 11.9 mmol O_2 (g biomass)$^{-1}$ h^{-1} was measured that was above the expected steady state value of 10 mmol O_2 (g biomass)$^{-1}$ h^{-1} (not shown).

Response time of *Acinetobacter* sp. during transient growth after different shifts in carbon source. The response time necessary for the dodecane induced oxygen uptake rate to increase above the background was different after each type of shift (Fig. 4.13). When a shift from acetate to the substrate mixture of dodecane and acetate was performed, dodecane induced oxygen uptake rate started to increase within less than 1 h. When a non-hydrocarbon substrate was replaced by dodecane as the sole carbon source, longer time periods were observed before dodecane induced oxygen uptake rates were detectable. After the shift from succinate to dodecane, the longest response time of nearly 6 h was observed.

Different response times were possibly caused by different requirements for intracellular compounds that are utilized for the redirection of metabolism. Endogenous oxygen uptake rates maybe a good measure for this kind of biochemical activities. For a shift from acetate to a mixture of acetate and dodecane, only minor changes in the endogenous oxygen uptake rate were observed during transient growth (Fig. 4.14A). In contrast, when dodecane alone was used after the shift, the endogenous uptake rates changed considerably (Fig. 4.14B and C). Maximal endogenous oxygen uptake rates of 5.5 mmol O_2 (g biomass)$^{-1}$ h^{-1} were observed in these cases, which indicated substantial biosynthetic activity. After one volume change, endogenous oxygen uptake rates returned to low and constant values of 1.9 (±0.3) mmol O_2 (g biomass)$^{-1}$ h^{-1} in all cases tested.

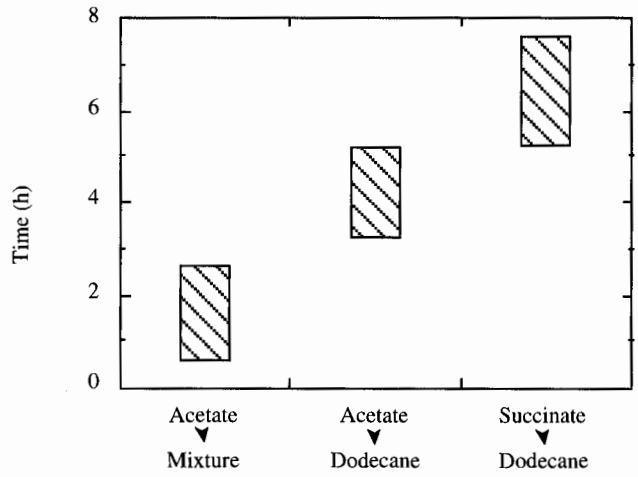

Figure 4.13. Response time of *Acinetobacter* sp. H01-N to the presence of dodecane during transient growth at a dilution rate of 0.1 h^{-1}. Shifts were performed from acetate to dodecane, from acetate to a mixture of dodecane and acetate, and from succinate to dodecane. Symbols: Time periods during which major increases in dodecane induced oxygen uptake rates occured.

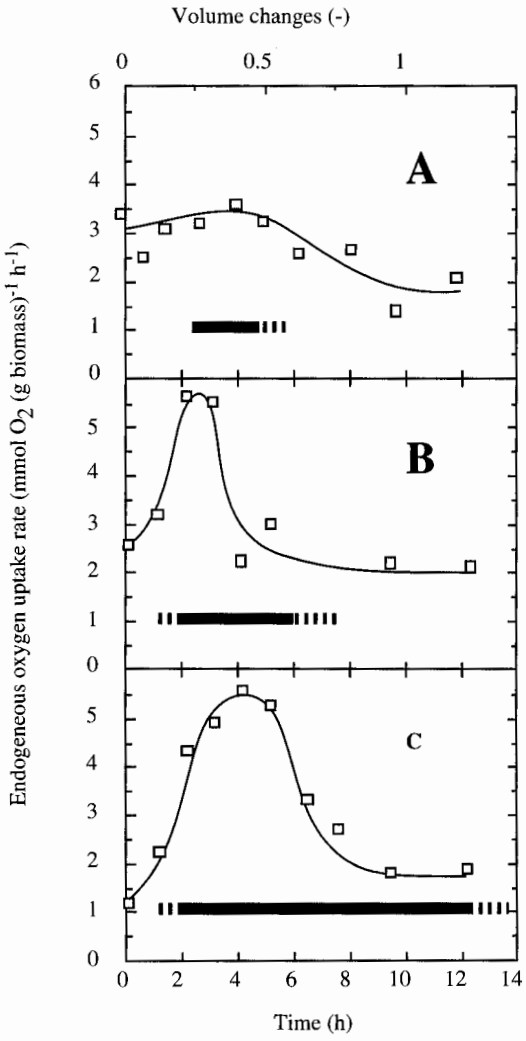

Figure 4.14. Endogenous oxygen uptake rates during transient growth of *Acinetobacter* sp. H01-N at a dilution rate of 0.1 h^{-1}. Shifts were performed from acetate to a mixture of acetate and dodecane (A), acetate to dodecane (B), and succinate to dodecane (C). Symbols: endogeneous oxygen uptake rate(□), bars indicate the presence of high residual dodecane concentrations during the shift (■).

4.5. DISCUSSION

Results showed that *Acinetobacter* sp. H01-N utilized substrate mixtures of acetate and dodecane sequentially when grown in batch culture under carbon excess conditions. In contrast, during carbon limited growth in continuous culture, these substrates were utilized simultaneously at low dilution rates. This strain was able to rapidly adapt to the presence of dodecane in carbon limited chemostat culture at low dilution rates. Results also showed that enhanced cell surface hydrophobicity, which was required for alkane uptake, was measured at high dodecane induced oxygen uptake rates.

4.5.1. Utilization of dodecane is repressed by acetate during batch growth

Regulation of enzyme synthesis. *Acinetobacter* sp. H01-N pregrown on dodecane first utilized acetate during batch growth on mixtures of acetate and dodecane while utilization of dodecane was repressed during growth on acetate. This substrate preference correlated with the results by Kennedy et al. (Kennedy and Finnerty, 1975) for the same organism pregrown on acetate for batch growth on a mixture of hexadecane and acetate. Although details of the regulation mechanism for repression of alkane metabolism by acetate is not yet known, the occurrence of a lag phase after depletion of acetate indicated that alkane metabolism was regulated at the level of enzyme synthesis (Harder, 1982). Since alkanes freely penetrate cellular membranes (Sikkema et al., 1995), it was likely that dodecane catabolic genes were repressed directly by catabolite repression rather than regulation by inducer exclusion. Such catabolite repression has been shown for an alkane degrading *Pseudomonas aerugionsa*, where tetradecane utilization was repressed by glucose and glucose-6-P (Dalhoff and Rehm, 1976).

After depletion of acetate, acetate catabolic enzyme activity decreased as indicated by decreasing acetate induced oxygen uptake rates. This decrease proceeded at an initial rate of 10 to 13 % per hour, which approximates to the turnover rate of cellular protein during lag phase (4 to 7% per hour) (Goldberg and St. John, 1976). During subsequent dodecane utilization, acetate induced oxygen uptake rates remained low. Since complete utilization of dodecane involves acetyl-CoA as an intermediate, either acetate uptake was less active during dodecane utilization or less carbon was metabolized via acetyl-CoA when dodecane was the carbon source.

Unusual secondary growth phase. A striking observation was that no biomass was formed during dodecane utilization. The first-order degradation kinetics of dodecane indicated nevertheless that enzymatic transformation of dodecane was taking place. Accumulation of intermediary products during early stages of batch growth has been described before (Bitzi, 1986). However, the absence of growth even after complete degradation of dodecane suggested accumulation of an end-product of alkane metabolism rather than an intermediary product. Although not measured here, it is known that *Acinetobacter* sp. H01-N produces wax-esters during growth on hydrocarbon substrates (Geigert et al., 1984). Usually, such wax-esters accumulate under carbon excess growth conditions as reserve material, and are used during periodes of starvation. Some wax esters are dead-end products (Singer et al., 1985). For example, recalictrant wax esters were formed during growth of *Acinetobacter* sp. H01-N on hexadecanol, a putative intermediate during hexadecane metabolism (Singer et al., 1985). It is therefore conceivable that intermediary accumulation of dodecanol in *Acinetobacter* sp. H01-N may favor production of the corresponding wax ester. Alternatively, dodecanoic acid may have accumulated, which then may have precipitated as a Ca-salt (T. Egli, personal communication). In contrast, under carbon limited conditions, growth proceeded rapidly after shifts from acetate to dodecane with the corresponding formation of biomass. Since accumulation of intermediary products appears unlikely under such conditions, these results are not in disagreement with the postulated product formation during diauxic batch experiments.

These results were in marked contrast to the previous results by Kennedy et al. (Kennedy and Finnerty, 1975) who observed "normal" diauxic growth on a mixture of acetate and hexadecane with formation of biomass during hexadecane utilization. By comparing alkane induced oxygen uptake rates, no clear difference in alkane metabolism was apparent between the two experiments. Kennedy et al. measured hexadecane induced oxygen uptake rates between 13 and 23 mmoles h^{-1} g^{-1} during growth on hexadecane in diauxic batch experiments (Kennedy and Finnerty, 1975). By correcting these data for the observed differences in maximum specific growth rates on the two alkane substrates and the different amount of oxygen required to oxidize one molecule of dodecane or hexadecane, dodecane induced oxygen uptake rates between 4 and 7 mmoles h^{-1} g^{-1} would be expected during exponential growth. The measured dodecane induced oxygen uptake rate remained below 4 mmoles h^{-1} g^{-1} during the entire experiment. It is clear though that this bulk parameter is not sufficiently accurate for the detailed analysis of metabolic routes, and more information at the molecular level is required. Furthermore, the influence of both alkane substrate and culture history

on diauxic growth of *Acinetobacter* sp. H01-N in general and on synthesis and activity of relevant dodecane catabolic enzymes needs more attention.

4.5.2. Simultaneous utilization of acetate and dodecane at low dilution rates

Whereas sequential utilization of acetate and dodecane was observed during batch growth, simultaneous utilization of these carbon sources was apparent at low dilution rates in carbon limited chemostat cultures. This observation was in agreement with experimental evidence from other systems where, under carbon limited growth conditions, simultaneous utilization of carbon sources is the rule rather than the exception (see for example (Egli et al., 1993; Harder, 1982)).

Growth yield. Growth yields on the substrate mixture were additive during mixed substrate utilization. Both additive and non-additive behavior has been observed before (Gommers et al., 1988). Often, combination of a carbon-limited growth substrate and an energy-limited growth substrate results in increased yields during mixed substrate growth (Dijkhuizen and Harder, 1979; Müller et al., 1983; Müller et al., 1988). Under such conditions, the energy limited substrate may serve as a carbon source only and the carbon limited substrate as an energy source. In such substrate mixtures, the carbon source is assimilated more efficiently which leads to increased yield coefficients during growth on the substrate mixture.

Carbon limited substrates are defined as substrates where the energy gain during assimilation is sufficient for growth processes (Gommers et al., 1988). From a chemical point of view, substrates with a degree of reduction above 5 can theoretically be carbon limited (Gommers et al., 1988). Therefore, dodecane (degree of reduction (v) $\approx$ 6.2) is potentially carbon limited, whereas acetate (v = 4.0) is energy limited. However, from this simple calculation it is not clear whether the energy in dodecane is available biochemically. Dodecane is assimilated via acetyl-CoA and 3-phospoglycerate. A P/O coefficient of 2 or higher is required for the energy gain from this assimilative pathway to be sufficient for growth (Müller and Babel, 1988). However, during growth on hexadecane, a P/O coefficient of 0.2 was measured in *Acinetobacter* sp. H01-N (Ensley et al., 1981). Indeed, the theoretically calculated yield according to this low P/O coefficient (Roels, 1980) was in fair agreement with the experimental data for growth on dodecane, indicating that hexadecane was energy limited. This assumption was supported by the observed yield coefficients. If only assimilation of dodecane occurred, 29 % of dodecane carbon would be lost due to assimilative decarboxylation, and, 50

mM of carbon would be required for the production of 1 g of bacterial biomass (Gommers et al., 1988). Experimentally, it was found that 72 to 79 mM of dodecane carbon were required for the production of 1 gram of biomass. This difference could not be explained by incomplete carbon recovery, since at least 65 mM of substrate carbon were detected in carbon dioxide and biomass. This implies that dodecane was not assimilated completely and, consequently, that this compound was energy limited. Therefore, both carbon sources were energy limited substrates, which is consistent with the observed additive behavior of the growth yields. However, despite the observation that both substrates were probably used simultaneously as a carbon and energy source, it might still be that acetate and dodecane were at least partially channelled through other pathways during growth on the substrate mixture compared to growth on the individual carbon sources. For example, during growth on the substrate mixture, fatty acid synthesis from acetate may be repressed (Gill and Ratledge, 1973; Rehm et al., 1983; Sampson and Finnerty, 1974). For the final proof of such effects, either experiments with labeled substrates or suitable assays for key enzymes woud be required.

Maximal dilution rate at which simultaneous utilization occurred. Simultaneous utilization of acetate and dodecane occurred at dilution rates below 0.3 h^{-1}. At a dilution rate of 0.3 h^{-1}, wash-in of dodecane was observed. In agreement with this observation was the fact that dodecane induced oxygen uptake rates in "acetate/dodecane" grown cells were at the same level as in acetate grown cells at a dilution rate of 0.3 h^{-1}. As shown by a D-shift experiment where the dilution rate was increased from 0.1 h^{-1} to 0.3 h^{-1} during growth on the substrate mixture, repression of dodecane catabolic enzymes occurred immediately after the shift (results not shown). In contrast to the observation in many other systems (Brinkmann and Babel, 1992; Dijkhuizen and Harder, 1979; Eggeling and Sahm, 1981; Egli et al., 1986; Kovárová, 1996), simultaneous utilization of dodecane and acetate was therefore not possible above the maximum specific growth rate due to repression of dodecane enzymes at higher dilution rates.

Influence of the dilution rate on enzyme activities. During growth on the substrate mixture, dodecane induced oxygen uptake rates decreased with increasing dilution rates for all dilution rates tested. Such repression may either be due to the presence of higher acetate concentrations at higher dilution rates or due to global regulation of cellular metabolism (Harder, 1982). Indeed, similar patterns were also observed for succinate induced oxygen uptake rates during growth on acetate or on the substrate mixture which indicated a general regulation mechanism since succinate utilization was not repressed by acetate (not shown). Such repression of catabolic enzymes at higher dilution rates

has also been observed in several other microbial systems during growth on both substrate mixtures and single substrates (see for example (Bally, 1994; Eggeling and Sahm, 1981; Egli et al., 1982), for review see (Harder, 1982)). In contrast, an atypical behavior was observed for acetate catabolic enzymes as indicated by acetate induced oxygen uptake rates. These enzymes increased with increasing dilution rates between 0.1 and 0.3 h^{-1} and remained constant at higher dilution rates.

Influence of the carbon source on enzyme activities. As indicated by the substrate induced oxygen uptake rates, enzyme activities in *Acinetobacter* sp. H01-N were dependent on the relative fluxes of each substrate. During growth on the substrate mixture, the relative flux of each substrate was half of the value of the total substrate flux, and enzyme activities of both acetate and dodecane metabolism were regulated respectively. Similarly, Bally (Bally, 1994) observed that the expression of NTA-dehydrogenase was dependent on the mixture composition of NTA and glucose in carbon limited chemostat. In an earlier work, Egli and co-workers (Egli et al., 1982) observed that methanol catabolic enzymes in the yeast *H. polymorpha* were dependent on the mixture composition of glucose and methanol during carbon limited growth.

Although repression of alkane catabolic enzymes by acetate was overcome during carbon limited growth at low dilution rates, dodecane induced oxygen uptake rates were only slightly above background during carbon limited growth in the absence of dodecane. This observation further established that dodecane degradation was an inducible process, which is consistent with previous results (Finnerty, 1990; Fox et al., 1992). Although the mechanism and regulation of the initial step in alkane metabolism have not yet been fully elucidated, it has been shown that long-chain aldehydes, which are putative intermediates of alkane metabolism, are oxidized by inducible deghydrogenases (Fox et al., 1992).

Constitutive levels of acetate induced oxygen uptake rates remained relatively high even during growth on dodecane as the only carbon source. This was consistent with the observation that acetate utilization occurred without lag during batch cultivation with dodecane pre-grown cells. As in other *Acinetobacter* sp. (Kleber and Aurich, 1973), both acetate and dodecane are probably metabolized via acetyl-CoA with both glyoxylate and the tricarboxilic acid cycle being active (Kleber and Göbel, 1975; Whitworth and Ratledge, 1975). Therefore, one would expect the activity of the enzymes of these pathways to be high during growth on dodecane. It was somewhat surprising that acetate induced oxygen uptake rates were reduced at all during growth on the substrate mixture or on dodecane alone. Such a reduction of acetate induced

oxygen uptake rates was also observed after depletion of acetate during mixed substrate batch growth. This observation suggests that the maximum specific acetate uptake rate was regulated by the relative acetate flux.

4.5.3. Transient growth

Acinetobacter sp. H01-N adapted rapidly to growth on dodecane. For shift experiments where dodecane was the only carbon source after the shift, a period of 3 to 6 h (0.3 to 0.6 volume changes) was required before induction of dodecane induced oxygen uptake started to increase. The observed pattern of biomass increase and substrate utilization during such shifts was typical for transient growth which required an induction process, and similar results have been observed previously (Bally and Egli, 1996; Standing et al., 1972). However, compared to other substrate mixtures, shifts from acetate to dodecane proceeded relatively fast. Standing et al. (Standing et al., 1972), for example, performed transient growth experiments from glucose to xylose at a dilution rate of 0.1 h^{-1}. As indicated by biomass concentrations, they observed a recovery time of the culture of approximately 2.5 volume changes after the shift. This was about twice the time that was observed here for shifts from acetate to dodecane.

The response time after shifts in carbon source was dependent on the carbon source that was used before the shift, and on the presence of an additional carbon source during transient growth, which has been observed for other microbial systems as well (Bally and Egli, 1996; Spånning and Neujahr, 1991). Shifts from succinate to dodecane were more difficult to achieve than shifts from acetate to dodecane. This was indicated by the prolonged time until dodecane induced oxygen uptake rates increased, and by longer recovery times of the culture after shifts from succinate to dodecane. Also, most pronounced maxima of the endogenous oxygen uptake rates were observed during transient growth after shifts from succinate to dodecane, which implied that more severe redirections of the intracellular mechanism were required before *Acinetobacter* sp. H01-N was able to degrade dodecane. Unlike with acetate as the non-hydrocarbon substrate, switches from succinate to dodecane required induction of the gyloxylate cycle. However, since only two enzymes are needed for this cycle (isocitrate lyase and malate synthetase) it seems likely that induction of the oxygenase system also influences the adaptation process and may add to the observed differences in shift responses.

The results also showed that *Acinetobacter* sp. H01-N responded faster to the presence of dodecane, when acetate was present during transient growth. The response time decreased seven-fold from 4.5 h to 0.5 h when acetate was present during the

whole experiment compared to shifts from acetate to dodecane as the only carbon source. In this case, endogenous oxygen uptake rates showed only minor changes during transient growth which indicated that acetate was used for carbon and energy source to synthesise dodecane enzymes rather than use of intracellular compounds. Bally et al. (Bally and Egli, 1996) found for *Chelatobacter heintzii* a correlation between the time period that was required before induction of NTA degradation started and the fraction of glucose in glucose/NTA mixtures during carbon limited growth on glucose and NTA, where lower fractions of glucose led to an increased adaptation time.

4.5.4. Physiological response to the presence of dodecane

Cell surface hydrophobicity during carbon limited growth at different dilution rates exhibited a similar pattern as the catabolic dodecane enzymes. Additionally, in a similar manner to the dodecane induced oxygen uptake rate, cell surface hydrophobicity seemed to be regulated by the relative flux of dodecane at a fixed dilution rate of 0.1 h^{-1}. These results further established that regulation of both alkane metabolism and physiological changes were tightly connected. Such physiological changes were not only apparent during carbon limited growth. During late exponential batch growth on acetate in the presence of dodecane, only a minor fraction of cells was bound to dodecane droplets, whereas during batch growth on dodecane, more than 90% of the cells were bound to dodecane (see Chapter 2). Therefore, as with dodecane metabolism, morphological and physiological changes were repressed by high concentrations of acetate. This was also observed by Kennedy et al. (Kennedy and Finnerty, 1975), who showed that hexadecane inclusion and intracytoplasmic membranes, which are physiological adaptations of this strain to growth on alkanes, were absent during batch growth on acetate in the presence of hexadecane.

Both these results and the results by Kennedy et al. (Kennedy and Finnerty, 1975) clearly indicated a strong interdependency between alkane degradation and certain morphological and physiological adaptations. However, it has not yet been determined whether all of these changes are required for growth on alkanes. It would be interesting in this context to develop mutations of *Acinetobacter* sp. H01-N that exhibit low surface hydrophobicities and to test whether these cells can still grow on alkanes. It is also of interest whether mutants that are blocked in alkane metabolism are able to become hydrophobic.

4.5.5. Concluding remarks

Alkane degrading microorganisms are present in virtually any environment where they have been searched for (Blackburn et al., 1993). Consequently, one would expect that alkane degradation is limited by abiotic factors (Atlas, 1981; Atlas, 1995) and by the ability of specialist strains to degrade alkanes under environmental growth conditions. Generally, catabolic genes for xenobiotics are organized in operons and are inducible (Sayler et al., 1990). Experimental data showed that alkane degradation in *Acinetobacter* sp. H01-N was dependent on the presence of dodecane, and was thus inducible, as is alkane degradation in other alkane degrading species (Dalhoff and Rehm, 1976; Eggink et al., 1988; Grund et al., 1975). Therefore, biodegradation of such compounds involves induction of relevant enzymes. Important questions that arise are how fast this induction process proceeds and how it is influenced by other carbon sources. For the system tested here, fast induction was observed that was even accelerated by the presence of other carbon sources. Since "natural" carbon sources are typically present at very low concentrations (Münster, 1993), one would expect that similar utilization pattern of alkanes and natural carbon sources are possible under natural conditions as was shown for the laboratory system used here. The relative ease at which alkanes are degraded in many natural environments seems to support this assumption (Al-Hadhrami et al., 1995; Blackburn et al., 1993; Sugiura et al., 1997; Whyte et al., 1996).

CHAPTER 5 Development and Characterization of a Whole Cell Bioluminescent Sensor for Bioavailable Middle-chain Alkanes in Contaminated Groundwater Samples

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A microbial whole cell biosensor was developed and characterized for its potential to measure water-dissolved concentrations of middle-chain length alkanes and some related compounds by use of bioluminescence. The biosensor strain E. coli DH5α (pGEc74, pJAMA7) carried the regulatory gene alkS from Pseudomonas oleovorans and a transcriptional fusion of P_{alkB} from the same strain with the promoterless luciferase genes luxAB from Vibrio harveyi on two separately introduced plasmids. In standardized assays, the biosensor cells were readily inducible with octane, a typical inducer of the alk system. The light emission after induction periods of more than 15 min correlated well with the octane concentration. In well-defined aqueous samples, a linear relationship between light output and the octane concentration for octane concentrations between 24 nM and 100 nM was found. The biosensor responded to middle-chain alkanes, but neither to alicyclic nor to aromatic compounds. In order to test its applicability to analyze environmentally relevant samples, the biosensor was used to detect the bioavailable concentration of alkanes in heating oil contaminated groundwater samples. By extrapolating calibrated light output data to low octane concentrations using a hyperbolic function, a total inducer concentration of about 3 nM in octane equivalents could be

estimated. The whole cell biosensor tended to underestimate the alkane concentration in the groundwater samples by about 25%, possibly due to the presence of unknown inhibitors. This was corrected for by spiking the samples with a known amount of an octane standard. Biosensor measurements of alkane concentrations were further verified by comparison with chemical analyses.

5.1. INTRODUCTION

Despite the fact that many contaminants are readily biodegradable, they often persist in the environment or are degraded at rates too slow for efficient cleanup. One major factor that limits biodegradation in the environment is insufficient availability of the pollutants for microbial attack. This is especially striking for hydrophobic compounds, such as those occurring in diesel oil contamination. Diesel oil consists mostly of linear and branched alkanes with different chain lengths and, in addition, contains a variety of aromatic compounds. Many of these compounds, especially linear alkanes, are known to be easily biodegradable (Watkinson and Morgan, 1990). Due to their low water-solubility, however, their biodegradation is often limited by the rates of dissolution, desorption or transport (Geerdink et al., 1996; Miller and Bartha, 1989). The bioavailability of hydrophobic compounds in general is determined by their sorption characteristics (Geerdink et al., 1996; Guha and Jaffe, 1996; McGroddy et al., 1996; Mihelic et al., 1993; Pignatello and Xing, 1996), dissolution or partitioning rates (Efroymson and Alexander, 1991; Guha and Jaffe, 1996; Stucki and Alexander, 1987; Thomas et al., 1986), and by transport processes to the microbial cell (Geerdink et al., 1996; Harms, 1996).

Microorganisms themselves can be applied as specific and sensitive devices for sensing the bioavailability of a particular pollutant or pollutant class. This is based on the properties of pollutants (as for most "normal" compounds) to invoke non-specific (e.g. toxicity or stress) or specific responses (e.g. activation of a degradative pathway) in microorganisms. The signalling pathway thus activated will regulate gene expression of one or more (sets of) genes. The extent of this gene expression is then a measure for the available ("sensed") concentration of the compound.

A rapid and sensitive way to measure such gene expression can be achieved by creating a gene fusion of the relevant promoter sequences and promoterless reporter genes such as those for bacterial luciferases of *Vibrio* spp. ((Engebrecht et al., 1985), for recent reviews see (Steinberg, 1995; Stewart, 1990)). Researchers who have used bacterial luciferase as a gene reporter have either applied the complete *luxCDABE*-operon or the *luxAB*-genes for the two subunits of luciferase only. Fusions with *luxAB* require the addition of a long-chain aliphatic aldehyde, preferably decanal, as a substrate for the luciferase reaction, whereas *luxCDABE* fusions intrinsically produce and regenerate the aldehyde substrate. The approach to use microbes to sense and report the presence of chemical compounds has recently gained great interest. Whole cell biosensors were developed that could detect naphthalene and salicylate (Heitzer et al., 1994; Heitzer et al., 1992), toluene, (Burlage et al., 1994) and mercury (Selifonova et al., 1993). A fusion of the *lux* genes and the regulatory elements of the isopropylbenzene catabolism operon was used to detect various hydrophobic pollutants such as alkylbenzenes and several other aromatic and aliphatic hydrocarbons (Selifonova and Eaton, 1996). Other biosensor strains were constructed to detect toxic compounds in general by coupling the *lux* genes with a stress inducible promoter (van Dyk et al., 1994).

Unfortunately, the light emission signal measured from whole cell biosensors is not only dependent on the available concentration of the inducing substance, but also on the stability of the luciferase in this particular strain (Korpela et al., 1989), the strain's physiological state, (Heitzer et al., 1992) and the presence of other stimulating or inhibitory substances in the sample to be measured (Burlage et al., 1994). The stability of the signal and the physiological state of the biosensor cells can be reasonably well controlled by optimized and standardized assay conditions. The sample composition is more difficult to control and can lead to false-positive or false-negative measurements (Burlage et al., 1994). Misinterpretation of false-positive results, i.e. partial or full induction of the reporter without its cognate inducer can be avoided by a clear understanding of the biosensor specificity and knowledge of potential other inducers in typical pollutant mixtures. False-negative results, i.e. partial or full inhibition of the signal expected from a particular concentration of inducer are mainly due to toxic compounds in the sample. They can be accounted for by suitable control experiments, such as spiking samples with a known amount of cognate inducer (Burlage et al., 1994) or by applying an additional, constitutively expressed reporter system to control the biosensor activity (Heitzer et al., 1992; Wood and Gruber, 1996).

Here we describe the construction of an *E. coli* whole cell biosensor for the detection of linear alkanes by creating a transcriptional fusion between the *alkB* promoter of

Pseudomonas oleovorans and the promotorless *luxAB* genes of *Vibrio harveyi*. In addition, the strain contained the gene for AlkS, which is the transcriptional activator of the *alkB* promoter (Eggink et al., 1988). The performance of the biosensor to detect available concentrations of octane was carefully analyzed in optimized and standardized assays. Furthermore, the response of the sensor to other possible inducers and potential inhibitors was tested. Finally, we describe application of the sensor to measure the bioavailable concentration of linear alkanes in oil-contaminated groundwater samples. The usefulness of such strains to analyze bioavailability of pollutants in the environment is discussed.

5.2. MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* DH5α (Sambrook et al., 1989) was used for routine cloning experiments with plasmids. The plasmids used are listed in Table 5.1. Plasmid pGEM7Zf(+) (Promega Corporation, Madison, Wis.) was used as a general cloning vehicle. Plasmid pUC18Not (Herrero et al., 1990) is similar to pUC18 (Yanish-Perron et al., 1985) but contains a multiple cloning site flanked by two *Not*I sites. Plasmid pKK232-8 (Brosius, 1984) contains the *rrnB* ribosomal RNA T1 terminator (Brosius et al., 1981). Plasmid pGEc47 (Eggink et al., 1987) contains the *alkST* and the *alkBFGHJKL* genes of *Pseudomonas oleovorans*. Plasmid pGEc74 (Eggink et al., 1988) contains only the *alkST* genes. Plasmid pHG171-luxAB is a promoter-probe construct based on the *luxAB* genes of *Vibrio harveyi* (Cohn et al., 1985; Johnston et al., 1986), and was a gift from Dr. J. Kuhn (Israel Institute of Technology, Israel). The plasmid was constructed by cloning the *luxAB* genes into plasmid pKA4 (Almashanu et al., 1996). Upstream of the *luxAB* genes there are a multiple cloning site, stop codons in all three reading frames, and a ribosome binding site (Fig. 1). Downstream lays a stem and loop structure which stabilizes the mRNA after transcription.

For the construction of a *luxAB*-based biosensor for alkanes, the following strategy was applied. A PCR reaction was performed with plasmid pGEc47 as template DNA and with primers ALKB1 (5'-GGCGTTGGTACCCCGGCTGCTTCG-3') and ALKB2 (5'-TTTATCTACGTCGACTGGAGCGGAATCC-3'). The reaction was done in a total volume of 50 µl of 20 mM Tris-HCl (pH 8.4), containing 10 pmol of each primer, 1 ng of plasmid pGEc47, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (Gibco BRL, Life Technologies, Inc., Gaithersburg, Md.), 0.05% W1 (Gibco BRL), and 0.8 U of *Taq* polymerase (Gibco BRL). Amplification was carried out in a Crocodile II thermocycler (Appligene, Illkirch, France) for 35 cycles of 30 s at

93.5°C, 30 s at 50°C and 45 s at 72°C, with a final extension of 4 min at 72°C. In this way a 0.6-kb fragment was obtained. This fragment contained the promoter region (P_{alkB}) and a small part of the open reading frame (*alkB'*) of the *alkB* gene, flanked by a *Kpn*I and a *Sal*I site. The authenticity of this fragment was confirmed by sequencing. Next, the fragment was cut by *Kpn*I and *Sal*I and inserted into pGEM7Zf(+), digested with *Kpn*I and *Xho*I, resulting in plasmid pJAMA1. Plasmid pJAMA2 was obtained by cloning the 0.6-kb *Hin*dIII-*Xba*I fragment with P_{alkB} from pJAMA1 into plasmid pHG171-luxAB. To prevent possible transcription from more upstream located promoters, a transcription terminator was cloned upstream of P_{alkB}. Hereto, a 0.2-kb *Eco*RI fragment containing the *rrnB* ribosomal RNA T1 terminator was isolated from plasmid pKK232-8. The fragment's 3'-recessive ends were filled in by using the Klenow fragment of DNA polymerase I. Subsequently, this fragment was inserted into pJAMA2 which was first partially digested with *Asp*718I and then subjected to Klenow polymerase treatment. This resulted in plasmid pJAMA4. The whole P_{alkB}-*luxAB* fusion was then recovered from pJAMA4 as a 3.0-kb *Hin*dIII-*Bgl*II fragment and cloned into pUC18Not which was digested with *Hin*dIII and *Bam*HI. The resulting plasmid is referred to as pJAMA7 (Fig. 5.1).

DNA manipulations. Plasmid DNA isolations, ligations and transformations were carried out according to well-established procedures (Sambrook et al., 1989). Restriction enzymes and other DNA-modifying enzymes were obtained from Amersham International plc. (Little Chalfont, England), Boehringer GmbH (Mannheim, Germany), Life Technologies, Inc. (Gaithersburg, Md.), and Pharmacia (Uppsala, Sweden). DNA fragments were isolated from agarose gels by using the Geneclean kit (Bio 101, Inc., LaJolla, Calif.) or QIAquick spin columns (Qiagen GmbH, Hilden, Germany).

Medium and growth conditions. *E. coli* strains were grown in Luria Bertani Broth (LB) medium. Cell growth was monitored by measurement of the optical density at 600 nm (OD_{600}) in an Uvikon 800 spectrophotometer (Kontron Instruments AG, Basel, Switzerland). The following antibiotics were added to the medium when required: tetracycline (Tc) at 10 µg/l; ampicilin (Ap) at 100 µg/l. Strains were routinely grown at 37°C if not otherwise specified.

TABLE 5.1. Plasmids used in this work

Plasmid	Relevant characteristics[a]	Source or reference
pGEM7Zf(+)	Ap^r	Promega
pUC18Not	MCS flanked by two *Not*I sites; Ap^r	(Herrero et al., 1990)
pKK232-8	pBR322 derivative containing the *rrnB* ribosomal RNA T1 terminator; Ap^r	(Brosius, 1984)
pHG171-luxAB	promoter-probe vector based on the *luxAB* genes; Ap^r	Dr. J. Kuhn
pGEc47	pLAFRI (RK2) with *alkBFGHJKL/alkST*; Ap^r, Tc^r	(Eggink et al., 1987)
pGEc74	pLAFRI (RK2) with *alkST*; Ap^r, Tc^r	(Eggink et al., 1988)
pJAMA1	pGEM7Zf(+) carrying 0.6 kb P_{alkB} PCR fragment; Ap^r	This work
pJAMA2	pHG171-luxAB carrying the 0.6 kb *Hind*III-*Xba*I fragment of pJAMA1; Ap^r	This work
pJAMA4	pJAMA2 carrying the 0.2 kb *Eco*RI fragment of pKK232-8; Ap^r	This work
pJAMA7	pUC18Not carrying the 3.0 kb *Hind*III-*Bgl*II fragment of pJAMA4; Ap^r	This work

[a] Abbreviations: MCS, multiple cloning site; Ap, ampicillin; Tc, tetracycline; r, resistance

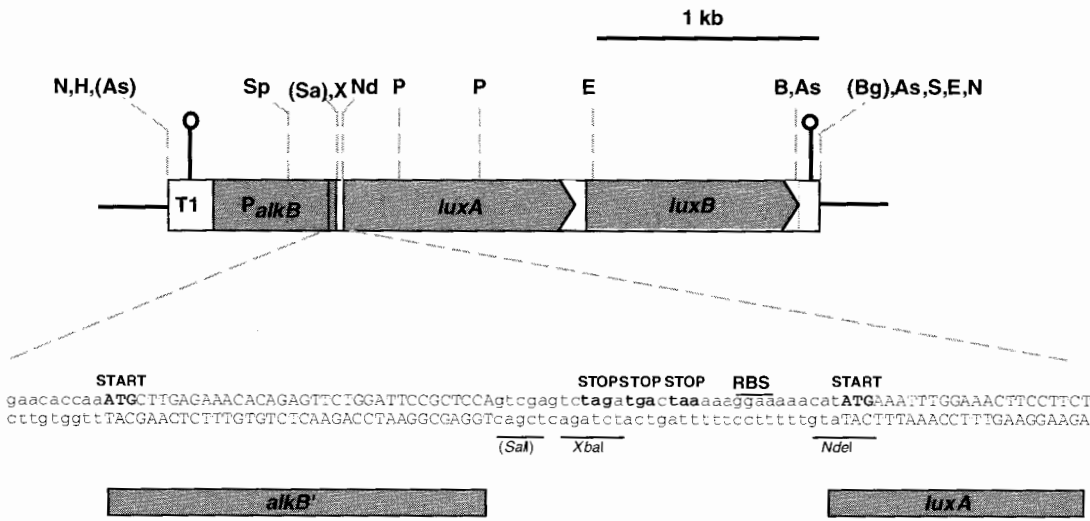

Figure 5.1. Organisation of the 3.0-kb *Not*I insert of pJAMA7. The remaining coding sequence of *alkB* is indicated with *alkB'*. Abbreviations: As, *Asp*718I; B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; N, *Not*I; P, *Pst*I; S, *Sst*II; Sa, *Sal*I; Sp, *Sph*I; X, *Xba*I; T1, rrnB ribosomal RNA T1 terminator; P_{alkB}, promotor of *alkB* gene; STOP, stop codons; RBS ribosome binding site. Sites in parentheses were destroyed during cloning. Sequences of *alkB* and *luxA* operon reading frames are in capital letters.

DNA manipulations. Plasmid DNA isolations, ligations and transformations were carried out according to well-established procedures (Sambrook et al., 1989). Restriction enzymes and other DNA-modifying enzymes were obtained from Amersham International plc. (Little Chalfont, England), Boehringer GmbH (Mannheim, Germany), Life Technologies, Inc. (Gaithersburg, Md.), and Pharmacia (Uppsala, Sweden). DNA fragments were isolated from agarose gels by using the Geneclean kit (Bio 101, Inc., LaJolla, Calif.) or QIAquick spin columns (Qiagen GmbH, Hilden, Germany).

Medium and growth conditions. *E. coli* strains were grown in Luria Bertani Broth (LB) medium. Cell growth was monitored by measurement of the optical density at 600 nm (OD_{600}) in an Uvikon 800 spectrophotometer (Kontron Instruments AG, Basel, Switzerland). The following antibiotics were added to the medium when required: tetracycline (Tc) at 10 µg/l; ampicilin (Ap) at 100 µg/l. Strains were routinely grown at 37°C if not otherwise specified.

Preparation of the inoculum for induction experiments. The finally constructed strain *E. coli* DH5α (pGEc74, pJAMA7) was used for induction experiments and for the quantification of linear alkanes. The inoculum for induction experiments was prepared in the following way. 2 ml of an overnight culture of *E. coli* DH5α (pGEc74, pJAMA7) was transferred into 100 ml of LB (Tc, Ap) medium. The cells were incubated at 30°C for about 6 hours until an OD_{600} of 0.55 was reached. Afterwards, the culture was immediately put on ice for 15 min and 20 ml of ice-cold sterile glycerol (87% vol/vol) was added. The mixture was kept on ice while it was divided into 650 µl portions in 1.5 ml Eppendorf tubes, which were then frozen in liquid nitrogen for 3 seconds and stored at -80°C.

Induction experiments with the whole cell biosensor *E. coli* DH5α (pGEc74, pJAMA7). Induction experiments were carried out in 10 mL glass tubes that were tightly closed with glass stoppers to avoid evaporation of volatile compounds. Each assay contained 5.7 ml of antibiotic-free LB medium, 0.3 or 0.6 ml of biosensor cells and 60 µl of a stock solution of a potential inducer compound dissolved in ethanol. Assay concentrations of octane were between 24 nM and 6.3 µM. Other compounds were added at concentrations of 5 µM. The negative control contained 60 µl of ethanol. Positive controls were amended with 60 µl of freshly prepared octane stock solution of the desired concentration, usually 11.2 µg/l (98 nM).

The assay was carried out as follows. Frozen stocks of *E. coli* DH5α (pGEc74, pJAMA7) biosensor cells were thawed in a water bath at 25°C for 2 min. The biosensor

cells were then placed back on ice until immediately prior to the inoculation of the assay. Induction experiments were started by the addition of the biosensor cells. During induction time, the glass tubes with the assay mixtures were incubated at 30°C on a rotary shaker at 200 rpm. Samples of 200 µl were taken after every 15 min during 75 min for determination of the light emission. If not otherwise stated, the sample taken after 1 h was used for comparison of inducer activities and for octane measurements.

Measurement of the light emission. 200 µl samples of assay mixtures were placed in a Microlite™1 microtiter plate (Dynatech Industries, Inc., McLean, Va.). The light emission was measured at 30°C in a Microlumat LB960 luminometer (Berthold AG, Regensdorf, Switzerland). The measurement was started after the automated injection of 25 µl of a decanal stock solution to the reaction assay. Along with the injection of decanal, air was dispersed into the assay in order to supply the luciferase reaction with oxygen. The decanal stock solutions were prepared in a 1:1 mixture of water and ethanol. To optimize the decanal concentration in the assay, decanal was added at final concentrations between 0 and 5 mM to the reaction assay. After the addition of the decanal substrate, the kinetic light emission was monitored for 60 seconds. Subsequent routine measurements were done at a final decanal concentration of 2 mM. The light output was integrated from 20 to 30 seconds after the decanal injection.

Groundwater samples. Groundwater samples were collected from a diesel oil contaminated site. Except for one sample (S6), 40 to 300 l were pumped from each well before a sample was taken. Sample flasks were completely filled with groundwater without leaving a headspace and stored at 4°C until further treatment. Sample S6 was collected from the bore hole without prior pumping of water. This sample was visibly covered with an oil film.

Detection of short-chain alkanes in contaminated groundwater with *E. coli* DH5α (pGEc74, pJAMA7). Groundwater samples were assayed for linear alkanes in glass tubes containing 4.7 ml of groundwater sample, 1 ml of 6-fold concentrated LB and 0.3 ml of biosensor cells. The blank and the positive control contained nanopure water instead of the groundwater sample and the positive control contained in addition 11.2 µg/l (98 nM) of octane. The assay and the measurement were then carried out according to the standard routine procedure as described above. In order to quantify inhibitory effects on the luciferase activity, each groundwater sample was spiked with 11.2 µg/l (98 nM) of octane and light emission was compared to a positive control containing the same concentration of octane in water.

Chemical analysis. For chemical analysis, the ground water samples were extracted with distilled pentane that had been spiked with 1.15 µM of Cl-octane as the internal standard. Samples were 30-fold concentrated upon extraction. 2 µl were injected into a GC-MS (Carlo Erba Strumentazione, Milan, Italy) equipped with a 30 m DB-XLB (inner diameter, 0.25 mm; film width, 0.25 µm) column (J & W Scientific, Folsom, Calif.). The following operating conditions were applied: The GC was temperature programmed from 313 K to 498 K at a heating rate of 10 K/min. The MS-detector was operated in the single ion mode recording ions of M/Z of 43 ± 0.2, 57 ± 0.2, 71 ± 0.2, 85 ± 0.2 typical for alkanes and 69 ± 0.2, 91 ± 0.2, 93 ± 0.2 and 105 ± 0.2 for the Cl-octane standard. Linear alkanes were identified by retention time and by their mass spectra. Aromatic compounds were identified by MS analysis.

Chemicals. Agarose was obtained from Gibco BRL (Life Technologies Inc., Gaithersburg, Md.). Yeast extract and tryptic casein were purchased from Biolife S. r. l. (Milan, Italy). Sodium chloride and glycerol were obtained from Fluka Chemie A. G. (Buchs, Switzerland) in analytical grade. Antibiotics were purchased from Fluka. Decanal was purchased from Sigma Chemical Co. (St. Louis, Mo.). All inducer substances and solvents were purchased from Fluka in analytical grade. Diethylether was further purified by distillation over sodium and subsequent filtration through Alox (I).

Data analysis. Quantification of alkane concentrations was done on experiments which were performed at least in triplicate. Student t-test analysis on the 0.05-level was performed to check results for significance. Curve fits were done by linear or non-linear least square analysis.

5.3. RESULTS

5.3.1. Construction of a whole cell biosensor for measuring availability of linear alkanes

As a tool for measuring alkane concentrations in aqueous solutions, we constructed an *E. coli* recombinant strain carrying the cloned *alkS* regulator gene and the *alkB* promoter region of *P. oleovorans* fused to the *luxAB* genes of *V. harveyi*. *alkS* and P_{alkB}-*alkB'*-*luxAB* were introduced on separate plasmids in this *E. coli*. The fusion P_{alkB}-*alkB'*-*luxAB* was tailor-made by using PCR and transcriptionally shielded by transcription terminators upstream of the *alkB*-promotor and downstream of *luxAB* (Fig. 5.1).

The luciferase activity in this *E. coli* was readily inducible with octane, a typical inducer of the *alk*-genes in *P. oleovorans*, indicating that both *alkS* and *alkB'-luxAB* transcription were functioning as expected. Fig. 5.2 shows the typical light output of strain *E. coli* DH5α (pJAMA7, pGEc74) after 1 hour of incubation without octane and in the presence of 0.288 or 1.44 μM octane. It can be noted that the system was expressed in *E. coli* DH5α (pGEc74, pJAMA7) to a small extent in the absence of octane, even though a transcription terminator was present in front of P_{alkB}- . It was also obvious from the data in Fig. 5.2 that the light output increased with higher assay concentrations of octane. The luciferase responded immediately to the addition of decanal. The light emission reached a maximum after about 5 s and remained nearly stable afterwards.

In order to exclude unspecific activation of the *luxAB* genes, control experiments were performed using *E. coli* DH5α carrying plasmid pJAMA7 only, thus lacking the transcriptional activator AlkS. No induction of luciferase expression by octane was observed in this strain (results not shown). This showed that expression of *luxAB* was not induced unspecifically by, for example, a general stress response, and required the presence of AlkS.

5.3.2. Optimization of the decanal substrate concentration for the luciferase activity of *E. coli* DH5α (pGEc74, pJAMA7)

One important prerequisite for reliable alkane measurements is the saturation of the cellular luciferase with decanal. To test the influence of the decanal concentration on the luciferase activity, we investigated the kinetic response in more detail. From kinetic measurements of the light output, we calculated the cumulative light emission as the product of the luciferase reaction sequence. As can be seen from Fig. 5.3, the cumulative light emission increased linearly with time, except for the first 5 s, which was possibly due to the transport processes needed to achieve steady-state reaction rates.

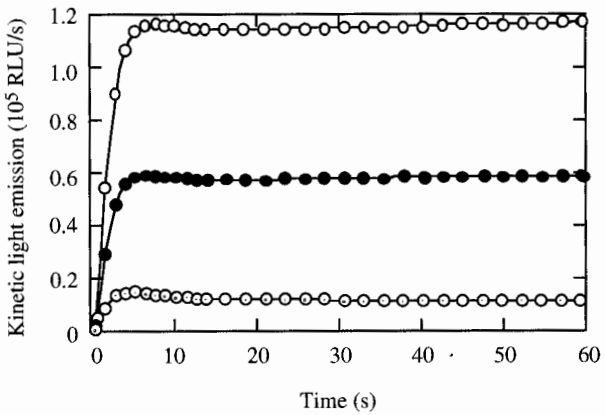

Figure 5.2. Light emission kinetics immediately after injection of 2 mM decanal to octane induced whole cell biosensors *E. coli* DH5α (pGEc74, pJAMA7). Octane concentrations were: 0.288 μM (●), and 1.44 μM (○). Control: no octane (⊙).

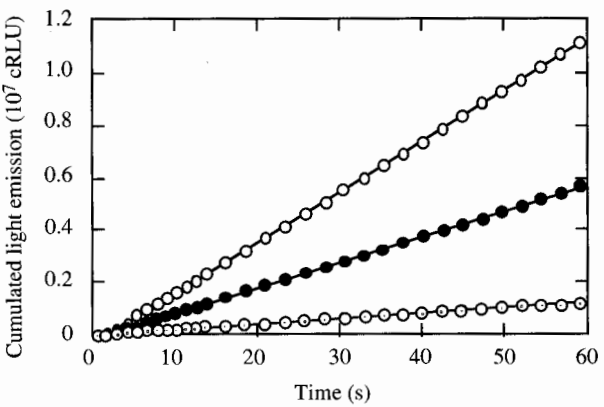

Figure 5.3. Cumulated light emission after injection of 2 mM decanal to an induced culture of *E. coli* DH5α (pGEc74, pJAMA7). Symbols: no octane (⊙), 0.288 μM octane (●), and 1.44 μM octane (○). cRLU, cumulated relative light units.

Subsequently, we determined the light production rates (i. e. the slopes of the linear increase in the cumulative light production) with different decanal concentrations with cultures that had been induced for 1 hour with 0, 0.288 and 1.44 μM octane. The light production rates showed a dependency on the decanal concentration that could be fitted reasonably well by a Michaelis-Menten type kinetics (Fig. 5.4). Saturation of the luciferase activity was observed for decanal concentrations above 2 mM for all octane concentrations tested. The half-saturation constant (K_M) was 0.2 mM for cultures induced with octane, and 0.1 mM for cultures incubated without octane. V_{max} values depended on the octane concentrations and probably reflected the amount of luciferase enzyme present in the cells after induction.

For practical reasons of decanal solubility, we chose to use a decanal assay concentration of 2 mM (stock concentration of 18 mM in ethanol-water) in all further experiments.

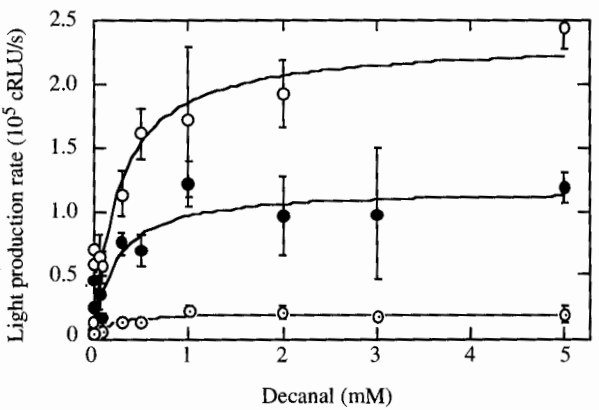

Figure 5.4. Light production rate as a function of the decanal concentration. Octane concentrations used to induce *E. coli* DH5α (pGEc74, pJAMA7) were: no octane (⊙), 0.288 μM (●), and 1.44 μM (○). Induction time was 60 min.

5.3.3. Time dependent induction of whole cell biosensors *E. coli* DH5α (pGEc74, pJAMA7) with octane

The whole cell biosensor *E. coli* DH5α (pGEc74, pJAMA7) was further characterized by analyzing the light response of this strain after induction with three different octane concentrations and by following growth of the assay culture at the same time. We used frozen batches of *E. coli* DH5α (pGEc74, pJAMA7) throughout this study, which had been pregrown under well defined conditions in order to standardize our measuring method (see Materials and Methods). Freshly thawed batches were immediately used for measurements.

A typical induction experiment is shown in Fig. 5.5. During the first 80 min of induction, an increase in light emission was detected, which started almost immediately after 15 min incubation time. The optical density after 80 min increased slightly from 0.06 to about 0.08. After about 60 to 80 min, the total light output levelled off, whereas the specific light emission (corrected for the number of cells) decreased (not shown). The background activity remained constant at about 3×10^5 RLU during the whole experiment. With water-saturated octane concentrations (720 µg/l; 6.3 µM), a maximum 20-fold increase for the luciferase activity compared to a control measurement without octane was observed after about 80 min incubation time.

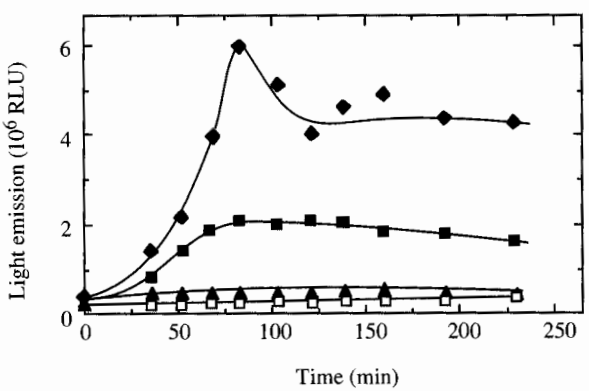

Figure 5.5. Time dependent light emission of *E. coli* DH5α (pGEc74, pJAMA7) after induction with different octane concentrations. Symbols: 6.3 µM octane (◆), 0.63 µM octane (■), 63 nM octane (▲), no octane (□).

5.3.4. Calibration of the biosensor light emission for water-dissolved octane concentrations

To allow quantification of octane concentrations in aqueous samples, we calibrated the light output against the octane concentration at different induction periods shorter than 75 minutes. The light emission showed a saturation type dependency on the octane concentration, at any induction time. The insert in Fig. 5.6 shows a typical curve for 60 min induction. Data points could be fitted with a hyperbolic equation with a good correlation for induction times above 15 min (r>0.95). At low octane concentrations, a linear correlation was found to fit the data as well. The linear range was most extended for an induction time of 60 min and reached from 2.8 to 90 µg octane per liter (24.5 to 790 nM) (Table 5.2 and Fig. 5.6). With this induction period, the background light emission without octane was 2.7 × 10^5 RLU. The lowest octane concentration tested (2.8 µg/l; 24.5 nM) resulted in a 1.4-fold increase of the light emission after 1 hour induction time. This increase was significant on the 5% level (n=5, P<0.05). For 90 µg/l (790 nM), the light emission was 8.4 times the background.

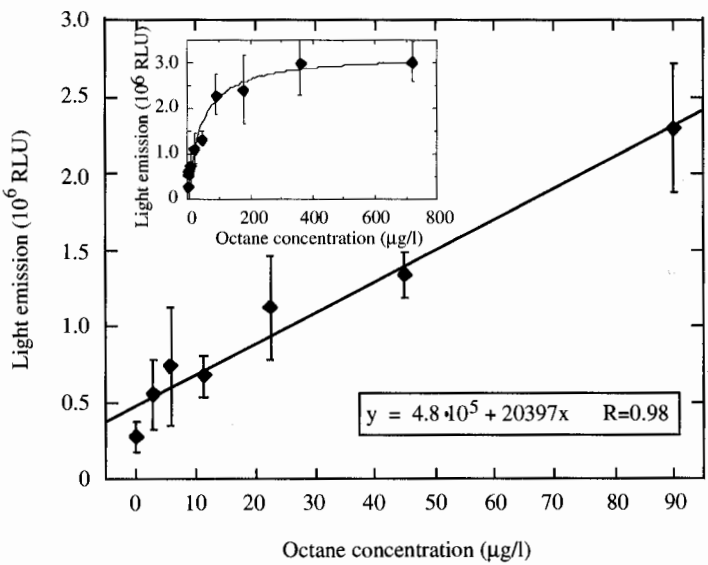

Figure 5.6. Light emission by *E. coli* DH5α (pGEc74, pJAMA7) after a 60 minute induction period as a function of the octane concentration.

TABLE 5.2. Linear correlations for the calibration of the whole cell biosensor *E. coli* DH5α (pGEc74, pJAMA7)

Incubation time (min)	Range (μg/l)	Correlation r[a]	a[b]	b[c]
15	0-5.6	0.99	9748	1.14×10^5
30	2.8-22.5	0.93	14138	2.49×10^5
45	2.8-90.0	0.95	13653	4.74×10^5
60	2.8-90.0	0.98	20397	4.83×10^5
75	2.8-45.0	0.99	39310	4.07×10^5

[a] r, correlation coefficient
[b] a, slope
[c] b, y-intercept

5.3.5. Induction and inhibition of the luciferase activity in *E. coli* DH5α (pGEc74, pJAMA7) with various alkanes, aromatic hydrocarbons and related compounds

It is known that AlkS responds to many other compounds (Wubbolts, 1994). We therefore tried to induce the luciferase activity in *E. coli* DH5α (pGEc74, pJAMA7) with a range of alkanes, alkane mixtures and several other compounds. All compounds were tested at concentrations of 5 μM. Table 5.3 shows the results in comparison with the octane induced luciferase activity at the same concentration. Significant induction was found for the alkanes from pentane to decane, whereas long-chain linear alkanes such as dodecane and hexadecane did not induce the luciferase activity in the biosensor. High boiling petroleum ether, a relatively undefined alkane mixture, led to a weak induction (16% of that of octane). A relatively strong induction was observed for the singly branched alkane 3-methylheptane (36 %). The poly-branched alkane heptamethylnonane and the long-chain branched alkane pristane did not lead to detectable induction. None of the alicyclic or aromatic compounds tested induced the luciferase activity. No induction

was found with dicyclopropylketone (DCPK) at a concentration of 5 µM. This compound had been successfully employed as a gratuitous inducer of the *alkB* expression previously at a concentration of 440 µM (0.05 %) (Wubbolts, 1994). At this concentration, induction was also found for our bioreporter (results not shown).

Several of the compounds that were tested for their potential to induce the luciferase activity in *E. coli* DH5α (pGEc74, pJAMA7) were then mixed as groups of substance classes and added to induction assays together with octane in order to determine their influence on the biosensor response to octane (Table 5.4). Since octane was present at a sufficiently high concentration of 5 µM to induce maximal light emission (Fig. 5.4), one would expect the mixtures to exhibit, if any, a reducing effect on the induction. This was indeed observed (Table 5.4). Linear alkanes, branched alkanes, and the polyaromatic hydrocarbons (PAH) had either no effect or resulted only in a minor inhibition. The luciferase activity was considerably reduced after induction in the presence of petroleum ether, alicyclic and aromatic hydrocarbons, alkylbenzene and DCPK. The most potent inhibitor was 2-hydroxybiphenyl leading to a 84% decrease in luciferase activity.

TABLE 5.3. Relative luciferase activity after the induction with different compounds.

Compound[a]	Rel. induction[b]		Compound	Rel. induction	
Linear alkanes			Alicyclic hydrocarbons		
Pentane	**13**[c]	(n.d)[d,e]	Cyclohexane	9	(≤2)
Hexane	**44**	(10)	Methylcyclohexane	11	(≤2)
Heptane	**81**	(85)	Dimethylcyclohexane	11	(n.d.)
Octane	**100**	(100)	Cycloheptane	11	(n.d.)
Nonane	**100**	(90)	Aromatic hydrocarbons		
Decane	**69**	(36)	Benzene	10	(≤2)
Undecane	6	(32)	Toluene	10	(≤2)
Dodecane	11	(2-3)	*m*-Xylene	11	(≤2)
Hexadecane	11	(n.d.)	Trichlorbenzene	9	(n.d.)
Petroleum ether[f]			Alkylbenzenes		
low boiling (30-45°C)	10	(n.d.)	Hexylbenzene	12	(≤2)
high boiling (50-70°C)	**16**	(n.d.)	Dicyclopropylketone	9	(86)[g]

- continued next page

- Table 5.3. continued

Compound	Rel. Induction		Compound	Rel. Induction	
Branched alkanes			PAHs		
Heptamethylnonane	11	(n.d.)	Naphthalene	10	(n.d.)
3-Methylheptane	**36**	(88)	1-Methylnaphtalene	8	(n.d.)
Pristane	11	(n.d.)	Background	8[h]	(≈2)

[a] Assay concentration of individual compounds was 5 µM. Compounds were added to the assay as ethanol stock solutions.
[b] Induction was measured after 69 minutes. The light output was related to the octane induced light emission that had arbitrarily been set to 100 %.
[c] Bold face numbers indicate significant induction on the 5% level ($5<n_1+n_2<8$, $P<0.05$, unpaired, one-tailed test)
[d] not determined
[e] Values between brackets refer to the data obtained by Wubbolts (Wubbolts, 1994) using an P_{alkB}-*cat* reporter.
[f] Petroleum ether refers to commercially available alkane mixtures. Boiling temperatures are given in brackets. These mixtures were added in the same amount as pentane (low boiling petroleum ether) and hexane respectively (high boiling petroleum ether).
[g] Assay concentration of DCPK was 0.05 % (440 µM).
[h] Background light emission was determined by adding the same amount of pure ethanol to an assay instead of inducer solution.

TABLE 5.4. Inhibition of the octane induced light emission in *E. coli* DH5α (pGEc74, pJAMA7) by different groups of related compounds.

Substance class	Compounds[a]	Inhibition (%)[b]
Linear alkanes	Pentane, Hexane, Heptane, Decane, Dodecane	6
Alkane mixtures	Petroleum ether (low and high boiling)	11
Branched alkanes[c]	Heptamethylnonane, 3-Methylheptane, Pristane	0
Alicyclic hydrocarbons	Cyclohexane, Methylcyclohexane, Cycloheptane	19
Aromatic hydrocarbons	Benzene, Toluene, *m*-Xylene	12
PAH[c]	1-Methylnaphthalene	0
Alkylbenzenes	Hexylbenzene	14
Biphenyls	2-Hydroxybiphenyl[d]	85
Other	Dicyclopropylketone (DCPK)	37

[a] Assay concentration for each individual compound was 5 μM.
[b] Inhibition refers to the percentage inhibition of the luciferase activity with octane in the presence of these compounds as compared to an assay with only octane present. Light response was measured after 69 minutes.
[c] Inhibition was measured after 54 minutes of incubation time.
[d] 2-hydroxybiphenyl was added in crystalline form to the assay.

5.3.6. Application of the whole cell biosensor for the measurement of alkane concentrations in contaminated groundwater

We used the alkane biosensor to measure the alkane concentrations available to the cells in samples from a heating oil contaminated groundwater site. In this area, 30 m^3 of diesel oil reached the groundwater table due to an undetected leak in a pipeline. A detectable induction of the luciferase activity of *E. coli* DH5α (pGEc74, pJAMA7) was observed with most samples and were expressed as octane equivalents (Table 5.5). Compared to a negative control without any additional alkanes or groundwater present, the induction was significant except for samples P7 and P9 ($n_1=n_2=3$, $P<0.05$). The sample from well S6 clearly contained the highest available alkane concentration. This sample had been the only one which visibly contained small oil droplets that were probably transferred into the assay. The lowest biosensor response was obtained with sample P7. In this sample, the total light output was even smaller than in the negative control containing nanopure water only, most probably due to inhibitory effects of unidentified contaminants on the biosensor performance. In order to quantify inhibitory effects, we spiked all samples with a known amount of octane (98 nM) and measured the reduction in the total light output as compared to a positive control containing the same octane concentration in deionized water. In all samples, a significant average reduction of 23 % of the light emission was observed ($4<n<6$, $P<0.05$). We used this finding to correct the calculated octane equivalent concentrations accordingly (Table 5.5).

We also analyzed the samples by GC-MS in order to verify the outcome of the induction experiments. Typical gas chromatograms of diethylether extracts from sample S6 are shown in Fig. 5.7A and B. It was possible to separately analyze the oil film floating on the groundwater sample S6. This clearly showed that middle and long-chain linear alkanes were present as dominant components of this oil film (Fig. 5.7B). Alkanes with chain lengths above C8 were clearly identified, among them the inducer compounds C9 to C11 for the luciferase activity in *E. coli* DH5α (pGEc74, pJAMA7). When the oil emulsion was carefully removed in a separation funnel, the resulting water fraction contained practically no alkanes. The dominant peaks in sample S6 were identified by mass spectroscopy as alkyl-substituted aromatic compounds (Fig 5.7A). In all aqueous samples, the concentrations of linear alkanes were below detection limit with the applied GC-MS method, which was between 5 and 50 nM depending on the measured compound. Middle-chain length alkanes (C8 to C11) were present at concentrations below 20 nM each and longer alkanes (C12 to C16) at concentrations below 50 nM each.

The chemical analysis of inducing alkanes was in agreement with the biosensor measurements, that indicated a group concentration of inducer compounds below 10 nM in most samples and below 30 nM in sample S6.

TABLE 5.5. Analysis of the groundwater at the diesel oil contaminated site with *E. coli* DH5α (pGEc74, pJAMA7).

Sample	Inhibition (%)[a]	octane equivalents (nM)	
		uncorrected[b]	corrected[c]
P1	20 (±9)	3.04 (±0.52)	3.79 (±1.00)
P4	14 (±9)	3.23 (±0.61)	3.77 (±1.03)
P5	18 (±11)	2.94 (±1.31)	3.61 (±1.84)
P7	25 (±14)	<0	<0
P9	27 (±15)	0.52 (±1.55)	0.71 (±1.73)
PS9	36 (±12)	1.82 (±0.97)	2.82 (±1.61)
S6	25 (±8)	14.35 (±4.28)	19.10 (±6.61)
S7	15 (±9)	2.61 (±0.68)	3.08 (±1.04)

[a] Inhibitory effect of the groundwater samples on the biosensor performance was measured by spiking samples with 98 nM of octane and determining the reduction of the total light output

[b] values were calculated by extrapolating standard light output data (Fig. 5.6) to very low octane concentrations using a hyperbolic function.

[c] corrected for the inhibitory effect of the sample on the biosensor performance

[d] not determined

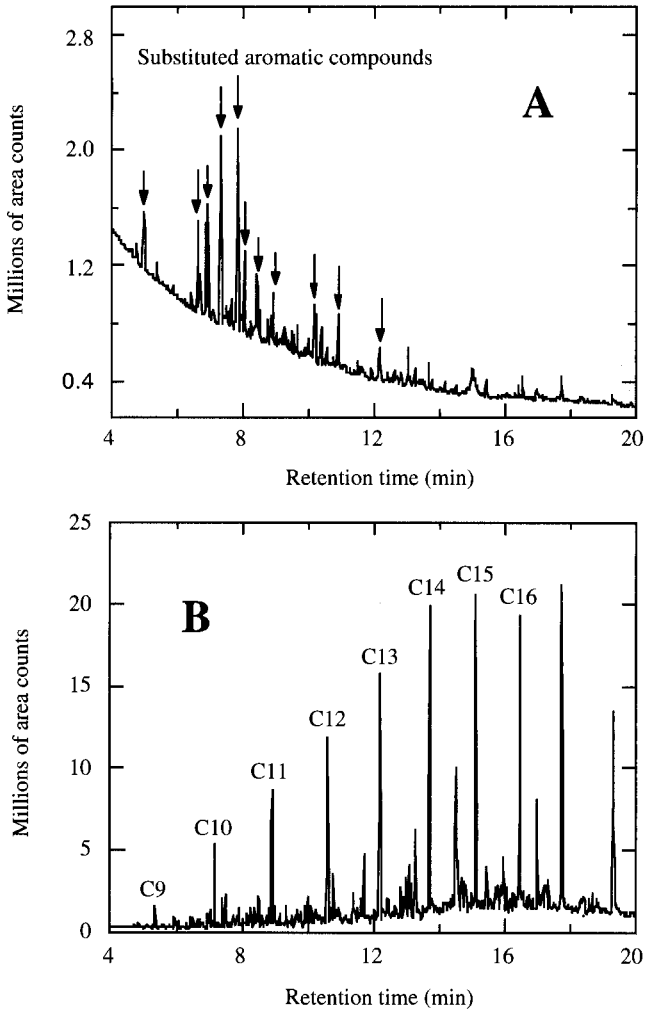

Figure 5.7. GC analysis of groundwater from sample S6. (A) Water without oil film. Arrows indicate alkyl substituted aromatic compounds according to GC-MS analysis. (B) Oil-water emulsion. Linear alkanes are indicated by their number of carbon atoms.

5.4. DISCUSSION

We described the construction, characterization and application of a bioluminescent whole cell biosensor to measure water dissolved concentrations of linear alkanes. Our biosensor system was based on the gene for the sensor protein AlkS and on a transcriptional fusion between P_{alkB} and the promoter free *luxAB* genes, which was transcriptionally activated by AlkS. Among the compounds tested, *E. coli* DH5α (pGEc74, pJAMA7) showed a specific response and a good sensitivity for middle-chain length linear alkanes and one branched alkane.

The biosensor was capable of sensing and reporting concentrations of octane as low as 24.5 nM. This concentration corresponded to about 20 molecules of octane present in the cytoplasm of each cell, when assuming a cell volume of 1.6 μm^3 and equal distribution of octane between bulk liquid and cytoplasm. In a similar calculation, DiMarco and co-workers observed that roughly 50 molecules of *p*-hydroxybenzoate per cell were sufficient to lead to an activation response of the transcriptional activator PobR (DiMarco et al., 1993). Other transcriptional regulators for catabolic gene expression typically responded to inducer concentrations below a few micromoles per liter (Table 5.6). Clearly, each detection limit is governed by intrinsic characteristics of the reporter system such as the sensor protein's binding affinity for the inducer compound, the transcription efficiency of the reporter gene and the specific activity of the reporter protein. Additionally, the transport rates of the inducer compounds into the biosensor cell probably play an important role, too. Hydrophobic compounds are known to accumulate in biological membranes and to penetrate the cytoplasmic membrane relatively easily (Sikkema et al., 1995). By using the equation by Sikkema et al. (Sikkema et al., 1994) and assuming that membranes occupy 7% of the total cell volume, we calculated that the octane concentration in the membranes of the whole cell biosensor *E. coli* DH5α (pGEc74, pJAMA7) was about 0.6 mM at a bulk concentration of 24.5 nM. This value corresponds to approximately 4×10^4 octane molecules accumulated in the membranes of each cell. Thus, if biosensor cell membranes are saturated with octane, small changes in cytoplasmic octane concentrations will rapidly be compensated by exchange with membrane bound octane. Since AlkS seems to be a soluble protein and is, if at all, only loosely associated with the membrane via hydrophobic stretches or ionic interactions (Wubbolts, 1994), this protein probably recognizes cytoplasmic octane only, and, consequently, efficient transport into the cell, as one would expect for octane according to the above calculations, is especially important for recognition efficiency of this inducer molecule.

TABLE 5.6. Comparison of different bacterial sensor specifities

Compound	Regulator	Reporter	Strain	Detected (μM)	Reference
naphthalene	NahR	*nahG-luxCDABE*	*P. fluorescence*	0.35[a]	(Heitzer et al., 1992)
salicylate	NahR	*nahG-luxCDABE*	*P. fluorescence*	2.9[a]	(Heitzer et al., 1992)
isopropylbenzene	IbpR	*ibpRo/pA'-luxCDABE*	*E. coli*	1	(Selifonova and Eaton, 1996)
o-cresol	DmpR	*Pdmp-luxAB*	*P. putida*	3.2[a]	(Shingler and Moore, 1994)
p-hydroxybenzoate	PobR	*pobA-lacZ*	*A. calcoaceticus*	0.1	(DiMarco et al., 1993)
m-toluate	XylS	*xylS/Pm::luxAB*	*P. putida*	5[a]	(de Lorenzo et al., 1993)
octane	AlkS	*alkB'-luxAB*	*E. coli*	0.024	This work

[a] lowest concentration tested

The use of *E. coli* as host strain for the alkane sensing and regulated luciferase response has all the advantages that working with *E. coli* has. The host strain apparently expresses *alkS* and recognizes the *alkB* promoter sufficiently well to allow a workable system. However, a relatively high background expression from P_{alkB} occurred under uninduced conditions even though the expression of *alkB'-luxAB* was transcriptionally shielded. Perhaps this is due to an incomplete repression or to the presence of weak *E. coli* promoters in this part of the *alkB* sequence. In addition, the current host strain carries the *alkB'-luxAB* fusion on a high copy number plasmid. Consequently, a weak basal level expression of *alkB'-luxAB* would lead to a high background luciferase activity.

Our biosensor strain carried the regulatory elements of the *alk* operon only, and was consequently unable to metabolize linear alkanes. Such a biosensor is a good tool for the rapid, unequivocal measurement of specific pollutants in contaminated waters or soils and shows the instantly available concentration of inducing compounds. For measuring substrate fluctuations rather than momentary concentrations, a biosensor which both senses and degrades a particular inducer compound is favourable. Such a biosensor system is the extensively studied naphthalene/salicylate degrading sensor strain *Pseudomonas fluorescence* HK44 (Burlage et al., 1990; Heitzer et al., 1994; Heitzer et al., 1992; King et al., 1990). In a recent work, an optical biosensor based on this reporter strain was successfully applied to on-line monitor changing concentrations of naphthalene and salicylate in an aqueous waste stream (Heitzer et al., 1994). Besides the ability of the biosensor strain to degrade the inducing compound, turnover rates of the cellular luciferase activity play an important role for such applications (Heitzer et al., 1994).

As expected from other studies (Blouin et al., 1996), we observed that the light emission of *E. coli* DH5α (pGEc74, pJAMA7) was strongly dependent on the decanal concentration in the assay. We could determine from testing different decanal concentrations, that at concentrations of 2 mM and above, the available luciferase amount in the cells was saturated with substrate. Therefore, at those substrate concentrations, the light emission is independent of the decanal concentration and directly reflects the amount of luciferase enzyme. Although high decanal concentrations are known to inhibit the luciferase enzyme (Holzman and Baldwin, 1983) or to be cytotoxic (Blouin et al., 1996), we did not observe such effects in our assay. Interestingly, the typical time-kinetic response (Fig. 5.1) of the light emission by our biosensor to higher decanal concentrations differed from the responses observed in other studies (Blouin et al., 1996). After the injection of decanal, we observed an immediate strong increase in light emission, which was not followed by a sharp decrease, but rather remained relatively stable for at least one minute (Fig. 1).

It has been observed previously, that transcriptional regulators often exhibit relaxed specificity towards structurally more or less related compounds (Selifonova and Eaton, 1996; Shingler and Moore, 1994; Wubbolts, 1994). The AlkS-*alkB'-luxAB* system in *E. coli* DH5α showed a specificity of induction for short and middle chain length alkanes. These results were mostly in agreement with those from a previous study (Wubbolts, 1994) where the inductive effect of several compounds on P_{alkB} had been studied in *E. coli* W3110 (pGEc289, pGEc74). This strain harboured a P_{alkB}-*cat* (chloramphenicol acetyl transferase) transcriptional fusion. Somemore chemicals were tested in that study such as chlorinated and brominated alkanes, some alkenes and selected branched alkanes, as well as a few ketones and ethers, to which AlkS also responded. However, it is not whether these compounds would also be detectable by our biosensor strain *E. coli* DH5α (pGEc74, pJAMA7). The effective range of inducer concentrations varied considerably among the inducer compounds tested. Wubbolts (Wubbolts, 1994) added 1 % vol/vol of each potential inducer compound tested or 0.05 % vol/vol (440 µM) of the gratuitous inducer DCPK to the assay , whereas we applied assay concentration of 5 µM only for each compound.

In contaminated sites, where hundreds of different chemicals besides the inducer compounds are present and may interact with a biosensor, it is clearly impossible to check the effect of groups of these compounds or of all compounds individually, even if they have all been identified by chemical analysis. Possible inhibitory effects can be addressed by adding a known amount of optimal inducer, in our case octane, to the unknown samples and measure induction differences. In the groundwater samples tested, we determined that the biosensor underestimated the bioavailable alkane concentration probably due to inhibition by approximately 25%. For routine measurements, some research groups (Burlage et al., 1994) considered spot checking as probably sufficient. An alternative strategy to account for effects of non-inducing compounds on the biosensor performance would be the use of either isogenic biosensor strains that exhibit constitutive bioluminescence (Heitzer et al., 1992) or the introduction of a second, constitutively expressed luciferase that emits another wavelength (Wood and Gruber, 1996). However, the use of such systems invokes other uncertainties, since the AlkS system might respond differently to the presence of non-inducing compounds than a constitutively expressed *luxAB* construct.

By applying our biosensor strain we could monitor the presence of an inducible fraction of, most likely, middle chain length alkanes in heating oil contaminated groundwater samples. Due to the specifities of the sensor's response, it could not be

excluded that some related compounds added to the inductive effect. It was evident from chemical analyses of sample S6 that the organic phase still present on some groundwater samples, contained the largest quantity of the alkane pool. Only a very small fraction of these alkanes were available to the biosensor cells. Since the total bioavailable concentration of these compounds was far below their water solubility, this implied that dissolution rates of these compounds rather than microbial uptake rates may limit biodegradation of these compounds at the site. Despite the low aqueous concentrations of the alkanes, the sensor was sufficiently sensitive to detect them in most samples. The two samples that did not induce luciferase activity were possibly outside of the contaminated zone.

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CONCLUDING REMARKS

Many pollutants have been found to be biodegradable in well-defined laboratory setups where pure cultures of specialist strains are cultivated on the pollutant as the only source for carbon and energy. Although many of these specialist strains are found to be ubiquitously distributed in natural ecosystems, it has been observed that pollutants often persist for a long time. This indicates that environmental factors often limit the extent and rate of biodegradation rather than the lack of specialist strains. Various factors have been identified that influence pollutant biodegradation, some of which have been investigated more closely in this thesis.

As far as the biodegradation of hydrophobic compounds is concerned, bioavailability has been recognized as a parameter of primary importance. Therefore, decisions concerning bioremedation strategy should also involve monitoring of the "bioavailable" pollutant concentration. However, the bioavailable concentration of pollutants by chemical analysis is difficult to assess. In order to measure the bioavailable concentrations of various pollutants, whole cell biosensors have proven to be a suitable tool. As an additional advantage, such biosensors can be made to be specific for a narrow range of compounds and measurements are easily performed. Here, a biosensor for the measurement of middle-chain alkanes and their bioavailable concentration was developed. Although the sensor could be used for the fast and easy detection of alkanes in contaminated ground waters, it is understood that more work is still required for the further optimization of the sensor system. It would be of particular interest to develop a biosensor with the original strain *Pseudomonas oleovorans* rather than using *E. coli* as a host strain.

A genetically engineered strain of *E. coli* was used for the construction of the whole cell biosensor. This is a relatively new application of genetically engineered microorganisms (GEMs) in environmental biotechnology. Other applications of GEMs for bioremediation have been considered before, including the use of specially designed strains for the biodegradation of xenobiotic compounds. However, the application of such strains often failed due to unsuitable conditions of growth. Additionally, the potential risks involved in the release of GEMs have been recognised and have become a matter of public concern. This resulted in legal restrictions for the application and release of GEMs, as it is for example the case in Switzerland (Swiss Environmental Law (USG), Art. 29). Should a biosensor such as the one mentioned be further

developed for the market and for routine testings, safety tests and a sensor setup that prevents unintentional release of GEMs during measurements are required.

In case of limited bioavailability, bioremediation efficiency can be increased by vigourous mixing. Biomass productivity during continuous cultivation of *Acinetobacter* sp. H01-N, for example, was clearly dependent on the stirrer speed and thus on the extent of mixing. In natural systems, it is not possible to increase the power input in a similar way. Therefore, other strategies such as the use of surfactants have been proposed to enhance pollutant bioavailability. However, the fact that there are controversial reports on the effect of surfactants shows that it is difficult to suggest a generally applicable strategy for bioremediation and how difficult it is to asses the success of such strategies. A biosensor - such as the one mentioned above - would be a useful tool for monitoring mass transport and bioavailability due to changes in operational conditions during field trials, and could provide useful insights into the mechanisms involved.

In nature, microbial growth usually takes place in the presence of a wide variety of potential growth substrates, which are usually present at very low concentrations, and the strategy for mixed substrate utilisation will depend on the prevailing growth conditions. Often, it is found that simultaneous utilisation is typical at low carbon concentrations, whereas at higher concentrations of individual carbon sources, sequential utilisation is observed. Since low carbon concentrations are typical in natural environments, it is most probable that specialist microbial strains will not feed on the contaminant only but will utilise several substrates at a time. Such a behaviour was also observed for *Acinetobacter* sp. H01-N growing on a mixture of acetate and dodecane. From these results, it would be expected that easily degradable carbon sources at typical concentrations - such as acetate - do not inhibit pollutant biodegradation. It was clear though, that alternate carbon sources nevertheless influenced both cell physiology and catabolic enzyme activity.

It is also of particular interest, how acclimation to pollutant biodegradation is influenced by the presence of other carbon sources. Our experiments showed that the presence of acetate as an alternate carbon source accelerated the adaptation of *Acinetobacter* sp. H01-N to growth on dodecane. This observation might be of use for the improvement of start-up efficiency in bioremediation programmes and might be an alternative to the initial seeding with specialist strains.

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