Physiology and biochemistry of the anaerobic biodegradation of isopropanol and acetone

Dissertation

zur Erlangung des Akademischen Grades eines Doktors der Naturwissenschaften (Dr. rer. nat.) im Fachbereich Biologie der Mathematisch-Naturwissenschaftlichen Sektion an der Universität Konstanz

vorgelegt von

Carlos Henrique Dullius

Tag der mündlichen Prüfung: 05.07.2011
1. Referent: Prof. Dr. Bernhard Schink, Universität Konstanz, Germany
2. Referent: Prof. Dr. Peter Kroth, Universität Konstanz, Germany

“A curiosidade do espírito na busca de princípios certos
é o primeiro passo para a conquista da sabedoria”

(Sócrates)

Für meine Familie

Für die Überlassung des Themas und für die Betreuung meiner Doktorarbeit bedanke ich mich ganz herzlich bei Herrn Prof. Dr. Bernhard Schink.

Im Besonderen bedanke ich mich auch bei allen die mich während der Zeit meiner Doktorarbeit begleitet und unterstützt haben. Dazu gehören meine Eltern Sirio und Marlene, meine Frau Anja und meine Kinder Leon und Viviana.

Den Arbeitskollegen des LS/Schink, Julia Röder, Antje Wiese, Sylke Wichmann und Joachim Schott und Frau Renate Gimmi, danke ich für die freundschaftliche Arbeitsatmosphäre. Ein Spezielles Dankeschön an Nicolai Müller und Jörg Deutzmann für die Unterstützung bei der Anreicherung von Enzymen und der Erstellung von phylogenetische Stammbäumen.

Dem Brasilien-Zentrum der Universität Tübingen und den Landesgraduiertenkolleg Baden-Württemberg danke ich für das Promotionsstipendium und der Deutscher Forschungsgemeinschaft (DFG) für die Bereitstellung der Forschungsmittel.

INDEX

1 Summary……...8

Zusammenfassung……..10

2 Abbreviations……13

3 Introduction……15

3.1 Isopropanol metabolism……15

3.2 Acetone metabolism……16

3.3 Aims……..18

***Syntrophic methanogenic enrichments*……19**

***Sulfate-reducing bacteria* ……..21**

***Nitrate-reducing bacteria*……23**

4 Material and Methods……..….24

4.1 Chemicals……..24

4.2 Microbiological methods……24

4.2.1 Source of microorganisms……..……..24

4.2.2 Preparation of liquid medium……25

4.2.2.1 Bicarbonate-buffered medium……..25

4.2.2,2 CO_2-free medium……..25

4.2.2.3 Sodium-free medium……...25

4.2.2.4 Phosphate-buffered medium……26

4.2.2.5 Aerobic phosphate-buffered medium (modified by Widdel, 1980)…….26

4.2.2.6 Sporulation medium (DSMZ, Catalog 1993, Medium Nr. 12).……26

4.2.2.7 Microelement solution SL 10 (Widdel et al., 1983)……..26

4.2.2.8 Selenite-tungstate solution (Widdel et al., 1983)…….27

4.2.2.9 Seven-vitamin solution (Widdel and Pfennig, 1981)……...27

4.3 Preparation of substrates……...27

4.4 Preparation of electron acceptor solutions……...27

4.5 Cultivation of microorganisms……..28

4.6 Isolation of microorganisms……..28

4.6.1 Isolation of methanogenic archaea…….28

4.6.2 Isolation of sulfate-reducers…….29

4.6.2.1 Sporulation test…….29

4.6.2.2 Pasteurization test……30

4.6.3 Isolation of nitrate-reducing bacteria……..30

4.7 Characterization of microorganisms……..……30

4.7.1 Determination of Gram reaction…….……30

4.7.2 Preparation of agar slides..……31

4.8 Analytical methods……...31

4.8.1 Gas Chromatography (GC)……....31

4.8.2 High-performance liquid chromatography (HPLC)…….32

4.8.3 Preparation of samples for HPLC and GC……32

4.9 Biochemical methods……..33

4.9.1 Protein determination……..33

4.9.2 Preparation of cell extracts……33

4.9.3 Detection of the acetone-carboxylase activity in nitrate-reducing bacteria…….……33

4.9.4 Coupled continuous enzyme tests……..……34

4.9.4.1 Propan-2-ol: $NADP^+$ oxidoreductase (EC 1.1.1.180)……34

4.9.4.2 Acetone carboxylase enzyme (EC 6.4.1.6)……..35

4.9.4.3 Carbon monoxide dehydrogenase (EC 1.2.99.2)……..35

4.9.4.4 3-hydroxybutyratyl-CoA dehydrogenase (EC 1.1.1.157)…….36

4.9.4.5 Butyrate-acetoacetate CoA-transferase (EC 2.8.3.9)…….……..36

4.9.4.6 Acetyl-CoA C Acetyltransferase (EC 2.1.3.9)….....36

4.9.5 Enrichment of the acetone carboxylase enzyme…….37

4.10 Molecular biological methods…….37

4.10.1 Extraction of DNA……37

4.10.2 Polymerase chain reaction (PCR)……38

4.10.3 Purification and sequencing of PCR products……38

4.10.4 DNA sequence analysis……..38

4.10.5 SDS-PAGE (SDS-PolyAcrylamid Gel Electrophoresis), NMR……..……38

5 Results.....40

5.1 Syntrophic methanogenic enrichments…….40

5.1.1 Characterization of syntrophic methanogenic enrichment cultures, KN-Ipr and KN-Act..……..40

5.1.2 Degradation experiments……...41

5.1.3 Sodium dependence of acetone degradation……...43

5.1.4 Sodium dependence of acetate degradation……44

5.1.5 Dependence of acetone degradation on carbon dioxide……..44

5.1.6 Isolation of isopropanol and acetone-degrading bacteria……46

5.1.7 Isolation and characterization of acetone-fermenting bacteria from the enrichment-culture KN-Act……...46

5.2 Sulfate-reducing bacteria……...50

5.2.1 Acetone degradation by *Desulfococcus biacutus*……..50

5.2.1.1 Enzymes of the carbonylation reaction system (Reppe carbonylation of alkenes)……..50

5.2.2 Addition of fumaric acid…….51

5.2.3 Labelling experiments…….51

5.3 Nitrate-reducing bacteria……52

5.3.1 Isolation of strain KN Bun08……52

5.3.2 Physiological characterization of strain KN Bun08……53

5.3.3 Phylogenetic characterization of strain KN Bun08…….55

5.3.4 Enrichment and characterization of acetone carboxylase……56

5.3.5 Comparison with *Alicycliphilus denitrificans*, strains BC and K601…..59

6 Discussion……..……..61

6.1 Methanogenic enrichments……...61

6.2 Sulfate-reducing bacteria……...62

6.3 Nitrate-reducing bacteria……64

6.3.1 Isolation of a novel acetone-degrading nitrate-reducer…….64

6.3.2 Measurement of acetone-carboxylase activity…….65

6.3.3 Characterization of acetone-carboxylase……..67

6.3.4 Comparison with acetone-carboxylases……67

7 Literature……..……69

1 Summary

The present work focused on the physiology and biochemistry of the anaerobic biodegradation of isopropanol and acetone and the elucidation of reactions and mechanisms which are involved in acetone degradation by anaerobic bacteria. For the investigation of physiological and biochemical aspects that are involved in these anaerobic degradation processes, syntrophic methanogenic enrichments, sulfate-reducing bacteria and nitrate-reducing strains were enriched and isolated.

Isopropanol and acetone degradation was investigated in growing cultures and in dense cell suspensions of methanogenic syntrophic co-cultures enriched and pre-cultivated with isopropanol or acetone. Bacterial organisms present in the enrichment co-cultures which are responsible for degradation of isopropanol and acetone were isolated and identified. An isolated isopropanol-degrading organism was identified as *Methanospirillum hungatei* and the analysis of the 16S rDNA of the unknown acetone fermenting organism present in the enrichment co-culture KN-Act indicated high similarity with an organism of the genus *Desulfosporosinus.*

In biodegradation experiments with growing cultures or dense cell suspensions possible metabolic pathways which are involved in the anaerobic isopropanol and acetone degradation could be demonstrated. The existence of a supposed membrane-bounded, sodium ion (Na^+)-dependent system which could provide the energy for the anaerobic acetone activation was determined. Acetate degradation by the methanogenic bacteria in the co-culture KN-Act was independent of the presence of sodium-ions. Accumulation of acetate in the medium was observed only when the methanogenic partner was inhibited by bromoethane sulfonate.

A strain of *Desulfococcus biacutus* was used for the investigation of the acetone-degrading pathways applied by sulfate-reducing bacteria. The incorporation of a carbon monoxide molecule (Reppe carbonylation of alkenes) in organic compounds as acetone appeared to be feasible for anaerobic acetone degradation by *D. biacutus*. The occurrence of specifically induced enzymes which are involved in the

acetone metabolism and the possible involvement of a carbonylation system was investigated in cell-free extracts of *D. biactus* after anaerobic growth with acetone and sulfate. Two further strategies were investigated in dense cell suspensions of *D. biacutus*. One is the increasing of acetone degradation rates parallel to the addition of fumaric acid on acetone with the respective formation of 2-oxopropyl succinate similar to the beta-oxidation of pyruvate and succinate.

The detection of an acetone carboxylation reaction was investigated in cell-free extracts of three different nitrate-reducing strains: *Paracoccus denitrificans*, *P. pantotrophus,* and the newly isolated acetone-degrading, nitrate-reducing strain KN Bun08. Initially, *in vitro* experiments were performed with crude cell-free extracts of the nitrate-reducing organisms mentioned above after anaerobic growth on acetone with reduction of nitrate. For detection of the acetone carboxylation reaction a coupled continuous, a modified enzyme test based on the use of the help enzymes Adenylate kinase (EC 2.7.4.3), also called Myokinase, Pyruvate kinase (EC 2.7.1.40) and Lactate dehydrogenase (EC 1.1.1.27) with the concomitant oxidation of NADH was used. The results show that an acetone carboxylation reaction was detected in cell-free extracts with the enriched acetone carboxylase enzyme from acetone grown cells of *P. denitrificans, P. pantotrophus* and strain KN Bun08. The activity of the ATP-dependent acetone carboxylase enzyme was measured by the formation of ADP and by the oxidation of NADH in a continuous spectrophotometric assay after chromatographic enrichment of the acetone carboxylase enzyme with two enrichment steps on a DEAE-sepharose column. Enzyme activities of 0.4, 0.03 and 0.2 U/mg protein were measured, respectively, for *P. denitrificans, P. pantotrophus* and KN-Bun08 after the second enrichment with DEAE-sepharose column. The activity was dependent on the addition of acetone and ATP, and was correlated with the amount of protein in the reaction mixture. After the last enrichment step, three subunits (alpha, beta and gamma subunits) of the acetone carboxylase enzyme were visible with SDS-PAGE and proteins were identified by mass spectrophotometry.

Zusammenfassung

Diese Arbeit konzentrierte sich auf die Physiologie und Biochemie des anaeroben Abbaus von Isopropanol und Aceton, sowie auf die Aufklärung von Reaktionsmechanismen, welche am Acetonabbau in anaeroben Bakterien beteiligt sind. Untersucht wurden diese physiologischen und biochemischen Aspekte in syntrophen, methanogenen Anreicherungen, sulfatreduzierenden Bakterien und nitratreduzierenden Stämmen, welche dafür angereichert und isoliert wurden.

Die Abbaureaktionen von Isopropanol und Aceton wurden mit wachsenden Kulturen und in dichten Zellsuspensionen von methanogenen, syntrophen Co-Kulturen, welche auf Isopropanol oder Aceton angereichert und vorkultiviert wurden, nachgewiesen. Bakterielle Organismen aus den angereicherten Co-Kulturen, welche für den Abbau von Isopropanol und Aceton verantwortlich waren, wurden isoliert und phylogenetisch eingeordnet. Ein isolierter, isopropanolabbauender Organismus konnte durch Sequenzierung und Alignment des Gens der 16S rRNA als *Methanospirillum hungatei* identifiziert warden. Die Analyse der 16S rDNA eines unbekannten, acetonfermentierenden Organismus aus der angereicherten Co-Kultur KN-Act zeigte phylogenetische Ähnlichkeit mit einem Organismus der Gattung *Desulfosporosinus.*

In Abbauexperimenten mit wachsenden Kulturen und dichten Zellsuspensionen konnten mögliche Abbauwege, welche am anaeroben Isopropanol- und Acetonabbau beteiligt sind, nachgewiesen werden. Das Vorhandensein eines bereits vermuteten, membrangebundenen, von Natriumionen abhängigen Transportsystems, welches die Energie zur anaeroben Aktivierung des Acetonmoleküls zur Verfügung stellt, konnte gezeigt werden. Der Abbau von Acetat durch methanogene Bakterien in der Co-Kultur KN-Act war Natrium-abhängig. Eine Akkumulierung von Acetat im Kulturmedium fand nur statt, wenn der methanogene Partner durch Bromethansulfonat gehemmt wurde.

Ein Stamm von *Desulfococcus biacutus* wurde verwendet, um die acetonabbauenden Reaktionswege in sulfatreduzierenden Bakterien aufzuklären. Das Einfügen eines Kohlenstoffmonoxidmoleküls in organische Verbindungen wie Aceton (Reppe Carbonylierung von Alkenen) erschien plausibel für den anaeroben Acetonabbau durch *D. biacutus*. Das Vorhandensein von spezifisch induzierten Enzymen, welche im Acetonmetabolismus beteiligt sind, und die mögliche Beteiligung eines Carbonylierungssystems konnte in zellfreien Extrakten von *D. biactus* nach anaerober Kultivierung mit Aceton und Sulfat nachgewiesen werden. Zwei weitere Strategien wurden in dichten Zellsuspensionen von *D. biacutus* nachgewiesen. Eine davon ist der Anstieg der Acetonabbauraten durch Zugabe von Fumarat mit der nachweislichen Bildung von 2-Oxopropylsuccinat ähnlich wie in der β-Oxidation von Pyruvat und Succinat.

Eine Aceton carboxylierende Reaktion wurde in zellfreien Extrakten aus drei verschiedenen nitratreduzierenden Stämmen nachgewiesen: *Paracoccus denitrificans*, *P. pantotrophus,* und dem neu isolierten, acetonabbauenden, nitratreduzierenden Stamm KN Bun08. Anfangs wurden *in vitro* Experimente mit zellfreiem Rohextrakt aus nitratreduzierenden Organismen, welche zuvor anaerob auf Aceton und Nitrat kultiviert wurden, gemacht. Für den Nachweis der Acetoncarboxylierungsreaktion wurde ein modifizierter, mit den Hilfsenzymen Adenylatkinase (EC 2.7.4.3), auch Myokinase genannt, Pyruvatkinase (EC 2.7.1.40) and Lactatdehydrogenase (EC 1.1.1.27) gekoppelter Enzymtest entwickelt, in dem NADH oxidiert wird. Die Ergebnisse zeigten, dass eine Acetoncarboxylierungsreaktion in zellfreien Extrakten mit angereicherter Acetoncarboxylase aus auf Aceton gewachsenen Zellen von *P. denitrificans, P. pantotrophus* und Stamm KN Bun08 zu sehen war. Die Aktivität der ATP-abhängigen Acetoncarboxylase wurde durch die ATP-Bildung und NADH-Oxidation in kontinuierlichen, spektrophotometrischen Untersuchungen nach chromatographischen Anreicherungen der Acetoncarboxylase mit zwei Anreinigungsschritten mit einer DEAE-Sepharose Säule nachgewiesen. Enzymaktivitäten von 0,4; 0,03 und 0,2 U/mg Protein wurden nachweislich in Extrakten aus *P. denitrificans, P. pantotrophus* and KN-Bun08 nach der zweiten Aufreinigung mit der DEAE-Sepharose Säule gemessen. Die Aktivität war abhängig

von der Aceton- und ATP- Zugabe und hing zusammen mit dem Proteingehalt im Reaktionsmix. Nach dem letzten Aufreinigungsschritt konnten drei Untereinheiten der Acetoncarboxylase (Alpha, Beta und Gamma Untereinheiten) mit SDS-PAGE sichtbar gemacht werden und mit Hilfe der Massenspektroskopie identifiziert werden.

Abbreviations

Acetyl-CoA	Acetyl coenzyme A
AMP	Adenosine monophosphate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
bidest.	Bidistilled water
BES	2-Bromo ethane sulfonic acid
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CoA-SH	free Coenzyme A
d	Day(s)
Da	Dalton
DH	Dehydrogenase
DNA	Desoxyribonucleic acid
rDNA	Ribosomal DNA
ε	Molar extinction coefficient
∆E	Absorption
eq.	Equation
E.C.	Enzyme Comitte Number
FID	Flame ionization detector
Fig.	Figure
g	Gravitational constant
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
GC	Gas chromatography
h	Hour(s)
HPLC	High performance liquid chromatography
IMP	Inosine monophosphate
ITP	Inosine triphosphate
l	Liter

LDH	Lactate dehydrogenase
min	Minute(s)
ml	Milliliter
mg	Milligram
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
OD	Optical density
PAGE	Polyacrylamid gelelectrophoresis
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate
rpm	Rotations per minute
SDS	Sodium dodecylsulfate
T	Time
µl	Microliter
U	Enzyme unit
UMP	Uridine monophosphate
UTP	Uridine triphosphate
vol	Volume
W	Weight
% (v/v)	Volume percent
% (w/v)	Weight percent

3 Introduction

3.1 Isopropanol metabolism

Isopropanol (also isopropyl alcohol, propan-2-ol) is the most widely used volatile organic compound and is applied by the chemical and pharmaceutical industry for the manufacture of a variety of commercial and consumer products. It is used as antifreeze agent, as antiseptic and cleaning solution and as solvent for cosmetics (Harris, 1991). Due to its high production and application, isopropanol enters the environment via various emissions. The world production of isopropanol in the year 2000 was 2.1 x 10^6 tonnes (Weissermel and Arpe, 1978).

In nature, isopropanol is released during microbial degradation of animal wastes by bacteria as a metabolic side product of some clostridial fermentations as for example by *Clostridium beijerinckii*. The degradation of isopropanol was first studied by Forster (1940) in the photosynthetic bacterial strain *Rubrivivax gelationosus*. These bacteria utilize isopropanol as the sole carbon source. Subsequent studies on isopropanol degradation, described by Siegel (1950), demonstrated that isopropanol is degraded trough a dehydrogenation reaction by a variety of bacterial strains that utilize isopropanol as a unique energy and carbon source. Some of these organisms have the capacity to support high concentrations of isopropanol. For example, *Sphingobacterium mizutaii* can grow in the presence of 38 g $\cdot$ l^{-1} of isopropanol and produce acetone as an intermediary product (Mohammad, 2006). Only little information existed about the biochemical characteristics of the isopropanol dehydrogenase enzyme (EC 1.1.1.80) which is involved in the isopropanol degradation reaction. Isopropanol is also used to produce acetone industrially in large-scale plants via dehydrogenation (Saito et al., 1994).

3.2 Acetone metabolism

Acetone is an organic compound which is chemically allocated to the group of aliphatic ketones. It is released as a metabolic end product of fungi, of bacteria and can be produced and excreted by mammals during starvation of cells or by diabetic mammalians (Widmark, 1920). In bacteria acetone is produced, for example, due to fermentation of carbohydrates by *Bacillus macerans* (Schardinger, 1905) and by *Clostridium acetobutylicum* (Northrop et al., 1919; Davies and Stephenson, 1941).

In large-scale plants, acetone is produced industrially via dehydrogenation of isopropanol and extensively applied in the chemical and cosmetic industry as a solvent. Due to its high production and applicability, acetone enters into natural environments via various emission sources. Some bacteria were shown to be tolerant to the presence of acetone in aquatic environments (some bacterial species are tolerant to acetone from 530 to 1.700 mg $\cdot$ l^{-1} and some green algae tolerate higher acetone concentrations up to 7.500 mg $\cdot$ l^{-1}). In aquatic systems, acetone can be easily transformed by organisms and its toxic effect is very weak compared with other water pollutants (Bringmann and Kühn 1977, 1978).

The physiology and biochemistry of bacterial acetone degradation were considered as a central interesting point during several years for a remarkable number of authors. The aerobic acetone degradation process was considered as a simple process (Roth, 1988). Bacteria such as *Rhodococcus rhodochrous* and the phototrophic *Rhodobacter capsulatus* were used to study the aerobic acetone degradation. These organisms were grown in the presence of acetone and utilized it as carbon and energy source (Ensign et al., 1996, Ensign et al., 1997, Ensign et al., 2002). As reported by Clarke and Ensign (1999), some of these organisms can also oxidize isopropanol and use it for growth.

The major part of the information about the acetone carboxylation reaction comes from a series of studies with the obligate aerobe *Xanthobacter autotrophicus* strain Py2 (Sluis and Ensign, 1996, 1997 and 2002). The acetone carboxylase enzyme was

induced after growth of *X. autotrophicus* strain Py2 with acetone, and was composed of three different polypeptides that were purified and characterized. Aerobes, nitrate reducers and phototrophs activate acetone by a carboxylation reaction which is coupled to the hydrolysis of ATP to AMP plus 2 inorganic phosphates with the expenditure of two ATP equivalents, to produce acetoacetate (eq. 1) (Ensign et al., 1998).

$$CH_3COCH_3 + CO_2 + ATP \rightleftharpoons CH_3COCH_2COO^- + H^+ + AMP + 2\ P_i \quad \text{(eq. 1)}$$

Some mechanisms which are involved in bacterial acetone production have been studied in details. One of them is the production of acetone through dehydrogenation of isopropanol by isopropanol dehydrogenase (EC. 1.1.1.80). As mentioned above, the characteristic of this enzyme is still unknown. Much more is known about the production of acetone via decarboxylation of acetoacetate by the acetoacetate decarboxylase enzyme (EC 4.1.1.4) (Davis, 1943; Zerner et al., 1966). The involvement of a carboxylation reaction was first observed by Robertson and Kühn (1983) with acetone and isopropanol grown cells of *Paracoccus pantotrophus*. The degradation of acetone and formation of acetoacetate occur under aerobic and anaerobic conditions. The reaction was dependent on the exogenous supply of carbon dioxide (Bonnet - Smits, 1988).

In anoxic habitats acetone can be formed as a metabolic product via bacterial fermentation, for example in *Clostridium acetobutylicum* (Davies and Stephenson, 1941) and in *Bacillus macerans* (Schardinger, 1905; Northrop et al, 1919) or by the dehydrogenation of isopropanol in methanogenic bacteria (Widdel, 1996; Zellner and Winter, 1987; Widdel et al., 1988; Zellner et al., 1989). Acetone degradation under anaerobic conditions has been studied in the past with nitrate-reducing strains and methanogenic, syntrophic co-cultures. In both cases, acetone was activated through a carboxylation reaction (Platen and Schink, 1987; 1989). Whereas aerobes and

phototrophic bacteria invest 2 ATP equivalents into the acetone carboxylation reaction, acetone-degrading, fermenting organisms have less than one ATP equivalent per reaction to allow the carboxylation of acetone and formation of acetoacetate (eq. 2).

$$CH_3COCH_3 + CO_2 \rightleftharpoons CH_3COCH_2COO^- + H^+, \Delta G^{0'} = +17.1 \text{ kJ/ mol acetone} \quad \text{(eq. 2)}$$

In syntrophic methanogenic co-cultures, acetoacetate is cleaved to acetate which is fermented to methane. In this context, anaerobic acetone degradation occurs in a way different from that used by aerobes and phototrophic organisms (Schink, 1997). Most studies on anaerobic acetone degradation via carboxylation reaction were done with intact cells of nitrate-reducing bacteria (Platen, 1989, 1990), sulfate-reducers (Platen, 1990 and Janssen, 1995) and phototrophic anaerobic bacteria (Birks, 1997). The partial characterization of the acetone carboxylation enzyme indicated that the biodegradation of acetone depends on the availability of CO_2 (Platen and Schink, 1987; Bonnet-Smits et al., 1988; Birks and Kelly, 1997). In cell-free extracts it was possible to detect the CO_2- and ATP-dependent acetone carboxylation reaction and the production of acetoacetate, respectively (Sluis et al., 1996). The acetone-carboxylating enzyme of *X. autotrophicus* has been isolated and purified, but the reaction mechanism is not completely understood (Sluis and Ensign, 1997; Sluis et al., 2002; Boyd et al., 2004).

3.3 Aims

The central aim of this work was focused on the study of physiological and biochemical aspects of the anaerobic biodegradation of isopropanol and acetone. The first part of this work was centered on the enrichment, isolation and characterization of bacteria with the metabolic ability to utilize isopropanol and acetone under anoxic conditions. The second part contained the identification of

anaerobic acetone degradation pathways through detection of specific enzymes in cell extracts of acetone-grown cells of sulfate- and nitrate-reducing strains.

The objectives of this work were focused on the elucidation of reaction mechanisms that are involved in the activation of acetone by anaerobic bacteria. Sulfate-reducing and fermenting bacteria live under energy-limited conditions that do not permit to use a carboxylation reaction for acetone activation as described for aerobic and phototrophic bacteria.

Syntrophic, methanogenic enrichments

The physiology of the anaerobic isopropanol and acetone biodegradation was investigated in two syntrophic methanogenic enrichment co-cultures, pre-cultivated with isopropanol or acetone as the sole energy and carbon source. Bacteria present in the enrichment cultures which are responsible for degradation of isopropanol and acetone were isolated and identified. According to the scheme depicted in Fig. 01, isopropanol should be first oxidized to acetone by a hydrogen-utilizing organism. In this scheme, acetone is activated to acetoacetate and degraded to acetate by an unknown acetone-fermenting organism. Acetate is then converted to methane and carbon dioxide by a methanogenic partner. This possible metabolic pathway was tested in biodegradation experiments with growing cultures or dense cell suspensions.

Furthermore in biodegradation tests with growing cultures or dense cell suspensions the formation of degradation products was followed and compared with biodegradation tests with an inhibition of the methanogenic partner.

A system based on the translocation of sodium ions (Na^+) through the cell-membrane which could provide the energy for the anaerobic acetone activation was tried to determine in intact cells of the enrichment culture KN-Act.

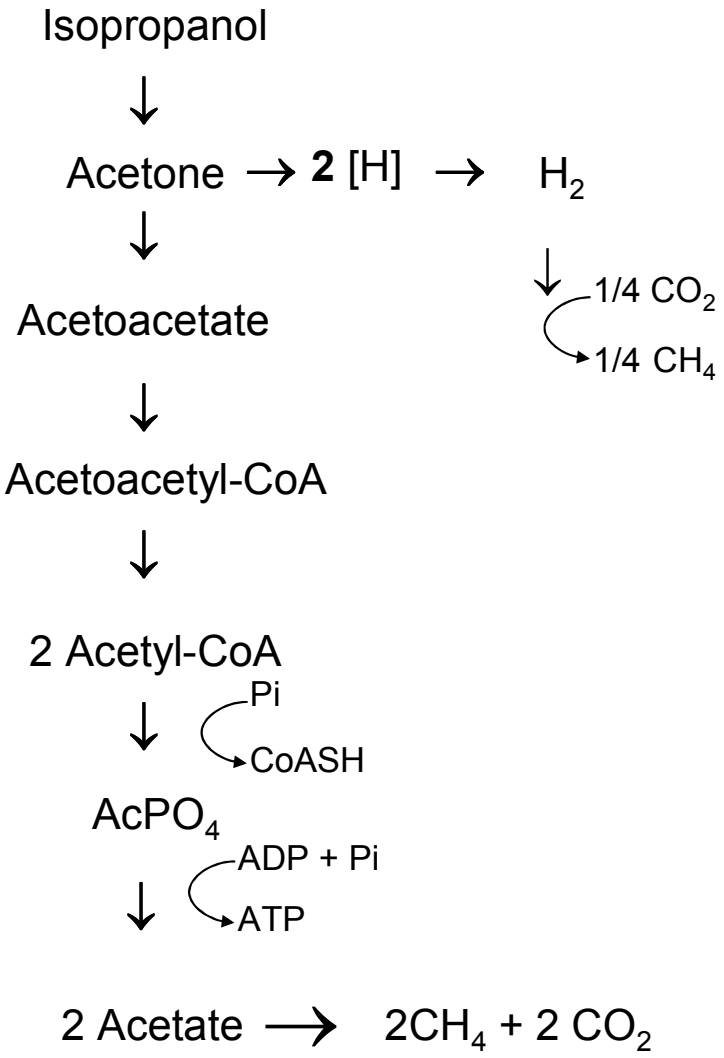


Fig. 01: Reaction scheme of anaerobic isopropanol and acetone biodegradation by syntrophic, methanogenic enrichment cultures. Isopropanol is degraded to acetone that is converted further to acetate by fermenting bacteria. Acetate is then degraded to methane and carbon dioxide by methanogenic, syntrophic partners.

Sulfate-reducing bacteria

The ability for anaerobic biodegradation of acetone was tested in pure cultures of the sulfate reducer *Desulfococcus biacutus*. Earlier assumptions from studies in the past indicated that acetone is degraded to an acetyl-CoA derivate. Biochemical characteristics suggested that the anaerobic acetone degradation by *D. biacutus* does not proceed through a carboxylation reaction. In this case the existence of a different metabolic pathway was tested through the measurement of enzyme activities in cell-free extracts and in dense cell suspensions.

One of the alternative concepts for acetone activation was an acetone carbonylation reaction, which was proposed and investigated in cell-free extracts of *D. biacutus* by identification of specific enzymes. Analoguous to the Reppe carbonylation reaction of alkenes, such a reaction should insert carbon monoxide into an acetone molecule and in this case form a 3-hydroxybutyryl derivate. Other studies indicated that sulfate-reducing organisms were able to oxidize acetyl residues through the carbon monoxide dehydrogenase pathway. In the context of a proposed acetone carbonylation concept (Fig. 02) the activity of the carbon monoxide dehydrogenase enzyme (EC 1.2.99.2) was tried to measure after growth of *D. biacutus* cells on acetone, 3-hydroxybutyrate, and pyruvate.

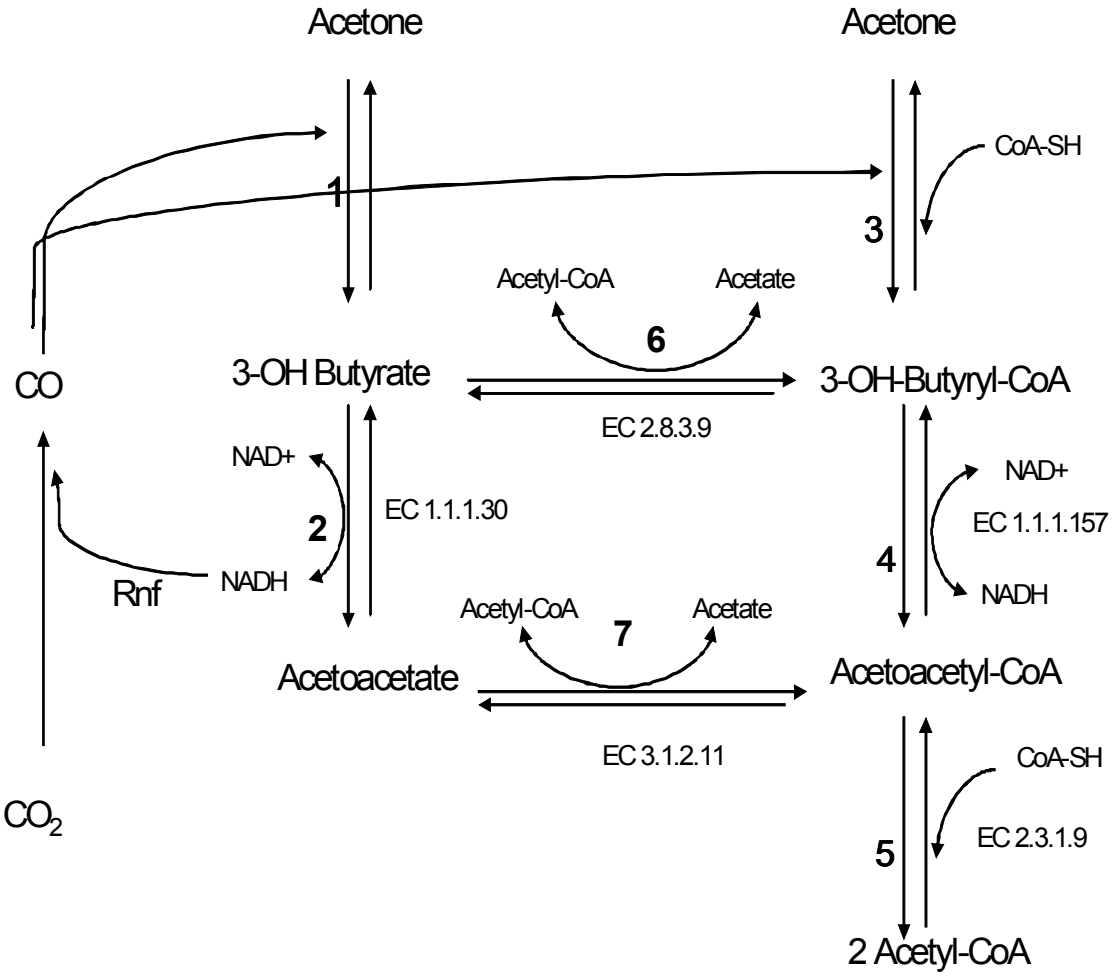


Fig. 02 Proposed scheme for the detection of enzymes involved in a hypothetical acetone carbonylation system through incorporation of carbon monoxide into acetone metabolism by *D. biacutus*.

Other strategies like the addition of acetone to the C-C double bond of fumarate with formation of 2-oxopropyl-succinate, analogous to the initial reaction in toluene degradation by *Thauera aromatica*, should be demonstrated in experiments with ^{13}C-labeled acetone in intact cells of *D. biacutus* after growth with acetone for identification of intermediate metabolites formed during acetone metabolism.

Nitrate-reducing bacteria

Biodegradation of acetone under denitrifying conditions was studied with the nitratereducing bacterial strains *Paracoccus denitrificans* and *Paracoccus pantotrophus*. In addition, an acetone-degrading, nitrate-reducing strain KN Bun08 was enriched and characterized.

Experiments for detection of the acetone carboxylation reaction were done, the carboxylation of acetone and its back reaction, the decarboxylation of acetoacetate, to determine optimal reaction conditions as buffer type, pH, and adequate nucleotides. Cell-free extracts of *Paracoccus denitrificans*, *Paracoccus pantotrophus* and strain KN Bun08 were used for *in vitro* detection of the acetone-carboxylating enzyme activity. The test was based on the measurement of substrate consumption rates and on the dependence of ATP with formation of derivates such as AMP or ADP.

A modified continuous enzyme test based on the coupling of helping enzymes with subsequent oxidation of NADH was used for the detection of the acetone carboxylase enzyme (EC 6.4.1.6) in enriched enzyme fractions obtained from cells of *Paracoccus denitrificans, Paracoccus pantotrophus* and strain KN-Bun08 after anaerobic growth on acetone plus nitrate. The enriched acetone carboxylase enzyme was excised from SDS polyacrylamide gels, and analyzed by molecular mass fingerprinting.

4 Materials and Methods

4.1 Chemicals

Analytical reagents, chemicals, and biochemicals were obtained from Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), Sigma (Deisenhofen, Germany), Pharmacia (Freiburg i. B., Germany) and Boehringer (Mannheim, Germany). Gases were obtained from Sauerstoffwerke Friedrichshafen (Friedrichshafen, Germany).

4.2 Microbiological methods

4.2.1 Source of microorganisms

The methanogenic enrichment cultures KN-Ipr and KN-Act were enriched from a anaerobic sludge sample of the municipal wastewater treatment plant of Constance, Germany. *Methanospirillum hungatei* was isolated from the methanogenic enrichment culture KN-Ipr. *Desulfosporosinus sp*. was isolated from the enrichment culture KN-Act. *Desulfococcus biacutus*, strain KMRActS was originally enriched from an anaerobic sludge sample of the municipal wastewater treatment plant of Marburg, Germany.

Paracoccus denitrificans (DSM ^{T45}) and *Paracoccus pantotrophus* were obtained from the DSMZ (German Collection of Microorganisms and Cell Culture, Braunschweig, Germany). Strain KN Bun08 was enriched from a small tarn sediment sample close to the University of Konstanz, Konstanz, Germany. *Alicycliphilus sp.* Strains BC1 and K601 were obtained from the group of Prof. Alfons Stams from the University of Wageningen, Wageningen, The Netherlands.

4.2.2 Preparation of liquid medium

4.2.2.1 Bicarbonate-buffered medium

Sulfate-reducing bacteria and methanogenic enrichment cultures were cultivated in anoxic bicarbonate-buffered (30 mM), sulfide-reduced, freshwater mineral medium (Widdel and Bak, 1992) containing (in $g \cdot l^{-1}$): $MgCl_2 \cdot 6\ H_2O$ (0.4); KH_2PO_4 (0.2); NH_4Cl (0.5); $CaCl_2 \cdot 2\ H_2O$ (0.15), KCl (0.5) and NaCl (1.0). The medium was reduced with $Na_2S \cdot 9\ H_2O$ (2 $ml \cdot l^{-1}$) and resazurine (0.4 $mg \cdot l^{-1}$) was added as a redox indicator. The medium was prepared in a Widdel flask (5 or 10 l volume) and autoclaved for 60 min at 121°C. After autoclaving the medium cooled down to room temperature under constant gas phase exchange with N_2/CO_2 gas mixture (80:20). Seven-vitamin solution (0,5 $ml \cdot l^{-1}$), selenite and tungstate solution (1 $ml \cdot l^{-1}$) and microelement solution SL10 (1 $ml \cdot l^{-1}$) were added to the medium and the pH was adjusted ranging from 7.1 to 7.3 with the addition of 1 M HCl (Widdel et al., 1983). For cultivation of organisms the medium was filled (50 to 60 ml) into 125 ml Müller-Krempel bottles (Müller & Krempel, Bülach, Switzerland) or into 120 ml serum bottles (Pharmapack, Königswinter, Germany) and sealed tightly with butyl rubber septa (Maag Technic, Dübendorf, Switzerland). The headspace atmosphere was filled with N_2/CO_2 gas mixture (80:20).

4.2.2.2 CO_2-free medium

A CO_2-free medium was prepared as described above in 4.2.2.1. Sodium bicarbonate was omitted and the medium was buffered with potassium phosphate (20 mM). N_2 gas was used for the gas phase exchange.

4.2.2.3 Sodium-free medium

Sodium-free medium was buffered with potassium hydrogen bicarbonate (30 mM) and reduced with sodium-free cysteine (2 mM). To minimize sodium presence in

medium all bottles and necessary materials were immersed in HCl solution 5% (v/v) and washed three times in ultra-pure water. The final sodium concentration in the medium was 100 µM.

4.2.2.4 Phosphate-buffered medium

For cultivation of nitrate-reducing bacteria, a phosphate-buffered (30 mM) non-reduced anoxic freshwater mineral medium was prepared. The medium contained (in $g \cdot l^{-1}$): $MgCl_2 \cdot 6\ H_2O$ (0.4); KH_2PO_4 (0.2); NH_4Cl (0.5); $CaCl_2 \cdot 2\ H_2O$ (0.15); NaCl (1.0) and KCl (0.5). After autoclaving, N_2 gas was used for gas exchange. Seven-vitamin solution (0.5 $ml \cdot l^{-1}$), selenite and tungstate solution (1 $ml \cdot l^{-1}$) and microelements solution SL10 (1 $ml \cdot l^{-1}$) were added to the medium and the pH was adjusted ranging from 7.2 to 7.4 with 1 M HCl.

4.2.2.5 Aerobic phosphate-buffered medium (modified by Widdel, 1980)

Medium containing (in $g \cdot l^{-1}$): $MgCl_2 \cdot 6\ H_2O$ (0.4); KH_2PO_4 (1.4); NH_4Cl (0.5); $CaSO_4$ (0.15); NaCl (1.0) and KCl (0.5). The medium was buffered with potassium phosphate (30 mM) and contained seven-vitamin solution (0.5 $ml \cdot l^{-1}$), selenite and tungstate solution (1 $ml \cdot l^{-1}$) and microelements solution SL10 (1 $ml \cdot l^{-1}$). The pH was adjusted ranging from 7.2 to 7.4 with 1 M HCl.

4.2.2.6 Sporulation medium (DSMZ, Catalog 1993, Medium Nr. 12)

To prepare a soil extract, air-dried garden soil (400 g) with high organic material content was sterilized in 1000 ml distilled water for 1 hour at 121°C. After sedimentation (3 to 4 hours) the supernatant was taken off and centrifuged at room temperature. Anoxic, bicarbonate-buffered sulfide-reduced medium (4.2.2.1), containing 10 % of the soil extract was complemented with 100 µM $MnCl_2$.

4.2.2.7 Microelement solution SL 10 (Widdel et al., 1983)

Microelement solution contained (in $mg \cdot l^{-1}$): $FeCl_2 \cdot 4\ H_2O$ (1500); $ZnCl_2$ (70); $MnCl_2 \cdot 4\ H_2O$ (100); $CoCl_2 \cdot 6\ H_2O$ (190); $CuCl_2 \cdot 2\ H_2O$ (2); $NiCl_2 \cdot 6\ H_2O$ (2); $NaMoO_4 \cdot 2\ H_2O$ (36); H_3BO_3 (6); HCl 25% solution (10 ml) in 1000 ml distilled water. The solution was sterilized in the autoclave for 25 min at 121°C.

4.2.2.8 Selenite-Tungstate solution (Widdel et al., 1983)

Solution contained (in mg · l^{-1}): NaOH (500); Na_2SeO_3 · 5 H_2O (3) and Na_2WO_4 · 2 H_2O. The solution was sterilized in the autoclave, 25 min, at 121°C.

4.2.2.9 Seven-vitamin solution (Widdel and Pfennig, 1981)

The seven-vitamin solution contained (in mg · l^{-1}): cyanocobalamine (10); p-aminobenzoate (10); D(+)-biotin (2); nicotinic acid (20); Ca-D(+)-pantothenic acid (100); pyridoxamine-di-hydrochloride (300) and thiamine-di-hydrochloride (200). The solution was filter-sterilized and stored at 4°C in dark.

4.3 Preparation of substrates

Anoxic substrate solutions were prepared in 120 ml serum bottles at concentrations ranging from 0.5 to 1 M. Substrate solutions (isopropanol, acetone, acetate, 3-hydroxybutyrate, butanone, butyrate, hexanone, malate, pyruvate, formate, fumarate, succinate, DL-lactate, propionate and citrate) were prepared with ultra-pure water and sterilized in the autoclave.

4.4 Preparation of electron acceptor solutions

Anoxic sodium nitrate and sodium sulfate solutions (1 to 2 M) were prepared in 125 and 500 ml Müller-Krempel bottles and sterilized in the autoclave for 25 min at 121°C. For sodium-free experiments a potassium sulfate solution was prepared.

4.5 Cultivation of microorganisms

The methanogenic enrichment cultures KN-Ipr and KN-Act were cultivated in anoxic, bicarbonate-buffered and sulfide-reduced mineral medium (4.2.2.1) (Widdel et al., 1983), which contained, respectively, 5 to 15 mM isopropanol and acetone as substrate. *Methanospiriullum hungatei* was cultivated under the same conditions with isopropanol (10 to 20 mM).

The sulfate-reducing strain *Desulfococcus biacutus* was cultivated under the same conditions with acetone (10 mM) as the sole energy source and sulfate (20 mM) as an electron acceptor.

Nitrate reducers were cultivated in anoxic, non-reduced, phosphate-buffered freshwater medium which contained acetone (10 mM) and nitrate (20 mM). Bacteria were grown in 120 ml, 500 ml, or 1000 ml Müller-Krempel bottles (Müller & Krempel, Bülach, Switzerland) or in 120 ml serum bottles. The headspace was filled with N_2 or N_2/CO_2 (80:20) and the bottles were sealed with butyl rubber (Bellco Glass Inc., Vineland, USA) septa. Bacteria were cultivated in the dark at 28°C.

4.6 Isolation of microorganisms

4.6.1 Isolation of methanogenic archaea

An isopropanol-degrading, hydrogen-utilizing organism was isolated from the methanogenic enrichment culture KN-Ipr. Culture aliquots (100 to 200 µl) of the methanogenic enrichment culture KN-Ipr were inoculated in 27 ml Hungate tubes (125 mm, ø 16,5 mm), filled with anoxic, bicarbonate-buffered, sulfide-reduced freshwater medium (4.2.2.1) (9 ml) containing isopropanol (10 to 20 mM) as the sole carbon and energy source.

4.6.2 Isolation of sulfate-reducers

100 to 250 µl samples of the enrichment culture KN-Act were taken anoxically to inoculate agar dilution series in tubes. For the agar dilution series, an agar solution (3% w/v) was prepared as described by Pfennig and Trüper (1981) and filled (3 ml per tube) in sterile tubes which were primed with anoxic, carbonate-buffered, sulfide-reduced freshwater medium (6 ml per tube) containing acetone (10 mM) and sulfate (20 mM). Tubes containing N_2/CO_2 (80:20) in the headspace were sealed with butyl rubber septa and incubated upside down in the dark at 28°C.

After 2 weeks of incubation, single colonies were picked anoxically and inoculated into tubes with anoxic, carbonate-buffered, sulfide-reduced freshwater medium (5 ml) containing acetone (10 mM) and sulfate (20 mM). 500 µl of the acetone-degrading organisms were taken sterile and anoxically from the highest dilution series and inoculated into serum bottles (120 ml) containing anoxic, bicarbonate-buffered sulfide-reduced freshwater medium (50 ml) and incubated in the dark at 28°C. To isolate acetone-degrading bacteria the agar dilution technique for isolation of methanogens was applied as described above.

Aliquots of the enrichment culture KN-Act (100 to 250 µl) were diluted in tubes with anoxic bicarbonate-buffered, sulfide-reduced medium (5 ml) containing acetone (10 mM) and sulfate (20 mM). The tubes were incubated in the dark at 28°C and the formation of spores was observed. To isolate spore-forming bacteria from contaminants, the aliquots were pasteurized as described in 4.6.2.2.

4.6.2.1 Sporulation test

Aliquots (3 ml) of a defined culture of acetone-degrading fermenting bacteria were inoculated in tubes containing sporulation medium (7 ml). The tubes were incubated in the dark at 28°C. The formation of spores was constantly verified with light-microscopy during the incubation time ranging from 1 to 3 weeks.

4.6.2.2 Pasteurisation test

For the elimination of non-sporulating contaminants, samples (10 ml) of the acetone-degrading sulfate-reducing bacteria were distributed in anoxic glass tubes sealed with butyl rubber septa. Tubes were immersed in a water bath for 15 min at 80°C. Culture samples were inoculated immediately in 120 ml Müller & Krempel bottles with anoxic, bicarbonate-buffered, freshwater medium (60 ml) containing acetone (10 mM) and sulfate (20 mM). Bottles were incubated in the dark at 28°C.

4.6.3 Isolation of nitrate-reducing bacteria

A nitrate-reducing bacterial strain was enriched from a small tarn sediment sample (20 cm depth) localized close to the University of Konstanz, Konstanz, Germany. 1 g sediment was diluted in 10 ml of sterile distilled water and 1 ml was inoculated in anoxic bicarbonate buffered, non-reduced freshwater medium containing butanone (10 mM) and nitrate (20 mM), and incubated in the dark at 28°C. After enrichment on butanone the cells were transferred successively and isolated under anoxic conditions on acetone (10 mM) and nitrate (20 mM).

4.7 Characterization of organisms

4.7.1 Determination of Gram reaction

The method described by Bartholomew (1962), was used for the determination of the Gram reaction. Additionally a potassium hydroxide test was used as described by Gregersen (1978). As reference organisms *Acetobacterium woodii* (Gram-positive) and *Desulfovibrio vulgaris* (Gram-negative) were used.

4.7.2 Preparation of agar slides

A modified method described by Wagener et al. (1986) was used for the preparation of agar slides for the microscopy and photography of bacteria. With this method, the cells become immobilized on glass slides which are coated with agarose. For preparation of the slides, 1.6% agarose (w/v) solution was prepared and autoclaved for 20 min at 121°C. Glass slides were immersed first in 97% ethanol and 99% acetone solution to remove fat and dust. Slides were dried at 60°C and stored in appropriate boxes. Each slide was coated with approx. 2 ml of 1.6% agarose solution that was distributed uniformly with a pipette on the slide surface. Slides were placed appropriately on a clean and dust-free place and dried covered at room temperature for 24 hours.

4.8 Analytical methods

4.8.1 Gas Chromatography (GC)

The concentration of methane was measured with the gas chromatograph Carlo Erba GC Vega Series 6000 (Carlo Erba Instruments, Milan, Italy) with a flame-ionization detector (FID) by injection of 200 µl samples from the headspace of growing cultures or from the headspace of dense cell suspensions in biodegradation tests. Nitrogen (60 ml $\cdot$ min^{-1}) was used as carrier gas on a packed (60/80 mesh) CarboSieve (5 Ă, Serva, Heidelberg, Germany) column (2 m x 4 mm), heated to 120°C. The temperature of injector and detector was 150°C. Samples were taken and injected by a gas-tight syringe (Hamilton Company, Nevada, USA). The detection signal was analysed by an integrator system and the concentration of methane was calculated and compared with standards (0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 and 20%) in a calibration curve.

4.8.2 High-performance liquid chromatography (HPLC)

High-performance liquid chromatography or High pressure liquid chromatography (HPLC) is a chromatographic resource that is used intensively in analytical chemistry and biochemistry. HPLC-methods can be applied for separation, identification or quantification of a variety of liquid compounds.

For quantitative determination of isopropanol, acetone, acetate and acetoacetate samples (1 ml) were taken sterile and anoxic from growing cultures or from dense cell suspensions, and analyzed by HPLC. Samples were injected by an autoinjector (Gilson 234, Abimed, Langenfeld, Germany) and eluted in 5 mM H_2SO_4. A constant flow rate (0.6 ml min^{-1}) was maintained by a High Pressure Pump LC-10AT (Shimadzu, Duisburg, Germany). Samples were separated on an ion exchange column Aminex HPX-87H (BioRad Laboratories GmbH, Munich, Germany) at 60°C. Compounds were detected by light refractive index UV/Vis detector ERC 7512 (ERC Gesellschaft für den Vertrieb wissenschaftlicher Geräte mbH, Regensburg, Germany). The concentration of the compounds was calculated and compared with standards (0.05, 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 8, 10, 12, 15 and 20 mM).

4.8.3 Preparation of samples for HPLC and GC

For HPLC analysis the samples (950 µl liquid culture) were mixed with 50 µl 1M H_2SO_4 to stop the reaction and centrifuged (5 min at 13.000 rpm) at room temperature. The supernatant was taken (200 to 500 µl) and filled into HPLC vials.
For GC analysis the samples (950 µl of headspace) were mixed with 50 µl 10 M formic acid and centrifuged (5 min at 13.000 rpm) at room temperature. 20 µl of the supernatant was injected into the gas chromatograph.

4.9 Biochemical methods

4.9.1 Protein determination

Protein concentrations were determined by the microprotein BCA-Protein Assay Kit, Pierce (Thermo Science, Inc).

4.9.2 Preparation of cell extracts

Cells of the late exponential growth phase (OD_{600} = 0.4 to 0.54 after 7 days) of nitrate-reducing cultures of *P. denitrificans, P. pantotrophus* or KN Bun08 were harvested by centrifugation (20 min, 8.500 x *g*, at 4°C) in a RC-5B Sorvall Refrigerated Superspeed Centrifuge (DuPont Instruments, Bad Homburg, Germany). Cells of the sulfate-reducing strain *D. biacutus* were harvested in the late exponential growth phase (OD_{600} = 0.45 after 18 days) in an anoxic chamber (Coy, Ann Arbor, MI, USA) and centrifuged in 350 ml polypropylene beakers. Cells were washed (1 to 3 times) and suspended in anoxic Tris-HCl Buffer (50 mM), pH 7.0 to 8.0, or in anoxic potassium-phosphate-buffer, pH 7.0 to 8.5, and centrifuged for 20 min at 1.500 x *g* (for serum-bottles) and 4.000 x *g* for polypropylene cups, at 4°C. After centrifugation the pellet was suspended in buffer and the cells were disrupted with a French Press (Aminco, Silver Spring, USA) with 4 to 8 passages at 130 MPa. The cell debris was separated by centrifugation in an ultracentrifuge with a rotor GS-A for 20 min at 27.000 rpm at 4°C.

4.9.3 Detection of acetone carboxylase activity in nitrate-reducing bacteria

Non-photometric tests were performed for the detection of the acetone carboxylase reaction in crude cell extracts of *P. denitrificans* and *P. pantotrophus*, and of the acetone-degrading strain KN Bun08. The assay mixtures (3 ml) contained 100 mM Tris-HCl buffer, pH 8.0; 5 mM $MgCl_2$; 100 mM NH_4Cl and 1 mM Mn^{2+}.

The activity of acetone carboxylase was tested in both reaction directions. For the acetone carboxylation reaction, 10 mM ATP, 20 mM $NaHCO_3$ and 1.5 mM acetone

was added to the assay mixture. Assays for acetoacetate decarboxylation contained 10 mM AMP, 2 mM inorganic phosphate, and 1.5 mM acetoacetate. All assay mixtures contained approximately 1.5 to 2 mg protein/ ml. In control assays, acetone and acetoacetate, ATP and AMP, $NaHCO_3$ and inorganic phosphate and protein was omitted. Bottles were sealed with butyl rubber stoppers and incubated in a water bath at 28°C. After time intervals (0, 10, 20, 30, 60, 120 and 240 min) samples (1 ml) were taken, acidified and centrifuged (5 min, 13.000 rpm) at room temperature. The consumption and formation of acetone or acetoacetate, in both reaction ways, was determined by HPLC. For optimization of the reaction conditions the assay described above was performed at different pH ranges (6.5, 7.0, 7.5, 8.0, 8.5 and 9.0) and with different nucleotides like ITP, GTP and UTP.

4.9.4 Coupled continuous enzyme tests

Enzyme tests were assayed in anoxic quartz cuvettes sealed with rubber stoppers, in a spectrophotometer model 100-40 (Hitachi, Tokyo, Japan) which was connected to an analogous recorder (SE 120 Metrawatt, BBC Goerz, Vienna, Austria). To the assay mixture (1 ml) the substrates, co-enzymes and extracts were added anoxically with syringes. Two different perspectives of coupled enzyme tests for the detection of the acetone carboxylase reaction in cell-free extracts of nitrate-reducing bacteria were tested.

4.9.4.1 Propan-2-ol: $NADP^+$ oxidoreductase (EC 1.1.1.180)

The activity of the propan-2-ol:$NADP^+$ oxidoreductase, also called isopropanol dehydrogenase, was measured in cell-free extracts of isopropanol-grown cells of *P. denitrificans*, *P. pantotrophus* and strain KN Bun08. Isopropanol dehydrogenase ($NADP^+$) catalyzes the chemical reaction (eq. 01):

$$\text{propan-2-ol} + NADP^+ \rightleftharpoons \text{acetone} + NADPH + H^+ \qquad \text{(eq. 03)}$$

The transformation of substrates (propan-2-ol and $NADP^+$) and products (acetone, NADPH and H^+) was measured at 340 nm and registered on millimeter paper.

4.9.4.2 Acetone carboxylase enzyme (EC 6.4.1.6)

The acetone carboxylase enzyme was detected with a continuous coupled enzyme test modified by Ziegler and Fuchs (1987). Enzyme tests were performed for the detection of an ATP-dependent acetone carboxylase reaction with enriched acetone carboxylase enzyme from cell extracts of *P. denitrificans, P. pantotrophus* and from strain KN Bun08 after anaerobic growth on acetone (10 mM) and nitrate (20 mM). The cells were harvested in the late exponential growth phase (OD = 0.5 to 0.75). Cell pellets were washed in 100 mM Tris-HCl buffer, pH 7.0. An ATP-dependent acetone carboxylase reaction was detected by coupling the AMP- or ADP-formation to the oxidation of NADH by the Adenylate kinase (EC 2.7.4.3), Pyruvate kinase (EC 2.7.1.40) and Lactate dehydrogenase (EC 1.1.1.27).

Assay mixtures (0.8 to 1 ml) contained 2 mM phosphoenolpyruvate, 1 Unit/ml pyruvate kinase, and 4 Units/ml of lactate dehydrogenase. The cuvettes were incubated previously at 25°C for 3 min after addition of enriched acetone carboxylase (0.3 to 0.5 mg/ml). The reaction was started with addition of 5 mM acetone. The decrease of absorbance was measured with the oxidation of NADH (0.4 mM) by spectrophotometer at 340 nm. All assays were prepared anoxically and in triplicates. In controls assays ATP, acetone and HCO_3^- were omitted, and NADH oxidation rates were measured with different concentrations of enriched acetone carboxylase enzyme.

4.9.4.3 Carbon monoxide dehydrogenase (EC 1.2.99.2)

The activity of carbon monoxide dehydrogenase was assayed in 1.5 ml glass cuvettes containing 1 ml of 50 mM potassium phosphate buffer, pH 7.0 and 4 mM methyl viologen. The cuvettes were flushed with 100% carbon monoxide gas and stoppered with rubber septa. The assay mixture was reduced with sodium disulfide to

turn the reaction blue and started by the addition of enzyme. The dye reduction was monitored at 578 nm.

4.9.4.4 3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157)

3-hydroxybutyryl-CoA dehydrogenase catalyzes de reaction of 3-hydroxybutanoyl-CoA + $NADP^+$ to form 3-acetoacetyl-CoA + NAD(P)H + H^+. The substrates for 3-hydroxybutyryl-CoA dehydrogenase are 3-hydroxybutanoyl-CoA and $NADP^+$. 3-hydroxybutyryl-CoA dehydrogenase was assayed by measuring acetoacetyl-CoA dependent NAD(P)H oxidation. The assay was performed with 1 ml of 50 mM sodium phosphate buffer, pH 7.3, containing 0.25 mM NADPH and 1 mM acetoacetyl-CoA. The measurement of the reversible reaction was followed by the oxidation of NADH at 365 nm (Bergmeyer, 1974).

4.9.4.5 Butyrate-acetoacetate CoA-transferase (EC 2.8.3.9)

The enzyme catalizes the reaction of butanoyl-CoA + acetoacetate to form butanoate and acetoacetyl-CoA. The assay mixture was performed with 1 ml of 50 mM sodium phosphate buffer, pH 7.3, containing. Butyrate-acetoacetate CoA-transferase was measured in assay mixtures of 1 ml of 50 mM phosphate buffer, pH 8.0, containing 2 mM butanoyl-CoA and 2.5 mM acetoacetate. The reaction was started with the addition of enzyme.

4.9.4.6 Acetyl-CoA *C*-acetyl-transferase (EC 2.3.1.9)

The assay was performed in 1 ml of 50 mM phosphate buffer, pH 8.0, in anoxic glass cuvettes with 2 mM acetoacetyl CoA and 1 mM coenzyme A. The reaction was started with the addition of enzyme and the formation of acetyl-CoA was followed by measurements in the HPLC.

4.9.6 Enrichment of acetone carboxylase enzyme

For the enrichment of the acetone carboxylase enzyme, cells (11 g wet weight) of *P. denitrificans*, *P. pantotrophus* and strain KN Bun08 were applied to a DEAE-Sepharose column (Amersham Biosciences; diameter, 26 mm; volume, 70 ml). The column was equilibrated with buffer A (10 mM Tris/HCl, pH 7.0) and washed with three column volumes of buffer A while eluting in step gradients with 100, 150, and 220 mM KCl at a constant flow rate of 3 ml · min^{-1}. Enzyme fractions were applied in a coupled enzyme test as described above, and selected for successive enzyme enrichment steps. Selected fractions were pooled and concentrated by ultrafiltration (Amicon concentrator with YM-70 membrane).

The preconcentrated enzyme (pool I) was used for a second enrichment step and applied to a DEAE-Sepharose column (Amersham Biosciences; diameter, 26 mm; volume, 70 ml) equilibrated with buffer B (10 mM Tris/HCl, pH 8.0) and washed with three column volumes. Fractions were eluted with buffer B in step gradients from 150 to 300 mM KCl at a constant flow rate of 3 ml · min^{-1}. Fractions containing acetone carboxylase enzyme were selected by coupled enzyme tests and concentrated (pool II) by ultrafiltration (Amicon concentrator with a YM-30 membrane).

Aliquots of the concentrated pool II (1 to 1.5 ml) were further used for purification on a gel filtration column (Amersham Biosciences; diameter, 16 mm; volume 120 ml). The rest of concentrated pool II (300 µl) was used for identification of protein bands in SDS-PAGE.

4.10 Molecular biological methods

4.10.1 Extraction of DNA

DNA was extracted from the enrichment cultures KN Bun08 and an acetone-degrading, sulfate-reducing enrichment culture. Samples (1 to 3 ml) were centrifuged (10.000 x *g*) for 15 min at room temperature. After centrifugation, the supernatant was discarded and the DNA from the pellets was extracted using a Gentra Puregene

Tissue Core Kit (Qiagen, Hilden, Germany). Extracted DNA was eluted in 50 to 100 µl sterile water and stored at 4°C.

4.10.2 Polymerase chain reaction (PCR)

Bacterial 16S rDNA was amplified using the universal primers 27F (5`-AGA GTT TGA TCC TGG CTC AG-3´) and 1492R (5´-GGT TAC CTT GTT ACG ACT T-3´). 1 to 2 µl of extracted bacterial DNA was used as a template for the PCR reaction (25 cycles), as described by Henckel et al., 1999.

4.10.3 Purification and sequencing of PCR products

The PCR product was purified using a QIAquick PCR purification Kit (Qiagen, Hilden, Germany) and sequenced using the 27F, 533F, 907F and 1492R primers (GATC, Konstanz, Germany).

4.10.4 DNA sequence analysis

All sequences were analyzed using LASERGENE version 5.5 from DNAstar (Madison, Wisconsin, USA). Alignment of sequences was done using the NCBI public database. All sequences were assembled using DNAStar (www.dnastar.com) and analyzed by ARB software (version 2.5b http://www.arb-home.de, Ludwig et al., 1997). Phylogenetic tree was constructed using the MEGA4 Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 as described previouslyby (Tamura et al., 2007).

4.10.5 SDS-PAGE (SDS-PolyAcrylamid Gel Electrophoresis), NMR

For analysis of the enriched enzyme acetone carboxylase an SDS polyacrylamide gel was prepared. The gel was casted in large gel chambers (Protean xi, BioRad) containing a resolving and a stacking gel with, respectively, 12% and 4% polyacrylamide as described by Laemmli (1970). All samples were mixed 1:2 in

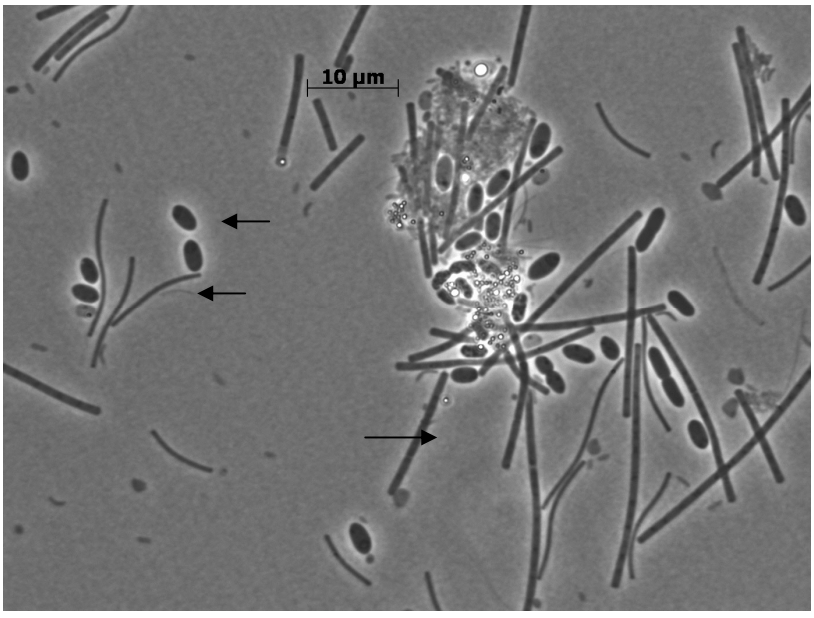
loading buffer (0.125 mM Tris-HCl, pH 6.8, 25% glycerol, 2% (w/v) SDS, 5% mercaptoethanol and 0.01% (w/v) bromophenolblue), and heated at 100°C for 5 min. After the runs (20 mA) gels were stained with Coomassie Brillant Blue R-250 as described by Neuhoff et al. (1988). Peptides from excised protein bands were analyzed by mass fingerprinting by the Proteomics Facility of the University of Konstanz. Peptides were analyzed by Mascot search engine and compared with the sequenced genome of *P. denitrificans*.

5 Results

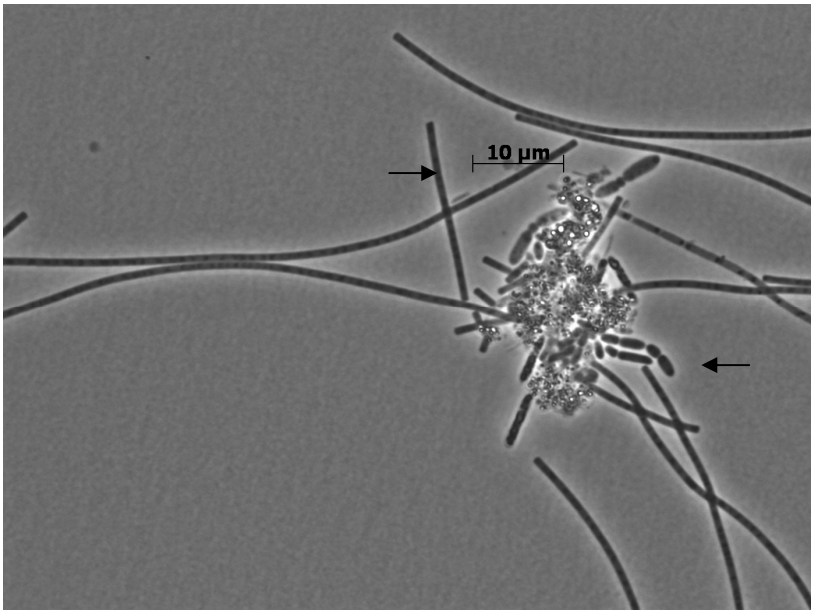
5.1 Syntrophic methanogenic enrichments

5.1.1 Characterisation of syntrophic methanogenic enrichment cultures, KN-Ipr and KN-Act

The isopropanol-degrading enrichment culture KN-Ipr utilized isopropanol and acetone as sole carbon and energy source. This culture (Fig. 3a) was composed of three types of dominant morphologically distinct microorganisms, (1) a spiral-shaped motile organism resembling *Methanospirillum hungatei*, (2) a rod-shaped acetone-degrading fermenting bacterium (0.9 x 1.5-2.0 µm in size) that was Gram-positive and non-motile and formed large aggregates with (3) a methanogenic acetate-degrading filamentous *Methanosaeta sp.*. The acetone-degrading enrichment culture KN-Act (Fig. 3b) was composed basically of two dominant microorganisms, (1) an acetone-fermenting rod-shaped bacterium and (2) methanogenic acetate- degrading filamentous *Methanosaeta*-like organisms. The formation of 100 to 300 µm large aggregates involving supposedly acetone-fermenting and the acetate-oxidizing methanogen was a common characteristic for both cultures.



(3a)

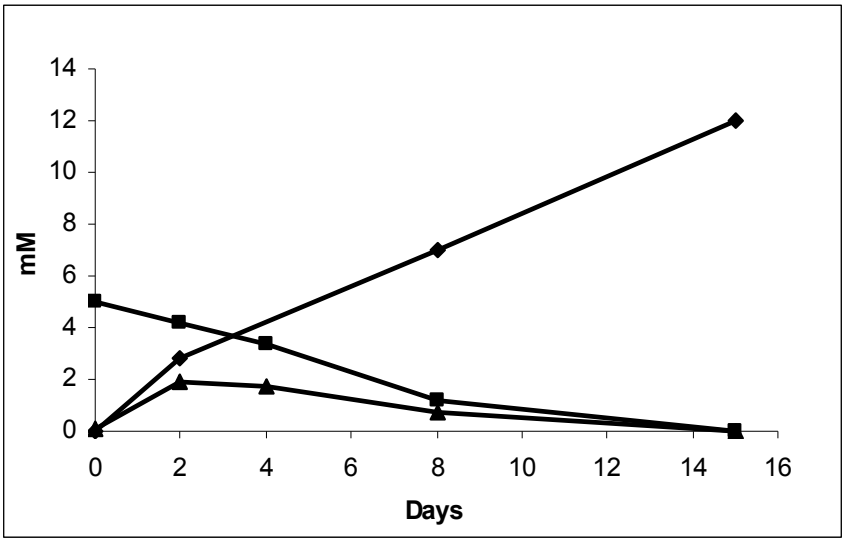


(3b)

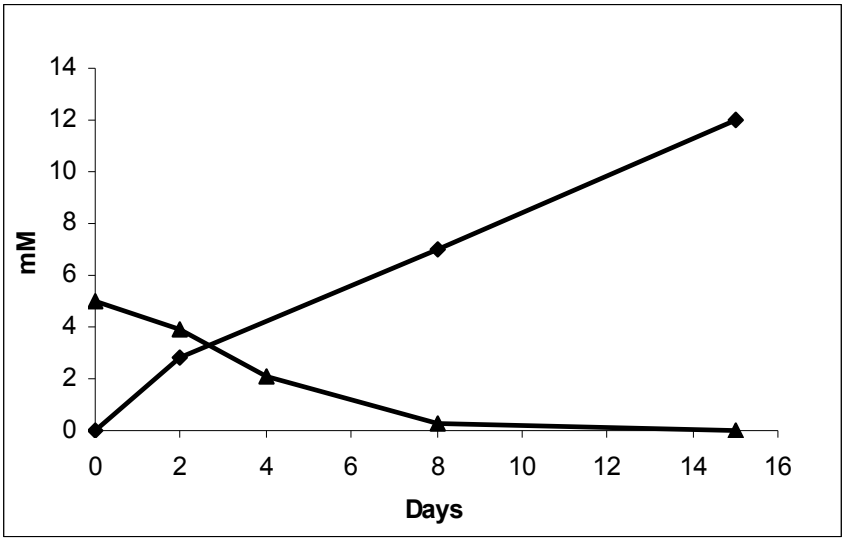
Figure 3: (3a) Phase contrast microphotographs of enrichment cultures. (3a) Isopropanol-degrading enrichment culture KN-Ipr showing (1) *Methanospirillum*-like organisms, (2) short rods and (3) *Methanosaeta*-like rods. (3b) Acetone-degrading enrichment culture KN-Act, showing (1) short rods and (2) *Methanosaeta*-like rods.

5.1.2 Degradation experiments

Isopropanol and acetone were completely degraded to methane in dense cell suspensions of the methanogenic enrichment culture KN-Ipr within 8 days (Fig. 4)



(a)



(b)

Figure 4: (a) Anaerobic degradation of isopropanol (a) and acetone **(b)** in dense cell suspensions of the enrichment culture KN-Ipr. Symbols: isopropanol (■), acetone (▲), methane (♦).

Accumulation of acetate in the medium was observed only in growing enrichment cultures in which the methanogenic partner was inhibited by bromoethane sulfonate (Fig. 5).

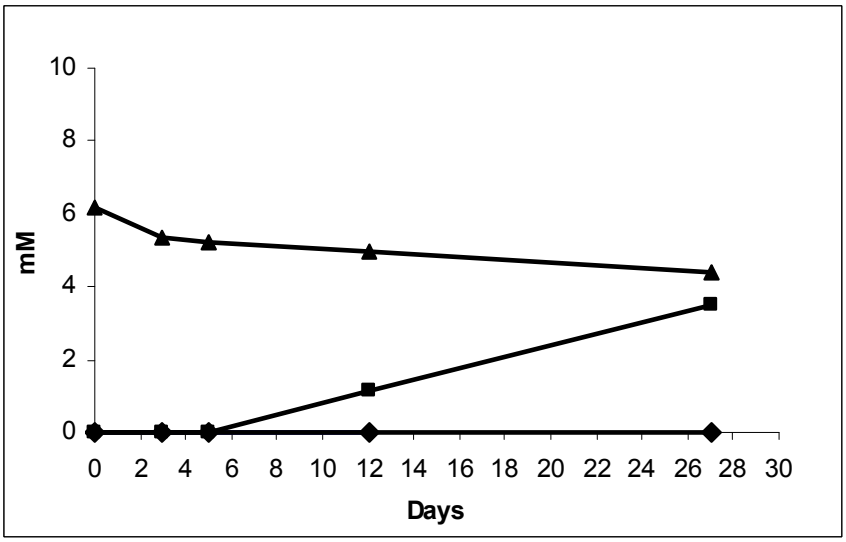
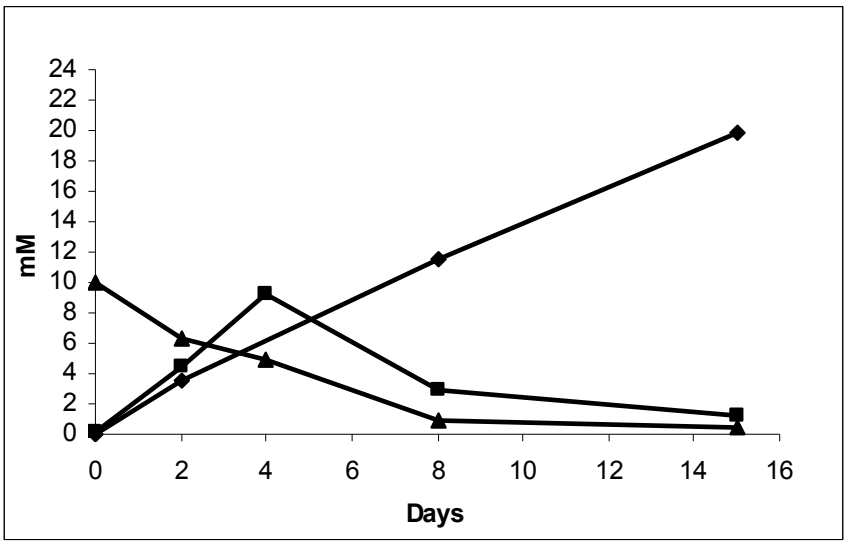


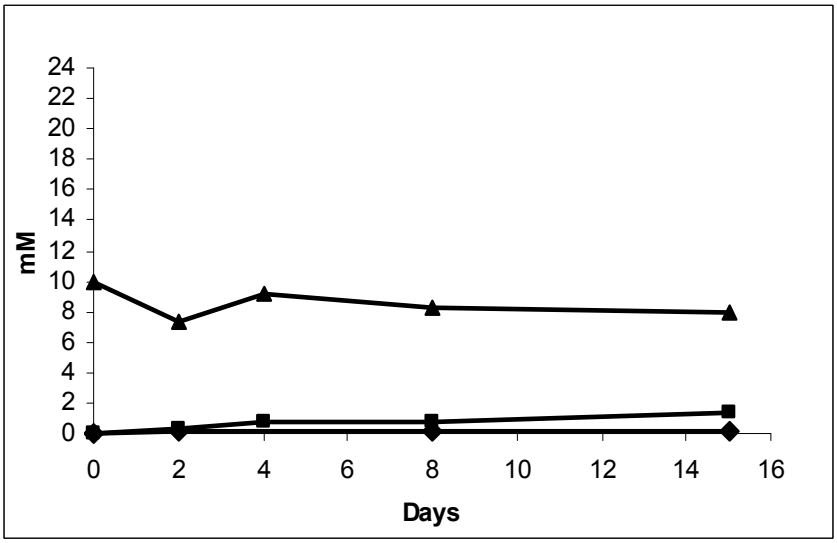
Figure 5: Anaerobic degradation of acetone by the enrichment culture KN-Act in the presence of 5 mM of bromoethane sulfonate. Symbols: acetone (▲), acetate (■).

5.1.3 Sodium dependence of acetone degradation

Acetone degradation in dense cell suspensions of the enrichment culture KN-Ipr was sodium dependent. In sodium-free medium, acetone degradation was slow (Fig. 6), and isopropanol was not degraded at all (not shown). Degradation of acetate in these enrichment cultures was not impaired in sodium-free medium (results not shown) indicating that lack of sodium ions inhibited specifically acetone degradation.



(a)



(b)

Figure 6: Acetone degradation in the presence of 30 mM NaCl (a) and in sodium-free medium (b). Symbols: acetone (▲), acetate (■), methane (♦).

5.1.4 Sodium dependence of acetate degradation

Considering that acetone degradation in the enrichment culture KN-Ipr occurred by a syntrophic cooperation, the absence of sodium could be a disturbing factor for the successive degradation step. The influence of sodium on acetate degradation was studied with methanogenic organisms of the enrichment culture KN-Ipr (Figure 7).

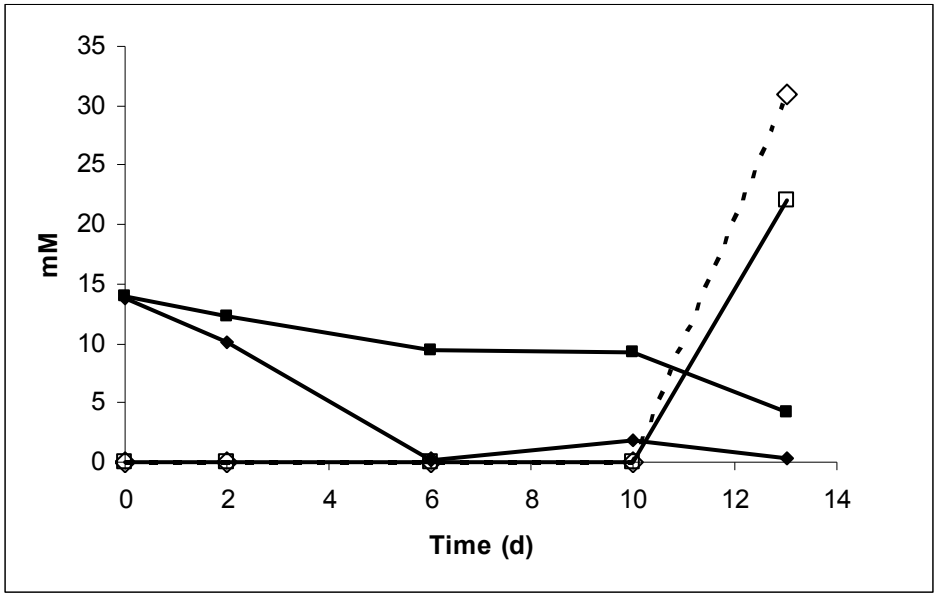


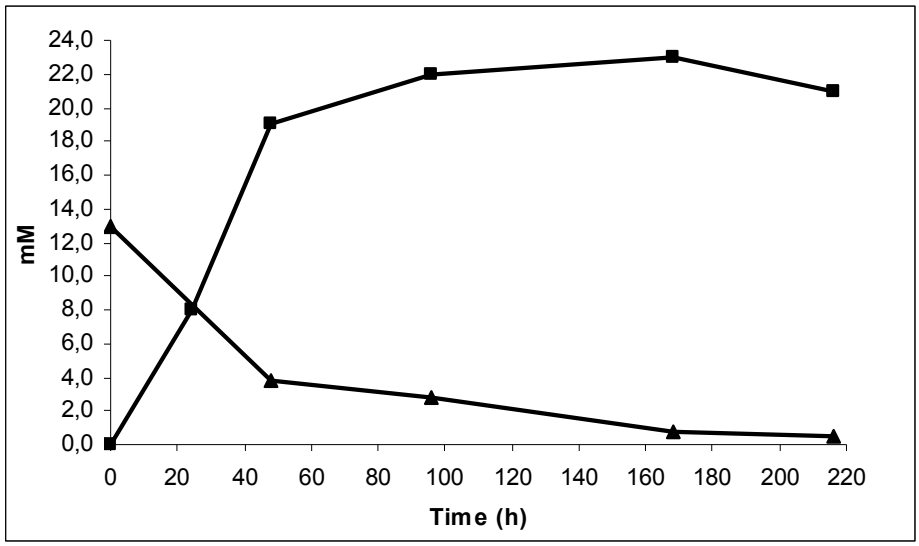
Figure 7: Acetate degradation in dense cell suspensions of the enrichment culture KN-Ipr, in the presence of 30 mM NaCl (■) and in sodium-free medium (♦). Formation of methane (◊) in the presence of 30 mM NaCl and in sodium-free medium (□).

The results indicate considerable differences in acetate degradation and methane formation rates in the presence and absence of sodium ions. The results indicate that sodium ions play an important role in acetone degradation

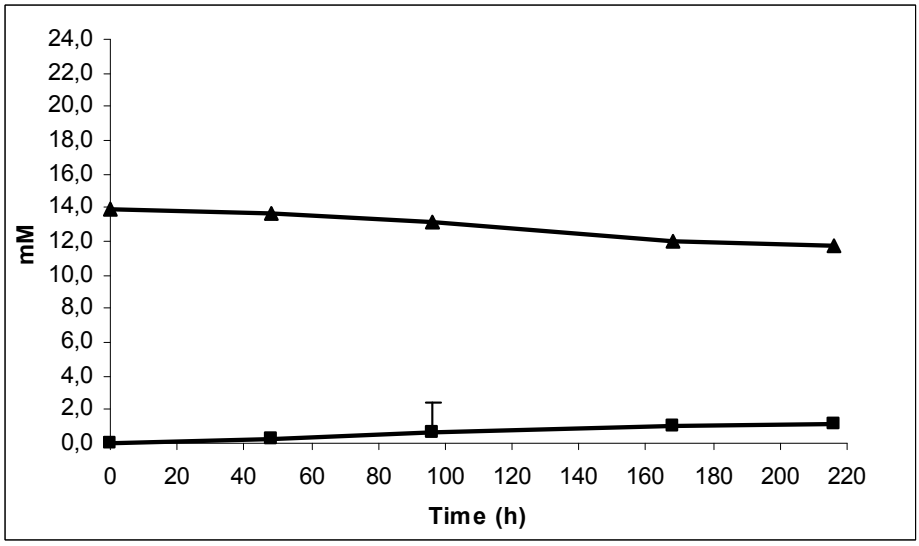
5.1.5 Dependence of acetone degradation on carbon dioxide

Acetone was degraded completely to methane in assays supplied with 20% carbon dioxide. In the absence of carbon dioxide, only a small amount of acetone was

degraded at the beginning of the reaction, but no further acetone degradation could be measured (Fig. 8).



(a)



(b)

Figure 8: Acetone degradation in dense cell suspensions of the enrichment culture KN-Ipr in medium supplied with 20 % of carbon dioxide (a) and in carbon dioxide-free medium (b). Symbols: acetone acetate (♦), methane (■).

5.1.6 Isolation of isopropanol- and acetone-degrading bacteria

The isopropanol-degrading methanogen *M. hungatei* was isolated from the enrichment culture KN-Ipr. After 7 to 14 days, 1 to 2 mm whitish transparent colonies could be detected. From these colonies, a *M. hungatei*-like methanogen was isolated in a liquid dilution series with isopropanol as substrate. This strain converted isopropanol stoichiometrically to acetone which accumulated in the medium (Fig. 9).

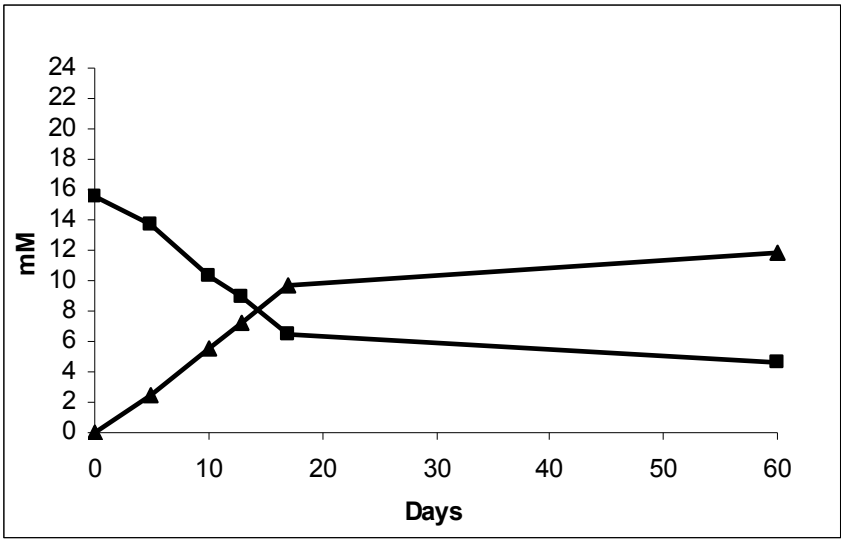
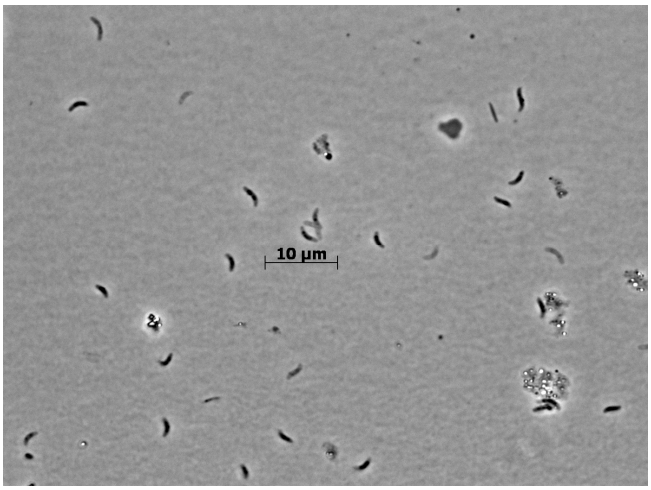


Figure 9: Degradation of isopropanol and formation of acetone by a pure culture of the isopropanol-oxidizing organism *M. hungatei* isolated from the enrichment co-culture KN-Ipr. Symbols: isopropanol (■),acetone (▲).

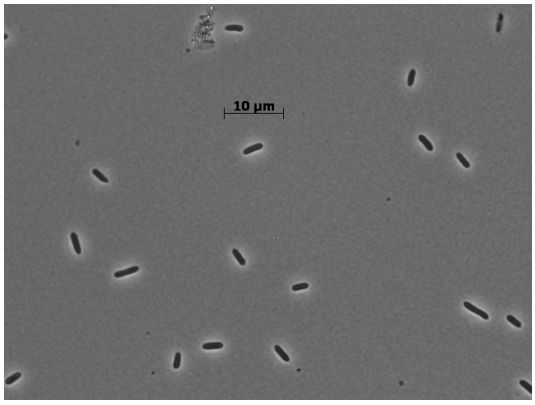
5.1.7 Isolation and characterization of acetone-fermenting bacteria from the enrichment-culture KN-Act

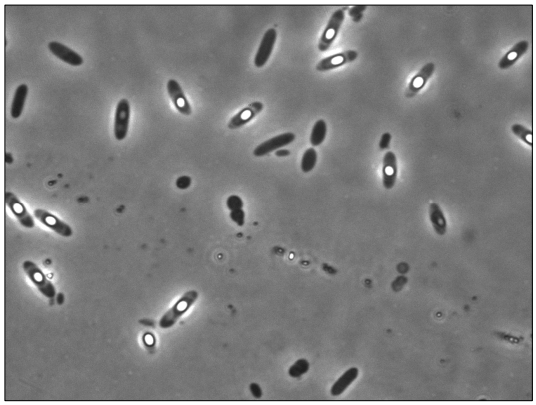
The acetone-degrading bacteria from the enrichment cultures could not be cultivated in agar, thus, purification had to proceed in liquid medium. Two acetone-fermenting bacteria were isolated from the methanogenic enrichment culture KN-Act in consecutive dilution series in anoxic bicarbonate-buffered mineral medium with 10

mM acetone and 20 mM sulfate. After elimination of the methanogenic partner, a second acetone-utilizing organism with the main acetone-fermenting bacteria was observed in growing cultures with acetone and sulfate. This organism was a Gram-positive, small curved motile rod, 0.2 x 0.6 µm in size. This organism was stimulated in pure culture after cultivation with acetone (10 mM) and sulfate (20 mM) by addition of yeast extract (0.1 %) to the medium (Fig. 10a). The acetone-fermenting organism was isolated in pure culture by further cultivation in anoxic medium containing acetone and sulfate. This organism grew slowly with acetone in the absence of the methanogenic syntrophic partner. Cells were Gram-positive, spore-forming, non-motile rods with rounded ends, 0.6 x 4-5 µm in size (Fig. 10b).



(10a)

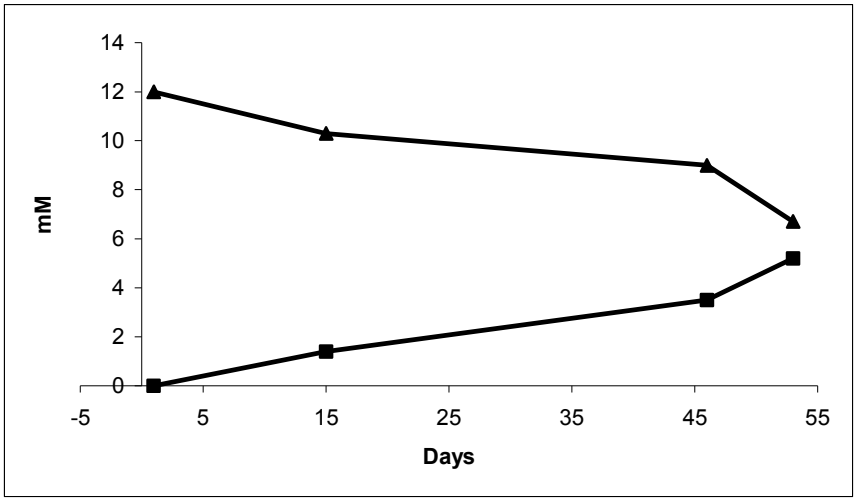




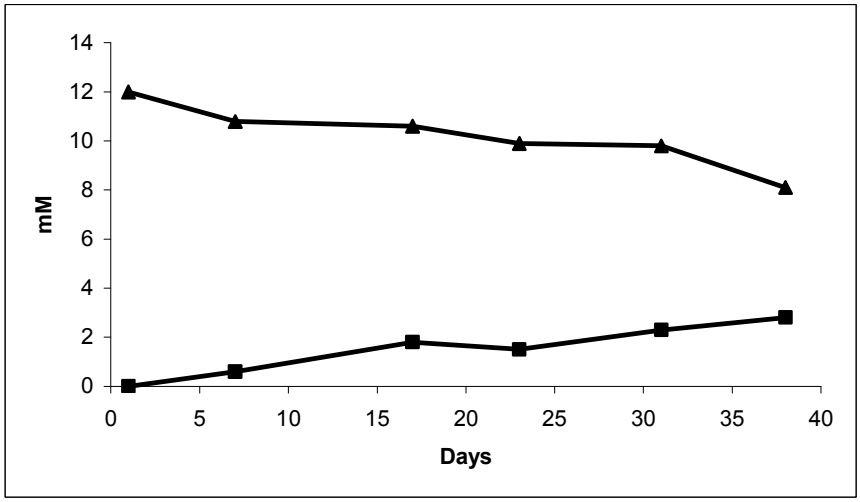
(10b)

Figure 10: Phase contrast microphotographs of acetone-degrading bacteria isolated from the enrichment cultures. (10a) KNActY 07, (10b) strain KNActS 07

Finally two acetone-degrading organisms were isolated from the enrichment culture KN-Act. Cells of the curved organism isolated first with the addition of 0.1 % of yeast extract (a) and cells of the acetone-fermenting organism (b) converted acetone under anoxic conditions stoichiometrically to acetate (Fig. 11).



(a)



(b)

Figure 11: Anaerobic acetone degradation in growing cultures of two isolated acetone-degrading organism KN-ActS 07 from the enrichment co-culture KN-Act. The first one **(a)** was purified on acetone and sulfate in the presence of yeast extract, and the acetone-fermenting organism **(b)** was purified by successive cultivation on acetone combined with pasteurization.

The isolated acetone-fermenting organism grew slowly with butanone, 3-hydroxybutyrate, 2-pentanone, and 2-hexanone (each 6 mM) with thiosulfate (10 mM) as electron acceptor and no growth on 2-pentanone and 2-hexanone could be

detected when sulfate was used as an electron acceptor. Comparison of the 16S rDNA of the isolated acetone-fermenting bacterium with the sequence of the genomic 16S rRNA of representatives of the genus *Desulfosporosinus* indicated 99% similarity with the sulfate-reducing organism of the genus *Desulfosporosinus sp.* Analysis of the complete sequence of the isolated organism indicated only minor similarity with *D. auripigmenti*, *D. orientis*, *D. lacus* and *D. meridiei* (Fig. 12). The results suggest that the isolated acetone-fermenting organism was probably a new unknown sulfate-reducer.

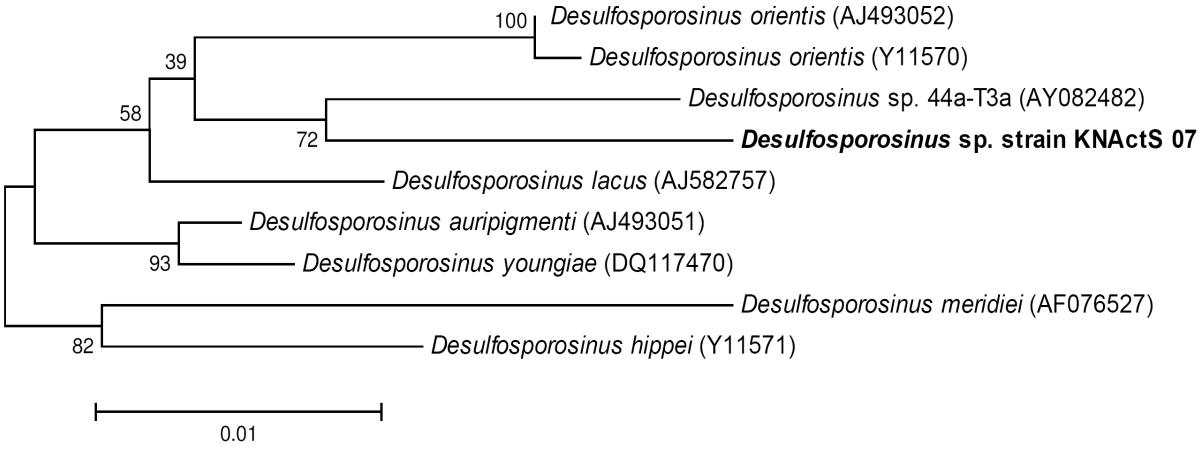


Figure 12: Phylogenetic tree of strain KNActS 07 and closely related strains. The tree was constructed using the Minimum Evolution method. Bootstrap values (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method based on 649 nucleotide positions. Bar represents 1% estimated phylogenetic divergence.

5.2 Sulfate-reducing bacteria

5.2.1 Acetone degradation by *Desulfococcus biacutus*

Under anoxic conditions, acetone (13 mM) was completely degraded after 18 to 20 days by cells of *D. biacutus*, in the presence of sodium sulfate (20 mM), at 28°C. The formation of sulfide was detected after 2 weeks. Aliquots of 5 to 10 ml of the culture were used for further cultivation of cells of *D. biacutus*.

5.2.1.1 Enzymes of the carbonylation reaction system (Reppe carbonylation of alkenes)

The presence of enzymes involved in a hypothetical carbonylation reaction was followed by spectrophotometric assays for the measurement of the activity of induced enzymes after growth of *D. biactus* on acetone. Activities of 0.3 to 0.8 U/mg protein of carbon-monoxide dehydrogenase (EC 1.2.99.2) were measured in cell-free extracts of *D. biacutus* after growth on acetone. This enzyme was also measured in cell-free extracts of *D. biacutus* after growth on 3-hydroxybutyrate and on pyruvate.
The results indicated that no activity of the intermediary enzymes, 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30), butyrate-acetoacetate CoA-transferase (EC 2.8.3.9), acetyl-CoA C-acetyl transferase (EC 2.3.1.9) and acetoacetyl-CoA hydrolase (EC 3.1.2.11) were measured in cell-free extracts of *D. biacutus,* after growth on acetone with exception of 3-hydroxybutyryl CoA dehydrogenase (EC 1.1.1.157).

Activities of 3-hydroxybutyryl CoA dehydrogenase of 0.3 to 0.5 U/mg protein were measured in cell-free extracts of *D. biacutus* after growth on acetone, on pyruvate and after growth on 3-hydroxybutyrate indicating that this enzyme was not specific expressed by growth on acetone.

5.2.2 Addition of fumaric acid

A possible addition of acetone to the CC double bond of fumaric acid was followed by the increasing of acetone degradation rates with the respective formation of 2-oxopropyl succinate by cell of *D. biacutus* after anaerobic growth on acetone and sulfate. The results indicated no increasing of the acetone degradation rate in the tests that contained fumaric acid. The HPLC analyses indicated that no 2-oxopropyl succinate was formed by cells of *D. biacutus* in the presence of fumaric acid.

5.2.3 Labelling experiments

To understand the initial step of acetone degradation under anaerobic conditions, labeling experiments were performed with ^{13}C-labeled bicarbonate added in assays containing dense suspension of intact cells of *D. biacutus* after growth on acetone.
The results indicated that no labeled CO_2 was incorporated to acetone and no acetyl-CoA derivative was detected during the reaction time.

Experiments with cell-free extracts of acetone grown cells of D. biacutus in the presence of carbon monoxide, ATP and acetone indicated the formation of an aldehyde derivative, most probably acetoacetaldehyde (data no available).

5.3 Nitrate-reducing bacteria

5.3.1 Isolation of strain KN Bun08

An acetone-degrading, nitrate-reducing bacterium, strain KN Bun08, was isolated from an acetone-degrading co-culture enriched from a small tarn sediment sample with 10 mM butanone and 10 mM nitrate as the electron acceptor. Strain KN Bun08 was purified by dilution in agar series containing acetone and nitrate. After 7 days of incubation, small whitish colonies were observed. After a second dilution run, pure cultures were obtained in liquid medium with acetone and nitrate. Cells were motile short rods, 0.5-1.0 x 1.5-2 µm in size (Fig. 13). Cells stained Gram-positive in the logarithmic growth phase and Gram-negative in the stationary growth phase and reacted positive in the oxidase and catalase test.

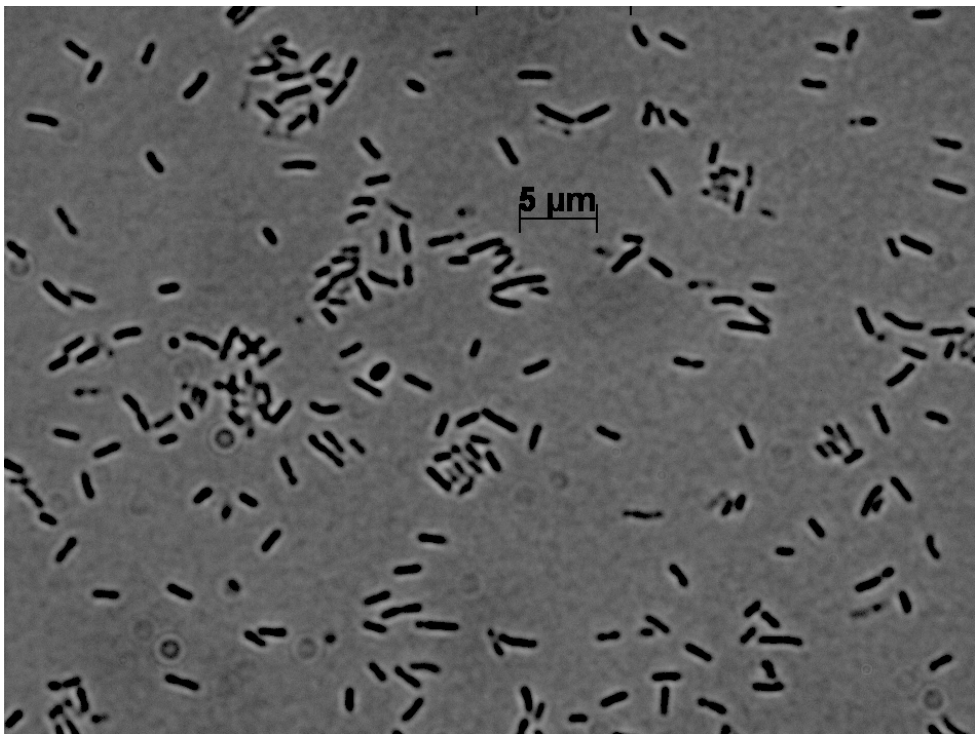


Figure 13: Phase-contrast photomicrograph of cells of strain KN Bun08, after growth with acetone and nitrate. Bar equals 10 µm.

5.3.2 Physiological characterization of strain KN Bun08

In batch cultures, strain KN Bun08 grew with acetone both aerobically and anaerobically with nitrate. It also grew with acetate, *DL*-lactate, pyruvate, succinate, propionate, butyrate, malate, citrate, fumarate and 3-hydroxybutyrate under anoxic conditions with nitrate and with oxygen as the electron acceptor; no growth was observed on formate. Growth on ethanol and sugars was observed with nitrate and with oxygen as electron acceptor. No growth on alicyclic and aromatic compounds was observed when nitrate was used as electron acceptor. Strain KN Bun08 grew on different aromatic compounds, and slow growth was observed on alicyclic compounds with oxygen as electron acceptor (Table 1).

Molar yields of nitrate-dependent growth of strain KN Bun08 with acetone and acetate were compared with those of *P. denitrificans* and *P. pantotrophus*. Yields of aerobic and nitrate-dependent anaerobic growth of strain KN Bun08 on acetone and on acetate were compared with those of *P. denitrificans* and *P. pantotrophus*. Calculated yields (Y_m) for anaerobic and aerobic growth on acetone and acetate demonstrated lower yield values of strain KN Bun 08 for acetone (9.3 mg . $mmol^{-1}$) and acetate (9.2 mg . $mmol^{-1}$) were compared with yields obtained for anaerobic growth of *P. denitrificans* (28.2 and 11. 5 mg . $mmol^{-1}$) and by *P. pantotrophus* (27.8 and 13.6 mg . $mmol^{-1}$), respectively for acetone and acetate. Under aerobic growth conditions strain KN Bun 08 showed similar yield values for growth on acetate and lower yield value for growth on acetone if compared with *P. denitrificans* and *P. pantotrophus*. Growth yield (Y_m) was based on biomass production and on substrate consumption rates (ΔS) (Tab. 2).

Table 1: Growth of strain KN Bun08 in the presence of different compounds under anaerobic conditions with nitrate and aerobically with oxygen as electron acceptor.

Compounds	Conc. mM	Electron acceptors	
		NO_3^-	O_2
Acetate	10	+	+
Formate	10	-	-
DL-Lactate	10	+	+
Pyruvate	10	+	+
Succinate	10	+	+
Propionate	10	+	+
Butyrate	10	+	+
Malate	10	+	+
Citrate	10	+	+
Fumarate	10	+	+
3-Hydroxybutyrate	10	+	+
Cyclohexanone	1	-	+-
1,3-Cyclohexadiol (cis-trans)	1	-	+-
1,2-Cyclohexanediol	1	-	+-
1,2-Cyclohexanedione	1	-	+-
1,4-Cyclohexanedione	1	-	+-
Cyclohexanol	1	-	+-
Phenol	1	-	+
Aniline	1	-	+
Benzoate	1	-	+
4-Hydroxybenzoate	1	-	+
Resorcinol	1	-	+
Hydroxyquinol	1	-	+
m-Cresol	1	-	+
o-Cresol	1	-	+
p-Cresol	1	-	+
Vanillate	1	-	+
Indole	1	-	+
Glucose	10	+	+
Fructose	10	+	+
Xylose	10	+	+
Ethanol	10	+	+

(+) = growth; (-) = no growth; (+-) = slow growth

Table 2: Growth yield of strain KN Bun08, *P. denitrificans* and *P. pantotrophus*, under anaerobic and aerobic conditions with nitrate as electron acceptor.

	anaerobic (Y_m)		aerobic (Y_m)	
Strain	acetone	acetate	acetone	acetate
KN Bun 08	*9.3*	*9.2*	*11.4*	*10.3*
P.denitrificans	*28.2*	*11.5*	*24.1*	*11.4*
P. pantotrophus	*27.8*	*13.7*	*17.2*	*9.4*

5.3.3 Phylogenetic characterization of strain KN Bun08

Sequence analysis of the partially amplified 16S rDNA gene indicated a relationship of strain KN Bun08 with representatives of the genus *Alicycliphilus sp.,* together with members of the genera *Comamonas* and *Acidovorax*. It showed 99 % similarity with the genus *Alicycliphilus denitrificans* (Fig. 14).

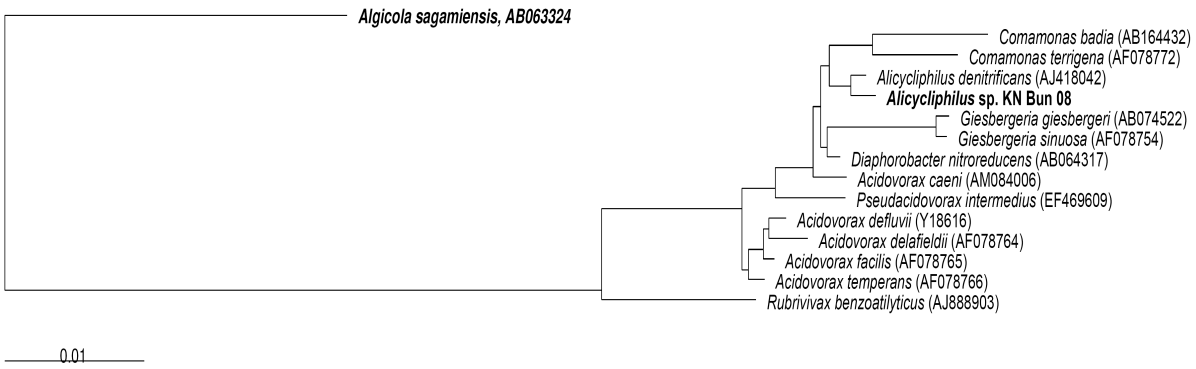


Figure 14: Phylogenetic tree based on the sequencing of the 16S rDNA of the acetone-degrading, nitrate-reducing strain KN Bun08. Phylogenetic parameters were analyzed and a phylogenetic tree was constructed with ARB software adjusted with filter for *Betaproteobacteria* indicating 1 % estimated sequence divergence.

5.3.4 Enrichment and characterization of acetone carboxylase

Carboxylation of acetone to acetoacetate with simultaneous conversion of ATP to AMP, and AMP-dependent decarboxylation of acetoacetate could be documented by discontinuous analysis by HPLC in cell-free extracts of strain KN Bun08, *P. denitrificans*, and *P. pantotrophus*. With the latter two, also ADP was formed as a co-product. Alternatively, the respective reactions could be followed by using GTP, ITP and UTP. As an alternative, continuous test system, conversion of ATP to ADP could be followed via pyruvate formation from phosphoenolpyruvate and NADH-dependent lactate formation (modified after Ziegler and Fuchs, 1987), especially with enriched enzyme preparations. This test was applied in the presence of adenylate kinase (myokinase) in order to convert AMP plus ATP to ADP. Addition of adenylate kinase was not necessary in assays with crude extracts or enriched enzyme fractions of *P. denitrificans* and *P. pantotrophus*, but was needed with extracts of strain KN Bun08. Adenylate kinase activities in the range of 0.2 to 0.5 U/ mg protein were measured with enriched acetone carboxylase (pool II) of acetone-grown cells of *P. denitrificans* and *P. pantotrophus*. Adenylate kinase (myokinase) activities of >0.9 U (mg protein)$^{-1}$ were measured in cell-free extracts of acetone-grown cells of *P. denitrificans* and *P. pantotrophus*. No myokinase activity was measured in enriched enzyme fractions (pool I and pool II) and in crude cell extracts of strain KN Bun08.

The acetone carboxylase enzyme was enriched from cells of *P. denitrificans*, *P. pantotrophus* and strain KN Bun08 after nitrate-dependent growth with acetone. The enzyme was enriched by chromatography on a DEAE-sepharose column under two slightly different separation conditions, and concentrated in enzyme pools after the first and second enrichment steps (pool I and pool II). Specific activities increased in the first enrichment step, but decreased occasionally in the second enrichment step (Tab. 3), simultaneously with the disappearance of the gamma subunit (see below).

Table 3: Activities of acetone carboxylase enriched from cell-free extracts of *P. denitrificans*, *P. pantotrophus* and strain KN Bun08 after anaerobic growth with acetone plus nitrate, indicating the decreasing of the enzyme activity after the first enrichment step by DEAE-sepharose column.

	U/mg protein								
	KN Bun08			*P. denitrificans*			*P. pantotrophus*		
enzyme	cell-free extract	pool I	pool II	cell-free extract	pool I	pool II	cell-free extract	pool I	pool II
acetone carboxylase	*0.02*	*0.6*	*0.2*	*0.05*	*0.9*	*0.4*	*0.04*	*0.2*	*0.03*
myokinase	*0*	*0*	*0*	*0.9*	*0.8*	*0.5*	*0.9*	*0.5*	*0.2*

Acetone carboxylase activity of all three strains was ATP-dependent and was correlated with the protein content in the assay mixture. A decrease of the specific activity by 30% to 60% was observed in enriched enzyme pools, respectively, after 3 and 7 days under N_2 gas. No enzyme activity could be measured in the absence of HCO_3^- or of acetone. All assays were performed under anoxic conditions although oxygen did not impair the enzyme activity. Protein bands of about 85, 78, and 20 kDa were specifically expressed during growth with acetone, and were not found after growth with 3-hydroxybutyrate. The same bands were selectively enriched during enrichment of acetone carboxylase by DEAE column chromatography, as shown in SDS-polyacrylamide gel electrophoresis (Fig. 15).

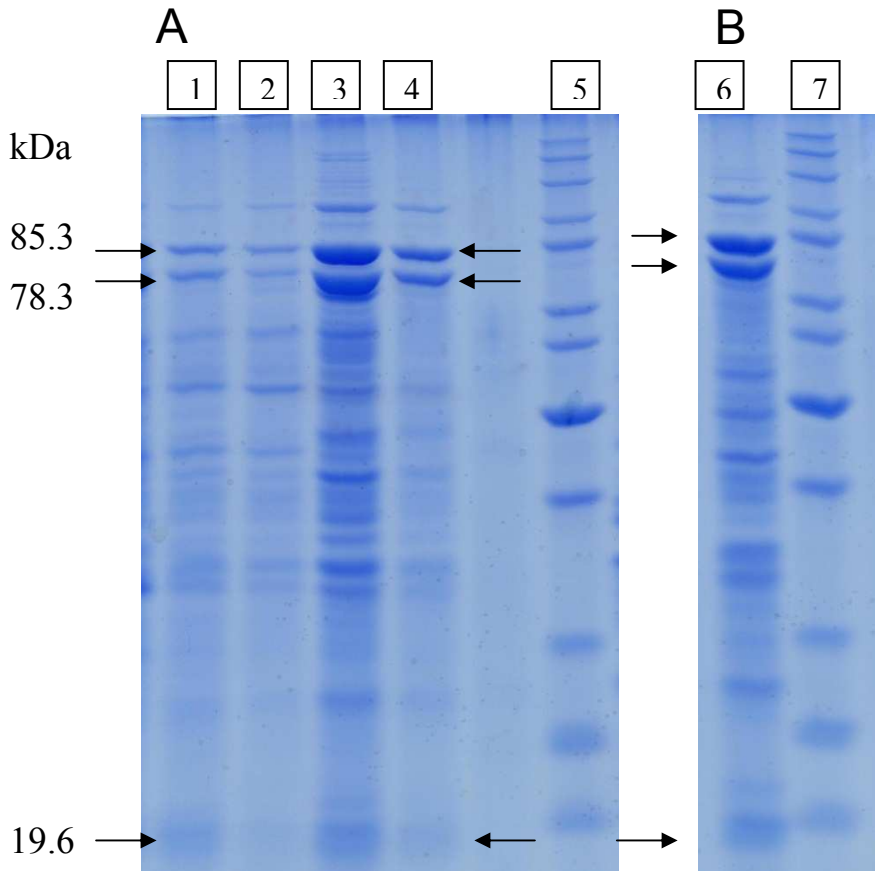


Figure 15. SDS-polyacrylamide gel electrophoresis of acetone carboxylase preparations enriched from cell-free extracts of *P. denitrificans*, *P. pantotrophus* and *Alicycliphilus sp.* strain KN Bun08. Putative acetone carboxylase subunits are marked with the respective molecular masses. A: Lanes 1 and 2, *P. denitrificans* preparation pools I and II; lanes 3 and 4, *P. pantotrophus* preparation pools I and II. B: Lane: 6, *Alicycliphilus sp.* strain KN Bun08 preparation pool II; Lanes 5 and 7, marker proteins.

Acetone carboxylase subunits were enriched from cell extracts and from enriched enzyme fractions, (pool I and pool II) from *P. denitrificans* (Lanes 1 and 2), *P. pantotrophus* (Lanes 3 and 4) and from enzyme fraction, (pool II) from *Alicycliphilus sp*. strain KN Bun08 (Lane 6), after anaerobic growth on acetone. Protein profiles were nearly identical to that of the three subunits of acetone carboxylase by *Xantobacter autotrophicus* strain Py2 (i. e., 85.3, 78.3, and 19.6 kDa) studied in detail for the characterization of acetone carboxylase enzyme (Sluis and Ensign, 2002).

In cell-free extracts of *Alicycliphilus denitrificans* strain K601^T, two subunits of the acetone carboxylase enzyme (α and β) were induced after anaerobic growth with

acetone plus nitrate. Both subunits were visible in SDS-PAGE, but the third subunit (γ) was not visible. Excised protein bands of putative acetone carboxylase subunits of *P. denitrificans, P. pantotrophus*, strain KN Bun08, and *A. denitrificans* strain K601 were subjected to mass spectrometric analysis for comparison with the gene sequences of *P. denitrificans* and *A. denitrificans* strain K601^T. The results indicated that in both cases the sequences were 95-98% identical with the respective sequences annotated as acetone carboxylase (*acxABC* genes).

The enzymes that represented significant identity with acetone carboxylase are hydantoinases. Hydantoinases are involved in the hydrolysis of cyclic amide bond of substituted pyrimidines and hydantoins to form *N*-carbamyl amino acids (May et al., 1998 and Syldatk et al., 1999). Subunits of hydantoinase enzymes show 15-30% identity with the alpha and beta subunits of acetone carboxylase (Sluis et al., 2002). However, no significant identity with other known enzymes was related for the gamma subunit of acetone carboxylase enzyme. We observed that the gamma subunit was lost after enzyme enrichment by DEAE-sepharose column. The loss of the gamma subunit appears to be related to the decreasing of the enzyme activities in continuous coupled enzyme assays.

5.3.5 Comparison with *Alicycliphilus denitrificans,* strains BC and K601

Two subunits of acetone carboxylase enzyme (α and β) were induced in cells of *A. denitrificans*, strain K601 after anaerobic growth on acetone and nitrate. In cell-free extracts and in enriched enzyme fractions of strain K601 both subunits (α and β) were visible in SDS-PAGE, and the third subunit (γ) was not identified by reversed-phase liquid chromatography. No activity of acetone carboxylase enzyme was measured in cell-free extracts and in enriched enzyme fractions of strain K601, indicating that the γ subunit was lost in the preparation simultaneous with loss of enzyme activity.

Neither acetone carboxylase subunit from crude cell extract of *A. denitrificans*, strain BC, was identified in cell-free extract and in enriched enzyme fractions of strain BC indicating that no acetone carboxylase subunit was induced by strain BC after growth onacetone.

6. Discussion

6.1 Methanogenic enrichments

The anaerobic degradation of acetone was investigated and compared between two methanogenic enrichment cultures, KN-Ipr and KN-Act grown with isopropanol or acetone, respectively. In both enrichment cultures, acetone was degraded in the absence of external electron acceptors. In both enrichments, an acetone-fermenting rod-shaped bacteria that converted 1 mol acetone to 2 mol acetate was identified. From the enrichment culture KN-Ipr the isopropanol-utilizing organism was purified and characterized as *Methanospirillum hungatei*. In degradation experiments, *M. hungatei* oxidized 1 mol isopropanol to 1 mol acetone and no involvement of these bacteria in acetone degradation was observed, indicating that acetone was degraded by other bacteria.

Acetone was degraded in both enrichment cultures by a rod-shaped acetone-fermenting bacterium that converted 1 mol acetone to 2 mol acetate that was further converted to methane and carbon dioxide by a methanogenic partner, a filamentous *Methanosaeta*-like rod. The bacterial composition of both enrichments, cultures KN-Ipr and KN-Act, was very similar with the acetone-degrading methanogenic enrichment culture WoAct that was used in earlier studies on acetone degradation (Platen and Schink, 1987).

In this enrichment culture, acetone was converted to acetate by a rod-shaped bacterium, and acetate was further degraded to methane by a methanogenic prokaryote identified as *Methanothrix. sp*. that is known to have a high affinity for acetate. Cultivation of the enrichment culture WoAct in dialysis culture with inhibition of methanogenic bacteria *Methanothrix sp*. demonstrated that the removal of the produced acetate is of major importance for the acetone-degrading bacteria, although slow logarithmic growth of the enrichment culture was observed in the presence of 5 mM acetate. Also in the present study, we show that acetone degradation in dense cell suspension of the purified acetone-fermenting strain KN-

ActS 07 was impaired by accumulation of acetate in medium. Characterization of the purified acetone-fermenting, sulfate-reducing strain KN-ActS 07 indicated high similarity with *Desulfosporosinus sp.* This bacterium grew in pure culture and degraded acetone slowly. The slow growth with acetone was probably caused by the accumulation of acetate (2 mM), in the medium that could not be removed further after exclusion of the methanogenic partner *Methanosaeta concilii.* The intolerance to acetate is distinctly characteristic and resembles the behavious of the acetone-degrading bacteria present in a methanogenic enrichment culture although those bacteria could stand up to 5 mM acetate (Platen and Schink, 1987). The ability of *Desulfosporosinus sp.* to reduce sulfate was a characteristic that supported the idea of alternative concepts for acetone activation under anaerobic conditions.

6.2 Sulfate-reducing bacteria

The cultivation of the acetone-fermenting, sulfate-reducing bacterium *Desulfosporosinus sp.*, strain KNActS 07 in the absence of the methanogenic partner *M. concilii* was characterized by very slow growth. Cultivation with different ketones such as 2-pentanone, 3- pentanone, 2-hexanone, 3-hexanone and butanone, as substrate and the use of thiosulfate as electron acceptor did not stimulate growth of the isolated sulfate-reducing strain KNActS 07, indicating the necessity of further experiments for optimization of growth conditions.

Considering these aspects a different acetone-degrading, sulfate-reducing bacterium *Desulfococcus biacutus* was selected and used as reference organism to test different concepts and to characterize the conditions for the initial acetone degradation with sulfate as electron acceptor.

In this work different alternative concepts were investigated in cell-free extracts or in intact cells of *D. biacutus* after growth with acetone plus sulfate. Initially the possibility was investigated that acetone could be activated via acetone carbonylation, analogous to the Reppe carbonylation reaction of alkenes. This

hypothesis was supported by the characteristic of these sulfate-reducing bacteria to oxidize acetyl derivates through the carbon monoxide dehydrogenase pathway.

The presence of enzymes specifically involved in acetone carbonylation was proposed and investigated in acetone-grown cell-free extracts of *D. biacutus* by measurement of enzyme activities. A carbon monoxide dehydrogenase activity was measured in cell-free extracts of *D. biacutus* after growth with acetone plus sulfate. In all tests, no activity of the expected enzymes was measured, with the exception of 3-hydroxybutyryl-CoA dehydrogenase. Unfortunately, higher enzyme activities (0.5 to 0.8 U/mg protein) were measured in cell-free extracts of *D. biacutus* after growth on 3-hydroxybutyrate and pyruvate and lower activity (0.05 to 0.1 U/mg protein) of this enzyme was measured in cell-free extracts of *D. biacutus* after growth with acetone.

Analogous to the addition of toluene to the C-C double bound of fumarate by the initial reaction in toluene degradation by *Thauera aromatica* (Biegert, 1996), we investigated if this type of reaction could be involved also in the activation of acetone. Addition of acetone to the C-C double bond of fumarate with formation of 2-oxopropyl succinate was an alternative strategy investigated with intact cells of *D. biacutus* after growth with acetone. However, acetone degradation was not stimulated in the presence of fumarate, and no formation of 2-oxopropyl-succinate was detected in measurements by HPLC. Parallel to these experiments a third concept based on experiments with ^{13}C labeling experiments were performed with the intention to indentify labeled acetone derivates by nuclear magnetic resonance spectroscopy. The results of the experiments with ^{13}C-labeled bicarbonate and labeled acetone added in intact cells of *D. biacutus* indicated that no acetyl-CoA or acetyl CoA derivates were formed during the reaction time.

Parallel investigations with cell-free extracts of *D. biacutus* used in photometric assays indicate that an aldehyde derivative was formed in the presence of ATP, carbon monoxide, and acetone. The involvement of a reaction that possibly forms acetoacetaldehyde and its inclusion in the metabolic pathway to form acetyl-CoA is a new hypothesis that will be examined in a separate study.

Based on the results obtained by the investigation of different strategies and on the energy limitation of sulfate-reducing and fermenting bacteria it is possible to conclude that these organisms apply an acetone carboxylation system different from those described for aerobes and phototrophs.

One alternative hypothesis investigated in this work with the acetone-fermenting bacteria present in the enrichment culture KN-Ipr was the possible involvement of membrane-bound proteins in acetone activation, e. g., by translocation of sodium ions across the cytoplasmic membrane. A dependence of acetone degradation on sodium ions was observed with cells of the acetone-degrading fermenting bacteria in sodium-free medium containing acetone. The dependence on sodium could be an indication for the existence of a membrane-bound enzyme system that probably provided the necessary energy for acetone carboxylation.

6.3 Nitrate-reducing bacteria

6.3.1 Isolation of a novel acetone-degrading nitrate reducer

In this work, the isolation and characterization of, strain KN Bun08 is described, a bacterium that grows on acetone with nitrate as electron acceptor. In batch cultures strain KN Bun08 grew with carboxylic acids (acetate, *DL*-lactate, pyruvate, succinate, propionate, butyrate, malate, citrate, fumarate and 3-hydroxybutyrate), with sugars (xylose, glucose and fructose) and with ethanol with either nitrate or oxygen as electron acceptor. Aromatic compounds were used only aerobically, and slow growth was observed on alicyclic compounds. No growth was observed on aromatic and alicyclic compounds in the absence of oxygen. Analysis of the 16S rDNA sequence of strain KN Bun 08 revealed 99% similarity with *Alicycliphilus denitrificans,* a cyclohexanol-degrading, nitrate-reducing beta-proteobacterium (Mechichi et al., 2003). Thus, although strain KN Bun08 differs from *A. denitrificans* substantially with respect to its substrate range, it has to be grouped with this species due to the high 16S rRNA sequence similarity. Earlier work in our group on nitrate-dependent acetone degradation was carried out with strain BunN which was not assigned

taxonomically at that time and, unfortunately, got lost. We therefore re-enriched such a strain under basically similar conditions as originally with strain BunN, resulting in strain KN Bun08. Both isolates appear to resemble each other as far as substrate utilization spectra are concerned. However, strain KN Bun08 allows to measure acetone carboxylation in vitro which could never be observed with strain BunN in the past.

6.3.2 Measurement of acetone carboxylase activity

Activity of the acetone carboxylase reaction was observed in *in vitro* assays containing cell-free extracts of strain KN Bun08 after anaerobic growth with acetone plus nitrate. This activity was measured either as consumption of ATP or as consumption of AMP or ADP, in acetone carboxylation or acetoacetate decarboxylation, respectively. Since acetone is volatile and acetoacetate is unstable in aqueous solution a reliable quantitative analysis of these reactants by HPLC was difficult. Since also ATPases and other enzymes present in the cell-free extracts interfered with our measurements further analysis required enriched enzyme preparations. For comparison, we prepared similar enrichments from two further acetone-degrading, nitrate-reducing bacteria, *P.denitrificans* and *P. pantotrophus*. Activities of the acetone carboxylases were measured in a continuous spectrophotometric enzyme assay after chromatographic enrichment in two enrichment steps on a DEAE sepharose column. Enzyme activities of 0.45, 0.03 and 0.22 U per mg protein were measured, respectively, for *P. denitrificans, P. pantotrophus* and strain KN Bun08 after the second enrichment with DEAE-sepharose column. The activity was dependent on the addition of acetone and ATP, and was correlated with the amount of protein in the reaction mixture.

Carboxylation of acetone to acetoacetate by the enzymes studied here required ATP as co-substrate, and converted it to AMP plus P_i. With this, the reaction mechanism of these enzymes appears to be similar to that of *X. autotrophicus* strain Py2 which was studied in detail (Sluis and Ensign, 2002). Also the subunit patterns were identical, suggesting that also the acetone carboxylases of the nitrate-reducing

bacteria form a hexamer of an $\alpha_2\beta_2\gamma_2$ structure with subunits of 85.3, 78.3, and 19.6 kDa. The gamma subunit was not detected in extracts of *Alicycliphilus denitrificans* strain K 601^T, and was partially lost also during enrichment of acetone carboxylases of the other strains, with concomitant loss of activity.

Sequence analysis of the enriched protein fractions indicated 15-30% identity of the alpha and beta subunits of acetone carboxylase with hydantoinases that are involved in the hydrolysis of cyclic amide bond of substituted pyrimidines and hydantoins to form *N*-carbamyl amino acids (May et al., 1998 and Syldatk et al., 1999). However, no significant identity with other known enzymes was found for the gamma subunit of acetone carboxylase. This subunit appears to be an essential constituent of acetone carboxylase since loss of the gamma subunit always went in hand with decreased enzyme activities.

The purification of acetone carboxylase from acetone-grown cells of *R. capsulatus* was possible by the use of an assay developed for *X. autotrophicus* strain Py2. Biochemical studies on acetone carboxylase in fractionated extracts of acetone-grown cells of *R. capsulatus* strain B10 indicated some differences if compared with the acetone carboxylases enriched from acetone-grown cells of nitrate-reducers. In cell extracts of *R. capsulatus,* the acetone carboxylase activity was dependent on the addition of ATP, Mg^{2+}, coenzyme A (CoA) or acetyl-CoA to the assay mixture (Sluis and Ensign, 2002). A dependence of acetone carboxylase activity on coenzyme A (CoA) or acetyl CoA was not observed in assays performed with enriched acetone carboxylase from the different nitrate-reducing bacteria or with purified acetone carboxylase from acetone- and isopropanol-grown cells of *X. autotrophicus* strain Py2.

A major part of work about carboxylation reactions were investigated on aerobes and phototrophs. This work was centered on the study of acetone carboxylation enzyme induced during anaerobic acetone metabolism. Studies in the past have provided *in vivo* and *in vitro* data about the carboxylation of acetone by anaerobic bacteria, strain BunN (Platen and Schink, 1989, Platen and Schink, 1990, Platen and Schink, 1994,

Janssen and Schink, 1995). However, no acetone carboxylase enzyme was enriched or purified from anaerobic acetone-degrading cells.

6.3.3 Characterization of acetone carboxylase

It was possible to conclude that the obtained sequences of enriched acetone carboxylase and the identification of the subunits (alpha, beta and gamma) of this enzyme in enriched enzyme fractions from strain KN Bun08, *P. denitrificans*, *P. pantotrophus* and *Alicycliphilus denitrificans* strain K601 corresponded to the subunits of acetone carboxylase that was purified and characterized from aerobic cells of *X. autotrophicus*, strain Py2 (Sluis and Ensign, 1997). Accurate analysis of identified polypetides by mass spectrometry indicated exact molecular mass of 85.3, 78.3 and 19.6 kDa for the acetone carboxylase subunits (Sluis and Ensign, 2002). Biochemical studies with purified acetone carboxylase from X. autotrophicus, strain Py2 shown that 1 mmol ATP was converted to 1 mmol AMT plus Pi. A similar biochemical characteristic was observed in enzyme assays containing enriched enzyme fractions indicating that the enriched ATP-dependent acetone carboxylase enzyme induced after growth on acetone from cells of strain KN Bun08, *P. denitrificans, P. pantotrophus* and from an *Alicycliphilus denitrificans*, strain K601 catalyzed the acetone-dependent hydrolysis of ATP to form AMP plus Pi.

This work provides more information about the acetone carboxylase enzyme that was induced by nitrate-reducing cells after anaerobic growth on acetone, specially by a newly nitrate-reducing bacteria, strain KN Bun08 that remember the loss strain Bun N investigated in the past in studies about anaerobic acetone metabolism.

6.3.4 Comparison with other carboxylases

Studies on acetone metabolism by the phototrophic bacterium *Rhodobacter capsulatus* demonstrated that two specific proteins were induced after growth on acetone and butanone. Partial protein purification indicated that two most abundant

proteins with molecular mass of 70 and 85 kDa were identified respectively as two subunits of acetone carboxylase (beta and alpha). However, the results of the N-terminal sequences indicated no similarity with other known carboxylases involved in CO_2 metabolsim and described in the database suggesting that the purified enzyme was a novel type of carboxylase (Birks and Kelly, 1997).

A recent study revealed the involvement of a carboxylase enzyme in the anaerobic ethylbenzene metabolism (Jobst et al., 2010). The purification of the acetophenone carboxylase showed that this enzyme was composed of five subunits (*apcABCDE*) with molecular masses of 70, 15 87, 75 and 34 kDa. The activity of acetophenone carboxylase was restored by mixing the purified subcomplexes. The purified subunits demonstrated hight similarity with to N-methyl-hydantoinases involved in the ATP-dependent hydrolysis of cyclic amide bonds (Ogawa et al., 1995).

Aerobic and nitrate-reducing anaerobic bacteria activate acetone by carboxylation to acetoacetate by an enzyme system requiring two ATP equivalents (Sluis et al., 2002, Boyd et al. 2004). It is obvious that bacteria fermenting acetone with CO_2 to acetate cannot afford so much ATP in the carboxylation reaction. In this context, it is of major interest that the acetone-fermenting bacteria in our culture obviously depended specifically on sodium ions for activity. One could speculate that acetone carboxylation is driven by sodium ions, analogous to the reversal of oxaloacetate decarboxylation of *Klebsiella aerogenes* (Dimroth and Hilpert, 1984), or that a redox reaction is involved which depends on a sodium ion-driven Rnf system as described recently for the homoacetogen *Acetoabcterium woodii* (Biegel and Muller, 2010).

7. Literature

Bartholomew JW: Variables influencing results, and the precise definition of steps in Gram staining as a means of standardizing the results obtained. Stain Technol 37: 139-155 (1962)

Biegert T, Fuchs G, Heider J: Evidence that anaerobic oxidation of toluene in the denitrifying bacterium *Thauera aromatica* is initiated by formation of benzylsuccinate from toluene and fumarate. Eur J Biochem 238: 661-668 (1996)

Birks SJ, Kelly DJ: Assay and properties of acetone carboxylase, a novel enzyme involved in acetone-dependent growth and CO_2 fixation in *Rhodobacter capsulatus* and other photosynthetic and denitrifying bacteria. Microbiol 143: 755-766 (1997)

Bonnet-Smits EM, Robertson LA, van Dijken JP, Senior E, Kuenen JG: Carbon dioxide fixation as the initial step in the metabolism of acetone by *Thiosphaera pantotropha*. J Gen Microbiol 134: 2281-2289 (1988)

Boyd JM, Elllsworth H, Ensign SA: Bacterial acetone carboxylase is a manganese-dependent metalloenzyme. J Biol Chem 279: 46644-46651 (2004)

Boyd JM, Ensign SA: ATP-dependent enolization of acetone carboxylase from *Rhodobacter capsulatus*. Biochem 44: 8543-8553 (2005)

Chemical and Enginnering News. Production by the U.S. chemical industry. In: Chronic Toxicity Summary Isopropanol. (http://oehha.ca.gov/air/chronic_rels/pdf/67630.pdf). Edited by M Heylin, American Chemical Society. Washington D.C., June 26:44 (1995)

Clark DD, Ensign SA: Evidence for an inducible nucleotide-dependent acetone carboxylase in *Rhodococcus rhodochrous* B276. J Bacteriol 181: 2752-2758 (1999)

Davies R, Stephenson M: Studies on the acetone-butyl-alcohol fermentation. I. Nutritional and other factors involved in the preparation of active suspensions of Clostridium acetobu tylicum (Weizmann). Biochem J 35: 1320-331 (1941)

Dow Chemical Company: Acetone Safety Data Sheet. Product Safety Assessment (http://www.dow.com/productsafety/finder/acetone.htm) 04.20.2006.

Ensign SA, Small FJ, Allen JR, Sluis MK: New roles for CO_2 in the microbial metabolism of aliphatic epoxides and ketones. Arch Microbiol 169: (3), 179-187 (1998)

Foster JW: The role of organic substrates in photosynthesis of purple bacteria. J Gen Physiol 24: 123-134 (1940)

Harris JW. Hydrocarbon production handbook. Hydrocarbone Processes. 64 : 154 (1991)

Janssen PH, Schink B: Metabolic pathways and energetics of acetone metabolism by the sulfate-reducing bacterium *Desulfobacterium cetonicum*. Arch Microbiol 163: 188-194 (1995)

Jobst B, Schühle K, Linne U, Heider J: ATP-dependent carboxylation of acetophenone by a novel type of carboxylase. J Bacteriol 192: 1387-1394 (2010)

May O, Habenicht A, Mattes R, Syldatk C, Siemann M: Molecular evolution of hydantoinases. Biol Chem 379: 743-747 (1998)

Mohammad BT, Wright PC, Bustard MT: Bioconversion of isopropanol by a solvent tolerant *Sphingobacterium mizutae* strain. J Ind Microbiol Biotechnol 33: (12), 975-983 (2006)

Northrop JH, Ashe LH, Senior JK: Biochemistry of *Bacillus acetoethylicum* with reference to the formation of acetone. J Biol Chem 39: 1-21 (1919)

Platen H, Schink B: Methanogenic degradation of acetone by an enrichment culture. Arch Microbiol 149: 136-141 (1987)

Platen H, Schink B: Anaerobic degradation of acetone and higher ketones via carboxylation by newly isolated denitrifying bacteria. J Gen Microbiol 135: 883-891 (1989)

Platen H, Temmes A, Schink B: Anaerobic degradation of acetone by *Desulfococcus biacutus* spec. nov. Arch. Microbiol. 154: 355-361 (1990)

Saito Y, Yamashita M, Ito E: Hydrogen production from 2-propanol as a key reaction for a chemical heat pump with reaction couple of 2-propanol dehydrogenation/acetone hydrogenation. Int J Hydrog En 19: 223-232 (1994)

Schardinger F: *Bacillus macerans*, ein Acetone-bildender Rotebacillus. Zbl Bakteriol Parasitenkd Insfektionskr Hyg Abt II 14: 772-781 (1905)

Schink B: Energetics of syntrophic cooperation in methanogenic degradation. *Microbiol Mol Biol Rev* 61: 262-280 (1997)

Siegel JM, Kamen MD: Studies on the metabolism of photosynthetic bacteria. VI. Metabolism of isopropanol by a new strain of *Rhodopseudomonas gelatinosa*. J Bacteriol 59: (5), 693-697 (1950)

Sluis MK, Small FJ, Allen JR, Ensign SA: Involvement of an ATP-dependent carboxylase in a CO_2-dependent pathway of acetone metabolism by *Xanthobacter* strain Py2. J Bacteriol 14: 4020-4026 (1996)

Sluis MK, Ensign SA: Purification and characterization of acetone carboxylase from *Xanthobacter* strain Py2. Proc Nat Acad Sci 94: 8456-8461 (1997)

Sluis MK, Larsen RA, Krum JG, Anderson R, Metcalf WW and Ensign SA: Biochemical, molecular, and genetic analyses of the acetone carboxylases from *Xanthobacter autotrophicus* strain Py2 and *Rhodobacter capsulatus* strain B10. J Bacteriol 11: 2969-2977 (2002)

Syldatk C*,* May O, Altenbuchner J*,* Mattes R, Siemann M: Microbial hydantoinases - industrial enzymes from the origin of life*?* Appl Microb Biotechnol 51: 293-309 (1999)

Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596-1599 (2007)

Weissermel K, Arpe HJ: Industrielle Organische Chemie: bedeutende Vor- und Zwischenprodukte. Verlag Chemie, Weinheim, Deutschland (1978)

Widdel F, Pfennig N: Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. I. Isolation of a new sulfate-reducer enriched with acetate from saline enwironments. Description of *Desulfobacter postgatei* gen. nov. sp. nov. Arch Microbiol 129: 395-400 (1981)

Widdel F, Kohring GW, Mayer F: Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. III. Characterization of the filamentous gliding *Desulfonema limicola* gen. nov. sp. nov., and *Desulfonema magnum* sp. nov. Arch Microbiol 134: 286-294 (1983)

Widmark FMP. Studies on the acetone concentration in blood, urine and alveolar air. II. The passage of acetone in aceto-acetic acid into the urine. Biochem J. 14: 364-378 (1920)

Ziegler K, Braun K, Böckler A, Fuchs G: Studies on the anaerobic degradation of benzoic acid and 2-aminobenzoic acid by a denitrifying Pseudomonas strain. Arch Microbiol 149: 62-69 (1987)

Erklärung

Ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung andere als der angegebenen Hilfsmittel angefertig habe. Die aus anderem Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet.

Weitere Personen, insbesondere Promotionsberater, waren an der inhaltlich materiellen Erstellung dieser Arbeit nicht beteiligt. Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Konstanz, den_____ _____

(Unterschrift)